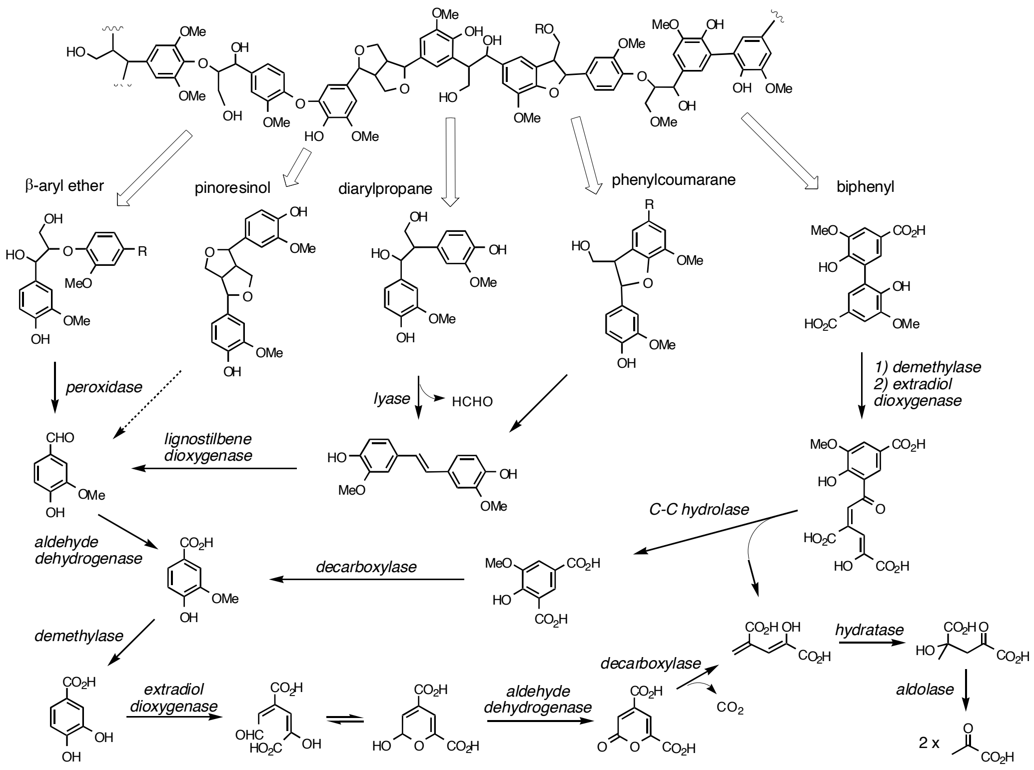
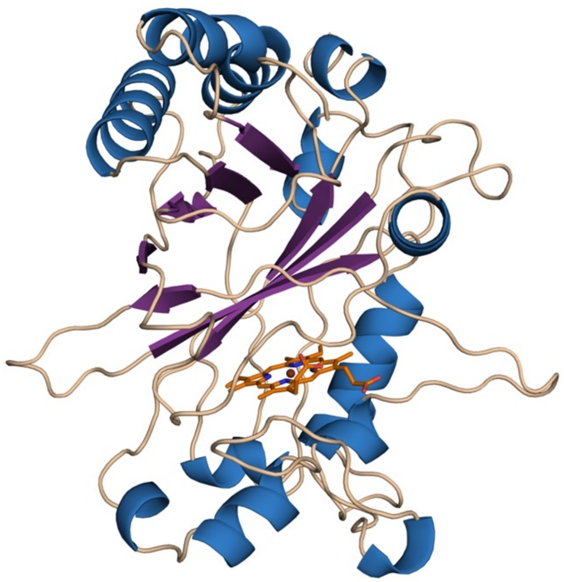
Bacterial Enzymes for Lignin Degradation: Enzymatic and Microbial Conversion to High-Value Products

Prof. Tim Bugg

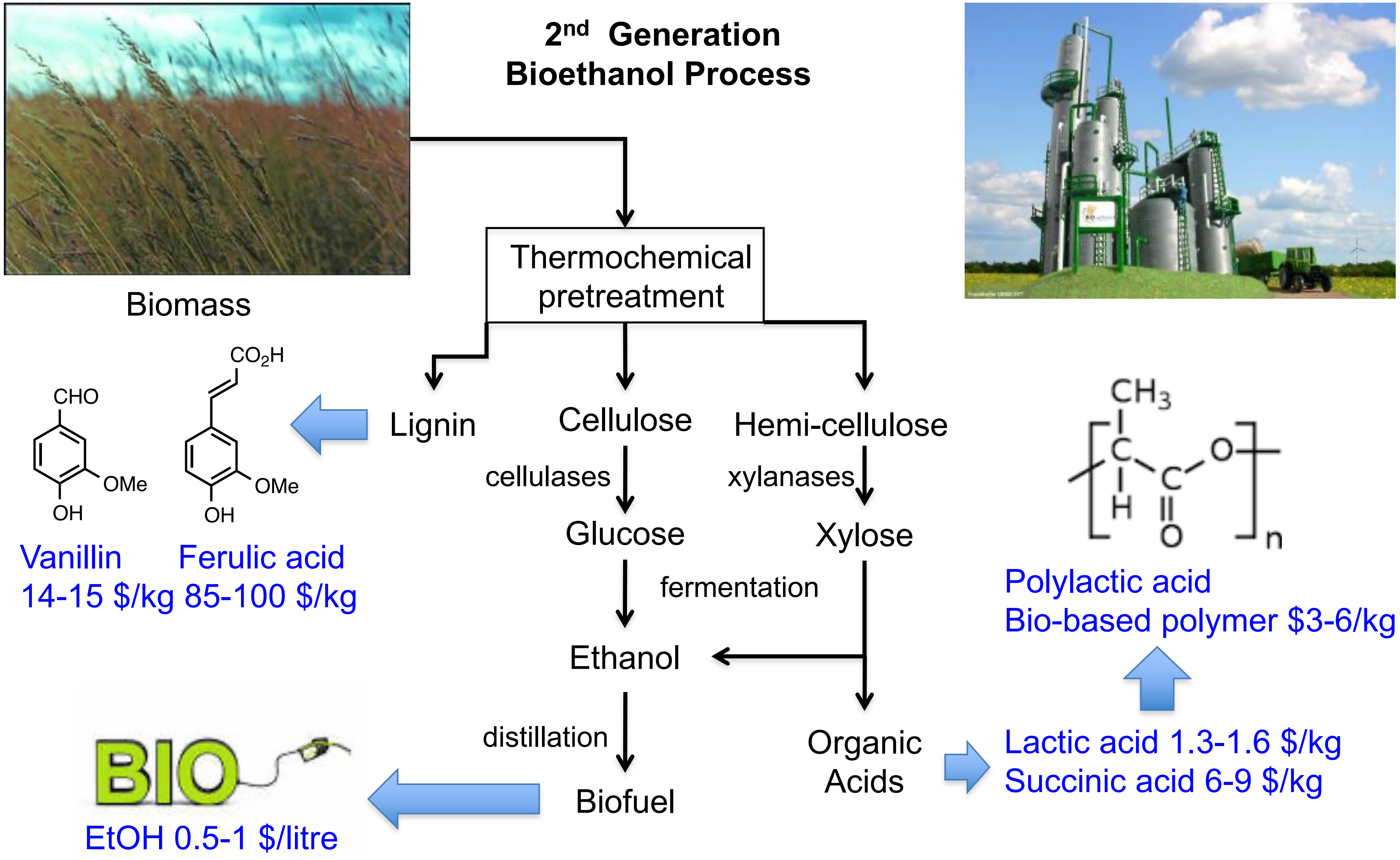
Department of Chemistry, University of Warwick





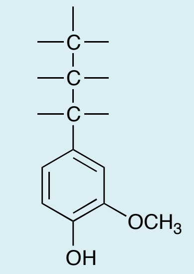
THE UNIVERSITY OF WARWICK

Lignocellulose-Based Bio-refinery Concept

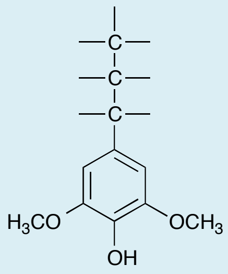


Molecular Structure of Lignin

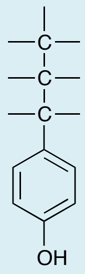
- High molecular weight aromatic polymer
- Heterogenous structure, highly cross-linked
- Contains aryl-C3 units:
- Different substitution patterns in different plant types:



guaiacyl unit (G)

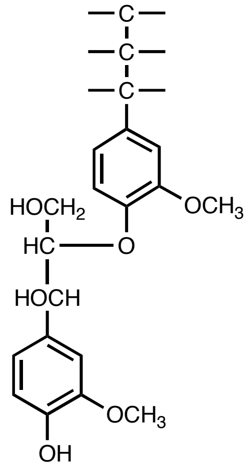


syringyl unit (S)

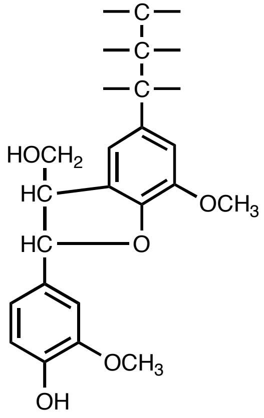


p-hydroxyphenyl unit (H)

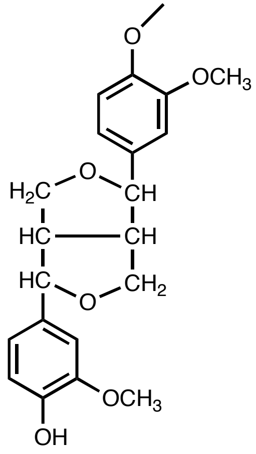
Potential source of aromatic products



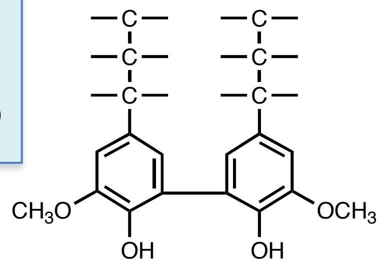
β-aryl ether



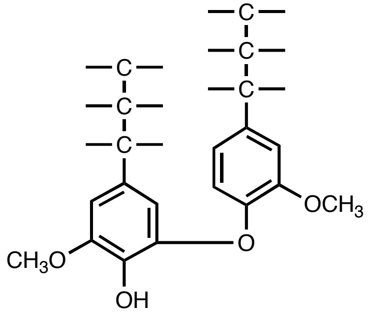
phenylcoumarane



pinoresinol

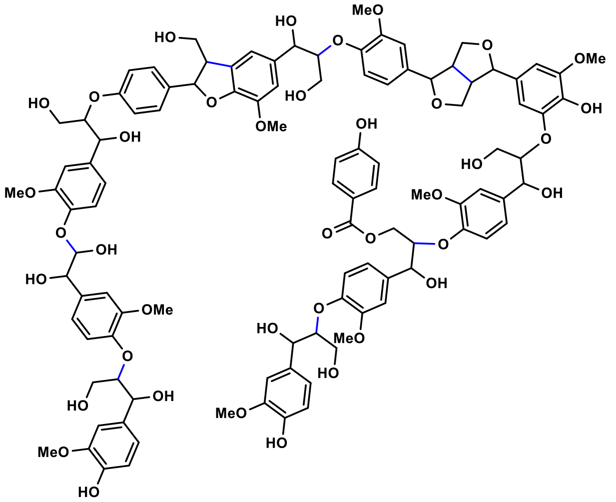


biphenyl

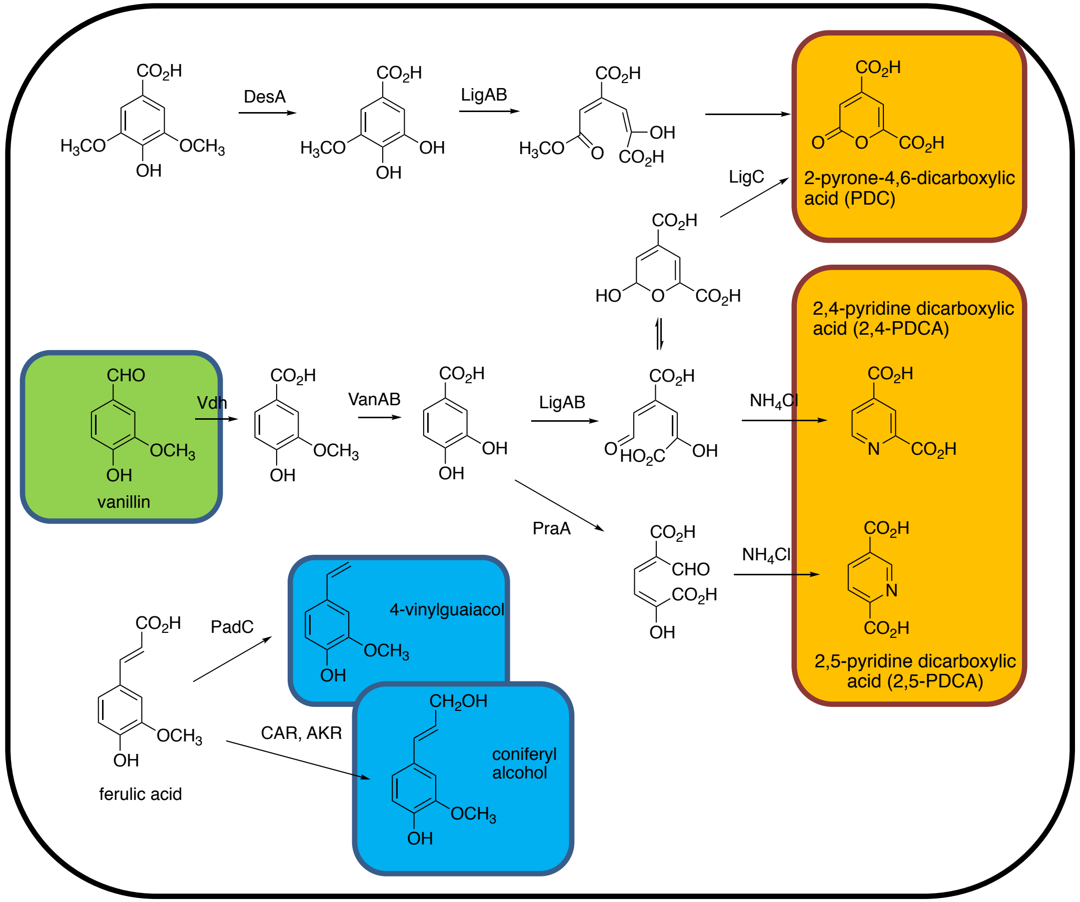


diaryl ether

Two Strategies for Biocatalytic Lignin Conversion to Low Molecular Weight Products

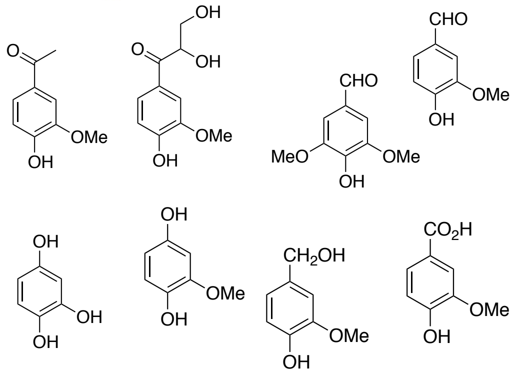


Microbial transformation by engineered microbe



- Choice of microbial host, genetic tools
- Competing pathways, understanding of metabolism

In vitro conversion by lignin-oxidizing enzymes & enzyme combinations

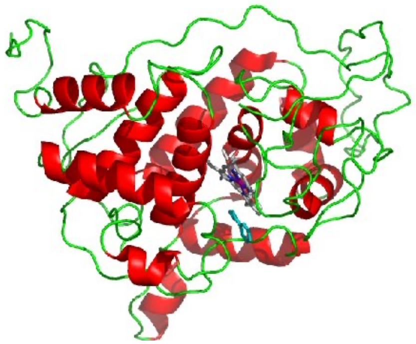


- Mixtures of products
- Lignin repolymerisation

Fungal Enzymes for Lignin Oxidation

Lignin peroxidase

P. chrysosporium

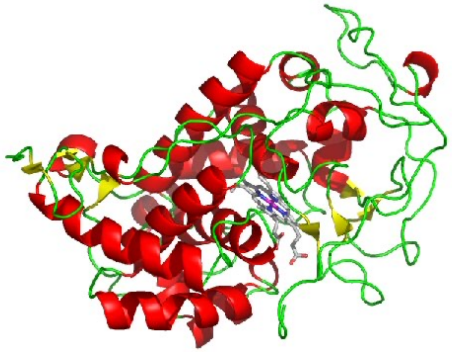


Heme Fe enzyme
Oxidant H_2O_2

Able to oxidise
Lignin model cpds
$C\alpha$-$C\beta$ cleavage
$C\alpha$ oxidation

Manganese peroxidase

P. chrysosporium

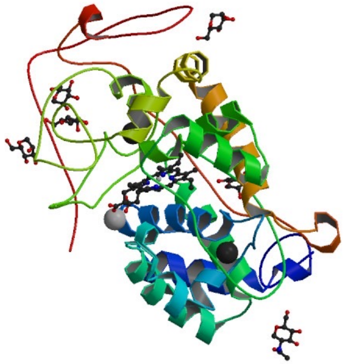


Heme Fe enzyme
Oxidant H_2O_2

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

Versatile peroxidase

Pleurotus eryngii

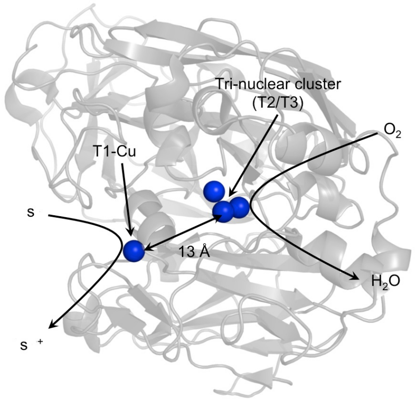


Heme Fe enzyme
Oxidant H_2O_2

Oxidises Mn(II) or lignin

Laccase

Trametes versicolor



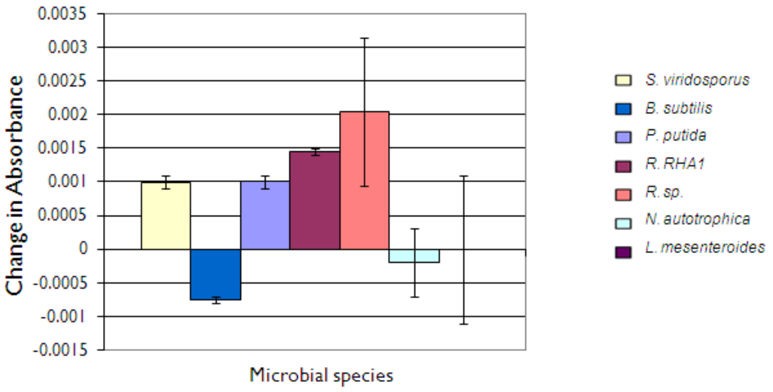
Multi-copper enzyme
Oxidant O_2

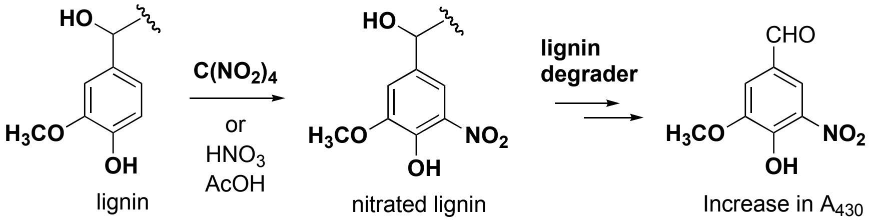
Able to oxidise wide range of phenols, using redox mediator

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?

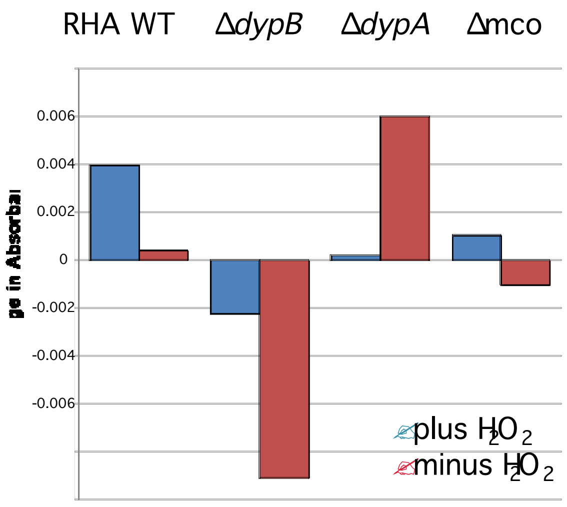
Identification of *Rhodococcus jostii* Peroxidase DypB



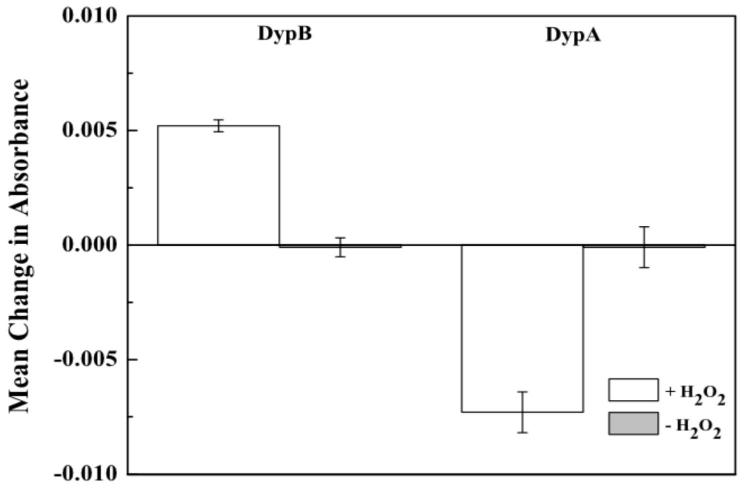


M. Ahmad et al, *Mol. Biosystems*, 2010, **6**, 815-821

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

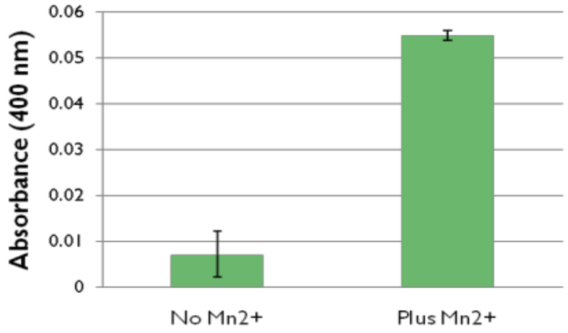


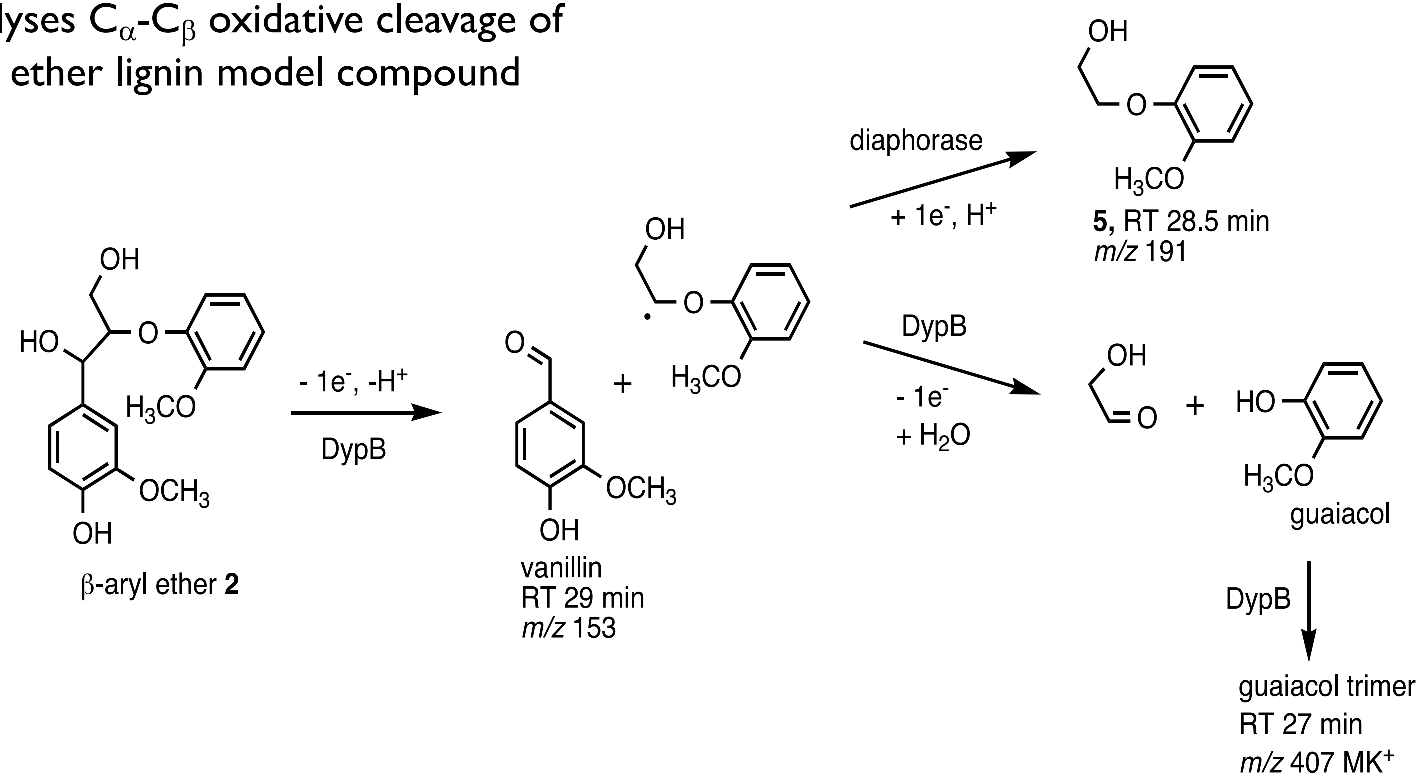
Biochemical Properties of DypB

- 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



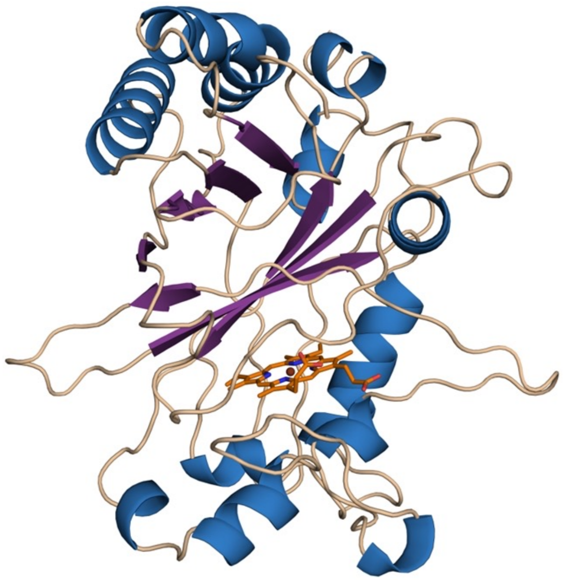


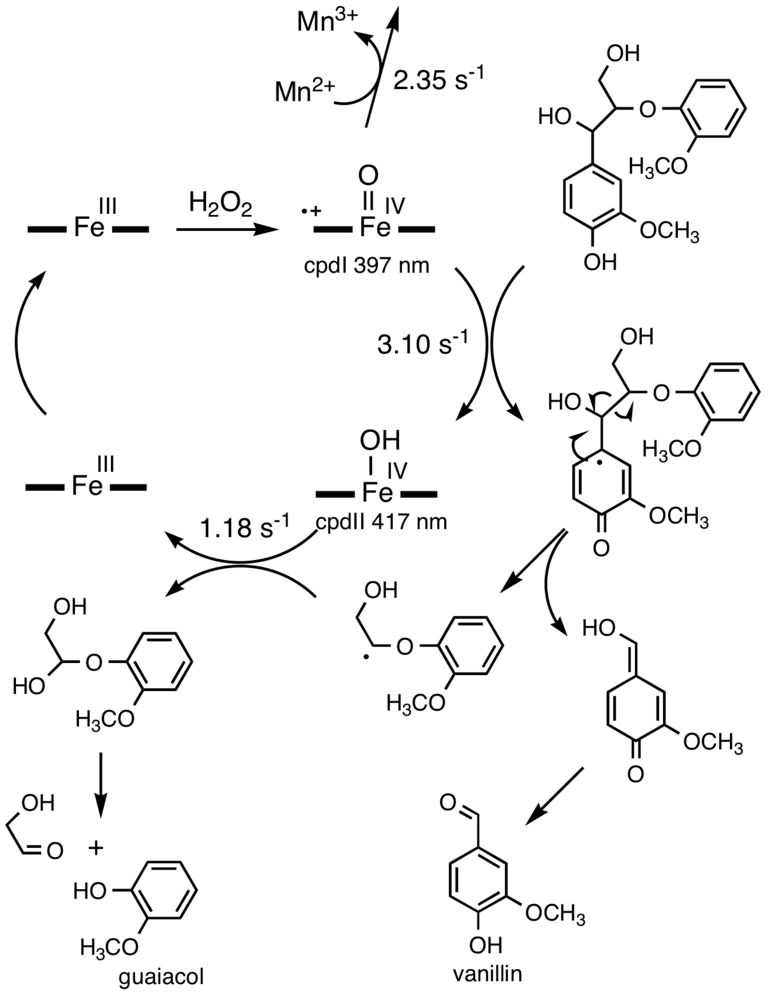
Catalytic cycle for DypB

• 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)

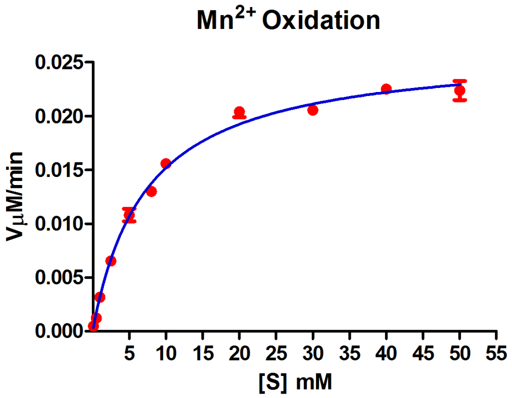
•Crystal structure of *R. jostii* DypB determined in collaboration with Prof. L. Eltis (UBC, Canada)

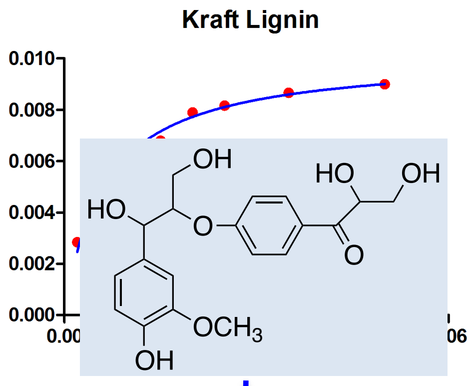




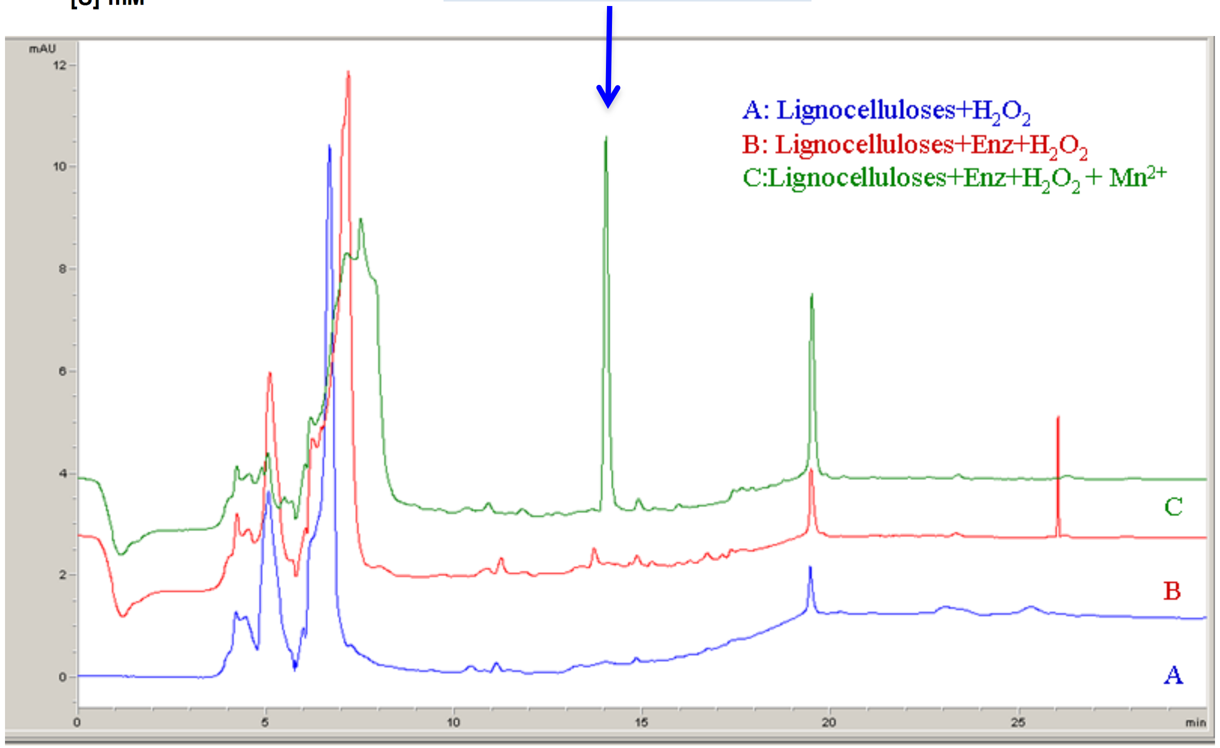
M. Ahmad, E.M. Hardiman et al., *Biochemistry*, 2011, **50**, 5096
J.N. Roberts et al., *Biochemistry*, 2011, **50**, 5108.

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



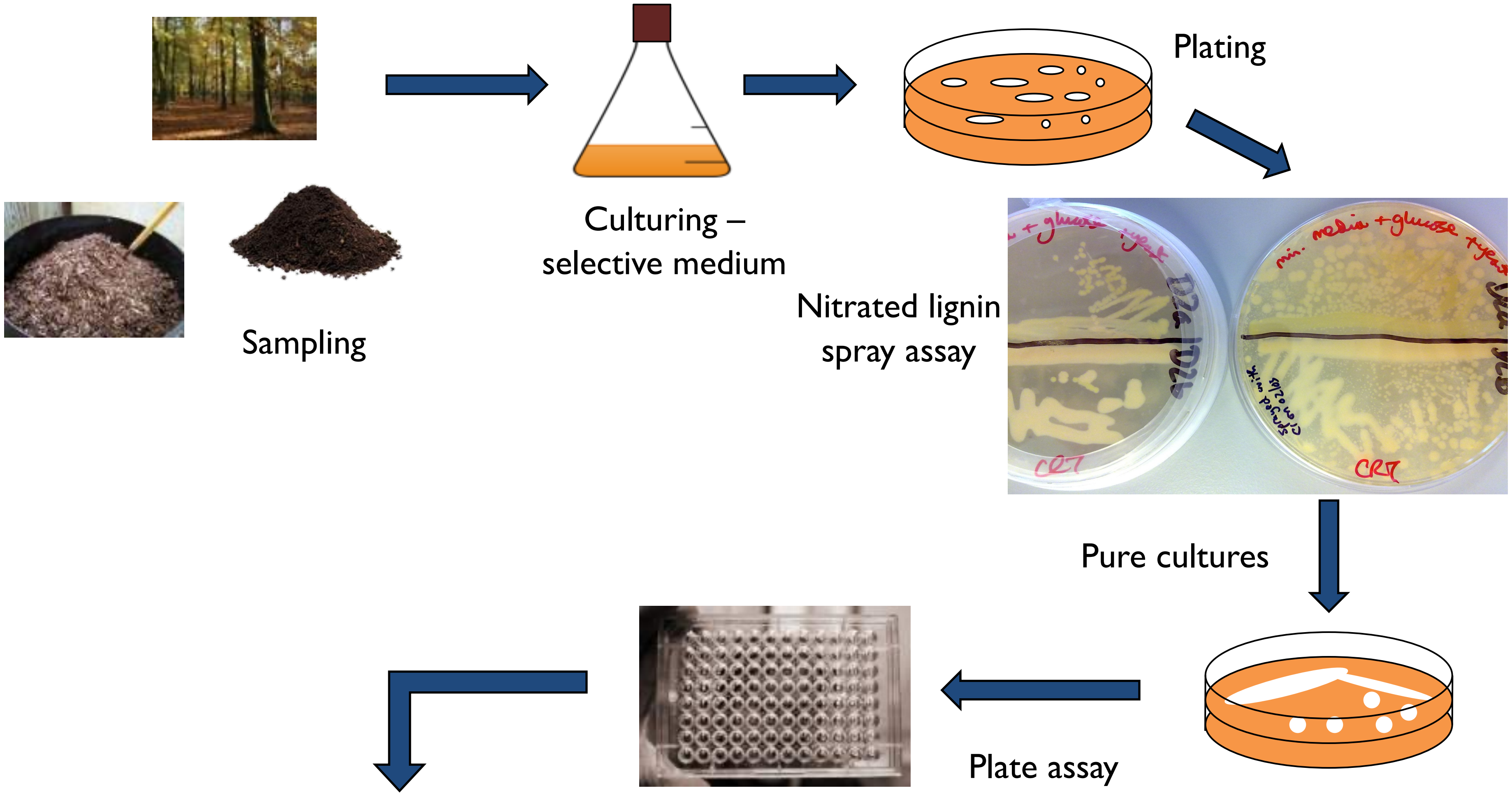


Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
ABTS	1.13	13.5	1.2×10^4
Mn(II)	7.3	2.4	3.3×10^2
Phenol	1.02	1.2	1.2×10^3
Kraft lignin	0.006	0.9	1.4×10^5



R. Rahmanpour & T.D.H. Bugg, *Arch. Biochem. Biophys.* **574**, 93-98 (2015)

Screening for Novel Lignin Degrading Strains



- 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

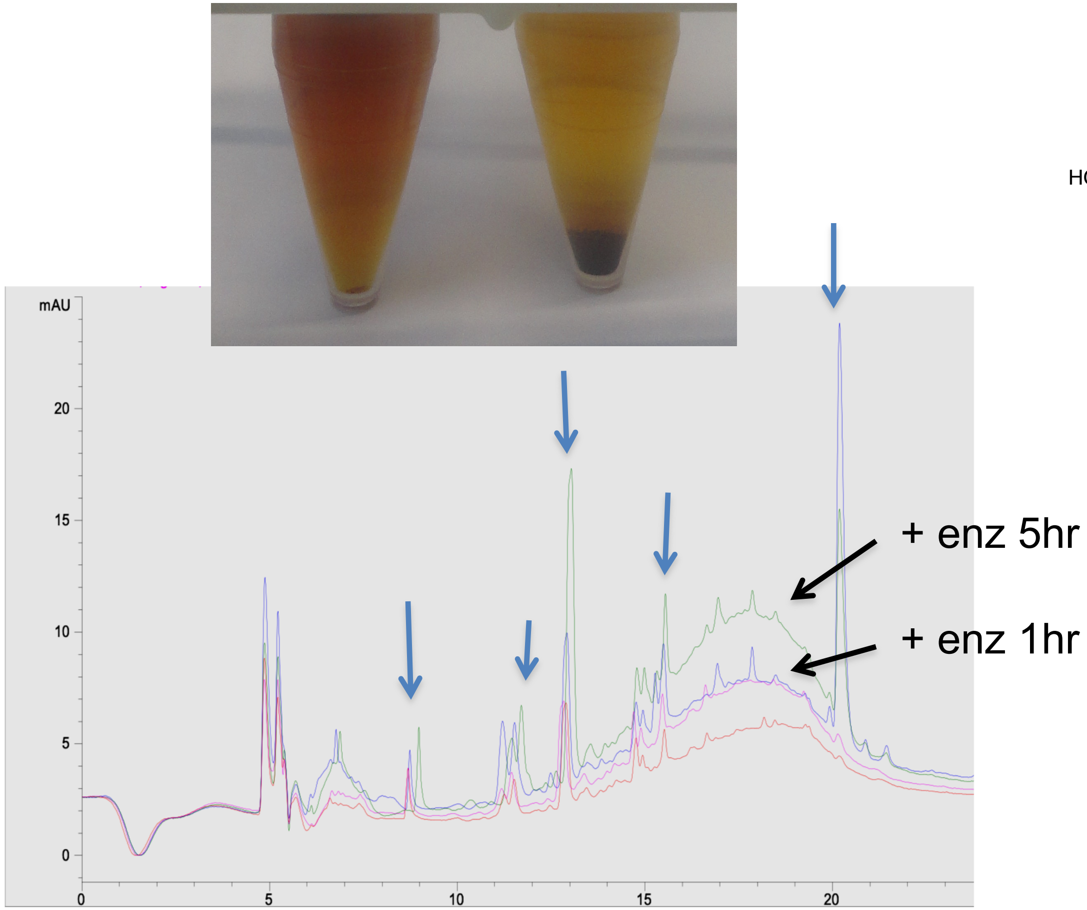
2 thermotolerant isolates from screening of composted wheat straw at 45 °C

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

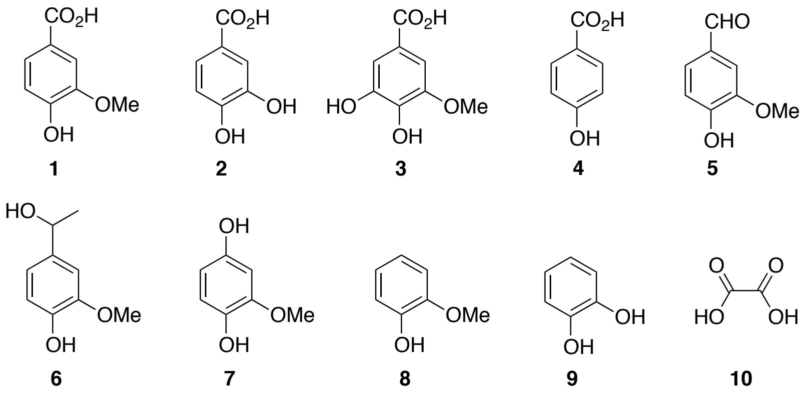
C.R. Taylor, E.M. Hardiman et al., *J. Appl. Microbiol.*, 2012, **113**, 521.

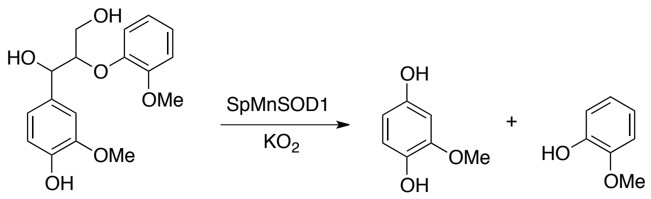
Reaction of *Sphingobacterium* Manganese Superoxide Dismutase with Organosolv lignin

Reaction of MnSOD with Organosolv Lignin
+ Enzyme -Enzyme



Aromatic products detected by LC-MS:

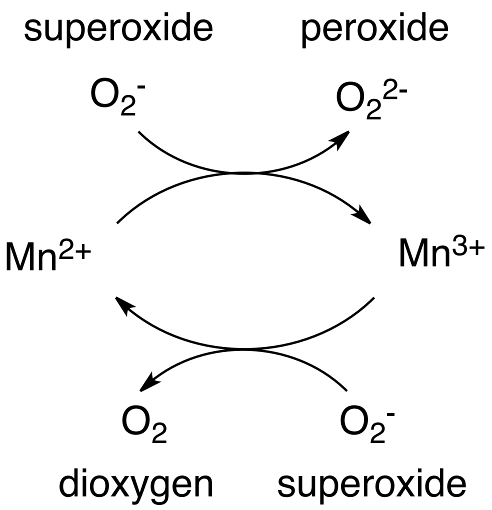




G.M.M. Rashid et al, *ACS Chem Biol* **2015**, *10*, 2286-2294

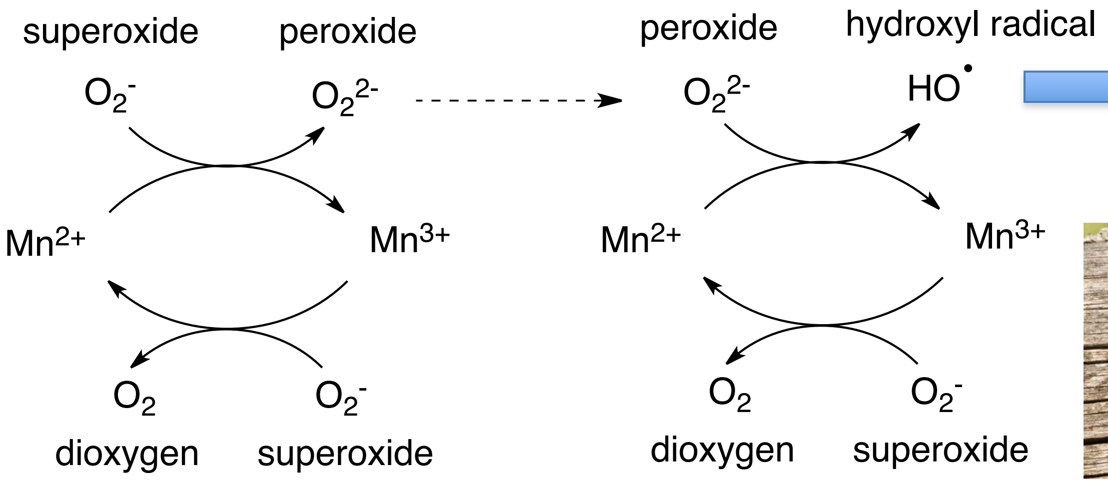
How could Superoxide Dismutase Attack Lignin?

Catalytic cycle of MnSOD



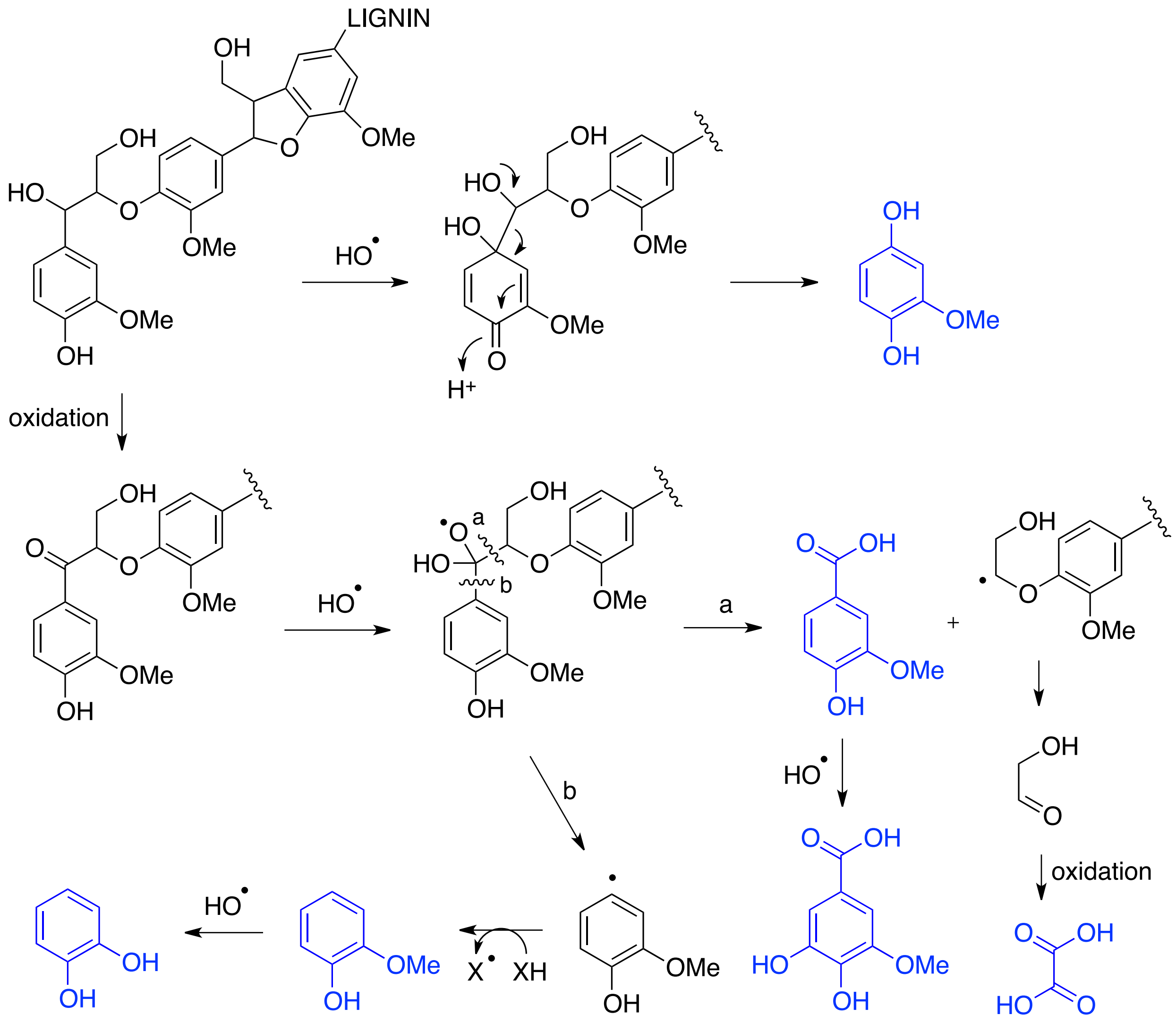
attacks lignin?

Possible modification:



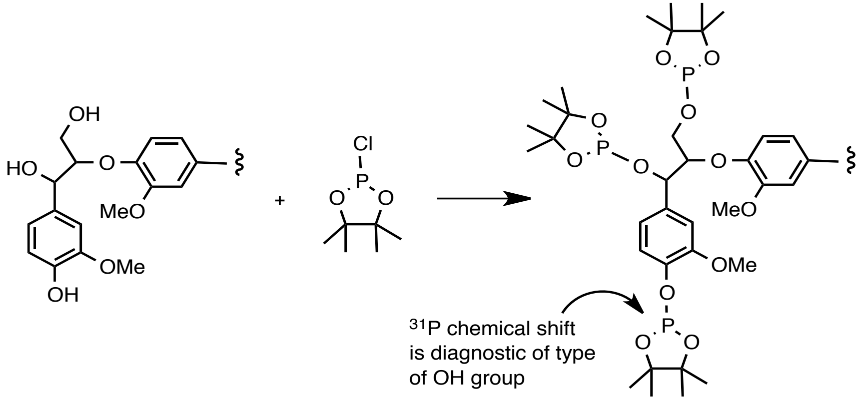
attacks lignin?

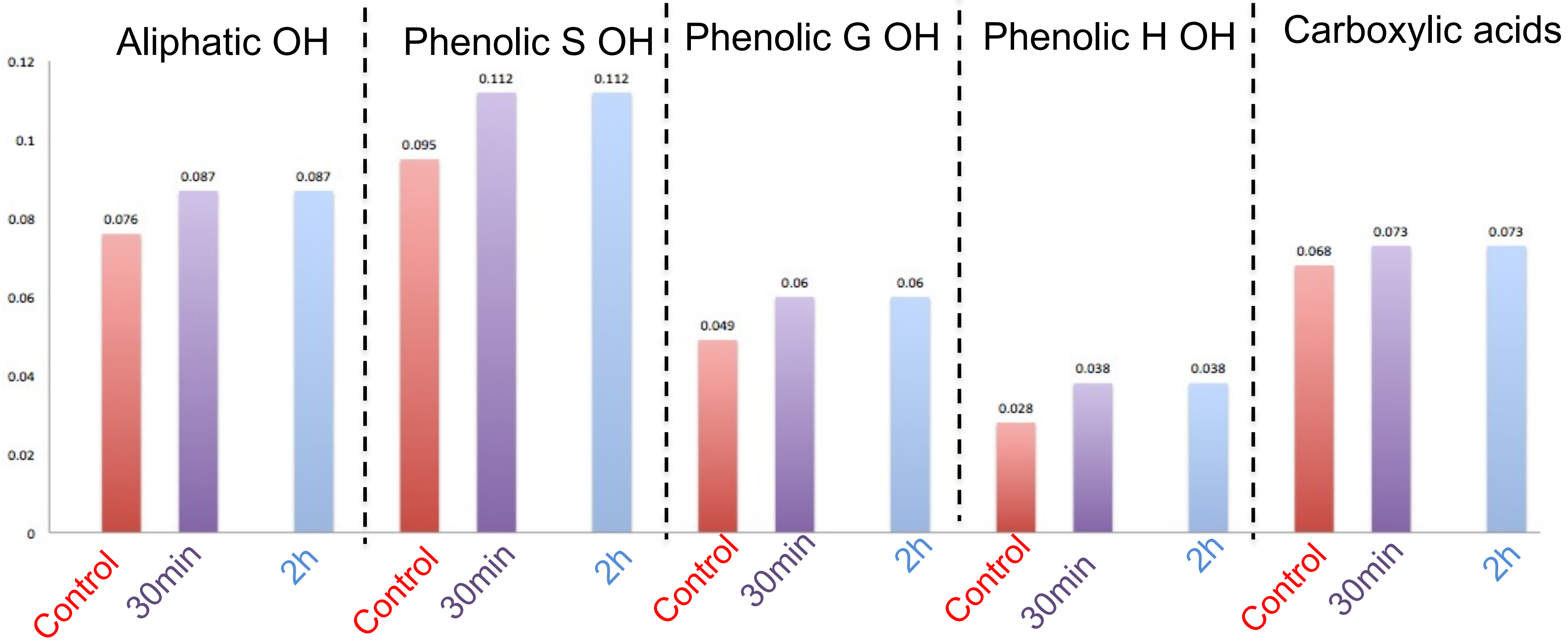
Hypothesis for MnSOD-catalysed Cleavage of Organosolv Lignin



Quantitative ^{31}P NMR Analysis of OH content

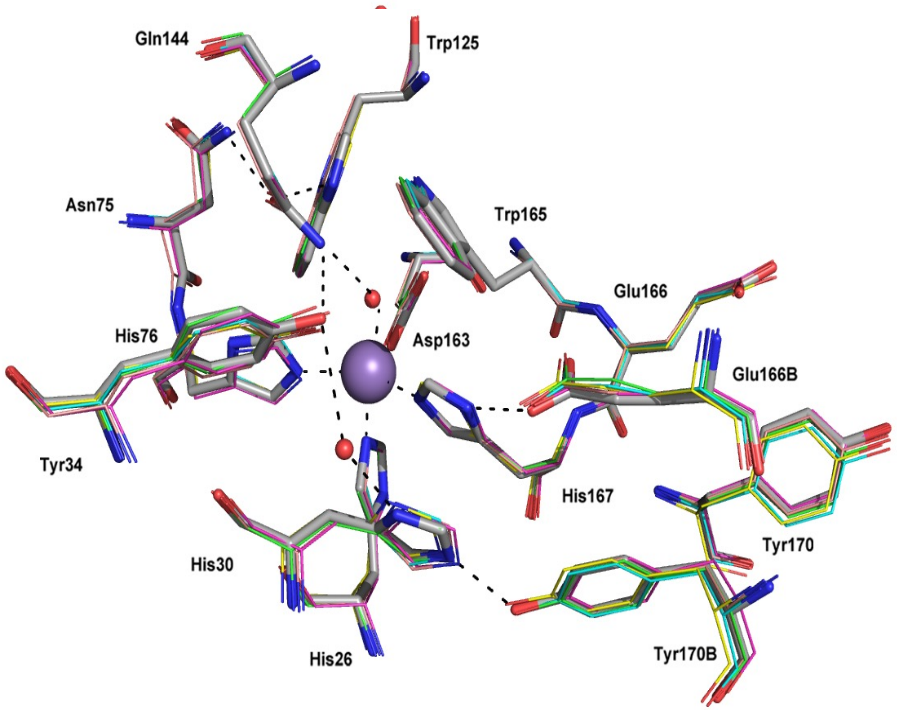
Prof. Stéphanie Baumberger
INRA Versailles

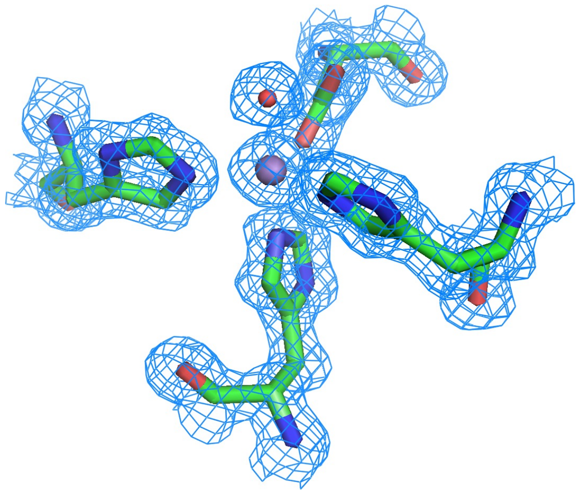




	Aliphatic OH	Phenolic S OH	Phenolic G OH	Phenolic H OH	Carboxylic acids
Batch 1	**+15%**	**+18%**	**+22%**	**+35%**	**+7%**
Batch 2	**+41%**	**+40%**	**+30%**	**+35%**	**+5%**

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

G.M.M. Rashid et al, *ACS Chem Biol* **2015**, *10*, 2286-2294

Amino Acid Replacements in SpMnSOD1 Close to Mn ligands

Leu4Gln His27Tyr His31Ala

```
Sphingobacterium MnSOD1    TTFAQFKQTPLPYAYDALEGAIDAKTMEIHYSKHAAGYTANLNKAIAGTP 70
sp|P61503|SODM_THET8       -MPYPFKLPDLGYPYEALEPHIDAKTMEIHHQKHHGAYVTNLNAALEKYP 49
sp|P0C0I0|SODM_STRPY       ---MAIILPELPYAYDALEPQFDAETMTLHHDKHHATYVANTDAALEKHP 47
sp|P00448|SODM_ECOLI       ---MSYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALESLP 47
tr|H6MT45|H6MT45_GORPV     --MAEYTLPDLPYDYAALEPHISGRIMELHHDKHHATYVKGANDTLDKLA 48
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His-26

Ser77Glu

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Sphingobacterium MnSOD1    AEK-ESIENILAKVSQY----SDAVRNNAGGHYNHELFWSILTPNKGTKP 115
sp|P61503|SODM_THET8       YLHGVEVEVLLRHLAALPQDIQTAVRNNGGGHLNHSLFWRLLTPGGAKEP 99
sp|P0C0I0|SODM_STRPY       EIG-ENLEELLADVPKIPEDIRQALINNGGGHLNHALFWELLSPEK-QDV 95
sp|P00448|SODM_ECOLI       EFANLPVEELITKLDQLPADKKTVLRNNAGGHANHSLFWKGLKKGT--TL 95
tr|H6MT45|H6MT45_GORPV     EAR--ADGSIAGKVYGL----SATLSFHLGGHTNHSIFWKNLSPNGGDKP 92
```
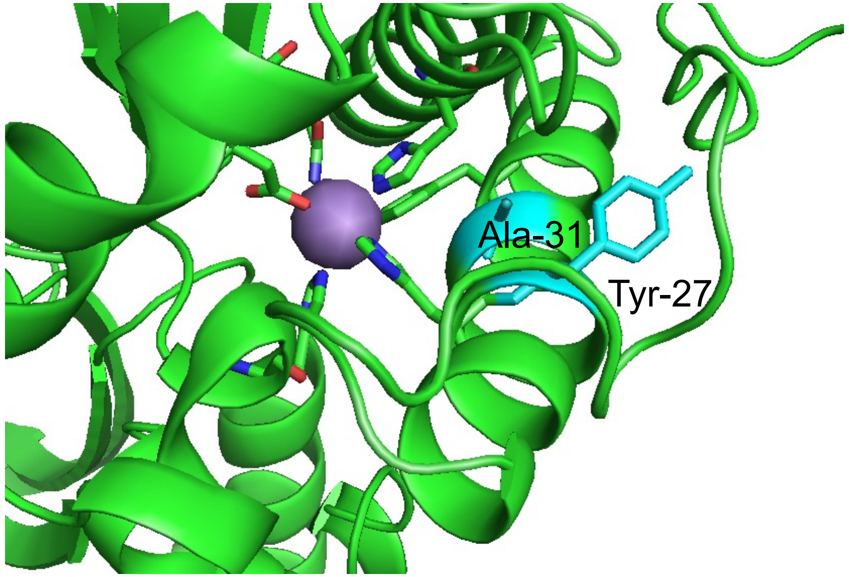
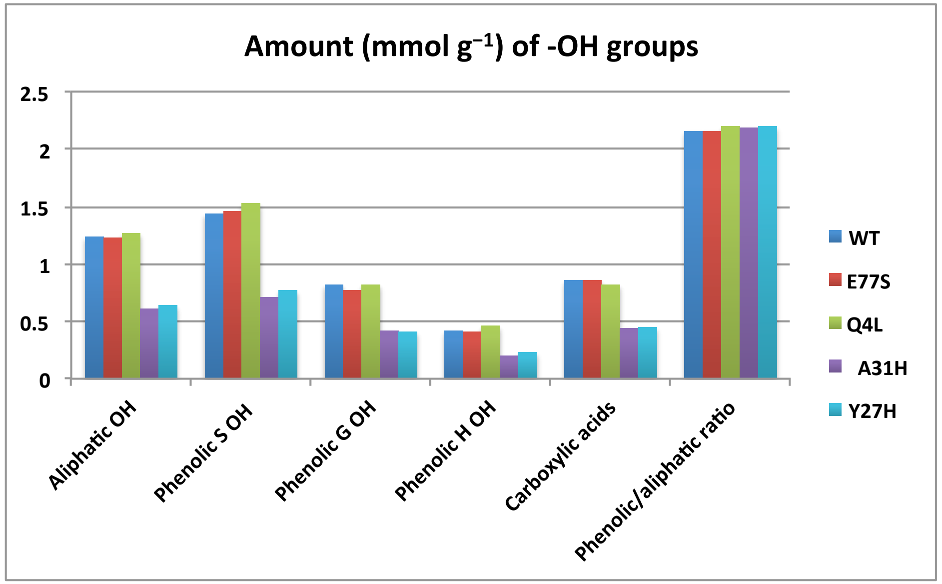

His-76

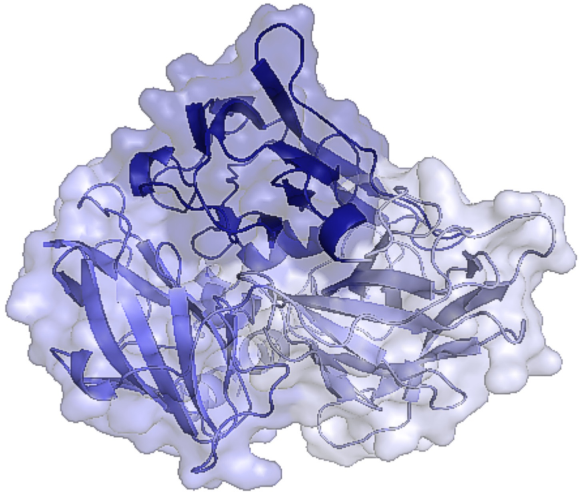


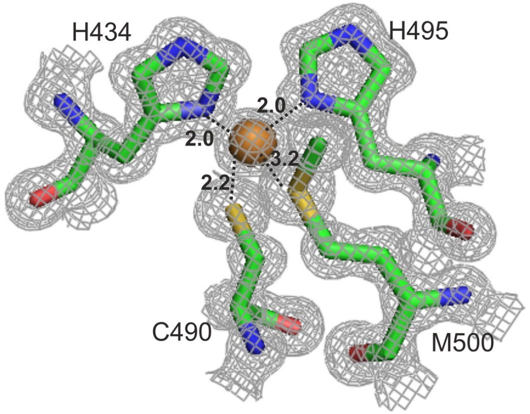


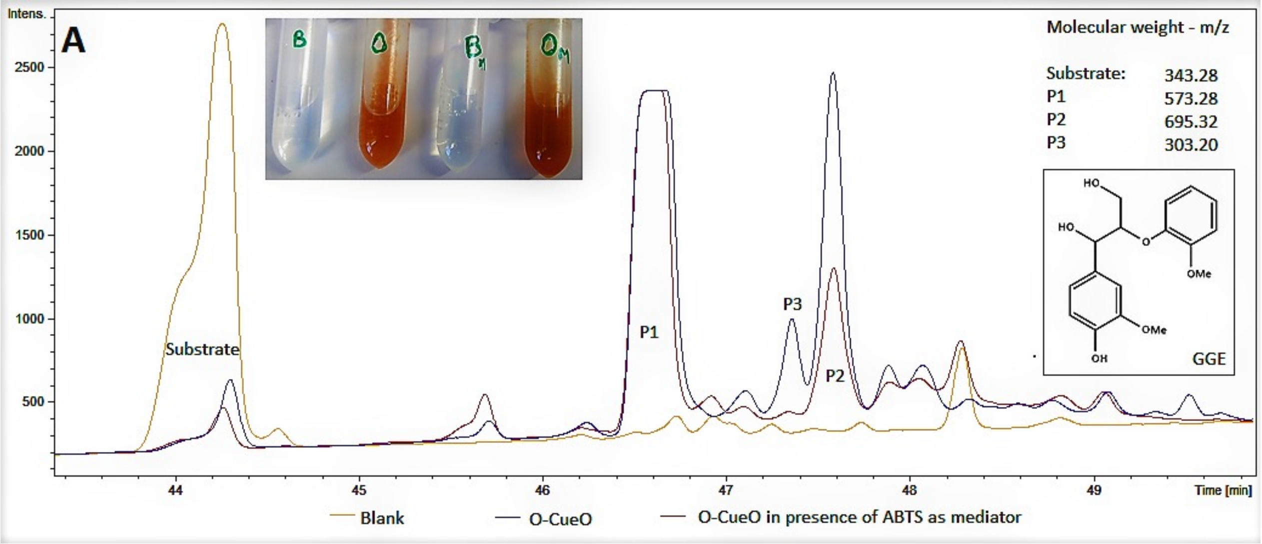
Ala31 AND Tyr27 are required for lignin demethylation activity!

A multicopper oxidase CueO from *Ochrobactrum* sp. active towards lignin model compounds and lignosulfonate

Dr Fabio Squina
(Univ Sorocaba, Brazil)

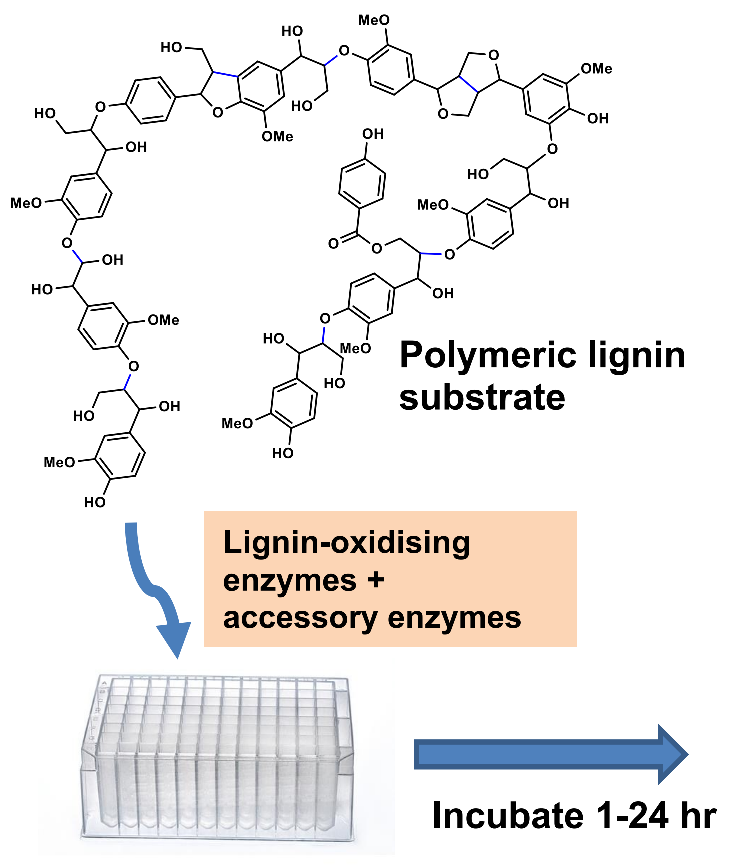


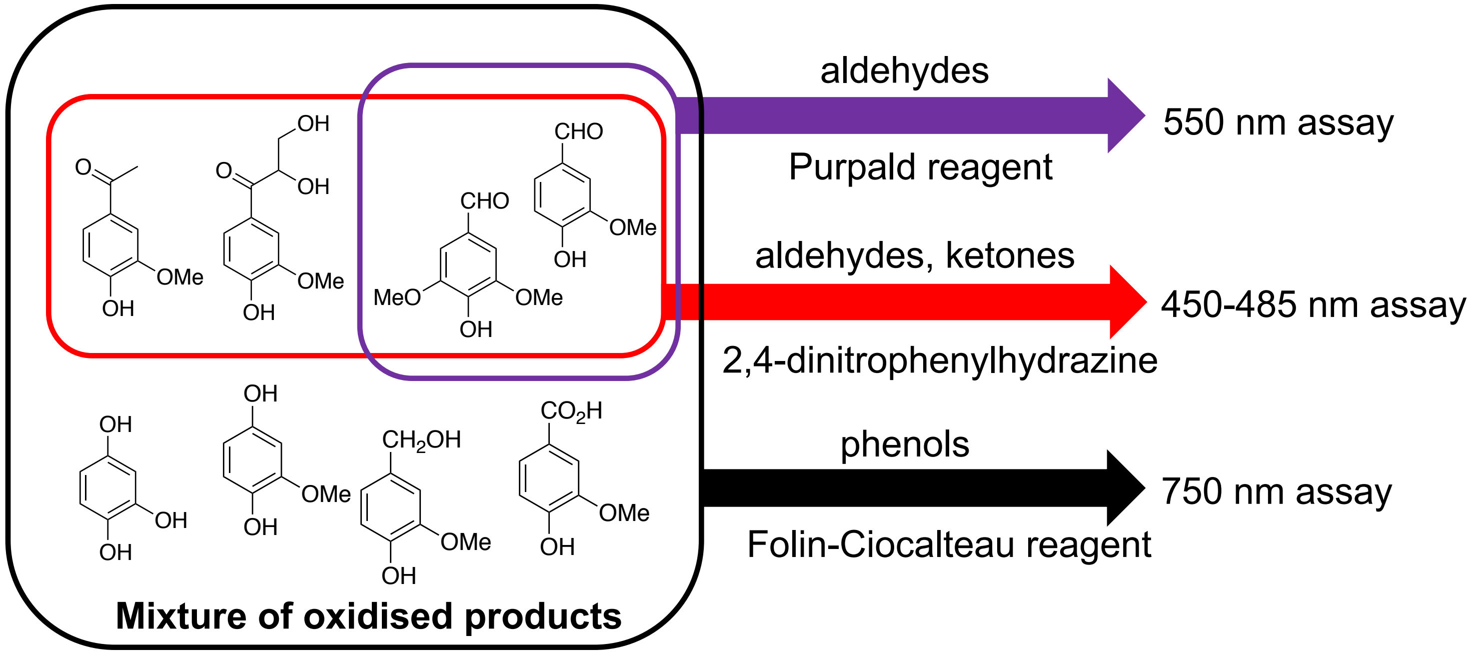


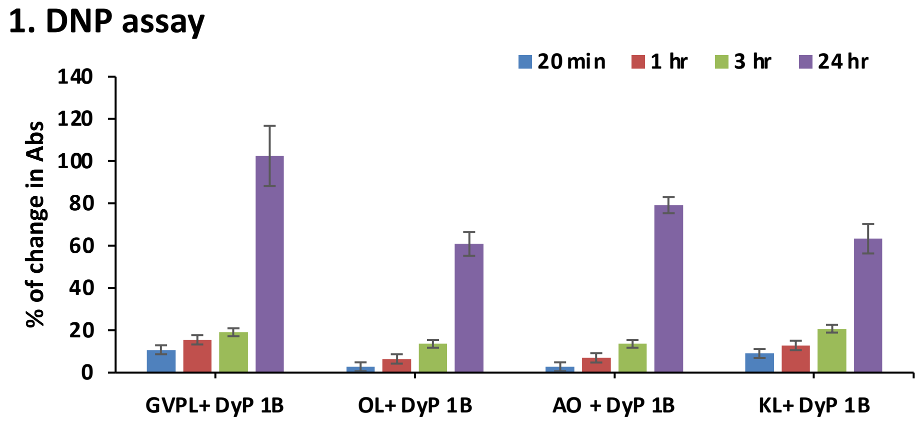


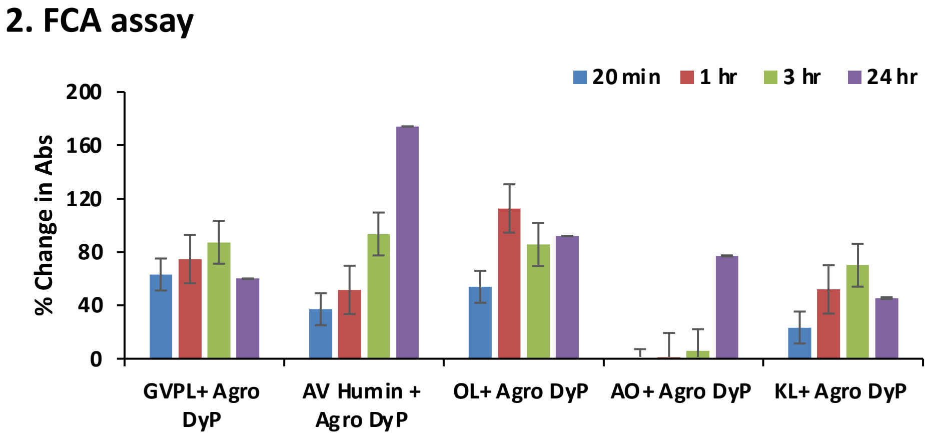
R.S. Granja-Travez, R.C. Wilkinson, G.F. Persinoti, F.M. Squina, V. Fulop, T.D.H. Bugg, *FEBS J.* 2018, **285**, 1684-1700

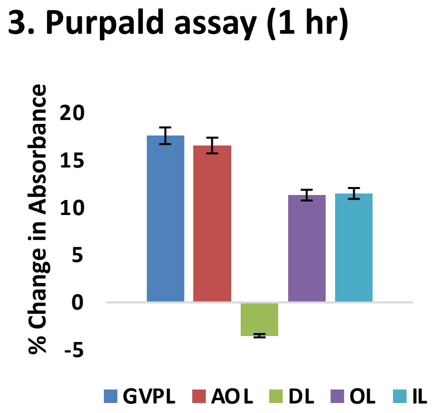
How to test combinations of lignin-degrading enzymes?
Colorimetric assays for low molecular weight product formation:











Accessory enzymes for lignin breakdown: bacterial dihydrolipoamide dehydrogenase prevents repolymerization of phenoxy radicals

Dimerisation of lignin dimer model compound GGE by *P. fluorescens* Dyp1B is prevented by *Thermobifida fusca* dehydrolipoamide dehydrogenase:

1 electron redox chemistry assisted by flavin coenzyme, and active site cysteine disulfide:

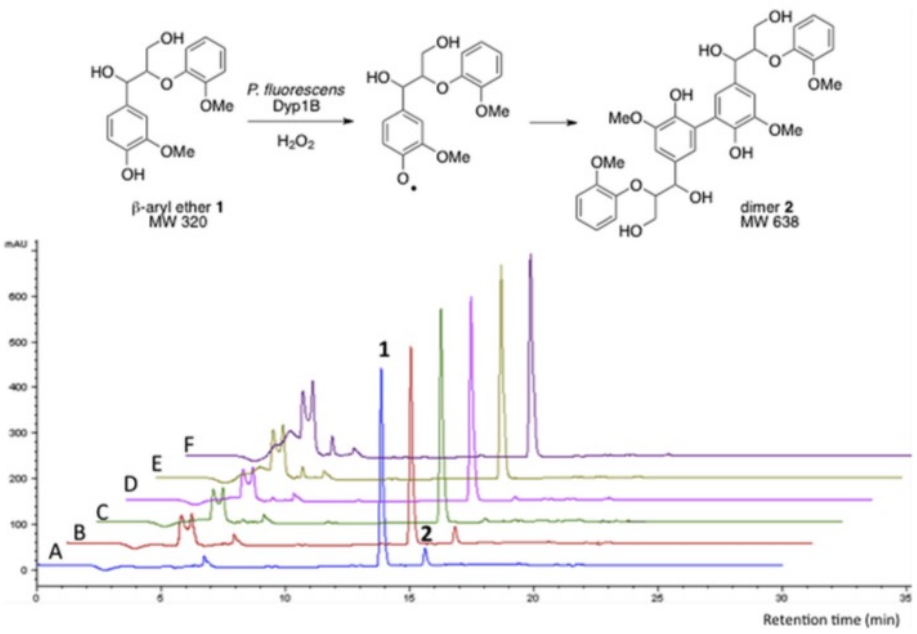


Fig. 1. HPLC traces for conversion of **1** to **2**. **A**, model compound, Dyp1B, DHLDH and H_2O_2 (no NADH); B, model compound, Dyp1B, H_2O_2 and NADH (no DHLDH); C-F, addition to trace **A** of 25 μM (C), 50 μM (D), 100 μM (E) or 200 μM (F) NADH.

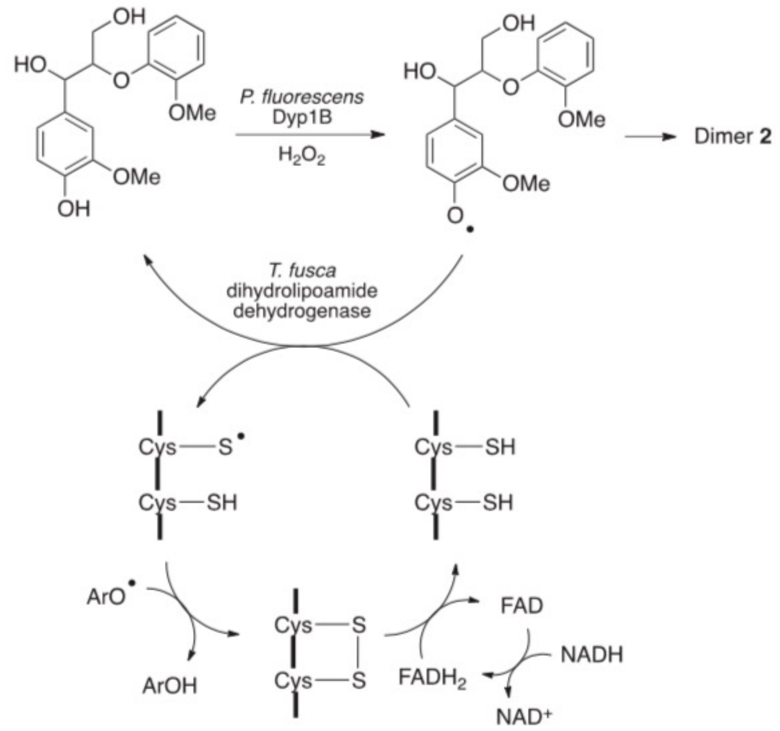


Fig. 2. Catalytic cycle for reduction of phenoxy radicals by *T. fusca* dihydrolipoamide dehydrogenase, via an active site disulfide intermediate.

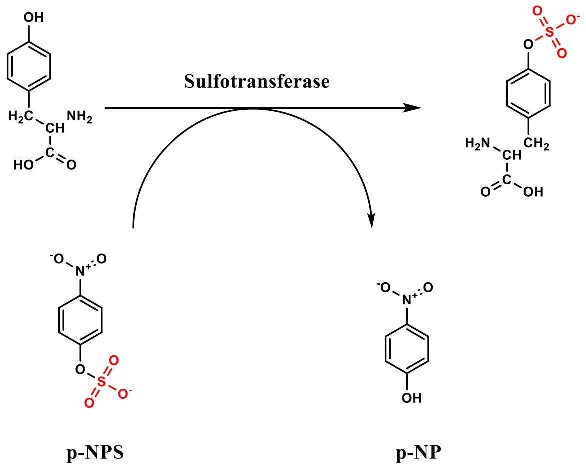
R. Rahmanpour, L.D.W. King & T.D.H. Bugg, *Biochem. Biophys. Res. Commun.* **2017**, *482*, 57-61.

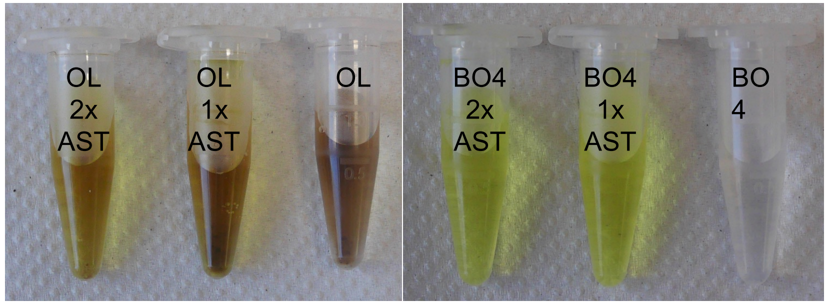
Two dihydrolipoamide dehydrogenase enzymes cloned from *Sphingobacterium* sp. T2
Also tested *Burkholderia cenocepacia* peroxiredoxin (reacts with hydrogen peroxide via Cys nucleophile).

Arylsulfate sulfotransferase (AST) from *Desulfitobacterium hafniense*

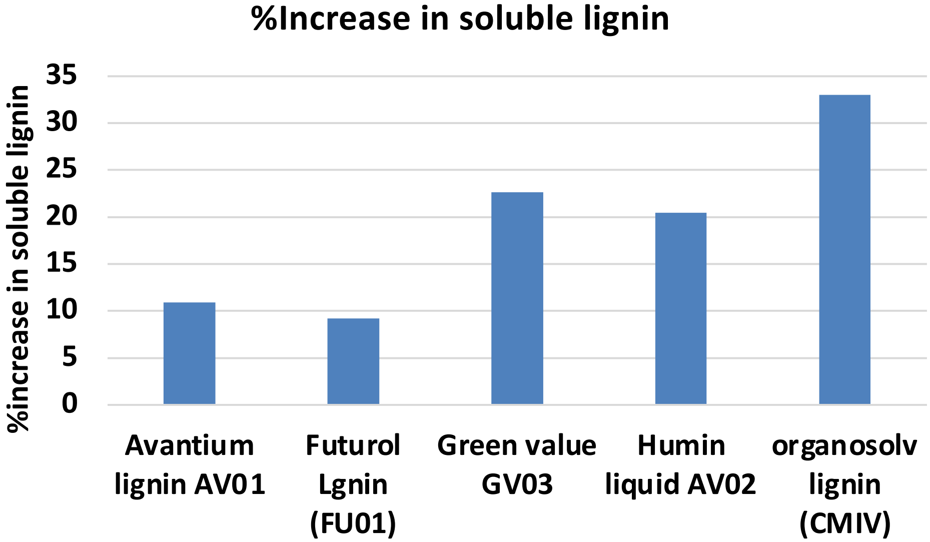
Reported to have activity for sulfation of lignin:
P. Prinsen, A. Narani, A. F. Hartog, R. Wever and G. Rothenberg, *ChemSusChem*, **2017**, *10*, 2267–2273.

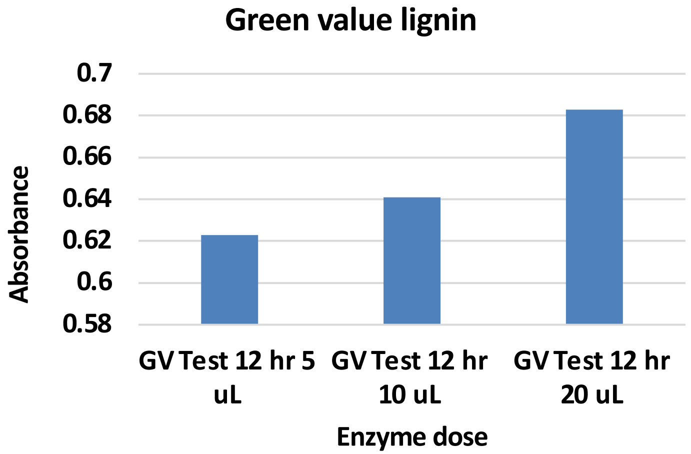
Synthetic p-nitrophenyl sulfate donor, colorimetric assay





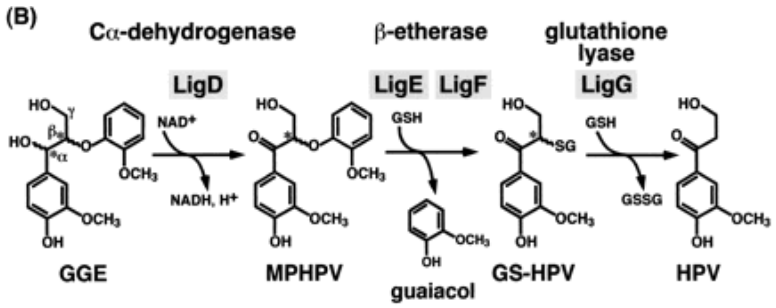
Incubation of AST with organosolv lignin (OL) and β-aryl ether lignin module compound (βO4)



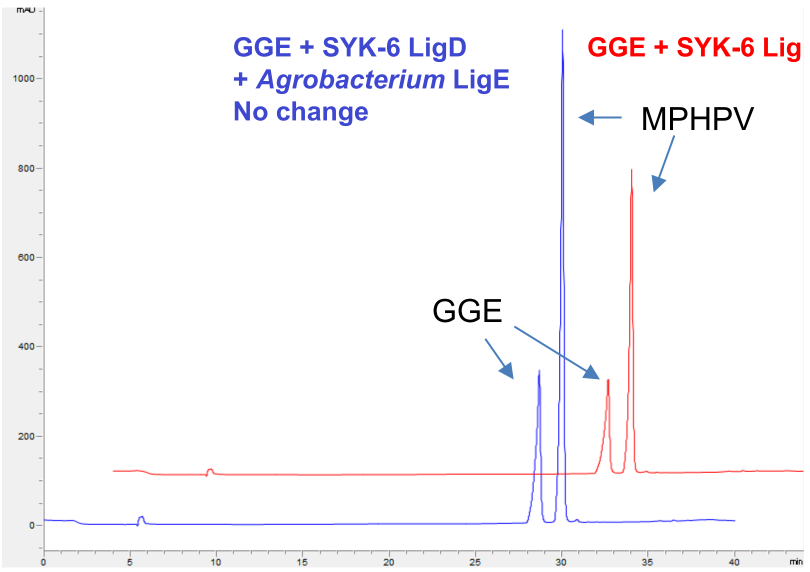


Glutathione-S-transferase LigE From *Agrobacterium* sp.

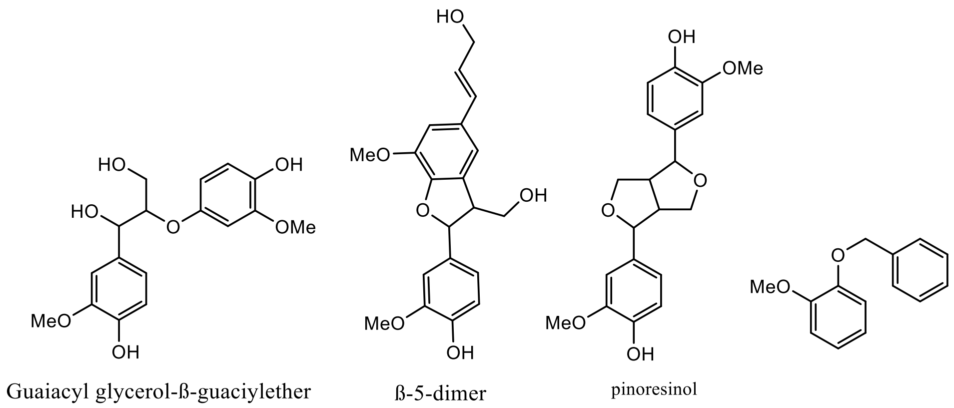
Reactions catalysed by *Sphingobium* SYK-6 LigDEFG



BUT *Agrobacterium* LigE does not convert MPHPV:



Other substrates tested for *Agrobacterium* LigE conversion:



X

Partial conversion, but no glutathione adduct formed

X

Consumed (HPLC)

Colorimetric assays show enhancement in product yield using enzyme combinations:

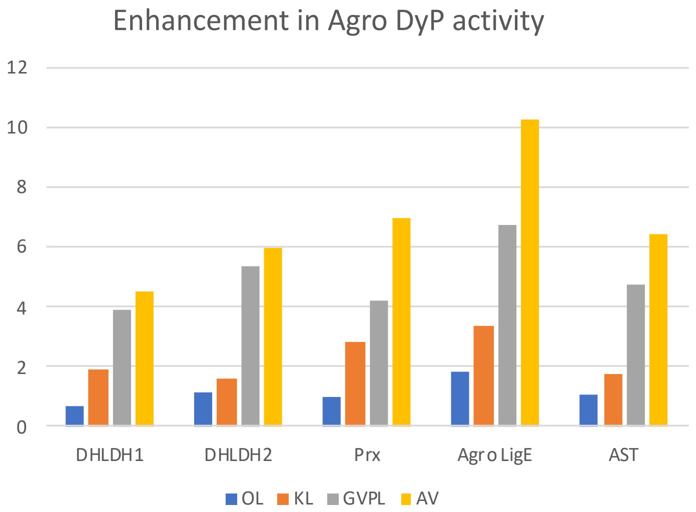
FCA Assay

Lignin prep	Green Value Protobind Lignin (soda lignin)						Wheat Straw Organosolv lignin						Alkali Kraft Lignin						
Ox Enz	DHLDH1	DHLDH2	Prx	Lig E	AST	No Acc-Enz	DHLDH1	DHLDH2	Prx	Lig E	AST	No Acc-Enz	DHLDH1	DHLDH2	Prx	Lig E	AST	No Acc-Enz	Ox Enz
Agro DyP	0.65	0.89	0.70	1.12	0.79	0.16	0.23	0.36	0.31	0.58	0.34	0.32	0.56	0.46	0.83	0.97	0.52	0.29	Agro DyP
Ct DyP	0.70	0.79	0.72	0.99	0.76	0.14	0.26	0.34	0.29	0.49	0.40	0.25	0.50	0.43	0.74	0.78	0.54	0.13	Ct DyP
DyP 1B	0.58	0.72	0.49	0.84	0.71	0.11	0.25	0.36	0.24	0.54	0.39	0.33	0.44	0.42	0.76	0.75	0.60	0.11	DyP 1B
SOD1	0.39	0.47	0.38	0.34	0.57	0.11	0.16	0.22	0.19	0.62	0.42	0.53	0.32	0.36	0.65	0.72	0.49	0.15	SOD1
CopA	0.40	0.25	0.23	0.69	0.66	0.34	0.17	0.27	0.18	0.19	0.22	0.14	0.31	0.37	0.34	0.33	0.37	0.28	CopA
CueO	0.33	0.26	0.31	0.46	0.54	0.28	0.16	0.22	0.16	0.13	0.18	0.11	0.26	0.36	0.32	0.35	0.34	0.23	CueO
No Ox Enz	0.28	0.44	0.33	1.10	0.68	0.25	0.11	0.11	0.11	0.10	0.50	0.02	0.10	0.10	0.04	0.04	0.46	0.24	No Ox Enz

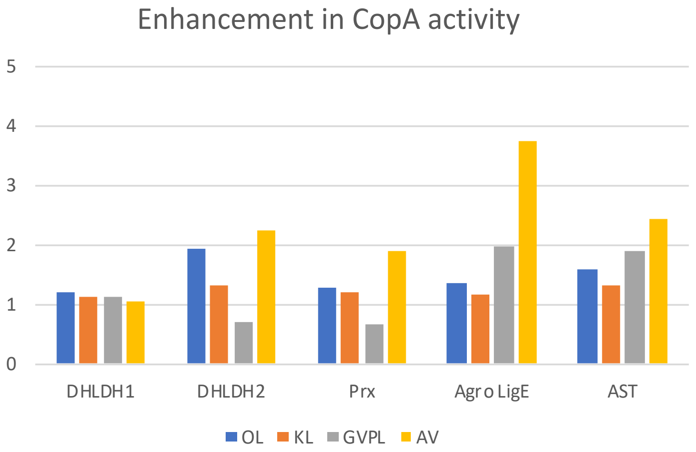
DNP assay

Lignin prep	Green Value Protobind Lignin (soda lignin)						Wheat Straw Organosolv lignin						Alkali Kraft Lignin						
Ox Enz	DHLDH1	DHLDH2	Prx	Lig E	AST	No Acc-Enz	DHLDH1	DHLDH2	Prx	Lig E	AST	No Acc-Enz	DHLDH1	DHLDH2	Prx	Lig E	AST	No Acc-Enz	Ox Enz
Agro DyP	0.42	0.38	0.36	0.37	0.37	0.21	0.48	0.48	0.56	0.53	0.61	0.16	0.46	0.47	0.58	0.55	0.67	0.02	Agro DyP
Ct DyP	0.39	0.37	0.39	0.38	0.38	0.12	0.48	0.47	0.50	0.48	0.59	0.12	0.46	0.49	0.49	0.48	0.60	0.07	Ct DyP
DyP 1B	0.38	0.39	0.37	0.38	0.42	0.12	0.46	0.47	0.49	0.45	0.61	0.07	0.44	0.48	0.50	0.50	0.64	0.02	DyP 1B
SOD1	0.36	0.42	0.37	0.34	0.44	0.11	0.29	0.29	0.29	0.28	0.58	0.07	0.33	0.33	0.32	0.31	0.63	0.10	SOD1
Cop A	0.25	0.26	0.30	0.23	0.38	0.25	0.54	0.47	0.50	0.51	0.68	0.05	0.58	0.52	0.55	0.58	0.69	0.06	CopA
CueO	0.23	0.23	0.30	0.24	0.39	0.25	0.50	0.49	0.48	0.50	0.60	0.08	0.53	0.47	0.48	0.54	0.60	0.20	CueO
No Ox Enz	0.22	0.23	0.34	0.23	0.42	0.23	0.12	0.11	0.14	0.11	0.13	0.01	0.10	0.11	0.13	0.10	0.11	0.05	No Ox Enz

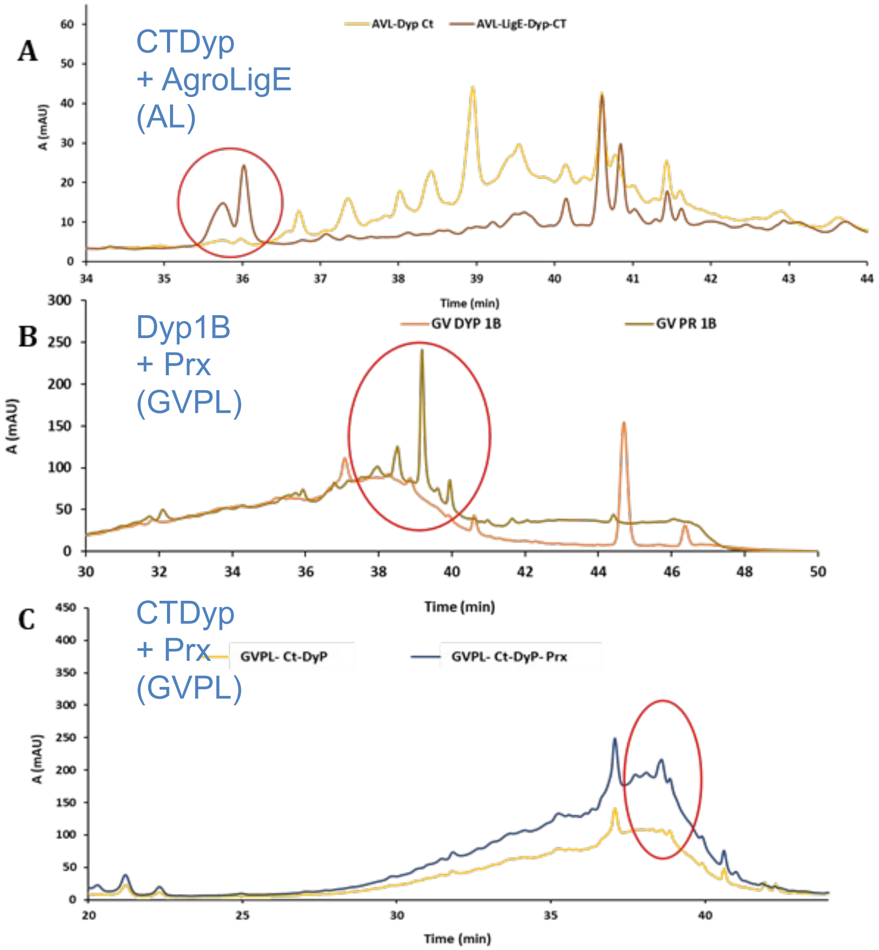
A



B

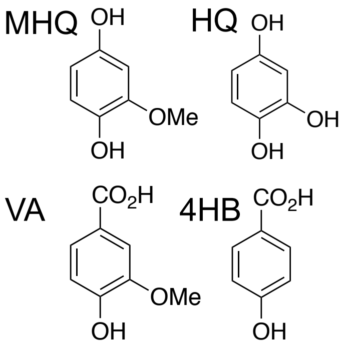


New or enhanced product peaks from lignin biotransformation by enzyme combinations



Estimation of product yield (200 mg scale)

Enzymes	Soluble lignin[a] (mg)	% conversion of soluble lignin[a]	% products extracted into EtOAc (w/w)	New product peaks identified by LC-MS, total %
No enzyme	180 ± 2	-	15.9	-
Agro DyP	184 ± 2	(-2.4[b])	17.6	MHQ, HQ, ∑ 7.5%
LigE	155 ± 2	13.9	18.9	VA, 8.7%
Agro DyP + LigE	148 ± 2	17.8	22.4	MHQ, HQ, VA, 4HB, ∑ 9.9%
Agro DyP + Prx	182 ± 2	-	16.5	MHQ, HQ, ∑ 8.0%
Agro DyP + DHLDH2	178 ± 2	1.2	18.7	MHQ, HQ, ∑ 7.1%



Method 1: Amount of soluble lignin (by assay, % reduction)

Method 2: Total products extracted into EtOAc (w/w %)

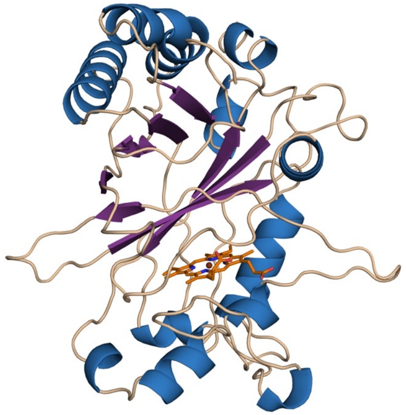
Method 3: Yield of individual products by LC-MS (calibrated vs standards, total %)

G.M.M. Rashid and T.D.H. Bugg, *Catalysis Sci. Technol*. **11**, 3568-3577 (2021)

Bacterial Enzymes for Lignin Conversion

Dye decolorizing peroxidase

Rhodococcus jostii, Pseudomonas

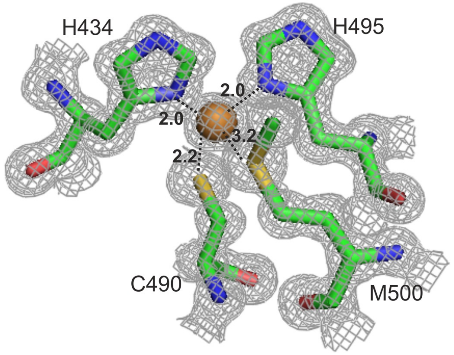


Heme Fe enzyme
Oxidant H_2O_2

Able to oxidise lignin model cpds and Mn(II)
$C\alpha$-$C\beta$ cleavage

Multicopper oxidase

Streptomyces coelicolor, Ochrobactrum sp.

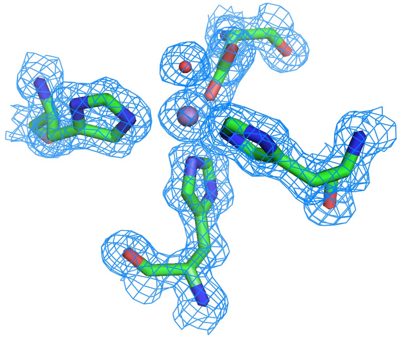


Multi-copper enzyme
Oxidant O_2

Able to oxidise wide range of phenols, using redox mediator

Manganese superoxide dismutase

Sphingobacterium sp. T2

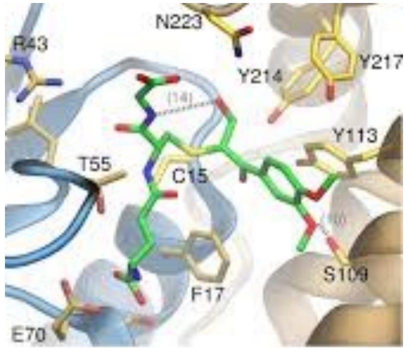


Mononuclear Mn
Generates HO radical

Oxidises and demethylates polymeric lignin

Beta-etherase

Sphingobium SYK-6, Novosphingobium

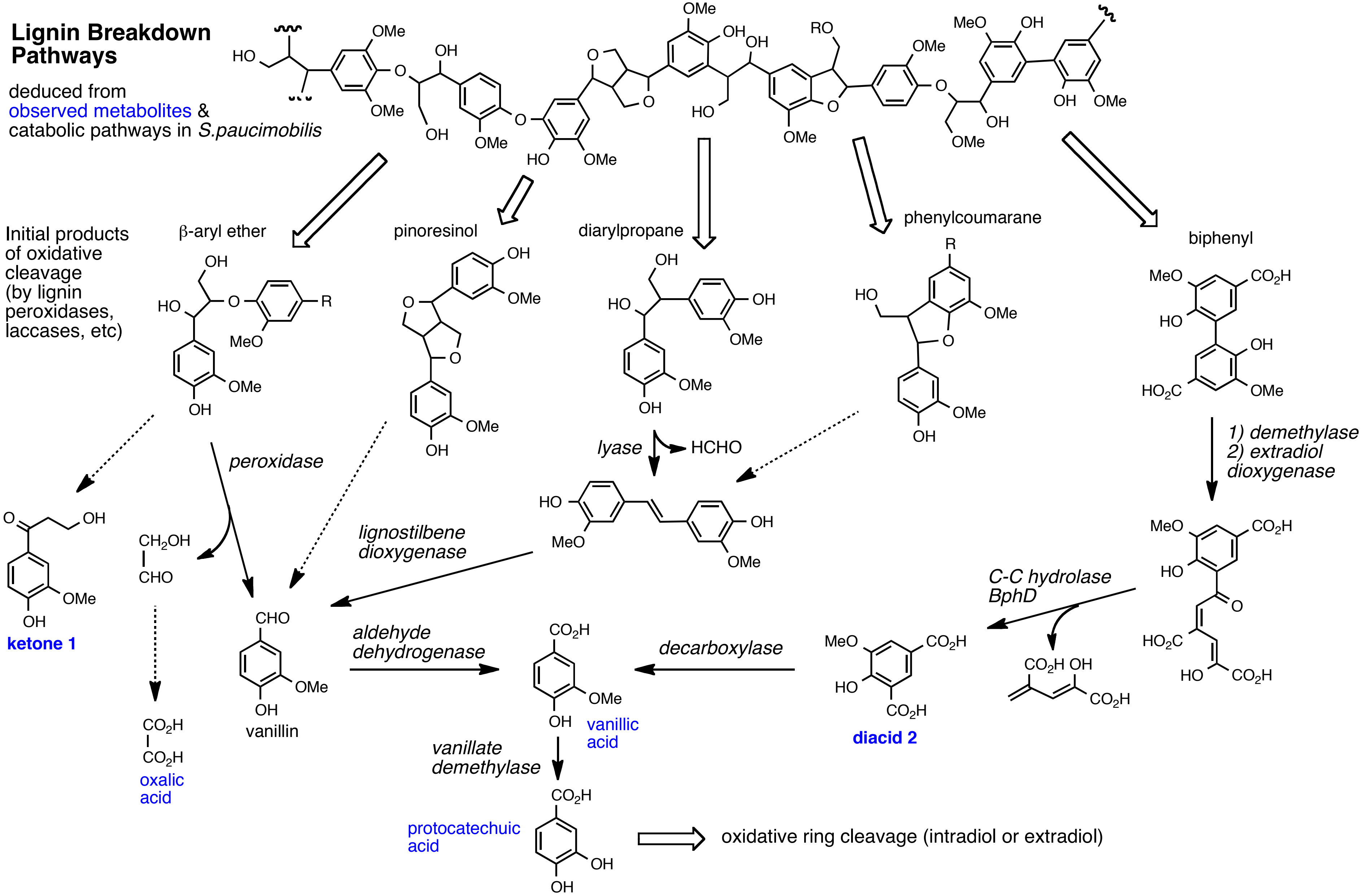


Reduced glutathione cosubstrate

Hydrolytic cleavage of β-aryl ether dimers and lignin oligomers

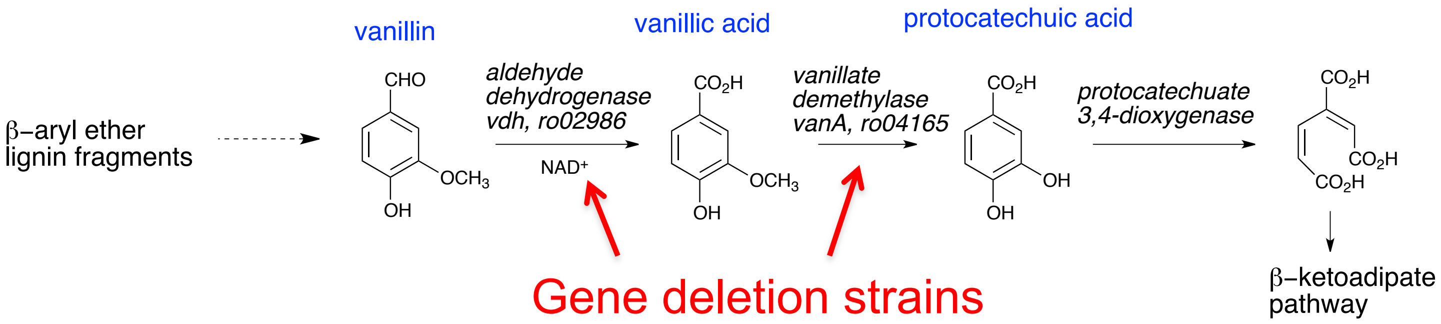
Lignin Breakdown Pathways

deduced from observed metabolites & catabolic pathways in *S.paucimobilis*

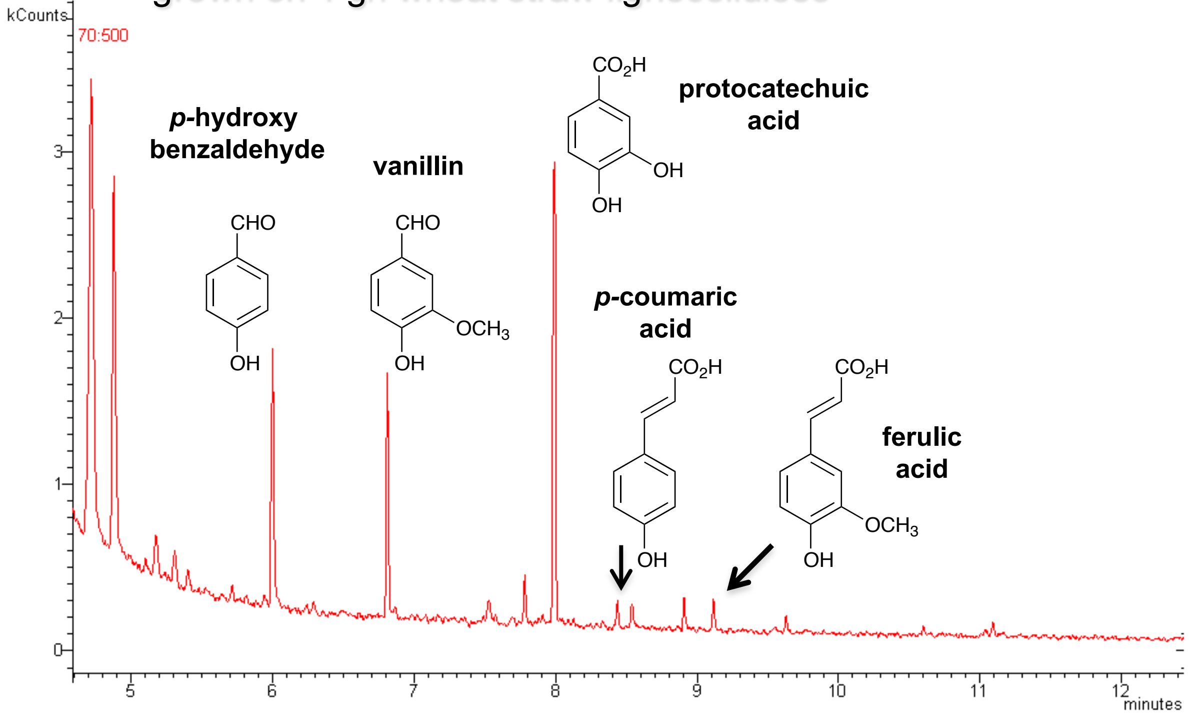


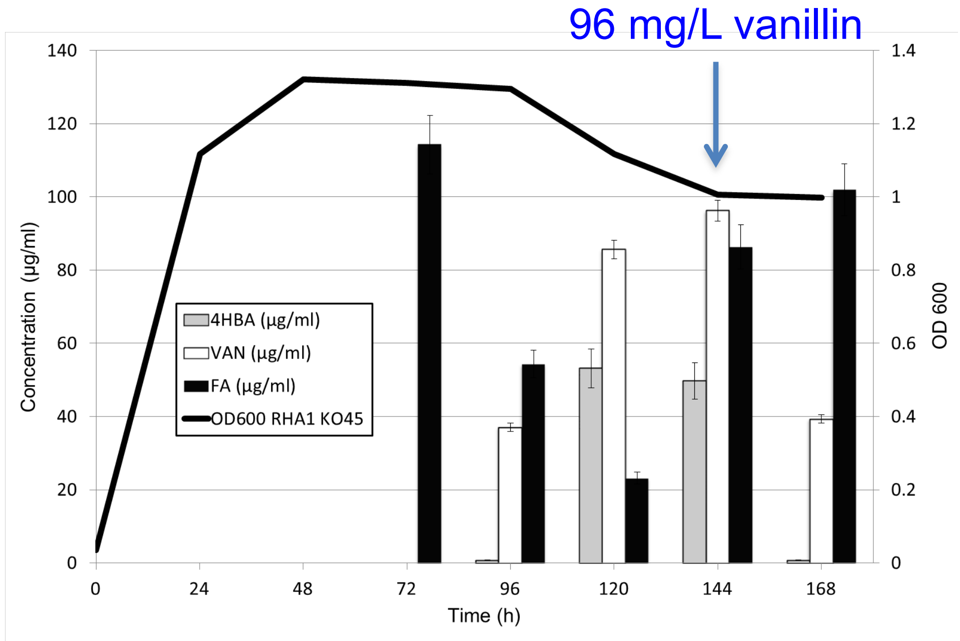
Can these pathways be manipulated via gene knockouts or enzyme inhibition?

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

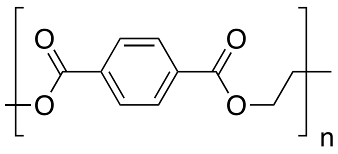




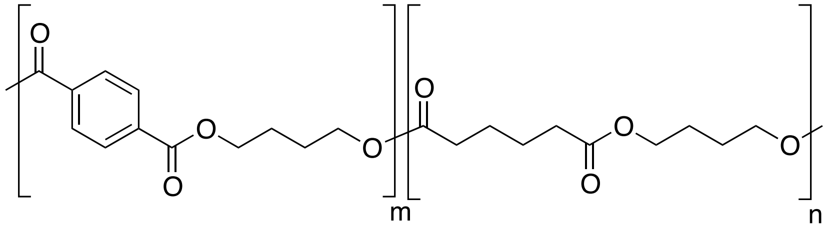
P.D. Sainsbury et al,
ACS Chem Biol **2013**, *8*, 2151-2156.

Generation of Aromatic Dicarboxylic Acids from Microbial Lignin Breakdown

PET (polyethylene terephthalate)

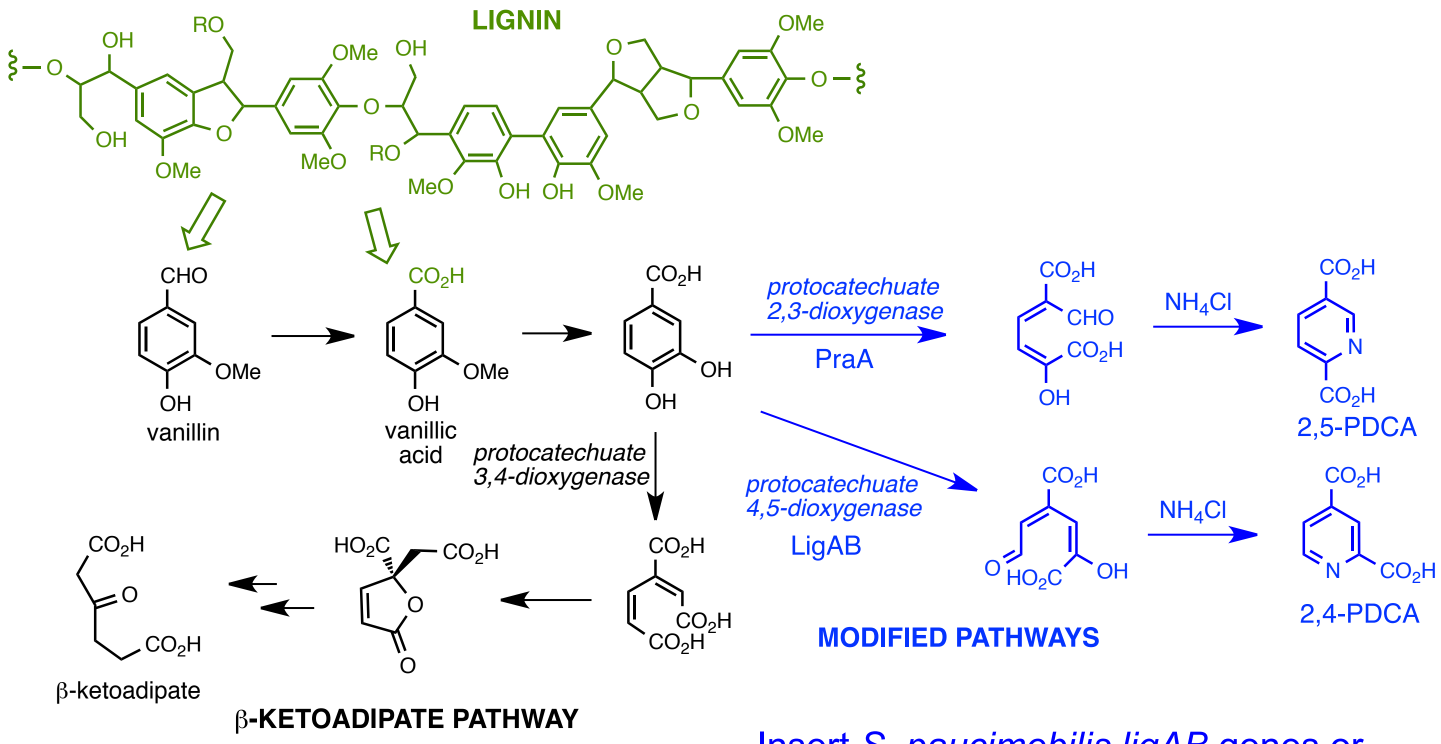


PBAT (polybutyrate adipate terephthalate)

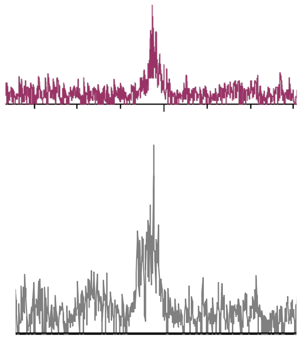


with Biome Bioplastics Ltd

biome bioplastics

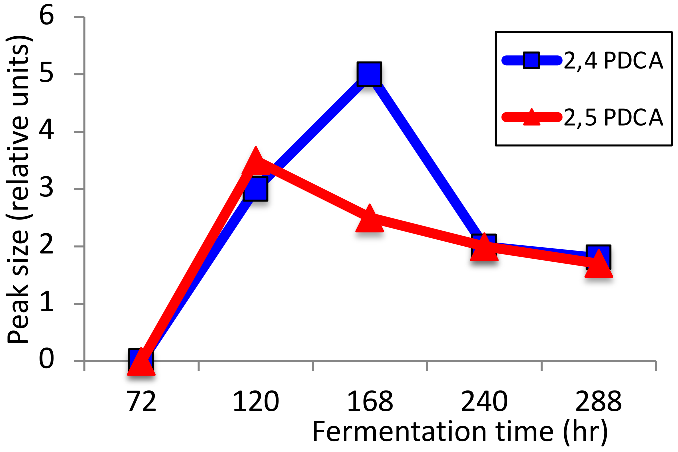


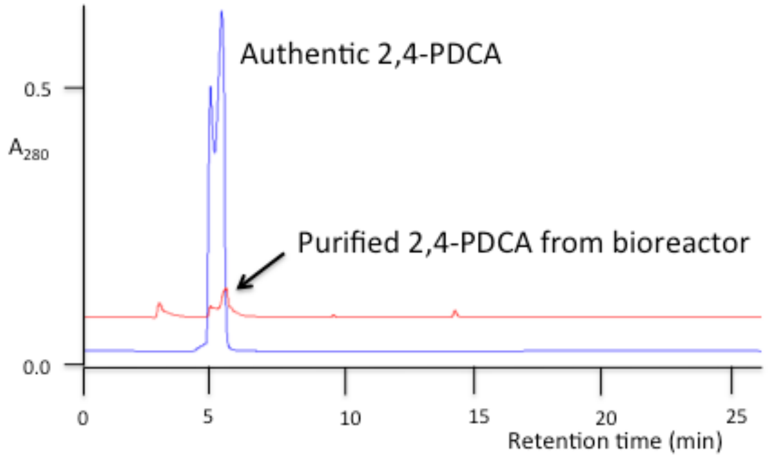
Identification by LC-MS from M9/1% wheat straw:



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)

Z. Mycroft et al, *Green Chemistry* **2015**, *17*, 4974-4979.

Genetic modification of *Rhodococcus jostii* RHA1 to produce pyridine-dicarboxylic acids

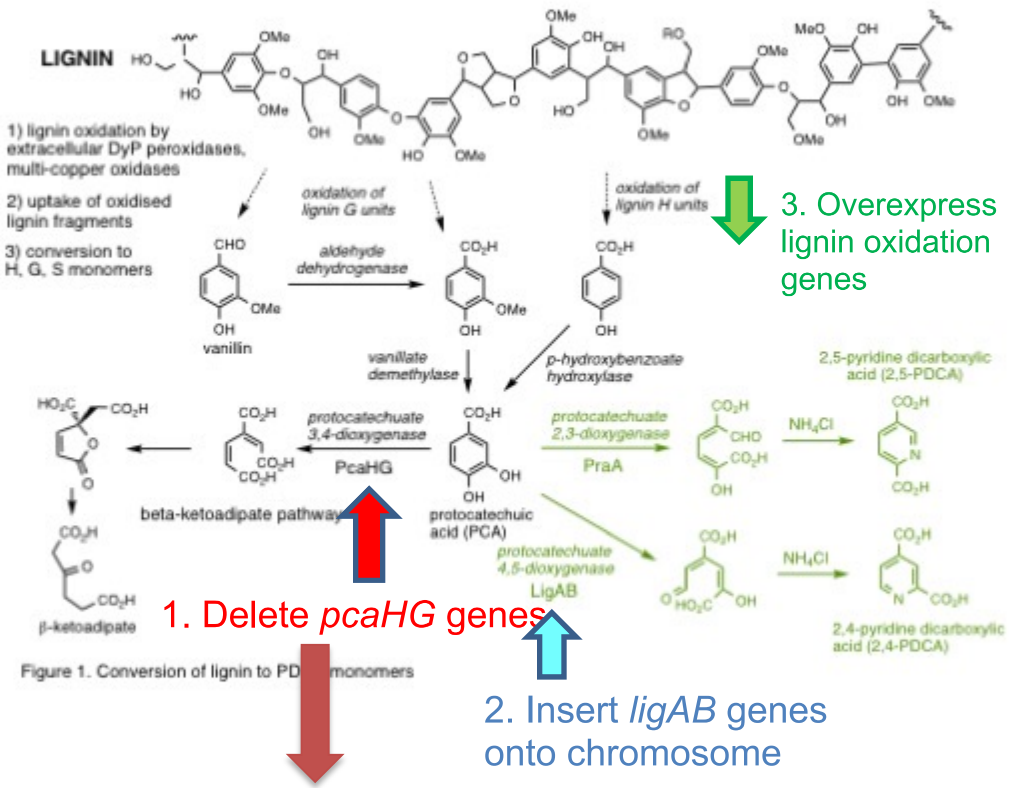
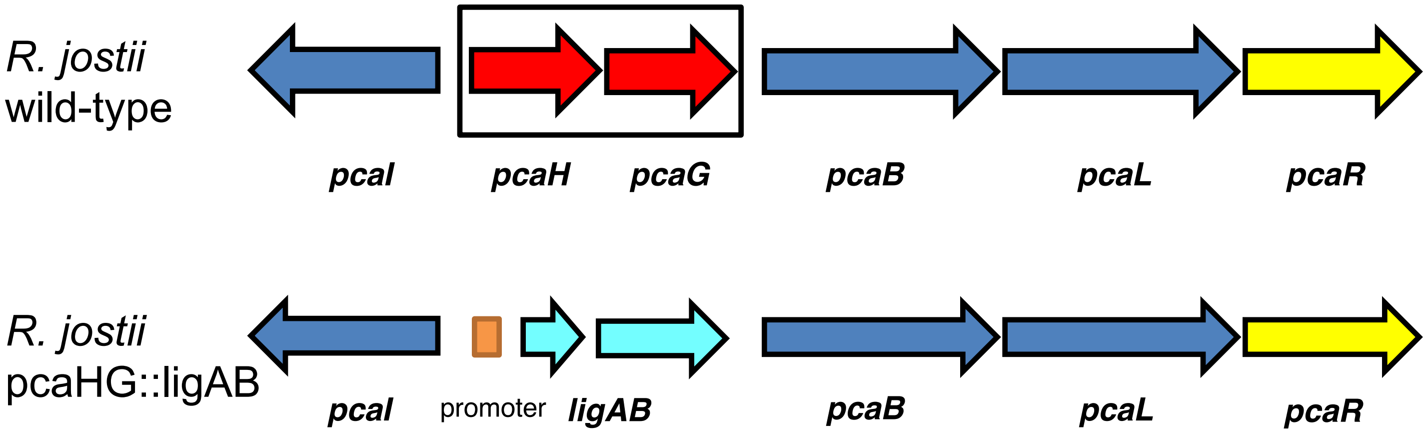


Figure 1. Conversion of lignin to PDCA monomers

2. Integration of *ligAB* genes onto chromosome:



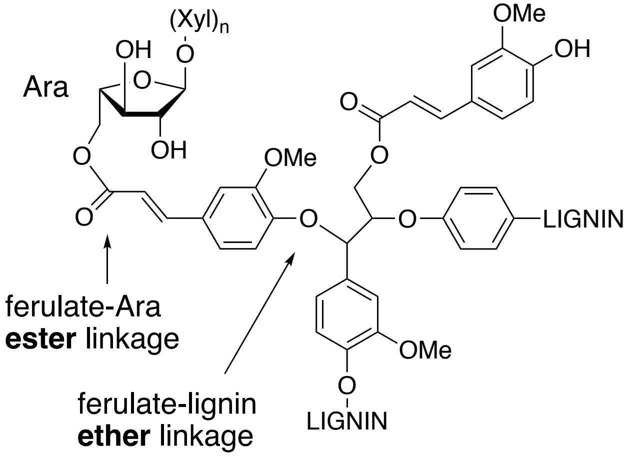
1. Deletion of *pcaHG* improves PDCA titre by 2-3 fold:
ΔpcaHG pTipQC2-ligAB 200 mg/L 2,4-PDCA
ΔpcaHG pTipQC2-praA 287 mg/L 2,5-PDCA

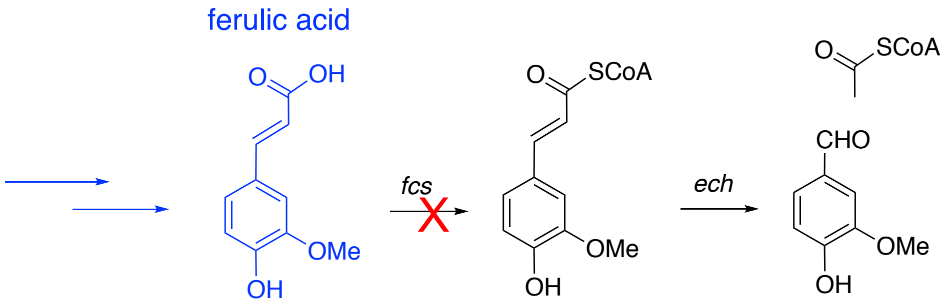
Promoter	Type	Inducer	PDCA titre 1% wheat straw	PDCA titre 1% Green Value lignin
PicI	Inducible	methanol	ND	70 mg/L
PnitA	Inducible	ε-caprolactam	79 mg/L	100 mg/L
Ptpc5	constitutive	-	290 mg/L	164 mg/L
		with pTipQC2-dyp2	*330 mg/L*	*240 mg/L*

3. Overexpression of *Amycolatopsis dyp2* gives enhanced PDCA t

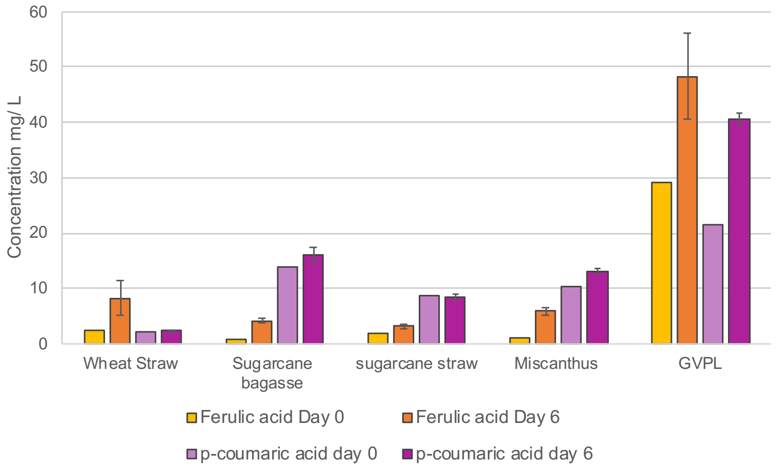
E.M. Spence, L. Calvo-Bado, P. Mines, and T.D.H. Bugg, *Microb. Cell Fact.*, **20**, article 15 (2021).

Generation of substituted styrenes via ferulic acid via metabolic engineering

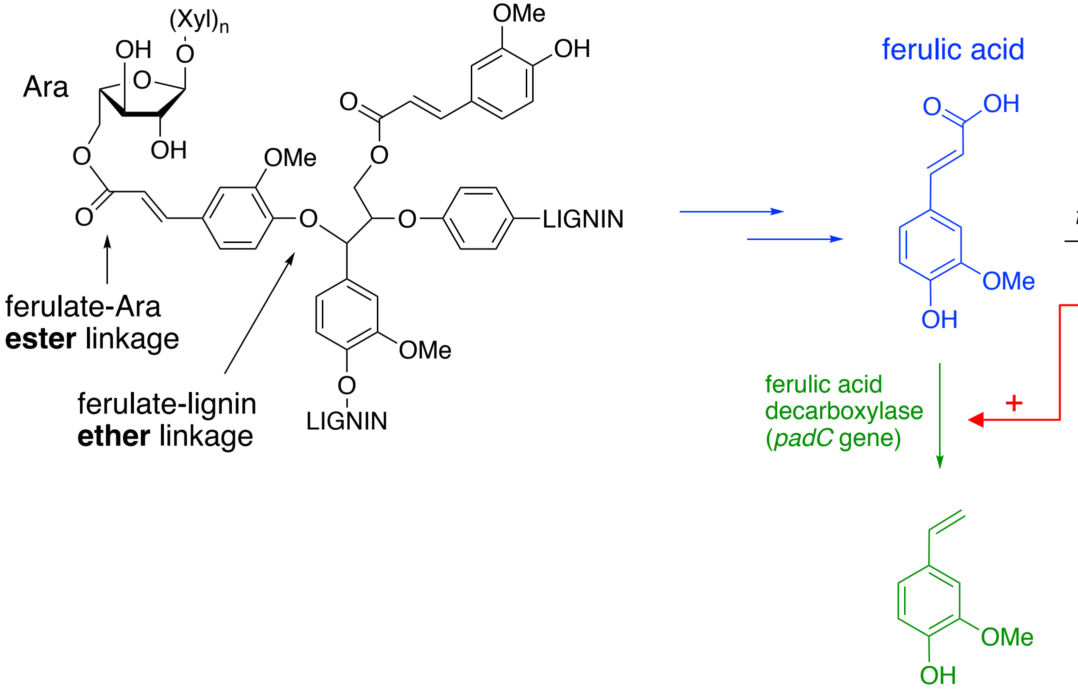


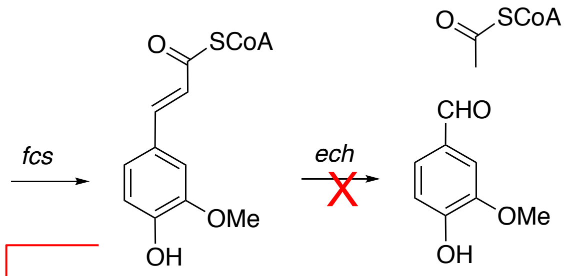


P. putida fcs or *ech* gene deletion mutants accumulate 10-50 mg/L ferulic acid or p-coumaric acid from lignocellulose or soda lignin feedstocks.



Generation of substituted styrenes via ferulic acid via metabolic engineering





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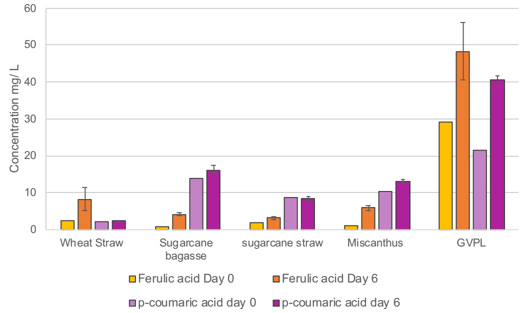
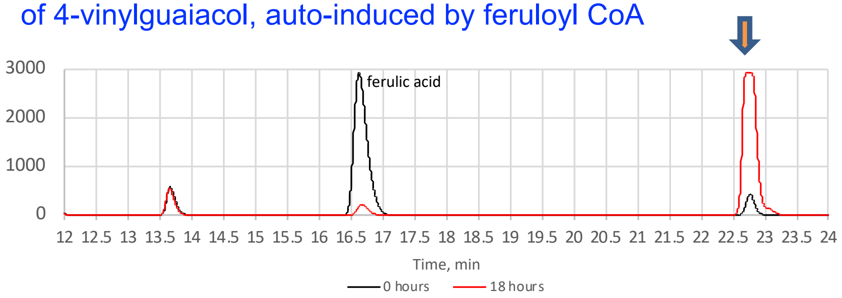
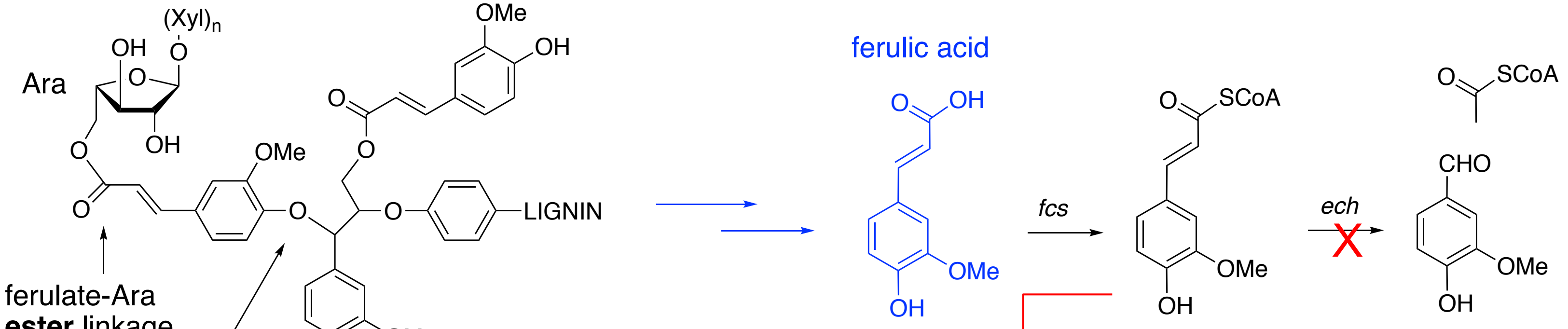


4-vinylguaiacol

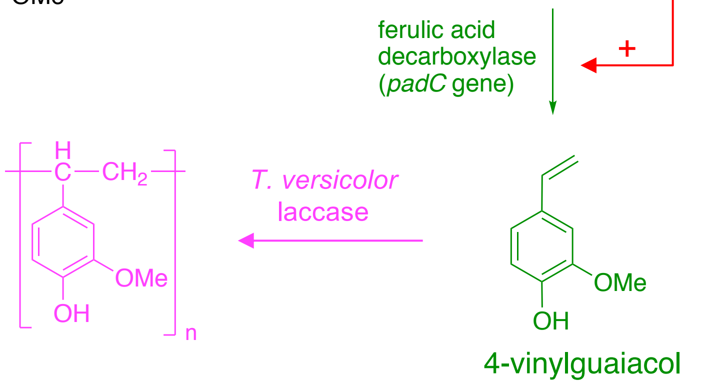
Replacement of *ech* by padC leads to formation of 4-vinylguaiacol, auto-induced by feruloyl CoA

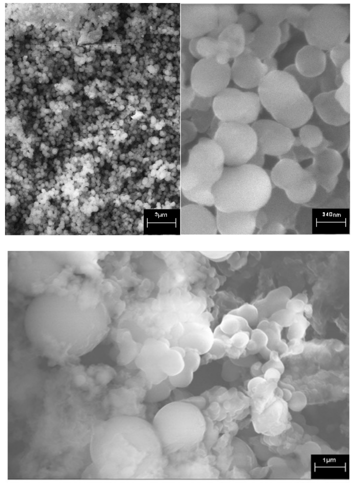


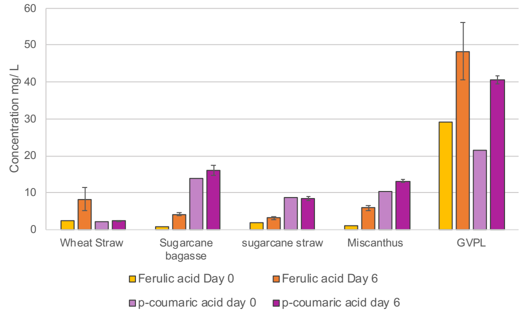
Generation of substituted styrenes via ferulic acid via metabolic engineering



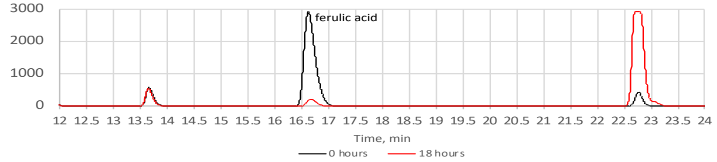
P. putida fcs or *ech* gene deletion mutants accumulate 10-50 mg/L ferulic acid or p-coumaric acid
from lignocellulose or soda lignin feedstocks.





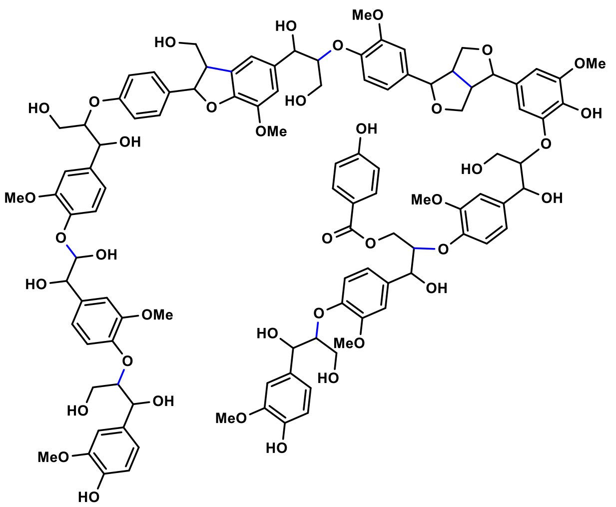


Replacement of *ech* by padC leads to formation of 4-vinylguaiacol, auto-induced by feruloyl CoA

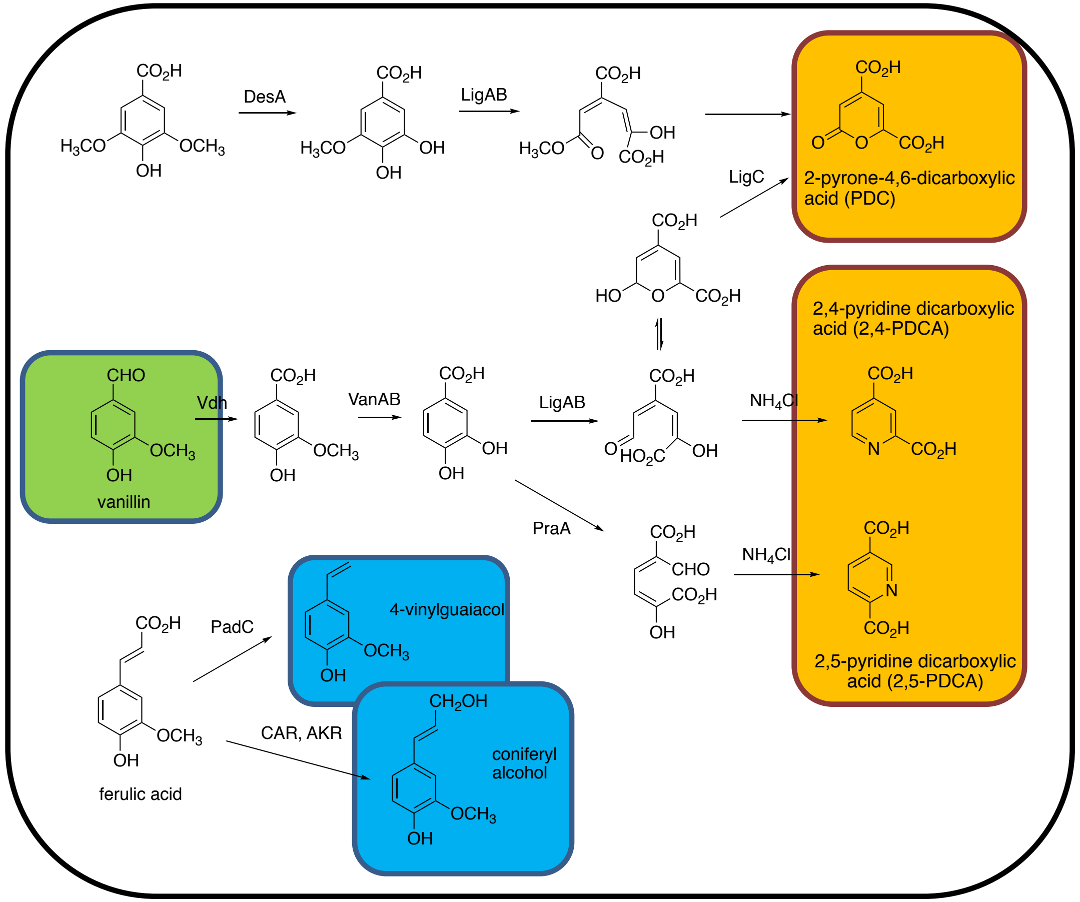


J.J. Williamson, N. Bahrin, E.M. Hardiman, and T.D.H. Bugg, *Biotechnol. J.* **2020**, *15*, 1900571.

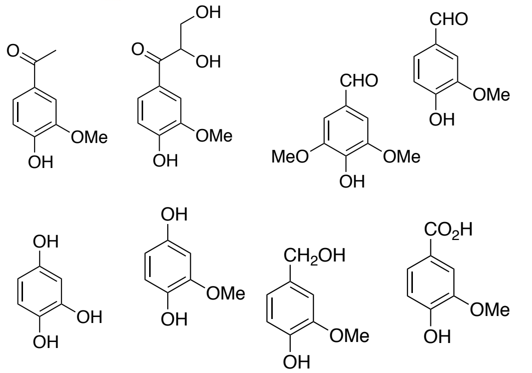
Two Strategies for Biocatalytic Lignin Conversion to Low Molecular Weight Products



Microbial transformation by engineered microbe



In vitro conversion by lignin-oxidizing enzymes & accessory enzymes



- Mixtures of products
- Lignin repolymerisation

- Choice of microbial host, genetic tools
- Competing pathways, understanding of metabolism

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