SHORT COMMUNICATION

Production of Ligninolytic Enzymes by Cultures of White Rot Fungi

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Abstract

Some *Basidiomycota* were chosen for studies of key ligninases synthesis (25°C, 30 days) in modified medium (shaken or not cultures) with added wheat straw. Liquid Czapek medium with straw yielded a higher amount of laccase than peroxidase, ground straw induced enzyme worse than chopped straw. With peroxidase the reverse dependencies were observed. Laccase of *Lentinus edodes* synthesized two enzyme isoforms (*ca* 30 and 16 kDa). In *T. versicolor* culture active laccase protein with highest molecular mass *ca* 65 kDa was found. *P. sajor-caju* yielded three different peroxidase isoforms. Ligninase biosynthesis depended on strain, straw fragmentation extent, culture method and growth medium.

K e y w o r d s: *Basidiomycota*, degradation of straw, laccase, peroxidase

Wood-degrading white rot fungi belonging to the phylum *Basidiomycota* degrade lignin in the natural environment under aerobic conditions by virtue of synthesizing ligninolytic enzymes that include the laccases (polyphenol oxidases EC 1.10.3.2) and lignin peroxidases (E.C.1.11.1.7) (Jung *et al.*, 2002; Sanchez, 2009). Both enzymes, depending on the part of the world, are produced using fungal cultures and lignocellulose substrates of varied origin. Laccases are secreted by higher plants, fungi and some bacteria (Claus, 2003; Hutterman *et al.*, 2001). These enzymes are characterized by broad substrate specificity, they oxidize phenolic compounds, aromatic amines and lignins, amongst others, reducing molecular oxygen to water (Riva, 2006). Laccase is capable of performing not only oxidation but other reactions which include even aromatic ring breaking. In the presence of appropriate mediators they are also capable of delignification of wood pulp and carrying out the oxidation of aromatic alcohols with high redox potential (Du *et al.*, 2013), In recent years a number of papers have described insects as laccase producers. Fungi, mainly the *Basidiomycota*, produce more than two types of peroxidases that play a key role in the degradation of lignins: a manganese peroxidase and a lignin peroxidase (Martinez, 2007). Fungi usually synthesize many isoforms of these enzymes, thus ensuring the degradation of various substrates, including lignins, aromatic and humic compounds as well as xenobiotics, including phenanthrene and pyrene (Hofrichter, 2002). Interest in ligninolytic enzymes grown from year to year because of their potential broad application in various industrial fields (Kunamneni *et al.*, 2008). Consequently, the considerable amounts of lignocellulose side or waste products from agriculture, forestry, sawmill industry, woodworking industry, furniture industry, milling industry, papermaking industry and the fermentation, fruits and vegetables industry, may find use in biotechnology for the large scale production of ligninases. One of such lignocellulose substrates is straw from grain crops, which depending on the type of plant can contain from about 30 to over 40% of crude fiber (mainly lignin and cellulose) as dry weight, which when added do culture media for *Basidiomycota* fungi is used to induce the synthesis of ligninases (Koroleva

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et al., 2002; Mayer and Staples, 2002; Michael *et al.*, 1991; Morais *et al.*, 2009; Shah *et al.*, 2005). The aim of these studies was to determine the level of ligninase production in cultures of fungi classified to the phylum *Basidiomycota* supplemented with wheat straw, a byproduct of the main grain crop cultivated in Poland, as an inducer of ligninases, depending on the degree of fragmentation of lignocellulose substrate and composition of culture medium.

The enzymatic studies embraced fungi belonging to the phylum *Basidiomycota* from the collection of the Department of Biochemistry, Maria Curie-Skłodowska University in Lublin: *Pleurotus ostreatus* 246 and *Lentinus edodes* 169 (Fungal Culture Collection of Forest Products Chemistry Laboratory, Tokyo, Japan); *Pleurotus ostreatus* 251 and *Lentinus edodes* 279 (Forest Products College of Agriculture Changbuk National University of Choengju, South Korea), Medical University in Warsaw *Pleurotus sajor-caju; A*sia Pacific Natural Agriculture Network, Bangkok, Thailand – *Pleurotus ostreatus* 6, University of Alexandria, Egypt – *Trametes versicolor* and *Phanerochaete chrysosporium.* Fungal strains were grown and stored at 5°C on potato-glucose agar (PDA) with slants or/in Czapek's agar medium (Burbianka *et al.*, 1983). The activity of select ligninolytic fungal enzymes was determined in cultures set up in modified liquid Czapek (Burbianka *et al.*, 1983) or potato-glucose media (PDE), in which saccharose or glucose, respectively, were replaced with 1% straw from Korweta winter wheat. Before preparing the growth media straw that was either ground or cut into 3 do 5 cm lengths was sterilized three times at 121°C. Fungi were grown on PDA at 25°C. After 10 day incubation ten 10 mm diameter discs were cut using sterile precautions out of each medium media overgrown with mycelium, which were transferred under sterile conditions to 500 ml Erlenmayer flasks containing 100 ml liquid of potato-glucose medium. After 10 days of incubation at 25°C in shaking water bath (130 rev./min) 10 ml of homogenized of fungal mycelium suspension was removed from the culture (inoculum) and transferred to 100 ml of modified production medium in which saccharose or glucose, respectively, were replaced with 1% straw from Korweta winter wheat (containing straw with varying degree of fragmentation from 3 to 5 cm length). Fungi were grown in three parallel cultures under stationary conditions without agitation or agitated in shaking water bath (150 rev./min) at 25°C. After 30 days the cultures were passed through Schott G1 filter coupled to vacuum filtration setup. Long time incubation is connected with decomposition of cellulose substances by lignolytic enzymes. The choice of the term was confirmed preliminary research where the highest activity of enzymes was observed after 30 day of incubation. Because breeding was carried out in a large volume of 100 ml volumetric flasks, of capacity 500 ml

of a suitable with narrow neck what stopped evaporation and in an air-conditioned room with a constant temperature and humidity. Laccase activity in culture filtrates was determined with the use of syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) as the substrate and peroxidase activity was assayed using the substrate pyrogallol (Leonowicz and Grzywnowicz, 1998; Toczko and Grzelińska, 1997). A unit (U) of laccase activity was taken as the amount of enzyme releasing 1 μmol products formed by the oxidation of 1 μmol syringaldehyde in one minute calculated per 100 ml filtrate, whereas a unit of peroxidase activity was taken as the amount of enzyme releasing 1 μmol purpurgallin in one minute calculated per 100 ml filtrate. The results of enzymatic analyses were verified by single- and multi-factor variance analysis using the program Statgraphics plus 4.0. Homogeneous sets were determined using Tukey's test for $\alpha = 0.05$. Laccase activity was detected on zymograms following electrophoretic separation under reducing conditions of 5-fold concentrated (Viva spin, Sigma) culture filtrates showing highest enzymatic activity. To detect laccase isoforms for the individual fungal strains electrophoresis was carried out under denaturing conditions in 12% polyacrylamide gel, after which the gels were incubated in 40 mM ABTS 100 mM Mc Ivaine buffer, pH 4.7; green bands against the background of unstained gel indicated the presence of active enzyme in the culture filtrates (Niku-Paavola *et al.*, 1990). The location of laccase activity bands on the gel with respect to standard proteins allowed the estimation of molecular masses.

The studied fungi grown in modified Czapek medium synthesized different amounts of laccase and peroxidase regardless of the degree of fragmentation of the straw and culture conditions (Table I). It was observed that whereas agitation of the fungal cultures increased the production of laccase, the lack of agitation reduced the synthesis of the enzyme and vice versa. Similar relations were found in the case of peroxidase production. Of the eight studied fungal strains only *P. ostreatus 246* produced the highest amount of laccase in medium containing ground straw that was not shake (Table I). In shaken cultures strains *P. ostreatus* 251, *P. sajor-caju* and *T. versicolor* produced the highest amounts of the studied enzyme compared to the remaining studied fungi (Table I). On average, wheat straw with lesser degree of fragmentation (cut) was a better inducer of laccase synthesis in both fungal culture conditions in Czapek medium than ground straw. The only exception was *P. ostreatus* 246, which produced half as much laccase in unshaken culture supplemented with cut straw compared to the ground substrate. In shaken cultures supplemented with wheat chopped straw *P. ostreatus* 251 synthesized the highest amounts of laccase; comparable enzyme activity was observed only in the case of *P. ostreatus* 246 (Table I).

Table I

Mean laccase and peroxidase activity ($U \times 100 \ ml^{-1}$) in culture filtrates of the studied fungi grown using various methods in Czapek medium containing 1% wheat straw with different degree of fragmentation (different letters indicate significantly differing means in columns for the type of culture)

Basidiomycota strains	Ground straw		Cut straw	
	Culture			
	unshaken	shaken	unshaken	shaken
Laccase				
1. *P. ostreatus* 246	1024.0 b	25.6 a	5424 cd	574.5 bc
2. *P. ostreatus* 251	8.4 a	102.5 ab	136.2 ab	727.8 c
3. *P. ostreatus* 6	62.2 a	15.8 a	480.8 bcd	122.8 ab
4. *P. sajor-caju*	129.7 a	162.9 b	274.1 d	152.4 ab
5. *L. edodes* 169	22.7 a	15.1 a	68.2 a	110.2 a
6. *L. edodes* 279	10.9 a	36.0 a	88.8 a	102.3 a
7. *T. versicolor*	8.0 a	63.3 ab	183.1 abcd	14.5 a
8. *Ph. chrysosporium*	5.2 a	40.5 a	160.1 abc	97.4 a
$LSD_{\alpha=0.5}$	389.964	106.064	382.446	462.316
Peroxidase				
1. *P. ostreatus* 246	0.91 ab	1.00 a	2.30 c	0.72 d
2. *P. ostreatus* 251	2.06 ab	0.93 a	0.10 a	2.27 e
3. *P. ostreatus* 6	0.88 a	2.10 a	0.05 a	0.54 bcd
4. *P. sajor-caju*	0.61 a	3.40 a	0.22 a	0.27 ab
5. *L. edodes* 169	3.75 abc	0.94 a	2.20 c	0.73 d
6. *L. edodes* 279	6.13 bcd	3.50 a	0 a	0.07 a
7. *T. versicolor*	10.60 d	5.10 a	1.42 b	0.34 abc
8. *Ph. chrysosporium*	7.36 cd	30.00 b	0.12 a	0.61 cd
$LSD_{\alpha=0.5}$	5.24828	14.1296	0.36819	0.28898

The results presented in Table I show that the fungal strains produced lower amounts of the peroxidase, compared to laccase, regardless of culture conditions employed. *Phanerochaete chrysosporium* was found to be the best peroxidase-producing strain in shaken culture with ground straw.

No peroxidase activity was found in *L. edodes* 279 cultures in Czapek medium containing chopped straw. The presented zymogram of culture filtrates shows that the studied strains released to the culture medium laccase with varied molecular mass. Under the conditions used only strains of *L. edodes* secreted two isoforms of laccase with low molecular mass each, respectively, molecular mass approx. 95 kDa and 50 kDa, in case of *P. chrysosporium* only single form of enzymes of mass approximately 90 kDa was secreted. Active laccase protein with highest molecular mass of approx. 65 kDa was observed for *T. versicolor*. *P. ostreatus* strains synthesized a single form of laccase with mass approx. 60 kDa and for *P. sajor-caju* with mass approx. 25 kDa.

At the same time the fungi were grown in PDE medium with the addition of 1% ground straw wheat. Most of the investigated strains grown in this medium produced higher amounts of laccase and peroxidase than in Czapek medium, regardless of culture method used (Table II). *P. sajor-caju* proved the best fungus of all those studied in the production of laccase in potato medium regardless of culture conditions whereas it synthesized peroxidase the best in shaken culture. Similarly as in Czapek medium, shaking of fungal cultures in PDE medium increased the production of laccase and peroxidase, whereas lack of agitation reduced the synthesis of enzymes (Table II).

For many years *in vitro* studies have been carried out on the synthesis and activity of inducible ligninolytic enzymes, the most important of which are laccase and peroxidase produced by so-called white rot fungi, which degrade lignins the most effectively. Different lignocellulosic substrates are used to induce the synthesis of ligninases, including straw from grain crops (Koroleva *et al.*, 2002; Morais *et al.*, 2001; Shah *et al.*, 2005). Enzymatic studies utilizing zymograms and colorimetric methods demonstrated that eight species of fungi belonging to the phylum *Basidiomycota*, produced laccase and peroxidase in both using medium supplemented with the wheat straw. The mean amount of laccase in submerged fungal cultures was significantly higher, regardless of method, than that of peroxidase, which is compatible with the results of Koroleva *et al.* (2002), Mayer and Staples (2002).

Table II
Activity of studied ligninases (U × 100 ml⁻¹) in culture fluids of fungi grown using different methods in
PDE medium supplemented with 1% ground wheat straw (different letters indicate significantly differing
means in the type of culture columns)

Basidiomycota strains	Laccase		Peroxidase	
	Culture			
	unshaken	shaken	unshaken	shaken
1. *P.ostreatus* 246	537.9 ab	15.8 a	1.72 ab	1.45 a
2. *P.ostreatus* 251	15.4 a	36.4 a	1.88 ab	0.85 a
3. *P.ostreatus* 6	375.5 ab	28.5 a	4.20 b	1.33 a
4. *P. sajor-caju*	2781.9 c	2028.3 c	2.60 ab	74.60 b
5. *L. edodes* 169	9.2 a	39.7 a	1.00 a	3.10 a
6. *L. edodes* 279	17.7 a	725.2 b	24.20 c	1.10 a
7. *T. versicolor*	1026.0 b	30.0 a	1.80 ab	5.20 a
8. *Ph. chrysosporium*	31.0 ab	146.6 a	10.20 b	3.80 a
LSD$_{\alpha=0.5}$	1000.11	465.14	36.0849	55.0441

The reason for this may be, among others, the ability of laccase to degrade lignins without synergistic cooperation with other ligninases, which has not been observed for peroxidase activity (Galliano *et al.*, 1991; Mayer and Staples, 2002). For this reason fungi that synthesize laccase do not have to produce significant amounts of peroxidases. The extent of fragmentation of wheat straw significantly affected the biosynthesis of the studied ligninases by fungal cultures in Czapek medium, which is compatible with the results obtained by Criquet *et al.* (1999), who showed that ground substrate increased laccase activity. Our studies have shown reverse relations for the production of both laccase and peroxidase (Table I). The biosynthesis of laccase was higher in fungal culture with chopped straw, whereas that of peroxidase was higher in medium with ground straw. This can be explained among others by different access to oxygen of the mycelium in culture with straw with varying degrees of fragmentation. The chopped straw did not form compact pellets with the mycelium, which are known to limit the access of oxygen the hyphae of the mycelium (Nazareth and Sampy, 2003). This is why media supplemented with cutting straw ensured optimal conditions for the biosynthesis of laccase. Ground straw together with hyphae of the mycelium forms aggregates that could restrict the access of oxygen for inside of aggregates, which in consequence could reduce the production of laccase. At the same time ground straw, by limiting laccase production and thus the intensity of the degradation of lignin, impeded the access of the mycelium to nitrogen compounds in the straw of grain crops after the depletion of the mineral nitrogen from the medium, which increased the secretion of peroxidase to the medium (Krajewski and Witomski, 2003). Since microorganisms degrade lignin oxidatively, the fungal cultures were set up using two methods – with and without agitation. Although agitation of the culture medium ensured the similar oxygen concentration in the whole culture volume, this did not always result in increased enzymatic activity. However, on the average biosynthesis of laccase in unshaken cultures of the studied fungi was higher than in shaken cultures, whereas in the case of peroxidase the reverse relationship was determined (Table I). Nazareth and Sampy (2003) demonstrated the negative effect of agitation of fungal cultures on laccase production. The authors attributed this to mechanical damage to the mycelium during agitation and its negative effect on fungal growth and synthesis of enzymes as well as to changes in CO_2 concentration in the medium (Zadrazil, 1975). However, this is not a rule that relates to all lignin-degrading fungi, as shown by our own studies. Agitation of *P. ostreatus 251* culture increased the biosynthesis of laccase about 12-fold compared to unshaken culture (Table I), which is in agreement with those of Jung *et al.* (2002). The higher production of the enzyme under agitation conditions may be the result of varied resistance of fungal hyphae to mechanical damage caused by shaking the culture as well as different requirements regarding oxygen and CO_2 concentration in the mycelium growth phase. In the literature there are several papers describing the fungi of the phylum *Basidiomycota* producing different numbers of laccase isoforms (Bertrand *et al.*, 2013; Karp *et al.*, 2012; Lettera *et al.*, 2010; Libardi *et al.*, 2012), which has been confirmed by the results of our studies. All the fungal strains investigated by us with the exception of *Lentinus edodes* produced only one isoform of the laccase. *Lentinus edodes* strains synthesized two isoforms of laccase, which is similar to the isoforms produced by *T. versicolor* HEMIN-9 (Bertrand, 2013). Libardi *et al.* (2012) using banana peels as a source of carbon observed the

synthesis of two laccase isoforms with mass 58.7 kDa and 21 kDa by *P. ostreatus* DSM 1833. The isoform of laccase with size 21 kDa described by these authors is similar in size to the isoforms produced by *P. ostreatus* 246, *P. ostreatus* 251 and *P. ostreatus* 6 (ok. 25 kDa) in our studies. The intensity of the biosynthesis of ligninolytic enzymes by *Basidiomycota* cultures depends on the biologically active substances present in the growth environment of fungi, as demonstrated by Eriksson and Blanchette (1990), as well as by the authors of the results presented herein. On average higher activity of ligninases in post-culture fluids of fungi maintained in modified PDE medium containing ground wheat straw compared to enzyme activity in Czapek medium was obtained. Modified potato medium (PDE) is richer in nutrients than modified Czapek medium since it contains 4 g potato extract per 1000 ml medium. Besides potato extract the medium was also supplemented with a small amount of starch and the amino acids aspartic acid, glutamic acid, leucine, lysine, phenylalanine, serine, threonine, arginine, tyrosine and others. The added chemical compounds stimulate the growth of heterotrophic microorganisms, including fungi, as well as their enzymatic activity, which has been confirmed in laboratory studies by Ruttimann-Johnson *et al.* (1993), Shi *et al.* (1997). Wheat straw, which is an agricultural byproduct, is a good inducer for the synthesis of key ligninolytic enzymes (laccase and peroxidase) in cultures of fungi from the phylum *Basidiomycota* selected for the study. The synthesis of ligninolytic enzymes is affected by choice of strain, as well as by various abiotic factors (degree of straw fragmentation, culture method, composition of culture medium).

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