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Supporting Information

Creating Oxidase–Peroxidase Fusion Enzymes as a Toolbox for Cascade Reactions

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Supporting information

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Materials and methods

Table S1: Overview of the enzymes, the host organisms, the original plasmids, and the plasmids created in this study for the heterologous overexpression of the fusion enzymes.

Enzyme	**Organism**	**Original plasmids**	**Plasmids of the fusions**
*Svi*DyP	*Saccharomonospora viridis* DSM 43017	pBAD His-*Svi*DyP [1]	
ChitO*	*Fusarium graminearum*	pET SUMO-ChitO_Q268R/G270E/S410R [2]	pBAD His-*Svi*DyP-ChitO*
EugO	*Rhodococcus sp.* strain RHA1	pBAD EugO (pEUGOA [3])	pBAD His-*Svi*DyP-EugO
HMFO	*Methylovorus sp.* strain MP688	pET His-SUMO-HMFO [4] (*E. coli* codon optimized)	pBAD His-*Svi*DyP-HMFO
HotAldO	*Acidothermus cellulolyticus* 11B	pBAD HAS [5] (*E. coli* codon optimized)	pBAD His-*Svi*DyP-HotAldO-His

Results: supplementary tables and figures

Expression and purification: yield and Reinheitszahl

Table S2: Expression yield and Reinheitszahl (Rz-value) of the purified enzymes.

Enzyme	Expression yield (mg/L culture broth medium)	Rz-value (Abs $_{406\ nm}$ / Abs $_{280\ nm}$)
*Svi*DyP	76	1.84
P-ChitO*	26	0.82
P-EugO	37	0.61
P-HMFO	60	0.75
P-HotAldO	29	0.97

Expression and purification: SDS-PAGE

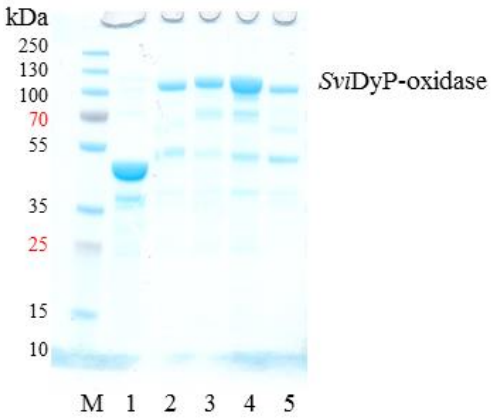
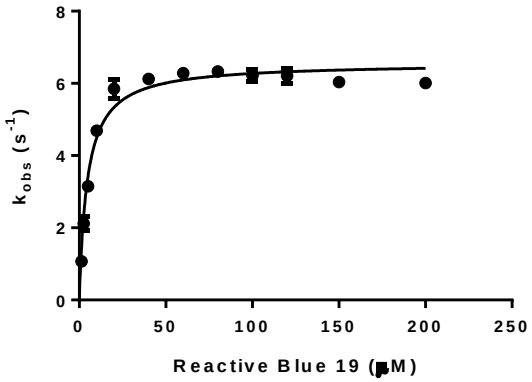


Figure S1: Analysis of the purified proteins by SDS-PAGE. M, PageRuler prestained plus protein ladder ThermoFisher Scientific. Lines: 1, *Svi*DyP; 2, P-ChitO*; 3, P-EugO; 4, P-HMFO; 5, P-HotAldO.

Michaelis-Menten kinetics of *Svi*DyP towards Reactive Blue 19

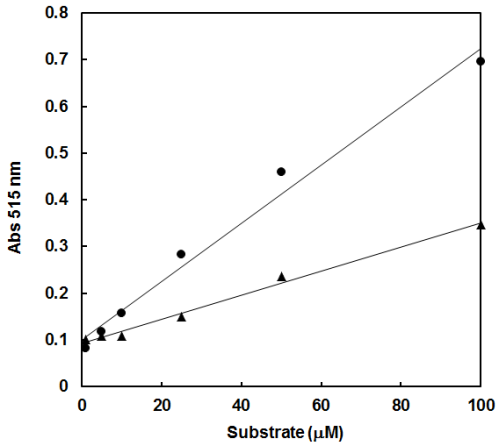


k_{cat}	6.57 ± 0.08 s^{-1}
K_M	4.63 ± 0.33μM

Figure S2: Michaelis-Menten kinetics of *Svi*DyP towards Reactive Blue 19 at pH 4.0.

Analysis of the sensitivity of the *Svi*DyP-assay

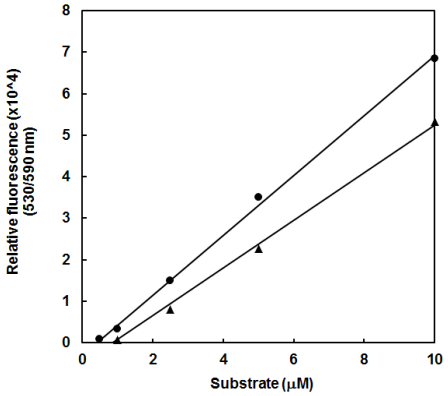
a. Signal response of the *Svi*DyP-coupled assay with AAP/DCHBS for detection



Enzyme	Substrate	Sensitivity (µM)	Slope	R^2
P-ChitO*	Cellobiose	25	0.0026	0.992
P-HotAldO	Xylitol	10	0.0062	0.984

Figure S3: Signal response of the coupled-assay with AAP and DCHBS for detection. Product formation was measured at 515 nm after 15 minutes of incubation at ambient temperature. Circles: P-HotAldO with substrate xylitol. Triangles: P-ChitO* with substrate cellobiose.

b. Signal response of the *Svi*DyP-coupled assay with Amplex Red for detection



Enzyme	Substrate	Sensitivity (µM)	Slope (x10^4)	R^2
P-ChitO*	Cellobiose	2.5	0.573	0.996
P-HotAldO	Xylitol	2.5	0.721	0.998

Figure S4: Signal response of the coupled-assay with Amplex Red for detection. Fluorescence of product resorufin was measured at varying substrate concentrations after 15 minutes of incubation at ambient temperature, excitation 530 nm, emission 590 nm. Circles: P-HotAldO with substrate xylitol. Triangles: P-ChitO* with substrate cellobiose.

***Svi*DyP-coupled assay**

Table S3: *Svi*DyP-coupled assay. Coupled activity was measured in 50 mM sodium citrate buffer pH 5.0 or 50 mM potassium phosphate buffer pH 6.0, with 0.10 mM AAP, 1.0 mM DCHBS and 150 nM enzyme. 23.8 mM cellobiose was added as substrate for ChitO* and 1.4 mM xylitol for HotAldO. Peroxidase activity was measured under the same conditions in the absence of the substrate for the oxidases, with 100 μM H_2O_2.

Enzyme	pH	Coupled activity k_{obs} (s^{-1})	Peroxidase activity k_{obs} (s^{-1})
P-ChitO*	5	0.35	0.54
	6	0.27	0.38
P-HotAldO	5	0.11	0.64
	6	0.32	0.44

One-pot divanillin synthesis using P-HMFO and P-EugO

Table S4: Conversion of vanillyl alcohol to vanillin, divanillin and related dimers and oligomers by P-HMFO and P-EugO. Samples were analyzed by reverse phase HPLC. Conversion of 1 and 2 mM vanillyl alcohol in respectively 50 mM potassium phosphate buffer pH 6.0 and 50 mM sodium citrate buffer pH 5.5 at 30 ºC, 100 rpm. Reactions at pH 6.0 were incubated for 2h, reactions at pH 5.5 for 21h. The table shows the percentages of vanillyl alcohol left after the reaction and the percentages of vanillyl alcohol that was converted to either vanillin or to divanillin and related dimers and oligomers.

Enzyme	Reaction conditions	Vanillyl alcohol (%)	Vanillin (%)	Divanillin, related dimers and oligomers (%)
P-HMFO	pH 5.5, 2h	37	46	17
	pH 5.5, 3h	31	50	19
	pH 5.5, 21h	10	69	21
	pH 6.0, 2h	3	89	8
P-EugO	pH 5.5, 2h	50	24	26
	pH 5.5, 3h	43	28	29
	pH 5.5, 21h	8	53	39
	pH 6.0, 2h	30	39	31

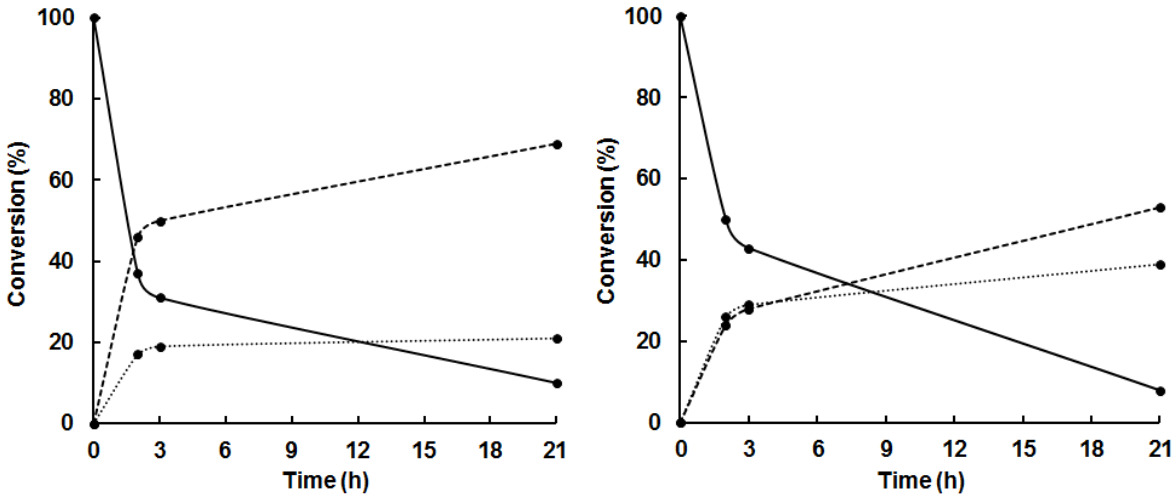


Figure S5: Conversion of 2 mM vanillyl alcohol by P-HMFO (left panel) and P-EugO (right panel) in 50 mM sodium citrate pH 5.5, 30 ºC and 100 rpm. Samples were analyzed by reverse phase HPLC for vanillyl alcohol depletion (solid line), vanillin production (dashed line) and formation of other products e.g. divanillin (main product of P-HMFO), mixed dimers and oligomers (dotted line).

One-pot divanillin synthesis using P-HMFO and P-EugO: LC-MS analysis

a. Reaction of *Svi*DyP with vanillyl alcohol at pH 5.5

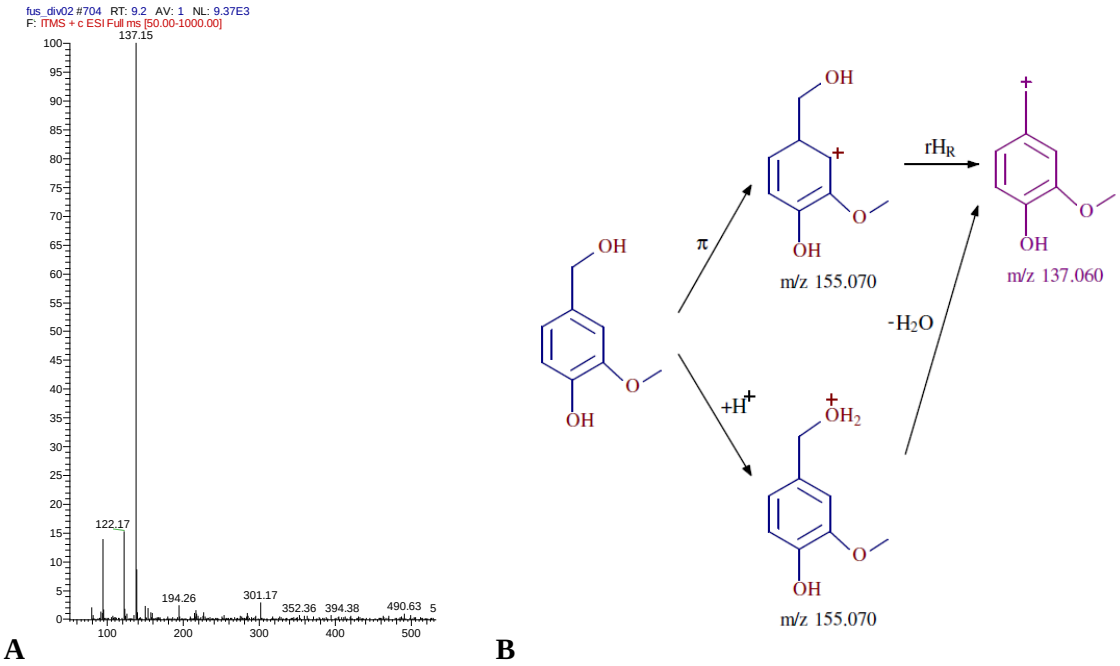


Figure S6: Mass spectrum corresponding to the peak of vanillyl alcohol. Observed peak (A) of highest abundance 137.15 correspond to expected fragmentation for vanillyl alcohol in positive mode (B). Figure B was generated using Mass Frontier 5.0 software from Thermo Scientific.

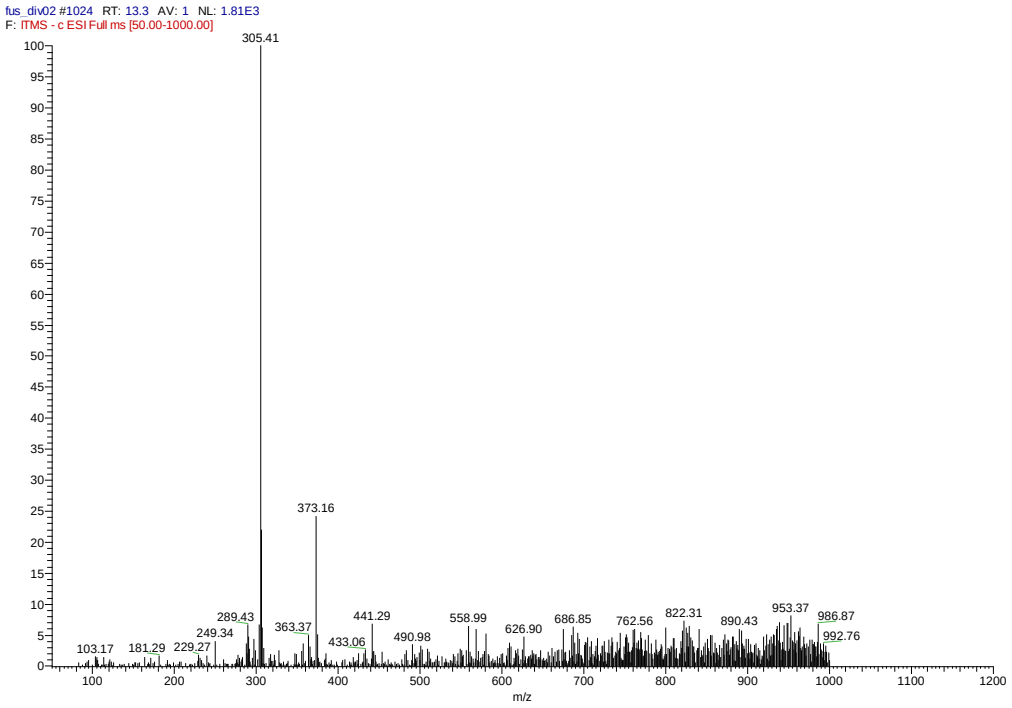


Figure S7: Mass spectrum corresponding to the peak of vanillyl alcohol dimer produced when only *Svi*DyP is present in reaction mixture with vanillyl alcohol. This is a known product of peroxidases as a result from oxidative phenolic coupling and keto-enol tautomerization.[6,7]

b. Reaction of P-HMFO with vanillyl alcohol at pH 5.5

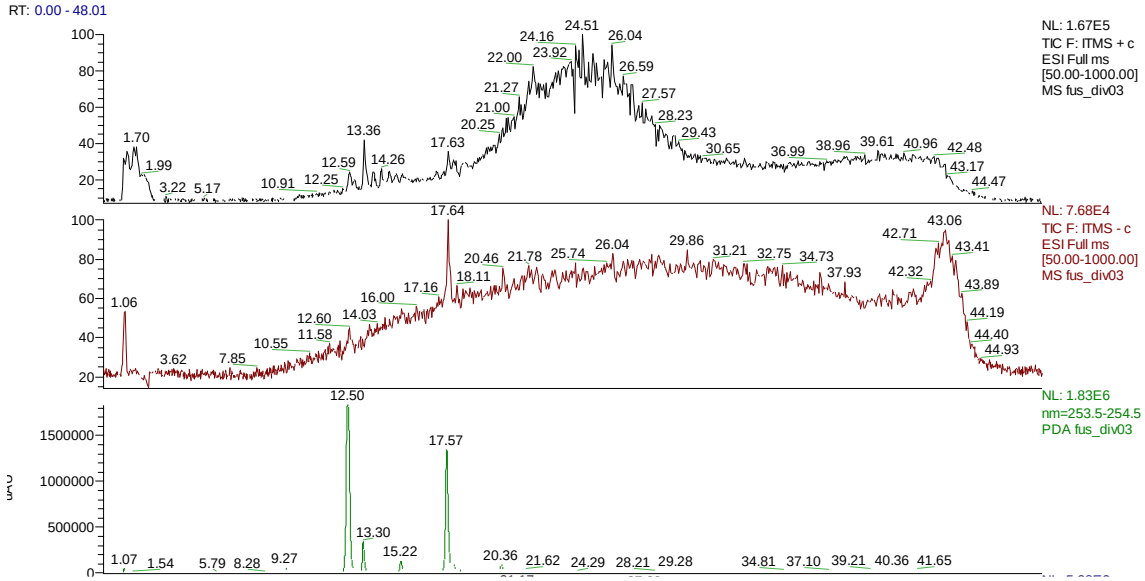


Figure S8: Chromatogram of reaction mixture of P-HMFO with vanillyl alcohol at pH 5.5. Total ionic current in positive mode (upper panel), negative mode (middle panel) and UV at 280 nm (lower panel) are shown.

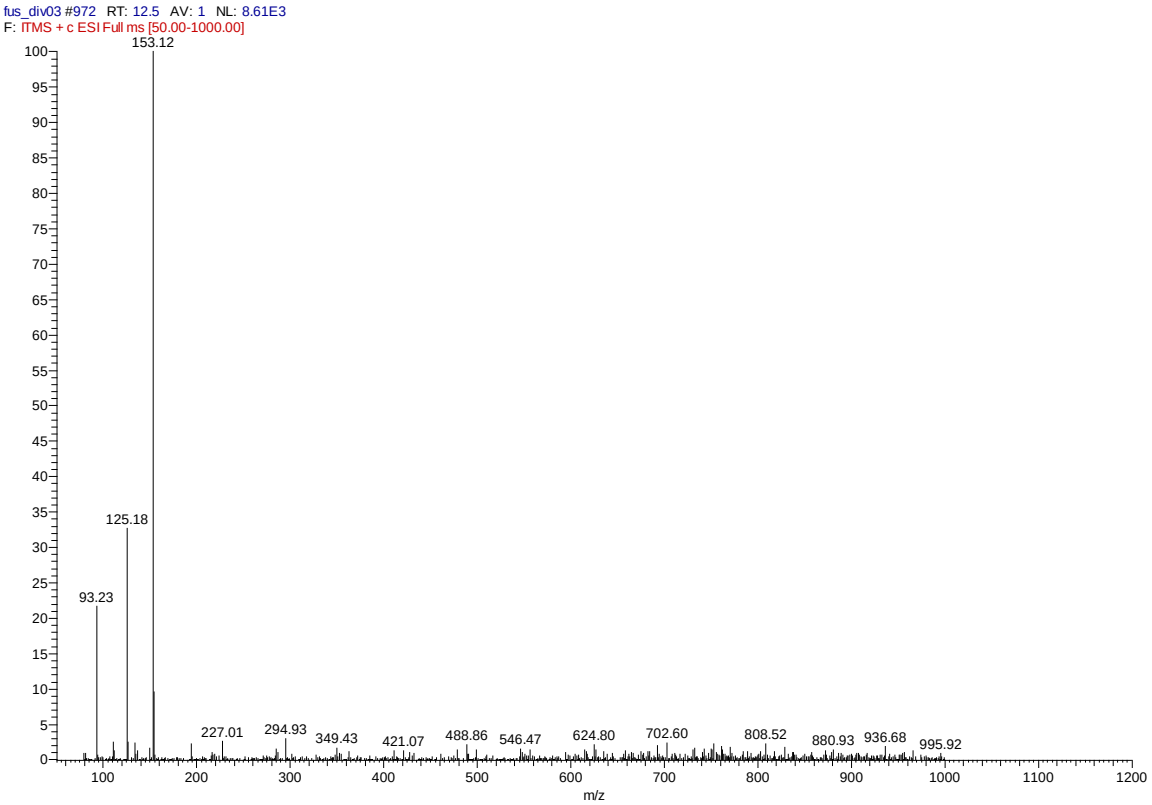


Figure S9: Mass spectrum corresponding to the peak with a retention time of 12.5 min was identified as vanillin.

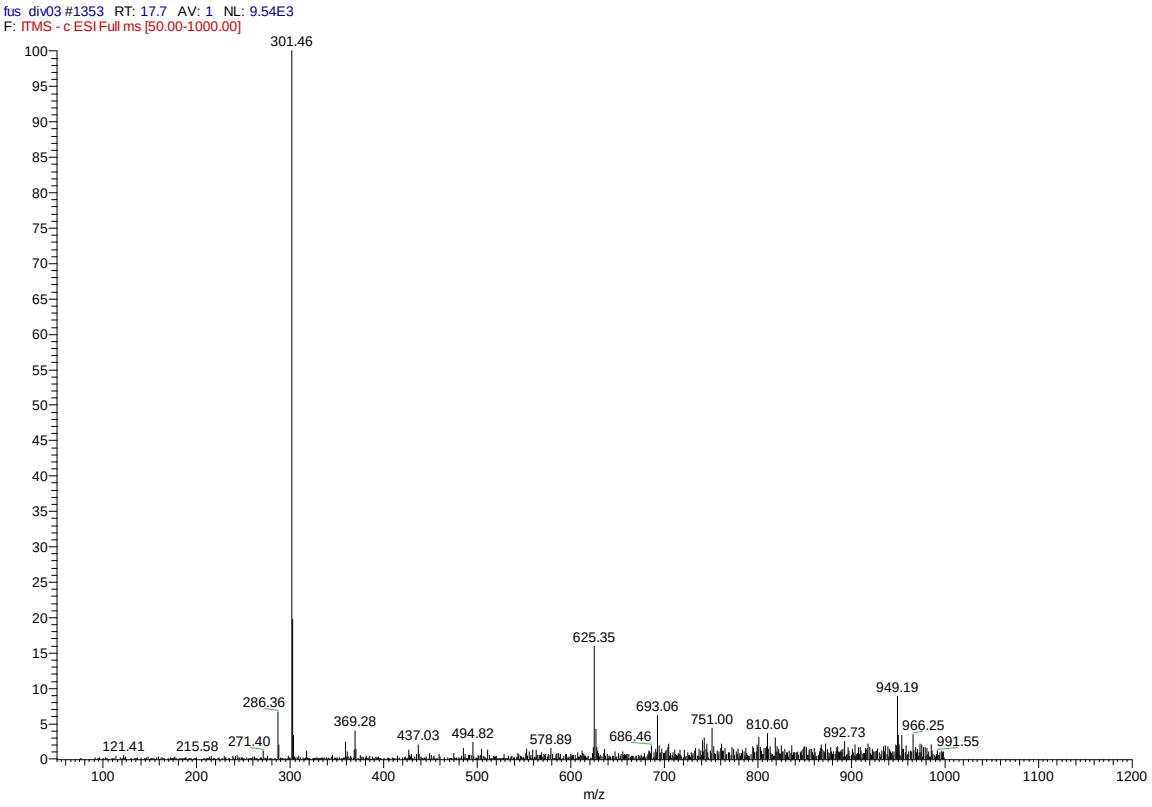


Figure S10: Mass spectrum corresponding to the peak with a retention time of 17.7 min was identified as divanillin.

c. Reaction of P-EugO with vanillyl alcohol at pH 5.5

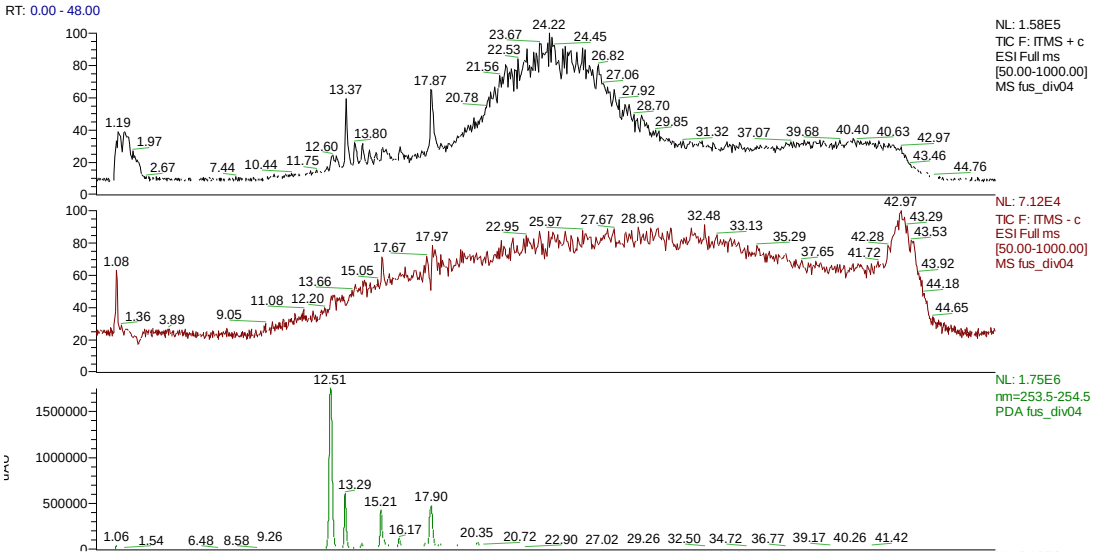


Figure S11: Chromatogram of reaction mixture of P-EugO with vanillyl alcohol at pH 5.5. Total ionic current in positive mode (upper panel), negative mode (middle panel) and UV at 280 nm (lower panel) are shown.

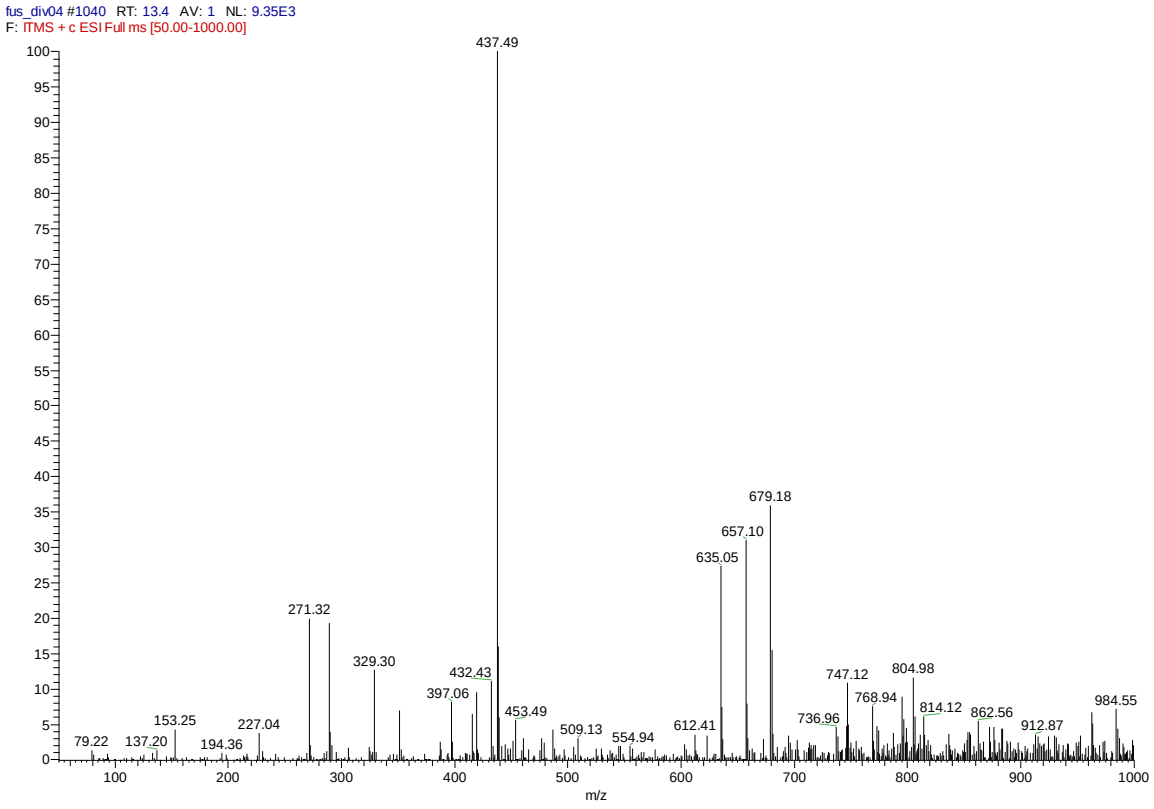


Figure S12: Mass spectrum corresponding to the peak with retention time of 13.4 min shows a mass of 437.49 which indicates that the product is likely a trimer of two vanillin and one vanillyl alcohol units.

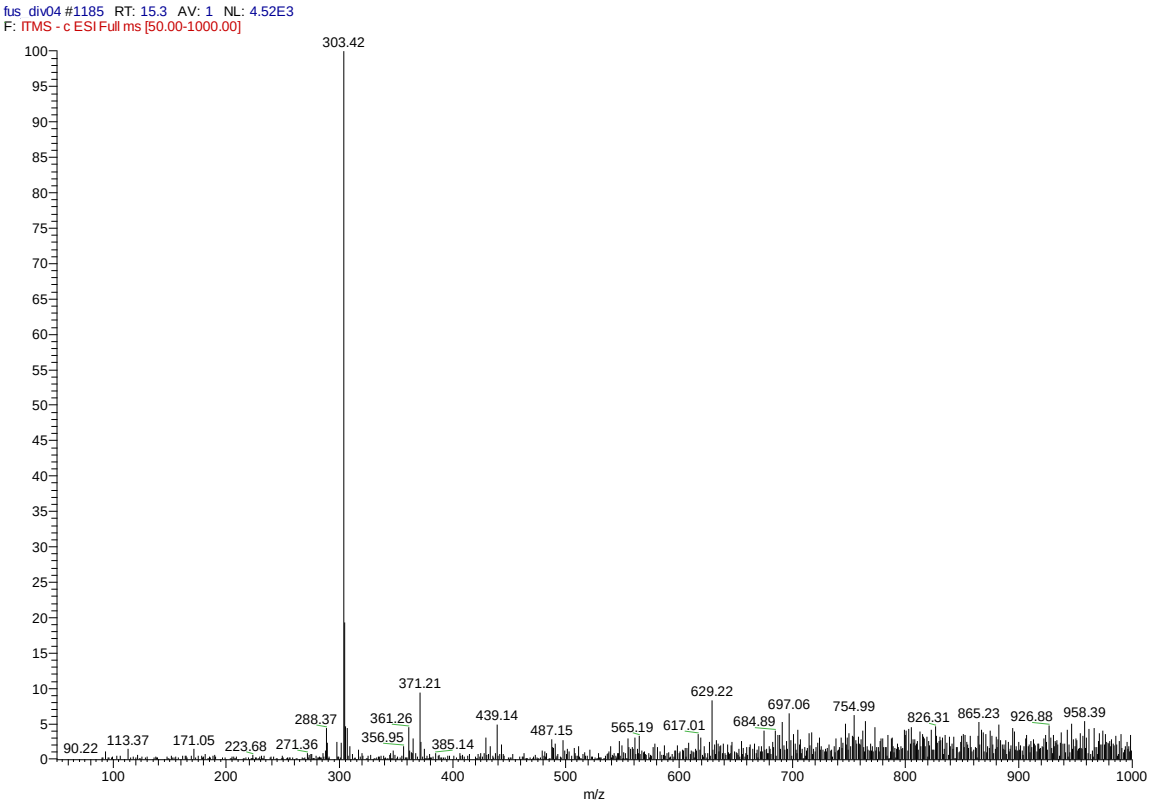


Figure S13: Mass spectrum corresponding to the peak with retention time of 15.3 min shows a mass of 303.42 which indicates that the product is a dimer of vanillyl alcohol and vanillin.

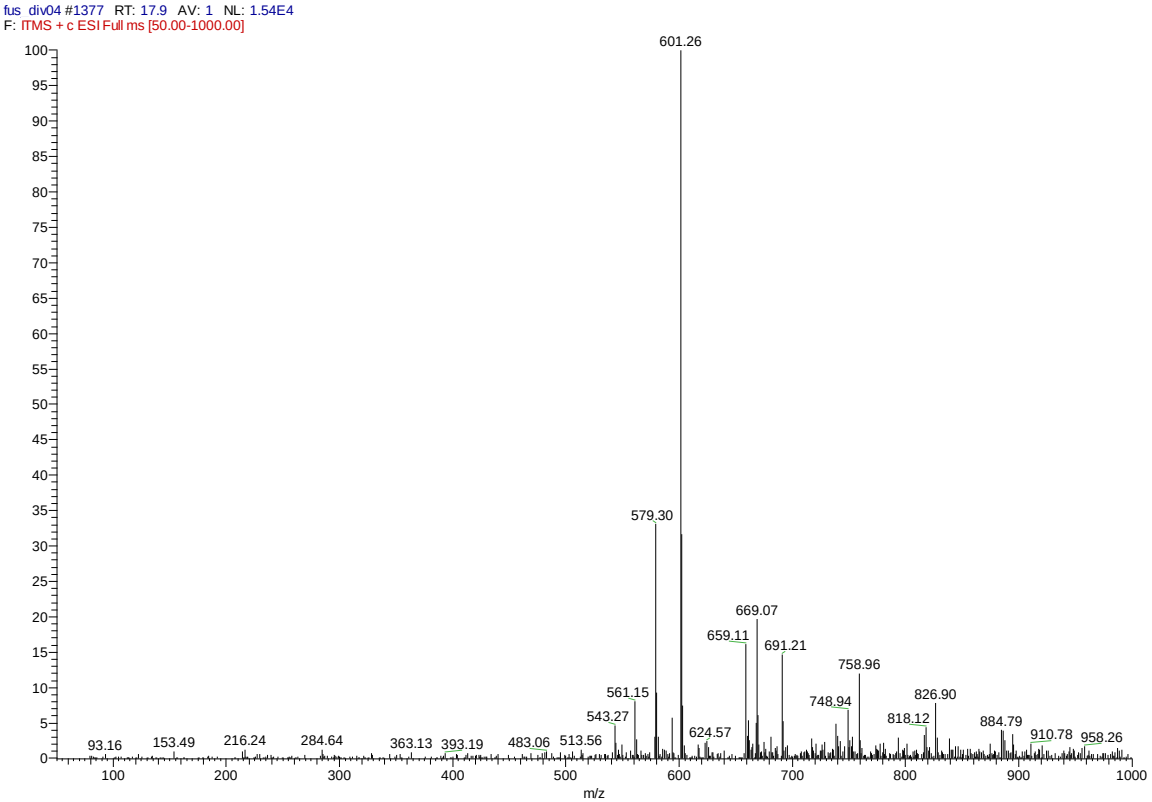
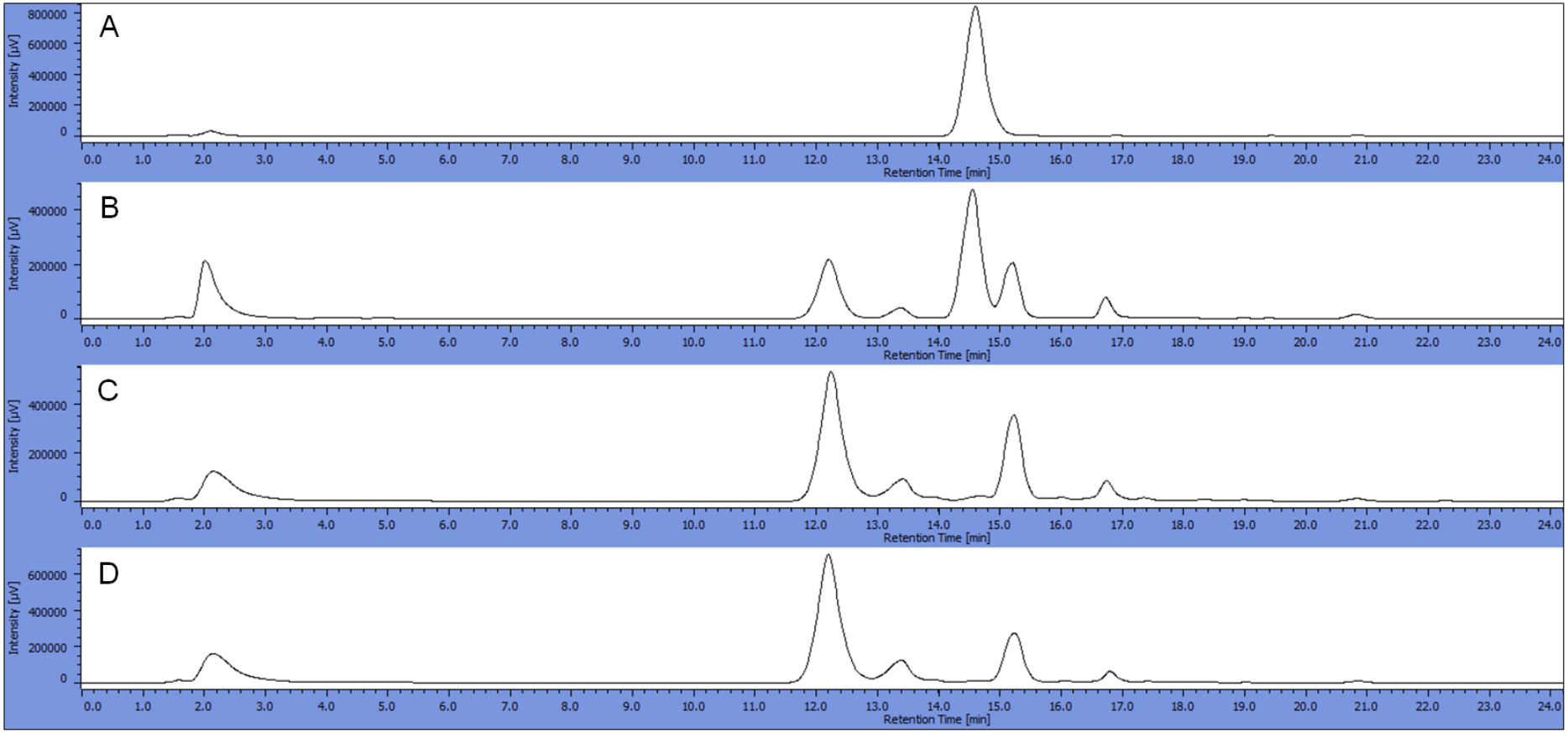


Figure S14: Mass spectrum corresponding to the peak with retention time of 17.9 min shows a mass of 601.26 which indicates that the product is a tetramer.

One-pot cascade reaction for the synthesis of lignin-like oligomers from eugenol

Figure S15: Reverse phase HPLC analysis of the synthesis of low molecular weight lignin-like oligomers from eugenol by fusion enzyme P-EugO and the non-fused enzymes. Panel A, eugenol incubated under the same conditions without the addition of enzyme. Panel B and C show the results after incubation of eugenol with 1.0 µM P-EugO fusion enzyme after 24 hours and 96 hours respectively. Panel D shows the results of eugenol incubation with the non-fused enzymes (1.0 µM *Svi*DyP and 1.0 µM EugO) after 96 h. Peaks at retention times 2.1, 12.2, 13.4, 14.6, 15.2, 20.8 minutes correspond to coniferyl alcohol, phenyl coumaran, pinoresinol, eugenol, lignin-like tetramer and dieugenol, as observed before.[8]



References

[1] D. I. Colpa, M. W. Fraaije, *J. Mol. Catal. B. Enzym.* **2016**, *134*, 372–377.

[2] A. R. Ferrari, M. Lee, M. W. Fraaije, *Biotechnol. Bioeng.* **2015**, *112*, 1074–1080.

[3] J. Jin, H. Mazon, R. H. H. van den Heuvel, D. B. Janssen, M. W. Fraaije, *FEBS J.* **2007**, *274*, 2311–2321.

[4] W. P. Dijkman, M. W. Fraaije, *Appl. Environ. Microbiol.* **2014**, *80*, 1082–1090.

[5] R. T. Winter, D. P. H. M. Heuts, E. M. A. Rijpkema, E. van Bloois, H. J. Wijma, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2012**, *95*, 389–403.

[6] N. Lončar, D. I. Colpa, M. W. Fraaije, *Tetrahedron* **2016**, *72*, 7276–7281.

[7] R. T. Nishimura, C. H. Giammanco, D. A. Vosburg, *J. Chem. Educ.* **2010**, *87*, 526–527.

[8] M. H. M. Habib, P. J. Deuss, N. Lončar, M. Trajkovic, M. W. Fraaije, *Adv. Synth. Catal.* **2017**, 10.1002/adsc.201700650.