

## Elucidation of microbial lignin degradation pathways using synthetic isotope-labelled lignin

Awatif Alruwaili, Goran M.M. Rashid, Victoria Sodr , James Mason, Zainab Rehman, David Cheung, Steven Brown and Timothy D.H. Bugg\*

### Supporting Information

Figure S1.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of  $^{13}\text{C}$ -labelled ferulic acid

Figure S2.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of  $^{13}\text{C}$ -labelled coniferyl alcohol

Figure S3 Purification of recombinant *Rhodococcus jostii* RHA1 glycolate oxidase

Figure S4. Gel permeation chromatography of unlabelled and  $^{13}\text{C}$ -labelled DHP lignin

Figure S5 Solid state  $^{13}\text{C}$  NMR spectrum of poly-ferulic acid

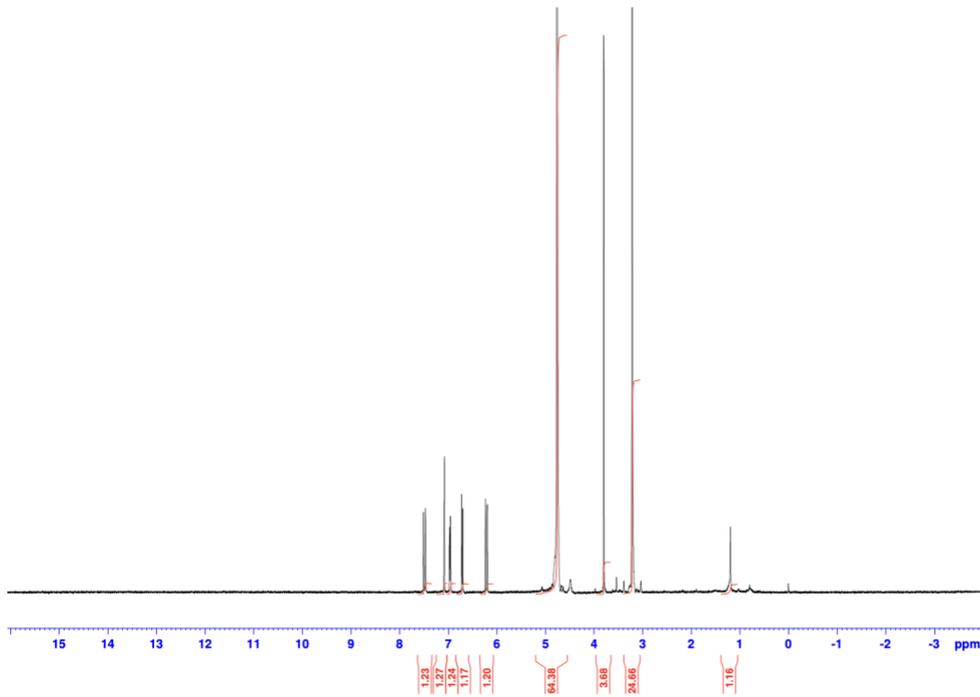
Figure S6,S7. Extracted ion chromatogram LC-MS data for the formation of unlabelled oxalic acid from unlabelled DHP lignin (S6), and  $^{13}\text{C}$ -labelled oxalic acid from  $^{13}\text{C}$ -labelled DHP lignin (S7), by *Rhodococcus jostii* RHA1, with control incubations lacking bacteria, and authentic oxalic acid standard.

Figure S8,S9. Extracted ion chromatogram LC-MS data for the formation of unlabelled homovanillic acid from unlabelled DHP lignin (S8), and  $^{13}\text{C}$ -labelled homovanillic acid from  $^{13}\text{C}$ -labelled DHP lignin (S9), by *Rhodococcus jostii* RHA1, with control incubations lacking bacteria, and authentic oxalic acid standard.

Figure S10. Extracted ion chromatogram LC-MS data for the formation of  $^{13}\text{C}$ -labelled oxalic acid from  $^{13}\text{C}$ -labelled polyferulic acid by *Rhodococcus jostii* RHA1, with control incubation lacking bacteria, and authentic oxalic acid standard.

Figure S11 HPLC analysis of reaction products from incubation of 4-hydroxyphenylacetic acid with *Rhodococcus jostii* RHA1 glycolate oxidase enzyme.

sample Unlabelled Ferulic acid H-NMR  
 PROTON.w T\_MeOD /opt/topspin3.5pl2 AA2 2

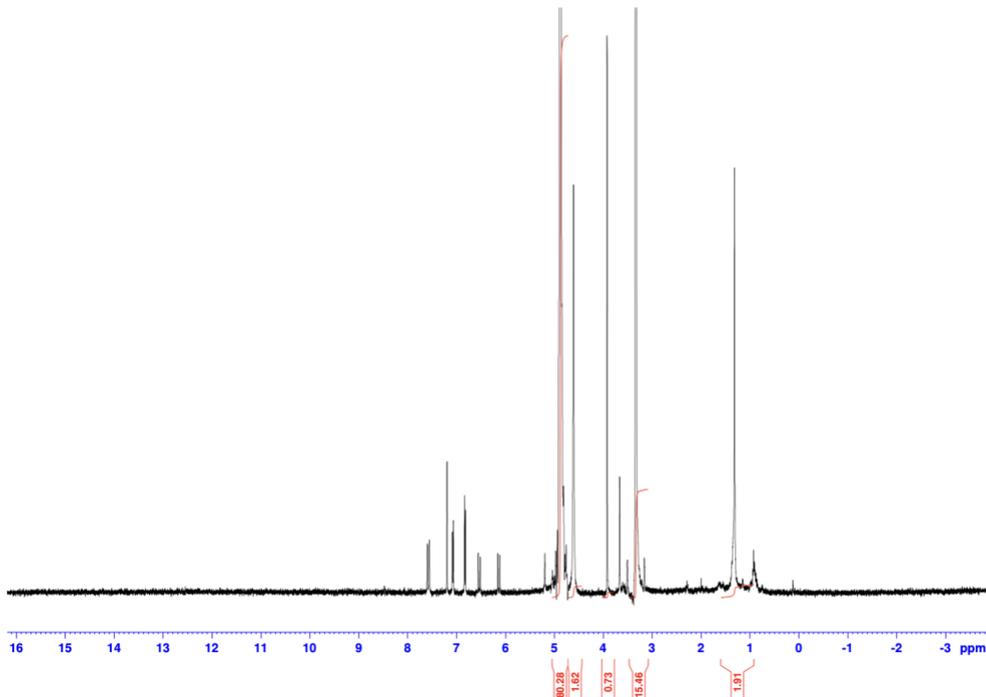


```
Current Data Parameters
NAME      Feb18-2022
EXPNO    10
PROCNO   1

F2 - Acquisition Parameters
Date_    20220218
Time     10.21 h
INSTRUM  spect
PROBHD   Z108618_0844 (
PULPROG  zg30
TD        65536
SOLVENT  T_MeOD
NS        16
DS        2
SWH       8012.820 Hz
FIDRES    0.122266 Hz
AQ        4.0894465 sec
RG        179.42
DW        62.400 usec
DE        6.50 usec
TE        298.0 K
D1        1.00000000 sec
TD0       1
SFO1     400.1324708 MHz
NUC1     1H
P1        14.00 usec
PLW1     12.92099953 W

F2 - Processing parameters
SI        65536
SF        400.1300477 MHz
WDW       EM
SSB       0
LB        0.30 Hz
GB        0
PC        1.00
```

13C Ferulic acid 1 Labelled  
 PROTON.w T\_MeOD /opt/topspin3.5pl2 AA2 5



```
Current Data Parameters
NAME      Feb22-2022
EXPNO    10
PROCNO   1

F2 - Acquisition Parameters
Date_    20220222
Time     11.14 h
INSTRUM  spect
PROBHD   Z108618_0844 (
PULPROG  zg30
TD        65536
SOLVENT  T_MeOD
NS        16
DS        2
SWH       8012.820 Hz
FIDRES    0.122266 Hz
AQ        4.0894465 sec
RG        145.76
DW        62.400 usec
DE        6.50 usec
TE        298.0 K
D1        1.00000000 sec
TD0       1
SFO1     400.1324708 MHz
NUC1     1H
P1        14.00 usec
PLW1     12.92099953 W

F2 - Processing parameters
SI        65536
SF        400.1300000 MHz
WDW       EM
SSB       0
LB        0.30 Hz
GB        0
PC        1.00
```

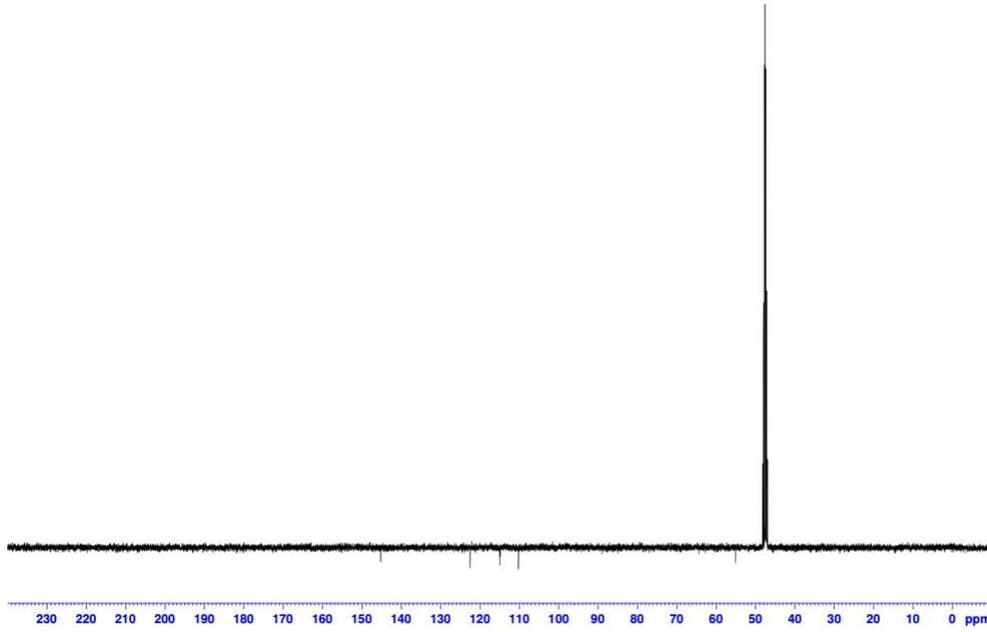
sample unlabelled Ferulic acid  
C13APTlong.w T\_MeOD /opt/topspin3.5pl2 AA2 2



```
Current Data Parameters
NAME      Feb18-2022
EXPNO    11
PROCNO   1

F2 - Acquisition Parameters
Date_    20220218
Time     11.50 h
INSTRUM  spect
PROBHD   Z108618_0844 (
PULPROG  jmod
TD        65536
SOLVENT  T_MeOD
NS        512
DS        4
SWH       26041.666 Hz
FIDRES   0.397364 Hz
AQ        1.2582912 sec
RG        201.62
DW        19.200 usec
DE        6.50 usec
TE        298.0 K
CNS12    145.0000000
CNS111   1.0000000
D1        2.00000000 sec
D20      0.00689655 sec
TD0       1
SF01     100.6248421 MHz
NUC1      13C
P1        10.00 usec
P2        20.00 usec
PLW1     47.37500000 W
SFO2     400.1316005 MHz
NUC2      1H
CPDPRG2  waltz16
PCPD2    90.00 usec
PLW2     12.92099953 W
PLW12    0.30953279 W

F2 - Processing parameters
SI        32768
SF        100.6127685 MHz
WDW       EM
SSB       0
LB        1.00 Hz
GB        0
PC        1.40
```



13C Ferulic acid 1 Labelled  
C13APT.w T\_MeOD /opt/topspin3.5pl2 AA2 5



```
Current Data Parameters
NAME      Feb22-2022
EXPNO    11
PROCNO   1

F2 - Acquisition Parameters
Date_    20220222
Time     11.23 h
INSTRUM  spect
PROBHD   Z108618_0844 (
PULPROG  jmod
TD        65536
SOLVENT  T_MeOD
NS        128
DS        4
SWH       26041.666 Hz
FIDRES   0.397364 Hz
AQ        1.2582912 sec
RG        201.62
DW        19.200 usec
DE        6.50 usec
TE        298.0 K
CNS12    145.0000000
CNS111   1.0000000
D1        2.00000000 sec
D20      0.00689655 sec
TD0       1
SF01     100.6248421 MHz
NUC1      13C
P1        10.00 usec
P2        20.00 usec
PLW1     47.37500000 W
SFO2     400.1316005 MHz
NUC2      1H
CPDPRG2  waltz16
PCPD2    90.00 usec
PLW2     12.92099953 W
PLW12    0.30953279 W

F2 - Processing parameters
SI        32768
SF        100.6127685 MHz
WDW       EM
SSB       0
LB        1.00 Hz
GB        0
PC        1.40
```

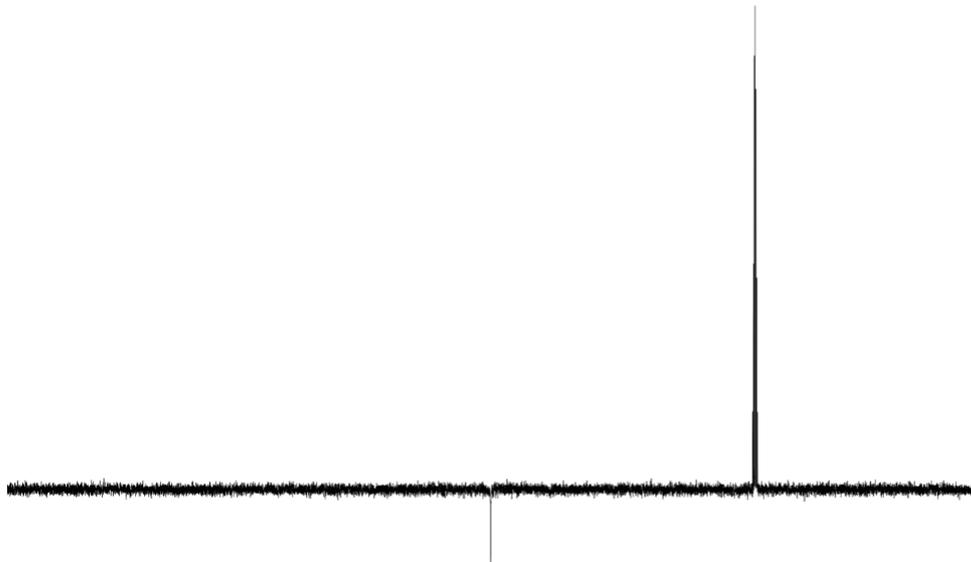


Figure S1. <sup>1</sup>H NMR (400 MHz) and <sup>13</sup>C NMR (125 MHz) spectra of <sup>13</sup>C-labelled ferulic acid

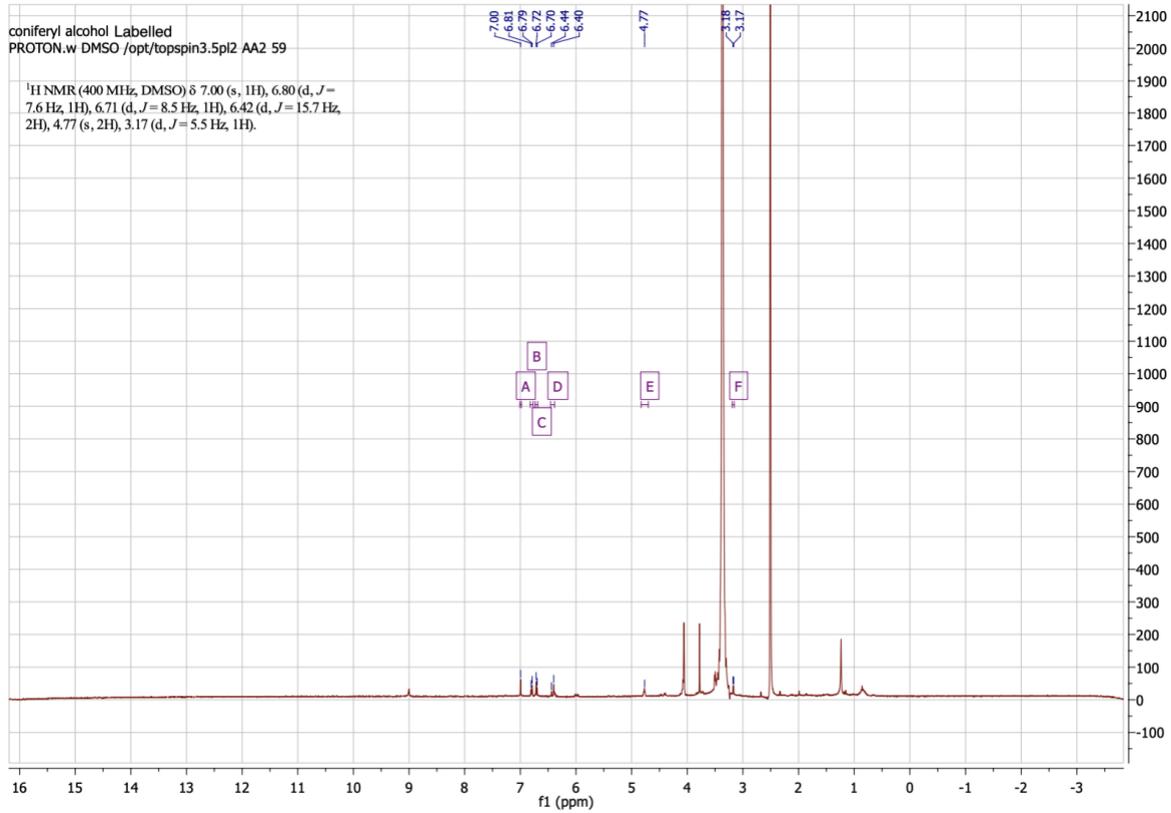
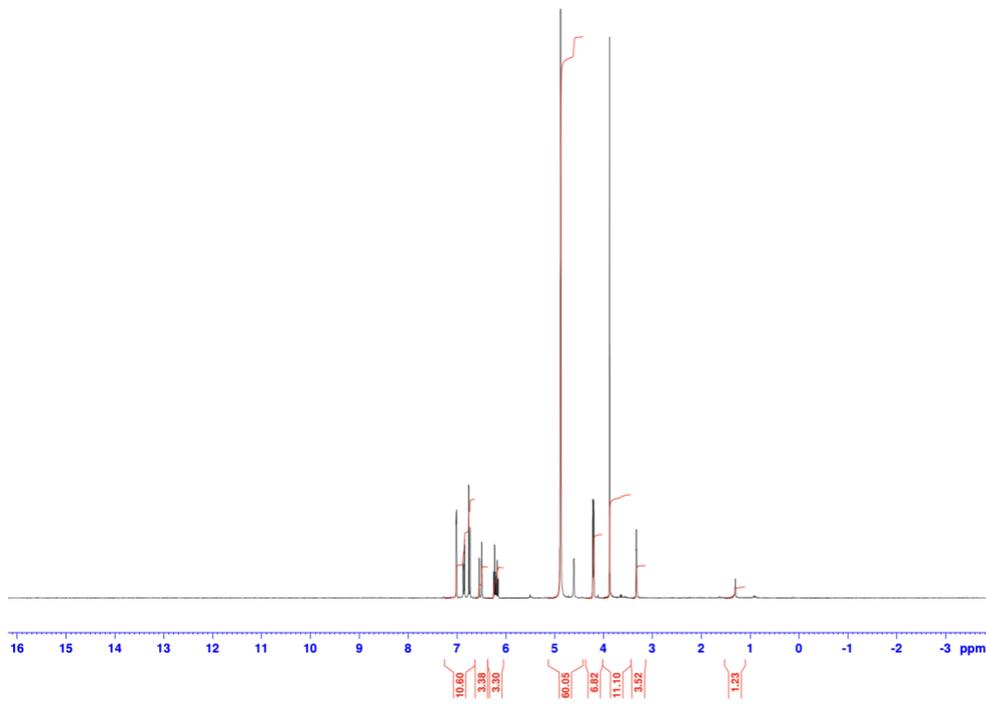
unlabelled coniferyl alcohol



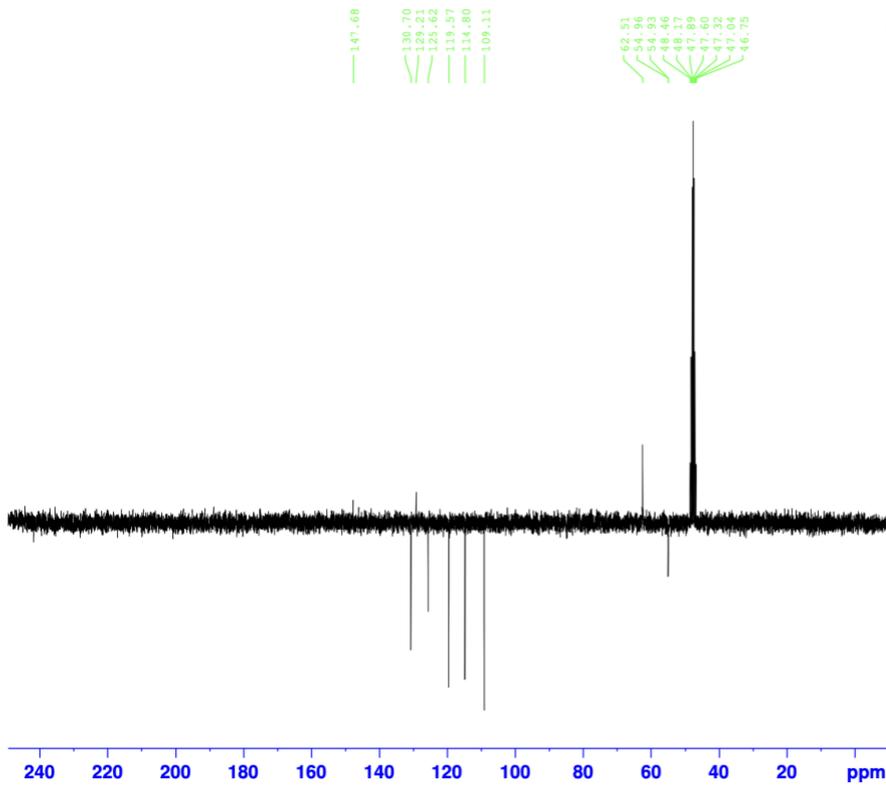
```
Current Data Parameters
NAME      Sep30-2022
EXPNO     10
PROCNO    1

F2 - Acquisition Parameters
Date_     20220930
Time      11:43 h
INSTRUM   spect
PROBHD    z104275_0340 (
PULPROG   zg30
TD         65536
SOLVENT   MeOD
NS         16
DS         2
SWH        6009.615 Hz
FIDRES     0.091699 Hz
AQ         5.4525952 sec
RG         200.94
DW         83.200 usec
DE         6.50 usec
TE         298.0 K
D1         1.00000000 sec
TDO        1
SFO1      300.1318533 MHz
NUC1       1H
P1         14.00 usec
PLM1       8.74460039 W

F2 - Processing parameters
SI         65536
SF         300.1300000 MHz
WDW        EM
SSB        0
LB         0.30 Hz
GB         0
PC         1.00
```



unlabelled coniferyl alcohol

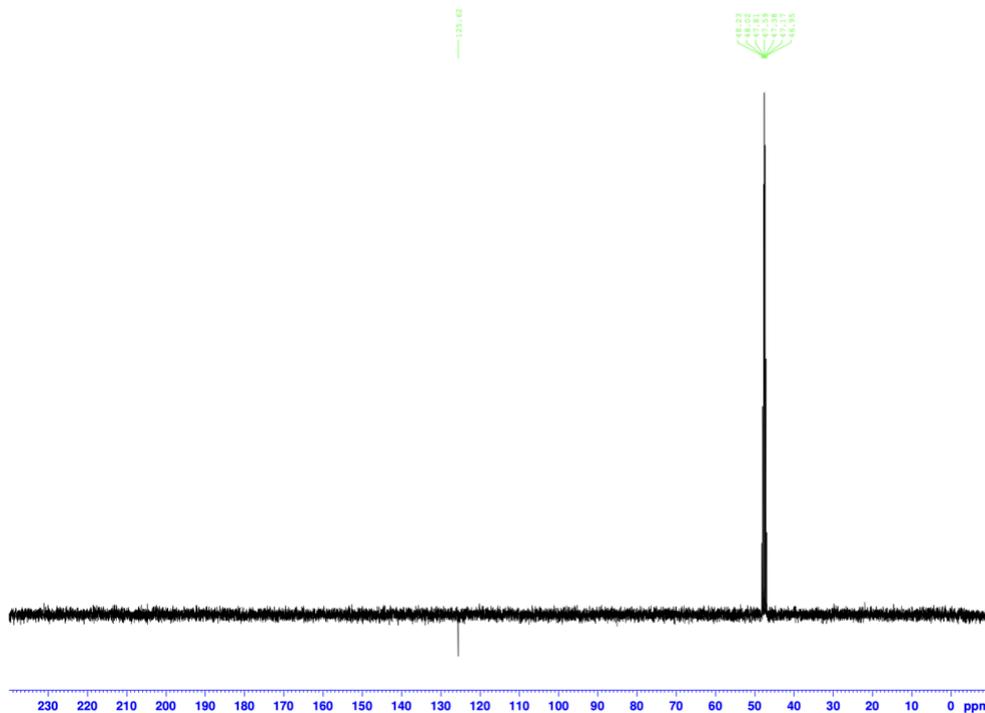


Current Data Parameters  
NAME Sep30-2022  
EXPNO 11  
PROCNO 1

F2 - Acquisition Parameters  
Date\_ 20220930  
Time 11.52 h  
INSTRUM spect  
PROBHD Z104275\_0340 (  
PULPROG jmod  
TD 65536  
SOLVENT MeOD  
NS 128  
DS 4  
SWH 19531.250 Hz  
FIDRES 0.298023 Hz  
AQ 1.6777216 sec  
RG 200.94  
DW 25.600 usec  
DE 6.50 usec  
TE 298.0 K  
CNST2 145.000000  
CNST11 1.000000  
D1 2.0000000 sec  
D20 0.00689655 sec  
TD0 1  
SF01 75.4768047 MHz  
NUC1 13C  
P1 10.00 usec  
P2 20.00 usec  
PLW1 36.59700012 W  
SFO2 300.1312005 MHz  
NUC2 1H  
CPDPRG[2] waltz16  
PCPD2 90.00 usec  
PLW2 8.74460030 W  
PLW12 0.20996240 W

F2 - Processing parameters  
SI 32768  
SF 75.4677485 MHz  
WDW EM  
SSB 0  
LB 1.00 Hz  
GB 0  
PC 1.40

coniferyl alcohol 13C bio Labelled  
C13APT.w MeOD /opt/topspin3.5pl2 AA2 59



Current Data Parameters  
NAME Apr29-2022  
EXPNO 21  
PROCNO 1

F2 - Acquisition Parameters  
Date\_ 20220429  
Time 14.56 h  
INSTRUM spect  
PROBHD Z108618\_0844 (  
PULPROG jmod  
TD 65536  
SOLVENT MeOD  
NS 128  
DS 4  
SWH 26041.666 Hz  
FIDRES 0.397364 Hz  
AQ 1.2582912 sec  
RG 201.62  
DW 19.200 usec  
DE 6.50 usec  
TE 298.0 K  
CNST2 145.000000  
CNST11 1.000000  
D1 2.0000000 sec  
D20 0.00689655 sec  
TD0 1  
SF01 100.6248421 MHz  
NUC1 13C  
P1 10.00 usec  
P2 20.00 usec  
PLW1 47.37500000 W  
SFO2 400.1316005 MHz  
NUC2 1H  
CPDPRG[2] waltz16  
PCPD2 90.00 usec  
PLW2 12.92099953 W  
PLW12 0.30953279 W

F2 - Processing parameters  
SI 32768  
SF 100.6127685 MHz  
WDW EM  
SSB 0  
LB 1.00 Hz  
GB 0  
PC 1.40

Figure S2.  $^1\text{H}$  NMR (400 MHz) and  $^{13}\text{C}$  NMR (125 MHz) spectra of  $^{13}\text{C}$ -labelled coniferyl alcohol

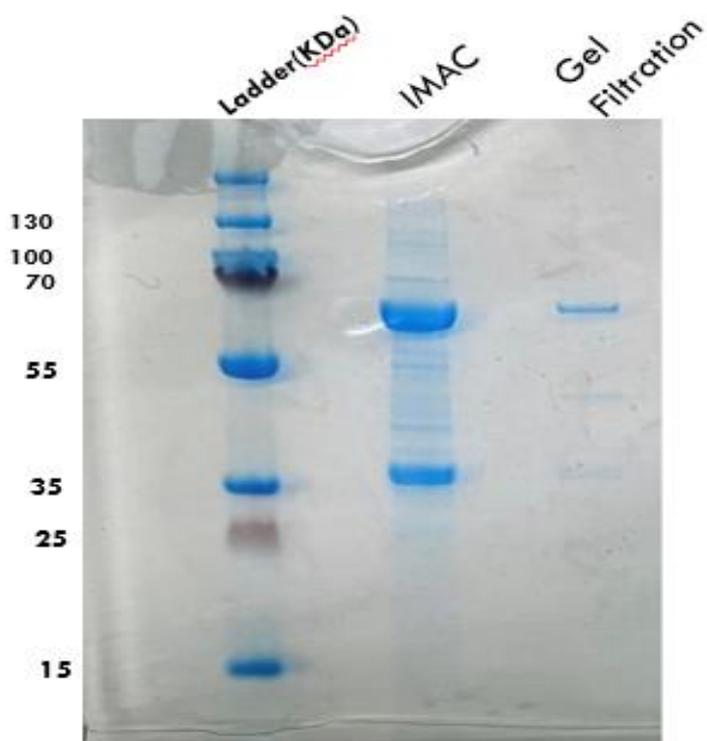
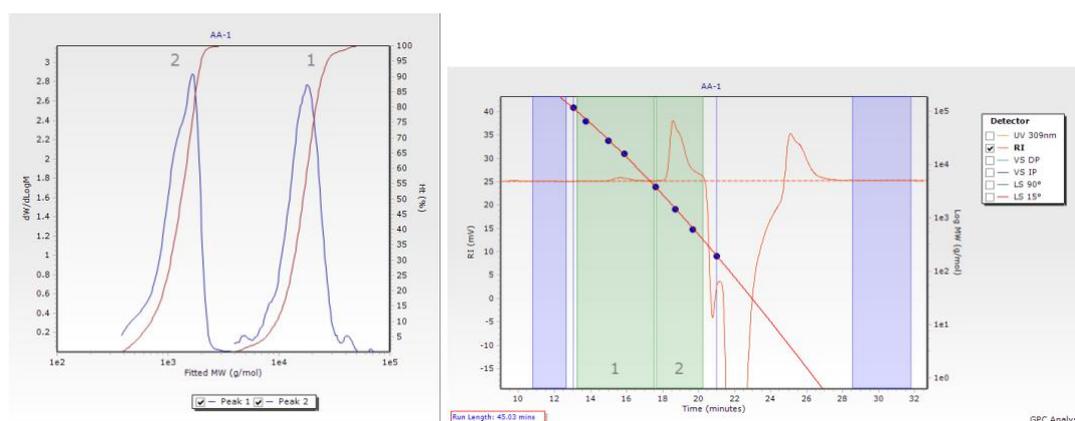


Figure S3. Purification of recombinant *Rhodococcus jostii* RHA1 glycolate oxidase (predicted  $M_r$  68 kDa) by Immobilized Ion Affinity Chromatography (IMAC, Ni-NTA column), followed by Superdex 200 Gel Filtration chromatography.

### GPC analysis of unlabelled DHP lignin

	Mp (g/mol)	Mn (g/mol)	Mw (g/mol)	Mz (g/mol)	Mz+1 (g/mol)	Mv (g/mol)	PD
Peak 1	5102	3471	5154	6961	9016	6682	1.484875

### GPC analysis of <sup>13</sup>C-Labelled DHP lignin



There are two distributions, at ~1500 g/mol (Peak 2) and a much smaller one at ~18000 g/mol (Peak 1)

	Mp (g/mol)	Mn (g/mol)	Mw (g/mol)	Mz (g/mol)	Mz+1 (g/mol)	Mv (g/mol)	PD
Peak 1	17950	14519	17032	19558	22543	19162	1.173084
Peak 2		1129	1308	1456	1577	1437	1.158547

Lignin samples were acetylated using acetic anhydride/pyridine. The acetylated samples were analysed on an Agilent 1260 Infinity II-MDS analyzer, on a 2 x PLgel Mixed-D column, using DMF/5mM NH<sub>4</sub>BF<sub>4</sub> as solvent, and polystyrene molecular weight standards.

Figure S4. Gel permeation chromatography of unlabelled and <sup>13</sup>C-labelled DHP lignin

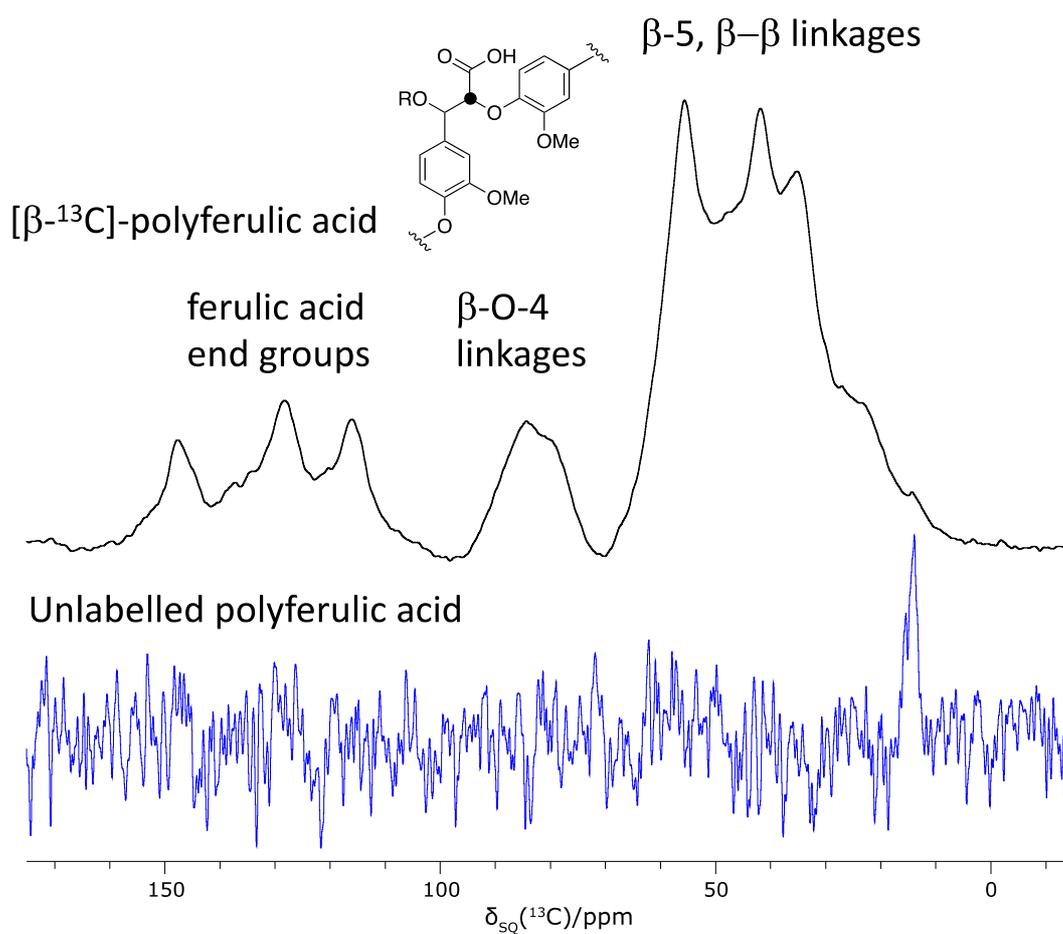


Figure S5.  $^{13}\text{C}$  solid state NMR spectrum of [ $\beta$ - $^{13}\text{C}$ ]-polyferulic acid. Data collection as described in Experimental section.

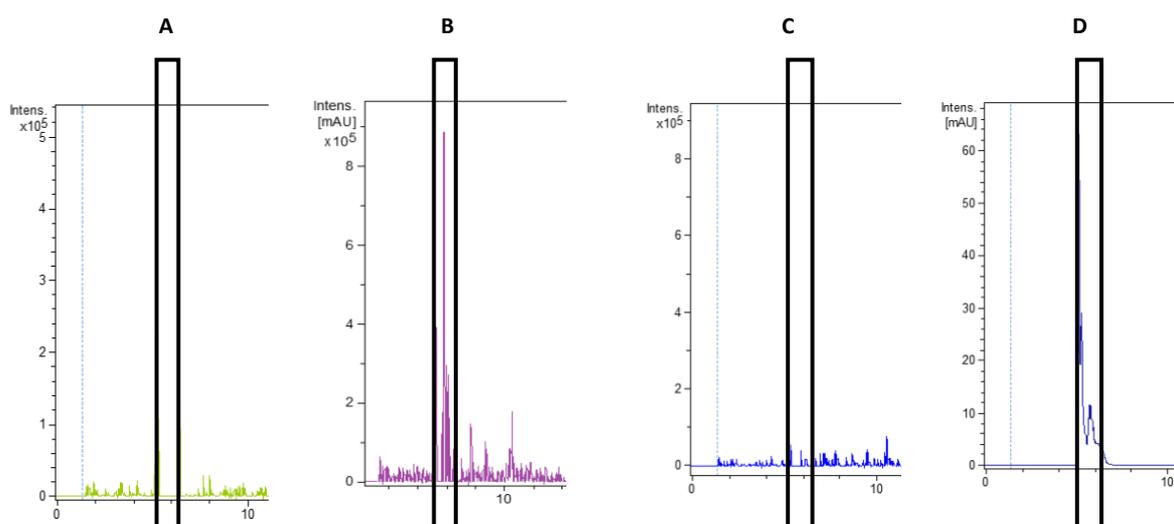


Figure S6. Extracted ion chromatogram LC-MS data for the formation of unlabelled oxalic acid (calculated 91.0 for  $MH^+$ ) at retention time 5.8 min from unlabelled DHP lignin by *Rhodococcus jostii* RHA1 (Panel B,  $m/z$  91.0; Panel C,  $m/z$  92.0). Panel A, control incubation lacking bacteria ( $m/z$  91.0). Panel D, authentic oxalic acid standard.

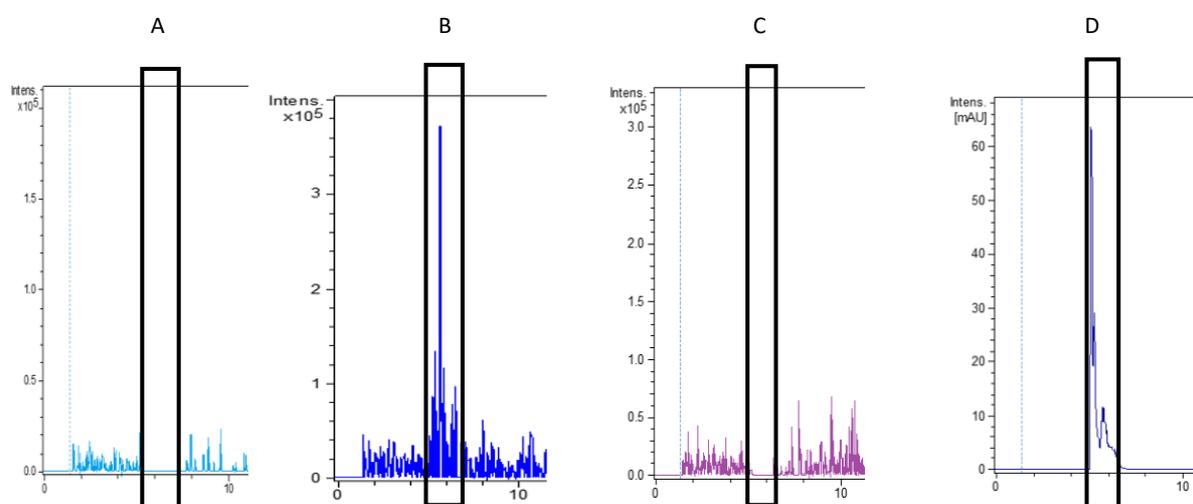


Figure S7. Extracted ion chromatogram LC-MS data for the formation of  $^{13}C$ -labelled oxalic acid (calculated 92.0 for  $MH^+$ ) at retention time 5.8 min from unlabelled DHP lignin by *Rhodococcus jostii* RHA1 (Panel B,  $m/z$  92.0; Panel C,  $m/z$  91.0). Panel A, control incubation lacking bacteria ( $m/z$  92.0). Panel D, authentic oxalic acid standard.

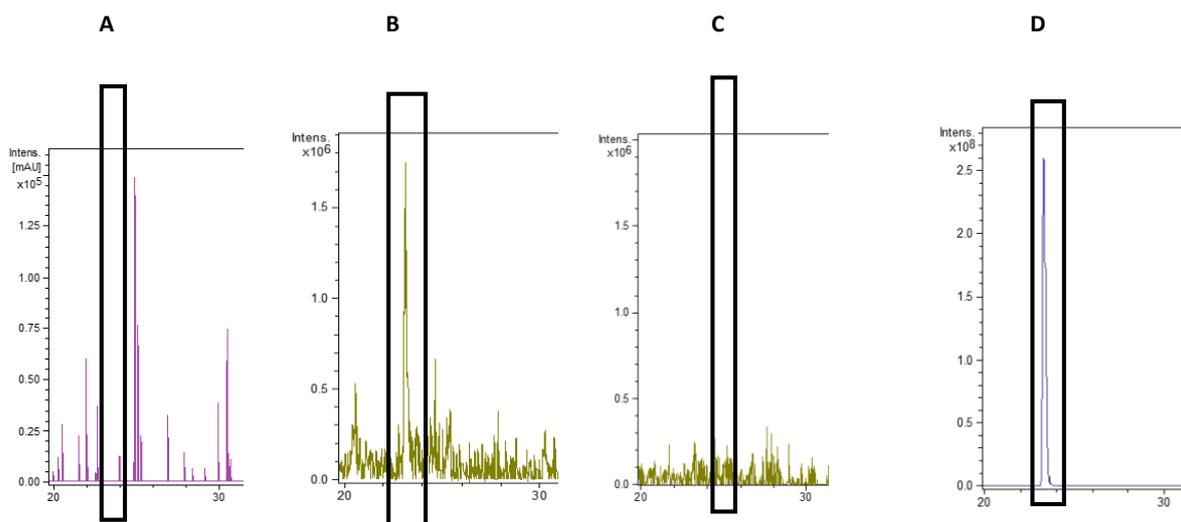


Figure S8. Extracted ion chromatogram LC-MS data for the formation of unlabelled homovanillic acid (calculated 205.0 for  $MNa^+$ ) at retention time 23.0 min from unlabelled DHP lignin by *Rhodococcus jostii* RHA1 (Panel B,  $m/z$  205.0; Panel C,  $m/z$  206.0). Panel A, control incubation lacking bacteria ( $m/z$  205.0). Panel D, authentic homovanillic acid standard.

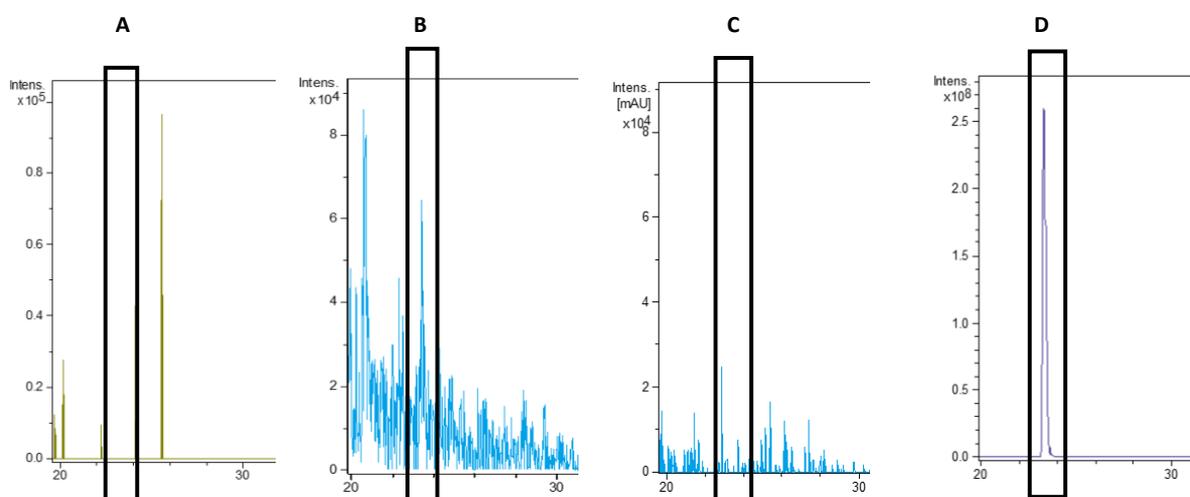


Figure S9. Extracted ion chromatogram LC-MS data for the formation of  $^{13}C$ -labelled homovanillic acid (calculated 206.0 for  $MNa^+$ ) at retention time 23.0 min from unlabelled DHP lignin by *Rhodococcus jostii* RHA1 (Panel B,  $m/z$  206.0; Panel C,  $m/z$  205.0). Panel A, control incubation lacking bacteria ( $m/z$  206.0). Panel D, authentic homovanillic acid standard.

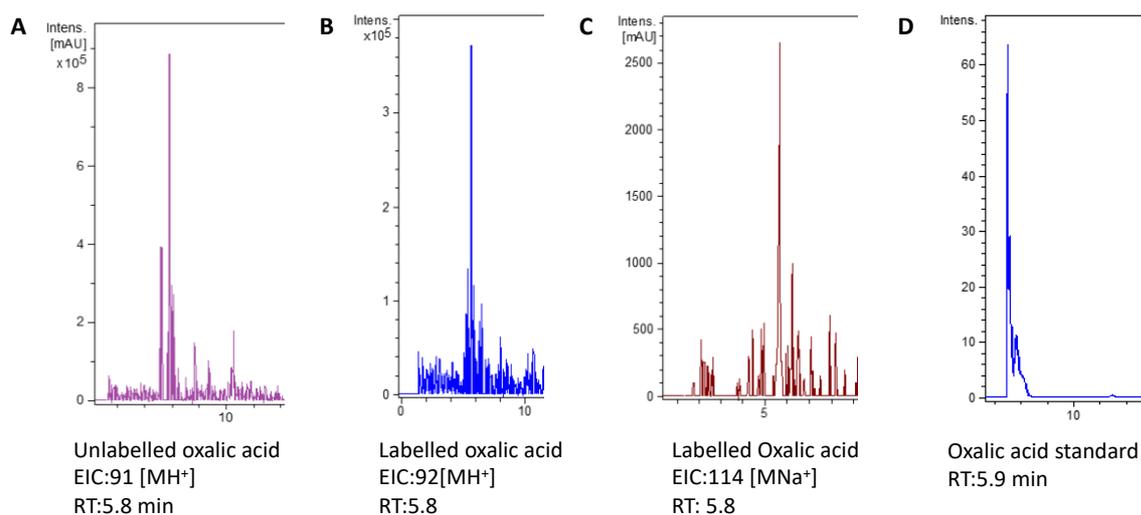


Figure S10. Extracted ion chromatogram LC-MS data for formation of  $^{13}\text{C}$ -labelled oxalic acid from  $[\beta\text{-}^{13}\text{C}]$ -poly-ferulic acid ( $m/z$  114.0,  $\text{MNa}^+$ ) by *Rhodococcus jostii* RHA1. Panel A, unlabelled oxalic acid formed from unlabelled DHP lignin ( $m/z$  91.0,  $\text{MH}^+$ ). Panel B,  $^{13}\text{C}$ -labelled oxalic acid ( $m/z$  92.0,  $\text{MH}^+$ ) formed from  $[\beta\text{-}^{13}\text{C}]$ -DHP lignin. Panel D, oxalic acid standard.

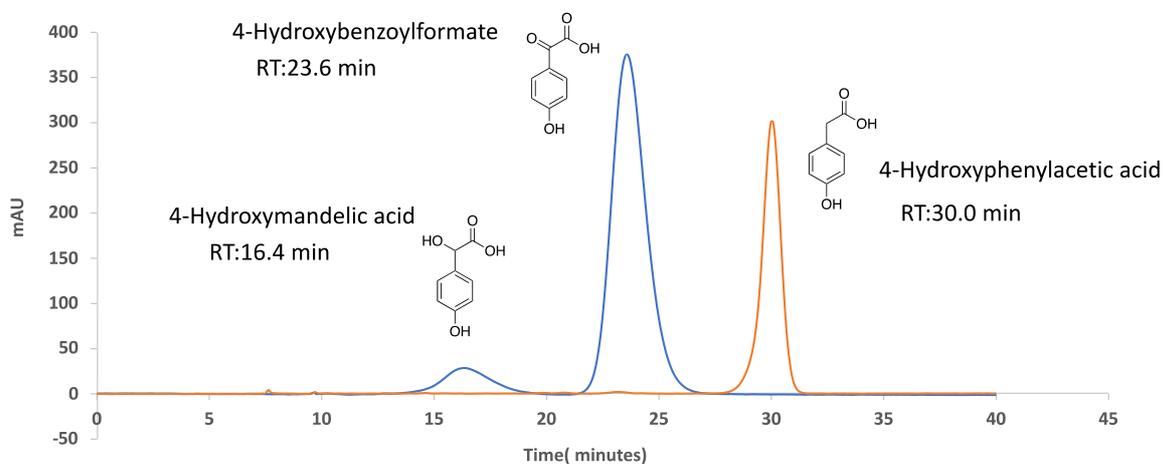


Figure S11. HPLC analysis of reaction products from incubation of 4-hydroxyphenylacetic acid with *Rhodococcus jostii* RHA1 glycolate oxidase enzyme. Blue line, sample treated with *R. jostii* RHA1 glycolate oxidase; orange line, control lacking glycolate oxidase enzyme. Reaction mixtures were separated on an Aminex HPX-87H Organic Acids column (300 x 7.8 mm) (Bio-Rad) at 45°C, with 5 mM sulfuric acid as mobile phase and a flow rate of 0.5 mL/min.