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

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Abstract

This work looks for a better understanding of the lignocellulolitic potential of crude enzyme extracts obtained from solid-state cultivation of four white-rot fungi (*Trametes versicolor*, *Bjerkandera adusta*, *Ganoderma applanatum* and *Phlebia rufa*) using the lignocellulosic substrate wheat straw. At different fermentation times, enzyme activities such as manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), laccase, carboxymethylcellulase (CMCase), avicelase, xylanase and feruloyl esterase were screened and the content of lignin as well as hydroxycinnamic acids in fermented straw were determined. In general, our results showed that MnP predominate among ligninolytic activities and xylanase among polysaccharide hydrolase activities. 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. Among hydroxycinnamic acids, ferulic acid content of *T. versicolor* and *P. rufa* fermented straw exhibited significant lower titers. The levels of esterified hydroxycinnamic acids degradation were considerably higher than previous reports with lignocellulosic biomass. The data show that *Phlebia rufa*, may be considered for more specific studies as higher ferulic and *p*-coumaric acids degradation was observed for earlier incubation times.

Resumo

O desenvolvimento deste trabalho teve como objectivo a aumentar o conhecimento do potencial lenhinocelulolítico de extractos enzimáticos obtidos através de fermentação sólida de quatro fungos da podridão branca (*Trametes versicolor*, *Bjerkandera adusta*, *Ganoderma applanatum* e *Phlebia rufa*) utilizando a palha de trigo como substrato durante 28 dias. As actividades enzimáticas da manganês-peroxidase (MnP), lenhina peroxidase (LiP), lacase, carboximetilcelulase (CMCase), avicelase, xilanase e *feruloíl* esterase foram analisadas com intervalos de 7 dias. A composição química da parede celular da palha de trigo, em cada um dos intervalos de tempo previamente mencionados, foi analisada medindo a concentração de lenhina e de ácidos hidroxicinâmicos esterificados. Os resultados obtidos mostraram que a actividade da MnP era predominante, relativamente às restantes actividades lenhinocelulolíticas Do mesmo modo, a actividade enzimática da xilanase era superior à das outras hidrolases. Todos os fungos produziram a enzima *feruloíl* esterase, enquanto que a LiP só foi detectada no extracto que continha *B. adusta*. Como não foram observadas diferenças significativas ($P > 0.05$) relativamente ao teor de lenhina da palha de trigo após a fermentação, a actividade da LiP não deverá ser considerada como um factor limitante do processo enzimático envolvido na ruptura das unidades constituintes da lenhina. Relativamente aos ácidos hidroxicinâmicos esterificados, especificamente na palha de trigo fermentada com os fungos *T. versicolor* e *P. rufa,* o ácido ferúlico e o ácido *p*-cumárico apresentaram valores de degradação mais elevados especialmente durante os primeiros sete dias de incubação. Foi possível identificar um mecanismo sinergético de degradação dos diferentes constituintes da parede celular em que a hidrólise dos ácidos hidroxicinâmicos e de parte das hemiceluloses poderá ser determinante para a degradação posterior da molécula de lenhina. Os níveis de degradação dos ácidos hidroxicinâmicos esterificados foram mais elevados quando comparados com

outros estudos com material lenhinocelulósico. Os dados indicam que o fungo *P. rufa,* poderá revelar-se bastante promissor, visto que apresentou valores elevados na degradação do ácido ferúlico e *p*-cumárico nos tempos iniciais da incubação.

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List of Abbreviations

ABSL	acetyl bromide soluble lignin	MnP	manganese peroxidase
ABTS	2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate	N	nitrogen
Avicelase	Avicell digesting cellulase	4NP	4-nitrophenol
BA	*Bjerkandera adusta*	4NPF	4-nitrophenyl ferulate
CafA	Caffeic acid	PcoumA	*p*-coumaric acid
CMCase	carboxymethylcellulase	PDA	potato dextrose agar
CP	crude protein	PR	*Phlebia rufa*
DM	dry matter	rpm	revolutions per minute
DNS	dinitrosalicylic acid	S	syringyl
FAO	Food and Agriculture Organization	SSF	solid state fermentation
FAE	feruloyl esterases	SyrA	Syringic acid
FerA	Ferulic acid	TV	*Trametes versicolor*
G	guaiacyl	WRF	White-rot fungi
GA	*Ganoderma applanatum*		
H	*p*-hydroxyphenyl		
HPLC	high-performance liquid chromatography		
LiP	lignin peroxidase		

Chapter 1

Introduction

1.1 Introduction

Straws, as well as other fibrous by-products, have been utilized as animal feed in the whole world for centuries. However, the constraints resulting from the low nitrogen (N) and the high fibre contents, as well as the increased importance of concentrate feeds has led to a continuous decrease in its utilization. When offered to livestock both dry matter intake and palatability are low. However, these by-products are most often the only available feed in tropical and subtropical areas where smallholder mixed crop livestock systems prevail. More recently, Shiere et al. (2004) have showed that straws and stovers are increasingly important, e.g. for animal feed, thatching, soil improvement, mushroom production and industrial use.

The annual production of cereals in 2004 was 470 million tons in Europe and 389 Mt in the USA and from the most common cereal crops around 149 Mt of wheat straw is produced in Europe (FAO, 2004). Although this agricultural by-product has a gross energy that is comparable to that of cereal grains, using straws as energy feed for ruminants is limited because their cell walls contain three-dimensional structures that are less available for microbial degradation in the rumen (Chaudhry, 2000).

Considerable effort has been expended in an attempt to improve the feeding value of cereal straws. Several treatments have been tested, and

most of the data published is related to the application of alkali treatments, such as sodium hydroxide or ammonia that have been shown to be effective for cleaving esterified bonds within the plant cell wall architecture, improving the ruminal digestion of cereal straw (Sundstøl, 1988; Wang et al., 2004). For instance, urea has been successfully applied to straws in order to improve their crude protein (CP) concentration and DM and fibre digestibility (Ibbotson et al., 1984, Dias-da-Silva and Sundstøl, 1986; Dias-da-Silva et al., 1988, Dias-da-Silva and Guedes, 1990).

In spite straw cell wall structure can be modified through chemical treatments, it should be noticed that until recently, in North America the majority of cereal straw was either reincorporated into the soil or used as bedding (Wang et al., 2004), giving us a clear and obvious idea of the general utilization of straw by-products in more developed countries.

According to Buranov et al. (2008), herbaceous crops are more recently receiving increasing attention because of the annual renewability and the largest annual biomass stock (549 million tons/year worldwide), this being the case of wheat in the EU, which is the preferred cereal grain for bioethanol production (Arifeen et al., 2007). Furthermore, in addition to the growing demand for traditional applications (paper manufacture, biomass fuels, composting, animal feed, etc.), novel markets for lignocellulosics have been identified in recent years (Malherbe et al., 2002).

Although several utilizations can thus be developed or identified for cereal straws one of the major limitations still present is related to the cell wall architecture.

1.2. Cell Wall Structure

Wheat straws have a complex cell wall structure in which the main components are cellulose, hemicelulose and lignin. These compounds are

closely associated and covalent cross-linkages have been suggested to occur between lignin and polysaccharides (Palonen, 2004).

Cellulose (Figure 1.1) is the predominant polymer in lignocellulosic biomass and is a linear homopolymer of anhydro D-glucose units linked together by β-1,4 glucosidic bonds (Foyle at al., 2007).

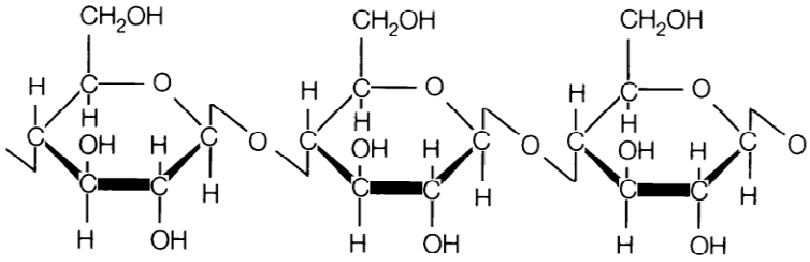

Figure 1. 1 - Structure of cellulose (retrieved from www.greenspirit.org.uk).

Hemicelluloses are generally classified according to the main sugar residue in the backbone, with xylans (Figure 1.2) and mannans being the main groups of hemicelluloses and forms hydrogen bonds with cellulose microfibrils, increasing the stability of the cellulose-hemicellulose-lignin matrix (Foyle et al., 2007).

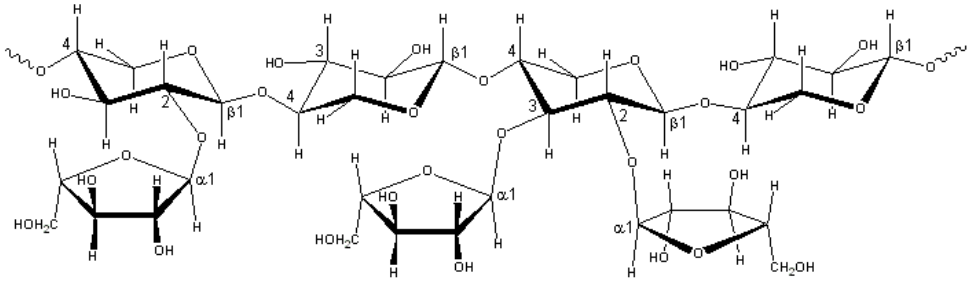

Figure 1. 2 - Structure of arabinoxylan (retrieved from www.lsbu.ac.uk).

Lignin is a phenolic polymer of the plant cell wall that provides rigidity and function as a binding and encrusting material. It is an amorphous polymer consisting of phenylpropane units, and their precursors are three aromatic alcohols (monolignols) namely *p*-coumaryl, coniferyl and sinapyl alcohols (Fig. 1.3), being the respective precursors of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties (Lewis and Yamamoto, 1990; Buranov et al. 2008).

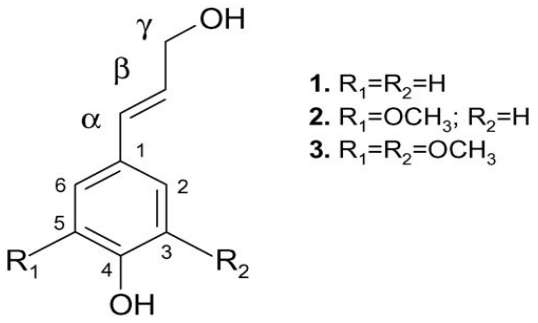

Figure 1. 3 – Lignin monoglicols (adapted from Buranov et al., 2008).

The strong carbon-carbon and ether linkages in lignin make it resistant to degradation. For this reason, lignin is generally accepted as the primary entity responsible for limiting digestion of forages, thereby reducing its nutritional value (Besle et al., 1994; Van Soest, 1994; Baurhoo et al., 2008).

Hydroxycinnamic acids, particularly ferulic acid and *p*-coumaric acid (Figure 1.4), and to some extent, caffeic and sinapic acids occur widely in the cell walls of wheat straw (Benoit et al., 2006) and their probable role is to decrease cell wall biodegradability and regulate the growth by cross-linking of cell wall polymers (Mastihuba et al., 2002). Furthermore, phenolic cross-links using ferulic or *p*-coumaric acids contribute to the

structural integrity of the cell wall by mediating direct covalent interactions between hemicellulose chains and lignin (Panagiotou et al., 2007; Buranov et al., 2008). *p*-Coumaric acid and lignin fractions in wheat straw are mainly connected with ester linkages and ferulic acid is associated with wheat straw lignin fractions through ether bonds and with polysaccharides through ester bonds (Scalbert et al., 1985, 1986; Fidalgo et al., 1993; Buranov et al., 2008). About 50–70% of ferulic acids are etherified to lignin in wheat straw cell walls and about 70% of *p*-coumaric acids are esterified to lignin (Sun et al., 1995; Lawther and Sun, 1996). In wheat straw cell walls, the majority of wheat straw lignin is directly linked to arabinose side chains of xylan by ether bonds without hydroxycinnamic acids.

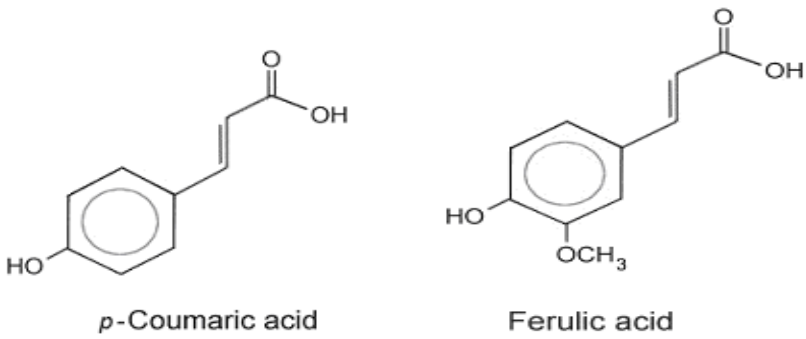

Figure 1. 4 – Structure of the *p*-coumaric and ferulic acids (retrieved from www.crscientific.com).

1.3. Possible future applications for straws

Currently, a large amount of studies regarding the utilization of lignocellulosic biomass as a feedstock for producing fuel ethanol is being

carried out worldwide (Tabka et al., 2006). For countries where the cultivation of energy crops is difficult, lignocellulosic materials are an attractive option for the production of biofuels and it is considered that lignocellulosic biomass will become the main feedstock for ethanol production in the near future (Saha, B. C., 2003). In the present time, the importance of bioethanol production from renewable biomass due to the higher oil prices has made the study of the composition of annual herbaceous crops imperative (Buranov et al. 2008). However, studies concerned with structure and properties of straw lignin are scarce. The structures of straw lignin are not yet well understood, nor are their precise interrelationships with other cell wall components (Buranov et al., 2008).

Lignocellulosic materials represent a promising option as a feedstock for ethanol production considering their output/input energy ratio, their great availability both in tropical and temperate countries, their low cost (primarily related to their transport), and their ethanol yields. One of the advantages of the use of lignocellulosic biomass is that this feedstock is not directly related to food production. In fact, several other alternatives are advanced by Chum and Overend (2001) referring to synthesis gas, methanol, hydrogen and electricity.

The pulp and paper industry discovered that lignocellulose biotechnology could improve process efficiency through savings in money and energy. However, the use of these by-products for papermaking presents several disadvantages due to the cost of collection and storage of the straws after harvest, the significant size of installation required, and the accelerated wear of paper machinery due to the high silica content. Therefore, the cost effectiveness of such a process depends on the development of additional value from the straw.

The production of phenolic compounds such as hydroxycinnamic acids should enhance the overall profitability of the process. Ferulic acid is

of economic interest as it is a vanillin precursor and a strong antioxidative molecule. It is a highly valuable additive that is used in the food industry and has pharmacological and cosmetic applications. (Tapin et al., 2006).

Commercial lignin is currently produced as a co-product of the paper industry, separated from fibre by a chemical pulping process. The usefulness of commercial lignins comes from their dispersing, binding, complexing and emulsifying properties.

Hemicelluloses, the second most common polysaccharides in nature, represent about 20–35% of lignocellulosic biomass. In recent years, bioconversion of hemicellulose has received much attention because of its practical applications in various agro-industrial processes, such as efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Saha, B. C., 2003).

The fractionation of herbaceous biomass into biopolymers (lignin, hemicelluloses, and cellulose) and the production of value-added products from these biopolymers depend, on many cases, on the efficiency of degradation and on the utilization of enzymes. For this purpose, white-rot fungi constitute a viable alternative as a biological treatment.

These fungi possess hydrolytic enzymes, which typically are induced by their substrates. All of these enzymes are industrially important; therefore, organisms able to produce them are interesting in view of the potential importance in industrial processes like bioremediation, biobleaching of pulp paper, degradation and detoxification of recalcitrant substances or in the food industry (Papinutti et al., 2007).

Among the enzymes being produced by these fungi special attention has been given to the different cellulases and endoglucanases that

hydrolyze internal bonds in the cellulose chain and act mainly on the amorphous parts of the cellulose, and exoglucanases that hydrolyze from the chain ends and produce predominantly cellobiose (Palonen et al., 2004). The production of hemicellulases such as xylanases as well as the synergic effect with esterases is also mentioned as one of the main features of these basidiomycetes (Faulds et al., 1995; Yu et al., 2002; Tabka et al., 2006). According to Panagiotou (2007), some investigations indicated that a complete degradation of cell wall structures co-forming xylans and arabinoxylans, besides activities for hydrolysis of glycoside bonds, requires the activity of appropriate esterases. Feruloyl esterases, members of the carboxylic acid esterases, have been found to break the ester linkage between hydroxycinnamic acids and the attached sugars present in the plant cell walls (Thibault et al., 1998; Crepin et al., 2004; Panagiotou et al. 2007).

White-rot basidiomycete fungi have also the ability to degrade lignin extensively (Kirk and Farrell, 1987). This capability to degrade lignin begins during the colonization of the substrate by the fungi and the easily digestible carbohydrates are converted into simpler sugars, a process known as fungus primary metabolism. The sugars are totally consumed by the fungus and then begins the secondary metabolism, which consists of the breakdown of structural carbohydrates and lignin from substrates by the extracellular enzymes (Moyson and Verachtert, 1991; Karunanandaa et al., 1995; Cohen et al., 2002; Valdez et al. 2008) composed of lignin peroxidases, laccases and manganese dependent peroxidases (Tekere et al., 2001; Martinez et al., 2002).

Considering the potential of the above mentioned enzyme complexes, the possible application of white-rot fungi to improve the nutritive values of fibrous feeds has also been studied. Nevertheless, available data on lignolytic enzymes is scarce, and most of the work was

conducted with fungi colonisation of cereal straws with good results in terms of digestibility increase, but with the loss of dry matter conditioning the efficacy of utilization of these fungi (Karunanandaa and Varga, 1996; Jalč et al., 1999). In addition, and supposing that the growth of fungi would not affect feed palatability, the appearance of the straw after the treatment is not appealing to the farmers.

The utilization of enzyme complexes in order to enhance feed digestion is one of the possible ways to increase the degradation of cellulose and hemicellulose, and several comprehensive reviews, regarding this subject, have been published (Beauchemin et al., 2003; Krause et al., 2003). According to these reviews the use of enzymes is considered to be very promising as increases in feed digestion have been reported. However, these enzymes are mainly constituted by cellulases and xylanases (Beauchemin et al., 2003).

A possible alternative to enhance the digestibility of fibrous feeds may than reside in the isolation and application of enzyme extracts, obtained from the cultivation of white-rot fungi on different substrates (Rodrigues et al., 2008). However, the available data on this subject is still sparse and several factors have already been pointed out that need further enlighten.

1.4. Objectives

The objective of this study was to evaluate the effect of the enzyme complexes produced by *Bjerkandera adusta, Ganoderma aplanattum, Phlebia rufa and Trametes versicolor* (Figures 1.5 to 1.8), cultivated on solid state fermentation, on the kinetics of wheat straw cell wall degradation, more specifically on the concentrations of lignin and esterified hydroxycinnamic acids.

Figure 1.5 – Illustration of *Trametes versicolor* (retrieved from www.mushroomexpert.com).

Figure 1.6 – Illustration of the *Ganoderma applanatum* (retrieved from www.mushroomexpert.com).

Figure 1.7 Illustration of the *Phelebia rufa* (retrieved from www.aranzadi-sciences.org).

Figure 1.8 - Illustration of the *Bjerkandera adusta* (retrieved from www.flickr.com).

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Chapter 2

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

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2.1. 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 (Lechner and Papinuti, 2006; Zeng et al., 2006; 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 (Ghatora et al., 2006; 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.

2.2. Methods

2.2.1. 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 4ºC and periodically subcultured.

2.2.2. 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.

2.2.3. 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.

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

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

2.2.5. Determination of Lignin and of esterified hydroxycinnamic acids in wheat straw samples

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

For the determination of hydroxycinnamic acids an adaptation of the procedures described by Chien (1992) was used. Samples were milled to a particle size of 1mm and further dried at 55ºC for 48h and dewaxed with toluene/80% ethanol (2:1, v/v) in a Soxtec apparatus for 3 h. After this, samples were incubated with 30 ml of ethanol (80%) at 85ºC for 1h

with continuous agitation. Following centrifugation (14000 rpm) at 5ºC for 15 min, the residue was dried at 60ºC overnight. A 25 mg portion of the dewaxed samples was saponified with 5ml of 1M NaOH for 18h under N_2 at 25ºC. An internal standard solution (100 µl) of ß-resorcylic acid in a solution (1:1, v/v) of methanol and water was added. The samples were neutralized to pH 2.0 with 6M HCl. The acidified solution was extracted with 3 × 2 ml of ethyl ether. The extract was dried 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 µm, 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).

2.3. Results and discussion

2.3.1. Enzyme activities

Enzyme production during incubation of four different fungi is presented in Figure 2.1. 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 2.1).

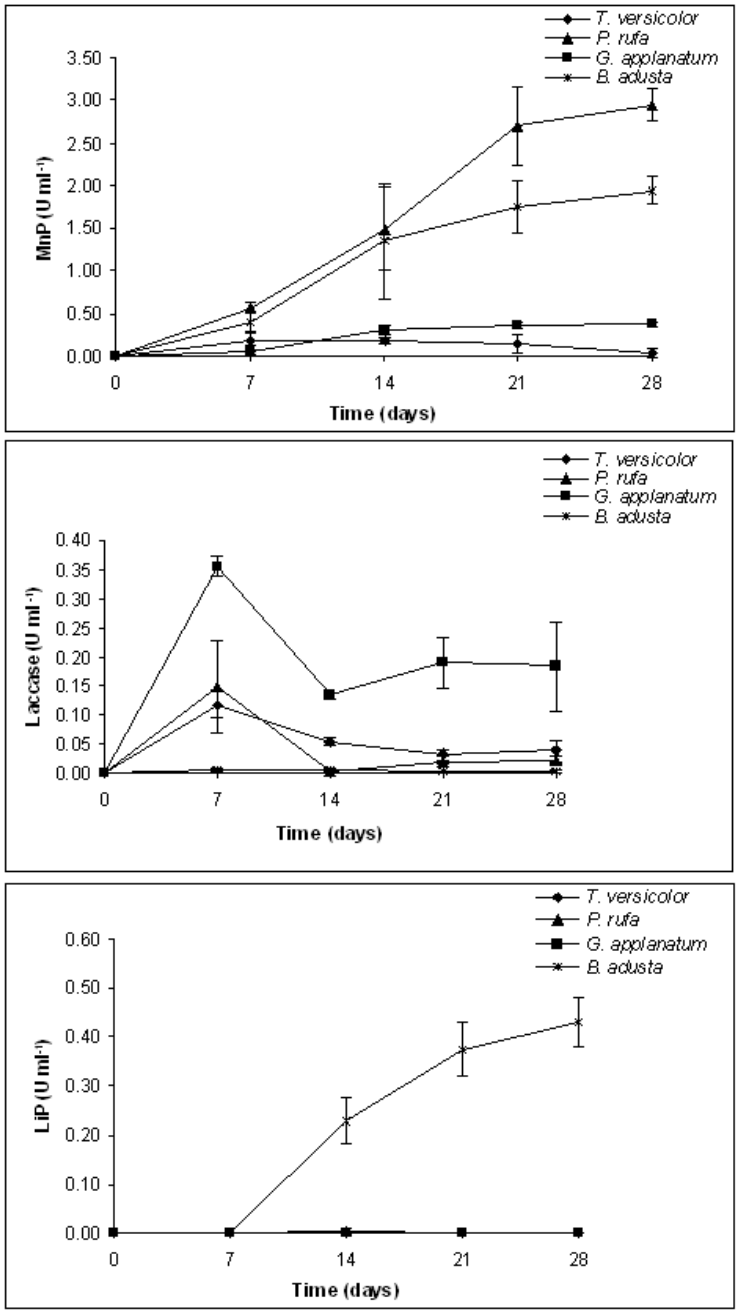

Figure 2.1 – Ligninolytic enzyme activities of white-rot fungi during the incubation period.

On the contrary, MnP production was lower for *G. applanatum* and *T. versicolor* for all incubation times (Table 2.1). With the exception of B. *adusta* that showed residual enzyme activity, laccase showed maximum activity at day 7 (Figure 2.1) for all the remaining fungi without any differences between the other incubation periods (Table 2.1). *G.* a*pplanatum* showed higher laccase activity for all incubation periods (Table 2.1). Regarding LiP, enzyme activity was only detected for *B. adusta*, showing the same tendency (Figure 2.1) already observed for MnP with increasing values until the end of the experiment.

The production of ligninolytic enzymes during wheat straw degradation by fungi has already been reported by several authors (Vares et al., 1995; Arora et al., 2002; Rodrigues et al., 2008; Zhang et al., 2008). However, the data regarding *P. rufa* and *G. applanatum* is being presented for the first time using a culture medium containing wheat straw. Laccase and MnP are considered to be the most common ligninolytic enzymes within white-rot fungi (Nerud et al., 1996). In several studies with different white-rot fungi strains activities of MnP and laccase are predominant (Vyas et al. 1994; Vares et al. 1995; Hofrichter et al. 1999; Tekere et al. 2001; Arora et al. 2002) and our results also show a general predominance on the activity of these two enzymes. Our data also show that MnP activity is much higher than that of laccase. This is in accordance to what was reported by Vyas et al. (1994) and Hofrichter et al. (1999) who refer to maximum levels of MnP 10 times higher than the maximum level of laccase activity. Production of LiP was only observed for *B. adusta* with a general increase along the incubation time. While LiP is not always detected (Tekere et al., 2001), Arora et al. (2002) and Vyas et al. (1994) detected LiP for *P. chrysosporium* and *T. versicolor* in SSF wheat straw mediums. One possible explanation for this may reside on the deficiency of identification of LiP activity when using the veratryl alcohol oxidation

Table 2.1 - 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.

assay (Arora et al. 2002) due to the presence of inhibitors or colour interference by aromatic compounds (Hofrichter et al. 1999).

While all fungi tested produced avicelase (Figure 2.2) its activity was very low, comparatively with CMCase. There were no significant differences for days 7, 14 and 21 (Table 2.1) and the maximum value observed (Figure 2.1) was on *T. versicolor* on day 28 (0.03 U/ml). In CMCase activity the most active producer appeared to be *G. applanatum*, with a maximum value (0.13 U/ml) on day14, while in *T. versicolor* (0.07 U/ml), *P. rufa* (0.06 U/ml) and *B. adusta* (0.02 U/ml) the peak was observed on day 7 (Figure 2.1). The xylanase activity (Table 2.1) was quite different for the four fungi ($P<0.05$). *G. applanatum* shows a first phase up to the second harvest (day 14) where it had a maximum value (0.22 U/ml), then declined, but it still existed with a lower value on day 28 (Figure 2.2). The activity of *P. rufa* between day 7 and 14 showed values fairly stable (Figure 2.2). Finally, *B. adusta* and *T. versicolor* showed a variable production for xylanase with no appreciable change over the 28 days of the trial. In line with our findings, CMCase activity usually presents values greater than avicelase independently of culture medium and fungi strains (Tanaka et al., 1999; Valášková and Baldrian, 2006; Rodrigues et al., 2008). Xylanase and Cellulolytic activities presented in this study are not within a high range of values. However, these relatively low values must be interpreted in relation to the overall objectives. In fact, if the utilization of these enzyme complexes is to be directed to the improvement of fibrous feed nutritive value, than we want to achieve maximum lignin degradation but not an extensive utilization of cellulose and hemicelluloses that must remain as energy sources for ruminant animals.

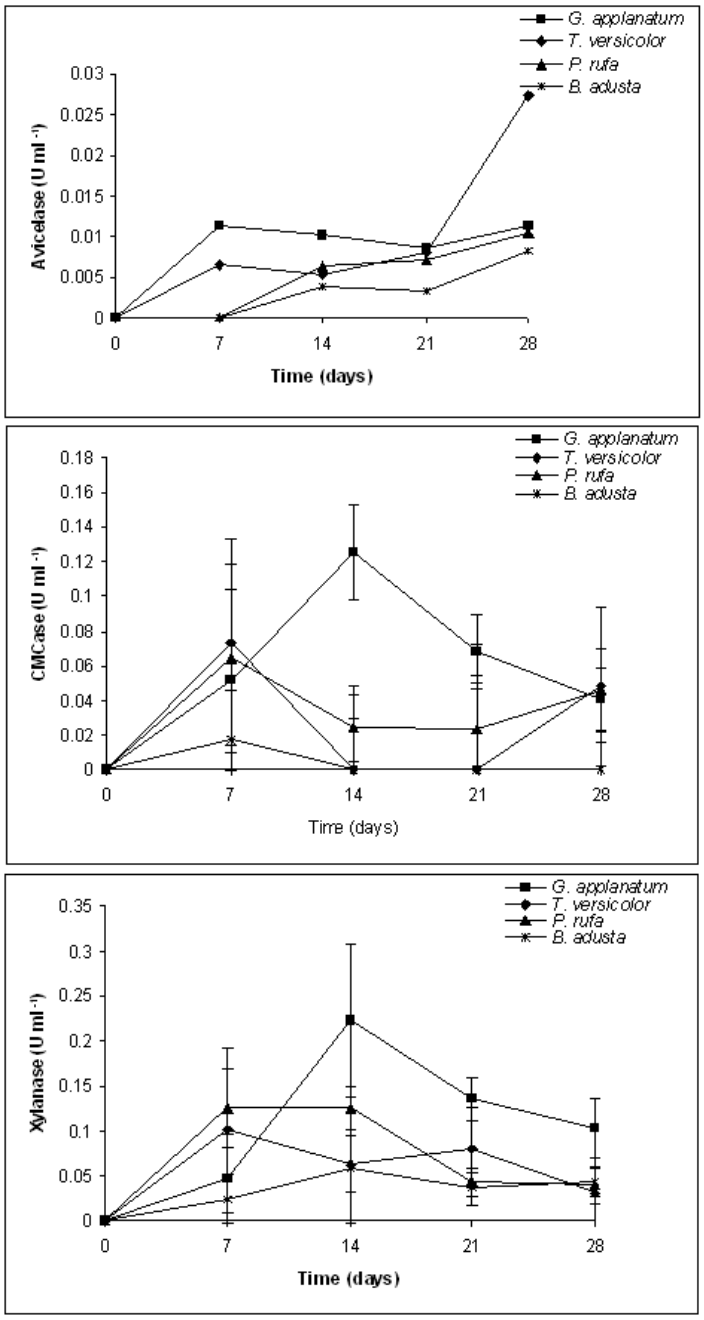

Figure 2.2 - Cellulolytic and hemicellulolytic enzyme activities of white-rot fungi during the incubation period.

Feruloyl esterase activity was quite similar (Table 2.1) for all fungi ($P<0.05$) with the exception of *G. applanatum*. There were no differences between day 7 and 28 (Table 2.1). Feruloyl esterase peak activity was detected on day 7 in all fungi with the exception of *T. versicolor* with a higher activity on day 14 and *P. rufa* which presented a second peak at 21 days of incubation (Figure 2.3).

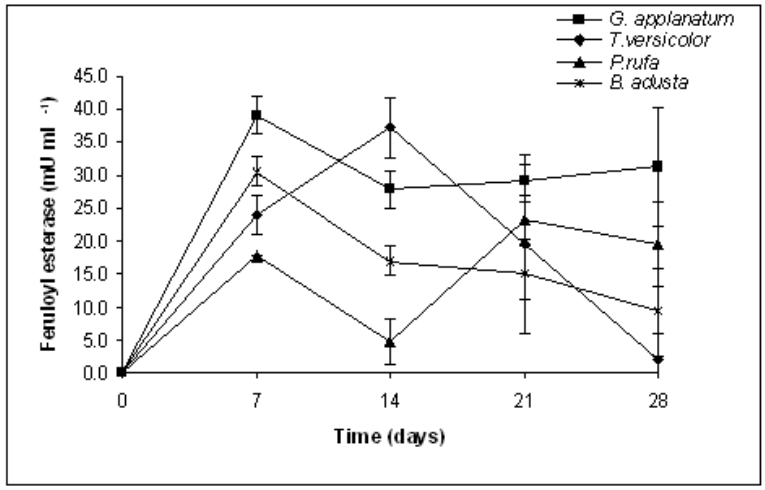

Figure 2.3 - Feruloyl esterase enzyme activity of white-rot fungi during the incubation period.

Data on feruloyl esterase activity for these fungi grown on wheat straw is scarce or at least it is not available. Generally the main fungi involved on feruloyl esterase production are *Aspergillus* and *Penicillium* and its activity is dependent on the substrate used, more specifically with the carbon source (Panagiotou et al., 2007). Nevertheless, our data seem to fall within the range of values reported by these authors who studied the production of feruloyl esterase by *P. brasilianum* grown on several substrates. Furthermore, *G. applanatum* showed the highest ($P<0.05$)

xylanase and feruloyl esterase activities (Table 2.1) indicating that the production of these two enzymes may be strongly related. This possible synergy among these enzymes, directly interrelated to the structural configuration of plant cell wall, has been evidenced by other authors (Ferreira et al., 2007; Panagiotou et al., 2007) and it will be discussed further on.

From the data we have obtained, and similarly to what was already reported for some of these fungi (Rodrigues et al., 2008), the differences between enzyme activities are quite high and must be interpreted by the different strains of fungi cultivated during different periods of incubation (Songulashvili et al., 2007) and different medium base substrates.

2.3.2. Lignin and esterified hydroxycinnamic acids

Phenolic composition of wheat straw cell wall is presented in Table 2.2. As expected results showed that ferulic and *p*-coumaric acids were the dominant esterified hydroxycinnamic acids and lignin concentrations are within the range of values normally reported for wheat straw. In spite of using different extraction methods, similar results have been reported by Pan et al. (1998), Yosef et al. (2000) and Sun et al. (2001).

Table 2.2 - Phenolic composition of wheat straw before incubation.

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

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

The effect of SSF in phenolic composition of wheat straw is presented in Table 2.3. While no significant effect (P>0.05) was observed in esterified *p*-coumaric acid content among the four fungi treatments, *B. adusta* gave the lowest decrease (P<0.05) in esterified ferulic acid concentration. For both esterified syringic and caffeic acids *P. rufa* treatment resulted in the highest values (P<0.05) in wheat cell wall after 28 days incubation. However, along the time of incubation there was a significant decrease (P<0.001) on the content of esterified hydroxycinnamic acids with no significant differences between 21 and 28 days of incubation (P>0.05) for esterified *p*–coumaric and ferulic acids concentrations. Already at 7 days of incubation (Figure 2.4) *T. versicolor* and *P. rufa* treated straw showed lower concentrations of esterified *p*-coumaric and ferulic acid, indicating that degradation was more effective with these two fungi in the beginning of incubation.

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

Table 2.3 - Phenolic composition of wheat straw incubated with four white-rot fungi in solid state fermentation.

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	-	*	-	***	-

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

[A] SyrA, Syringic acid; CafA, Caffeic acid; p-CoumA, *p*-coumaric acid; FerA, Ferulic acid; G, Guaiacyl, S, Syringyl; H, p-hydroxyphenyl.

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

[C] * P<0.05; ** P<0.01; ***P<0.001.

ferulic and *p*-coumaric acid respectively. However if this treatment was associated with a light alkaline extraction the recovery increased to 20 and 25%, respectively.

Taking in account the measurements we performed during the experimental period our results show that a decrease of around 80% for both esterified *p*-coumaric and ferulic acids was obtained until 28 days of incubation (Table 2.3). However, in relation to the initial concentrations of these hydroxycinnamic acids in wheat straw, a decrease of 72% and 77% was measured at 21 days of incubation for *p*-coumaric and ferulic acids, respectively, while for the period between 21 and 28 days of incubation the decrease was only around 20% for both esterified hydroxycinnamic acids. In this way it seems that there is a first phase up to the 14-21 days of growth on which there is a rapid decrease of esterified *p*-coumaric and ferulic acids, and a second phase in which values remain fairly stable indicating that probably fungi produced all the necessary enzymes to release these compounds in the first 21 days of incubation (Figure 2.4).

These results indicate that all the fungi treatments were able to reduce the content of esterified *p*-coumaric and ferulic acids in higher extent than the application of commercial enzymes, probably due to the synergism between the enzyme complexes produced by the fungi as we have mentioned in a previous work (Rodrigues et al., 2008). This is also valid to explain why *T. versicolor* and *P. rufa* treatments showed a more pronounced degradation of these hydroxycinnamic acids in the first 7 days of incubation (Figure 2.4).

The lignin content decrease did not differ widely between the different fungi treatments (Table 3). However there was a significant decrease ($P<0.001$) in the total amount of lignin from day 7 until the end of the incubation period (Table 2.3) reaching a value of 33%. When analysing

the content of wheat straw lignin without any fungal treatment (Table 2.2) it is possible to see that this decrease is around 43%, indicating that lignin loss was quite low within the first 7 days of incubation (13%). Differences were observed among the fungi treatments during the incubation period (Figure 2.5) with *T. versicolor* and *P. rufa* treatments leading to a higher decrease in lignin content between 14 and 21 days of incubation.

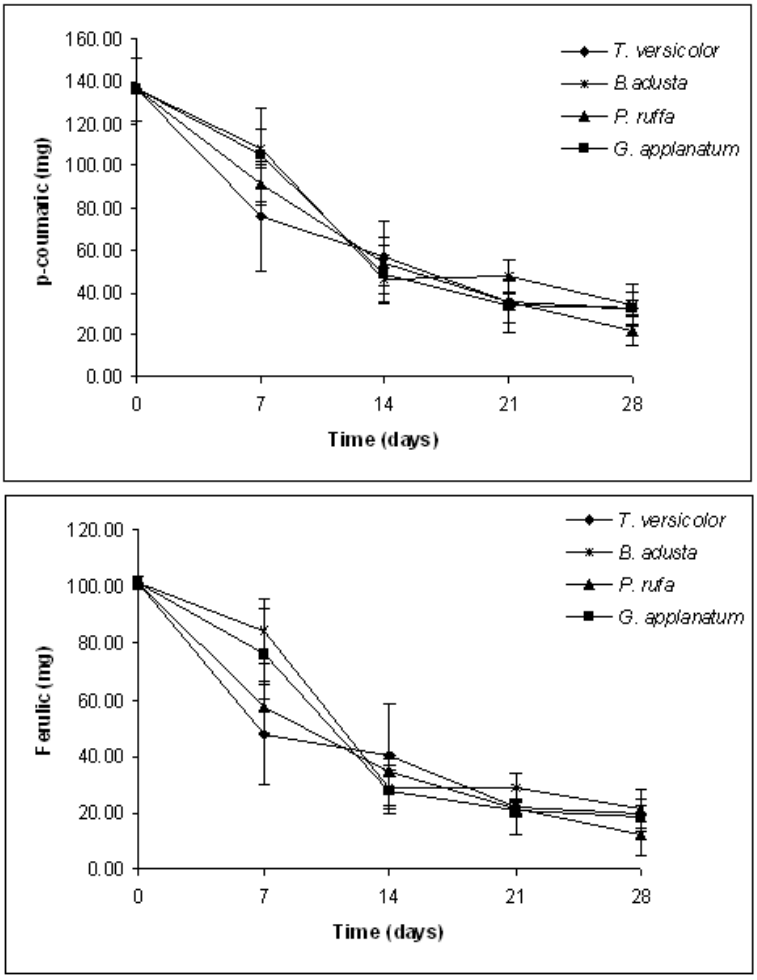

Figure 2.4 - Time course of hydroxycinnamic acids degradation during the incubation period.

Several authors have reported the potential for white-rot fungi to degrade lignin and presented values that are quite variable depending on the strain, type of fermentation as well as on the incubation period. For instance, Jalč (2002) on a review paper analysing the results of several authors presented values of wheat straw lignin degradation that varied between 2-65%. Arora et al. (2002) showed percentage loss of wheat straw lignin for *P. radiata* and *T. versicolor* of 18.5 and 12.5%, while other *Phlebia spp.* showed lignin losses that reached 25%. More recently, Zhang et al. (2008) when studying the effect of steam explosion pre-treatment on lignin biodegradation of wheat straw by *T. versicolor* reported a decrease around 30%.

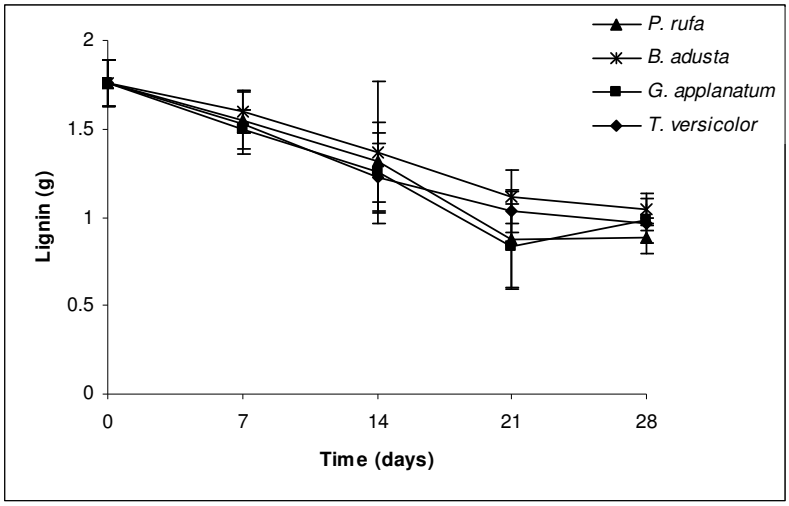

Figure 2.5 - Time course of lignin degradation during the incubation period.

As stated before lignin losses on the first 7 days of incubation were quite low with a more pronounced decrease afterwards. The same has been observed by Arora et al. (2002) who suggested a synergistic role in ligninolysis due to the fact that various enzyme maxima occurred prior to

maximum lignin loss. This sequential action of enzymes in lignin degradation was also pointed out by Zhang et al. (2008). According to these authors though the maximum of MnP and laccase activities were detected during the first 10 days of wheat straw incubation with *T. versicolor*, lignin degradation started later and tended to increase until the maximum degradation rate was reached on day 30. Our data also show a similar pattern in which significant lignin degradation seems to occur after an increase in the enzyme activities of laccase. However, the same was not observed for MnP activities as there was a general increase along the incubation period.

Synergistic effects are also attributed to the interaction between esterases and hemicellulases. Fungal feruloyl and *p*-coumaroyl esterases are capable of releasing feruloyl and *p*-coumaroyl units and play an important role in biodegradation of recalcitrant cell walls in grasses (Kuhad et al., 1997). These enzymes act synergistically with xylanases to disrupt the hemicellulose-lignin association, without mineralization of lignin *per se* (Borneman et al., 1990). Therefore, hemicellulose degradation seems to be required before efficient lignin removal can commence, at least for some substrates. This synergistic effect was also underlined by Panagiotou et al (2007) pointing out that the production of feruloyl esterase and arabinofuranosidase is co-regulated.

2.4. Conclusions

Our data show that the enzyme complexes produced by fungi seem to exert their effect in the cell wall structure due to the synergism between the different types of enzymes. In fact, the higher degradation of esterified hydroxycinnamic acids in the first 7-14days of incubation, directly related to the xylanase and feruloyl esterase activities during this period, precede a more intensive degradation of the lignin structures. The present study

also indicated that the fungi treatments were able to reduce to a considerable extent the content of esterified *p*-coumaric and ferulic acids. Considering that wheat straw is quite recalcitrant and that results from the application of commercial esterases do not normally approach such high values these results are of substantial interest.

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

2.5. References

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Chapter 3

Final Considerations

> "In the European Community, the amount of unexploited or badly exploited lignocellulosic by-products is considerable" (Zadrazil and Reiniger, 1988)

3.1. Final considerations

As mentioned before, in Chapter 1, the utilization of crop by-products faces new challenges that will definitively create new edible products in terms of its utilization in the bio-fuel industry, as a value added feed for ruminants or as a basal medium for fungal growth and posterior extraction of enzymes for food, pharmaceutical, pulp and paper industries.

For all these possibilities one of the main constraints still resides in limitations that cell wall structure poses to an efficient degradation process. In fact, the accessibility to fermentable carbohydrates trapped within a complex structural matrix, in which lignin and the hydroxycinnamic acids are considered to be the main *dramatis personae*, is not yet efficiently overcome.

White-rot fungi are considered to be unique in their capacity to degrade cell wall components of a variety of lignocellulosic materials. These fungi are able to increase selective delignification as well as to promote cellulose and hemicellulose biodegradation. For this purpose white-rot fungi produce a diversity of enzyme complexes, including cellulases (endoglucanases and cellobiohydrolases), hemicellulases

(xylanases), esterases and ligninolytic enzymes (laccase, Mn-peroxidase, lignin-peroxidase).

Following the research line on enzyme complexes isolated from white-rot fungi and its possible application to enhance the degradability of fibrous by-products, the work herein presented showed that the four fungi tested were able to efficiently degrade lignin and esterified ferulic and *p*-coumaric acids. However, the effect of the enzyme complexes is potentiated by the synergistic process along the degradation process. In fact, as it was discussed, several synergistic effects are attributed to the interaction between enzymes produced by white-rot fungi. Among these the integrative action between different ligninolytic enzymes as well as between esterases and hemicellulases are the most studied ones.

Data obtained suggest that lignin degradation is preceded by an intense degradation of esterified hydroxycinnamic acids, indicating that the cleavage of ester bonds between hemicellulose and ferulic acid is a key point for this process. Among the hydroxycinnamic acids, *p*-coumaric and ferulic acids cross-link to lignins and polysaccharides through ester and ether linkages and ferulates and differulates have also been found to form cross-links between lignin and hemicelluloses. If it is established that this structural arrangement is responsible for some of the main limitations to cell wall degradation, then a sequential enzyme degradation process that increases the ligninolytic enzyme accessibility to lignin is also a key factor in biodegradation processes of complex lignified substrates.

The majority of information so far available concerns the lignin-degrading enzyme systems of *Phanerochaete chrysosporium* and *Trametes versicolor*. However, other strains are now being studied and its potential is yet far from being described. In this study it is clear that different strains of white-rot fungi behave differently along the incubation period showing significant differences in enzyme kinetics and in cell wall biodegradation.

The data show that *Phlebia rufa*, may be considered for more specific studies as higher esterified ferulic and *p*-coumaric acids degradation was identified for earlier incubation times. Finally, it should also be referred that the levels of esterified hydroxycinnamic acids degradation were considerably higher than those presented elsewhere, also indicating the potential for *Phlebia rufa* utilization.