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3.1 Introduction

The experiments that were carried out in bags, as described in Technical Note 6.2, using the white-rot fungus *P. pulmonarius P17* did not show significant degradation of the lignins in the MELISSA plant material despite good growth of the fungus. The work described in present report was done to verify the results obtained earlier with the *Pleurotus* species (FOOD-1) and quantify lignin degradation.

Despite the variable results obtained within workpackage 7.1, one of the most extensively studied white rot fungi, i.e. *Phanerochaete chrysosporium*, is evaluated in this study as well in the fibre degradation experiments as a reference strain [Zadrazil (1993); Carlile and Watkinson (1994)].

In first instance, experiments were carried out on a small scale in petri-dishes using different substrates as starting material. Within workpackage 7.2 the lignin degrading properties of the selected fungi have been evaluated on different substrates, containing plant material, the output of the waste compartment (MELISSA cake, MC), or combinations thereof. The MELISSA plant material, i.e. the non-edible parts of wheat, lettuce and red beet, form the basis for those substrates. The plant substrates were blended to enlarge the accessible surface and lignin-enriched to evaluate whether the fungi use the lignins in an earlier growth stage due to the lack of readily available nutrients.

The effect of adding ferrulic acid to the growth medium is evaluated in the petri-dish assays. This phenolic compound is known to stimulate the induction of lignolytic enzymes in certain fungal strains, which takes place at RNA level [Leonowicz and Trojanowski, (1975); Leonowicz et al (1972); Leonowicz and Trojanowski (1975)], and accordingly may affect degradation of the plant fibres.

Both the *P. pulmonarius* and *P. chrysosporium* strain were cultured on pure MELISSA cake to compare their behaviour towards the plant substrates. Using this medium the effect of changing the growth conditions (e.g. temperature, addition of an inert material as a solid support) and reactor medium (solid vs. liquid medium) on the efficacy of the fungi to degrade lignin was investigated.

One major goal within the Food on Orbit Demonstration project 2 is to complete the mass balances of the degradation process induced by the fungi. Initial experiments to achieve this were carried out in a closed system. This system contains the strain *P. pulmonarius* growing on MELISSA cake. To complete the mass balances 'continuous' CO_2 measurements were done and related to changes in the substrate composition and fibre degradation.

3.1 Material and methods

3.1.1 Preparation of growth media

3.2.1.1 Different media for petri-dish experiments

Plant leaves and straw pieces were blended using a Waring Commercial Blender type 8011-S to obtain a much larger accessible surface area of the plant material to use in petri-dishes compared to the substrate used in the bag experiments (TN 6.2). Particle sizes below 1 mm were thus obtained. Three different substrates were prepared containing the dried MELISSA plant wastes, i.e. wheat straw, red beet, lettuce (1:1:1). Some ground plant material was washed with a large excess of water (10 L of water was used for approx. 200 g of dry plant powder) to remove soluble components that are readily available nutrients for the fungi. The material was filtered and the "lignin-enriched" residue was freeze-dried.

Both to the rich plant substrate (A), which was used as such, and the washed "lignin-enriched" plant material (B), MELISSA cake was added in 1:9 MC:plant ratio (based on dry matter content). To one batch of the washed plant substrate ferrulic acid (1 mM) is added (C). As a fourth medium (D) solely the output of the waste compartment (MELISSA cake, MC, Batch EWC Reactor 1, pH 5.8 – 6, 25/04/03) was used. The solid content of the MC was raised to 9 % dry matter content by centrifugation of the dispersion and collection of the precipitate.

Agar (1%) is added to 'solidify' the media in the plates for easier handling and storage. All media prepared as described above were autoclaved for 20 min at 121°C.

3.2.1.2 MELISSA cake as liquid medium

Liquid media containing the output of the waste compartment (MC) as obtained from EPAS (EWC concentrate supplied at 25/09/03) were prepared in erlenmeyers (1 liter). Approximately 150 ml of the MELISSA cake (solid content of ~37 g/l) was added to the erlenmeyers (medium 1). As an inert carrier material perlite was selected and added (0,48 g) to the MC (medium 2). All media prepared as described above were sterilized by autoclavation for 20 min at 121°C.

3.2.1.3 MELISSA cake as substrate for incubation in a closed system

Petri-dishes were prepared containing MELISSA cake, as obtained from EPAS (EWC concentrate supplied at 25/09/03) and agar (1 w/w %) to 'solidify' the medium in the plates. MC was selected from the experiments on petri-dishes as performed before, because of its high lignin content. The solid content of the MC was raised from solid content of ~50 g/l (as stated by EPAS) to a dry matter content of ~8% by centrifugation of the dispersion and collection of the precipitate. Accordingly, 6 petri-dishes in total could be prepared, which are collectively used in further experiments.

The medium prepared as described above was autoclaved for 20 min at 121°C before inoculation.

3.2.2 Fungal species and growth conditions

Pleurotus pulmonarius p17 and *Phanerochaete chrysosporium* were selected for evaluation of lignin degradation efficacy. Both strains were grown on petri-dishes containing malt mycological

peptone medium (MMP). Spawn of *P. pulmonarius* was prepared by adding sterilized rye grains on the MMP plates. *P. chrysosporium* appeared not to grow well on these grains. To inoculate the different media pieces of agar containing *P. chrysosporium* and *P. pulmonarius* colonized grains (one grain per plate unless stated otherwise) were added, respectively.

All samples were incubated at temperatures of 24°C or 37°C in the dark for at least 30 days. All solid media, i.e. the petri-dishes were left to stand, whereas the liquid cultures were shaken at 180 rpm. After incubation the fungal respiration and/or lignin degradation efficiency were determined according to the methods described in paragraph 3.2.5 and 3.2.4, respectively.

The MELISSA cake plates were incubated at 20°C in a closed system to determine fungal respiration (see paragraph 3.2.5). Subsequently, lignin degradation efficiency was measured according to the method described in 3.2.4).

3.2.3 Evaluation of fungal growth

The mycelial colonization was followed in time by visual examination. Other indications about the fungal growth were the substrate consumption after 30 days of incubation and changes in the substrate composition. The respiration activity of the fungus is another parameter to indicate fungal growth.

3.2.4 Methods for measuring substrate composition and lignin degradation

The colonized substrates (approx. 2-2.5 g) in the petri-dishes were freeze-dried and subsequently subjected to the fibre determination assay. The samples were submitted to a combined method to determine the contents of extractives, lignin, uronic acids and polysaccharides in fibrous materials.

Before chemical analysis the material was first grinded in a Waring Commercial Blender type 8011-S. The samples are twice extracted in a Soxtec apparatus, first with a mixture of ethanol and toluene (2:1 v/v), secondly with 96% ethanol (boiling for 30 minutes and rinsing for 75 minutes). A third extraction is done with hot water (1 hour at 100°C). These extractions were done according to TAPPI method T264 om-82 in order to remove proteins, waxes, fats, resins, tannins, gums, starches, free sugars and colouring materials (like chlorophyll etc.). The extracted materials are dried overnight at 60°C for quantification.

From the extracted material contents of lignin and polysaccharides, as well as uronic acids can be determined (the latter were not determined). Hereto, the samples were hydrolysed with sulphuric acid (12M, 1 hour at 30°C and 1M, 3 hours at 100°C). The formed monosaccharides were reduced and acetylated and measured on GLC. The acid insoluble lignin is determined gravimetrically and the acid soluble lignin in the hydrolysate is determined spectrophotometrically at 205 nm.

In Technical Note 6.2 (Appendix) an extensive protocol of the lignin degradation measurements can be found.

3.2.5 Continuous CO_2 production measurements

Pleurotus respiration rate and the accumulation of CO_2 were followed in time in a closed system attached to a CO_2 analyzing sensor (LI-COR, inc. LI6250 CO_2-analyser, Serie No: IRG620). Hereto, six petridishes containing solidified MELISSA cake inoculated with the

Pleurotus pulmonarius strain were left to grow for 30 days at 20°C. The total amount of substrate in the cuvet consisted of 14 g dry matter.
The CO_2-laser can measure respiration rates of about 5 ppm/4 litre/min reliably. CO_2 was measured during 5 minutes. CO_2 was measured in parts per million (ppm) and expressed in ml CO_2/kg/hour, according to:

$$CO_2 \text{ exchange (ml } CO_2\text{/kg/hour)} = \frac{(CO_2(T=5)-CO_2(T=0))}{M / 5*60} * 4.12E^{-3}$$

Where $CO_2(T=5)$ and $CO_2(T=0)$ are the levels of CO_2 at t=5 min and t=0 min (in ppm), M is the sample mass (in kg), and $4.12E^{-3}$ is used as calculation factor from ppm into ml CO_2, respectively. This constant depends upon the laser and tubing dimensions.

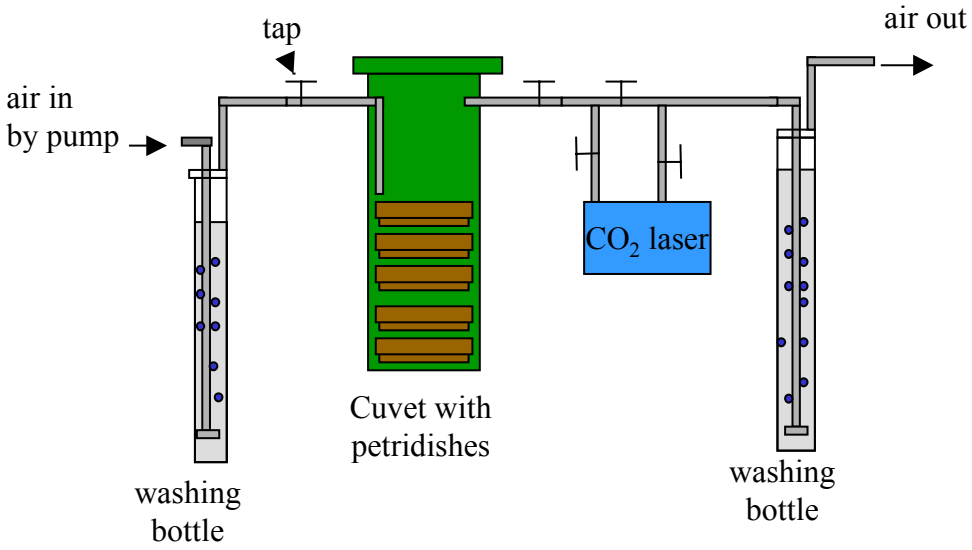

Figure 1: Experimental set-up of continuous CO_2 measuring system.

The closed system depicted in Figure 1 is used to measure the CO_2 production of the fungus. Incoming ambient air is pumped through two washing bottles containing 0.4 M $Ba(OH)_2$ solution to remove CO_2 and to humidify the air. The CO_2-free air is directed through the cuvet, which holds the petridishes containing the *Pleurotus Pulmonarius P17* cultures on MELISSA cake. The incoming air is directed from bottom to top in the cuvet to prevent CO_2 accumulation. At the other end of the incubation cuvet, all CO_2 generated by the fungus, is trapped in another two washing bottles filled with 0.4 M $Ba(OH)_2$ in the form of the $BaCO_3$. The two washing bottle sections are equal in dimensions.

At regular time intervals the CO_2-laser measures the respiration rate in the cuvet (V ~2 l) during 5 min to check the exchange process in time.

The relation between carbon dioxide production and dry weight decrease in a closed system will be established. The regular measurements will provide indications if the carbondioxide production is linear in time or not.

An alternative procedure to determine the CO_2 production rate in the cuvet was used to verify the data generated by the CO_2 analyzer. For measuring respiration (O_2 uptake and CO_2 production) the cuvette was closed temporary and headspace samples were taken directy after closing and after 4.5 hours. O_2 and CO_2 were measured with a Chrompack CP2002 gas chromatograph (GC), by injecting the samples from the cuvet.

To convert O_2 and CO_2 percentage to partial pressure, total pressure in the room was used. The difference in gas partial pressure between the measurement directly after closing and 4.5 hours later was converted to moles per weight per time.

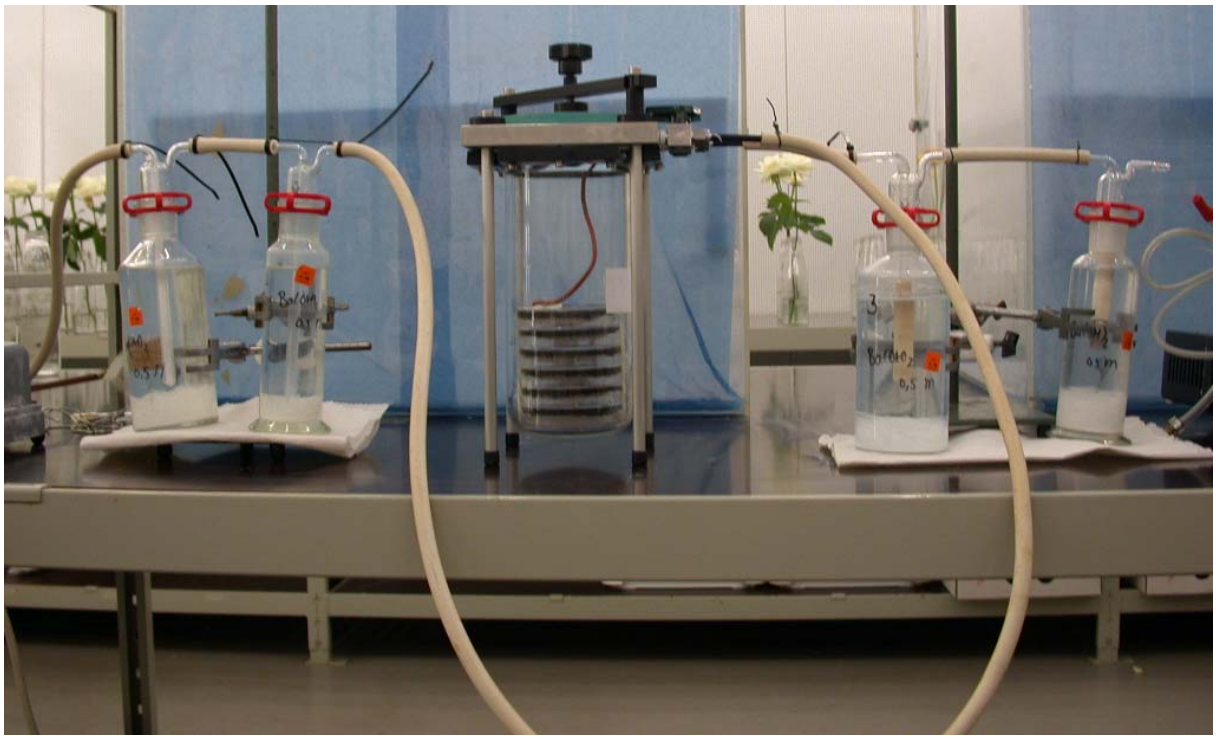

Figure 2:
Accumulation of CO_2 produced by the fungus *P. pulmonarius* is measured from the formation of the water insoluble $BaCO_3$ in a closed system.

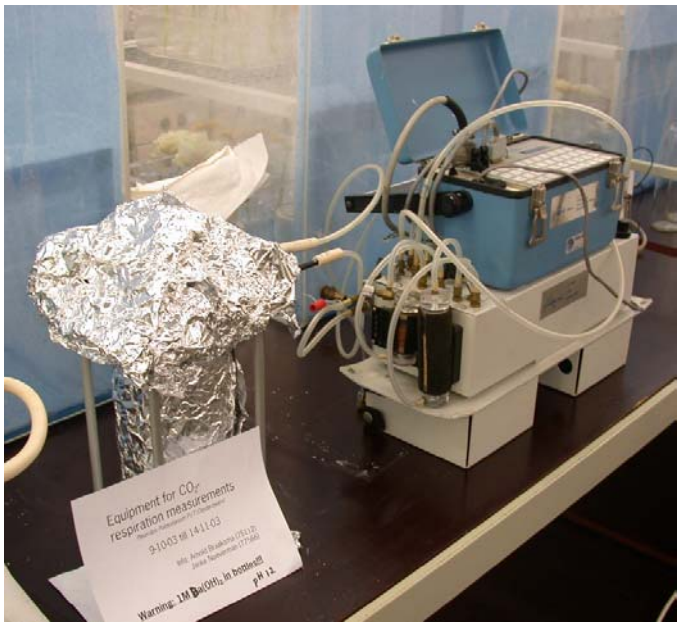

Figure 3:
The cuvet is directly connected to the CO_2 measuring device to assess the respiration rate.

3.3 Results and discussion

3.3.1 Verification and quantification of lignin degradation by fungi on petri-dishes

On petri-dishes containing media with different composition, and accordingly, varying lignin concentrations, both *P. pulmonarius* and *P. chrysosporium* were cultivated.

3.3.1.1 Evaluation of fungal growth on media with varying lignin content

In the Figures 4, 5, and 6 the growth of both the fungi *P. chrysosporium* and/or *P. pulmonarius* on the different plates is visible after 12, 19, and 29 days of incubation. *P. pulmonarius* grew well on the rich plant medium, as is shown in Figure 4. The mycelium fully colonized the plates within 8 days. No results are shown of *P. chrysosporium* on this medium, as this fungus does not grow well on rich media. To induce high ligninolytic activity, *P. chrysosporium* needs media that are relatively poor, especially in nitrogen sources [Zadrazil (1993); Carlile and Watkinson (1994)].

Determination of the solid content of the substrate after cultivation of *P. pulmonarius* on rich plant medium (Table 1) shows that the solid content slightly decreased from 9.97 in the starting material to 9.63 % after 47 days of incubation. This corresponds to a solid consumption of 3 % after 47 days, and is therefore estimated at 2 % after a 30-day incubation period. This value of 2 % is used as a correction factor for calculation of the lignin degradation rates described in section 3.3.1.2.

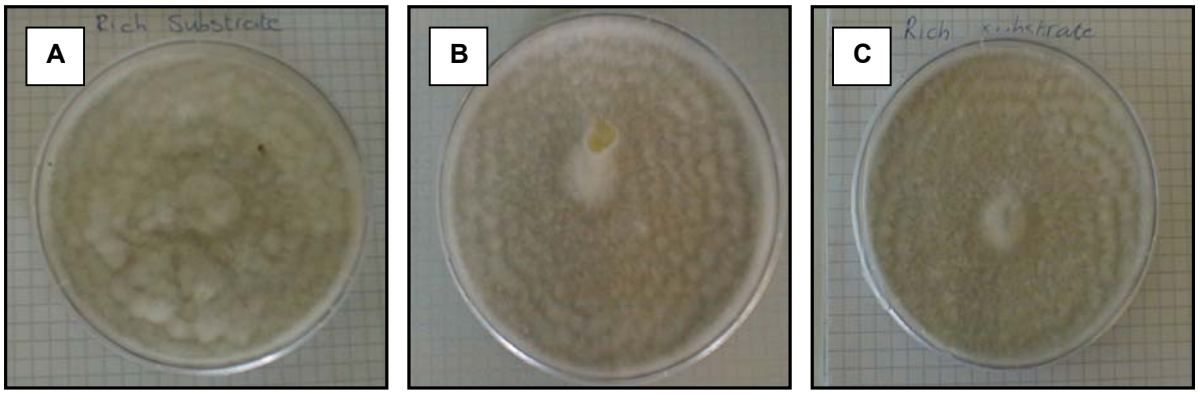

Figure 4: Fungal growth of *P. pulmonarius* on rich plant substrate after 12 (A), 19 (B) and 29 (C) days of incubation.

Secondly, the growth of *P. pulmonarius* and *P. chrysosporium* on MELISSA cake is discussed. The growth of the two fungi at MC on petridishes is shown in Figure 5. The MC plates are fully colonized after ~12 days of incubation, as is shown in Figure 5A and D. The difference in morphology, i.e. the physical appearance, of the two strains is clearly visible in this picture. In comparison with the rich plant medium *P. pulmonarius* seems to grow somewhat less thriving, which may be explained by the difference in composition of the substrates. In the rich medium a much higher content of readily available nutrients will be present than in the MELISSA cake plates.

Due to a limited number of plates containing the MELISSA cake substrate, the important value indicating the solid content of the substrate before and after incubation of the fungi could not be determined. Unfortunately, there were no plates left after the 30-day incubation period. Therefore, the correction factor as stated above needed to be estimated. This value was carefully chosen to represent substrate (MC) conversion of 5% after 30 days of incubation. However, in forthcoming experiments this percentage should be verified.

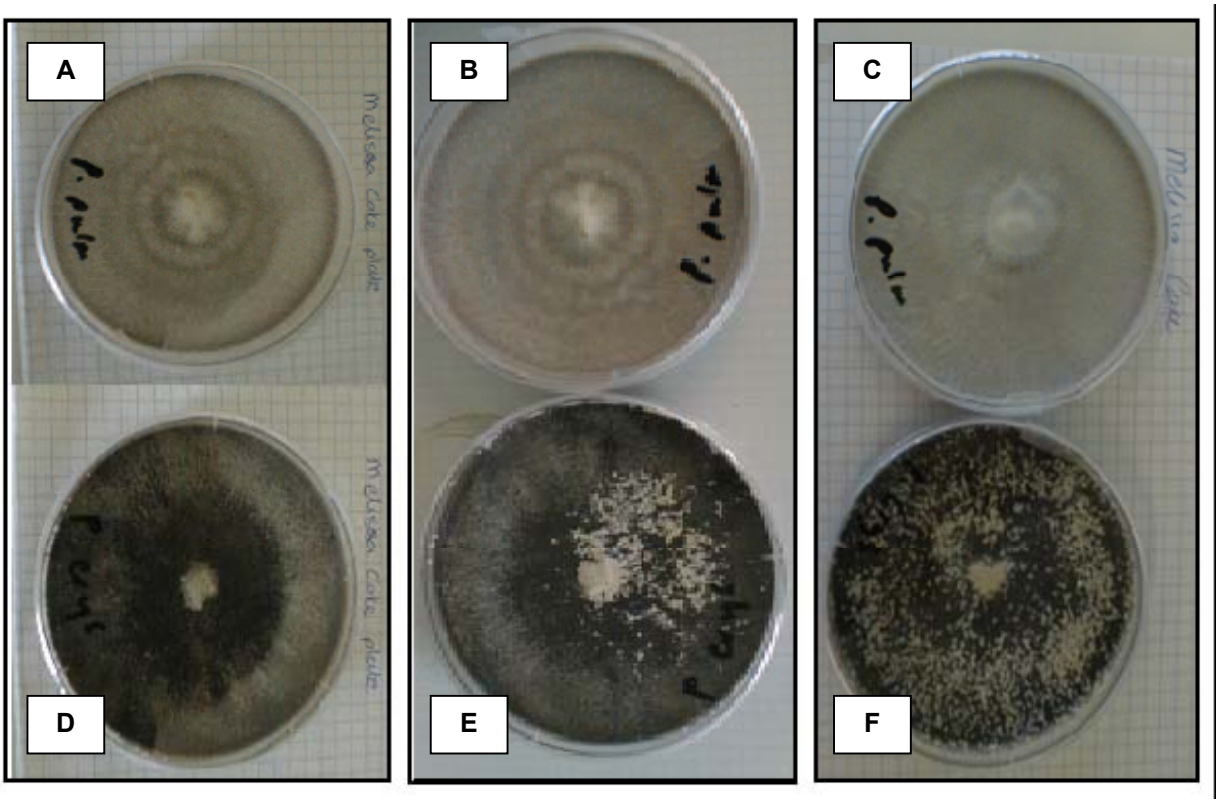

Figure 5: Fungal growth of *P. pulmonarius* (A, B, C) and *P. chrysosporium* (D, E, F) on MELISSA cake substrate after 12 (A, D), 19 (B, E) and 29 (C, F) days of incubation.

Table 1: Substrate consumption levels on petri-dishes by *P. pulmonarius* and *P. chrysosporium* incubated at 24°C.

T (days)	Strain	Substrate	Solid content (%)	Solid consumption (%) (estimation 30 d)
0	-	Rich	9.97	-
47	P17	Rich	9.63	-3 % (-2%)
0	-	Lignin enriched	9.78	-
47	P17	Lignin enriched	7.65	-22 % (-15%)
47	P chrys	Lignin enriched	7.15	-27 % (-18%)

The growth on lignin-enriched plant media is described and displayed in Figure 6 for *P. pulmonarius* and *P. chrysosporium*, respectively. Both fungi grow thrivingly on the substrates both in the absence and presence of ferrulic acid (the latter results are not shown, but were similar to the plates in Fig. 6). This suggests that the addition of the ferrulic acid does not affect fungal

growth. Interesting are the bottom and side views of the plates, which clearly show the substrate conversion and the distribution of the fungi throughout the substrate (Fig. 6B', C, and F).

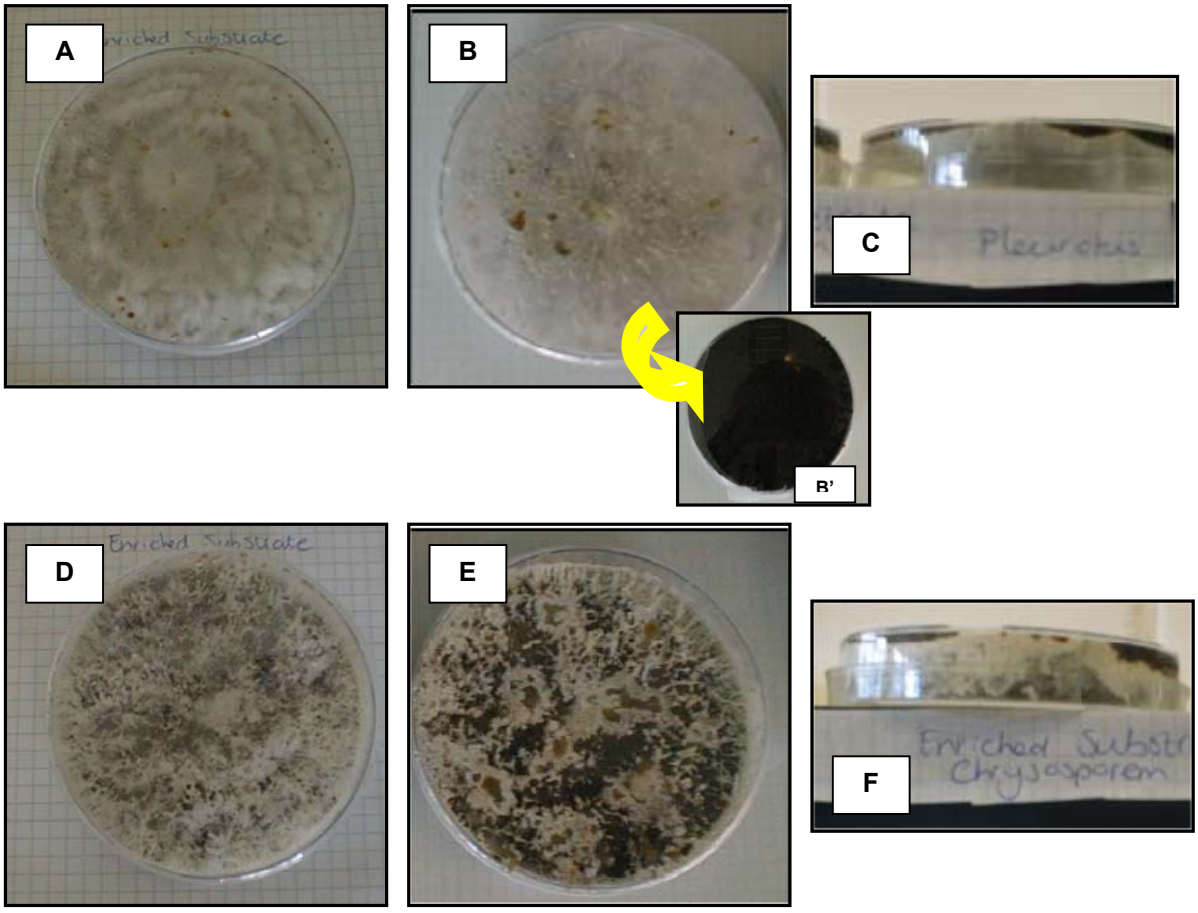

Figure 6: Fungal growth of *P. pulmonarius* (A-C) and *P. chrysosporium* (D-F) on lignin-enriched substrate after 12 (A, D), 19 (B, B' (bottom view), E) and 29 (C, F (side views)) days of incubation.

The solid content as determined after freeze-drying changed from 9.78 % of the starting lignin enriched material to 7.65 % and 7.15 % after 47 days of incubation of *P. pulmonarius* and *P. chrysosporium*, respectively (Table 1). This corresponds with a total solids consumption of 22 % and 27 % after 47 days for *P. pulmonarius* and *P. chrysosporium*, respectively. After 30 days, these values are estimated to be 15% and 18% for both fungi, respectively. These percentages are used in the calculations made in the lignin degradation measurements.

3.3.1.2 Changes in substrate composition

Investigation of the rich plant material samples after 0 and 30 days of cultivation of *P. pulmonarius*, respectively, leads to the results presented in Table 2. Most striking is the similarity in substrate composition at the different time points, indicating that the starting material was not essentially altered by the growth of this *Pleurotus* strain. The extractions with both the organic solvents, i.e. ethanol and toluene, and water showed slightly lower amounts after 30 days. The lignin content, both the acid insoluble and soluble fraction, hardly changed. The polysaccharide content of the day 30 samples was significantly higher than at t=0. This suggests that the fungus mainly uses readily available nutrients in the substrate.

There is no need to employ the more 'complex' metabolic routes for their survival. Therefore, lignin concentrations and polysaccharide contents seem unaltered.

Table 2: Substrate composition (%) of the rich plant medium after 0 and 30 days of cultivation of *P. pulmonarius* at 24°C.

T	strain	EtOH/Tol +EtOH soluble compounds (%)	Water soluble compounds (%)	AIL* (%)	ASL** (%)	Total polysaccharides (%) including *(xylose (%); glucose (%))***
0	-	17	26	33	2	13 *(4; 8)*
30	P17	10	24	31	2	30 *(9; 19)*

***** AIL = acid insoluble lignin, **ASL = acid soluble lignin, *** the percentages between brackets represent the fractions of xylose and glucose of the total polysaccharides (both sugars are the most abundant building blocks of the various polysugars that were detected via GC analysis).

The plant material used to prepare the rich medium in the petri-dishes was the same as the substrate used in the bag experiments. The composition and the lack of changes therein of the rich samples in the petri-dishes indicated in Table 2 may explain the results obtained in the bag experiments (TN 6.2). The media in the bags were too rich. Apparently, there was no trigger for the fungus to break down lignin and polysaccharides to any degree.

The results obtained for the MELISSA cake samples after cultivation of *P. pulmonarius* and *P. chrysosporium* are presented in Table 3. In these samples the lignin content decreases from 59 % of acid insoluble lignin, which clearly represents the largest part of the total lignin fraction, to 40 % and 44 % after cultivation of *P. pulmonarius* and *P. chrysosporium*, respectively. Of the total lignin fraction this comprehends a significant decrease of about 32 % and 26 %, respectively.

The polysaccharide content of the starting material was relatively low, indicating that most of the polysugars were degraded during anaerobic digestion. (It should be noted that no corrections were made for the deviation observed in the total amount of material that was retrieved during determination of the substrate composition; as can be seen >100% of the amounts of the MELISSA samples (Table 3) were detected).

Table 3: Substrate composition (%) of the MELISSA cake after 0 and 30 days of cultivation of *P. pulmonarius* and *P. chrysosporium*, respectively, at 24°C.

T	strain	EtOH/Tol +EtOH soluble compounds (%)	Water soluble compounds (%)	AIL* (%)	ASL** (%)	Total polysaccharides (%) including *(xylose (%); glucose (%))***
0	-	21	25	59	4	15 *(3; 9)*
30	P17	18	27	40	3	17 *(5; 10)*
30	P chrys	16	33	44	3	25 *(7; 15)*

***** AIL = acid insoluble lignin, **ASL = acid soluble lignin, *** the percentages between brackets represent the fractions of xylose and glucose of the total polysaccharides (both sugars are the most abundant building blocks of the various polysugars that were detected via GC analysis).

Whereas the experiments performed in present study were done to verify the results obtained earlier, some of the results from Table 3 are compared with the data obtained within the FOOD-1 project in Table 4. The latter are given in italic font.

Table 4: Substrate composition (%) of MELISSA cake after 0 and 30 days of cultivation of *P. pulmonarius* in comparison with the results obtained during the first Fungus on Orbit Demonstration (FOOD) project. Both experiments were carried out at 24°C.

T	Strain	project	EtOH/Tol +EtOH soluble compounds (%)	Water soluble compounds (%)	AIL *(%)	ASL **(%)
0	-	FOOD-2	21	25	59	4
30	P17	FOOD-2	18	27	40	3
30	P chrys	FOOD-2	16	33	44	3
0	*-*	*FOOD-1*	*31*	*4*	*23*	*3*
30	*P17*	*FOOD-1*	*20*	*5*	*21*	*2*

***** AIL = acid insoluble lignin, **ASL = acid soluble lignin.

It is important to notify the difference in starting material between the two projects, and accordingly, the discrepancy of the results between these two. The present samples contained much higher amounts of lignin, especially acid insoluble lignin (AIL). Whereas a 9% decrease in the AIL fraction was observed within FOOD-1 after 30 days of cultivation of *P. pulmonarius P17*, currently, one third of the AIL fraction is degraded by this microorganism. This increase in lignin degradation efficiency seems to be induced by the higher starting content of lignin. The latter is also observed for the lignin-enriched substrates and the results are presented in Table 5.

The washing step of the plant material resulted in starting substrates with AIL percentages around 50% of the total mass. In contrast with the rich plant substrates, conversions around 20-25% were observed for both the fungi in the lignin-enriched fractions. From the numbers given in Table 5, no stimulating effect on the lignin degradation efficiency could be derived for addition of ferrulic acid to the culture.

Note: Actually, the degree of degradation of lignin is higher, whereas the analyses of the composition are performed on the total biomass (substrate and fungi). It is impossible to separate the fungus from the substrate. Fungal biomass can be estimated, for instance, from ergosterol content or alternative secondary metabolites that are typical for these microorganisms. Such tests were not included in this study, however, in future work these may be incorporated.

Table 5: Substrate composition (%) of the lignin-enriched media after 0 and 30 days of cultivation of *P. pulmonarius* and *P. chrysosporium*, respectively, at 24°C.

T	Substrate/ *Strain*	EtOH/Tol + EtOH soluble compounds (%)	Water soluble compounds (%)	AIL* (%)	ASL** (%)	Polysaccharides (%)
0	Rich	17	26	33	2	13
0	Enriched	6	13	47	2	41
0	Enriched + FA	19	21	51	2	35
30	Rich/ *P17*	10	24	31	2	30
30	Enriched/ *P17*	7	14	35	2	37
30	Enriched/ *P chrys*	12	15	37	2	31
30	Enriched + FA/ *P17*	6	14	40	2	53
30	Enriched + FA/ *P chrys*	7	23	41	1	12

***** AIL = acid insoluble lignin, **ASL = acid soluble lignin. Although the depicted fractions represent by far the largest amount of the substrate, added the values (%) may differ from 100%. The deviation should be deminished by plural measurements.

In general, the lignin degradation efficiencies are hardly different for the two strains. The influence of the lignin start concentration on the degradation efficiency of the fungi becomes evident. In this respect, the results observed within the FOOD-1 project seem in line with the results obtained with the newly prepared substrate batches (an overview of the results is given in section 3.3.3).

Whereas the substrate composition, especially the lignin contents of the media, did hardly alter when *P. pulmonarius* was grown on rich plant medium, in the MC and lignin-enriched substrates the lignin content of the samples significantly decreased. No defined effect could be established from ferrulic acid. Enrichment of the lignin concentration in the substrate positively affects the lignin degradation. Whereas the output of the first waste compartment, the MELISSA cake, contains high amounts of lignin, this may be a good substrate for the fungi. It remains to be tested how the fungi perform in a liquid MC culture.

The change from the solid state fermentation approach to liquid phase originates from the disappointing results in earlier experiments. The plant material that was selected to form the solid support for the fungi, i.e. mainly wheat straw, during solid state fermentation, appeared too rich in non-lignin nutrients, that no degradation of the fibres was detected. MELISSA cake, on the other hand, contained high amounts of lignin. However, the particles within the MELISSA cake were too small to form some support in solid state fermentation. Liquid phase reactions were expected to yield better results. It was checked whether the fungi would benefit from addition of an inert support material (i.e. perlite).

3.3.2 Optimization of lignin degradation by fungi cultured in MELISSA cake

The possibilities to cultivate the fungi *Pleurotus pulmonarius* P17 and *Phanerochaete chrysosporium* in completely mixed reactors (liquid media) have been reviewed on the basis of their lignin biodegradation efficacy. As substrate, MELISSA cake (MC), the lignin-rich output of the waste compartment, has been used at a larger scale than in the experiments described in the petri-dishes (section 3.3.1). The fungi may profit from the addition of an inert solid support material. Therefore, perlite is selected as a porous siliceous material, as good results were obtained in other projects carried out by A&F.

The fungi so far have been grown at 24°C. In order to evaluate whether this temperature is most optimal for the lignin degradation mechanism by the fungi, 37°C was selected as another incubation condition.

3.3.2.1 Evaluation of fungal growth

Both *P. pulmonarius* and *P. chrysosporium* were cultured in liquid MELISSA cake medium (150 ml) at 24°C and 37°C (dry matter ~ 4%) in the absence and presence of the inert solid carrier perlite. Due to the dark brown color of the liquid suspension it is difficult to visually determine the growth. The viscosity of the growth medium containing *P. pulmonarius* did clearly increase, thus indicating the presence of the fungal mycelium. For *P. chrysosporium* the latter could not be observed. The morphology of *P. chrysosporium* appears to differ from that of *P. pulmonarius* as is visible upon culturing in petri-dishes (Figures 5 and 6). In liquid media upon shaking the morphology may differ again from that on agar plates.

Both fungi grow well in liquid MC medium at the two temperatures, as can be seen from the consumption of solids after analyzing the dry matter content of the different samples (Table 6 and 7). From the total solid material up to 15% and 25% has been consumed after 29 days of cultivation of *P. pulmonarius* and *P. chrysosporium*, respectively.

When perlite was added to the liquid MELISSA cake, the absolute solid content was reduced 5% (Table 7). Apparently, *P. chrysosporium* does not benefit by the addition of the support material. Unfortunately, no data could be shown from the *P. pulmonarius* culture, as the erlenmeyer which contained that culture broke during shaking in the 37°C incubator. The single experiments were pilot-trials, in future work multiple measurements (triplicate or more) should assure a solid experimental approach.

Table 6: Substrate consumption levels in liquid MELISSA cake after 0 and 29 days of culturing of *P. pulmonarius* and *P. chrysosporium*, respectively.

T (days)	Strain	Temperature (°C)	Solid content (%)	Substrate consumption (%) on dry matter basis
0	-	-	4.35	-
29	*P pulm*	24	3.82	- 15
29	*P chrys*	24	3.33	- 25
29	*P pulm*	37	4.14	- 13
29	*P chrys*	37	3.72	- 22

Table 7: Substrate consumption levels in liquid MELISSA cake containing perlite after 0 and 29 days of culturing of *P. chrysosporium.*

T (days)	Strain	Temperature (°C)	Solid content (%)	Substrate consumption (%) on dry matter basis
0	-	-	3.90	-
29	*P chrys*	37	3.91	- 5

3.3.2.2 Lignin degradation in liquid cultures

The substrate composition of the starting material and the cultures after incubation for 29 days was evaluated to assess the lignin degradation efficiencies of both the fungi. The results that were generated from the lignin determination assay are shown in the Tables 8 and 9.

Table 8: Substrate composition (%) of the liquid MELISSA cake after 0 and 29 days of culturing of *P. pulmonarius* and *P. chrysosporium*, respectively.

Sample	EtOH/toluene soluble compounds (%)	EtOH soluble compounds (%)	Water soluble compounds (%)	AIL** (%)	ASL*** (%)	Total poly saccharides (%) *(glucose %)***
t=0 Liquid medium MC	15	2	13	27	2	46 *(30)*
t=29 days 24°C *P pulm.*	11	4	12	25	1	41 (*27)*
t=29 days 24°C *P chrys.*	10	4	21	25	1	30 *(18)*
t=29 days 37°C *P pulm.*	5	n.d.*	19	25	1	40 *(27)*
t=29 days 37°C *P chrys.*	6	5	23	27	1	30 *(18)*

* n.d.=not determined, ** AIL = acid insoluble lignin, ***ASL = acid soluble lignin, **** The percentage between brackets represents the fractions of glucose, which is the most abundant building block of the polysugar fraction as detected via GC analysis.

The relative composition of the substrate did not change dramatically after 1 month of culturing of *P. pulmonarius* and *P. chrysosporium* at 24 and 37°C. Overall, the content of hydrophobic components (EtOH +/- toluene soluble fractions) decreased. The fraction of watersoluble compounds increased more or less proportional to the decrease in polysaccharide content. The lignin content of the substrate hardly changed due to growth of the fungi.

Upon addition of perlite as inert support material, similar results were obtained (Table 9).

Table 9: Substrate composition (%) of the liquid MELISSA cake after 0 and 29 days of culturing of *P. chrysosporium* in the presence of perlite as an inert support.

Sample	EtOH/toluene soluble compounds (%)	EtOH soluble compounds (%)	Water soluble compounds (%)	AIL* (%)	ASL** (%)	Total polysaccharides (%) *(glucose %)***
t=0 Liquid medium MC+ Perlite	14	2	12	31	2	39 *(26)*
t=29 days 37°C *P. chrys.* + Perlite	7	3	25	30	1	33 *(21)*

* AIL = acid insoluble lignin, **ASL = acid soluble lignin, *** The percentage between brackets represents the fractions of glucose, which is the most abundant building block of the polysugar fraction as detected via GC analysis.

The starting concentration of the lignin fraction of the MELISSA cake was relatively low, compared to the previous batch that was obtained from EPAS. The polysaccharide content in the present batch was much higher than in the previous batch (Table 3).

In absolute terms, however, the masses of the specific fractions of the fibrous material do change, as given in Table 10.

In Table 10 it is observed that the masses of hydrophobic components decrease due to fungal growth. The acid insoluble lignin fraction decreased 9% for *P. chrysosporium* in the presence of perlite up to 25% for *P. chrysosporium* grown at 24°C. The acid soluble lignin content is reduced with 33 to 57%. Only, the amount of watersoluble components increased with respect to the starting substrate, which may represent newly formed biodegradation products that originate from the other fractions.

Based on the absolute masses of constituents and changes herein, *P. chrysosporium* most effectively degraded the lignin fraction and the carbohydrates, i.e. celluloses and arabinoxylans, in the MELISSA cake using present experimental set-up in liquid media.

In general, we can conclude that both *P. pulmonarius* and *P. chrysosporium* grow well in liquid cultures. MELISSA cake is a good substrate for the fungi, as can be seen from the changes in the substrate composition and consumption of solids. Especially, when viewing the absolute values depicted in Table 10, *P. chrysosporium* seems more efficient in degrading lignin and polysaccharides than *P. pulmonarius*. Addition of perlite as solid support has no positive effect on *P. chrysosporium* growth in the liquid culture.

Above results indicate that the position of the fungal compartment is preferred after the first waste compartment. The concentration of lignin in the starting material is thus the highest and may promote lignin degradation by the fungi (see section 3.3.3 for a summary of the results).

Table 10: Absolute changes in the substrate composition of the MELISSA cake after incubation of *P. pulmonarius* and *P. chrysosporium*, for 29 days, respectively. The absolute values were calculated from the solid contents and the original sample masses.

Sample	EtOHl/toluene + EtOH soluble compounds g (decrease %) [B]	Water soluble compounds g (decrease %)	AIL g (decrease %)	ASL g (decrease %)	Poly-saccharides [C] g (decrease %)	rhamnose g (decrease %)	arabinose g (decrease %)	xylose g (decrease %)	mannose g (decrease %)	galactose g (decrease %)	glucose g (decrease %)
t=0 Liquid	1.11	0.74	1.60	0.12	2.69	0.01	0.13	0.68	0.05	0.06	1.77
t=29 days 24°C *P pulm*	0.81 (27%)	0.65 (12%)	1.38 (14%)	0.08 (33%)	2.23 (17%)	0.01 (0%)	0.12 (10%)	0.58 (16%)	0.02 (52%)	0.03 (44%)	1.47 (16%)
t=29 days 24°C *P chrys*	0.69 (38%)	1.02 (-38%)	1.19 (25%)	0.05 (57%)	1.45 (46%)	0.01 (0%)	0.08 (44%)	0.46 (32%)	0.04 (14%)	0.02 (62%)	0.85 (52%)
t=29 days 37°C *P pulm*	n.d. [A] n.d.	1.06 (-44%)	1.38 (14%)	0.06 (46%)	2.23 (17%)	0.01 (0%)	0.11 (20%)	0.58 (15%)	0.03 (35%)	0.02 (65%)	1.49 (16%)
t=29 days 37°C *P chrys*	0.53 (52%)	1.14 (-54%)	1.30 (19%)	0.05 (58%)	1.47 (45%)	0.01 (0%)	0.06 (52%)	0.46 (33%)	0.03 (36%)	0.02 (59%)	0.90 (49%)
t=0 Liquid + perlite	0.95	0.70	1.82	0.09	2.30	0.01	0.12	0.59	0.03	0.05	1.52
t=29 days 37°C *P chrys* + perlite	0.55 (42%)	1.36 (-94%)	1.66 (9%)	0.04 (53%)	1.85 (20%)	0.01 (0%)	0.08 (29%)	0.57 (4%)	0.02 (33%)	0.02 (52%)	1.15 (24%)

A) n.d.= not determined. B) (Decrease (%), relative to amount of material in starting substrate; negative values represent increased amounts due to degradation of other constituents). C) The polysaccharide content comprehends the total of the following sugar constituents, i.e. rhamnose, arabinose, xylose, mannose, galactose, en glucose (last 6 columns).

3.3.3 Overview of lignin degradation efficiency

From the petri-dish experiments using the various substrates, it could be derived that the lignin concentration of the starting material is an important parameter for actual lignin degradation by the two white-rot fungi used in this study. When the lignin content of the substrate after 1 month of incubation was plotted as a function of the start lignin concentration (Figure 7), this trend becomes even more evident.

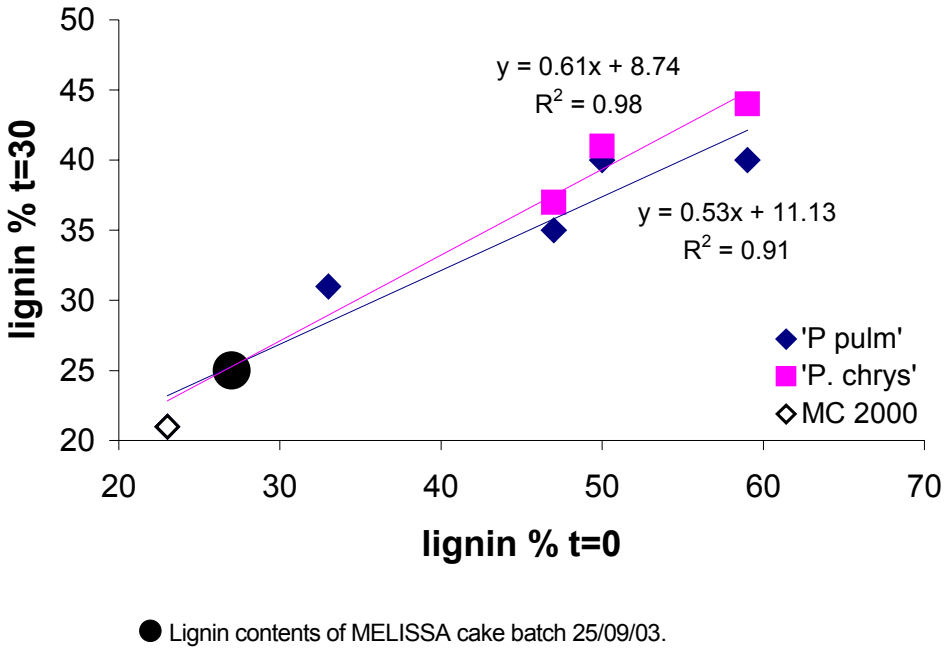

Figure 7: Lignin content of the substrate (AIL (%)) after 30 days of growth of fungi *P. pulmonarius* and *P. chrysosporium*, respectively, versus the lignin content of the various starting materials (AIL).

Both for *P. pulmonarius* and *P. chrysosporium* a good correlation between the two lignin concentrations after 0 and 30 days of culturing at 24°C is observed (Figure 7). For both the fungi the curves are almost comparable, however, in relative terms *P. pulmonarius* seems to perform the best with respect to lignin degradation.

It is interesting to compare the data from the petri-dish assays with the results obtained in the first Food on Orbit Demonstration project. The data-point MC2000 generated by cultivation of *P. pulmonarius* in liquid MC medium is in line with the results obtained in the present project on petri-dishes, but also with the incubation in liquid cultures. The latter is presented by the black dot in the graph for both the fungi *P. pulmonarius* and *P. chrysosporium,* respectively.

Figure 7 indicates that within the lignin start concentration range between 20 to 60% it is possible to predict the end lignin concentration after 30 days of incubation. The initial lignin concentration of the EWC batch obtained from EPAS was too low to expect significant degradation of this fraction in terms of composition (%). The values that were determined from the lignin analysis were similar to the values as could be interpolated using the trendlines given in Figure 7 for both *P. pulmonarius* and *P. chrysosporium*.

3.3.4 Completion of the mass balances

In the closed system, as depicted in the Figures 1, 2 and 3, *P. pulmonarius P17* was grown at 20°C on six petri-dishes containing MELISSA cake as substrate. The set-up was selected from its possibility to control the gas conditions within the system and evaluate gas exchange processes, i.e. fungal respiration. This investigation has been used to assess the mass balance of the culturing process. On the one hand, the substrate composition has been determined before and after incubation for 1 month as in the previous paragraphs. On the other hand, the gas exchange within the system was measured in time.

3.3.4.1 Substrate conversion in the closed system

P. pulmonarius grew very thrivingly on the petri-dishes at 20°C. Table 11 shows the nutrient intake of *P. pulmonarius* from the MELISSA cake samples during the 29 days incubation period. In total, 3% of all solid nutrients was converted by the fungus, as calculated from the difference in solid content in the petri-dishes between the start/end time and sample masses.

Table 11: Substrate consumption levels on petri-dishes containing MELISSA cake by *P. pulmonarius* after incubation for 29 days at 20°C in the closed system.

T (days)	Strain	Substrate	Solid content (%)	Solid consumption (%)
0	-	MC agar	7.91	-
29	P17	MC agar	7.67	-3 %

The composition of the MELISSA cake is depicted in Table 12. After 29 days of culturing *P. pulmonarius* the content of hydrophobic components as extracted using EtOH +/- toluene decreased. The relative amount of water soluble components increased, probably due to biodegradation of polysugars and other constituents from the medium. The content of the lignin fraction did hardly change.

Table 12: Substrate composition (%) of the MELISSA cake medium after 0 and 29 days of cultivation of *P. pulmonarius* at 20°C in the closed system.

T	strain	EtOH/Tol +EtOH soluble compounds (%)	Water soluble compounds (%)	AIL* (%)	ASL** (%)	Total polysaccharides (%) *(glucose %)***
0	-	14	10	27	2	45 *(29)*
29	P17	8	25	28	1	42 *(29)*

* AIL = acid insoluble lignin, **ASL = acid soluble lignin, *** The percentage between brackets represents the fractions of glucose, which is the most abundant building block of the polysugar fraction as detected via GC analysis.

Table 13: Absolute changes in the substrate composition of the MELISSA cake after 29 days of incubation of *P. pulmonarius* at 20°C in the closed system. The absolute values were calculated from the solid contents and the original sample mass.

Sample	EtOHl/toluene + EtOH soluble compounds g (decrease %) [A]	Water soluble compounds g (decrease %)	AIL g (decrease %)	ASL g (decrease %)	Poly-saccharides [B] g (decrease %)	rhamnose g (decrease %)	arabinose g (decrease %)	xylose g (decrease %)	mannose g (decrease %)	galactose g (decrease %)	glucose g (decrease %)
t=0 MC agar	2.01	1.49	4.01	0.25	6.63	0.01	0.39	1.78	0.08	0.09	4.30
t=29 days 20°C *P pulm*	1.07 (47%)	3.31 (-125%)	3.73 (7.1%)	0.19 (24%)	5.61 (15.4%)	0.01 (0%)	0.21 (46%)	1.52 (15%)	0.01 (85%)	0.03 (63%)	3.83 (11%)

A) (Decrease (%), relative to amount of material in starting substrate; negative values represent increased amounts due to degradation of other constituents). B) The polysaccharide content comprehends the total of the following sugar constituents, i.e. rhamnose, arabinose, xylose, mannose, galactose, en glucose (last 6 columns).

In absolute terms (Table 13) the nutrient conversion data are comparable with the results presented in Table 12. The absolute mass reduction of the lignin fractions due to growth of *P. pulmonarius* are 7% and 24% for the AIL and ASL fraction, respectively. Overall, a reduction of 0.5 gram of biomass has been observed due to *P. pulmonarius* growth on the MELISSA substrate.

3.3.4.2 Fungal respiration in the closed system

CO_2 production due to growth and metabolism of *P. pulmonarius* in the cuvet on MELISSA cake was evaluated in time. This was done by regular measurements of the CO_2 production rate using the CO_2-detection unit that could be attached to the cuvet (Figure 3). However, no changes were detected in the CO_2 production rate within the system during the incubation period using this analyzer. The measuring unit seems to be hindered by the high humidity of the air in the system, which may interfere with the CO_2 readings. Accordingly, current approach did not yield reliable results. This implies that integrated respiration values were not available. The fungal respiration is not constant during growth.

The CO_2 which was produced throughout the incubation period is captured in the $Ba(OH)_2$ solution in the washing bottles. From the formation of solid $BaCO_3$ in these bottles the total accumulation could be assessed. During the first 8 days and the total incubation period of 28 days, the amounts of $BaCO_3$ that were formed represented 1.67 grams and 8.72 grams, respectively (Table 14).

Table 14: CO_2 accumulation measurements after 8 and 28 days of incubation of *P. pulmonarius* on MELISSA cake agar at 20°C in the closed system. Values are calculated from the conversion of carbondioxide and oxygen and the actual formation of $BaCO_3$.

	Conversion (nmol.s-1)	Used/produced In 8 days (mmol)	$BaCO_3$ (g) Predicted/ formed	Traced (%)	Used/produced In 28 days (mmol)	$BaCO_3$ (g) Predicted/ formed	Traced (%)
O_2	19.34	13.4	2.64 [A]	63	46.8	9.22 [A]	95
CO_2	14.24	9.8	1.94 [A]	86	34.5	6.79 [A]	128
			1.67 [B]			**8.72** [B]	

A) values were predicted from the O2 use and CO2 production rate (conversion nmol.s^{-1}). B) Actual accumulation of CO2 formed in time as weighed back from washing bottles.

As an additional check at the end of the incubation the CO_2 production and O_2 consumption levels were evaluated using gas chromatography (GC) analysis. During 4 hours the cuvet was closed and the CO_2 and O_2 concentrations after the incubation period were compared with the levels before closure. Subsequently, the gas exchange rates in the cuvet that could be established represented values of 14.24 nmol.s^{-1} for CO_2 and 19.34 nmol.s^{-1} for O_2 gas (Table 14).

These values are used to determine the respiration coefficient (CO_2/O_2) which corresponds to a value of 0.74. This coefficient may vary in time, as it represents the fungal metabolism and type of nutrients that are being utilized. If a readily available sugar as glucose is used, for instance, the coefficient is equal to 1 (1 mol of O_2 is needed to produce 1 mol of CO_2). Lignin breakdown, just like fat breakdown, will probably result in values lower than 1.

Based on the CO_2 production and O_2 consumption levels determined at the end of the incubation (single measurement), the $BaCO_3$ production levels could be predicted and compared with the real levels formed (Table 14).

In this manner, about 95% of the $BaCO_3$ formed throughout the incubation period of 28 days could be predicted based on the O_2 consumption. Based on the CO_2 production rate, more $BaCO_3$ was actually accumulated than predicted. The predicted values were based on a single measurement of the rates of oxygen consumed and carbondioxide produced at the end of the incubation. The values that were thus predicted after 8 days, the initial growth phase of the fungus, are overestimated as can be seen in Table 14. The GC analyses should regularly be repeated throughout the incubation period of the fungus to estimate the growth, nutrient use and may contribute to complete the mass balances.

Overall, we can conclude that combined the experimental set-up to detect CO_2 accumulation and the CO_2 and O_2 analyses via GC functions well. In this first test, based on O_2 consumption level the expected amount could almost completely be traced. Repeated measuring of both rates is needed to estimate the respiration coefficient in time, optimal nutrient composition and should be related to (bio)masses. To discriminate between substrate and fungal biomass, the ergosterol content should be determined. Combined the information can lead to actual completion of the mass balances of the degradation of the plant fibres from the MELISSA cycle.

3.4 Conclusions

Generally, we observed that both *P. pulmonarius* and *P. chrysosporium* are able to degrade lignins from plant origin. Both fungi significantly and approximately to the same extent decrease the lignin content of lignin-rich MELISSA cake and lignin-enriched plant substrates. Apparently, the degradation of the lignin fraction largely depends on the lignin start concentration in the substrate. Therefore, it is suggested that the fungal compartment should be placed after the first waste compartment to optimally use the fungal biodegradation capacity on lignin-rich material. It remains to be tested what the optimal nutrient composition is.

For lignin start concentrations ranging between 20 and 60% a more or less linear relation is observed to predict the extent of degradation from the end lignin concentration (See Figure 7). Especially, the fungal behaviour towards substrates that contain even higher concentration of lignin or fixed lignin/sugar ratios would be an interesting aspect to evaluate.

Both *P. pulmonarius* and *P. chrysosporium* grow well in mixed reactors in liquid media. This suggests that liquid state fermentation could be done to degrade the plant fibres. The use of liquid fermentation reactors offers the possibility to fully optimize the system and define optimal growth conditions. So far, it was not possible to discriminate between the lignin degradation efficiency at 24 and 37°C. Furthermore, no positive effect could be established from the addition of ferrulic acid as inducer of ligninolytic enzymes, and from the addition of perlite as inert solid support material.

The 'continuous' CO_2 measurements in the closed system may contribute to the completion of mass balances of the fungal metabolism. Total CO_2 accumulation in combination with regular detection of CO_2 production and O_2 consumption rates via gas chromatography gives valuable information in this respect. When the gas exchange data are related to (absolute) substrate conversion numbers, substrate composition and (bio)mass within the system, it will be possible to establish a predictive model. However, extensive measurements are needed to take into account all relevant parameters for generating and verifying such mathematical model.

Overall, the experiments performed within workpackage 7.3 contribute to a coherent picture regarding the behaviour and performance of white-rot fungi in degradation of fibrous material from

plant origin. The work presented here forms a good basis for further construction of the fungal compartment as a valuable tool and environmentally friendly unit within the MELISSA cycle.

3.5 Suggestions for further research

In future work it is important to focus on optimization of the fermentation process. In liquid reactors the white-rot fungi may be cultivated with the main goal to degrade lignin. Mass balances should be completed via continuous/regular measurements of all relevant parameters, and accordingly contribute to generate and validate a predictive model.

Parameters that are important to assess in this respect:

- CO_2/O_2 exchange rates using gas chromatography measurements (regular detection)
- CO_2 accumulation in the system
- Selection of fungal strain (So far, both the edible mushroom strain *P. pulmonarius* and the non-edible fungal strain *P. chrysosporium* performed well with respect to lignin degradation in lignin-rich media)
- Substrate consumption (absolute values)
- Substrate conversion (absolute values, but also further discrimination of several substrate fractions. The watersoluble components represent, for instance, proteins and peptides, sugars, ions and other small hydrophilic compounds.)
- Substrate composition (what is the most optimal composition to achieve highest lignin degradation by the fungi? What would be optimal sugar/lignin content for fungal growth and lignin degradation? See previous point)
- Growth conditions (temperature, stirring, gas conditions)
- Reactor selection and design
- Biomass monitoring (via the detection of ergosterol content or alternative secondary metabolites that are typical for fungi).
- Antibiotic production by the fungi. As a defense mechanism all (micro)organisms protect themselves from other microorganisms that may be harmful to them. Often they produce antibiotic substances to eliminate those invading microorganisms. It will be important to evaluate whether the selected fungi do produce such components, and if so, whether these accumulate and may cause problems for the different stages of the MELISSA cycle. Do they, for instance, interfere with the bacteria used in the first waste compartment.

When taking into account above aspects, a system may be developed that works and is under control. The measurements related to process optimization can be performed at Agrotechnology and Food Innovations B.V. of Wageningen University and Research Centre.

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