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WP 7.1: Screening of fungi with ligninolytic activity

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1.1 Introduction

White-rot fungi produce many types of extracellular lignin-modifying enzymes. That is the reason why the excess of fibres in the waste compartment could be reduced by addition of an associated compartment colonised by one of these types of fungi.

The best-characterised enzymes produced by these fungi are laccase (Lac), lignin peroxidase (LiP) and manganese peroxidases (MnP). But other extracellular enzymes are mentioned such as glyoxal oxidases and aryl alcohol oxidases (AAO). These mushrooms were traditionally classified into three main groups:

1- LiP-MnP group: including *Phanerochaete chrysosporium*, and *Trametes versicolor* (Fig 1) which were mushrooms producing mainly these two enzymes to degrade lignin.

Fig. 1: *Trametes* versicolor in the nature.

2- MnP-Lac group: including *Pleurotus ostreatus, Pleurotus eryngii,* and *Lentinus edodes* which apparently the main enzymes were a manganesium dependent peroxidase and laccase.

3- LiP-Lac group: which were described as the most inefficient lignin degraders.

Some authors described a 4th group called Lac-AAO and included mushrooms as *Bjerkandera adusta* (Fig. 2).

Fig. 2: *Bjerkandera adusta* in the nature.

But controversial results have been obtained as researches tested different media and conditions, because, for instance, MnP activity was found in cultures of *B. adusta*, Lac and AAO were detected also in *T. versicolor* and LiP in some strains of *L. edodes*, *Pleurotus* could, if stimulated produces AAOs etc. Sometimes, the activity was not detected but the gene encoding for the enzyme was present in the fungi genome as e.g. the LiP gene in *B. adusta*.

LiP and MnP are heme-containing glycoproteins which require hydrogen peroxide as an oxidant. Fungi usually secrete several isoenzymes into their medium but others are cell-wall bound. LiP oxidises non-phenolic lignin substructures by abstracting one electron and generating cation radicals which are then decomposed chemically. But the precise mechanism is still not well determined.

MnP oxidises Mn(II) to Mn (III) which then oxidises phenol rings to phenoxy radicals which lead to decomposition of compounds. It is the enzyme with more evidences of playing a crucial role in the degradation of lignin.

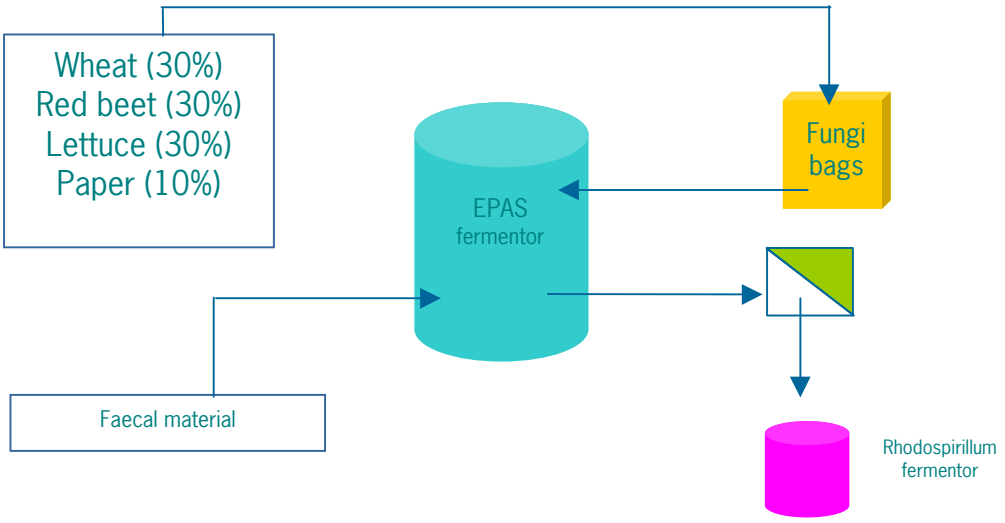
Laccase is a copper-containing oxidase which utilises molecular oxygen as oxidant and also oxidises phenolic ring to phenoxy radicals. It has also capacity to oxidise non-phenolic compounds under certain conditions, e.g. if the reaction mixture is supplemented with ABTS. The enzyme may also cause cleavage of phenolic rings when substituted with bulky groups. The physiological function of laccase is not clear and even now its role, in lignin biodegradation is uncertain.

Glyoxal oxidase is another extracellular enzyme supposedly involved in lignin biodegradation as producer of hydrogen peroxide, but this enzyme hasn't been studied a lot. A similar enzyme is AAO which oxidises substrates as veratryl alcohol to veratraldehyde without using H_2O_2. The latter may be produced for the subsequent action of peroxidases.

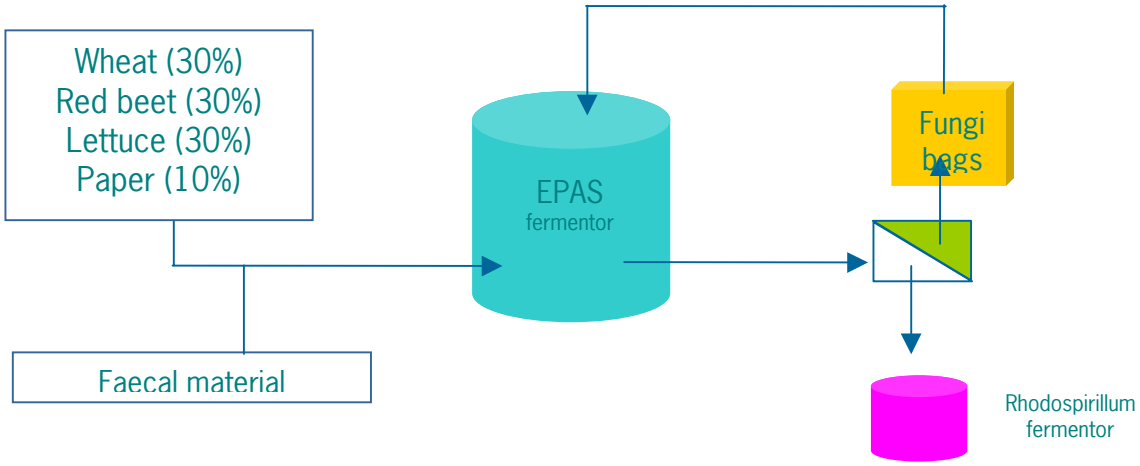
The aim of ATO in this first WP 7.1 was to screen and evaluate within the mushrooms of the ATO collection for a strain which could most efficiently degrade the lignin that disturb the MELISSA cycle. Since the production of ligninolytic enzymes is strongly affected by the medium in which the fungus is cultivated the precise location of the "fungal compartment" should be previously defined.

According to the loop design proposed for the waste compartment at the kick off meeting of FOOD 2, the "fungal compartment" could be located at two points which means two strategies to investigate:

1. **Before the waste compartment**: It would receive the plant waste, degrade the fibres and from this compartment a "more degraded fibre"-waste would go to the "waste compartment"



2. **After the waste compartment**: It would receive (after filtration unit) the material not degraded by the waste compartment (EPAS). This outcome will be mentioned as MELISSA cake (MC). Perhaps directly, or after mixing with the plants, would be added to the fungal compartment (ATO). After degradation in the fungal compartment, the output is transported back into the first waste compartment (EPAS fermentor) for further degradation of the organic material by bacteria.



Firstly, the first strategy was studied since the second needed the output from the waste compartment after the addition of the plants (MELISSA cake containing the plants mentioned in the outlines). The output (MC) had to be daily collected from the EPAS fermentor and stored until sufficient amounts are pooled to perform experiments with fungi at Petry scale. Since the fungi screening would request large amounts of material (from the first and second strategy), they were only tested using the plant material as cultivation medium (1st strategy) and the best suited species were further tested in the MC material (2nd strategy). In the mean time, while ATO tested the fungi in the plant wastes, EPAS used the same plant waste materials in the first waste compartment to prepare the MC.

1.2 Material and Methods

1.2.1 Preparation of standardised media

Media containing plant wastes

The non-edible parts of wheat, lettuce and red beet plants will be added to the MELISSA cycle to improve the carbon loops. Toilet paper will also be included and added together with the plants in the ratio: wheat straw (30%), lettuce waste (30%), red beet (30%) and toilet paper (10%) at the waste compartment. From various sources ATO collected the above mentioned plant material. Part of it was used in tests to grow fungi on by ATO, the other part was sent to EPAS and there further used in the waste compartment to prepare MC.

It was convenient to avoid extra variable to use the same batch of wastes at ATO and EPAS because of the differences from sample to sample. Therefore, ATO purchased all the plant wastes and supplied EPAS with the same material than ATO is going to use to cultivate the mushrooms (Table 1).

- **Wheat straw**: was purchased from the farms belonging to the University of Wageningen (The Netherlands). It consisted of wheat straw exclusively roots were not included.

- **Lettuce waste**: was collected at the fields belonging to the Universidad Miguel Hernandez of Orihuela (Alicante, Spain). Contained the roots, stem and upper part of leaves.

- **Red beet waste**: was kindly donated by Henk van Walderveen en Znn BV (Nieuwegein, The Netherland). Skin, peat and a little part of flesh of the red beet fruit. No roots or leaves.

- **Toilet paper**: was obtained at the local supermarket a international brand was selected that can also be available at Belgium (Lotus).

Waste material was distributed as follow:

Table 1: Plant waste material available to develop the project

Material (waste)	Total purchased (kg dry weight)	ATO	Sent to EPAS
Wheat straw	3	2.5 (small pieces)	0.5 (ground)
Lettuce	2	1.5 (ground)	0.5 (ground)
Red beet	1.7	1.2 (small pellets)	0.5 (ground)
Toilet paper	0.56	0.56 (small pieces)	

With the described materials the following media were prepared and poured in Petriplates to be inoculated with the different mushroom species:

- **MMP** (Malt Mycological Peptone): 10 g malt extract, 5 g mycological peptone and15 g agar / L tap water (control medium).

- **WS** (Wheat straw): 15 g ground wheat straw and 15 g agar / L tap water.

- **LW** (Lettuce waste): 15 g ground lettuce waste and 15 g agar / L tap water.

- **RBW** (Red beet waste): 15 g ground red beet waste and 15 g agar / L tap water.

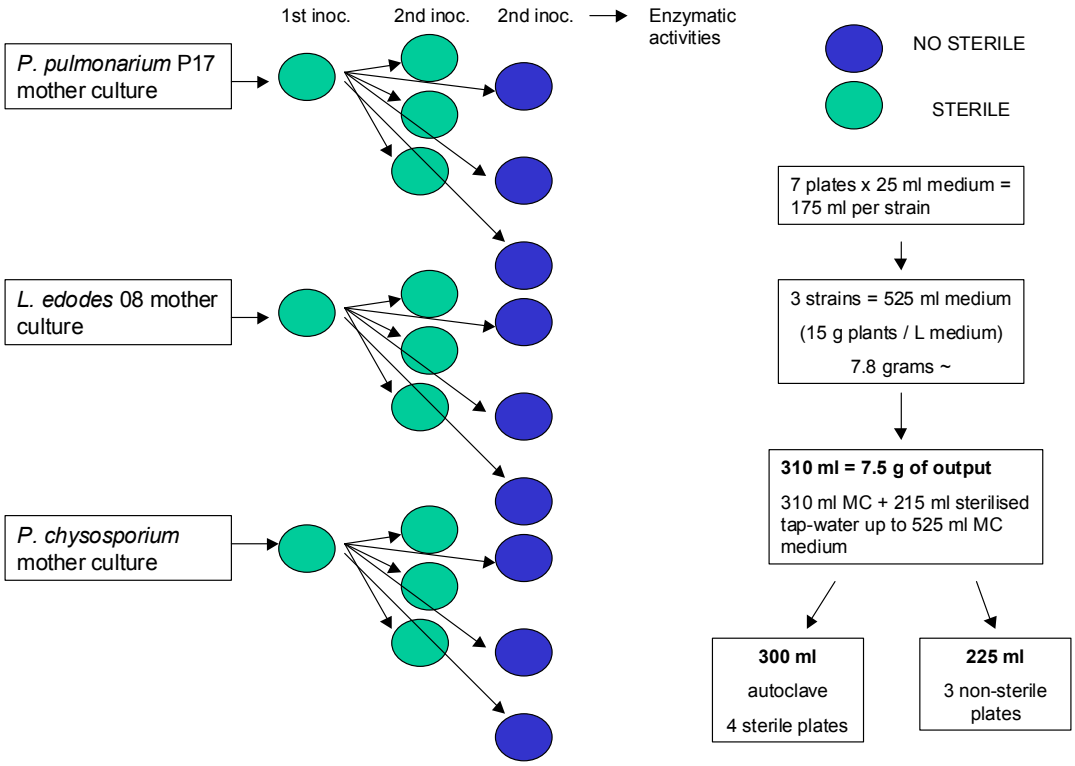
- **TP** (toilet paper): 15 g pieces of toilet paper (Lotus, 3 layers white) and 15 g agar / L tap water.

- **WLBP** (wheat, lettuce, beet, paper): 4.5 g wheat straw, 4.5 g lettuce waste, 4.5 g red beet waste and 1.5 g toilet paper and 15 g agar /L tap water.

Media containing the MELISSA cake (MC)

Two different types of media were prepared, one using sterile MC (sterilisation in autoclave) and the other directly obtained from the fermentor. EPAS kept the output from the fermentor as hygienically as possible to minimise contamination. EPAS sent MC to ATO as a total volume of 310 ml (distributed in sterile Falcon tubes, 24.09 g/L). To define precisely the minimal MC amount required, the media were prepared as follow:

A first inoculation had to be done with 3 of the mushrooms selected in the 1st strategy (see later) in sterilised MC plates to adapt the mycelium to the new medium. From these plates an inoculum was transferred to a second plate with sterilised and non-sterilised MC medium.



So the last prepared medium was:

- **MC** (MELISSA cake): 15 g dw output from the waste compartment (sterilised or not) and 15 g agar / L tap water or liquid phase from the output.

1.2.2 Fungal species

All the fungal strains available at ATO were cultivated in the described media (except for MC) to select the best lignin degrading strain. The fungi species and strains were:

Edible mushrooms:

- *Agaricus bisporus* A15, 17, 9.18, C45, B62
- *Pleurotus ostreatus* 30, 1111, 2171, 2191, 2204, K15
- *Pleurotus pulmonarius* 2204^{P}, P17
- *Pleurotus eryngi* 2600
- *Lentinus edodes* 8, S9, 4, S14, S92, 3102, 904

Non edible mushrooms (but also white root fungi):

- *Phanerochaete chrysosporium,*
- *Bjerkandera adusta,*
- *Trametes (coriolus) versicolor*

Fig. 3: *P. eryngi* fruitbodies

Fungi were cultivated in triplicate at 24°C except for *Phanerochaete chrysosporium* that should be cultivated at 37°C and *Bjerkandera adusta* and *Trametes versicolor* that should be cultivated at 26°C.

1.2.3 Methods for measuring mycelial growth

Petriplates were prepared using the media above described. A first inoculation in each medium was performed to adapt the mycelium to the new substrate and from this plate an inoculum was transferred to a second plate with the same medium. In this second plate sterilised cellophane circles were placed at the top of the semi-solid media. Fungi were inoculated and incubated until the petriplate was completely colonised.
The growth of the strains was monitored by measuring of the mycelial diameter during the incubation time and the final weight (fresh and dry) after harvesting mycelia from the plates when they were fully-grown. Percentage of retained water was estimated.

Diameter
Mycelial growth was measured each day at the same time with a ruler until the fungi colonise completely the petriplates. Minor and major diameters were calculated and the average used as measure of growth. Three plates were used per different strain and per different medium.

Weight
The mycelial growth was also analysed by weighing the fresh biomass produced on the cellophane when plates were fully colonised. Afterwards, mycelia were frozen and freeze-dried and weighed again to calculate the dry weight. Percentage of water was calculated from differences between fresh and dry weight.

1.2.4 Methods for measuring ligninolytic activities

Enzyme source preparation
Enzymes secreted by the fungi during their cultivation on the semi-solid media were extracted from the agar once the mycelium was removed together with the cellophane paper. Remaining media was cut in small pieces and left extracting during one hour in 25 ml of Milli Q water under agitation. The extract was filtered, centrifuged and the supernatant used as source of extracellular enzymes (triplicate).

Enzymatic activities
Peroxidase activity (POD) was measured separately as manganesium dependent peroxidase (MnP) and manganesium independent peroxidase (MiP). The sum of both activities was considered as total peroxidase activity (TP). POD was monitored as described by (Castillo, Stenström et al. 1994) using as substrate 3-(dimethylamino)benzoic acid (DMAB) which interact with 3-methyl-2-benzothiazolinone hydrazone (MBTH) producing a purple coloured reaction in the presence of the enzyme, H_2O_2 and Mn.

Laccase activity (Lac) was firstly measured using ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) as substrate as previously described by (Pelaez, Martinez et al. 1995) but *P. chrysosporium* cultures secreted a part of enzymes other compounds with antioxidant activity disturbing the enzymatic activities of laccase. Therefore, experiments were again performed using L-Dopa as substrate and increasing the sensibility of the test by adding a potent nucleophile molecule (MBTH) as described by (Espín, Morales et al. 1997).

Lignin Peroxidase activity (LiP) was also measured following two methods, firstly as the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde (Zhao, Theodorus et al. 1996) and secondly as the oxidation of Azure B as described by (Archibald 1992).

Aryl alcohol oxidase activity (AAO) was measure using the same substrate than for LiP (veratryl alcohol) but with no H_2O_2 present in the reaction medium according to (Guillen, Martinez et al. 1994).

Cellulase activity was determined using carboxymethyl cellulose as substrate which is degraded to glucose by the cellulases from the extracts. Afterwards glucose levels were determined using a commercial Glucose (HK) assay Kit (Sigma-Aldich NL). Proteases were measured as described in (Sarath, Motte et al. 1990) using azoalbumine as substrate. Protein was quantified by the Bradford method (BioRad) for protein determination as described in Bradford (1976) and Stoscheck, CM. (1990).

1.2.5 Detection of lignolytic activity in *P. chrysosporium*

To detect ligninolytic enzymes in *P. chrysosporium*, a medium was prepared including a dye which change colour in the presence of ligninolytic enzymes. WLBP medium was prepared with 0.02% (w/v) of Poly R-478 (see also report of FOOD 1). If ligninolytic enzymes are produce the dye change colour from purple to yellow (degradation products).
Enzymes were monitored during incubation time (2 - 8 days), and later on up to 30 days.

1.3 Results and discussion

1.3.1 Growth of mycelia on Petri dishes containing the plant waste (1st strategy)

Selected fungi species were cultivated on MMP medium as control (Fig 4) to define the typical growth curves of the species in a nutritive medium. Then, results could be compared to those obtained when cultivated on the specific media and noticed growth delays. When inoculated in media containing only one plant waste, for instance WS, no significant differences between strains were found (Fig 5), they could all grow with only a slight delay respect to the nutritive medium. Only a couple of strains, as for instance Lentinus S14 showed more problems to grow on WS than in MMP. If compared by species and not by strains (Table 2) could be observed that the fastest growing specie was *Pleurotus*, followed by *Lentinus* and the slowest was *Agaricus*.

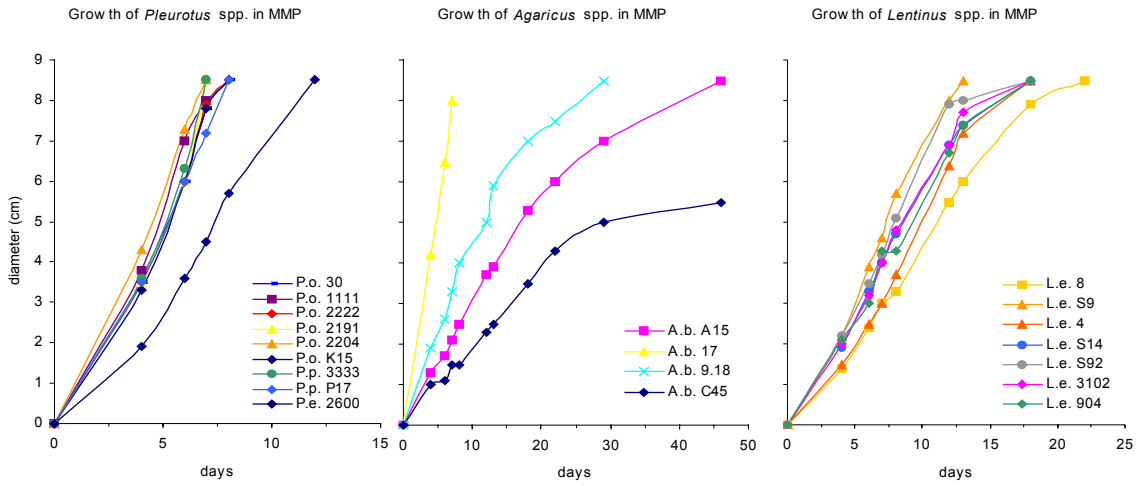


Fig 4: Growth of *Pleurotus* spp. strains (A), *Agaricus* spp. (B) and *Lentinus* spp. in MMP control medium

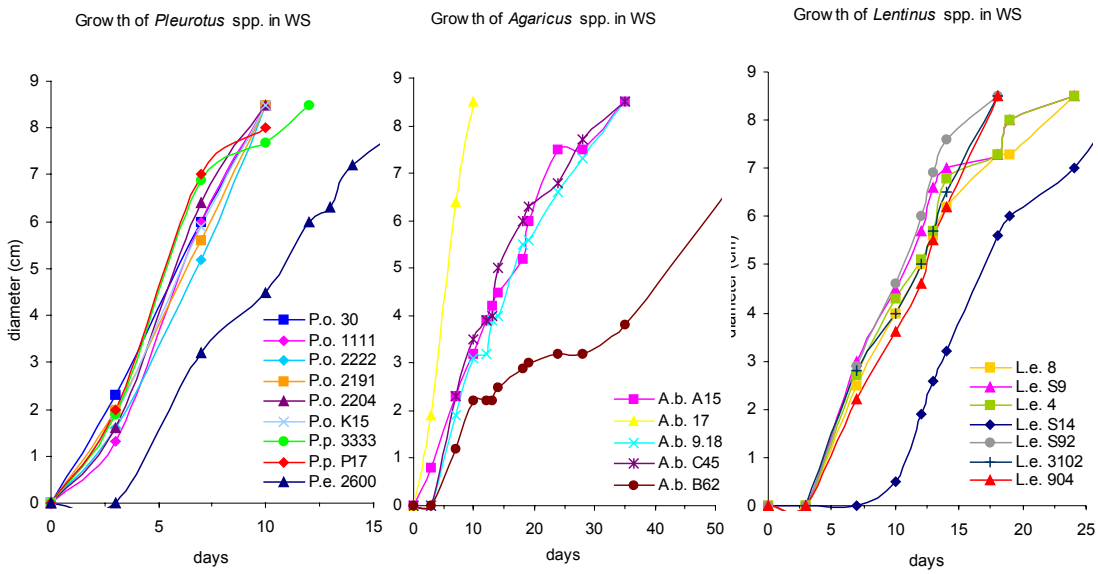


Fig 5: Growth of *Pleurotus* spp. strains (A), *Agaricus* spp. (B) and *Lentinus* spp. in WS medium

For all the strains, a small delay of few days was observed if compared to MMP but in red beet and lettuce wastes the delay was smaller, apparently these media were more nutritive than wheat straw. All the mycelia could grow on the media prepared by single plant waste except for the media containing only paper, which, as expected, did not have enough nutrients.

Table 2: Range of time (in days) needed to the complete Petri dish colonisation by the selected mushrooms strains in the media prepared using only one type of waste.

Mushrooms / Media	MMP	WS	LW	RBW	TP
Agaricus bisporus (strains A15, 17, 9.18, C45 and B62)	8 – 46	35 - 52	18	ND	NG
Pleurotus ostreatus (strains 30, 1111, 2171, 2191, 2204, and K15)	7 – 8	10	10	10	NG
Pleurotus pulmonarius (strains 2204^{P} and P17)	7 – 8	10 – 12	10	10	NG
Pleurotus eryngi 2600	12	18	ND	12	NG
Lentinus edodes (strains 8, S9, 4, S14, S92, 3102, 904)	13 - 22	18 – 28	ND	13 - 28	NG
Phanerochaete chrysosporium	ND	ND	ND	ND	ND
Trametes versicolor	ND	ND	ND	ND	ND
Bjerkandera adusta	ND	ND	ND	ND	ND

ND = no data NG = no growth

According to results, *A. bisporus* strains grew very slowly (the fastest strains needed only 8 days but the slowest almost 46 days on MMP) so they were discarded and not cultivated in the WLBP medium. The rest of fungi including the three non-edible were inoculated in plates containing the WLBP medium and on MMP.

Strains are usually stored on MMP plates so firstly they were inoculated on WLBP plates to provoke the re-arrangement of the biochemical pathways which allow the mycelia to adapt themselves to grow on new media (avoiding the lag phase). Later from these plates, fungi were

transferred to fresh WLBP plates, but anyway a lag phase could be observed in all the strains (Figs 6 to 8). This fact could not be explained so far.

Nevertheless, WLBP medium seemed to be a rich medium to cultivate *Pleurotus ostreatus* and *P. pulmonarius* strains. Some of them grew faster than in the standard MMP (Fig. 6). *P. eryngii* presented more difficulties than the other strains to grow on WLBP medium. A delay in the growth was also observed as compared to MMP.

Lentinus edodes grew slower than *Pleurotus* but in all the media the development of this specie is slower than *Pleurotus*. If the two media were compared, some *Lentinus* strains as for instance *L. edodes* 08 grew on the WLBP medium faster than on the ordinary MMP (Fig 7).

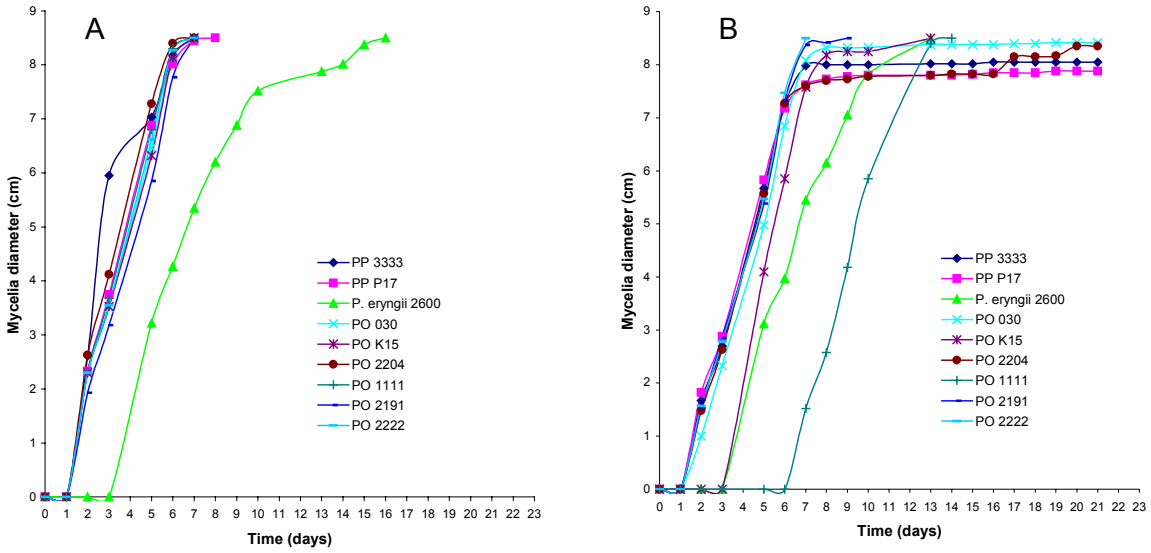


Fig. 6: *Pleurotus* strains grown on (A) WLBP medium and (B) MMP medium.

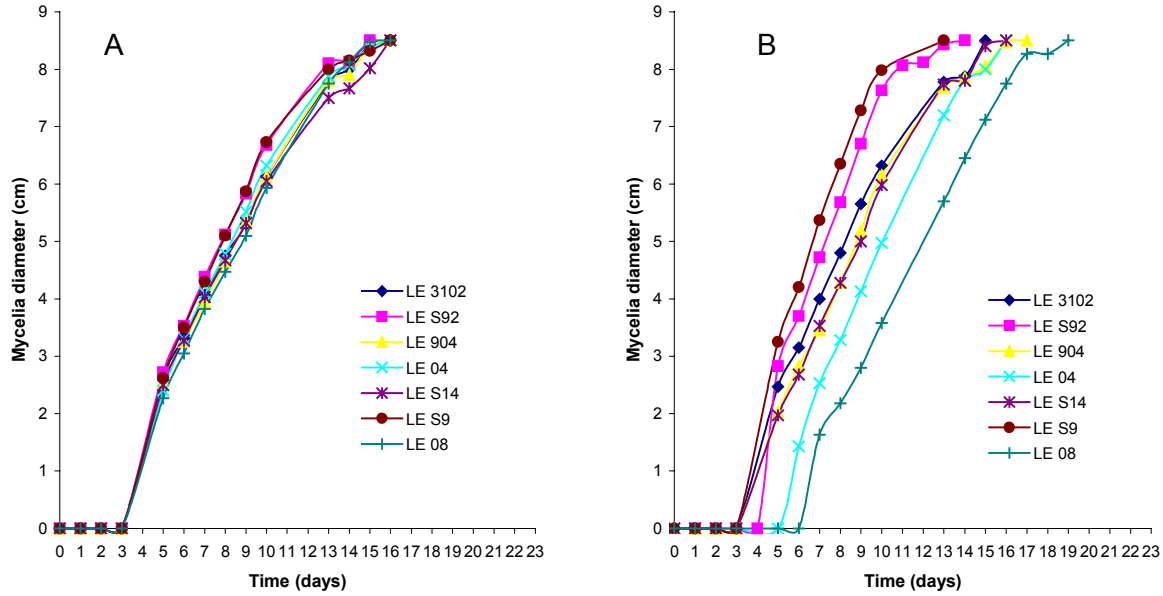


Fig. 7: *Lentinus* strains grown on (A) WLBP medium and (B) MMP medium

The fastest growing fungus was *Phanerochaete chrysosporium* which in two days covered the full plate (Fig. 8) using both media. The species is cultivated at its optimal growth temperature, i.e. at 37°C, which is higher than the cultivating temperatures used for the other fungi. Apparently, this temperature increase positively affects the growth rate.

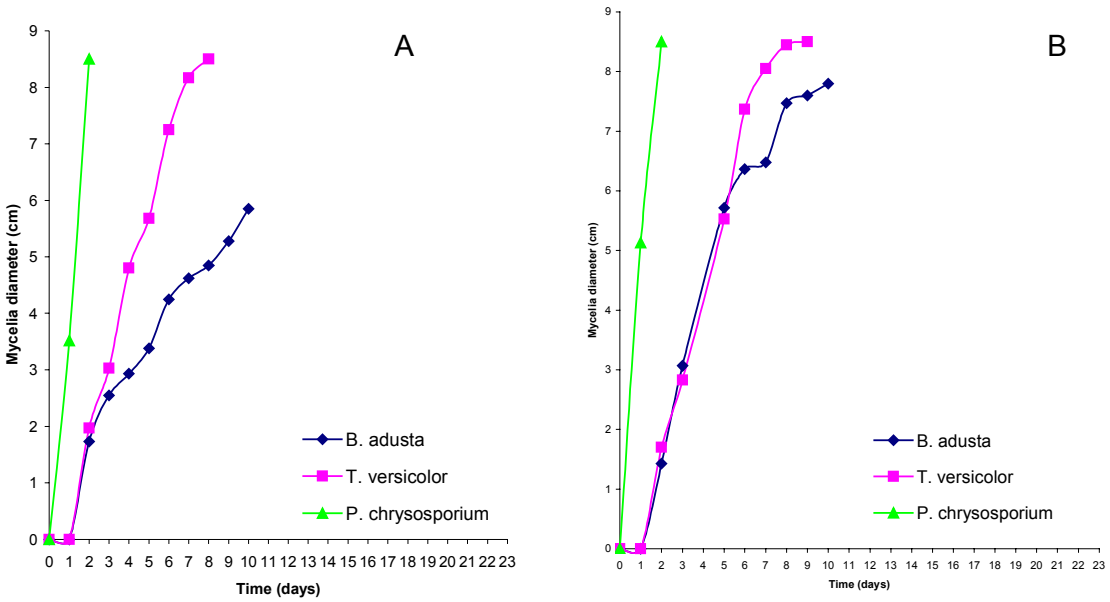


Fig. 8: *Phanerochaete chrysosporium, Bjerkandera adusta* and *Trametes (coriolus) versicolor* strains grown on (A) WLBP medium and (B) MMP medium

Phanerochaete chrysosporium is not edible. This fungus never form a mushroom for reproduction, but form effused, very flat, fruiting bodies that appear as no more than a crust on the underside of a log. It is a member of the Basidiomycota, which means it bears its meiotic spores externally on a structure called a basidium. There are many kinds of fungi that cause a white rot, but *Phanerochaete chrysosporium* has several features that might make it very useful. First of all, unlike some white rotters, it leaves the cellulose of the wood virtually untouched. Secondly it has a very high optimum temperature, which means it can grow on wood chips in compost piles, which attain a very high temperature. These characteristics point it as highly suitable for the purposes of this WP. When grown on WLBP showed an appearance a little different than on MMP (Fig. 9) but it was also able to fully colonise the plate in a record time.

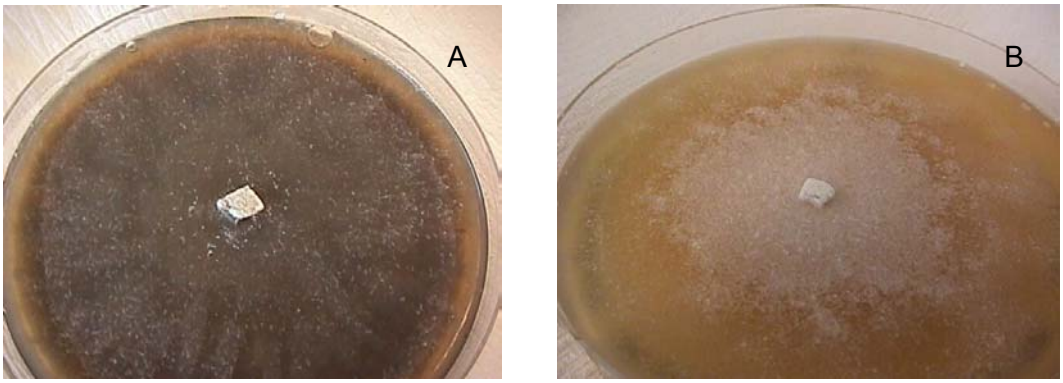


Fig. 9: *P. chrysosporium* mycelium grown on WLBP media (A) and MMP (B).

Bjerkandera adusta and *Trametes versicolor* they are also not toxic (poison) but also not edible, the mushrooms are too small as they can be seen in Figures 1 and 2 but they are described as excellent lignin degrading fungi. They form spores only after these fruitbodies have been produced. In the nature, usually induction of fruitbodies is related to a decrease in the temperatures. *Trametes* mycelium showed on both media a normal circular appearance but *Bjerkandera* mycelium appeared in some sectors as degeneration or change of properties of the hyphae because it had a different aspect and grew more in one direction rather than other when in fact the content of the plate was homogeneous whithin the plates (Fig. 10).

Fig. 10: Mycelium of *Bjerkandera* (A) and *Lentinus* (B) grown on WLBP medium and mycelium of *P. pulmonarius* grown on MMP medium (C).

Another parameter to take into consideration when evaluating the growth of a fungus is the biomass produced. Some mushrooms spread their hyphae very long searching for a better source of nutrients and they seem to growth very fast but in fact the "body" of the fungus is fragile and not very energetic. Therefore, the fresh and dry weight of the produced mycelia were also monitored.

In WLBP medium, *P. chrysosporium* and *T. versicolor* were the fungi which produced more fresh mycelia followed by some of the *Lentinus* strains and *Bjerkandera* (Table 3). *Lentinus* strains were in general the fungi with higher biomass (dry basis) *Trametes* showed also a high value followed by *Phanerochaete*.

Table 3: Fresh and dry mycelial weight (g) from fungi harvested when plates were fully colonised. Water content (in %) was also calculated for fungi cultivated in the WLBP and MMP media.

Strain / Media	WLBP			MMP		
	Fresh weight	Dry weight	Water content	Fresh weight	Dry weight	Water content
P. chrysosporium	0.552	0.034	93.65	0.824	0.097	88.48
B. adusta	0.448	0.029	92.36	1.105	0.124	87.23
T. versicolor	0.503	0.039	93.44	1.485	0.167	88.13
P. ostreatus K15	0.275	0.036	87.10	0.924	0.108	87.95
P. ostreatus 1111	0.285	0.033	88.44	0.486	0.078	83.89
P. ostreatus 2191	0.335	0.037	88.71	0.591	0.100	82.83
P. ostreatus 2222	0.313	0.031	90.26	0.617	0.072	88.30
P. ostreatus 030	0.269	0.034	85.13	0.629	0.093	85.25
P. ostreatus 2204	0.314	0.031	90.00	0.727	0.135	80.85
P. pulmomarius 3333	0.294	0.034	88.21	0.705	0.104	84.34
P. pulmonarius P17	0.216	0.021	90.46	0.684	0.132	80.33
P. eryngii	0.183	0.018	89.99	0.341	0.064	81.18
L. edodes 3102	0.330	0.041	87.53	0.551	0.064	88.39

L. edodes S92	0.417	0.047	88.88	0.665	0.081	87.90
L. edodes 904	0.277	0.036	86.98	0.524	0.066	87.31
L. edodes 04	0.278	0.032	88.06	0.555	0.077	86.01
L. edodes S14	0.301	0.032	89.44	0.617	0.114	81.51
L. edodes S9	0.495	0.058	88.16	0.587	0.071	87.89
L edodes 08	0.278	0.035	87.25	0.55	0.096	83.09

Weighs in general were higher in MMP plates indicating that the fast growth of the fungi in the other WLBP media was not because the media was nutritive but because fungi spread their hyphae fast searching for a more suitable media. For some of the strains the fresh weight was almost double in MMP. On MMP the mushrooms that produced more fresh mycelium were *Trametes*, *Bjerkandera*, *Phanerochaete* and some strains from *Pleurotus* confirming the importance of testing the fungi in the correct media for the selection of the best lignin degrading fungi. In this medium *Pleurotus* were the mushrooms with lower percentage of water so they would be considered as the mushrooms with higher biomass.

1.3.2 Enzymatic activities of mycelia grown on plant wastes

Lignocellulosic materials as the plant waste from the MELISSA cycle should be degraded in mushrooms by lignolytic and cellulolytic enzymes. The enzymes involved in the lignin degradation were mentioned before and cellulases are enzymes responsible for cellulose degradation. But, other enzymes can also interfere the activity of these two types of enzymes, *i.e.* proteases. The composition of some media, usually media rich in proteins, induces the production and secretion of proteases. If the selected strains produce also proteases the rest of the exo-enzymes could be degraded and they would not be able to degrade properly the lignin residues. Therefore, the enzymatic activity of all these enzymes were studied:

Peroxidases

This enzyme is considered as the most important in the degradation of lignin. Each specie produce different peroxidases. According to most of the publications *Pleurotus* species produce two types of peroxidases. One request Mn in the medium to catalyse the reaction and it is called MnP and the other no (MiP). In recent publications this last enzyme is also called VP (versatile peroxidase) because apparently it can catalyse many other reactions.

In fungi as *Phanerochaete* and *Trametes* another type of peroxidase is described as the major produced, lignin peroxidase (LiP), but also MnP is secreted. *Bjerkandera* could produce LiP, MnP and MiP and *Lentinus* was described as only able to produce MnP but, recent studies indicated the presence of the LiP gene in its genome. Apparently the lack of LiP activity is due to a possible artefact produced if veratryl alcohol is used as substrate for its determination (Crestini and Sermanni 1995).

All these peroxidases (POD) were measured in the selected species. In general, a tendency to produce more peroxidases in the WLBP media than in MMP was observed. But a large variation in between the different strains was also observed. For instance, of the *Pleurotus* strains (Fig. 11a), some of them as *P. ostreatus* 2204 and *P. pulmonarius* P17 showed high peroxidase activity (including MnP and MiP) while others as *P. ostreatus* O30 did not arise to levels higher than 5 µkat/ml. *P. eryngii* showed a high activity if the mushroom was cultivated on WLBP medium and no activity on MMP.

Peroxidases showed in the Figure 11b for *Lentinus* species is MnP. MiP was not described in this specie and when measured no activity was found (data not shown). *Lentinus edodes* 904 was the

strain with the highest peroxidase activity followed by *L. edodes* 3102 and *L. edodes* 08 (Fig. 11b). The activity was higher in WLBP medium than in the control MMP medium.

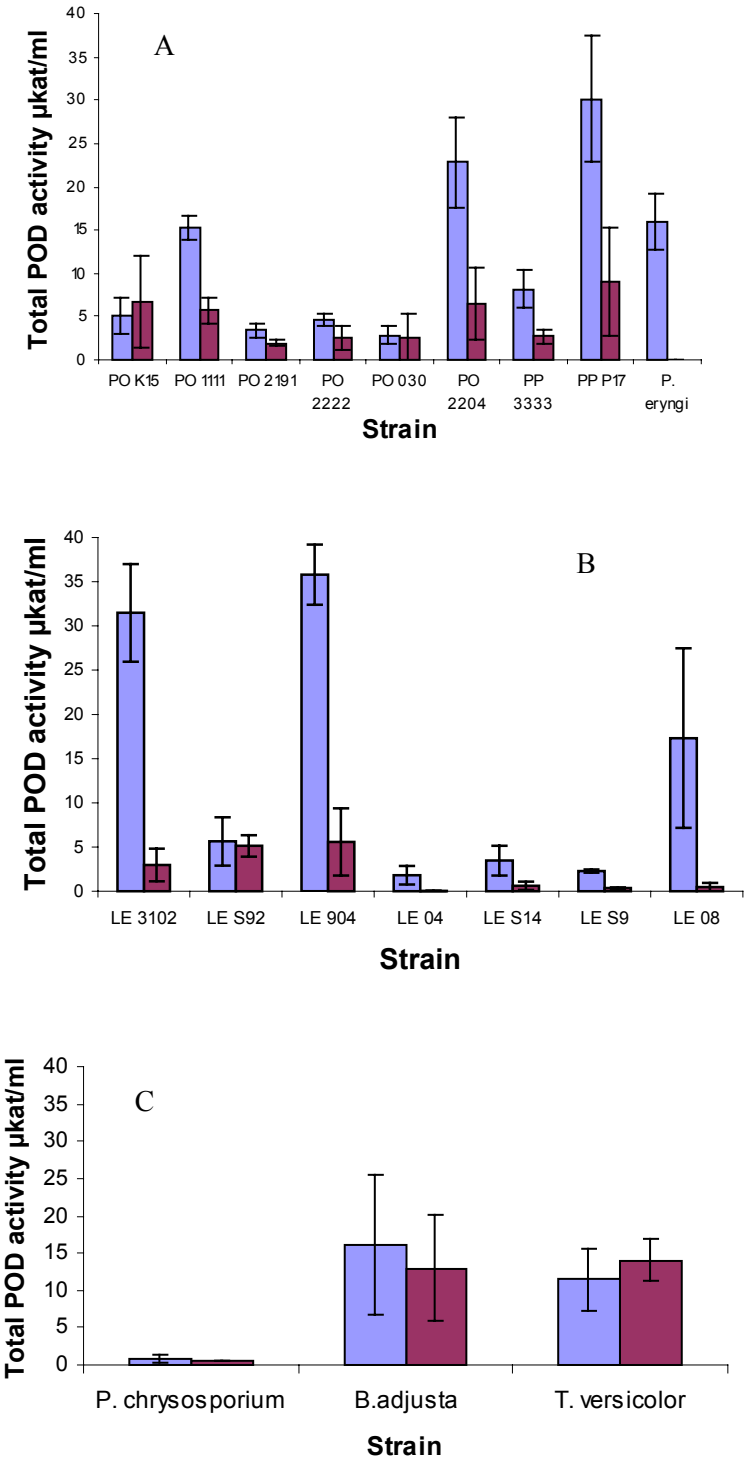


Fig. 11: Total peroxidase activity in the fungi cultivated on WLBP (■) and in MMP (■) media. *Pleurotus* mycelia (**A**), *Lentinus* mycelia (**B**), non-edible fungi (**C**).

The peroxidase activity in *P. chrysosporium* (MnP as for *Lentinus*) was insignificant. This result was surprising because this fungi is considered as one of the largest peroxidases producers of all the white-rot fungi. No explanation can be given so far and the experiments will be repeated. *B. adusta* and *T. versicolor* showed also peroxidase activity but to a lower extend than some of the *Pleurotus* and *Lentinus* strains. The influence of WLBP or MMP media was not as large as occurred in the other mushrooms (Fig. 11c).

In *Pleurotus* mushrooms grown on WLBP petriplates more MnP activity was noticed than in MMP plates where in some cases the MiP activity was higher than MnP (Fig. 12). As shown before, there was a large variation between strains in the secretion of both enzymes. WLBP medium induced the production of MnP more than MiP. This last enzyme was produced more or less in similar quantities in WLBP and MMP media.

P. chrysosporium, as well as *Lentinus* do not produce MiP but LiP. This is in concordance with the results found when this enzyme was measured in both media (Fig. 13). *B. adusta* and *T. versicolor* under certain cultivation conditions have been described as able to produce MiP. This activity was also detected in both WLBP and MMP media. *B. adusta* showed almost the same level in both media and *T. versicolor* showed more MiP in MMP medium.

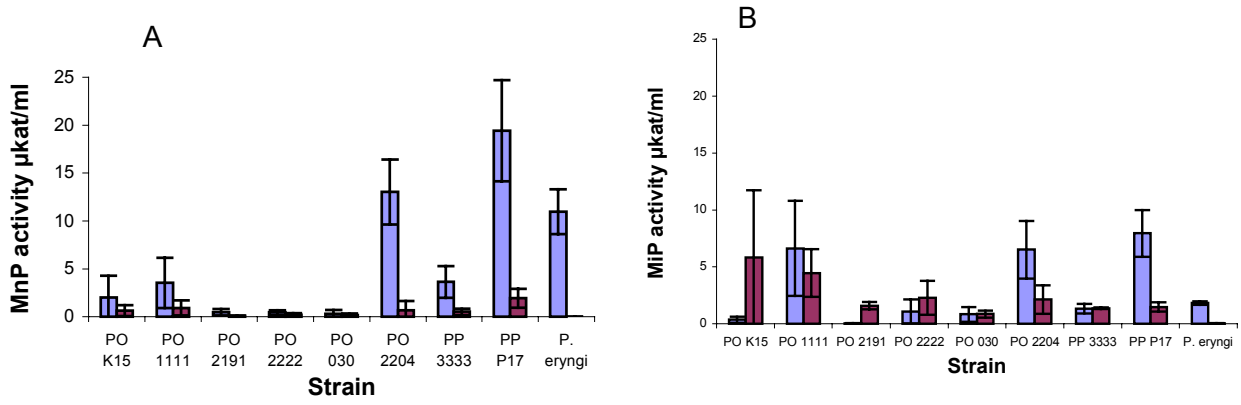


Fig. 12: MnP (A) and MiP (B) in *Pleurotus* strains. Fungi were cultivated on WLBP (■) and in MMP (■) media.

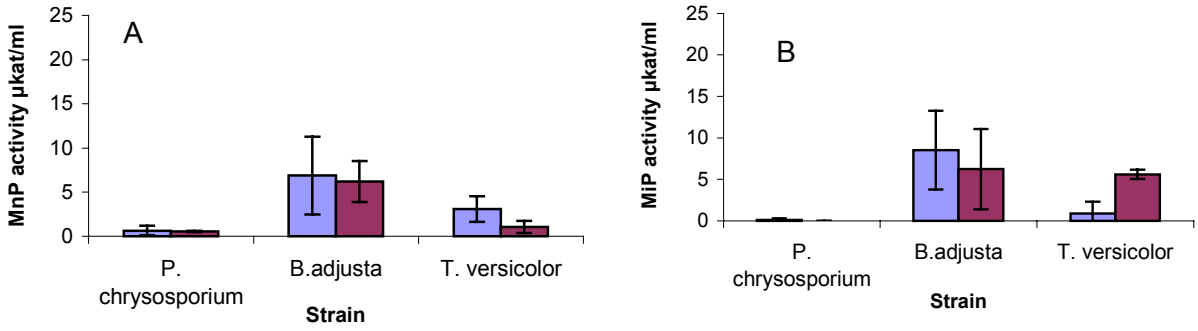
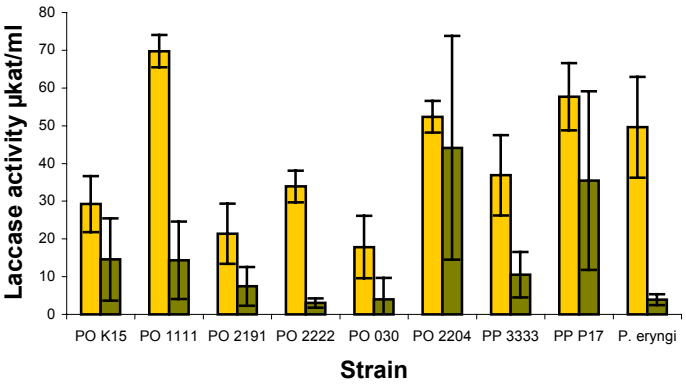


Fig. 13: MnP (A) and MiP (B) in non-edible mushrooms. Fungi were cultivated on WLBP (■) and in MMP (■) media.

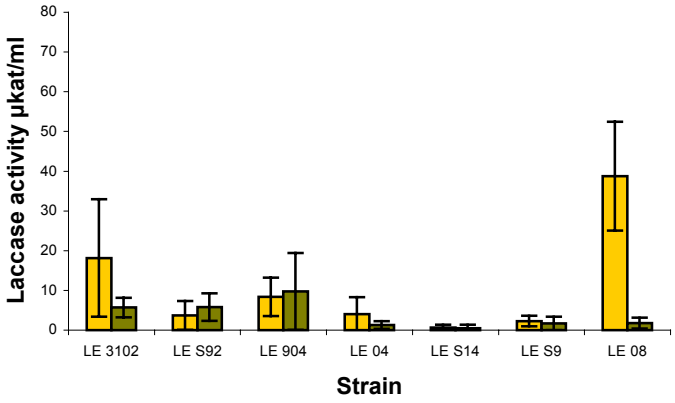
When LiP activity was measured no activity in any of the fungi species was detected neither with the widely used assay method that precise veratryl alcohol as substrate or the one that utilise Azure-B as substrate (with less interference).

Laccase

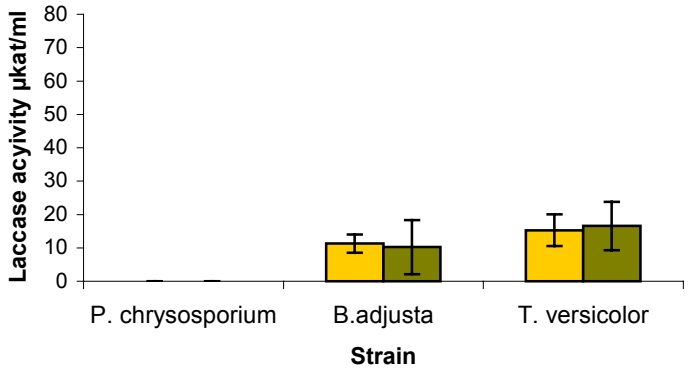
For some strains, the absence or low peroxidase activity was substituted by a high laccase activity, *Pleurotus ostreatus* 1111, for instance, showed a higher laccase activity rather than peroxidase. But other strains also showed high levels of laccase concomitant with high peroxidase activity as *P. ostreatus* 2204 and *P. pulmonarius* P17 (Fig. 14a). Laccase activity was, for all the *Pleurotus* strains, higher in WLBP medium than in MMP medium.



A



B



C

Fig. 14: Laccase activity of *Pleurotus* mycelia (**A**), *Lentinus* mycelia (**B**), *Phanerochaete chrysosporium , Bjerkandera adusta* and *Trametes versicolor* (**C**) in WLBP (■) and in MMP (■) media.

Lentinus species did not show high laccase activity except for the strains, *L. edodes* 3102 and *L. edodes* 08 (Fig. 14b). The latest showed also a high peroxidase activity. In MMP these strains did not show activity, only when the mushrooms were cultivated on the special WLBP medium the laccase activity was induced.

The non edible mushrooms showed also a low activity of laccase. *P. chrysosporium*, as it was previously described, lacked laccase (Fig. 14c), and *B. adusta* and *T. versicolor* showed low laccase activity in both WLBP and MMP media.

Aryl alcohol oxidases

This enzyme was measured in all the strains and in the WLBP and MMP media and no activity in any of the assays was detected. Only a little activity in *B. adusta* could be monitored but it was extremely low (figure not shown).

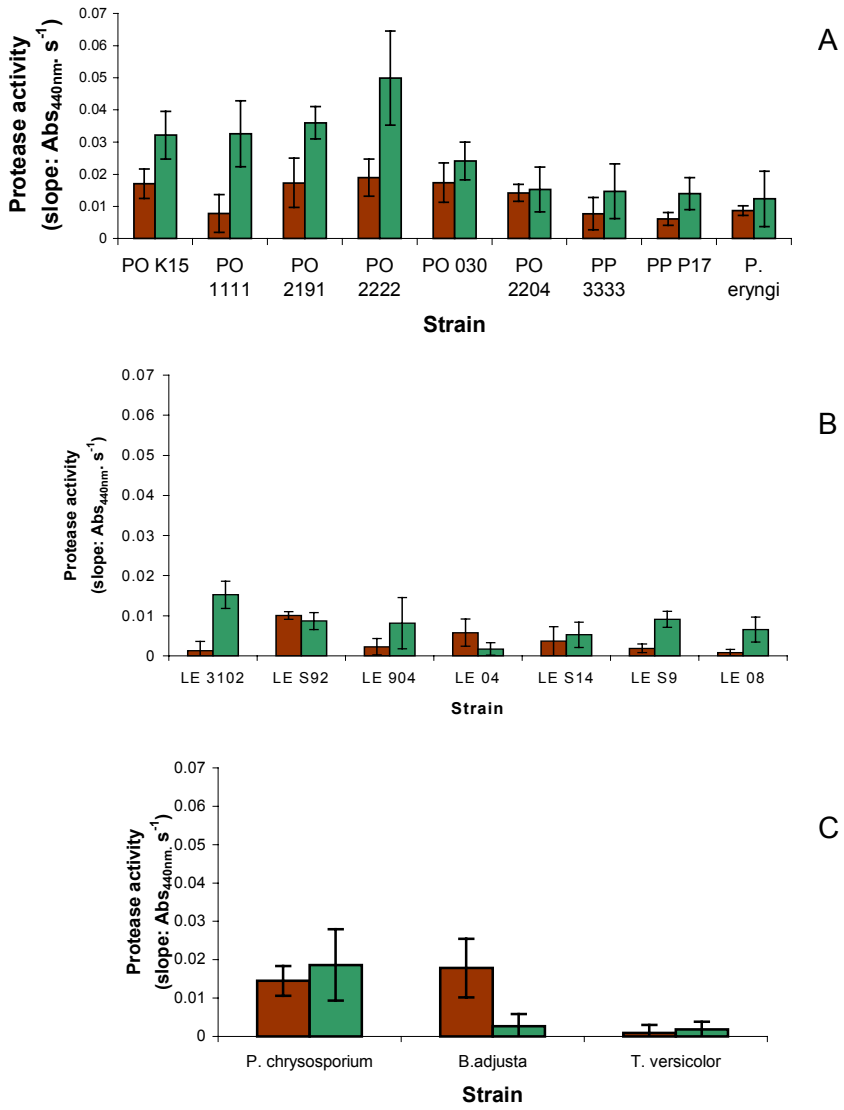


Fig. 15: Protease activity of *Pleurotus* mycelia (**A**), *Lentinus* mycelia (**B**), *Phanerochaete chrysosporium , Bjerkandera adusta* and *Trametes versicolor* (**C**) in WLBP (■) and in MMP (■) media.

Proteases

Sometimes if many proteins are present in the medium mycelium produces also proteases. These enzymes may degrade the exo-enzymes secreted by the fungus to degrade lignin. Therefore, it would be convenient to select a strain that it is not very proteolytic. Protease activity was measured in all the selected strains.

Protease production in all the mushrooms was low. The highest proteolytic activity was found in *Pleurotus* mushrooms but in those strains with not very high peroxidase or laccase activity (*P. ostreatus* 2222) (Fig. 15a). Proteases levels were also higher in MMP medium than in WLBP therefore not much degradation of the lignolytic enzymes is expected in the strains if cultivated in the MELISSA medium. None of the *Lentinus* strains showed a highly significant protease activity (Fig. 15b) as well as the non edible mushrooms (Fig. 15c). Only *P. chrysosporium* and *B. adusta* showed protease activity in the WLBP medium but was also not very high.

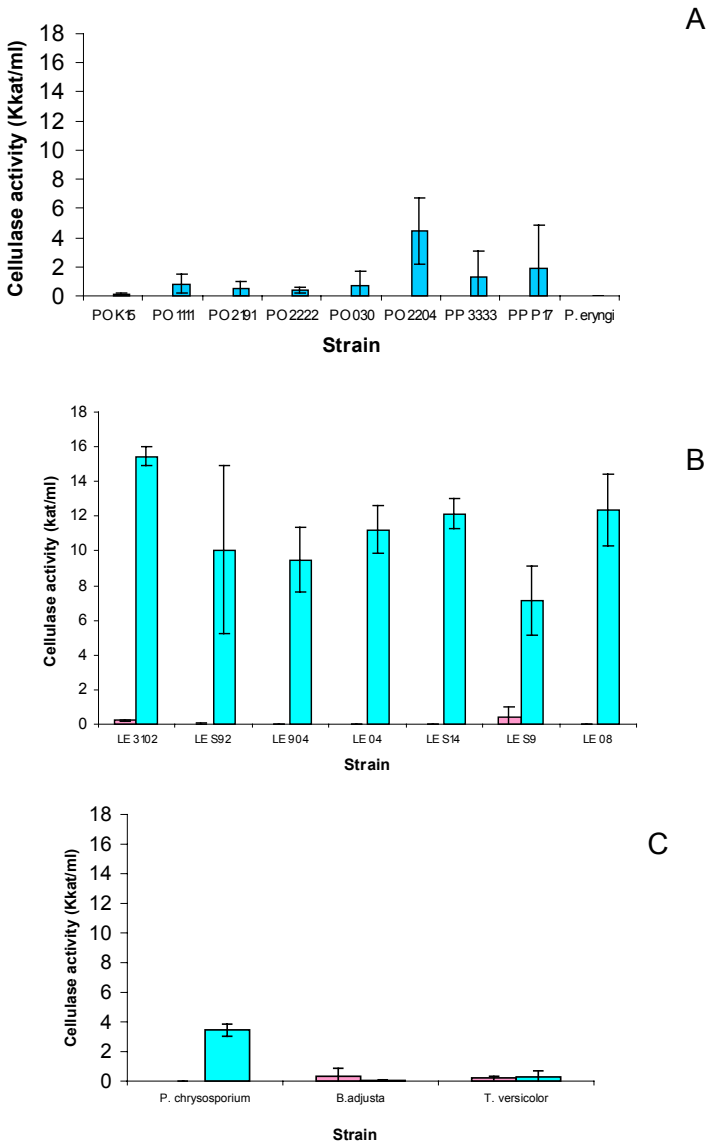


Fig. 16: Cellulase activity of *Pleurotus* mycelia (**A**), *Lentinus* mycelia (**B**), *Phanerochaete chrysosporium , Bjerkandera adusta* and *Trametes versicolor* (**C**) in WLBP (■) and in MMP (■) media.

Cellulases

Apart from the ligninolytic enzymes, some of the mushroom species are also able to produce cellulases that contribute to the degradation of the lignocellulosic fibres of the plants. Cellulase activity was measured in the WLBP and MMP media containing selected mushrooms. None of the *Pleurotus* mushrooms showed cellulase activity on WLBP medium (Fig. 16a). On MMP cellulases were produced to varying extent depending on strain. *Lentinus* mushrooms showed the highest cellulase activity (Fig. 16b), however, this was only noticed when the mycelia were cultivated on MMP medium. When testing the non-edible mushrooms, only low cellulase activities were noticed (Fig. 16c). *P. chrysosporium* produced low amounts of cellulases when cultivated on MMP medium. In general, this fungal strain is well known for the production of high amounts of ligninolytic enzymes, and the absense of cellulases. The activity observed in these experiments is therefore a bit difficult to explain. Additional enzyme activity measurements were performed (see later).

1.3.3 Pre-selection of the better suited strains

Apart from *P. chrysosporium* a comparison could be performed between all the strains to decided which mushroom strain should be selected for the further studies taken into consideration all the analysed parameters (Table 4). Mushrooms were ordered according to the best performance in the enzymatic activities and growth. The five best were market and results indicated that *P. ostreatus 2204, P. pulmonarius P17* and *L. edodes 08* were good candidates. The *P. pulmonarius* was better than the *P. ostreatus*. It was remarkable that a *Lentinus* specie scored so high even being a slow growing strain.

Table 4: Comparison of mushroom performance according to the measured parameters. The first mushrooms with the highest enzymatic activity (except for protease that was the lowest) and growth or biomass production were market in bold.

Fungi / Scores	Growth	Biomass	POD (MnP)	LiP	Laccase	AAO	Protease	Cellulase
P. chrysosporium	**1**	8	19	0	19	0	12	0
B.adusta	4	12	6	0	13	**1**	13	**2**
T. versicolor	3	**4**	9	0	12	0	**2**	4
P. ostreatus K15	**2**	6	12	0	8	0	13	6
P. ostreatus 1111	**2**	9	8	0	**1**	0	9	0
P. ostreatus 2191	**2**	**5**	15	0	9	0	13	0
P. ostreatus 2222	**2**	11	13	0	7	0	14	0
P. ostreatus 030	**2**	8	16	0	11	0	13	0
P. ostreatus 2204	**2**	11	**4**	0	**3**	0	12	0
P. pulmonarius 3333	**2**	8	10	0	6	0	9	0
P. pulmonarius P17	3	13	**3**	0	**2**	0	8	0
P. eryngii	4	14	7	0	**4**	0	10	0
L. edodes 3102	4	**3**	**2**	0	10	0	**3**	**3**
L. edodes S92	4	**2**	11	0	16	0	11	5
L. edodes 904	4	6	**1**	0	14	0	**5**	0
L. edodes 04	4	10	18	0	15	0	7	0
L. edodes S14	4	10	14	0	18	0	6	0
L. edodes S9	4	**1**	17	0	17	0	**4**	**1**
L. edodes 08	4	7	**5**	0	**5**	0	**1**	0

P. pulmonarius P-17 was also the best strain when the experiments on FOOD 1 project were developed. This strain could easily grow using the former MELISSA cake (the output of the first compartment before the addition of higher plants). Therefore, this strain or *Lentinus edodes* 08 should be pre-selected to be tested on FOOD 2 MELISSA cake plates (MC).

The three pre-selected candidates were *Pleurotus pulmonarius* P-17, *Lentinus edodes* 08 and even when results indicated that *P. chrysosporium* was not producing ligninolytic enzymes, the latter mushroom was selected because it is classified in literature as the best lignin degrading fungus.

1.3.4 Growth of mycelia on Petri dishes containing the MELISSA cake (2nd strategy)

Selected fungi species were plated in sterilised MC medium (Fig 17a). From this plate a second inoculation was performed on sterilised and non sterilised (Fig 17b) MC media (in triplicate). Plates (from the second inoculation) containing non-sterilised medium were contaminated and mycelia did not grow.

Fig. 17: MC aspect in a) sterile and b) non-sterile petriplates. a) *Pleurotus* mycelium inoculated from the WLBP medium (first inoculation).

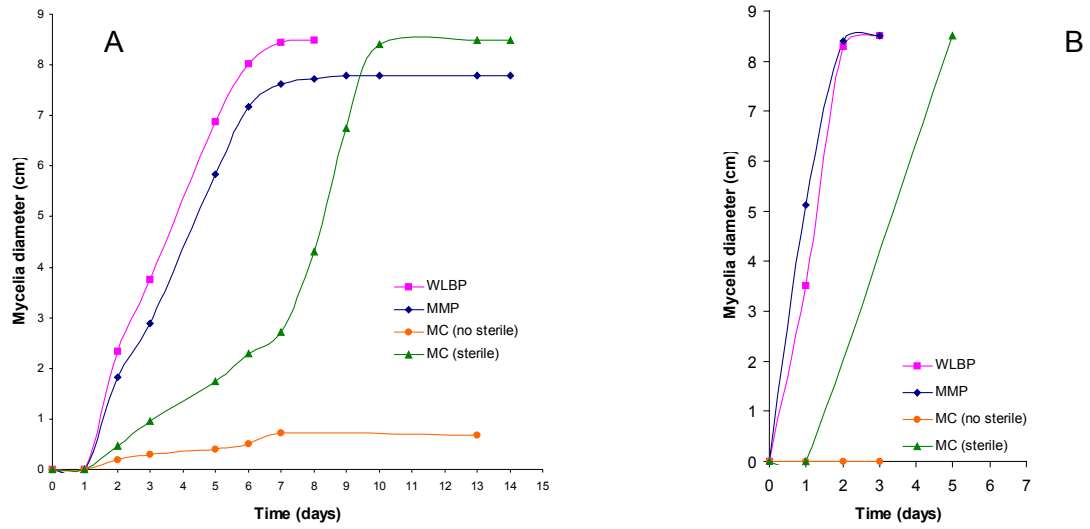


Fig. 18: Growth ratio of a) *Pleurotus pulmonarius* P-17 (average of triplicate) and b) *P. chrysosporium* (one plate for MC sterile) in various media.

From the sterilised MC medium only *Pleurotus* could grow (Fig. 18a). *Lentinus* could not develop its mycelium if it was transferred to another MC plate. One plate of *P. chrysosporium* from the triplicate grew but the other two not (Fig. 18b).

Pleurotus mycelium from the second inoculation (Fig 19a) seemed less vigorous than the mycelium from the first (Fig. 17a) indicating that 100% MC is not an optimal medium to cultivate the fungi.

From the MC plate where *P. chrysosporium* was able to grow, the other two plates were re-inoculated, but mycelium did not develop further. MC plate in which mycelia grew changed colour from dark brown to cream or light brown (Fig. 19).

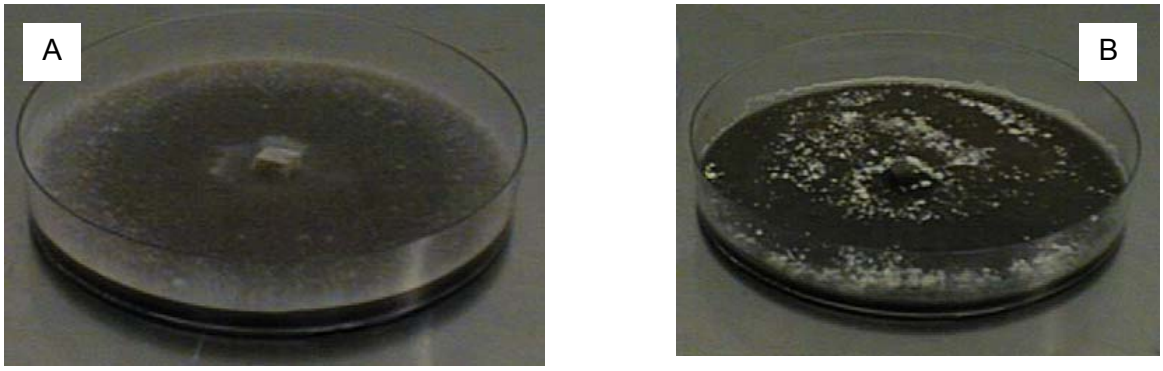


Fig. 19: Sterilised MC plates (second inoculation) fully grown with a) *P. pulmonarius* and b) *P. chrysosporium*.

P. chrysosporium was also inoculated again in WLBP plates containing a purple dye to try to explain the results discussed in previous paragraphs. If ligninolytic enzymes were produced, the dye would be broken into yellow degradation products. Two days after inoculation, *P. chrysosporium* completely colonised the plates but, they did not turn yellow. Only one of them did it after 21 days of incubation (Fig. 20).

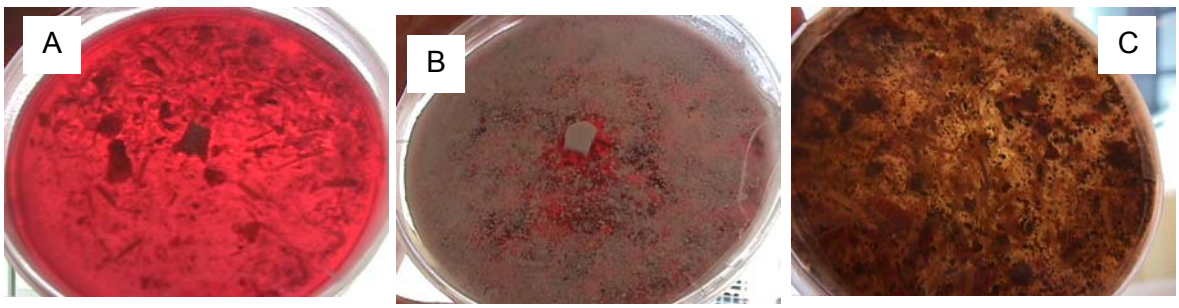


Fig. 20: WLBP medium stained with Poly R immediately after inoculation with *P. chrysosporium* (a), after 5 days incubation at 37°C (b) and after 21 days of incubation (this latest one occurred only in one petriplate).

Therefore, it could be concluded that *P. chrysosporium* could produce ligninolytic enzymes but the trigger to induce them is unknown and difficult to define since results differed so much between triplos.

1.3.5 Enzymatic activities of mycelia grown on MELISSA cake

Ligninolytic enzymes were analysed and compared in MMP, WLBP and sterilised MC media (in those plates in which mycelium could fully colonise). *Pleurotus* grew on MC so extraction of their enzymes could be performed (in the triplicate) but *P. chrysosporium* and *Lentinus* plates could not be analysed.

The selected *Pleurotus* strain produced higher amount of peroxidases in WLBP medium than MMP and MC (Fig. 21a). In MC peroxidases were produced to a higher extent than MMP medium but lower than WLBP. When laccases were measured, no significant differences were found between WLPB and MC media, but both produced higher amount of laccases than on MMP medium (Fig. 21b). WLBP seemed a better medium to produce peroxidases than MC but MC also induced the production of more laccases than WLBP. Since both enzymes are involved in the lignin degradation a medium combination of both media would be the best to grow the fungi. Aryl alcohol oxidases and lining peroxidases were not detected in any of the media.

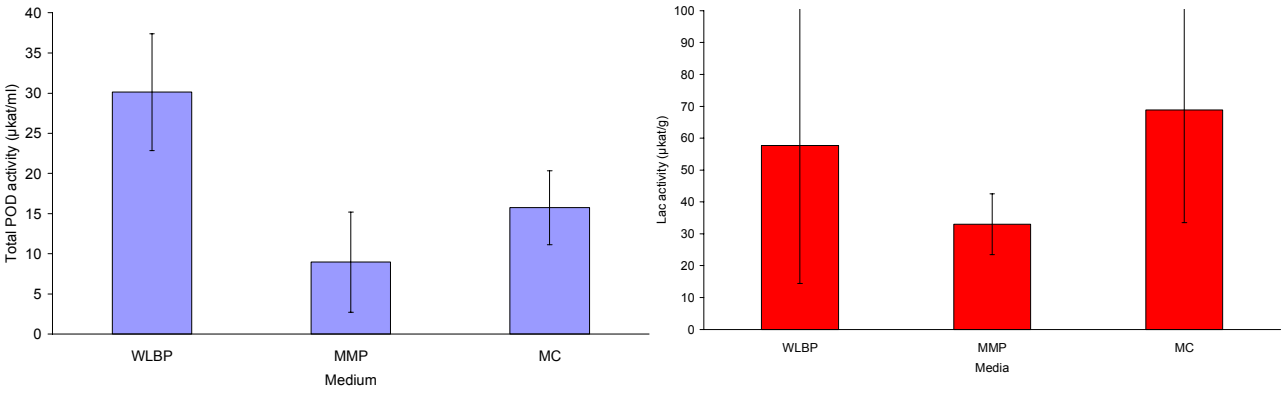


Fig. 21: Peroxidase (a) and laccase (b) activities of *Pleurotus* mycelia in WLBP, MMP and sterile MC media.

WLBP-Poly R plates where also screened for peroxidases, laccases, aryl alcohol oxidases and lignin peroxidases. After 2 days plates seemed fully colonised and enzymes were measured after 1, 2, 3, 4, 5 and 8 incubation days. Nevertheless, none of the mentioned enzymes was detected except for LiP. Ligning peroxidase was found but only in the plate in which *P. chrysosporium* turned the media yellow (after 21 days of incubation).

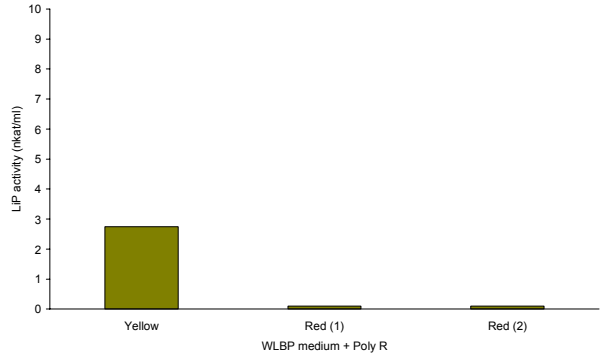


Fig. 22: Lignin peroxidase activity in *P. chrysosporium* grown on WLBP and Poly R.

The triplicate was screened separately taking into consideration whether de colour of the medium changed (to yellow) or not (Fig. 22). The detected LiP activity was of the magnitude order of nanokatals. Perhaps difficulties on extracting this enzyme may explain the results. Peroxidases, laccases and aryl alcochol oxidases were also not detected after 21 incubation days. Therefore, it

could be concluded that results with *P. chrysosporium* were too variable to try to work with this fungus at bag scale.

1.4 Conclusions

From the experiments carried out within the framework of workpackage 7.1, *Pleurotus pulmonarius* P17 has been selected as most promising fungus with regard to lignin degradation. The selection was made based on enzyme activity measurements performed after extracts of the growth medium of various edible and non-edible fungi. The *Pleurotus pulmonarius* strain displayed high peroxidase activities, which are related to lignin degradation mechanisms in fibrous material. On the other hand, the protease activity in the medium colonised by *Pleurotus pulmonarius* was relatively low. This may be an advantage for the enzyme activity in general, as proteases break down proteins (including enzymes), and accordingly may interfere with enzymatic processes that lead to degradation of lignin.

In future experiments *Pleurotus pulmonarius* P17 is used to perform lignin degradation measurements on the real non-edible parts of wheat, lettuce and red beet with and without MELISSA cake as medium. For the next experiments as experimental set-up it was chosen to grow the fungi at a larger scale than done so far at petridish level. In plastic bags, pasteurized MC and plant wastes were inoculated with the *Pleurotus* spawn (WP 7.2). The actual fungal growth, and lignin degrading capacity on this medium was monitored using CO_2 production rates and substrate composition measurements.

1.5 Bibliography

Archibald, F. S. (1992). “A new assay for lignin-type peroxidases employing the dye azure B.” *Applied and Environmental Microbiology* **58**(9): 3110-3116.

Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72:* 248-254. 1976.

Castillo, M. P., J. Stenström, et al. (1994). “Determination of Manganese Peroxidase activity with 3-Methyl-2-benzothiazolinone Hydrazone and 3-(Dimethylamino)benzoic acid.” *Analytical Biochemistry* **218**: 399-404.

Crestini, C. and G. G. Sermanni (1995). “Aromatic ring oxidation of vanillyl and veratryl alcohols by *Lentinus edodes*: possible artifacts in the lignin peroxidase and veratryl alcohol oxidase assays.” *Journal of Biotechnology* **39**: 175-179.

Espín, J. C., M. Morales, et al. (1997). “Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase.” *Journal of Agricultural and Food Chemistry* **45**(4): 1084-1090.

Guillen, F., A. T. Martinez, et al. (1994). “Hydrogen-peroxide-producing system of Pleurotus eryngii involving the extracellular enzyme aryl-alcohol oxidase.” *Applied Microbiology and Biotechnology* **41**(4): 465-470.

Pelaez, F., M. J. Martinez, et al. (1995). “Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation.” *Mycological Research* **99**(1): 37-42.

Sarath, G., R. S. d. l. Motte, et al. (1990). Protease assay methods. *Proteolytic enzymes a practical approach*. R. J. Beynon and J. S. Bond. Oxford, Oxford University press**:** 25-55.

Stoscheck, CM. Quantitation of Protein. *Methods in Enzymology 182:* 50-69 (1990).

Zhao, J., H. Theodorus, et al. (1996). “Comparative studies of lignin peroxidases and manganese-dependent peroxidases produced by selected white rot fungi in solid media.” *FEMS Microbiology Letters* **145**: 393-399.