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**Identification of an Extracellular Bacterial Flavoenzyme that can Prevent
Re-polymerisation of Lignin Fragments**

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Abstract. A significant problem in the oxidative breakdown of lignin is the tendency of phenolic radical fragments to re-polymerise to form higher molecular weight species. In this paper we identify an extracellular flavin-dependent dihydrolipoamide dehydrogenase from *Thermobifida fusca* that prevents oxidative dimerization of a dimeric lignin model compound, which could be used as an accessory enzyme for lignin depolymerisation.

Keywords: lignin degradation; dihydrolipoamide dehydrogenase; *Thermobifida fusca*.

Introduction

The aromatic heteropolymer lignin is a major component of plant lignocellulose cell walls, and is liberated during cellulosic bioethanol production, and pulp/paper manufacture. The valorisation of lignin to produce aromatic chemicals is an important problem in the design of a future biorefinery for production of fuels and chemicals from renewable sources [1], and several new approaches involving either chemocatalysis [2-6] or biocatalysis [7-10] have been published in recent years.

One significant technical obstacle in the valorisation of lignin is the tendency of lignin fragments formed during oxidative cleavage to “repolymerise” or “condense” to form higher molecular weight species, via polymerisation of radical intermediates formed during lignin breakdown [11]. This problem has been encountered using fungal lignin peroxidase [12-14] and laccase enzymes [15,16] and with bacterial Dyp peroxidase [17] and laccase [18] enzymes, and is also encountered in chemocatalytic valorisation of lignin, where a high molecular weight char is observed alongside depolymerised products [1]. Kirk & Farrell commented on this problem in their review of microbial lignin oxidation in 1987, noting that polymerisation of lignin is not prominent *in vivo*, therefore they suggested that phenoxy radical intermediates “are reduced back to the phenols by an undiscovered enzyme and/or mechanism that prevents polymerization” [19]. In basidiomycete *Pleurotus ostreatus*, a FAD-dependent aryl alcohol oxidase was shown in 1995 to reduce quinonoids and phenolic radicals, and to prevent polymerisation of laccase-generated phenolic compounds [20], but no further progress has been made to prevent lignin repolymerisation enzymatically, and no such enzyme has been identified in bacteria that can oxidise lignin.

In previous studies with bacterial *Rhodococcus jostii* peroxidase DypB, we found that addition of *C. kluyveri* diaphorase changed the distribution of products obtained upon conversion of a lignin model compound by DypB [17], consistent with an enzymatic single-electron reduction of phenolic radical intermediates by a reduced flavin coenzyme. We noted that in a proteomic study of thermophilic cellulose degrader *Thermobifida fusca* by Adav *et al* [21], the most abundant extracellular protein was found to be a dihydrolipoamide dehydrogenase (accession Q47R85), one of four dihydrolipoamide dehydrogenase sequences in the *T. fusca* genome (see Supplemental Figure S1). We therefore hypothesised that this extracellular flavin-dependent enzyme might be responsible for one-electron reduction of phenolic radical intermediates during lignin breakdown. We report here evidence that this enzyme can prevent lignin repolymerisation.

Materials and Methods

Cloning of T. fusca dihydrolipoamide dehydrogenase gene.

The thermophile *Thermobifida fusca* YX (DSMZ) was grown in Luria-Bertani broth overnight at 45 °C. Genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega). Forward (5' CACC GTG AGT GAA AGC GGC GGC ACA TTC 3') and Reverse primers (5' TCA GTC GTG GAC GTG CAA CGG C 3') for the DLDH gene were designed with the intention of cloning into a pET151/D-TOPO vector. The gene was amplified from genomic DNA by polymerase chain reaction using Pfx Taq polymerase, using 34 cycles of: 30 sec at 94 °C, 30 sec at 58 °C; 90 sec at 72 °C. The PCR product (1.4 kb) was excised from a 1% agarose gel and extracted using a GeneJET PCR Purification Kit (Thermo Scientific), and cloned into a pET151/D-TOPO vector (Invitrogen) using manufacturer's instructions, and transformed into One Shot TOP10 competent cells (Invitrogen). The DNA sequence was confirmed via DNA sequencing.

Overexpression of recombinant T. fusca dihydrolipoamide dehydrogenase.

A 1L culture of *T. fusca* DLDH pET151/TOP10 in Luria-Bertani broth containing 50 µg/ml ampicillin was grown at 37 °C for 2-3 hours. At OD₅₉₅ = 0.6, riboflavin (0.5 mM) and IPTG (0.5 mM) were added to the culture, which was then incubated in a shaker overnight at 15 °C at 200 rpm. After centrifugation at 6000 g, the cell pellet was re-suspended in 10ml of lysis buffer (50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 10mM imidazole). Cells were lysed using a cell disruptor at 21 kpsi. The cell lysate was then centrifuged at 13,000 g for 40 minutes. DLDH was then purified using nickel affinity chromatography. The supernatant was incubated with 2ml of Ni²⁺ Sepharose beads at 4 °C for 60 minutes. This cell supernatant-Ni²⁺-Sepharose mixture was then placed in disposable plastic column and the resin washed with 50ml of wash buffer (50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 20 mM imidazole), then DLDH was eluted with 7ml of elution buffer (50mM NaH₂PO₄ pH 8.0, 300mM NaCl and 250 mM imidazole). Fractions were collected and analysed by SDS-PAGE gel electrophoresis. Fractions containing DLDH (48 kDa band) were pooled and incubated overnight at room temperature with TEV protease (1mg/ml) in order to remove the His tag. The DLDH-TEV mixture was centrifuged at 13,000 g for 15 minutes, and the supernatant was concentrated using a 10 kDa Millipore Centricon. Native DLDH was purified by passing the solution through a 5ml HisTrap Ni²⁺ resin FPLC column, then desalted using a PD10 desalting column.

Dihydrolipoamide dehydrogenase assay.

All assays were run in 50mM sodium phosphate buffer pH 6.0 at 25 °C, in the presence of 0.2 mM NADH. Enzyme kinetics were measured through the decrease in the absorption spectrum of NADH at 340nm over 2 minutes, as previously described by Youn *et al* [22].

HPLC Assays:

Catalytic action of DHLDH toward oxidized lignin model compound was studied by reaction of model compound with *Pseudomonas fluorescens* Dyp1B enzyme in presence of *T. fusca* DHLDH enzyme and NADH. The reaction was performed in 50mM phosphate buffer pH 6.0 at 25 °C in presence of 0.4 mM model compound, 25-200 μ M NADH, 560 nM Dyp1B, 210 nM DHLDH and 0.5 mM hydrogen peroxide. The following reactions were set up: 1) model compound + buffer + H₂O₂; 2) model compound + Dyp1B + H₂O₂; model compound + Dyp1B + H₂O₂ + NADH; 3) model compound + Dyp1B + H₂O₂ + NADH + DHLDH. The solutions were incubated at 25 °C for 30 minutes. Aliquots (500 μ L) were taken and these fractions were analyzed by HPLC. Aliquots for HPLC were mixed with CCl₃COOH (100%, w/v, 50 μ L), and the solution was then centrifuged for 10 min at 10000 rpm. HPLC analysis was conducted using a Phenomenex Luna 5 μ m C18 reverse phase column (100 Å, 50 mm, 4.6 mm) on a Hewlett-Packard Series 1100 analyzer, at a flow rate of 0.5 mL/min, with monitoring at 270 nm. The gradient was as follows: 50% MeOH/H₂O for 5 min; 50-80% MeOH/H₂O from 5 to 12 min; 80% MeOH/H₂O from 12 to 25 min; 80-30% MeOH/H₂O from 25 to 30 min. Peaks of interest were collected for further analysis by mass spectrometry and MS-MS fragmentation.

Results

T. fusca dihydrolipoamide dehydrogenase was cloned from genomic DNA, and was expressed as a His₆ fusion protein from a pET151 vector in *Escherichia coli*. The recombinant protein expressed well as a 48 kDa protein, was purified by metal affinity chromatography, and the fusion tag removed by digestion with TEV protease (see Supplemental Figure S2). Addition of riboflavin to the growth media was found to yield recombinant enzyme with stoichiometric amounts of flavin cofactor (λ_{max} 460 nm, see Supplemental Figure S3). The recombinant enzyme was catalytically active for NADH-dependent reduction of lipoic acid, with k_{cat} 0.14 s⁻¹ and K_{m} 430 μ M, and showed maximal activity at pH 6.0. The enzyme also showed NADH-dependent quinone reductase activity with *p*-benzoquinone (k_{cat} 0.42 s⁻¹ K_{m} 540 μ M, see Supplemental Figures S4-S6), comparable with activity reported for dihydrolipoamide dehydrogenase from *Streptomyces seoulensis* [22].

We have previously reported that Dyp-type peroxidase DypB from *Rhodococcus jostii* RHA1 is able to oxidatively cleave β -aryl ether lignin model compound **1** [17]. Treatment of **1** with peroxidase Dyp1B from *Pseudomonas fluorescens* [23] or *Thermobifida fusca* Dyp peroxidase [24] leads to the formation of a new peak observed by C₁₈ reverse phase HPLC at retention time 15.5 min (see Figure 1, trace B). Analysis of this peak by electrospray mass spectrometry gave *m/z* 660.4, corresponding to an oxidative dimer of **1**, and further analysis of this species by MS-MS

fragmentation has identified this species as dimer **2** arising from oxidative dimerization of **1** [24]. The formation of compound **2** therefore represented a convenient model for oxidative repolymerisation of lignin fragments.

Addition of 210 nM *T. fusca* dihydrolipoamide dehydrogenase (DHLDH) and 100 μ M NADH to an incubation of 1.5 mM β -aryl ether **1** with *P. fluorescens* Dyp1B was found to completely prevent the formation of **2** (see Figure 1 trace E, and Supplemental Figure S7), whereas addition of NADH alone had no effect (trace B). Treatment of either the oxidised product **2** or lignin model compound **1** with *T. fusca* DHLDH gave no change as observed by HPLC, indicating that DHLDH acted upon an intermediate species formed during the oxidation of **1** to **2**. The disappearance of product **2** was found to be dependent upon the concentration of NADH, showing partial disappearance of **2** at 25 and 50 μ M NADH (traces C,D). Since the dimer **2** is formed in approximately 10% yield from **1** (trace A), and prevention of dimerization occurs at 50-100 μ M NADH, the stoichiometry of NADH required to prevent formation of **2** is in the range 1-2 nmoles NADH/nmole of dimer **2**.

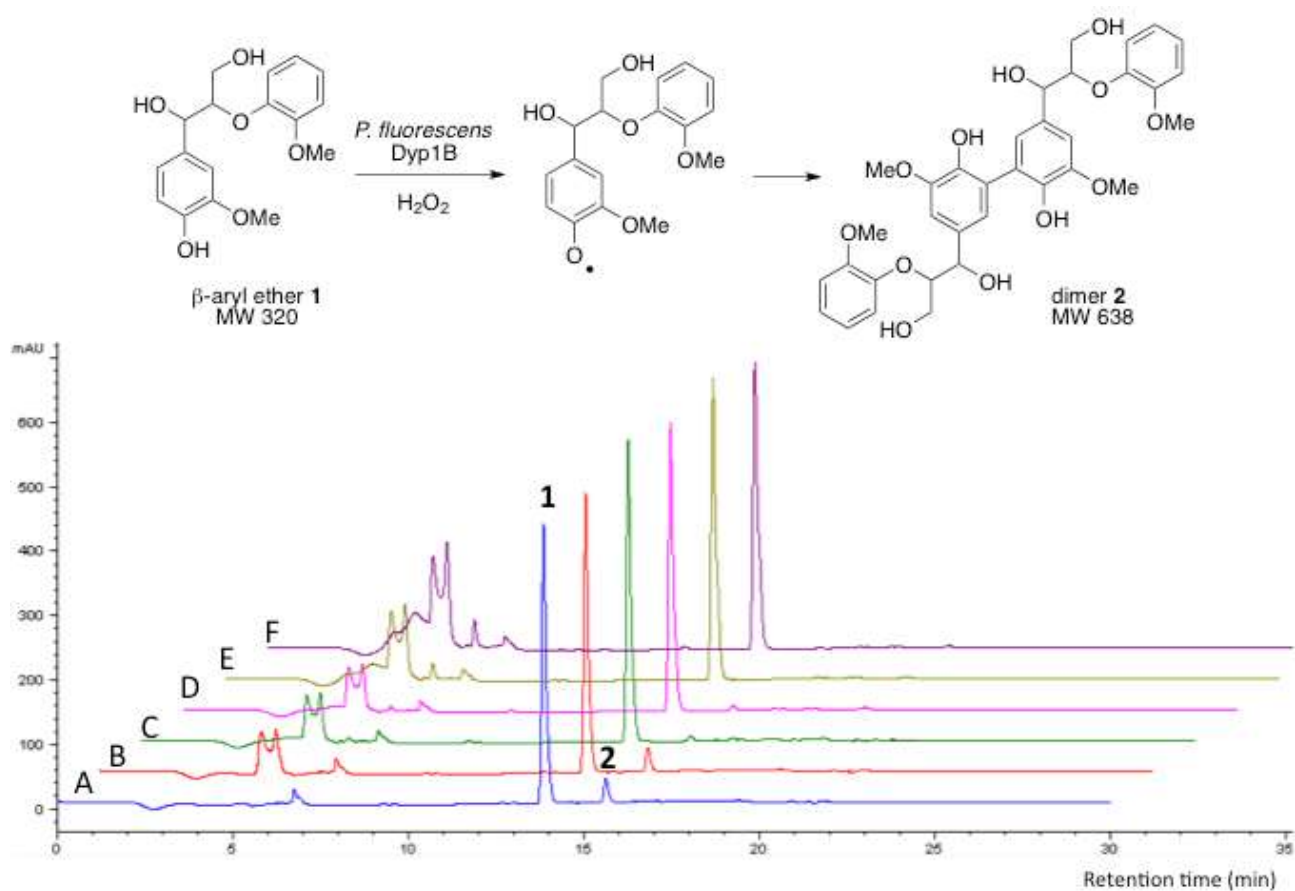


Figure 1. HPLC traces for conversion of **1** to **2**. A, model compound, Dyp1B, DHLDH and H₂O₂ (no NADH); B, model compound, Dyp1B, H₂O₂ and NADH (no DHLDH); C-F, addition to trace A of 25 μM (C), 50 μM (D), 100 μM (E) or 200 μM (F) NADH.

The formation of **2** from **1** could be rationalised by dimerization of a phenoxy radical formed from **1**. In support of this mechanism, the conversion of **1** to **2** was also observed upon addition of nitroxyl radical reagent TEMPO to **1**. The catalytic mechanism of dihydrolipoamide dehydrogenase is known to proceed via reduction of an active site disulfide cysteine pair by reduced flavin [25,26]. We therefore propose the catalytic cycle shown in Figure 2, where the reduced form of the active site disulfide reduces a phenoxy radical intermediate via a 1-electron transfer, to generate a cysteine radical intermediate. We have not observed any flavin semiquinone intermediate by pre-steady state kinetic analysis of *T. fusca* DHLDH, therefore we propose a further 1-electron transfer to a phenoxy radical, generating the oxidised active site disulfide, which is reduced by FADH₂, generating FAD which is in turn reduced by NADH.

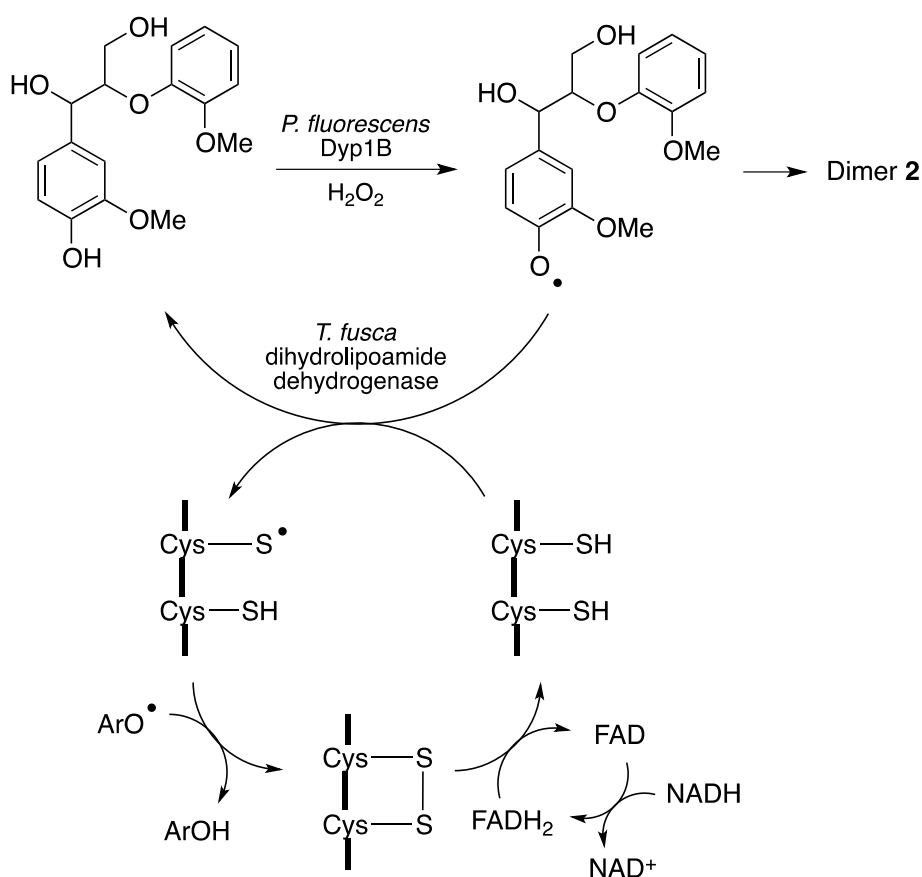


Figure 2. Catalytic cycle for reduction of phenoxy radicals by *T. fusca* dihydrolipoamide dehydrogenase, via an active site disulfide intermediate.

Preventing repolymerisation of phenoxy radical intermediates could therefore promote the formation of low molecular weight products from lignin breakdown. The processing of wheat straw

organosolv lignin by *P. fluorescens* Dyp1B and hydrogen peroxide was investigated in the presence and absence of *T. fusca* DHLDH: in the absence of DHLDH, no small molecule products were observed by C₁₈ reverse phase HPLC (see Figure 3B), but in the presence of *T. fusca* DHLDH and NADH, two new product peaks were observed (see Figure 3D). The larger product peak was identified by electrospray mass spectrometry (*m/z* 169.0495) and MS-MS fragmentation (see Supplemental Figure S8) as 3-methoxy-4,5-dihydroxybenzaldehyde (**4**). Previously we have found that treatment of wheat straw lignocellulose with PfDyp1B generates dimer lignin dimer **3** [23], hence the formation of **4** could be rationalised by oxidative C_α-C_β cleavage of **3**, known to be catalysed by *R. jostii* DypB [17], and phenolic hydroxylation at position 5.

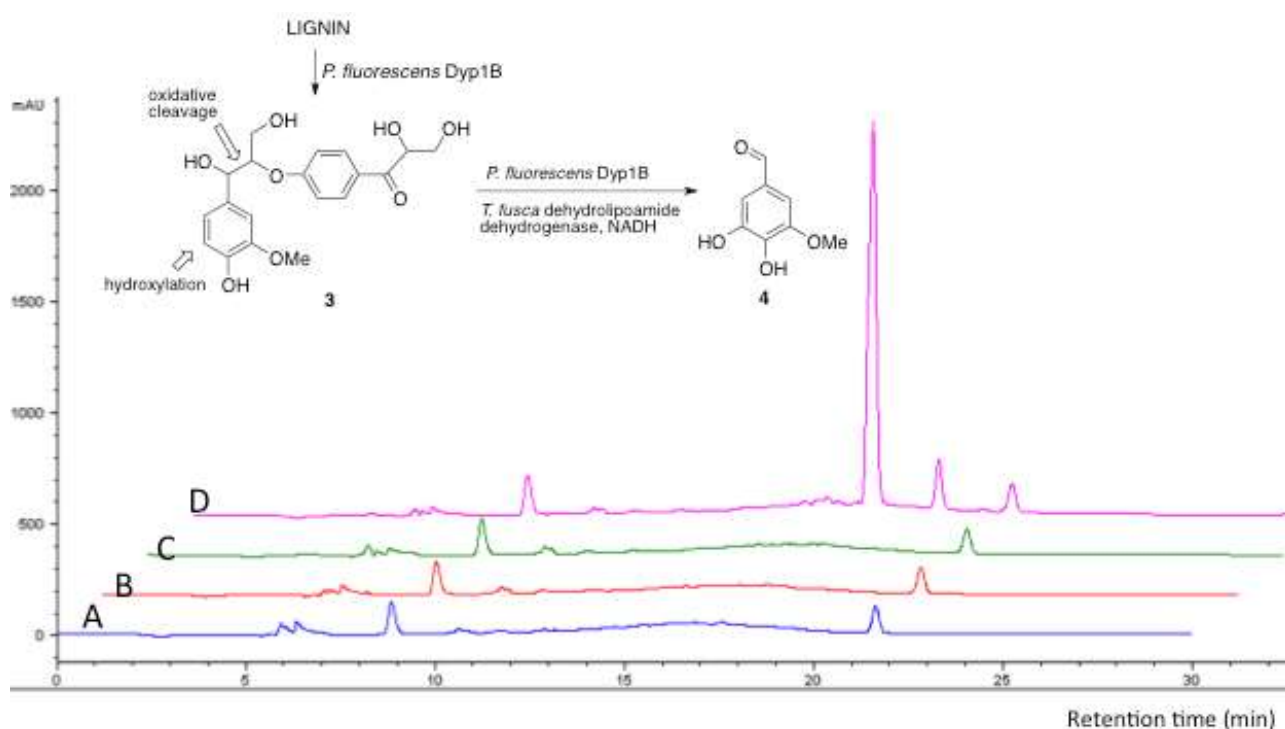


Figure 3. Processing of wheat straw organosolv lignin by *P. fluorescens* Dyp1B and *T. fusca* dihydrolipoamide dehydrogenase and NADH (trace D). A, organosolv lignin only; B, addition to A of Dyp1B and H₂O₂; C, addition to A of DHLDH and NADH.

Discussion

The results in this paper verify that *T. fusca* dihydrolipoamide dehydrogenase is able to prevent lignin repolymerisation *in vitro*, and aid the formation of low molecular weight aromatic products, fulfilling the prediction by Kirk & Farrell in 1987 [19]. Dihydrolipoamide dehydrogenase has also been implicated in reducing cellular nitric oxide [27], iron [28], and tellurite [29]. Although dihydrolipoamide dehydrogenase is normally an intracellular enzyme [22,25,26] there are reports of cell surface dihydrolipoamide dehydrogenase enzyme activities in *Pseudomonas aeruginosa* [30],

Neisseria meningitidis [31] and *Synechocystis* PCC6803 [32], and this enzyme has been detected in the extracellular proteome of *Xanthomonas campestris* [33], and in extracellular vesicles of *Streptomyces coelicolor* M110 [34].

The source of reducing equivalents *in vivo* on the cell surface for extracellular dihydrolipoamide dehydrogenase is uncertain, since NADH is an intracellular metabolite, but the flavin cofactor could be reduced via electron transfer, for example by thioredoxin disulphide reductase, which is known to transfer redox equivalents across the cell membrane for the maintenance of extracellular protein disulphide linkages [35]. Alternatively, dihydrolipoamide dehydrogenase could be involved in a redox cycling mechanism, as used by brown rot fungi to aid lignocellulose breakdown [36].

This observation suggests that an extracellular dihydrolipoamide dehydrogenase might fulfill a similar role as an accessory enzyme for lignin degradation to aryl alcohol oxidase in *Pleurotus ostreatus* [20], whose catalytic mechanism has been investigated subsequently in more detail [37]. Cellobiose dehydrogenase has also been suggested to fulfill this role in white-rot fungi [38]. These enzymes could be valuable biocatalysts for *in vitro* enzymatic conversion of lignin to aromatic products via biocatalysis.

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References

1. J. Zakzeski, P.C. Bruijninx, A.L. Jongerijs, B.M. Weckhuysen, The catalytic valorization of lignin for the production of renewable chemicals. *Chem. Rev.* 110 (2010) 3552–3599.
2. A. Rahimi, A. Azarpira, H. Kim, J. Ralph, S.S. Stahl, Chemoselective metal-free aerobic alcohol oxidation in lignin. *J. Am. Chem. Soc.* 135 (2013) 6415-6418.
3. A. Rahimi, A. Ulbrich, J.J. Coon, S.S. Stahl, Formic acid-induced depolymerization of oxidised lignin to aromatics. *Nature* 515 (2014) 249-252.
4. M.R. Sturgeon, M.H. O'Brien, P.N. Ciesielski, R. Katahira, J.S. Kruger, S.C. Chmely, J. Hamlin, K. Lawrence, G.B. Hunsinger, T.D. Foust, R.M. Baldwin, M.J. Bidy, G.T. Beckham Lignin depolymerisation by nickel supported double hydroxide catalysts. *Green Chem.* 16 (2014) 824-835.
5. C.S. Lancefield, O.S. Ojo, F. Tran, N.J. Westwood, Isolation of functionalized phenolic monomers through selective oxidation and C-O bond cleavage of the β -O-4 linkages in lignin. *Angew. Chem.* 127 (2015) 260-264.

6. P.J. Deuss, M. Scott, F. Tran, N.J. Westwood, J.G. de Vries, K. Barta, Aromatic monomers by in situ conversion of reactive intermediates in the acid-catalyzed depolymerization of lignin. *J. Am. Chem. Soc.* 137 (2015) 7456-7467.
7. P.D. Sainsbury, E.M. Hardiman, M. Ahmad, H. Otani, N. Seghezzi, L.D. Eltis, T.D.H. Bugg, Breaking down lignin to high-value chemicals: the conversion of lignocellulose to vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1. *ACS Chem. Biol.* 8, (2013) 2151-2156.
8. J.G. Linger, D.R. Vardo, M.T. Guarneri, E.M. Karp, G.B. Hunsinger, M.A. Franden, C.W. Johnson, G. Chupka, T.J. Strathmann, P.T. Pienkos, G.T. Beckham, Lignin valorization through integrated biological funneling and chemical catalysis. *Proc. Natl. Acad. Sci. USA* 111, (2014) 12013-12018.
9. D.R. Vardon, M.A. Franden, C.W. Johnson, E.M. Karp, M.T. Guarneri, J.G. Linger, M.J. Salm, T.J. Strathman, G.T. Beckham, Adipic acid production from lignin. *Energy Environ. Sci.* 8, (2015) 617-628.
10. Z. Mycroft, M. Gomis, P. Mines, P. Law, T.D.H. Bugg, Biocatalytic conversion of lignin to aromatic dicarboxylic acids in *Rhodococcus jostii* RHA1 by re-routing aromatic degradation pathways. *Green Chem.* 17 (2015) 4974-4979.
11. T.D.H. Bugg, R. Rahmanpour, Enzymatic conversion of lignin into renewable chemicals. *Curr. Opin. Chem. Biol.* 29 (2015) 10-17.
12. S.D. Haemmerli, M.S.A. Leisola, A. Fietcher, Polymerisation of lignins by ligninases from *Phanerochaete chysosporium*. *FEMS Microbiol. Lett.*, 35 (1986) 33-36.
13. P.J. Kersten, B. Kalyanaraman, K.E. Hammel, B. Reinhammar, T.K. Kirk, Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochem. J.* 268, (1990) 475-480.
14. K.E. Hammel, K.A. Jensen, Jr., M.D. Mozuch, L.L. Landucci, M. Tien, E.A. Pease, Ligninolysis by a purified lignin peroxidase. *J. Biol. Chem.* 268 (1993) 12274-12281.
15. O. Milstein, A. Hüttermann, R. Fründ, H-D. Lüdemann, Enzymatic co-polymerization of lignin with low-molecular mass compounds. *Appl. Microbiol. Biotechnol.* 40 (1994) 760-767.
16. K. Rittstieg, A. Suurnakki, T. Suortti, K. Kruus, G. Guebitz, J. Buchert, Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. *Enz. Microb. Technol.* 31 (2002) 403-410.
17. M. Ahmad, J.N. Roberts, E.M. Hardiman, R. Singh, L.D. Eltis, T.D.H. Bugg, Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. *Biochemistry* 50 (2011) 5096-5107.
18. S. Majumdar, T. Lukk, J.O. Solbiati, S. Bauer, S.K. Nair, J.E. Cronan, J.A. Gerlt, Roles of small laccases from *Streptomyces* in lignin degradation. *Biochemistry* 53 (2014) 4047-4058.

19. T.K. Kirk, R.L. Farrell, Enzymatic “combustion”; the microbial degradation of lignin. *Annu. Rev. Microbiol.* 41 (1987) 465-505.
20. L. Marzullo, R. Cannio, P. Giardina, M.T. Santini, G. Sannia, Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidised substrates. *J. Biol. Chem.* 270 (1995) 3823-3827.
21. S.S. Adav, C.S. Ng, M. Arulmani, S.K. Sze, Quantitative iTRAQ secretome analysis of cellulolytic *Thermobifida fusca*. *J. Proteome Res.* 9 (2010) 3016-3024.
22. H. Youn, J. Kwak, H-D. Youn, Y.C. Hah, S-O. Kang, Lipoamide dehydrogenase from *Streptomyces seoulensis*: biochemical and genetic properties. *Biochim. Biophys. Acta* 1388, (1998) 405-418.
23. R. Rahmanpour, T.D.H. Bugg, Characterisation of Dyp-type peroxidases from *Pseudomonas fluorescens* Pf-5: oxidation of Mn(II) and polymeric lignin by Dyp1B. *Arch. Biochem. Biophys.* 574, (2015) 93-98.
24. R. Rahmanpour, D. Rea, S. Jamshidi, V. Fülöp, T.D.H. Bugg, Structure of *Thermobifida fusca* DyP-type peroxidase and activity towards Kraft lignin and lignin model compounds. *Arch. Biochem. Biophys.* (2016) 594, 54-60.
25. V. Massey, Q.H. Gibson, C. Veeger, Intermediates in the catalytic action of lipoyl dehydrogenase (diaphorase). *Biochem. J.* 77 (1960) 341-351.
26. M.J. Danson, A. McQuattie, K.J. Stevenson, Dihydrolipoamide dehydrogenase from halophilic archaeobacteria: purification and properties of the enzyme from *Halobacterium halobium*. *Biochemistry* 25 (1986) 3880-3884.
27. A.U. Igamberdiev, N.V. Bykova, W. Ens, R.D. Hill, Dihydrolipoamide dehydrogenase from porcine heart catalyzes NADH-dependent scavenging of nitric oxide. *FEBS Letters* 568 (2004) 146-150.
28. F. Petrat, S. Paluch, E. Dogruöz, P. Dörfler, M. Kirsch, H-G. Korth, R. Sustmann, H.de Groot, Reduction of Fe(III) ions complexed to physiological ligands by lipoyl dehydrogenase and other flavoenzymes *in vitro*: implications for an enzymatic reduction of Fe(III) ions of the labile iron pool. *J. Biol. Chem.* 278 (2003) 46403-46413.
29. M.E. Castro, R. Molina, W. Diaz, S.E. Pichuantes, C.C. Vasquez, The dihydrolipoamide dehydrogenase of *Aeromonas caviae* ST exhibits NADH-dependent tellurite reductase activity. *Biochem. Biophys. Res. Commun.* 375 (2008) 91-94.
30. T. Hallström, M. Mörgelin, D. Barthel, M. Raguse, A. Kunert, R. Hoffman, C. Skerka, P.F. Zipfel, Dihydrolipoamide dehydrogenase of *Pseudomonas aeruginosa* is a surface-exposed immune evasion protein that binds three members of the Factor H family and plasminogen. *J. Immunology* 189 (2012) 4939-4950.

31. I.L. De la Sierra, L. Pernot, T. Prangé, P. Saludjian, M. Schiltz, R. Fourme, G. Padron, Molecular structure of the lipoamide dehydrogenase domain of a surface antigen from *Neisseria meningitides*. *J. Mol. Biol.* 269 (1997) 129-141.
32. A., Engels, U., Kahmann, H.G., Ruppel, E.K. Pistorius, Isolation, partial characterization and localization of a dihydrolipoamide dehydrogenase from the cyanobacterium *Synechocystis* PCC 6803. *Biochem. Biophys. Acta* 1340 (1997) 33-44.
33. S.A. Watt, A. Wilke, T. Patschkowski, K. Niehaus, Comprehensive analysis of the extracellular proteins from *Xanthomonas campestris* pv. *campestris* B100. *Proteomics* 5 (2005) 153-167.
34. H. Schrempf, I. Koebisch, S. Walter, H. Engelhardt, H. Meschke, Extracellular *Streptomyces* vesicles: amphorae for survival and defence. *Microb. Biotech.* 4 (2011) 286-299.
35. K. Ito, K. Inaba, The disulfide bond formation (Dsb) system. *Curr. Opin. Struct. Biol.* 18 (2008) 450-458.
36. K.A. Jensen, Jr, C.J. Houtman, Z.C. Ryan, K.E. Hammel, Pathways for extracellular Fenton chemistry in the brown rot Basidiomycete *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* 67 (2001) 2705-2711.
37. A. Hernández-Ortega, P. Ferreira, A.T. Martinez, Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation. *Appl. Microbiol. Biotechnol.* 93 (2012) 1395-1410.
38. M.D. Cameron, S.D. Aust, Cellobiose dehydrogenase – an extracellular fungal flavocytochrome. *Enz. Microb. Technol.* 28 (2001) 129-138.