
This is an electronic reprint of the original article.
This reprint may differ from the original in pagination and typographic detail.

Belt, Tiina; Mollerup, Filip; Hänninen, Tuomas; Rautkari, Lauri

Inhibitory effects of Scots pine heartwood extractives on enzymatic holocellulose hydrolysis by wood decaying fungi

Published in:
International Biodeterioration and Biodegradation

DOI:
[10.1016/j.ibiod.2018.03.004](https://doi.org/10.1016/j.ibiod.2018.03.004)

Published: 01/08/2018

Document Version
Peer-reviewed accepted author manuscript, also known as Final accepted manuscript or Post-print

Published under the following license:
Unspecified

Please cite the original version:
Belt, T., Mollerup, F., Hänninen, T., & Rautkari, L. (2018). Inhibitory effects of Scots pine heartwood extractives on enzymatic holocellulose hydrolysis by wood decaying fungi. *International Biodeterioration and Biodegradation*, 132, 150-156. <https://doi.org/10.1016/j.ibiod.2018.03.004>

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.

Inhibitory effects of Scots pine heartwood extractives on enzymatic holocellulose hydrolysis by wood decaying fungi

Authors: Tiina Belt*, Filip Mollerup, Tuomas Hänninen, Lauri Rautkari

Affiliations:

Aalto University, School of Chemical Engineering, Department of Bioproducts and Biosystems, P.O.Box 16300, 00076 Aalto, Finland

*Corresponding author

E-mail: tiina.belt@aalto.fi

Tel.: +358 456729190

Abstract

The heartwood of Scots pine contains extractives that protect it against wood decaying fungi. Pine extractives such as pinosylvins are fungicidal compounds, but they may also have other mechanisms of action. This experiment investigated whether pinosylvins and other heartwood extractives can act as inhibitors of holocellulose hydrolysis, similarly to many other biomass-derived phenolic compounds. The inhibitory properties of extractives were studied against enzymes secreted by a brown rot (*Coniophora puteana*) and a white rot fungus (*Trametes versicolor*), as well as against a commercial *Trichoderma reesei* enzyme cocktail. The extractives were studied as wood-free extracts and extractives-containing wood powders. In all experiments, the behaviour of the white rot differed from that of the other two. The white rot hydrolases were strongly inhibited and deactivated by extractives, particularly pinosylvins, whereas the others showed only mild or moderate inhibition and no deactivation. The white rot enzymes seemed to modify the pinosylvins, with further studies suggesting that the modified pinosylvins may form complexes with enzymes and cause their deactivation. These results suggest that pine heartwood extractives have potential to contribute to decay resistance as hydrolase inhibitors but only when the fungus produces enzymes capable of modifying the extractives.

Keywords: cellulase, heartwood, inhibition, pinosylvin, Scots pine, xylanase

1. Introduction

Wood extractives are biologically active secondary metabolites produced by trees. Extractives often accumulate in the heartwood (HW), where they increase the resistance of the wood material to decay and other forms of fungal and insect attack. Due to the great economic significance of decay resistance in wood products, the formation and properties of HW and its extractives have been extensively studied (Hillis. 1987, Taylor et al. 2002).

In Scots pine (*Pinus sylvestris*), the decay resistance of HW is typically classified as either moderate or slight, making the HW more durable than the sapwood or the woods of other common local trees such as spruce or birch (EN 350, Jebrane et al. 2014, Plaschkies et al. 2014,

42 Van Acker et al. 2003). The HW extractives of Scots pine consist primarily of resin acids and
43 pinosylvins (Willför et al. 2003, Fang et al. 2013), both of which have been implicated in the
44 decay resistance of pine HW (Harju et al. 2002, 2003, Venäläinen et al. 2003, 2004). While
45 there are conflicting results on the significance of resin acids, all of these studies have
46 demonstrated that pinosylvins play a role in decay resistance. Pinosylvins are fungicidal to a
47 range of wood decaying fungi (Hart and Shrimpton. 1979, Seppänen et al. 2004), but their
48 mechanism of action and other possible means of decay prevention are poorly characterised.
49 Pinosylvins may, for example, contribute to decay resistance as antioxidants, by interfering
50 with the oxidative wood degradation mechanisms of fungi (Belt et al. 2017). Pinosylvins may
51 also be capable of inhibiting the enzymatic hydrolysis of holocellulose to digestible sugars, but
52 this potential mechanism has not yet been explored.

53 While pinosylvins have not been studied as inhibitors of carbohydrate hydrolases, other
54 biomass-derived aromatics such as lignins and low molecular weight phenolics have been the
55 topic of extensive study. Lignins block the access of enzymes to holocellulose and cause the
56 unproductive adsorption of enzymes onto their surface, resulting in reduced hydrolysis
57 (Palonen et al. 2004, Rahikainen et al. 2011, Rollin et al. 2011). Smaller phenolics, whether
58 lignin derived or not, can also inhibit cellulases and hemicellulases. The smaller phenolics have
59 been shown to form soluble and insoluble complexes with enzymes, causing enzyme
60 deactivation and inhibition of hydrolysis (Boukari et al. 2011, Sharma et al. 1985, Tejirian and
61 Xu. 2011, Ximenes et al. 2011). As phenolic compounds the pinosylvins may have similar
62 effects, although the possibility of other extractives taking part in hydrolase inhibition cannot
63 be excluded. Resin acids, for example, have previously been found to inhibit cellulose
64 hydrolysis (Leskinen et al. 2015).

65 In this study, Scots pine HW extractives were tested for their ability to inhibit the enzymatic
66 hydrolysis of wood polysaccharides. The effects of the extractives were tested against
67 extracellular enzymes secreted by cultures of brown rot (*Coniophora puteana*) and white rot
68 (*Trametes versicolor*) fungi, which differ in their mechanisms of wood degradation and may
69 therefore differ in their susceptibility to inhibition by extractives. White rot fungi degrade wood
70 by means of a full cellulase system and lignin-degrading enzymes such as peroxidases and
71 laccases, whereas brown rots employ a non-enzymatic degradation system at the initial stages
72 of decay, followed by enzymatic attack using hemicellulases and typically an incomplete
73 cellulase system (Riley et al. 2014, Zhang et al. 2016). The effects of extractives were also
74 tested against a commercial *Trichoderma reesei* cellulase preparation. Extractives were studied
75 as inhibitors and deactivators of holocellulose hydrolysis and their mechanisms of action
76 investigated. In all experiments, the extractives were studied as wood-free extracts and as
77 extractives-containing wood powders, to determine whether the inhibitory effects are
78 significant also when the extractives are associated with their natural wood matrix.

79

80 **2. Materials and methods**

81 **2.1 Wood material and extract processing**

82 The wood material used in this experiment was obtained from a freshly felled 70 year old Scots
83 pine tree that was stored frozen until use. Discs were sawn from a section of the trunk at approx.
84 1 m height, and HW material was cut from the discs. The HW material was ground to a fine
85 powder in a Wiley mill (0.5 mm mesh) and freeze-dried. A portion of the powder was then
86 Soxhlet extracted (6 h, 300 mL solvent and approx. 10 g powder) with either acetone or

87 sequentially with n-hexane and MeOH. Small aliquots of each extract were taken for
88 composition analysis by GC and GC-MS. The remaining acetone and MeOH extracts were
89 evaporated to dryness and redissolved in EtOH, while residual solvents were evaporated from
90 the wood powders. The acetone (AE) and methanol (ME) extracts and the unextracted (UEW),
91 hexane extracted (HEW), and hexane + methanol extracted (HMEW) wood powders were used
92 to study the inhibitory properties of HW extractives.

93

94 **2.2 Enzyme production**

95 The brown rot fungus *Coniophora puteana* (strain BAM Ebw. 15, Federal institute for materials
96 research and testing, Germany) and the white rot fungus *Trametes versicolor* (strain PRL 572,
97 University of Helsinki culture collection, Finland) were maintained on 2% malt extract agar.
98 For enzyme production, five pieces of agar (approx. 1 mm² in size) from the growing edges of
99 mycelium were used to inoculate 50 mL batches of culture medium, which contained (per L) 3
100 g yeast extract, 3 g tryptone, 0.8 g NaH₂PO₄, 0.5 g MgSO₄ x 7H₂O, 0.4 g K₂HPO₄ x 3H₂O, and
101 50 g acetone-extracted Scots pine sapwood flour. The pH of the medium was set to 5.5 before
102 autoclaving. The fungi were grown stationary in the dark for 11 days at 28°C, after which the
103 culture medium was separated by filtration and concentrated in spin columns (10 kDa MW cut-
104 off). Celluclast 1.5L, a *Trichoderma reesei* cellulase preparation, was purchased from Sigma
105 Aldrich and used as received.

106

107 **2.3 Inhibition and deactivation experiments**

108 All hydrolyses were conducted in 2 mL capped tubes in an inverting mixer. In a total volume
109 of 1 mL, each hydrolysis reaction contained 50 mM acetate buffer (pH 5.0), 0.1 mg of enzyme,
110 and 10 mg of bleached softwood Kraft pulp as substrate. Hydrolyses with *C. puteana* and *T.*
111 *versicolor* enzymes were performed at 30°C, whereas hydrolyses with Celluclast were
112 performed at 30 and 50°C to determine whether an increase in hydrolysis temperature changes
113 the effects of extractives. Each hydrolysis was run for 24 h, after which the release of
114 monosaccharides was determined by high performance anion exchange chromatography
115 (HPAEC). All hydrolyses were performed in triplicate.

116 Inhibition of hydrolysis by extractives was studied by combining extractives, enzyme, and
117 substrate immediately at the beginning of hydrolysis. Extracts were studied at a concentration
118 of 2.5 mg mL⁻¹, and due to their hydrophobicity, they were added from an EtOH solution (final
119 EtOH concentration 2.5%, v v⁻¹) to produce a stable suspension. The wood powders were added
120 at a concentration of 25 mg mL⁻¹, because the extractives content of the UEW was approx. 10%.
121 The sugar yields of extract hydrolyses were compared to a reference hydrolysis containing an
122 equal volume of pure EtOH in place of extract, while the yields of wood powder hydrolyses
123 were compared to a reference containing 25 mg mL⁻¹ of HMEW.

124 Deactivation of enzymes by extractives was studied by incubating the enzymes with extractives
125 prior to hydrolysis. Enzymes and extractives were combined at the same concentrations as used
126 in hydrolysis and incubated at hydrolysis temperature for 24 h. After incubation, the enzyme-
127 extractives mixtures were combined with substrate and hydrolysis commenced. Samples with
128 pure EtOH and HMEW, also incubated at hydrolysis temperature for 24 h, served as references.

129

130 **2.4 Enzyme distribution experiments**

131 To study whether extractives bind or precipitate enzymes, the extractives and enzymes were
132 combined without substrate at the concentrations specified in section 2.3 and incubated at
133 hydrolysis temperature for 24 h. Two different kinds of enzyme distribution experiments were
134 performed: one studying protein content and the other studying hydrolytic activity. In the
135 protein content study, the samples were centrifuged at the end of the incubation period and the
136 supernatants collected. The supernatants were extracted with 1% (w v⁻¹) of
137 polyvinylpyrrolidone to remove solubilised phenolics and then filtered through 0.2 µm
138 syringe filters. The protein contents of the supernatants were measured and compared to that of
139 a similarly treated reference containing pure EtOH or HMEW.

140 In the activity experiment, the samples were again centrifuged after incubation and separated
141 into supernatant and solids fractions. The supernatants were extracted with 1%
142 polyvinylpyrrolidone and filtered, while the solids were resuspended in fresh buffer. The
143 extract solids were resuspended by first adding EtOH to the final 2.5% (v v⁻¹) concentration,
144 followed by the addition of buffer and manual disruption with a small spatula to disperse the
145 remaining solids. The extracted supernatants and the resuspended solids were then used to
146 hydrolyse the pulp substrate. Samples with pure EtOH and HMEW, also incubated at hydrolysis
147 temperature for 24 h, served as references. All enzyme distribution experiments were performed
148 in triplicate.

149

150 **2.5 Modification of pinosylvins**

151 The modification of pinosylvins by the enzyme preparations was studied in substrate-free
152 incubations using the same enzyme and extractives concentrations as in section 2.3. The
153 incubations were conducted at hydrolysis temperature, with samples withdrawn after 4, 8, and
154 24 hours of incubation. The ME reactions were conducted in a volume of 1 mL, and 250 µL
155 aliquots were withdrawn at the indicated times and directly processed for analysis by GC. The
156 UEW reactions were also conducted in a volume of 1 mL but in individual tubes. At each time
157 point, a set of tubes was withdrawn, centrifuged, and the supernatants discarded. The solid
158 powders were washed once with 1 mL of water, and then extracted twice with 1 mL of MeOH
159 in a sonicator (45°C, 30 min per extraction). The two MeOH extracts were collected and
160 combined, then 1 mL portions of the combined extracts were processed for analysis by GC. The
161 modification incubations were conducted in triplicate.

162

163 **2.6 Protein analyses and enzyme activities**

164 Protein contents were determined using the Bio-Rad protein assay reagent according to the
165 manufacturer's instructions, with bovine serum albumin as standard.

166 Total cellulase activity was measured according to Ghose (1987) using filter paper as substrate.
167 Endoglucanase and xylanase activities were measured with 1% CMC (low viscosity, DS 0.7)
168 and xylan (birchwood) as substrates, respectively, using the xylanase assay procedure of Bailey
169 et al. (1992). The release of reducing sugars was determined with the dinitrosalicylic acid
170 reagent against glucose and xylose standard curves. β-glucosidase and β-xylosidase activities
171 were measured with p-nitrophenyl β-D-glucopyranoside and p-nitrophenyl β-D-xylopyranoside
172 as substrates, respectively, following the β-glucosidase assay procedure of Li et al. (2012). The

173 buffer used in all assays was 50 mM acetate (pH 5.0). *C. puteana* and *T. versicolor* activities
174 were measured at 30°C, while the activities of Celluclast were measured at 50°C.

175 Laccase activity was measured with 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic
176 acid) as substrate in 50 mM citrate buffer (pH 3.5) at 25°C. Appropriately diluted enzyme was
177 mixed with the substrate solution, and the increase in absorbance was monitored at 420 nm (ϵ
178 = 36 mM⁻¹ cm⁻¹) for 5 min.

179

180 **2.7 Sugar analyses**

181 The monosaccharide content of the pulp hydrolysates was determined with HPAEC. The
182 hydrolysates were diluted with MilliQ water and an aliquot of the internal standard (fucose)
183 was added. The samples were analysed with a Dionex ICS-3000 instrument equipped with
184 pulsed amperometric detection and a CarboPac PA20 column. Water was used as the eluent
185 (0.37 mL min⁻¹), and pure monosaccharides were used as standards for quantification.

186

187 **2.8 Extractives analyses**

188 The composition of extractives in the Soxhlet extracts and degraded samples was determined
189 with GC and GC-MS. An aliquot of each extractives solution and the internal standard
190 (heneicosanoic acid) were evaporated to dryness under vacuum and then redissolved in 700 μ L
191 of pyridine. The extractives were silylated at 70°C for 20 min after addition of 300 μ L of N,O-
192 bis(trimethylsilyl)trifluoroacetamide with 5% chlorotrimethylsilane. The extractives were
193 quantified by GC-FID (Shimadzu GC 2010 Plus) with a HP-5 column (30 m x 0.23 mm i.d.,
194 0.25 μ m film thickness). The oven temperature program was 2 min at 100°C, 6°C min⁻¹ to
195 280°C, and 5 min at 280°C. Helium (1 mL min⁻¹) was used as the carrier gas. The extractives
196 were identified by GC-MS (Trace 1300 GC/Thermo Scientific ISQ single quadrupole MS),
197 using the same type of column and the same oven temperature programme as with GC. Mass
198 spectra (50-700 m/z) were recorded at 70 eV.

199

200 **3. Results and discussion**

201 **3.1 Characterisation of extractives and enzyme preparations**

202 Table 1 shows a summary of the chemical composition of the HW extracts and wood powders.
203 The analysis was limited to main component groups, but detailed descriptions of the
204 composition of Scots pine HW extractives are available elsewhere (Fang et al. 2013, Willför et
205 al. 2003). The acetone extract (AE), which can be considered a total HW extract, consisted
206 mostly of resin acids. Pinosylvins were the second most abundant component in the AE, while
207 fatty acids accounted for only 3.1% of the extract. In the MeOH extract (ME), the proportion
208 of pinosylvins was greatly increased due to the removal of resin acids and fatty acids in the
209 hexane extraction step. The unextracted wood powder (UEW) contained 9.8% extractives
210 removable by sequential extraction with hexane and MeOH. The relative amounts of the
211 different extractives were very similar to those in the AE, which shows that the AE is a good
212 representation of the total HW extractives. The hexane extracted wood powder (HEW)
213 contained only 2.1% of MeOH soluble extractives, but it had retained most of the pinosylvins

214 present in the UEW. The slight loss of pinosylvins seen in the HEW is due to the partial
 215 solubility of pinosylvin monomethyl ether in hexane.

216

217 Table 1. Composition of extractives in the acetone (AE) and methanol (ME) extracts (% g⁻¹
 218 of extract) and the unextracted (UEW) and hexane extracted (HEW) wood powders (mg g⁻¹ of
 219 oven-dry wood)

	AE	ME	UEW ^a	HEW
	%	%	mg g ⁻¹	mg g ⁻¹
Gravimetric yield	-	-	98.0	20.8
Pinosylvins	15.0	64.4	15.4	13.4
Resin acids	75.2	5.8	71.8	1.3
Fatty acids	3.1	0.0	2.9	0.0

220 ^aSum of the hexane and MeOH extractions

221

222 The protein contents and hydrolase activities of the brown rot, white rot, and commercial
 223 enzyme preparations are presented in Table 2. Celluclast, the commercial *T. reesei* cellulase
 224 cocktail, showed much higher specific cellulase activities than the *C. puteana* and *T. versicolor*
 225 secretomes, particularly in the case of total cellulase and endoglucanase activity. The cellulase
 226 activities of *T. versicolor* were lower than those of *C. puteana*, which is in agreement with
 227 earlier results (Irbe et al. 2014). All of the enzyme preparations also had significant xylanolytic
 228 activity, with *C. puteana* showing the highest and *T. versicolor* the lowest activity. The different
 229 activity profiles of the enzymes were also reflected in hydrolysis monosaccharide yields, which
 230 are given in Table 3 for the two reference hydrolyses (pulp + EtOH for extracts, pulp + HMEW
 231 for wood powders). As expected, Celluclast showed the highest hydrolytic efficiency,
 232 producing mostly glucose from the pulp or pulp-wood mixture and a proportionally small
 233 amount of xylose. *C. puteana* produced significantly more glucose than *T. versicolor*, which is
 234 likely to be due to the low hydrolase activity of the white rot enzyme preparation (Table 1). The
 235 natural preference of white rots for hardwood substrates is unlikely to be a significant factor, as
 236 the preference is believed to be mostly related to lignin (Faix et al. 1985, Highley 1982). In
 237 addition to glucose, the two wood decaying fungi produced proportionally large amounts of
 238 xylose and other hemicellulosic sugars, mostly mannose.

239

240 Table 2. Protein contents (mg mL⁻¹) and hydrolase activities (U mg⁻¹ protein) of the *C. puteana*
 241 (Cp), *T. versicolor* (Tv), and Celluclast (Cell.) enzyme preparations

		Cp	Tv	Cell.
Protein content	mg mL ⁻¹	3.8	4.5	44.6
Total cellulase	U mg ⁻¹	0.10	0.05	0.63
Endoglucanase	U mg ⁻¹	6.2	0.7	26.8
Xylanase	U mg ⁻¹	22.6	2.2	9.5
β-glucosidase	U mg ⁻¹	0.41	0.75	0.80
β-xylosidase	U mg ⁻¹	0.14	0.11	0.35

242

243

244 Table 3. Monosaccharide yields (mg L⁻¹) in pulp reference hydrolyses by the *C. puteana* (Cp),
 245 *T. versicolor* (Tv), and Celluclast (Cell.) enzyme preparations. Pulp + 2.5% EtOH is used as
 246 reference for hydrolyses containing extracts, pulp + 2.5% HMEW for those containing wood
 247 powders

		Cp	Tv	Cell. 30°C	Cell. 50°C
1% pulp + 2.5% EtOH	Glc	384.5	227.8	2933.8	3774.6
	Xyl	91.0	113.3	360.8	386.2
	Other	99.5	53.4	40.5	30.8
1% pulp + 2.5% HMEW	Glc	545.8	264.5	3098.9	4045.2
	Xyl	113.6	123.0	549.3	560.7
	Other	175.5	125.8	46.9	57.2

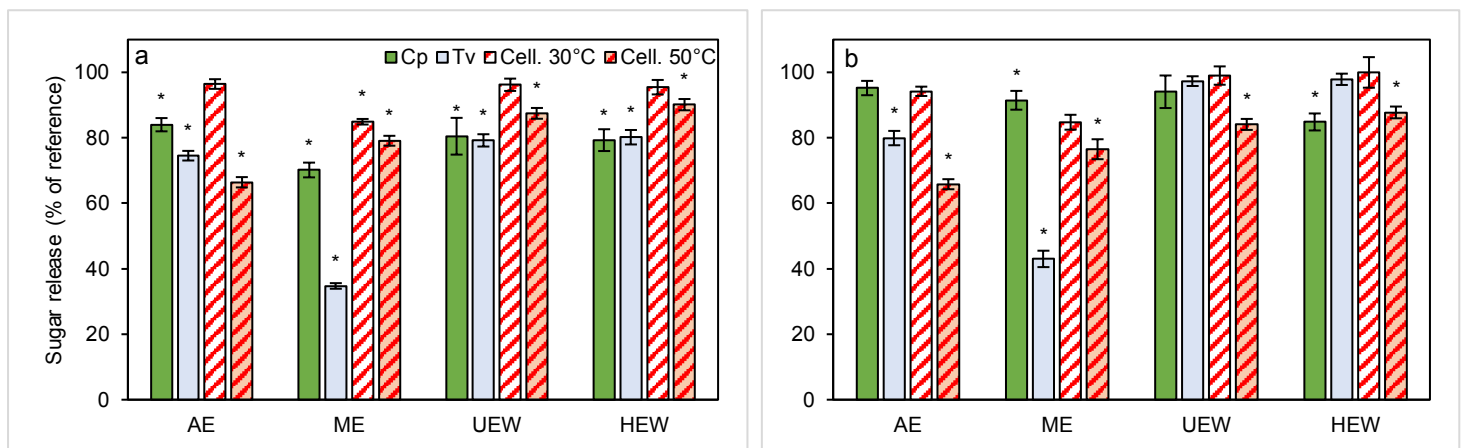
248

249

250 3.2 Inhibition

251 To study the effects of extractives on holocellulose hydrolysis, pulp was hydrolysed by the three
 252 enzyme preparations in the presence of extracts and wood powders. The sugar yields were then
 253 compared to the reference yields given in Table 3. As shown in Fig. 1, all enzyme preparations
 254 showed some degree of inhibition by extractives. Glucose and xylose (and mannose in the case
 255 of *C. puteana* and *T. versicolor*, data not shown) generally showed similar levels of inhibition
 256 by each extractives preparation, although the glucose yields tended to be slightly more affected
 257 than the yields of hemicellulosic sugars.

258



266 Figure 1. Inhibition of enzymatic glucose (a) and xylose (b) release from pulp by extractives.
 267 AE, acetone extract; ME, methanol extract; UEW, unextracted wood powder; HEW, hexane
 268 extracted wood powder; Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast. *statistically
 269 significant difference to reference ($p < 0.05$, Tukey's range test)

270

271 The release of glucose by the brown rot fungus *C. puteana* was mildly inhibited by both the
 272 extracts and wood powders. The pinosylvins-rich ME was more inhibitory than the AE, while
 273 the two wood powders did not differ in their effects, suggesting that the inhibition is primarily

274 due to pinosylvins. The extent of inhibition was similar for the extracts and wood powders,
275 which shows that extractives are capable of interacting with enzymes even in wood and can
276 thus be of relevance in wood decay. However, the release of xylose (and mannose, data not
277 shown) by *C. puteana* was not substantially inhibited by extractives. As brown rot fungi such
278 as *C. puteana* tend to preferentially degrade hemicelluloses (Highley. 1987, Irbe et al. 2006),
279 the slight inhibition of glucose release from wood is unlikely to be a significant factor in
280 preventing wood decay.

281 The hydrolases of the white rot fungus *T. versicolor* were also inhibited by both the extracts
282 and wood powders. The wood powders had only a small effect, with the release of glucose
283 slightly inhibited and the release of xylose unaffected. The pinosylvins-rich ME, however, was
284 found to have a strong influence on *T. versicolor* hydrolases. The release of glucose was
285 reduced to 35% of the reference, and even xylose release was affected, with the ME-containing
286 hydrolysis releasing 43% of the reference xylose.

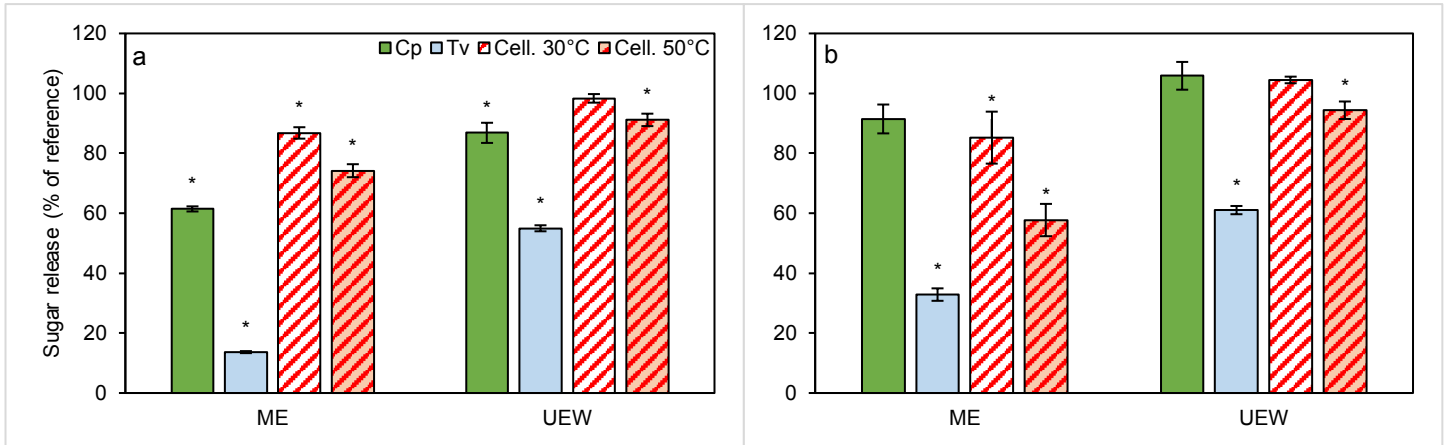
287 Celluclast, the commercial cellulase preparation, showed very little inhibition by extractives
288 when the hydrolyses were conducted at 30°C. At 50°C the AE became moderately inhibitory,
289 with the glucose and xylose yields reduced to approx. 65% of the reference. The effects of the
290 other extractives preparations on sugar release remained minor, which shows that hydrophobic
291 compounds are more inhibitory than pinosylvins at a higher hydrolysis temperature, at least in
292 the case of *T. reesei* hydrolases. The inhibiting component is likely to be the resin acids, which
293 accounted for 75% of the AE (Table 1). Cellulase inhibition by abietic acid, the most abundant
294 Scots pine HW resin acid, has been previously documented (Leskinen et al. 2015). However,
295 the inhibitory action of the hydrophobic fraction appears to require a wood-free extract: the
296 UEW was less inhibitory than the AE, even though both hydrolysis reactions contained
297 approximately the same amount of extractives in the same proportions (Table 1).

298

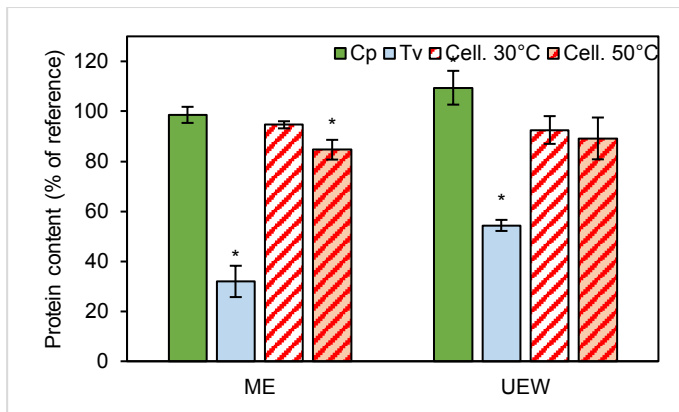
299 3.3 Deactivation

300 In addition to inhibition, the ability of extractives to deactivate enzymes was investigated. The
301 ME and UEW were selected for deactivation studies, as they represent the most inhibitory and
302 the most natural extractives preparations used in this experiment. The enzymes were first
303 incubated with extractives for 24 h, then the enzyme-extractives mixtures were used to
304 hydrolyse pulp. The protein contents of the incubation supernatants were also tested, to
305 determine whether the loss of hydrolytic activity is due to unproductive binding or precipitation
306 of enzyme protein, which are common deactivation mechanism with lignins and many simple
307 and oligomeric phenolics (Palonen et al. 2004, Rahikainen et al. 2011, Tejirian and Xu. 2011).
308 The effects of ME and UEW incubation on sugar yields and protein content are presented in
309 Figs. 2 and 3, respectively.

310 In the case of *C. puteana* and Celluclast, a 24 h pre-incubation of the enzymes with extractives
311 caused only small changes in sugar yield compared to the inhibition experiment (Fig. 1). The
312 ME caused moderate inhibition of glucose release by *C. puteana* (61% of reference glucose),
313 but apart from that, *C. puteana* and Celluclast remained only slightly or not at all affected by
314 extractives. The protein content determinations also showed that little to no supernatant protein
315 was lost during the 24h incubation period (Fig. 3). Thus, it appears that extractives do not
316 deactivate the *C. puteana* and Celluclast hydrolases and that the inhibition of sugar release seen
317 in their presence arises by some mechanism other than precipitation or unproductive binding of
318 enzymes.



327 Figure 2. Deactivation of enzymatic glucose (a) and xylose (b) release from pulp by
 328 extractives. AE, acetone extract; ME, methanol extract; UEW, unextracted wood powder;
 329 HEW, hexane extracted wood powder; Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast.
 330 *statistically significant difference to reference ($p < 0.05$, Tukey's range test)



331
 332 Figure 3. Loss of supernatant protein due to incubation with the methanol extract (ME) or the
 333 unextracted wood powder (UEW). Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast.
 334 *statistically significant difference to reference ($p < 0.05$, Tukey's range test)

335 Unlike *C. puteana* and Celluclast, *T. versicolor* showed almost complete deactivation. With
 336 pre-incubation, the glucose yield of the ME-containing reaction dropped to 14% of the
 337 reference, compared to the 35% obtained without pre-incubation (Fig. 1). The UEW had also
 338 become significantly more inhibitory with pre-incubation, with the glucose yield decreasing
 339 from 79% (Fig. 1) to 55% of the reference. Xylose yields were also reduced compared to
 340 hydrolyses without pre-incubation. Finally, protein content measurements (Fig. 3) showed a
 341 loss in supernatant protein following incubation with the ME and UEW, suggesting that the
 342 inhibitory and deactivating effects of the extractives may be due to complex formation between
 343 enzymes and extractives.

344 The deactivation of only the *T. versicolor* hydrolases suggests that the white rot enzyme
 345 preparation differs from the other two in some key way. While differences in the nature of the
 346 hydrolases are possible, another explanation is that the *T. versicolor* secretome contains
 347 enzymes that are capable of modifying the extractives to more inhibitory compounds.
 348 Supporting this modification hypothesis is the finding that the white rot enzyme preparation
 349 caused a gradual change in the colour of extractives-containing samples. Pinosylvins are likely

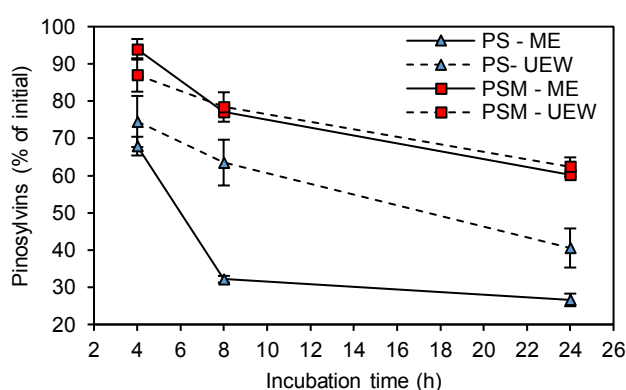
350 to be the modified extractives component: the pinosylvins-rich ME showed the strongest
 351 inhibition of *T. versicolor* (Fig. 1) and also the strongest colour change (from light brown to
 352 pink to orange over the course of several hours). *T. versicolor* produces a number of enzymes
 353 that act on phenolic substrates such as pinosylvins, most notably laccases and peroxidases
 354 (Moldes et al. 2004, Schlosser et al. 1997). Laccases in particular are known to act on a wide
 355 variety of compounds (Baldrian. 2006), including resveratrol and its derivatives, which are
 356 structural analogues of the pinosylvins (Nicotra et al. 2004, Ponzoni et al. 2007, Schouten et al.
 357 2002). The *T. versicolor* enzyme preparation used in this experiment had 4.6 U mg⁻¹ of laccase
 358 activity, while the *C. puteana* and Celluclast preparations showed no activity at all.

359

360 3.4 Mechanism of *T. versicolor* inhibition and deactivation

361 To determine whether *T. versicolor* causes modification of pinosylvins, the enzyme preparation
 362 was incubated with the ME and the UEW and samples collected at different time points for GC
 363 analysis. *C. puteana* and Celluclast were tested as well but were found to cause no changes in
 364 the concentration of extractives. The results of *T. versicolor* incubation are given in Fig. 4,
 365 which shows the changes in the pinosylvins content of the samples. The two primary HW
 366 pinosylvins, pinosylvin and pinosylvin monomethyl ether, were monitored separately. During
 367 the course of 24 hours, *T. versicolor* was able to cause significant degradation of both
 368 compounds. Pinosylvins were removed from both the extract and the wood powder, with the
 369 degradation of pinosylvin proceeding more rapidly than that of the methyl ether derivative.

370 The laccase-catalysed oxidation of phenolic compounds typically leads to the formation of
 371 oligomeric and polymeric products (Mita et al. 2003, Sun et al. 2013). A number of different
 372 oligomers can be produced from resveratrol by laccase oxidation (Nicotra et al. 2004, Ponzoni
 373 et al. 2007, Schouten et al. 2002), and given the structural similarity of resveratrol and
 374 pinosylvin, the formation of analogous oligomers from pinosylvin by the *T. versicolor* enzyme
 375 seems possible. No oligomers or other reaction products were detected in this experiment, but
 376 their lack may be due to the unsuitability of the employed GC method for the detection of
 377 oligomeric products.



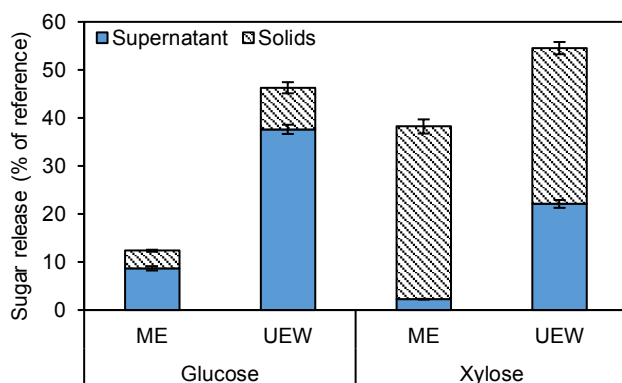
378

379 Figure 4. Disappearance of pinosylvins from the methanol extract (ME) or the unextracted
 380 wood powder (UEW) during incubation with the *T. versicolor* enzyme preparation. PS,
 381 pinosylvin; PSM, pinosylvin monomethyl ether

382 The results presented in Figs. 1, 2, and 4 suggest that the strong inhibition and deactivation of
 383 *T. versicolor* hydrolases by pine HW extractives is due to the modification of pinosylvins by *T.*
 384 *versicolor* enzymes, possibly laccases. Taken together with the protein content results (Fig. 3),

385 the modified pinosylvins then potentially complex and deactivate enzymes. However, the
386 simple protein content measurements do not provide information on the type of enzymes that
387 have been complexed, nor do they indicate whether the enzymes have been deactivated.
388 Therefore, to better understand the mechanism of inhibition, the loss and distribution of *T.*
389 *versicolor* enzyme activities in the ME and UEW samples was investigated in more detail.

390 The *T. versicolor* enzyme preparation was first incubated with the ME and the UEW and then
391 centrifuged and separated into supernatant and solid fractions. Both fractions were used to
392 hydrolyse pulp to determine whether any hydrolytic activity had been lost from the supernatant
393 and to see whether any activity remained in the solids. The glucose and xylose yields of the
394 fractionated hydrolyses are presented in Fig. 5, which shows a clear loss of hydrolytic activity
395 in the ME and UEW samples. Cellulase activity was lost from both supernatants, with the ME
396 supernatant releasing less than 10% and the UEW 38% of the reference glucose. The loss of
397 cellulase activity in the supernatants may be due to complex formation between enzymes and
398 extractives, as suggested by the reduction in supernatant protein content (Fig. 3), although
399 irreversible deactivation without complexation is also possible. Very little cellulase activity was
400 found in the ME and UEW solids, suggesting that if cellulases are present, they have been
401 almost completely deactivated. Xylanase activity was also lost from the supernatants, but
402 unlike the cellulases, a notable portion of the xylanase activity could be recovered from the
403 solids. The presence of active xylanase in the solid fraction could explain why xylose release
404 appears to be less affected by extractives than the release of glucose, at least in case of *T.*
405 *versicolor* and *C. puteana* (Figs. 1 and 2).



406

407 Figure 5. Residual glucose and xylose releasing enzyme activity in the supernatant and solid
408 fractions of *T. versicolor* after a 24 h incubation with the methanol extract (ME) or the
409 unextracted wood powder (UEW)

410 The results shown in Fig. 5 therefore demonstrate that the loss of protein seen in Fig. 3 is
411 accompanied by a loss of hydrolytic activity. Hydrolytic activity is lost from solution and
412 cannot be fully recovered from the solids, supporting the earlier hypothesis that the inhibition
413 of *T. versicolor* hydrolases is due to complex formation between enzymes and modified
414 pinosylvins. The enzymes may be complexed by the hypothetical pinosylvins oligomers,
415 resulting in enzyme deactivation. Alternatively, the enzymes may be attacked by the radical
416 intermediates of pinosylvins: experiments with resveratrol have shown that enzymatic
417 oxidation of the 3,5-OH structure produces an unstabilised m-semiquinone radical that can
418 attack and deactivate enzymes, becoming non-covalently incorporated into the enzyme in the
419 process (Szewczuk et al. 2004, 2005). In this experiment only the white rot fungus was capable
420 of modifying pinosylvins, but assuming that the modification is catalysed by laccase, the ability

421 will not be limited to just white rot fungi. Many brown rot fungi produce laccases, including
422 some strains of *C. puteana* (D'souza et al. 1996, Lee et al. 2004).

423

424 **4. Conclusions**

425 This experiment investigated the ability of Scots pine HW extractives to inhibit the enzymatic
426 hydrolysis of holocellulose. The hydrolases of the commercial cellulase preparation and the
427 brown rot fungus *C. puteana* were only slightly affected by the extractives, whereas the
428 enzymes of the white rot fungus *T. versicolor* showed strong inhibition and deactivation,
429 particularly by the pinosylvins-rich ME. Investigation of the causes of inhibition indicated that
430 the *T. versicolor* enzyme preparation was capable of modifying pinosylvins: the modified
431 pinosylvins might have complexed the enzymes and caused their deactivation. Modification of
432 pinosylvins appeared to be required for significant hydrolase inhibition by the HW extractives,
433 suggesting that hydrolase inhibition is unlikely to be effective against fungi that do not secrete
434 enzymes capable of modifying pinosylvins. Against fungi that do secrete such enzymes,
435 hydrolase inhibition may be an additional means of decay prevention by extractives.

436

437 **Acknowledgements**

438 This work was supported by a doctoral study grant to TB by the School of Chemical
439 Engineering, Aalto University.

440

441 Declarations of interest: none

442

443 **References**

444 Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of
445 xylanase activity. *Journal of Biotechnology* 23, 257-270.

446 Baldrian, P., 2006. Fungal laccases - occurrence and properties. *FEMS microbiology reviews*
447 30, 215-242.

448 Belt, T., Hänninen, T., Rautkari, L., 2017. Antioxidant activity of Scots pine heartwood and
449 knot extractives and implications for resistance to brown rot. *Holzforschung* 71, 527-534.

450 Boukari, I., O'Donohue, M., Remond, C., Chabbert, B., 2011. Probing a family GH11 endo- β -
451 1,4-xylanase inhibition mechanism by phenolic compounds: Role of functional phenolic
452 groups. *Journal of Molecular Catalysis B-Enzymatic* 72, 130-138.

453 D'souza, T.M., Boominathan, K., Reddy, C.A., 1996. Isolation of laccase gene-specific
454 sequences from white rot and brown rot fungi by PCR. *Applied and Environmental*
455 *Microbiology* 62, 3739-3744.

- 456 EN 350, 2016. Durability of wood and wood-based products – Testing and classification of
457 the durability to biological agents of wood and wood-based materials. European Committee
458 for Standardisation, Brussels, Belgium.
- 459 Faix, O., Mozuch, M.D., Kirk, T.K., 1985. Degradation of gymnosperm (guaiacyl) vs.
460 angiosperm (syringyl/guaiacyl) lignins by *Phanerochaete chrysosporium*. *Holzforschung* 39,
461 203-208.
- 462 Fang, W.W., Hemming, J., Reunanen, M., Eklund, P., Pineiro, E.C., Poljanšek, I., Oven, P.,
463 Willför, S., 2013. Evaluation of selective extraction methods for recovery of polyphenols
464 from pine. *Holzforschung* 67, 843-851.
- 465 Ghose, T.K., 1987. Measurement of cellulase activities. *Pure and Applied Chemistry* 59, 257-
466 268.
- 467 Harju, A.M., Kainulainen, P., Venäläinen, M., Tiitta, M., Viitanen, H., 2002. Differences in
468 resin acid concentration between brown-rot resistant and susceptible Scots pine heartwood.
469 *Holzforschung* 56, 479-486.
- 470 Harju, A.M., Venäläinen, M., Anttonen, S., Viitanen, H., Kainulainen, P., Saranpää, P.,
471 Vapaavuori, E., 2003. Chemical factors affecting the brown-rot decay resistance of Scots pine
472 heartwood. *Trees-Structure and Function* 17, 263-268.
- 473 Hart, J.H., Shrimpton, D.M., 1979. Role of stilbenes in resistance of wood to decay.
474 *Phytopathology* 69, 1138-1143.
- 475 Highley, T.L., 1982. Influence of type and amount of lignin on decay by *Coriolus versicolor*.
476 *Canadian Journal of Forest Research* 12, 435-438.
- 477 Highley, T.L., 1987. Changes in chemical components of hardwood and softwood by brown-
478 rot fungi. *Material Und Organismen* 22, 39-45.
- 479 Hillis, W.E., 1987. Heartwood and tree exudates. Springer-Verlag, Berlin.
- 480 Irbe, I., Andersons, B., Chirkova, J., Kallavus, U., Andersone, I., Faix, O., 2006. On the
481 changes of pinewood (*Pinus sylvestris* L.) Chemical composition and ultrastructure during the
482 attack by brown-rot fungi *Postia placenta* and *Coniophora puteana*. *International*
483 *Biodeterioration & Biodegradation* 57, 99-106.
- 484 Irbe, I., Elisashvili, V., Asatiani, M.D., Janberga, A., Andersone, I., Andersons, B., Biziks, V.,
485 Grinins, J., 2014. Lignocellulolytic activity of *Coniophora puteana* and *Trametes versicolor* in
486 fermentation of wheat bran and decay of hydrothermally modified hardwoods. *International*
487 *Biodeterioration & Biodegradation* 86, 71-78.
- 488 Jebrane, M., Pockrandt, M., Terziev, N., 2014. Natural durability of selected larch and Scots
489 pine heartwoods in laboratory and field tests. *International Biodeterioration & Biodegradation*
490 91, 88-96.

- 491 Lee, K.H., Wi, S.G., Singh, A.P., Kim, Y.S., 2004. Micromorphological characteristics of
492 decayed wood and laccase produced by the brown-rot fungus *Coniophora puteana*. *Journal of*
493 *Wood Science* 50, 281-284.
- 494 Leskinen, T., Salas, C., Kelley, S.S., Argyropoulos, D.S., 2015. Wood extractives promote
495 cellulase activity on cellulosic substrates. *Biomacromolecules* 16, 3226-3234.
- 496 Li, G., Jiang, Y., Fan, X.J., Liu, Y.H., 2012. Molecular cloning and characterization of a
497 novel β -glucosidase with high hydrolyzing ability for soybean isoflavone glycosides and
498 glucose-tolerance from soil metagenomic library. *Bioresource technology* 123, 15-22.
- 499 Mita, N., Tawaki, S., Uyama, H., Kobayashi, S., 2003. Laccase-catalyzed oxidative
500 polymerization of phenols. *Macromolecular Bioscience* 3, 253-257.
- 501 Moldes, D., Lorenzo, M., Sanroman, M.A., 2004. Different proportions of laccase isoenzymes
502 produced by submerged cultures of *Trametes versicolor* grown on lignocellulosic wastes.
503 *Biotechnology Letters* 26, 327-330.
- 504 Nicotra, S., Cramarossa, M.R., Mucci, A., Pagnoni, U.M., Riva, S., Forti, L., 2004.
505 Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from
506 *Myceliophthora thermophyla* and from *Trametes pubescens*. *Tetrahedron* 60, 595-600.
- 507 Palonen, H., Tjerneld, F., Zacchi, G., Tenkanen, M., 2004. Adsorption of *Trichoderma reesei*
508 CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated
509 lignin. *Journal of Biotechnology* 107, 65-72.
- 510 Plaschkies, K., Jacobs, K., Scheiding, W., Melcher, E., 2014. Investigations on natural
511 durability of important European wood species against wood decay fungi. Part 1: Laboratory
512 tests. *International Biodeterioration & Biodegradation* 90, 52-56.
- 513 Ponzoni, C., Beneventi, E., Cramarossa, M.R., Raimondi, S., Trevisi, G., Pagnoni, U.M.,
514 Riva, S., Forti, L., 2007. Laccase-catalyzed dimerization of hydroxystilbenes. *Advanced*
515 *Synthesis & Catalysis* 349, 1497-1506.
- 516 Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L., Kruus,
517 K., 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood - study of
518 enzyme binding and inactivation on lignin-rich surface. *Biotechnology and bioengineering*
519 108, 2823-2834.
- 520 Riley, R., Salamov, A.A., Brown, D.W., Nagy, L.G., Floudas, D., Held, B.W., Levasseur, A.,
521 Lombard, V., Morin, E., Otilar, R., Lindquist, E.A., Sun, H., LaButti, K.M., Schmutz, J.,
522 Jabbour, D., Luo, H., Baker, S.E., Pisabarro, A.G., Walton, J.D., Blanchette, R.A., Henrissat,
523 B., Martin, F., Cullen, D., Hibbett, D.S., Grigoriev, I.V., 2014. Extensive sampling of
524 basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for
525 wood decay fungi. *Proceedings of the National Academy of Sciences of the United States of*
526 *America* 111, 9923-9928.
- 527 Rollin, J.A., Zhu, Z.G., Sathitsuksanoh, N., Zhang, Y.H.P., 2011. Increasing cellulose
528 accessibility is more important than removing lignin: a comparison of cellulose solvent-based

- 529 lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnology and*
530 *bioengineering* 108, 22-30.
- 531 Schlosser, D., Grey, R., Fritsche, W., 1997. Patterns of ligninolytic enzymes in *Trametes*
532 *versicolor*. Distribution of extra- and intracellular enzyme activities during cultivation on
533 glucose, wheat straw and beech wood. *Applied Microbiology and Biotechnology* 47, 412-418.
- 534 Schouten, A., Wagemakers, L., Stefanato, F.L., van, d.K., van Kan, J. A. L., 2002.
535 Resveratrol acts as a natural antifungicide and induces self-intoxication by a specific laccase.
536 *Molecular microbiology* 43, 883-894.
- 537 Seppänen, S.K., Syrjälä, L., von Weissenberg, K., Teeri, T.H., Paajanen, L., Pappinen, A.,
538 2004. Antifungal activity of stilbenes in in vitro bioassays and in transgenic *Populus*
539 expressing a gene encoding pinosylvin synthase. *Plant Cell Reports* 22, 584-593.
- 540 Sharma, A., Milstein, O., Vered, Y., Gressel, J., Flowers, H.M., 1985. Effects of aromatic-
541 compounds on hemicellulose-degrading enzymes in *Aspergillus japonicus*. *Biotechnology*
542 *and bioengineering* 27, 1095-1101.
- 543 Sun, X.J., Bai, R.B., Zhang, Y., Wang, Q., Fan, X.R., Yuan, J.G., Cui, L., Wang, P., 2013.
544 Laccase-catalyzed oxidative polymerization of phenolic compounds. *Applied Biochemistry*
545 *and Biotechnology* 171, 1673-1680.
- 546 Szewczuk, L.M., Forti, L., Stivala, L.A., Penning, T.M., 2004. Resveratrol is a peroxidase-
547 mediated inactivator of COX-1 but not COX-2 - A mechanistic approach to the design of
548 COX-1 selective agents. *Journal of Biological Chemistry* 279, 22727-22737.
- 549 Szewczuk, L.M., Lee, S.H., Blair, I.A., Penning, T.M., 2005. Viniferin formation by COX-1:
550 Evidence for radical intermediates during co-oxidation of resveratrol. *Journal of natural*
551 *products* 68, 36-42.
- 552 Taylor, A.M., Gartner, B.L., Morrell, J.J., 2002. Heartwood formation and natural durability -
553 A review. *Wood and Fiber Science* 34, 587-611.
- 554 Tejirian, A., Xu, F., 2011. Inhibition of enzymatic cellulolysis by phenolic compounds.
555 *Enzyme and microbial technology* 48, 239-247.
- 556 Van Acker, J., Stevens, M., Carey, J., Sierra-Alvarez, R., Miltz, H., Le Bayon, I., Kleist, G.,
557 Peek, R.D., 2003. Biological durability of wood in relation to end-use - Part 1. Towards a
558 European standard for laboratory testing of the biological durability of wood. *Holz Als Roh-*
559 *Und Werkstoff* 61, 35-45.
- 560 Venäläinen, M., Harju, A.M., Kainulainen, P., Viitanen, H., Nikulainen, H., 2003. Variation
561 in the decay resistance and its relationship with other wood characteristics in old Scots pines.
562 *Annals of Forest Science* 60, 409-417.
- 563 Venäläinen, M., Harju, A.M., Saranpää, P., Kainulainen, P., Tiitta, M., Velling, P., 2004. The
564 concentration of phenolics in brown-rot decay resistant and susceptible Scots pine heartwood.
565 *Wood Science and Technology* 38, 109-118.

- 566 Willför, S., Hemming, J., Reunanen, M., Holmbom, B., 2003. Phenolic and lipophilic
567 extractives in Scots pine knots and stemwood. *Holzforschung* 57, 359-372.
- 568 Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M., 2011. Deactivation of cellulases by
569 phenols. *Enzyme and microbial technology* 48, 54-60.
- 570 Zhang, J., Presley, G.N., Hammel, K.E., Ryu, J., Menke, J.R., Figueroa, M., Hu, D., Orr, G.,
571 Schilling, J.S., 2016. Localizing gene regulation reveals a staggered wood decay mechanism
572 for the brown rot fungus *Postia placenta*. *Proceedings of the National Academy of Sciences*
573 of the United States of America 113, 10968-10973.

