Modification of wheat straw lignin by solid state fermentation with white-rot fungi

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Abstract

The potential of crude enzyme extracts, obtained from solid-state cultivation of four white-rot fungi (*Trametes versicolor*, *Bjerkandera adusta*, *Ganoderma applanatum* and *Phlebia rufa*), was exploited to modify wheat straw cell wall. At different fermentation times, manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), laccase, carboxymethylcellulase (CMCase), avicelase, xylanase and feruloyl esterase activities were screened and the content of lignin as well as hydroxycinnamic acids in fermented straw were determined. All fungi secreted feruloyl esterase while LiP was only detected in crude extracts from *B. adusta*. Since no significant differences ($P>0.05$) were observed in remaining lignin content of fermented straw, LiP activity was not a limiting factor of enzymatic lignin removal process. The levels of esterified hydroxycinnamic acids degradation were considerably higher than previous reports with lignocellulosic biomass. The data show that *P. rufa*, may be considered for more specific studies as higher ferulic and *p*-coumaric acids degradation was observed for earlier incubation times.

Keywords: Wheat straw, White-rot fungi, Biodegradation

1. Introduction

Wheat straw is one of the most abundant crop residues in the world, representing around 149 million tons per year on Europe according to FAO (2004). This huge amount of residues may constitute a promising raw material that could potentially be transformed into a more edible feed for ruminants (Rodrigues et al., 2008) or alternatively it could also be used for the production of ethanol (Fang et al., 2002). In either of these possibilities the main constraint to improve hydrolysis of this lignocellulosic material is the complexity of the cell wall structure.

The degradation of the plant cell wall is often inefficient because most polymers of cellulose and hemicellulose are either insoluble or too closely associated with the insoluble matrix (Panagiotou et al., 2007). Furthermore, hydroxycinnamic acids, particularly ferulic and *p*-coumaric acids, are covalently bound to cell wall pectins and polysaccharides (arabynoxylans, xyloglucans) through ester linkages and to lignin, mainly by ether bonds, thus influencing cell wall properties and its biodegradability.

The utilization of white-rot fungi enzyme complexes may be considered an alternative research field to increase the accessibility of cell wall structure. Research has shown that lignin is oxidised and degraded by a ligninase system (Elisashvili et al., 2008; Rodrigues et al., 2008) composed by lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. In addition, cellulases, hemicellulases and esterases are also considered to be extremely important in the degradation process of lignocellulosic biomass (Tabka et al., 2006; Panagiotou et al., 2007). In this way it is our opinion that these enzymes should act in synergy to facilitate the complete degradation of cell walls.

The aim of the present work was to (i) study the production of enzyme complexes by four different fungi - *Trametes versicolor* (TV), *Bjerkandera adusta* (BA),

Ganoderma applanatum (GA) and *Phlebia rufa* (PR) - at different times of incubation under solid state fermentation (SSF) of wheat straw; (ii) to evaluate its influence in the degradation of esterified hydroxycinnamic acids and (iii) removal of lignin, the most recalcitrant biopolymer of plant cell wall.

2. Methods

2.1. Fungal strains

Four fungal strains, *Trametes versicolor*, *Bjerkandera adusta*, *Ganoderma applanatum* and *Phlebia rufa*, were used to obtain the enzymatic extracts. Fungi were collected on the north of Portugal and were maintained on potato dextrose agar (PDA) plates at 4^{o}C and periodically subcultured.

2.2. Enzyme production

Enzymatic extracts were obtained from a solid culture medium containing 15 g of wheat straw with 0.5 g of glucose in 45ml of deionized water. Incubations were in 250 ml Erlenmeyer flasks containing the culture media and two 10 mm agar plugs removed from each isolated fungus. Flasks were incubated at 27°C and fermented straw from four flasks of each fungus was harvested every 7 days until 28 days after inoculation. After harvesting, contents of the culture flasks were suspended in 150 ml of deionized water and incubated on a rotary shaker (100 rpm) for 3 h. Extracts were filtered, centrifuged and aliquots were used to determine enzyme activities.

2.3. Experimental design and statistical analysis

All treatment combinations were completed in quadruplicate. The main and interaction effects of time of incubation and fungal species factors (4 x 4 factorial design), enzyme kinetics, hydroxycinnamic acids and lignin amount were evaluated using PROC GLM in SAS 9.1 software (SAS® Inc., Cary, NC) for analysis of variance and significance tests. When significant (i.e., $P<0.05$) differences were obtained using least square means procedures of SAS to compare means at the 5% level of confidence using a multiple comparison *t*-test.

2.4. Enzyme assays

Enzymatic activities were determined at 25°C using a Helios UV-Vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Manganese peroxidase (MnP) activity was determined according to the modified method of Heinfling et al. (1998) by the formation of Mn^{3+}-tartrate ($\varepsilon_{238} = 6.5\ mM^{-1}cm^{-1}$) from 1.5 mM $MnSO_4$ using 100 mM tartrate buffer (pH 5) and 10 mM H_2O_2. Lignin peroxidase activity was monitored at pH 3.0 according to Tien & Kirk (1988), and the formation of veratraldehyde was monitored at 310 nm ($\varepsilon_{310} = 9.3\ mM^{-1}cm^{-1}$). Laccase activity was determined according to Dias et al. (2003) by measuring the oxidation of 2mM 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) buffered with 100 mM citrate-phosphate (pH 4.0) and formation of ABTS cation radical was monitored at 420nm ($\varepsilon_{420} = 36.0\ mM^{-1}cm^{-1}$). Assay of feruloyl esterases was performed as reported by Mastihuba et al. (2002) through a spectrophotometric method by measuring the production of 4-nitrophenol (4NP) from 4-nitrophenyl ferulate (4NPF), which was obtained from the Institute of Chemistry of the Slovak Academy of Sciences (Slovakia). After 30 min incubation at 50ºC , 4NP released from 1 mM substrate (final concentration) buffered with 100 mM phosphate pH 6.5 was determined by absorbance readings at 410 nm.

117 Absorbance was converted into concentration through a standard curve prepared with
118 4NP (0.05-0.5 mM).

119 For cellulolytic enzyme assays, activities of carboxymethylcellulase (CMCase)
120 and Avicell digesting cellulase (avicelase) were measured according to the IUPAC
121 (Wood and Bhat, 1988). The reducing sugars released were determined by the
122 dinitrosalicylic acid (DNS), using glucose as a standard (Bezerra and Dias, 2004).
123 Xylanase activity was determined under similar conditions as described above, except
124 that 1 % xylan solution was used as the substrate (Mandels et al., 1974; Tabka et al.,
125 2006).

126 **2.5. Determination of Lignin and of esterified hydroxycinnamic acids in wheat**
127 **straw samples**

128 The acetyl bromide soluble lignin (ABSL) was used to determine lignin content of
129 samples as described by Fukushima and Hatfield (2001).

130 For the determination of hydroxycinnamic acids an adaptation of the procedures
131 described by Chien (1992) was used. Samples were milled to a particle size of 1mm and
132 further dried at 55ºC for 48h and dewaxed with toluene/80% ethanol (2:1, v/v) in a
133 Soxtec apparatus for 3 h. After this, samples were incubated with 30 ml of ethanol
134 (80%) at 85ºC for 1h with continuous agitation. Following centrifugation (14000 rpm)
135 at 5ºC for 15 min, the residue was dried at 60ºC overnight. A 25 mg portion of the
136 dewaxed samples was saponified with 5ml of 1M NaOH for 18h under N_2 at 25ºC. An
137 internal standard solution (100 µl) of ß-resorcylic acid in a solution (1:1, v/v) of
138 methanol and water was added. The samples were neutralized to pH 2.0 with 6M HCl.
139 The acidified solution was extracted with 3 × 2 ml of ethyl ether. The extract was dried
140 under nitrogen at 25ºC. The final residue was redissolved in 1 ml of water:methanol and

stored in the dark prior to analysis by high-performance liquid chromatography (HPLC). For the HPLC analysis a Dionex Ultimate 3000 with a PDA detector was used. The reverse phase column (Kromasil, 250x4 mm, particle size 5 □m, Teknokroma, Spain) was maintained at 30ºC during the runs. The samples (50 µl) were analysed by gradient elution of 5% formic acid (Solvent A) and methanol (Solvent B) (0 min, B = 5%; 2min, B = 5%; 65 min, B = 65%) at a flow rate of 1 ml/min and monitored between 200 and 400 nm. Phenolic acids were identified by comparison of retention time and UV-Spectrum of pure standards. Calibration curves were established with appropriate mixtures of ferulic, caffeic, syringic and coumaric acids. Results for lignin (g) and hydroxycinnamic acids (mg) are expressed in relation to wheat straw initial weight (15 g).

3. Results and discussion

3.1. Enzyme activities

Enzyme production during incubation of four different fungi is presented in Figure 1. MnP activity developed gradually over the incubation period showing maximum values for *P. rufa* and *B. adusta* (2.95 U/ml and 1.95 U/ml, respectively) on day 28, but no significant differences were observed between 21 and 28 days of incubation (Table 1).

On the contrary, MnP production was lower for *G. applanatum* and *T. versicolor* for all incubation times (Table 1). With the exception of B. *adusta* that showed residual enzyme activity, laccase showed maximum activity at day 7 (Figure 1) for all the remaining fungi without any differences between the other incubation periods (Table 1). *G. applanatum* showed higher laccase activity for all incubation periods (Table 1).

165 Regarding LiP, enzyme activity was only detected for *B. adusta*, showing the same
166 tendency (Figure 1) already observed for MnP with increasing values until the end of
167 the experiment.

168 The production of ligninolytic enzymes during wheat straw degradation by fungi
169 has already been reported by several authors (Arora et al., 2002; Rodrigues et al., 2008;
170 Zhang et al., 2008). However, the data regarding *P. rufa* and *G. applanatum* is being
171 presented for the first time using a culture medium containing wheat straw. Laccase and
172 MnP are considered to be the most common ligninolytic enzymes within white-rot fungi
173 (Nerud et al., 1996). In several studies with different white-rot fungi strains activities of
174 MnP and laccase are predominant (Vyas et al. 1994; Hofrichter et al. 1999; Tekere et al.
175 2001; Arora et al. 2002) and our results also show a general predominance on the
176 activity of these two enzymes. Our data also show that MnP activity is much higher
177 than that of laccase. This is in accordance to what was reported by Vyas et al. (1994)
178 and Hofrichter et al. (1999) who refer to maximum levels of MnP 10 times higher than
179 the maximum level of laccase activity. Production of LiP was only observed for *B.*
180 *adusta* with a general increase along the incubation time. While LiP is not always
181 detected (Tekere et al., 2001), Arora et al. (2002) and Vyas et al. (1994) detected LiP
182 for *P. chrysosporium* and *T. versicolor* in SSF wheat straw mediums. One possible
183 explanation for this may reside on the deficiency of identification of LiP activity when
184 using the veratryl alcohol oxidation assay (Arora et al. 2002) due to the presence of
185 inhibitors or colour interference by aromatic compounds (Hofrichter et al. 1999).

186 While all fungi tested produced avicelase (Figure 2) its activity was very low,
187 comparatively with CMCase. There were no significant differences for days 7, 14 and
188 21 (Table 1) and the maximum value observed (Figure 2.1) was on *T. versicolor* on day
189 28 (0.03 U/ml). In CMCase activity the most active producer appeared to be *G.*

190 *applanatum*, with a maximum value (0.13 U/ml) on day14, while in *T. versicolor* (0.07
191 U/ml), *P. rufa* (0.06 U/ml) and *B. adusta* (0.02 U/ml) the peak was observed on day 7
192 (Figure 2). The xylanase activity (Table 1) was quite different for the four fungi
193 ($P<0.05$). *G. applanatum* shows a first phase up to the second harvest (day 14) where it
194 had a maximum value (0.22 U/ml), then declined, but it still existed with a lower value
195 on day 28 (Figure 2). The activity of *P. rufa* between day 7 and 14 showed values fairly
196 stable (Figure 2). Finally, *B. adusta* and *T. versicolor* showed a variable production for
197 xylanase with no appreciable change over the 28 days of the trial. In line with our
198 findings, CMCase activity usually presents values greater than avicelase independently
199 of culture medium and fungi strains (Valášková and Baldrian, 2006; Rodrigues et al.,
200 2008). Xylanase and Cellulolytic activities presented in this study are not within a high
201 range of values. However, these relatively low values must be interpreted in relation to
202 the overall objectives. In fact, if the utilization of these enzyme complexes is to be
203 directed to the improvement of fibrous feed nutritive value, than we want to achieve
204 maximum lignin degradation but not an extensive utilization of cellulose and
205 hemicelluloses that must remain as energy sources for ruminant animals.

206 Feruloyl esterase activity was quite similar (Table 1) for all fungi ($P<0.05$) with
207 the exception of *G. applanatum*. There were no differences between day 7 and 28
208 (Table 1). Feruloyl esterase peak activity was detected on day 7 in all fungi with the
209 exception of *T. versicolor* with a higher activity on day 14 and *P. rufa* which presented
210 a second peak at 21 days of incubation (Figure 2).

211 Data on feruloyl esterase activity for these fungi grown on wheat straw is scarce
212 or at least it is not available. Generally the main fungi involved on feruloyl esterase
213 production are *Aspergillus* and *Penicillium* and its activity is dependent on the substrate
214 used, more specifically with the carbon source (Panagiotou et al., 2007). Nevertheless,

215 our data seem to fall within the range of values reported by these authors who studied
216 the production of feruloyl esterase by *P. brasilianum* grown on several substrates.
217 Furthermore, *G. applanatum* showed the highest (P<0.05) xylanase and feruloyl
218 esterase activities (Table 1) indicating that the production of these two enzymes may be
219 strongly related. This possible synergy among these enzymes, directly interrelated to the
220 structural configuration of plant cell wall, has been evidenced by other authors (Ferreira
221 et al., 2007; Panagiotou et al., 2007) and it will be discussed further on.

222 From the data we have obtained, and similarly to what was already reported for
223 some of these fungi (Rodrigues et al., 2008), the differences between enzyme activities
224 are quite high and must be interpreted by the different strains of fungi cultivated during
225 different periods of incubation and different medium base substrates.

226

227 **3.2. Lignin and esterified hydroxycinnamic acids**

228 Phenolic composition of wheat straw cell wall is presented in Table 2. As
229 expected results showed that ferulic and *p*-coumaric acids were the dominant esterified
230 hydroxycinnamic acids and lignin concentrations are within the range of values
231 normally reported for wheat straw. In spite of using different extraction methods,
232 similar results have been reported by Yosef et al. (2000) and Sun et al. (2001).

233 The effect of SSF in phenolic composition of wheat straw is presented in Table 3.
234 While no significant effect (P>0.05) was observed in esterified *p*-coumaric acid content
235 among the four fungi treatments, *B. adusta* gave the lowest decrease (P<0.05) in
236 esterified ferulic acid concentration. For both esterified syringic and caffeic acids *P.*
237 *rufa* treatment resulted in the highest values (P<0.05) in wheat cell wall after 28 days
238 incubation. However, along the time of incubation there was a significant decrease

239 ($P<0.001$) on the content of esterified hydroxycinnamic acids with no significant
240 differences between 21 and 28 days of incubation ($P>0.05$) for esterified *p*–coumaric
241 and ferulic acids concentrations. Already at 7 days of incubation (Figure 3) *T. versicolor*
242 and *P. rufa* treated straw showed lower concentrations of esterified *p*-coumaric and
243 ferulic acid, indicating that degradation was more effective with these two fungi in the
244 beginning of incubation.

245 The level of decrease in esterified *p*-coumaric and ferulic acid on grass lignocellulose
246 from Bermuda grass treated with two white-rot fungi (*Ceriporiopsis subvermispora* and
247 *Cyathus stercoreus*) reported by Akin et al. (1995) was around 50 and 65%,
248 respectively. More recently, Topakas et al. (2007) in a review on microbial production,
249 characterization and applications of feruloyl esterases (FAE) reported the release of
250 ferulic acid in several agroindustrial by-products and only one paper is referred for
251 wheat straw data. In it, Benoit et al. (2006) refer to quite lower levels of release pointing
252 values of 16% and 58% of *p*-coumaric and ferulic acids respectively in steam exploded
253 wheat straw. Nevertheless, these authors considered these results good due to the highly
254 resistant cell wall structure of wheat straw. Tapin et al. (2006) studied the potential of
255 wheat straw as raw material for papermaking and recovery of some phenolic
256 compounds with FAE from *A. niger* and xylanase and referred that treatment with FAE
257 released 7.5% and 12% of ferulic and *p*-coumaric acid respectively. However if this
258 treatment was associated with a light alkaline extraction the recovery increased to 20
259 and 25%, respectively.

260 Taking in account the measurements we performed during the experimental
261 period our results show that a decrease of around 80% for both esterified *p*-coumaric
262 and ferulic acids was obtained until 28 days of incubation (Table 3). However, in
263 relation to the initial concentrations of these hydroxycinnamic acids in wheat straw, a

264 decrease of 72% and 77% was measured at 21 days of incubation for *p*-coumaric and
265 ferulic acids, respectively, while for the period between 21 and 28 days of incubation
266 the decrease was only around 20% for both esterified hydroxycinnamic acids. In this
267 way it seems that there is a first phase up to the 14-21 days of growth on which there is
268 a rapid decrease of esterified *p*-coumaric and ferulic acids, and a second phase in which
269 values remain fairly stable indicating that probably fungi produced all the necessary
270 enzymes to release these compounds in the first 21 days of incubation (Figure 3).

271 These results indicate that all the fungi treatments were able to reduce the content
272 of esterified *p*-coumaric and ferulic acids in higher extent than the application of
273 commercial enzymes, probably due to the synergism between the enzyme complexes
274 produced by the fungi as we have mentioned in a previous work (Rodrigues et al.,
275 2008). This is also valid to explain why *T. versicolor* and *P. rufa* treatments showed a
276 more pronounced degradation of these hydroxycinnamic acids in the first 7 days of
277 incubation (Figure 3).

278 The lignin content decrease did not differ widely between the different fungi
279 treatments (Table 3). However there was a significant decrease ($P<0.001$) in the total
280 amount of lignin from day 7 until the end of the incubation period (Table 3) reaching a
281 value of 33%. When analysing the content of wheat straw lignin without any fungal
282 treatment (Table 2) it is possible to see that this decrease is around 43%, indicating that
283 lignin loss was quite low within the first 7 days of incubation (13%). Differences were
284 observed among the fungi treatments during the incubation period (Figure 3) with *T.*
285 *versicolor* and *P. rufa* treatments leading to a higher decrease in lignin content between
286 14 and 21 days of incubation.

287 Several authors have reported the potential for white-rot fungi to degrade lignin
288 and presented values that are quite variable depending on the strain, type of

289 fermentation as well as on the incubation period. For instance, Jalč (2002) on a review
290 paper analysing the results of several authors presented values of wheat straw lignin
291 degradation that varied between 2-65%. Arora et al. (2002) showed percentage loss of
292 wheat straw lignin for *P. radiata* and *T. versicolor* of 18.5 and 12.5%, while other
293 *Phlebia spp.* showed lignin losses that reached 25%. More recently, Zhang et al. (2008)
294 when studying the effect of steam explosion pre-treatment on lignin biodegradation of
295 wheat straw by *T. versicolor* reported a decrease around 30%.

296 As stated before lignin losses on the first 7 days of incubation were quite low with
297 a more pronounced decrease afterwards. The same has been observed by Arora et al.
298 (2002) who suggested a synergistic role in ligninolysis due to the fact that various
299 enzyme maxima occurred prior to maximum lignin loss. This sequential action of
300 enzymes in lignin degradation was also pointed out by Zhang et al. (2008). According
301 to these authors though the maximum of MnP and laccase activities were detected
302 during the first 10 days of wheat straw incubation with *T. versicolor*, lignin degradation
303 started later and tended to increase until the maximum degradation rate was reached on
304 day 30. Our data also show a similar pattern in which significant lignin degradation
305 seems to occur after an increase in the enzyme activities of laccase. However, the same
306 was not observed for MnP activities as there was a general increase along the incubation
307 period.

308 Synergistic effects are also attributed to the interaction between esterases and
309 hemicellulases. Fungal feruloyl and *p*-coumaroyl esterases are capable of releasing
310 feruloyl and *p*-coumaroyl units and play an important role in biodegradation of
311 recalcitrant cell walls in grasses (Kuhad et al., 1997). These enzymes act synergistically
312 with xylanases to disrupt the hemicellulose-lignin association, without mineralization of
313 lignin *per se* (Borneman et al., 1990). Therefore, hemicellulose degradation seems to be

314 required before efficient lignin removal can commence, at least for some substrates.
315 This synergistic effect was also underlined by Panagiotou et al (2007) pointing out that
316 the production of feruloyl esterase and arabinofuranosidase is co-regulated.

317

318 **4. Conclusions**

319 Our data show that the enzyme complexes produced by fungi seem to exert their
320 effect in the cell wall structure due to the synergism between the different types of
321 enzymes. In fact, the higher degradation of esterified hydroxycinnamic acids in the first
322 7-14days of incubation, directly related to the xylanase and feruloyl esterase activities
323 during this period, precede a more intensive degradation of the lignin structures. The
324 present study also indicated that the fungi treatments were able to reduce to a
325 considerable extent the content of esterified *p*-coumaric and ferulic acids. Considering
326 that wheat straw is quite recalcitrant and that results from the application of commercial
327 esterases do not normally approach such high values these results are of substantial
328 interest.

329 Nevertheless, more studies on the specificity of enzyme activities of fungi are still
330 necessary to improve our knowledge on the mechanisms that regulate its production and
331 specific interaction with the different substrates.

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338 **5. References**

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453 Figure Captions

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455 Figure 1 – Ligninolytic enzyme activities of white-rot fungi during the incubation period.

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457 Figure 2 - Cellulolytic, hemicellulolytic and feruloyl esterase enzyme activities of
458 white-rot fungi during the incubation period.

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460 Figure 3 - Time course of hydroxycinnamic acids and lignin degradation during the
461 incubation period.

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Table 1 - Enzyme activities from four white-rot fungi incubated with wheat straw during 28 days in solid state fermentation.

Source	Enzyme activities (U/ml)						
	MnP	LiP	Laccase	Avicelase	CMCase	Xylanase	Feruloyl
Fungi[A]							
BA	1.363^{b}	0.259^{b}	0.004^{a}	0.006^{a}	0.006^{a}	0.041^{a}	0.018^{ab}
GA	0.271^{a}	0.000^{a}	0.216^{c}	0.003^{a}	0.067^{c}	0.132^{d}	0.032^{c}
PR	1.928^{c}	0.000^{a}	0.049^{b}	0.010^{b}	0.028^{b}	0.063^{b}	0.016^{a}
TV	0.144^{a}	0.000^{a}	0.062^{b}	0.014^{c}	0.054^{c}	0.112^{c}	0.021^{b}
Time (days)							
7	0.307^{a}	0.000^{a}	0.157^{b}	0.008^{a}	0.020^{a}	0.025^{a}	0.027^{c}
14	0.831^{b}	0.058^{b}	0.049^{a}	0.008^{a}	0.016^{a}	0.106^{b}	0.022^{b}
21	1.242^{c}	0.094^{c}	0.061^{a}	0.007^{a}	0.054^{b}	0.109^{b}	0.022^{b}
28	1.326^{c}	0.108^{c}	0.062^{a}	0.011^{b}	0.065^{a}	0.107^{b}	0.016^{a}
Effects[B]							
Fungi	***	***	***	***	***	***	***
Time	***	***	***	***	***	***	***
Time*Fungi	***	***	***	***	***	***	***

Values within a column bearing the same superscript are not significantly different (P>0.05) according to Tukey's test.

[A] BA, *Bjerkandera adusta*; GA, *Ganoderma applanatum*; PR, *Phlebia rufa*; TV, *Trametes versicolor*.

[B] ** P<0.01; ***P<0.001.

479 Table 2 - Phenolic composition of wheat straw before incubation.

480

Phenolic composition [A]				
Esterified hydroycinnamic acids (mg)				Lignin (g)
SyrA	CafA	p-CoumA	FerA	
15.4	3.6	136.3	101.2	1.76

481 [A] SyrA, Syringic acid; CafA, Caffeic acid; p-CoumA, *p*-coumaric acid; FerA, Ferulic
482 acid.

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485 Table 3 - Phenolic composition of wheat straw incubated with four white-rot fungi in solid state fermentation.
486

Source	Phenolic composition [A]				
	Esterified hydroxycinnamic acids (mg)				Lignin (g)
	SyrA	CafA	p-CoumA	FerA	
Fungi [B]					
BA	11.0^{a}	1.5^{a}	59.2^{a}	41.0^{b}	1.3^{a}
GA	13.5^{b}	1.7^{a}	54.8^{a}	35.9^{ba}	1.4^{a}
PR	17.8^{c}	2.2^{b}	50.7^{a}	32.34^{a}	1.2^{a}
TV	12.3^{ab}	1.4^{a}	50.2^{a}	31.0^{a}	1.2^{a}
Time (days)					
7	15.6^{b}	2.6^{c}	95.1^{c}	66.4^{c}	1.5^{c}
14	14.2^{b}	1.6^{b}	51.4^{b}	32.7^{b}	1.3^{b}
21	13.9^{b}	1.5b	38.1^{a}	23.1^{a}	1.0^{a}
28	10.9^{a}	1.0^{a}	30.2^{a}	18.0^{a}	1.0^{a}
Effects [C]					
Fungi	***	**	-	*	-
Time	**	***	***	***	***
Time*Fungi	-	*	-	***	-

487 Values within a column bearing the same superscript are not significantly different (P>0.05) according to Tukey's test.
488 [A] SyrA, Syringic acid; CafA, Caffeic acid; p-CoumA, *p*-coumaric acid; FerA, Ferulic acid; G, Guaiacyl, S, Syringyl; H, p-hydroxyphenyl.
489 [B] BA, *Bjerkandera adusta*; GA, *Ganoderma applanatum*; PR, *Phlebia rufa*; TV, *Trametes versicolor*.
490 [C] * P<0.05; ** P<0.01; ***P<0.001.

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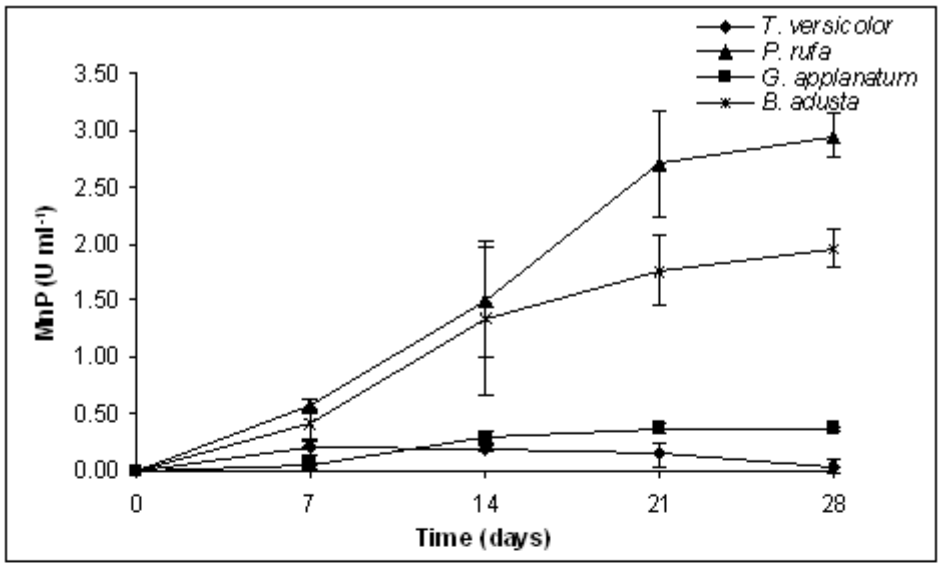
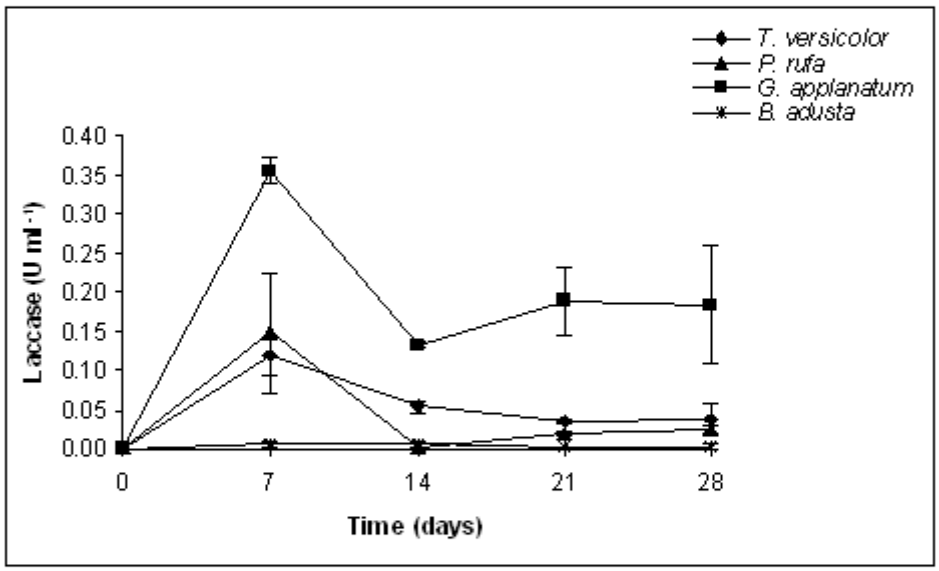
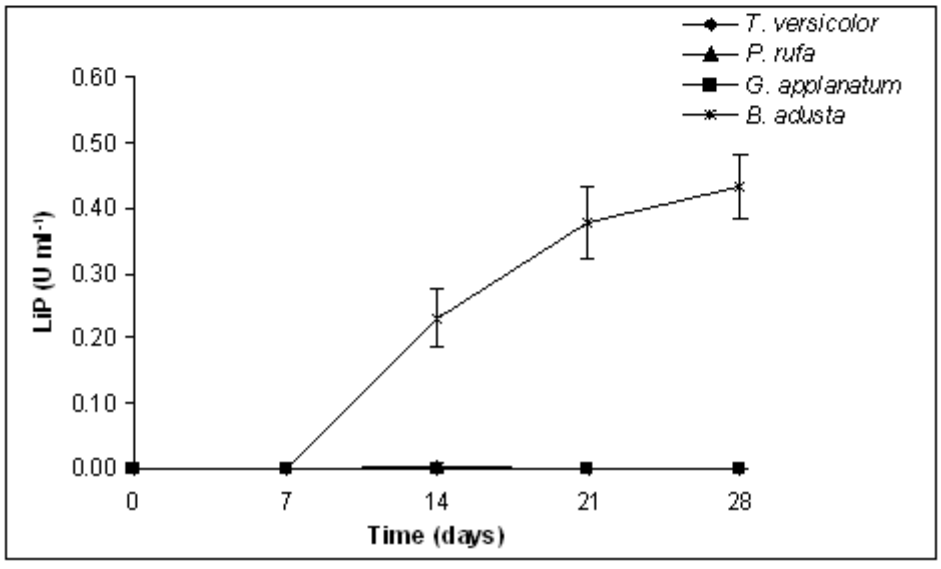
493 Figure 1

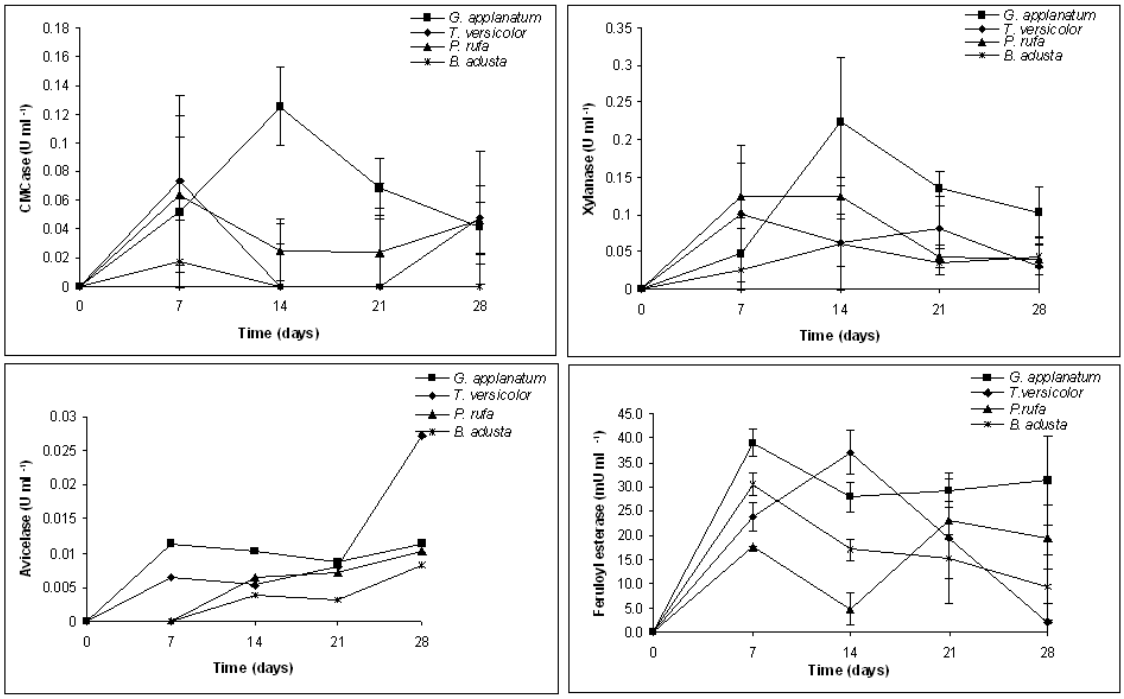






494

Figure 2


G. applanatum
T. versicolor
P. rufa
B. adusta
CMCase (U ml -1)
Xylanase (U ml -1)
Avicelase (U ml -1)
Feruloyl esterase (mU ml -1)
Time (days)

510 Figure 3

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