Supplementary information

Breaking down lingnin to high-value chemicals: the conversion of lignocellulose to vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1

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Table S1 Identification of lignin breakdown metabolites from *R. jostii* RHA1, *Pseudomonas putida*, *Microbacterium phyllosphaerae*, and *Sphingobacterium* T2, using GC-MS and LC-MS. Strains grown on Luria-Bertani broth containing 1 g/l wheat straw lignocellulose. GC-MS and LC-MS procedures as reported in references 5, 14.

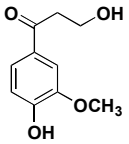
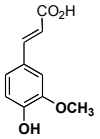
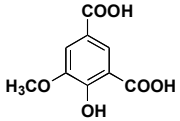
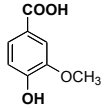
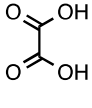
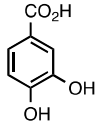
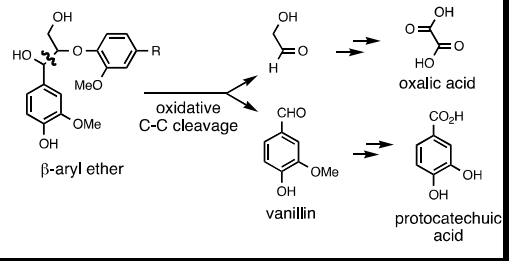
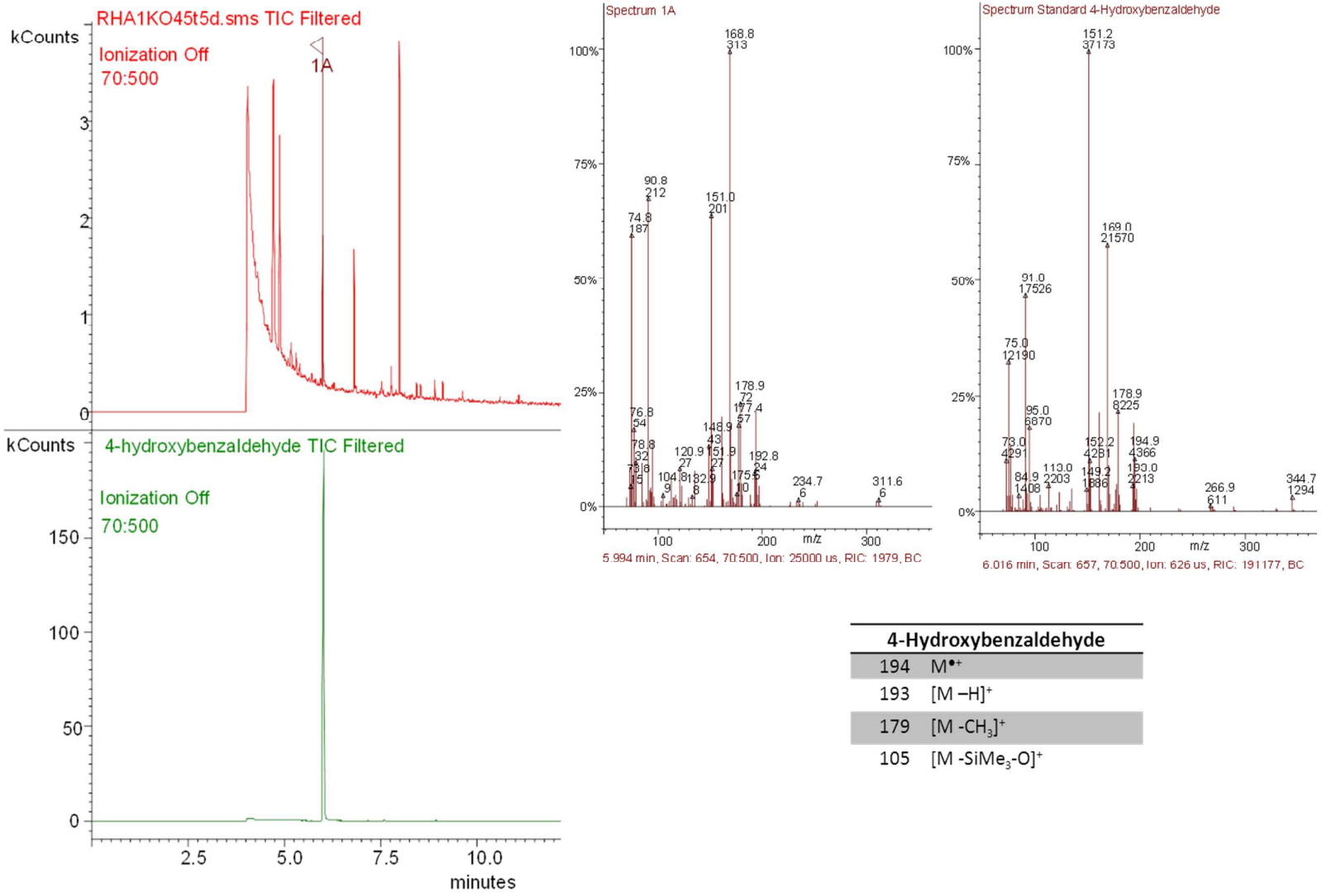
Compound	1 	2 Intradiol/extradiol ring cleavage product	3 Ferulic acid 	4 5-carboxy-vanillic acid 	5 vanillic acid 	6 oxalic acid 	7 proto-catechuic acid 
LC-MS (RT, *m/z*)	4.29 min 235 MK^+	4. 56 min 225 MK^+	5.25 min 195 MH^+	5.76 min 251 MK^+	9.09 min 169 MH^+		
GC-MS (RT, *m/z*, silylated)	7. 02 min 268 M^+ 253 -CH_3	7.71 min 243 M^+ 228 -CH_3	5.27 min 251 M-CH_3	6.03 min 341 M-CH_3		4.40 min 234 M^+	12.20 min 371 M^+
P. putida (ref 5)	Major 6hr, 1d, 3d	Minor 7d	Minor 6hr	Minor 6hr	-	Major 4hr, 24hr, 2d	
R. jostii RHA1 (ref 5)	Major 4 hr, 1d, 5d	Minor 1d, 2d	Major 1d, 5d	Minor 4hr, 6hr, 24 hr	Minor 6 hr	Major 4 hr - 7d	
Microbacterium A1.1 (ref 14)							Minor 5d
Sphingobacterium T2 (ref 14)						Minor 6d	
Probable origin	β-aryl ether breakdown	Oxidative ring cleavage product	Bound to hemi-cellulose, lignin	Intermediate on biphenyl breakdown pathway			

Table S2. Metabolite formation in *Rhodococcus jostii* RHA1 and RHA045 from various growth media. Quantitation via LC-MS except where observed by GC-MS as noted.

***Rhodococcus jostii* RHA1**	**Vanillin**	**p-hydroxy-benzaldehyde**	**Vanillic acid**	**Ferulic acid**	**p-coumaric acid**
Luria-Bertani broth + 1% wheat straw lignocellulose	Not observed	Not observed	Minor metabolite (GCMS, 6 days)	Major metabolite (GCMS, 5 days)	Not observed
M9 minimal media + 1% wheat straw lignocellulose	Not observed	Not observed	0.2 µg/ml (3 days)	20 µg/ml (5 days)	Not observed
M9 minimal media + 0.1% xylan	Not observed	Not observed	<0.01 µg/ml	0.01 µg/ml	Not observed

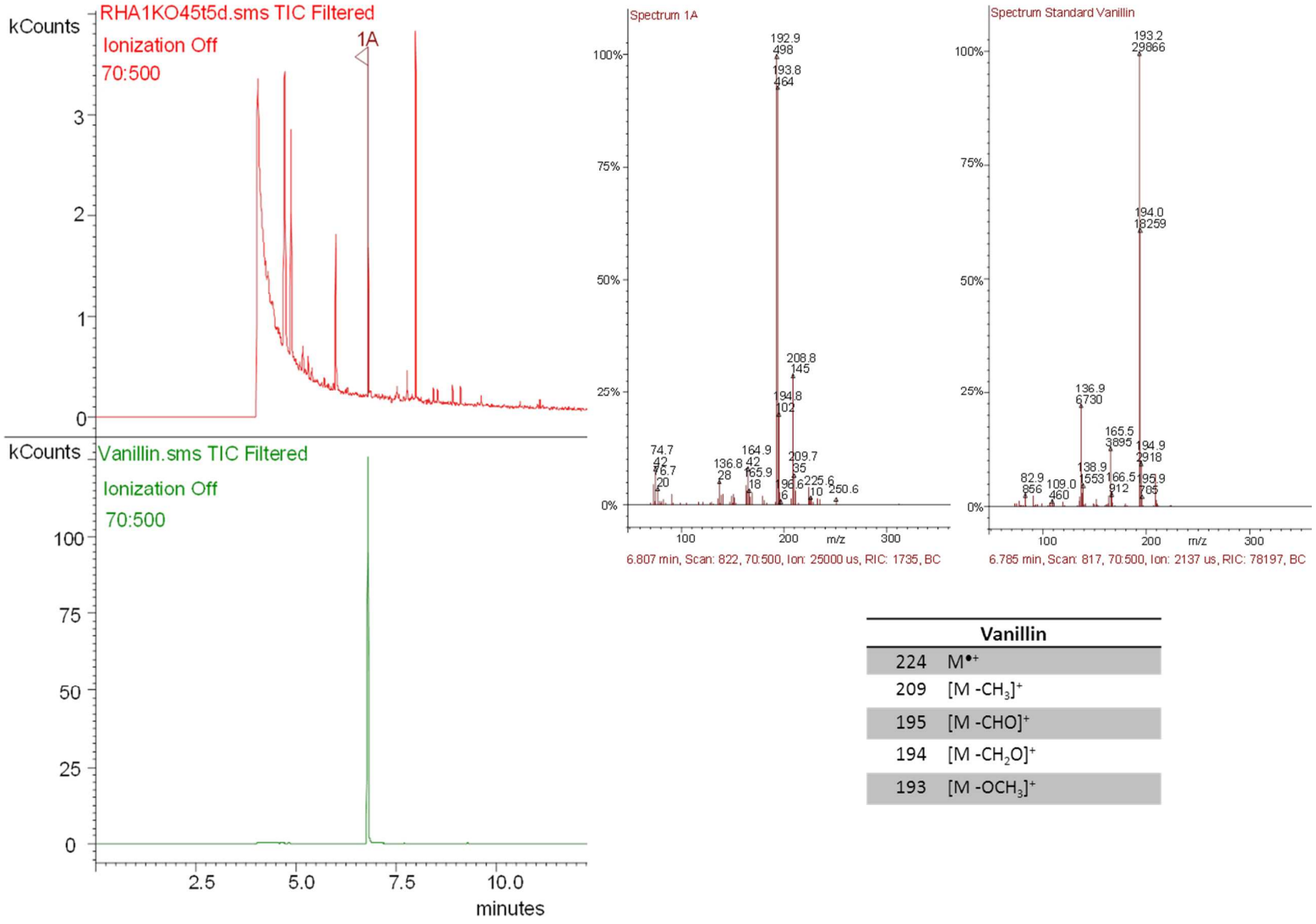
***Rhodococcus jostii* RHA045 (Δ*vdh* mutant)**	**Vanillin**	**p-hydroxy-benzaldehyde**	**Vanillic acid**	**Ferulic acid**	**p-coumaric acid**
M9 minimal media + 1% wheat straw lignocellulose	Major (GCMS, 5 days)	Major (GCMS, 5 days)	Minor (GCMS, 5 days)	Minor (GCMS, 5 days)	Minor (GCMS, 5 days)
M9 minimal media + 2.5% wheat straw lignocellulose	96 µg/ml (6 days)	53 µg/ml (5 days)	3-120 µg/ml (4-6 days)	23-86 µg/ml (4-6 days)	Minor peak, not quantified
M9 minimal media + 0.1% xylan	Not observed	Not observed	<0.01 µg/ml	0.01 µg/ml	Not observed

Figure S1 GC-MS data and standard for 4-hydroxybenzaldehyde (mono-$SiMe_3$ derivative)



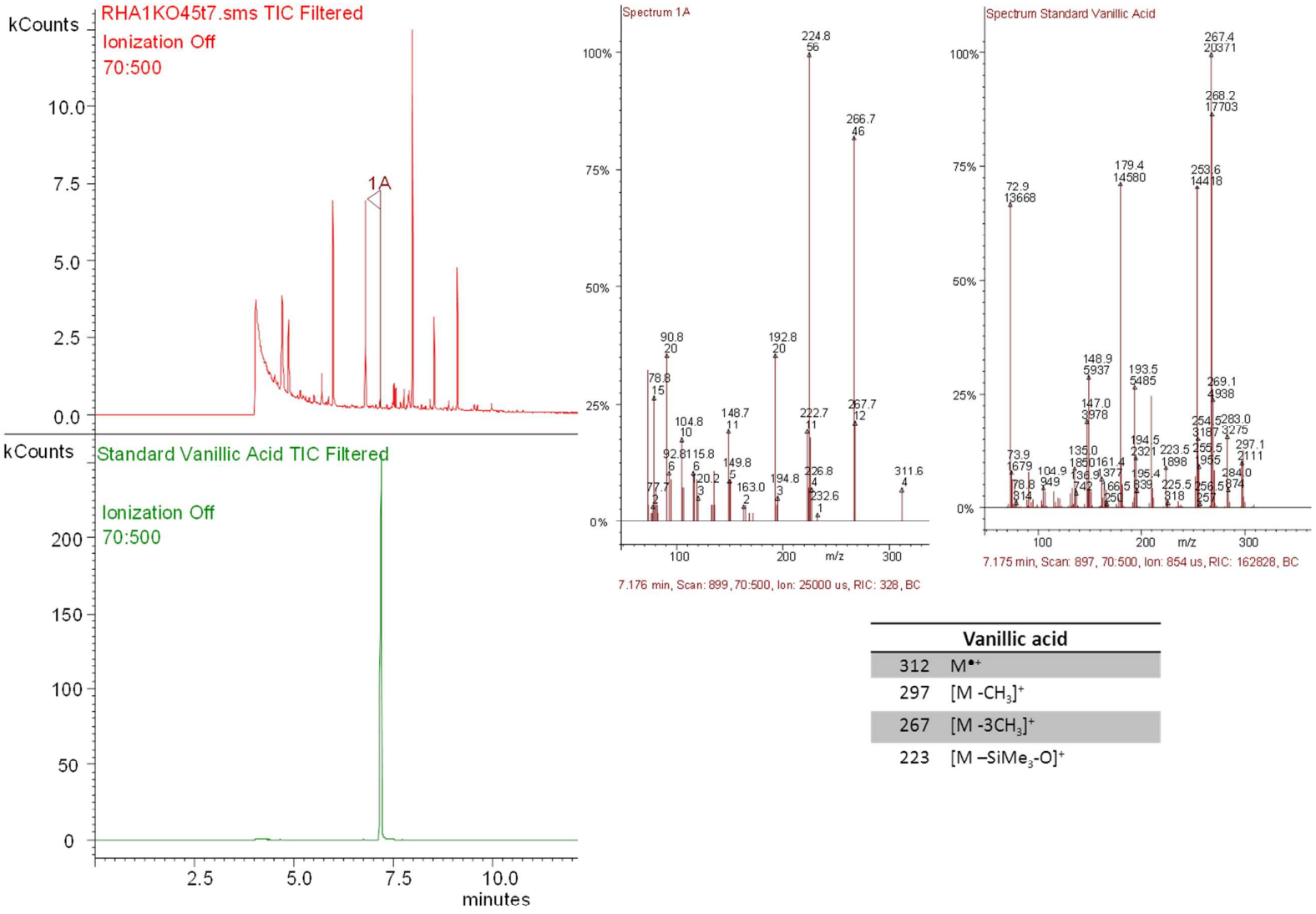
4-Hydroxybenzaldehyde	
194	$M^{\bullet+}$
193	$[M-H]^+$
179	$[M-CH_3]^+$
105	$[M-SiMe_3\text{-}O]^+$

Figure S2 GC-MS data and standard for vanillin (mono-$SiMe_3$ derivative)



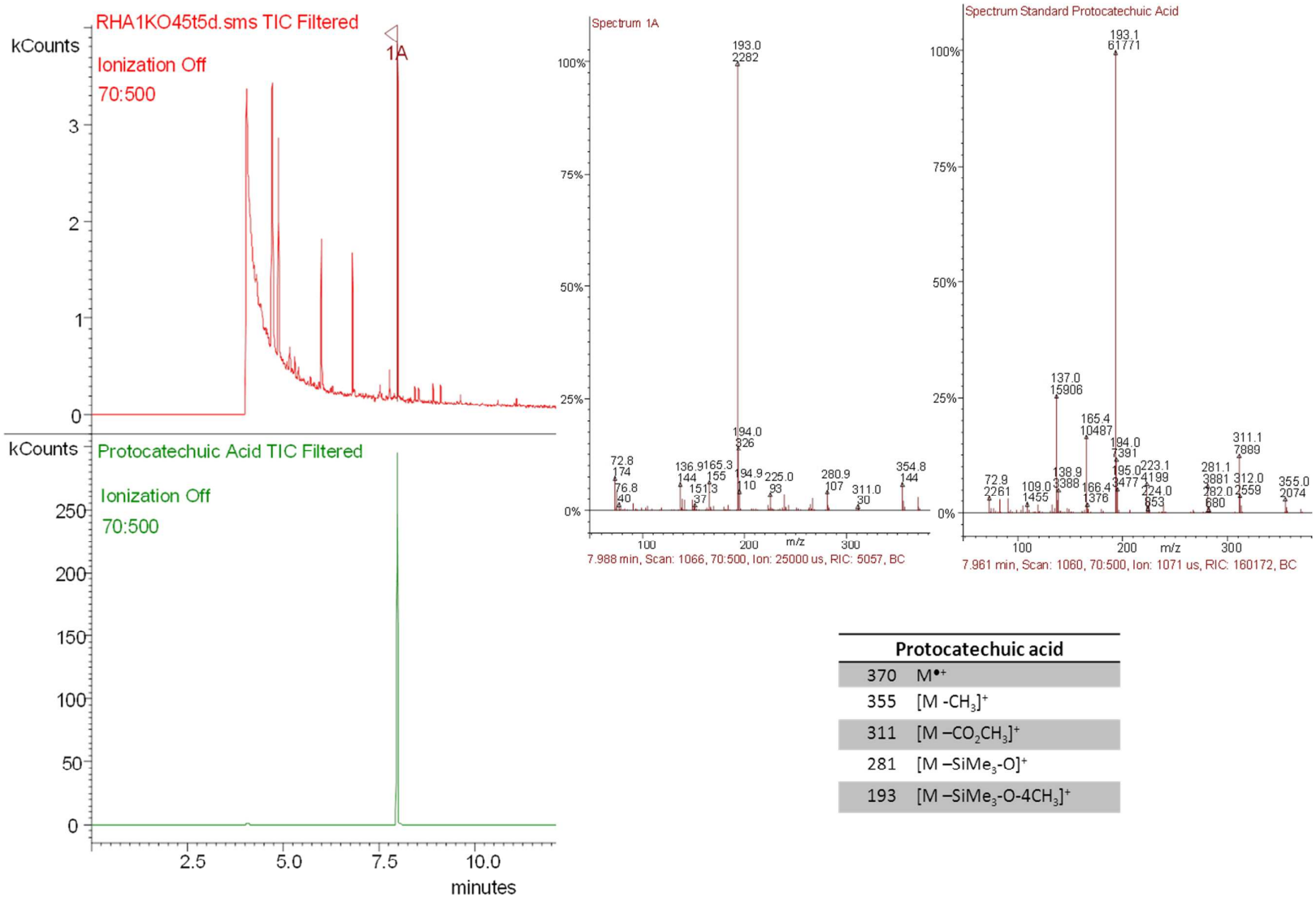
Vanillin	
224	$M^{\bullet+}$
209	$[M - CH_3]^+$
195	$[M - CHO]^+$
194	$[M - CH_2O]^+$
193	$[M - OCH_3]^+$

Figure S3 GC-MS data and standard for vanillic acid (mono-$SiMe_3$ derivative)



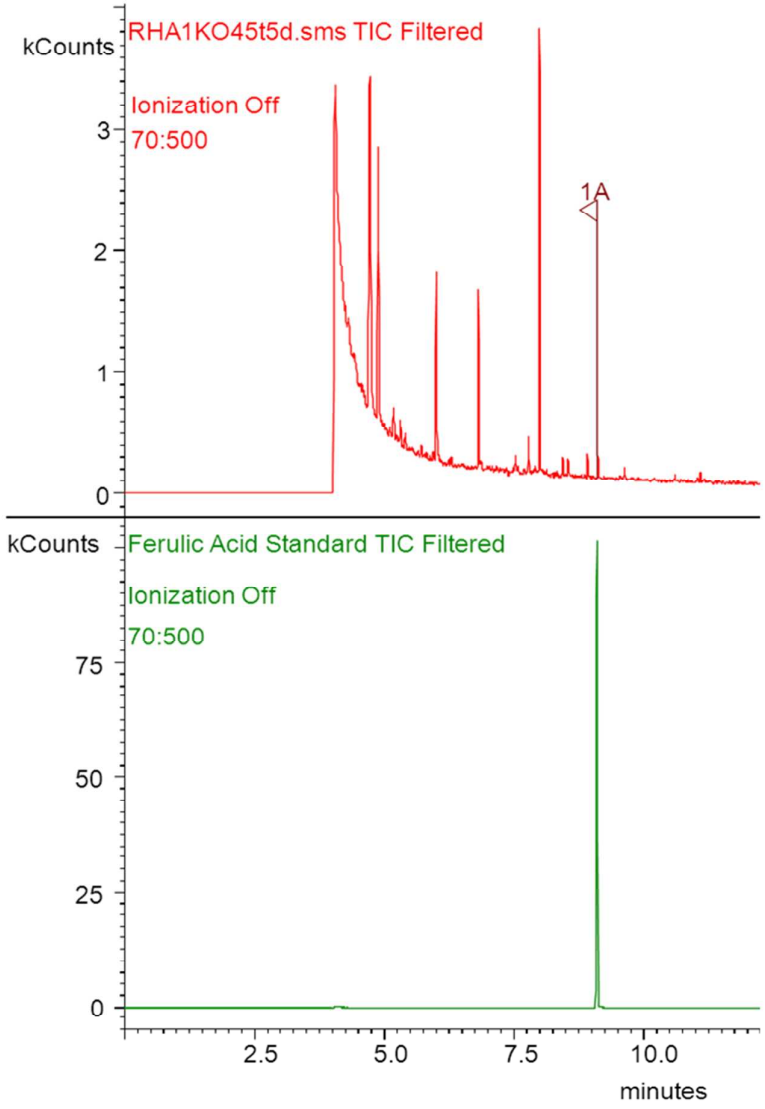
Vanillic acid	
312	$M^{\bullet+}$
297	$[M -CH_3]^+$
267	$[M -3CH_3]^+$
223	$[M –SiMe_3\text{-}O]^+$

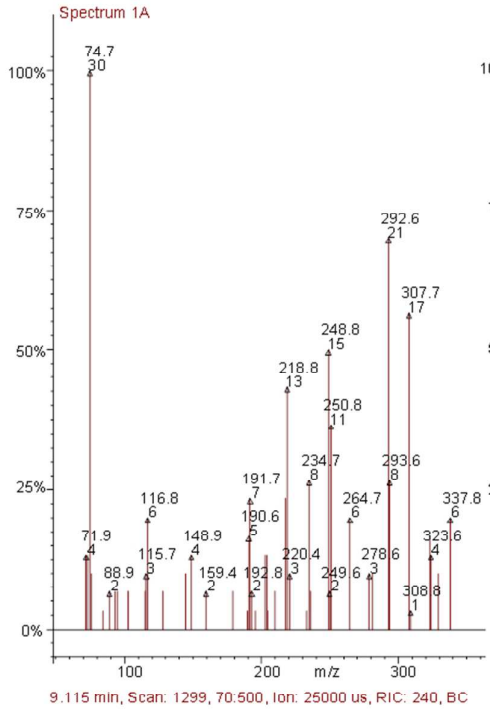
Figure S4 GC-MS data and standard for protocatechuic acid (di-$SiMe_3$ derivative)

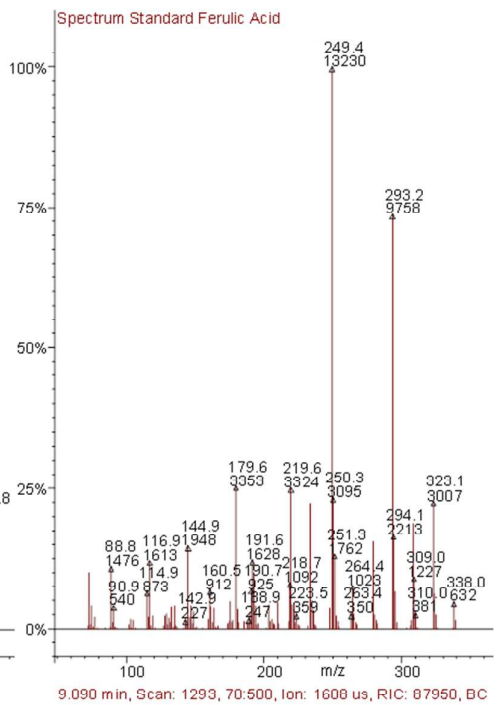


Protocatechuic acid	
370	$M^{\bullet+}$
355	$[M - CH_3]^+$
311	$[M - CO_2CH_3]^+$
281	$[M - SiMe_3\text{-}O]^+$
193	$[M - SiMe_3\text{-}O\text{-}4CH_3]^+$

Figure S5 GC-MS data and standard for ferulic acid (mono-$SiMe_3$ derivative)

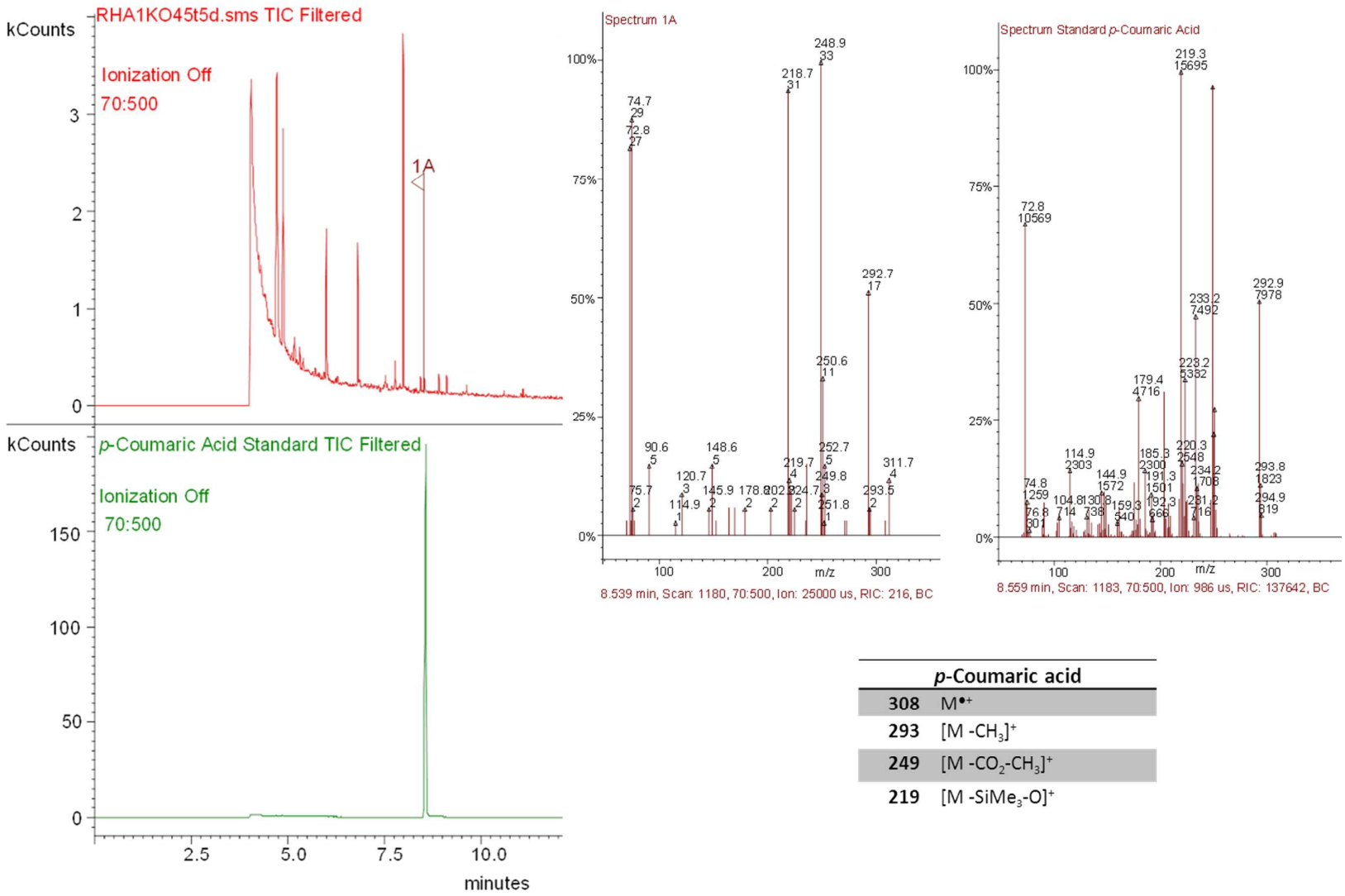






Ferulic acid	
338	$M^{\bullet+}$
323	$[M - CH_3]^+$
308	$[M - OCH_2]^+$
293	$[M - CO_2H]^+$
251	$[M - SiMe_3 - CH_3]^+$
249	$[M - SiMe_3 - O]^+$
219	$[M - SiMe_3 - O - 2CH_3]^+$

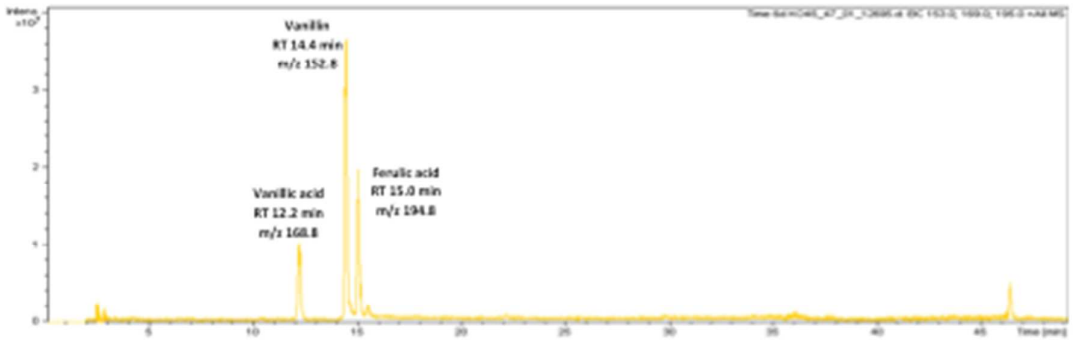
Figure S6 GC-MS data and standard for *p*-coumaric acid (mono-$SiMe_3$ derivative)



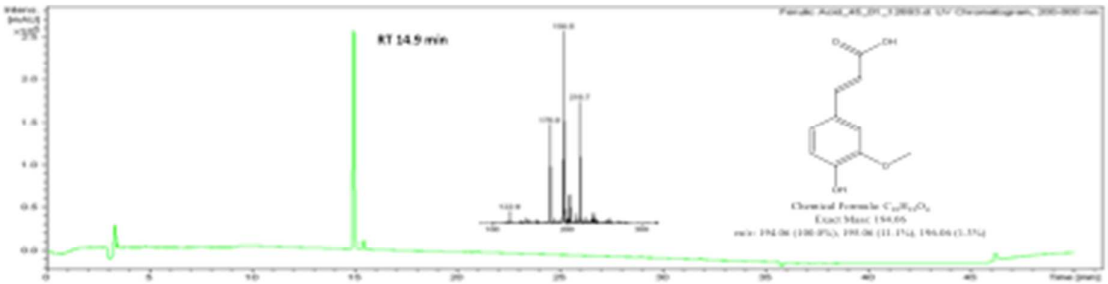
p-Coumaric acid	
308	$M^{\bullet+}$
293	$[M - CH_3]^+$
249	$[M - CO_2 - CH_3]^+$
219	$[M - SiMe_3 - O]^+$

Figure S7. LC-MS data and authentic standards for ferulic acid, vanillic acid and vanillin.

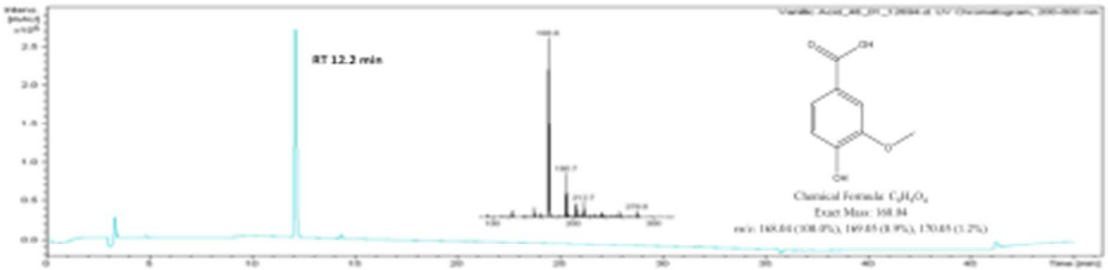
A. Extracted ion chromatogram +ve mode (*m/z* 153.0, 169.0 and 195.0) of sample taken from incubation of *R. jostii* RHA1 Δ*vdh* with lignocellulose at time 6 days.



B. LC-MS chromatogram and mass spectrum of ferulic acid standard (retention time 14.9 min, MH^+ 194.8)



C. LC-MS chromatogram and mass spectrum of vanillic acid standard (retention time 12.2 min, MH^+ 168.8)



D. LC-MS chromatogram and mass spectrum of vanillin standard (retention time 14.4 min, MH^+ 152.8)

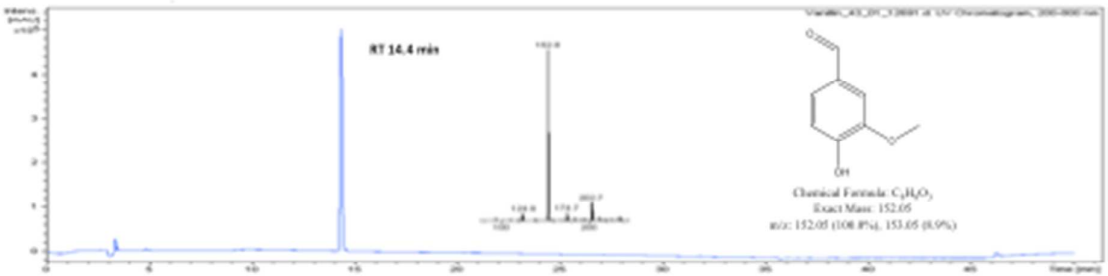


Figure S8 LC-MS data (extracted ion chromatograms) for *R. jostii* RHA045 in minimal medium containing 2.5% wheat straw lignocellulose and 0.05% glucose after 0, 72, 96, 120, 144, 168 hr, compared with wild-type *R. jostii* RHA1 after 96 hr. Metabolites: ferulic acid, 17.2 min; vanillin, 15.1 min; vanillic acid, 11.2 min; vanillyl alcohol, 15.6 min; 4-hydroxybenzaldehyde, 13.2 min.

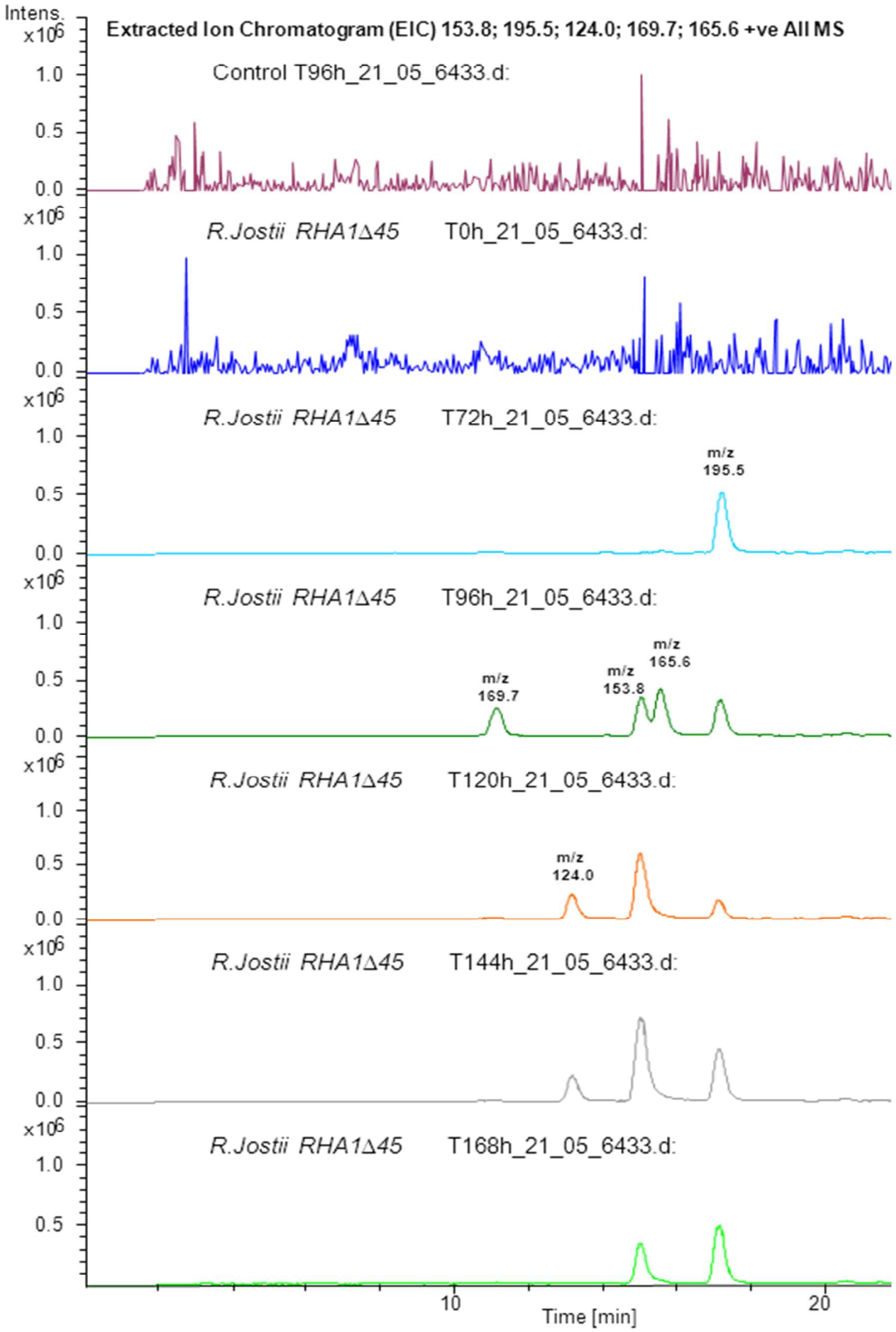


Figure S9. Growth curves for *R. jostii* mutants RHA045 *(Δvdh)* and RHA046 *(ΔvanA)* and wild-type RHA1 on M9 minimal medium containing 0.5 mM vanillic acid or ferulic acid, showing similar levels of growth for wild-type and RHA045, but low growth of RHA046 on ferulic acid, suggesting that ferulic acid is metabolized to vanillic acid, rather than vanillin (low growth of RHA046 on ferulic acid due to release of one equivalent of acetyl CoA).

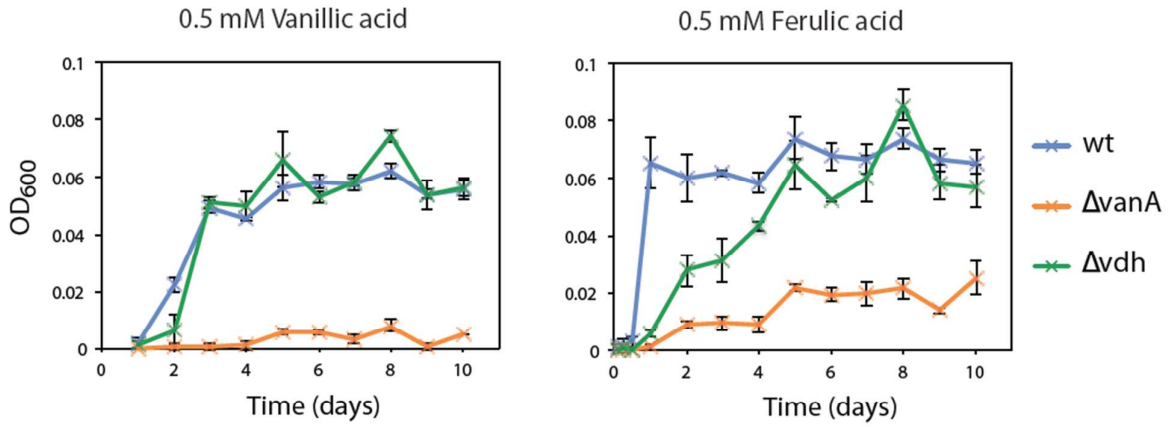


Figure S10. HPLC analysis of RHA046 *(ΔvanA)* and RHA045 *(Δvdh)* grown on M9 minimal medium containing 0.5 mM ferulic acid, showing conversion of ferulic acid to vanillic acid in RHA046. UV detection at 280 nm.

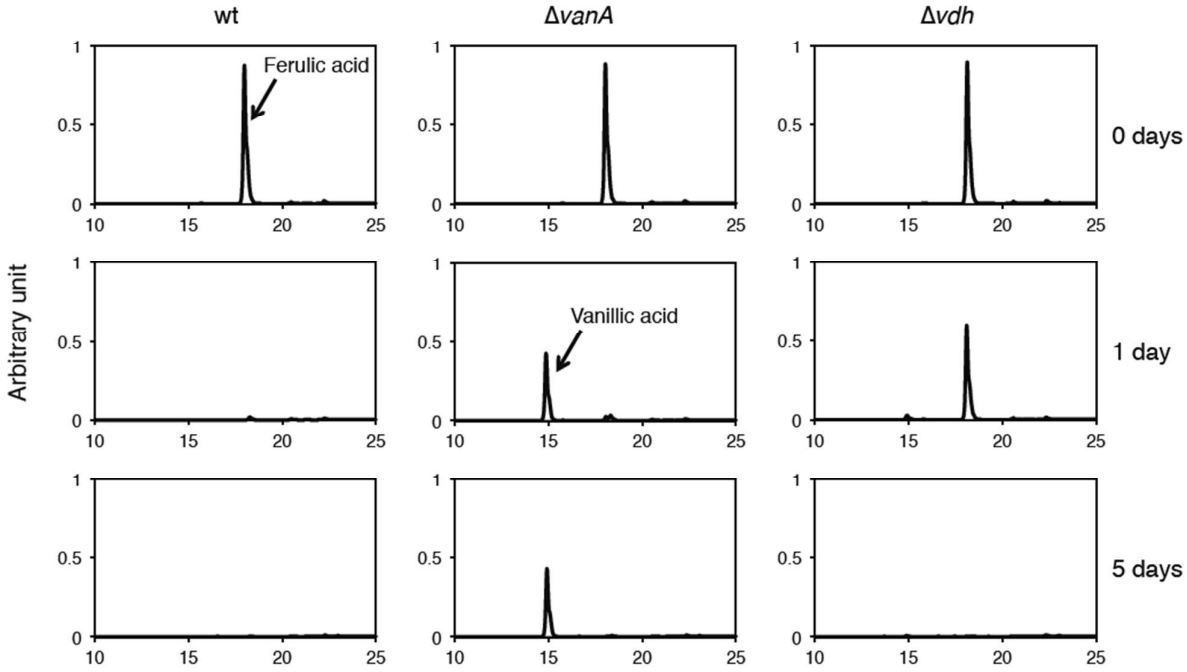


Figure S11. Time course of metabolite production from culture of *R. jostii* RHA045 (*Δvdh* mutant) on M9 minimal medium containing 0.5% w/v wheat straw alkali lignin (obtained as described in Materials and Methods) and 0.05% w/v glucose, over 5 days at 30 °C. Bacterial growth was assessed by OD600. VAN, vanillin; FA, ferulic acid; VAN AC, vanillic acid; 4HBA, 4-hydroxybenzaldehyde. Additional peaks containing higher molecular weight material (*m/z* > 1000) were also observed in this experiment, indicative of condensed or repolymerised lignin.