Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of *Pleurotus ostreatus* on wheat-straw-based substrate

Martin Rühl, Christoph Fischer and Ursula Kües*
Molecular Wood Biotechnology and Technical Mycology, Büsgen-Institute, Georg-August-University Göttingen, Büsgenweg 2, 37077 Göttingen, Germany
* For correspondence - ukupees@gwdg.de

**Abstract**

Two commercial *Pleurotus ostreatus* strains (030 and K12) were cultivated under industrial conditions on wheat straw blocks 17-18 kg in weight. Within 10 weeks of cultivation, three flushes of fruiting body production were obtained with total yields of 4.2 and 4.1 kg mushrooms/substrate block and a biological efficiency (BE = total yield of fresh mushrooms per dry weight of substrate) of about 95%, respectively. Ligninolytic activities were followed up within the substrate over the cultivation periods. Laccase, manganese peroxidase (MnP) and versatile (manganese-independent) peroxidase (VP) activities were high in vegetative growth phases within the substrate and dropped upon initiation of fruiting body formation to be hardly detectable at the harvest of the mature mushrooms. Directly after harvest at all observed flushes, there was a sharp increase in all three enzymatic activities in the substrate for both strains (maximum activities for laccase 1.1-1.3 U/ml, for MnP 15-16 U/ml and for VP 1.1-1.4 U/ml). Enzymes can be extracted by pressing the spent mushroom substrates (SMS). Potential applications for the press juice or the enzymes in the SMS are discussed.

**Key words**

Laccase, peroxidase, fruiting bodies, spent mushroom substrate (SMS), solid state fermentation (SSF)

**Introduction**

Worldwide, with constantly increasing production rates, *Pleurotus* species are currently the second most produced edible mushrooms (1, 2). Alone in China, the annual production in 2003 was about 2.5 million t (3). *Pleurotus ostreatus* (Jacq.) P. Kumm. 1871, known as hiratake in Japan and as oyster mushroom in Western countries, is a saprophytic white-rot basidiomycete growing in forests on hardwoods and less often on softwoods (4). For commercial production of the fruiting bodies, mainly straw from wheat (Western countries) and rice (Asia) is used as cheap basic substrate, but saw dust and wood chips and other agricultural wastes may also be used (5). To facilitate growth on such lignocellulosic substrates, white rot-fungi secrete different types of oxidative enzymes for lignin degradation (6, 7). *P. ostreatus* produces different types of peroxidases (MnP: manganese dependent peroxidase and VP: versatile peroxidase; synonym: manganese-independent peroxidase MiP) as well as laccases (8, 9).

Ligninolytic enzymes have also repeatedly been linked to fruiting body production in higher basidiomycetes but species appear to differ at timings of high enzyme production and there is no clear-cut picture on functional relevance of the enzymes in fruiting (1, 10). From small scale experiments in *P. ostreatus*, it has previously been reported that production of ligninolytic enzymes increases with vegetative biomass production on...
solid growth substrates and that it drops during the sexual fruiting stage (11 - 13). Here, we present a study on the ligninolytic activities of two commercial *P. ostreatus* strains during substrate colonisation and over three flushes of fruiting body production under industrial conditions. High yields of laccases and peroxidases were detected in the substrate blocks shortly after mushroom harvests offering the possibility to harvest from spent mushroom substrate (SMS) enzymes for biotechnological applications as an extra benefit from mushroom cultivation.

**Methods**

**Cultivation of *Pleurotus ostreatus* on solid substrate**

Industrial produced pasteurised wheat-straw-based substrate originally prepared and inoculated with a fungus by Substratbetrieb Horst (Horst, Netherlands) was provided by the commercial mushroom grower druid Austernpilze (Immichenhain, Germany). Two different production strains of *P. ostreatus* were used, 030 (Le Champignon, Loches, France) and K12 (Sylvan Inc., Horst, Netherlands). Commercial substrate blocks (50 x 40 x 17 cm³, around 17-18 kg each) wrapped in plastic foil were placed in a mushroom cultivation chamber.

During vegetative growth or for regenerative growth after mushroom harvest of the first flush, the temperature in the chamber was kept at 20-25 °C and the relative humidity (RH) at 90-95%. Once fruiting body primordia (sized 1 to 2 mm in Ø) became visible for the naked eye, the temperature was lowered to 16 to 18 °C and RH was increased to 95-98% as done in commercial mushroom production to improve fruiting body development and quality of the mushrooms (5). The lower temperature was hold up to the harvest of the mature mushrooms. Upon the first flush, the temperature was again raised for regenerative growth but not upon the second flush. The actual temperature setting throughout the cultivation was influenced by internal substrate conditions. During the whole cultivation periods, the internal temperature of substrate blocks was measured. The internal values were kept around 20 °C (20-21 ± 1 °C) in vegetative growth phases and around 19 °C (19 ± 2 °C) in mushroom production phases. Deviations from these internal values were compensated by smaller adjustments in temperature in the cultivation chambers (± 5 °C during mycelial growth phases, ± 2 °C during mushroom production phases). The moisture content of the substrate blocks was also controlled throughout the length of the cultivation period and found to be relatively constant (76 to 78%) at a level as wanted for mushroom cultivation (14).

**Determination of mushroom yields**

Fruiting bodies were harvested at maturation and weighted. Total mushroom yields were calculated as biological efficiency (BE) in percentage of the weight of fresh fruiting bodies per weight of dried substrate (5, 15).

**Enzyme detection in the substrate**

Every second day from day 21 after inoculation onwards when the substrate was fully colonised, samples of about 50 g (± 10 g) throughout colonised substrate were taken by cutting approximately 4 x 4 x 2 cm³ sized cuboids from the substrate blocks. The samples with the submerged mycelium were weighted and subsequently squeezed by a self-made hand press (contact surface 25 x 30 cm²) in order to extract all free liquid from the substrate. The residual substrate was dried at 80 °C for 48 h and the substrate moisture content was calculated from subtracting the dry weight (gram dried substrate [gds]) from the wet weight at sample harvest.

The liquids pressed from substrate samples were collected and measured in volume and their
pHs were determined. Upon centrifugation in order to eliminate any smaller solid parts from the liquids, aliquots were taken for enzyme measurements. Laccase activity was determined in 120 mM sodium acetate buffer (pH 5.6) with ABTS (2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonate]) as a substrate and the oxidation was determined at 420 nm according to Matsumura et al. (16). Laccase activities were calculated using an extinction coefficient of $\varepsilon_{420} = 36000 \text{ M}^{-1}\text{cm}^{-1}$ (17). Peroxidase activities were analysed by oxidation of phenol red under presence and absence of Mn$^{2+}$ for determining MnP and VP activities, respectively, according to the protocol of Kuwahara et al. (18). Peroxidase activities were calculated using an extinction coefficient of $\varepsilon_{610} = 4460 \text{ M}^{-1}\text{cm}^{-1}$ (19). Enzyme activities are given in units (U) defined as the amount of ABTS, respectively phenol red in $\mu$mol transformed per min.

Results

Laccase and mushroom production alternate during cultivation of *P. ostreatus* 030

In a first experiment, laccase activities were followed up within three commercial substrate blocks inoculated with *P. ostreatus* strain 030. Once the first primordia were observed (day 4 after fully colonising the substrate blocks), the temperature was set at 16-18 °C and RH to 95-98%. In all three substrate blocks, laccase activities were comparably low during subsequent primordia and fruiting body development (approximately 0.3-0.5 U/ml press juice) and at harvest of the mature fruiting bodies (approximately 0.1-0.2 U/ml press juice). 3.0 ± 0.4 kg mushrooms per substrate block were collected at the first flush lasting four days. The day after harvest, there was a sudden increase in enzyme activity with an average laccase activity of 0.6 U/ml press juice. For regenerative mycelial growth, two days later the temperature for cultivation was raised to 20-25 °C and there was a further increase in laccase activity to about 1.5 U/ml hold in this range over the following seven days. Afterwards, laccase activities in the substrate blocks gradually dropped to about 0.8 U/ml press juice for the next seven days. At this point, primordia of the second flush became visible and the temperature was lowered to 18°C. Laccase activities declined further in the next days of incubation. Mature fruiting bodies of the second, slightly prolonged flush were first harvested after one week (2.0 ± 0.7 kg mushrooms per substrate block; 125% BE in total calculated from the yields of the first and the second flush) when laccase activities were barely detectable. During the three subsequent days of flush of the second harvest, there was again a dramatic increase in laccase activity to about 0.8 U/ml at day 3 of harvest (last day of harvest) and about 1.4 U/ml press juice two days later. The observations suggested an inverted correlation between laccase activity and fruiting body maturation with the minimum of activity occurring when the mushrooms were mature.

Mushroom production in a large scale experiment with two different *P. ostreatus* strains

To follow this observation up in more detail, commercial wheat straw blocks inoculated with either *P. ostreatus* 030 or with *P. ostreatus* K12 were incubated in a cultivation chamber under conditions of industrial mushroom production. Per strain, six substrate blocks were analysed. All substrate blocks were completely colonised 21 days after inoculation (Fig. 1A). After three further days, when small primordia appeared (Fig. 1B), the temperature was lowered for fruiting body production (Fig. 1C) until mushrooms were harvested (Fig. 1D) upon which the temperature was raised for regenerative growth (Fig. 1E). Two further flushes of mushroom production were followed up (not
Fig. 1 Wheat straw substrate blocks at different stages of cultivation with *P. ostreatus* strain 030 (2nd experiment) shown in full sight (left panels) and representative detailed views with fungal structures (right panels). Days indicate the age of the substrate block after inoculation with the strain. 

A. A substrate block at day 21 when the whole substrate was colonised by the fungus. 

B. A substrate block at day 25 when the young primordia appeared. 

C. A substrate block at day 29 with maturating fruiting bodies. 

D. A substrate block at day 31 with mature sporulating fruiting bodies. 

E. A substrate block at day 33, after mushroom harvest.
Ligninolytic enzymes in the large scale experiment

The water content of the substrate blocks was relatively constant over the whole cultivation periods with values between 76% and 78% and the pH of the press juice from the substrate was stable at pH 5.0 ± 0.2, regardless of the 
P. ostreatus strain used. Activities of three types of enzymes (laccase, MnP, VP) were measured in eluates obtained from pressing representative samples of the substrate blocks. The activity of all the ligninolytic enzymes showed a periodical alteration with mushroom production (Fig. 2 and Fig. 3). For both strains, activities of all three enzymes gradually declined with progress of vegetative growth and primordia production to be lowest at the point of mushroom harvest. Also for both strains, there was a sharp increase in activities of all three enzymes shortly after each mushroom harvest. Over the time, the activities curves of all three enzymes in both strains followed similar patterns with maxima and minima occurring at comparable periods. However, highest laccase activities of 1.4 U and 1.1 U per ml press juice (5.45 U/gds and 4.30 U/gds) and 0.9 U and 1.1 U per ml press juice (3.55 U/gds and 3.88 U/gds) were reached for 
P. ostreatus strains 030 and K12, respectively, prior and after the first flush at vegetative growth phases where primordia were not yet formed. At mushroom harvests, laccase activities in the substrate were in the range of 0.2 to 0.5 U/ml (Fig. 2B, Fig. 3B). Maximum MnP and VP activities were observed shortly after the second and the third flush in regenerative phases prior to primordia formation. MnP activities were always about 10 times higher than VP activities (compare Fig. 2C and D, Fig. 3C and D). Maximum MnP activities were 15.0 U/ml and 16.0 U/ml (50.63 U/gds and 55.31 U/gds) (Fig. 2C, Fig. 3C), and maximum VP activities 1.9 U/ml and 1.5 U/ml (5.91 U/gds and 5.58 U/gds) for strains 030 and K12, respectively (Fig. 2D, Fig. 3D). At times of harvest, when all enzyme activities were lowest, minimum values between 0.0 U/ml to 4.5 U/ml were measured for MnP activities (Fig. 2C, Fig. 3C) and minimum values between 0.0 U/ml to 0.3 U/ml for VP activities (Fig. 2D, Fig. 3D), respectively. Parallel to the prolonged harvest time of the second flush (Fig. 2A, Fig. 3A), the period of low level enzymatic activities in the substrate was longer at the second flush than at the first flush (Fig. 2B-C, Fig. 3B-D). Since temperature shifts documented by photographs). For both strains, the three flushes occurred synchronised in weeks 5, 8 and 10 of cultivation with only slight time shifts between 
P. ostreatus strain 030 and K12 (Fig. 2A and Fig. 3A). Mushroom yields declined with the three flushes from 2.2 kg to 0.5 kg per substrate block (Table 1). Whilst the first flush was confined in both strains to one day, the second flush was spread over a few days and the third flush with one day was short (Fig. 2A, Fig. 3A) with little yield (Table 1). The total mushroom yield (in average 4.1 and 4.2 kg per substrate block) and the total BE (95%) did not differ significantly between the two strains (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>1st harvest</th>
<th>2nd harvest</th>
<th>3rd harvest</th>
<th>Total harvest</th>
<th>Total BE(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>030</td>
<td>2.2 ± 0.4 kg</td>
<td>1.4 ± 0.8 kg</td>
<td>0.5 ± 0.3 kg</td>
<td>4.1 ± 0.8 kg</td>
<td>95 ± 16%</td>
</tr>
<tr>
<td>K12</td>
<td>2.2 ± 0.2 kg</td>
<td>1.5 ± 0.6 kg</td>
<td>0.5 ± 0.3 kg</td>
<td>4.2 ± 0.6 kg</td>
<td>95 ± 19%</td>
</tr>
</tbody>
</table>

Table 1: Mushroom yields per substrate block (n=6)
Fig. 2 Fruiting body production (A) and ligninolytic enzyme activities (B. laccase, C. manganese peroxidase = MnP, D. versatile peroxidase = VP) during cultivation of *P. ostreatus* strain 030 on industrial wheat straw substrate blocks. Values are calculated averages and standard deviations from six substrate blocks. In A., the environmental settings in the climate chamber (relative humidity: solid line; temperature: dashed line) are given in addition to the biological efficiency (BE) of fruiting body production.
applied in the culturing regime were always done after a drastic change in enzyme activities, respectively not done after the second flush (Fig. 2A, Fig. 3A), we can conclude that changes in enzymatic activities were influenced by the internal physiological conditions of the fungus related to synchronisation within the mycelium of fruiting and non-fruiting periods. Some influence on the absolute level of enzymatic activities by the temperature of the air cannot be excluded but substrate temperature, moisture and pH did not differ significantly by the different settings of environmental conditions in the climate chamber (see above). The microclimate within the substrate might be expected to be more decisive for the enzymatic activities of the submerged mycelium. However, communication with the surface mycelium and the developing fruiting structures must exist in order to regulate enzymatic activities in the submerged mycelium appropriately up or down as required in the cycle of vegetative mycelial growth and sexual reproduction.

Discussion

Various types of oxidative enzymes are produced by white rot fungi in order to make use of lignocellulosic substrates for nutrition (6, 7). In this study, we found commercial P. ostreatus strains to produce laccase, MnP and VP in solid state fermentation on wheat straw as described previously for the species in the literature (12, 20, 21). Under conditions of industrial mushroom cultivation using 17-18 kg blocks of wheat straw, enzyme activities in the substrate blocks alternated with periods of mushroom production (Fig. 2, Fig. 3). Mata et al. (11) and Elisashvili et al. (13) reported a similar diurnal behaviour in enzymatic activities (laccase and MnP) and fruiting body production when strains were cultivated on other lignocellulosic substrates (sugarcane bagasse, tree leaves). Mata et al. (11) tested however only the first flush on 0.5 kg blocks with sugar cane bagasse as substrate and Elisashvili et al. (13) apparently also but on 2 kg substrate blocks of either tree leaves or wheat straw. In our study, we followed up the enzymatic behaviour in the substrate over three flushes on large industrial substrate blocks. As in mushroom industry (5), we obtained two flushes with reasonable yields whilst the output of the third flush dropped to a third of the yield of the second flush (Table 1). In industrial production, a third flush is usually not waited for since the earnings expected from a third flush do not outweigh the costs of the prolonged cultivation under a controlled temperature and humidity regime (5). Our total yields in both series of experiments were comparable to standard yields in commercial production (5).

The diurnal cycling pattern of enzymatic activities in the substrate and fruiting body production suggests a clear physiological distinction within cultures of P. ostreatus between phases of vegetative growth and phases of sexual reproduction. Lignocelolytic enzymes appear in P. ostreatus to be produced for regenerative vegetative hyphal growth but not for fruiting body development. Similarly, high enzymatic activities were observed in lignocellulosic substrates during growth phases in the white-rotting basidiomycetes Lentinus tigrinus [laccase and peroxidase (22)], Lentinula edodes [laccase and peroxidase (23)] and Grifola frondosa [laccase (24)] with drastic reductions in enzyme activities during the period of fruiting body formation. For these species, it is very likely that the enzymes are produced for lignocellulosic substrate degradation in order to provide nutrients for the growing organism. In contrast, in cultures of the compost fungus Agaricus bisporus, laccase and peroxidase activities increase in the substrate from vegetative growth to early stages of fruiting body development and drop strongly during fruiting body maturation (25, 26). Furthermore, laccase activities increase greatly in substrates
Fig. 3 Fruiting body production (A) and ligninolytic enzyme activities (B, laccase, C, manganese peroxidase = MnP, D, versatile peroxidase = VP) during cultivation of P. ostreatus strain K12 on industrial wheat straw substrate blocks. Values are calculated averages and standard deviations from six substrate blocks. In A, the environmental settings in the climate chamber (relative humidity: solid line; temperature: dashed line) are given in addition to the biological efficiency (BE) of fruiting body production.
after vegetative growth at fruiting body initiation of cultures of the straw fungus Volvariella volvacea and they stay at high level until the fruiting bodies matured (27). If not also for nutritional reasons produced under an alternative regulation regime, enzyme production might be oxidative stress-related (28, 29) and/or the enzymes might have functions in fruiting body morphogenesis and pigmentation (1, 10, 27). The discussion on biological functions of laccases and peroxidases in relation to fruiting body development of basidiomycetes remains still controversial (1, 10).

The anti-cyclic pattern of enzyme production and fruiting body development as observed in our study for P. ostreatus is however of a potential biotechnological interest. Industrial enzymes from fungi are mostly produced in bioreactors in liquid cultures but production by solid state fermentation (SSF) finds increasing attention (30-32). P. ostreatus has been tested in SSF with wheat straw, canola meal, sawdust based SMS, grapevine sawdust, sugarcane bagasse and tree leaves as substrates in production of phenol oxidases and/or also cellulases (9,11,13,33-37). In small scale production [using either 16 g substrate with grapevine sawdust (9), 500 g bagasse substrate (11), 10 g dry canola meal (35), or 16 g substrate of tree leaves (37)], yields between 0.12-32.2 U/gds and 18.0 U/gds were reported for laccases and yields between 0.37-6.9 U/gds and 1820 U/gds for MnP although values might not all be directly comparable due to the use of different substrates (Table 2). Only Stajic et al. (9) reported also VP activities (measured with DMP = 2,6-dimethoxyphenol). With values between 2.2 U/l to 38 U/l (0.03 – 0.58 U/gds), the VP activities were around 10% of the measured MnP activities. In our studies, with the drastic increase in enzymatic activities two to four days after mushroom harvests, we obtained enzymatic yields of up to 2.9 U/gds for laccase, 45 U/gds for MnP and 5.9 U/gds VP. Compared to the other studies where the same substrates were used as in our study (ABTS, phenol red, Table 2), our enzyme yields are thus evenly good or even better.

Trials of obtaining the free liquid from whole industrial wheat straw substrate blocks with a larger hydraulic press resulted in volumes of about 5-6 l (not shown). Taken at optimum enzymatic activities within the substrate, this volume of press juice should then contain a calculated total amount of about 5.5-6.6 kU of laccase, of about 75-80 kU of MnP and of 7.5-11.4 kU of VP. The raw liquid has however a brown colour, likely caused by a high content of phenolic compounds. Unfortunately, concentrating the enzyme activities by ammonium precipitation did not result in purification of the enzymes from these compounds since they co-precipitated (not shown). Potential applications for the raw enzyme solutions or of concentrated solutions are therefore restricted to processes where a brown coloration does not matter or were presence of extra phenolic compounds might even be of advantage. An example for such case can be the production of wood and paper composites by a two-component system in which the glue consists of ligninolytic enzymes (usually laccase) and phenolic compounds (e.g. lignosulphonate) [reviewed by (38, 39)]. Matcham and Wood (40), Singh et al. (34) and Ko et al. (36) published previously protocols as how to wash out enzymes with tap water and various buffers from SMS of various types of mushrooms (A. bisporus, Flammulina velutipes, Hericium erinaceum, L. edodes, P. ostreatus). However, procedures were only tested on small scale such as 100 ml and it remains to be shown whether these procedures will be economic enough for obtaining larger volumes of purified enzymes needed for other commercial applications such as in the food and in the pharmacy industries (41, 42).
Table 2: Laccase and MnP activities of *Pleurotus ostreatus* cultures on different lignocellulosic substrates in SSF

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Laccase</th>
<th>MnP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value in paper</td>
<td>U/gds</td>
<td>Value in paper</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>1.1-1.4 U/ml (ABTS)</td>
<td>3.9-5.5</td>
<td>15-16 U/ml (phenol red)</td>
</tr>
<tr>
<td>Grapevine sawdust</td>
<td>8–2145 U/l (SGZ)</td>
<td>0.12-32.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25-459 U/l (DMP)</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>0.04 U/g substrate (SGZ)</td>
<td>0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>455 U/g substrate (DMAB+MBTH)</td>
</tr>
<tr>
<td>Canola meal</td>
<td>300 nkat/gds (SGZ)</td>
<td>18.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>Tree leaves</td>
<td>15 U/flask (ABTS)</td>
<td>3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 U/flask (phenol red)</td>
</tr>
</tbody>
</table>

* U/gds were calculated from a) the weight of dry substrate and the measured activity given in the paper, b) the weight of the substrate deduced by the known moisture content and the measured activity given in the paper, and c) the conversion of nkat into international units as described in (32). Substrates used in enzymatic tests are given in brackets; ABTS = 2,2’-azino-bis [3-ethylbenzthiazoline-6-sulfonate], SGZ = syringaldazine, DMP = 2,6-dimethoxyphenol, DMAB = 3-dimethylaminobenzoic acid, MBTH = 3-methyl-2-benzothiazolinone hydrazone hydrochloride. nd = not determined.

Alternatives for beneficial usage of the high enzymatic levels within substrates shortly after mushroom harvest are to apply the SMS or the enzymatic crude extract of SMS in bioremediation of contaminated soils and water. The used up the spent substrate or the crude extract might be mixed with the soil or the water for promoting the degradation of phenolic and non-phenolic aromatic pollutants (43 - 50). For mushroom growers, it might therefore be useful to wait a few days after the final mushroom harvest before disposal of the SMS in order to recycle it in a best possible way at a stage of high enzymatic activities. Currently, schemes for a broad re-use of the mushroom cultivation residues have been established only in China which produces more than 4 million tons of SMS per year (51). In other countries, SMS is still considered as waste but recent changes in legislation in favour of ecological-friendly SMS disposal altered this view in a number of European countries. In a topical review on alternative uses of SMS, Oei et al. judge the extraction of specific proteins as one of the most beneficial potential applications of SMS (52).

Acknowledgements

We are very grateful to druid austermpilze for supplying inoculated industrial substrate blocks for the experiments. Research on *Pleurotus*...
cultivation in our laboratory was initiated by funds of the DBU (Deutsche Bundesstiftung Umwelt) and is now supported by a common project with Prof. Dr. R. Berger from the Leibniz-University Hannover in frame of the VW Vorab grant Lebensmittelnetzwerk Niedersachsen of the Ministry of Science and Culture of Lower Saxony (grant ZN 2145).

References


