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	Name	Signature
Prepared by:	Ellen van Kan	
Approved by:	Dries Demey	

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Quantity	Company/Department	Name
1	EPAS	Veronik Hermans
1	EPAS	Dries Demey
1	ESA	Christophe Lasseur
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2.1 Introduction

About 20 different edible and non-edible mushroom strains were cultivated for screening of their potential ligninolytic activity on plant tissues and Melissa cake as substrate, as described in TN 6.1. Those included subspecies of *Agaricus bisporus*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Pleurotus eryngii*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Bjerkandera adusta*, and *Trametes versicolor*. Based on the activities of diverse extracellular enzymes that are produced and excreted by the fungi, the lignin degradation activity has been estimated via standardized *in-vitro* test systems.

In the various enzyme assays *Pleurotus pulmonarius P17* performed the best, especially in those detecting peroxidase activity, which may be related to lignin degradation. The activity of proteases, on the other hand, which may interfere with enzyme activity in general, is low. Therefore, *Pleurotus pulmonarius* was selected as potentially most effective lignin degrading strain. *P. pulmonarius* belongs to the class of white-rot fungi, that are able to degrade lignin and/or lignocellulose complexes

Within workpackage 7.2 the lignin degrading properties of the selected fungus have been evaluated on different substrates, containing the non-edible parts of plants in the absence and presence of the output of the waste compartment (Melissa cake, MC).

2.2 Material and Methods

2.2.1 Preparation of growth media for bag experiments

Different substrates were used to cultivate the fungus. The solid state fermentation reaction within the fungal compartment was simulated in plastic bags.

Two types of substrate bags were prepared. One containing only non-edible parts of wheat, red beet and lettuce plants (33 % each plant) and the other containing the same plant material (~30% each) in combination with MELISSA cake (~10%) obtained from EPAS (06/12/02).

Bags containing only plants (WBL bags): 10 g dry weight (dw) wheat straw chopped in pieces of 1 to 3 cm were homogeneously mixed with 10 g dw red beet pieces and 10 g dw lettuce powder. Tap water (142 ml) was added to the mixture and left overnight until the material absorbed the water. Wet material was transferred to autoclave bags of approx. 20 x 15 cm, sealed and placed in a water bath at 80°C for 1 h. After cooling down, substrates were inoculated with 20 g of *Pleurotus* spawn. Grains were homogeneously mixed with the substrate and the bag was sealed again. Holes were made with a needle covering the whole surface of the bag to promote air exchange needed for fungal growth.

Bags containing the plants and MELISSA cake (MC bags): 9.5 g dw chopped wheat straw were mixed with 9.5 g dw red beet pieces and 9.5 g dw lettuce powder. MELISSA cake was added (42 ml) and poured on the plant mixture. The MC bags contained 1.37 g (= 4.6%) of solids (MC batch 32.8 g solids/L) and 31.8% of each plant. Tap water (100 ml) was also added and the mixture was treated as described above.

2.2.2 Fungus and growth conditions

Pleurotus pulmonarius p17 was selected for evaluation of lignin degradation efficacy. The strain was grown on a petri-dish containing minimal medium P (MMP) agar. Spawn of *P. pulmonarius* was prepared by adding sterilized rye grains on the MMP plates. To inoculate the different media *P. pulmonarius* colonized grains were added.

Inoculated bags were placed at 24°C in the dark for almost 1 month. During the incubation the growth rate and fibre degradation rate were monitored. Samples were taken after 2, 14, 28 and 38 days of incubation. After incubation the fungal respiration and/or lignin degradation efficiency were determined (see paragraph 2.2.5 and 2.2.4, respectively). CO_2 production was measured immediately after sampling and the bags were stored at –20°C until further analysis of the substrate composition.

2.2.3 Methods for evaluating mycelial growth

The growth of the fungal mycelium was done visually by following mycelial colonization. A digital camera is used to visualize the fungi in various stages of their growth. After incubation parameters such as respiration measurements, lignin degradation, enzyme activities etc. give indications about the growth.

2.2.4 Methods for measuring substrate composition and lignin degradation

The freeze-dried colonized substrates (approx. 2-2.5 g) were 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 of the fibrous materials.

Before chemical analysis the material is first grinded in a Retsch cutting mill with a 0.5 mm sieve according to TAPPI method T257 cm-85. The samples are extracted twice in a Soxtec apparatus with organic solvents. Firstly 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 are derived from TAPPI method T264 om-82 and are done to remove proteins, waxes, fats, resins, tannins, gums, starches, free sugars and colouring materials (like chlorophyl etc.). The extracted materials are dried overnight at 60°C for quantification.

The extracted material is further used to determine the contents of lignin and polysaccharides, and might as well be used for determination of uronic acids (not determined). Hereto, the samples are hydrolysed with sulphuric acid (12 M, 1 h at 30°C and 1 M, 3 hrs at 100°C). The monosaccharides that are thus formed are reduced, acetylated, followed by measuring using gas chromatography (GLC). The acid insoluble lignin is determined gravimetrically and the acid soluble lignin in the hydrolysate is determined spectrophotometrically at 205 nm.

In the Appendix (section 2.7) an extensive protocol of the lignin degradation measurements is given.

2.2.5 CO_2 measurements

Pleurotus respiration rate in the substrate bags was measured as indirect recording of the fungal growth. The substrate was homogeneously mixed and placed in a special chamber to measure the CO_2 exchange during 5 min (Figure 1). The CO_2-laser (LI-COR, inc. LI6250 CO_2-analyser, Serie No: IRG620) 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 calculated 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.

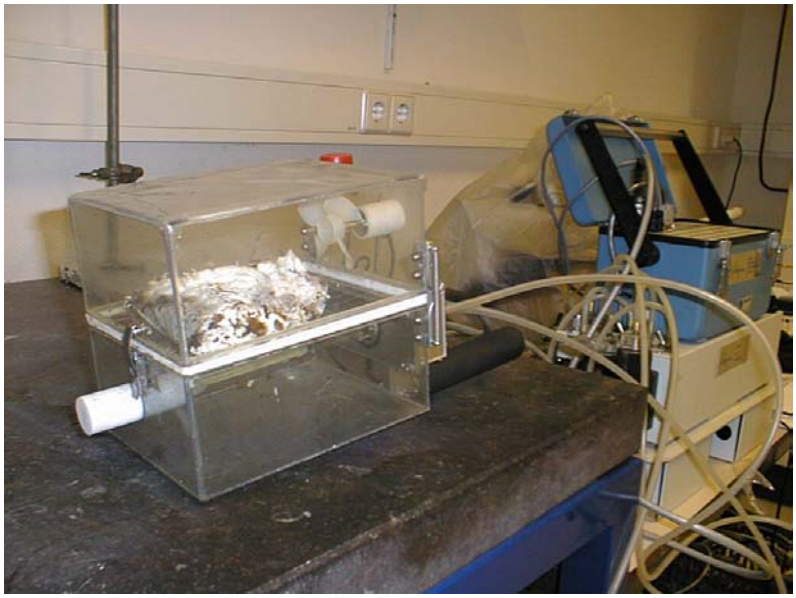

Figure 1: Experimental set-up to measure the fungal respiration rate.

2.2.6 Peroxidase activity measurements

Extracts of the substrates in bags were prepared by adding 10 ml (first extraction) and 5 ml (second extraction) demineralized water to ~1 g dry biomass. The suspensions were shaken for 30 min at room temperature and subsequently centrifuged for 10 min at 12500 rpm (Sorvall centrifuge).

Peroxidase activity (POD) was monitored as described by Castillo *et al* (1994). The substrate 3-(dimethylamino)benzoic acid (DMAB) in the presence of the enzyme, H_2O_2 and Mn interacts with 3-methyl-2-benzothiazolinone hydrazone (MBTH) to form a purple reaction product.

Laccase activity (Lac) in the extracts was measured using L-3,4-dihydroxyphenylalanine (L-Dopa) and MBTH as described by Espín *et al* (1997). The procedures used to determine the enzyme activities were the same as those used in Workpackage 7.1.

2.3 Results and Discussion

The behaviour of the fungus in the bags is described and discussed in current section. Possible changes in the substrate composition, focussing on lignin and polysaccharide content are monitored.

2.3.1 Fungal growth in bag experiments

After inoculation of the bag substrates *P. pulmonarius* mycelium was growing well and substrates were very soon fully colonized. This is visible in the WBL and MC bags in the Figures 2 and 3 after 14 days and 28 days of incubation, respectively. The *Pleurotus* mycelium is fully covering the surfaces of the substrate at day 14. The inside of the substrate is not completely colonized after 28 days of incubation as shown in Figure 3.

Figure 2: WBL and MC bags after 14 days of incubation at 24°C.

Figure 3: Inside of the A) MC and B) WBL substrates after 28 days of incubation at 24°C.

However, after 38 days of incubation the mycelium also covers the more internal parts of the substrates (Figure 4). However, it can be seen that degeneration processes start to take over the mycelial growth of Pleurotus. Also some contamination of other fungi is visible.

Figure 4: Detail of a substrate bag after 38 incubation days. In the upper left corner a grey fungus could be observed. *Pleurotus* mycelium started also to degenerate as observed in the lower left corner of the figure.

2.3.2 CO_2 measurements of fungi in substrate bags

Respiration data may represent the various stages of growth of the fungi. However, in these experiments respiration measurements did not follow the smooth profile of the mycelial growth as visually evaluated.

Respiration values indicated that the mycelium grew very fast at the beginning and when the second sample was taken (14 days) mycelium seemed to have started degenerating or fructification (Figure 5). In these stages generally the fungal respiration activities decrease and level off to constant values. Differences between WBL and MC seemed to indicate that mycelium was growing better on WBL than on MC substrate.

Large differences were found between the duplos (Fig. 5) when the CO_2 concentration was measured, in particular between the samples obtained at the second incubation day for the WBL control samples. After two days, the fungi in the WBL bags showed almost 7-fold higher respiration than in the MC bags. After 14 days these values were more similar and appeared almost constant during the 38 days of measuring.

Due to the poor reproducibility of the measuring points at day 2 and the need for a larger number of measuring points no definite conclusions can be drawn.

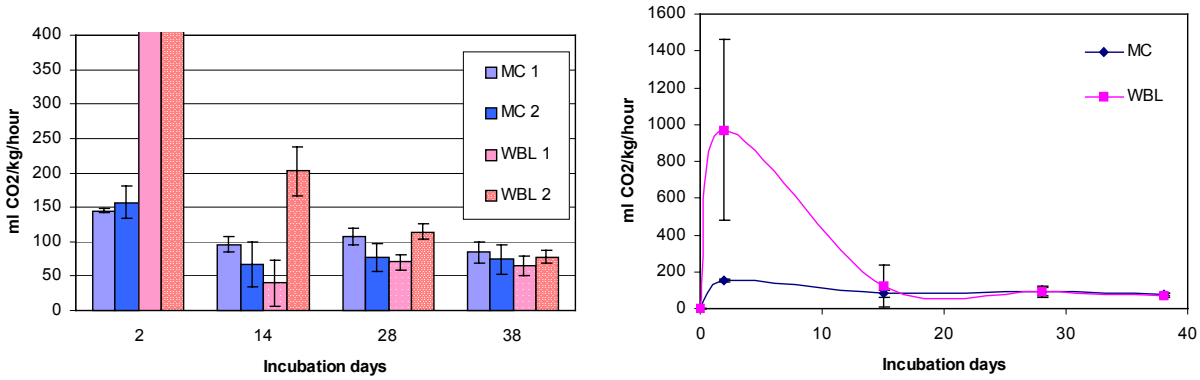

Figure 5: CO_2 concentration in WBL control bags and in MC bags. A) Differences between the duplos are shown. B) Differences between MC and WBL bags during the incubation time.

2.3.3 Quantification of lignin degradation in bags

In order to detect whether lignin is being degraded, contents of extractives, lignin, polysaccharides in the fibrous substrates were determined.

At first, the MC substrate bags were analysed according to the procedure described in paragraph 2.2.4 and Appendix (section 2.7).

Fibre analysis on the MC bags (Table 1 and 2) did not show significant differences in substrate composition due to *Pleurotus* growth. Both in the lignin content of the samples and in the sugar content of the samples no decrease is observed as a result of fungal growth. A slight decrease in glucose content, which indicates the cellulose content, may indicate some substrate conversion. This is also observed for readily available nutrients, represented by the EtOH +/- toluene and water extracted fractions.

Table 1: Extractives and acid soluble and insoluble lignin fractions in MC bags during *Pleurotus pulmonarius* growth.

Sample	Extractives		Lignin	
	ethanol/toluene + ethanol (%)	Water (%)	Acid Insoluble Lignin (%)	Acid Soluble Lignin (%)
MC 2 days bag 1	8	12	17	2
MC 2 days bag 2	9	12	16	2
MC 14 days bag 1	8	13	22	2
MC 14 days bag 2	9	16	20	2
MC 28 days bag 1	6	15	18	2
MC 28 days bag 2	7	13	21	2
MC 38 days bag 1	6	16	18	2
MC 38 days bag 2	7	14	20	2

Table 2: Sugar composition of MC substrate bags during *Pleurotus pulmonarius* growth.

Sample	Polysaccharide contents					
	rhamnose (%)	arabinose (%)	Xylose (%)	mannose (%)	galactose (%)	glucose (%)
MC 2 days bag 1	0.2	3	15	0.3	0.6	30
MC 2 days bag 2	0.2	3	14	0.3	0.7	28
MC 14 days bag 1	0.3	2	13	0.6	0.9	25
MC 14 days bag 2	0.2	2	14	0.6	0.7	25
MC 28 days bag 1	0.2	2	16	0.4	0.6	28
MC 28 days bag 2	0.2	2	14	0.5	0.8	25
MC 38 days bag 1	0.2	2	13	0.4	0.6	27
MC 38 days bag 2	0.3	3	14	0.4	0.6	25

No convincing lignin degradation could be established from determined substrate composition. Therefore it was decided to detect the enzyme activity in the sample bags, as an indirect measure of the potential to degrade fibrous material. The MC bag samples have been subjected to a screening for laccase and peroxidase activity, according to the methods mentioned in paragraph 2.2.6.

2.3.4 Peroxidase activities in the bags

In Figure 6 the outcome of the enzyme activity assays of the MC samples taken at several time points is presented. The laccase activities of the extracts are higher than the peroxidase activities. In comparison with the “time zero” samples (= 2 days), the enzyme activities increase in time. This is an “indirect” parameter to estimate the potential ligninolytic activity in

the fibrous samples. It is difficult to interpret the results, whereas they can't be compared with data of fungal growth on differently composed media.

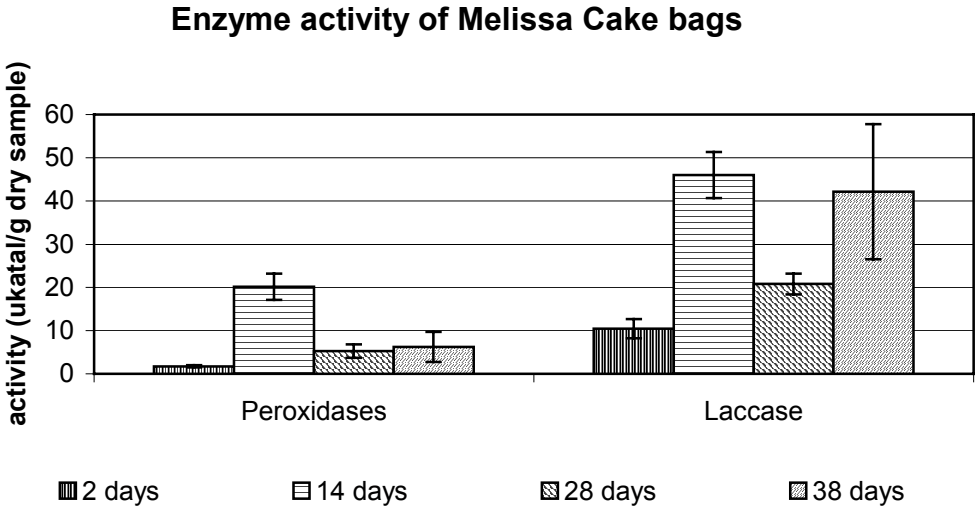

Figure 6: Peroxidase and laccase activity as measured in extracts of the dried samples in MC bags.

2.4 Concluding remarks

The substrate composition, especially the lignin contents of the media, did hardly alter when *P. pulmonarius* was grown on the plant substrate. Numerous arguments may be put forward for the low degree of lignin degradation due to the growth of *P. pulmonarius*. These may be related to the substrate as such, the substrate composition measurements or the experimental set-up.

In relation to the substrate, the surface of the solid material may not be accessible. The amount of substrate, i.e. 30 g of solids, was too high. Intermediate steps within the various project stages between the enzyme assays and the bag tests should have been evaluated.

The incubation time might have been too short, however, in the bag set-up this incubation could not be extended whereas degeneration processes prevailed around day 38 of the incubation period.

Substrate-wise low amounts of lignin (~17%) were detected. This could indicate that the substrate as such was too rich in readily available nutrients, like low molecular weight sugars, fatty acids etc. Alternatively, the lignin degradation measurements are not sensitive enough to detect changes in the substrate composition. However, for objective comparison of the analysis as used throughout the MELISSA project, it is decided not to change the procedure of evaluation.

The next experiments were carried out to verify the results obtained earlier with the *Pleurotus* species within FOOD-1 and were performed on a smaller scale in petri-dishes.

2.5 Suggestions for further research

The future experiments will contribute to finding a solid explanation of the above results. They will be carried out to verify the results obtained earlier with the *Pleurotus* species and quantify lignin degradation. As a reference, *Phanerochaete chrysosporium* is evaluated as well.

New experiments will be carried out on a smaller scale in petri-dishes using different substrates as starting material. These include lignin-enriched substrates which contain less readily available nutrients. Accordingly, lignins may be used by the fungi in an earlier growth stage.

Moreover, the effect of changing the growth conditions (e.g. temperature, activating reagents, addition of an inert carrier) and reactor medium (solid vs. liquid medium) will be investigated.

In a closed system it'll be focussed on completion of mass balances using continuous CO_2 measurements in combination with lignin degradation measurements.

2.6 Bibliography

Blumenkrantz, N., and Asboe-Hansen, G. Anal. Biochem., 1973, **54**, 484-489

Castillo, M. P., J. Stenström, et al. *Analytical Biochemistry,* 1994, **218**: 399-404.

Espín, J. C., M. Morales, et al. *Journal of Agricultural and Food Chemistry,* 1997, **45** (4): 1084-1090.

TAPPI Test Methods, handbook, 1996/1997, Atlanta (GA).

2.7 Appendix

Fibre determination

Samples were submitted to a combined method for determination of contents of extractives, lignin, polysaccharides and uronic acids in fibrous materials.

Before chemical analysis the material is first grinded in a Retsch cutting mill with a 0.5 mm sieve according to TAPPI method T257 cm-85. The powdered samples are 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 are derived from TAPPI method T264 om-82. The extracted materials are dried overnight at 60°C.

The extracted material is now ready to be used for determination of contents of lignin and polysaccharides and if desired uronic acids.

The samples are hydrolysed with sulphuric acid (12M, 1 hour at 30°C and 1M, 3 hours at 100°C). The formed monosaccharides are 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. Uronic acids in the hydrolysate can be determined spectrophotometrically according to the method described by Blumenkrantz et al.

Extractions

The following materials are needed to perform the extraction with a Soxtec apparatus:

Grinded fibrous material (< 0.5 mm particles)
Soxtec extraction unit HT6 with heating unit
Extraction cups (aluminium)
Extraction tubes (suitable for the Soxtec)
Tubes standard
Tubes holder
Tubes clamp
Cup holder
Analytical weighing device (resolution minimal 0.1 mg)
Stove 60°C and 105°C
Exsiccator
Fat-free cotton
Glass cooking beads
Toluene
Ethanol 96%
Demineralised water
Behrotest heater (including holder 24 tubes)
100 ml glass reaction tubes
Air coolers (according to DIN38409)
200 ml erlenmeyer flasks
Filter papers

Method:

1. Dry the extraction cups (including glass cooking beads) for 1 hour at 105°C.
2. Weight in accurately 1.5 – 2 g of sample into the extraction tubes (W1) (duplo extraction per material). Add a piece of cotton on top of the sample.
3. Also determine the dry weight content of the material.
4. Attach the tube holders on the extraction tubes and place these in the tube standard.
5. Put on the oilbath of the heating unit and the desired temperature to 200°C. Open the tap for the water-cooling.
6. Remove the extraction cups from the heating stove and place them in an exsiccator for cooling for half an hour. Weight the cups accurately (W2).
7. Add 45 ml ethanol (96%) into the cups and place the cups in the cup holder.
8. Switch the extraction units to “rinsing” and place the tube holder with the extraction tubes under the condensers.
9. Switch the extraction units to “boiling” and back to “rinsing” (the extraction tubes are placed upwards into the condensers).
10. Place the cup holder (with the extraction cups) under the condensers and push the black crank downward to fasten the cups.
11. Switch the extraction units to “boiling” and cook for 30 minutes.
12. Switch the extraction units to “rinsing” and flush for 75 minutes.
13. Close the taps of the condensers and evaporate the main part of solvent.
14. Press on “AIR” and open “EVAPORATION” to remove the rest of the solvent.
15. Close “EVAPORATION” and remove the cups and place them in a stove for 1 hour at 105°C.
16. Cool down the cups in an exsiccator and weight the cups (including the glass beads and the extracted material) (W3).
17. Place the tube standard under the condensers and switch the extraction units towards “boiling”. By turning the tubeholder a bit the tubes will be released.
18. Evaporate the remaining solvent in the tubes overnight in the suction hood.
19. Place a beaker under a condenser and open the tap and let the solvent flow out the condenser.
20. Close the cooling water tap and shut off “AIR” and “POWER”.
21. Put on the Behrotest heater to 105°C.
22. Place 200 ml erlenmeyer flasks in a stove for one hour at 105°C and determine weights after cooling down in an exsiccator.
23. Remove the entire extracted sample from the extraction tubes into 100 ml reaction tubes, add 50 ml demineralised water and place tubes in Behrotest heater.
24. Place air coolers on top of the reaction tubes and heat for one hour. Stir regularly.
25. Filter the dispersion over paper into a 200 ml erlenmeyer flask and wash the residue two times with boiling water. The residue should be dried in a stove at 60°C for 24 hour. The erlenmeyer flasks with the filtrates should be dried in a stove at 105°C until all water is evaporated. After cooling down in an exsiccator the flasks are weighted.
26. After cleaning the extraction tubes they can be used again for further extractions.

Calculation

$$\text{Extractives} = \frac{W3 - W2}{W1} \times 100\ \%$$

W3 = weight of cup+cooking beads or erlenmeyer flask + extractives (g)
W2 = weight of cup+cooking beads or erlenmeyer flask (g)
W1 = weight of sample (g as 100% dry matter)

Reference: Manual Soxtec System HT6, Tecator (Perstorp Analytical). Preparation of wood for chemical analysis, T264 om-88 (TAPPI 1996/1997)

Hydrolysis with sulphuric acid

Materials for hydrolysis with sulphuric acid:

72% sulphuric acid (= 12 M H_2SO_4)
Demineralised water
50 ml PP centrifugal tubes (Greiner)
Sealed Pasteur pipettes
G4 glass filter crucibles (dried at 105°C)
2 ml centrifugal tubes (Eppendorf)
Solution of monosaccharides (See E: Saccharides determination)
Solution of internal standard Inositol (See E: Saccharides determination)

Method:

1 Weight in 375 mg of sample into 50 ml PP-tubes (in triplo).
2 Add 3.75 ml 72 % sulphuric acid (cooling in ice bath).
3 Stir with sealed Pasteur pipettes.
4 Prehydrolyse at 30°C for 1 hour (stir every 15 minutes).
5 Add 36.25 ml demineralised water and carefully remove Pasteur pipettes without any sample).
6 Standard: Add 5 ml solution of monosaccharides.
Sample: Add 5 ml solution of internal standard (inositol).
7 Close tube and shake.
8 Hydrolyse in waterbath at 100°C during 3 hour (shake every half-hour).
9 Cool down in ice bath for 15 minutes.

Lignin determination

Method:

1 Filter over G4 glass filter crucible.
2 Separate filtrate into 4 Eppendorf centrifuge tubes (2 ml). (Samples are used for determination of acid soluble lignin, saccharides and uronic acids.)
3 Wash all residual lignin out of the PP tubes into the G4 crucible and wash until acid free (check with pH-indicator paper).
4 Dry crucible with residue overnight at 105°C, cool down in exsiccator and determine weight.
5 Acid soluble lignin is determined by spectrophotometry (UV absorption at 205 nm):
Dilute filtrate with 1M H_2SO_4 until absorption is between 0.1 to 0.8.
(Often a dilution of 10 or 20 times is sufficient.)
Do not forget: Measurements are done relative to a reference solution of 1M H_2SO_4.

Calculation acid insoluble lignin (AIL):

$$AIL = \frac{B - A}{C} \times 100\%$$

A = Weight glass filter crucible (g)
B = Weight glass filter crucible + residue after drying (g)
C = Weight sample before acid hydrolysis (g as 100% dry matter)

Calculation acid soluble lignin (ASL):

$$ASL = \frac{A * B * C}{D * E}$$

A = Absorption (relatively to 1 M H_2SO_4 at 205 nm)
B = Dilution factor
C = Volume filtrate (45 ml)
D = Extinction coefficient of lignin (110 g/l cm)
E = Weight sample before acid hydrolysis (g as 100% dry matter)

Reference: Acid-insoluble lignin in wood and pulp, T222 om-83 (TAPPI 1996/1997). Acid-soluble lignin in wood and pulp, UM-250 (TAPPI 1996/1997)

Saccharides determination

Materials:

10 ml glass tubes
Fresh (!) solution of 150 g/l sodiumborohydrid in 3 M NH_3 (0.224ml 25% NH_3 filled up to 1 ml and add 150 mg $NaBH_4$)
1-methylimidazole
Acetic acid
Acetic anhydride
Dichloromethane
Acetone

100 ml volume flask:	0.2 g rhamnose L(+) monohydraat (art.nr.7361 Merck)
(standard	0.4 g arabinose L(+) (art.nr.1494 Merck)
sugars	1.0 g xylose D(+) (art.nr.8689 Merck)
solution	0.2 g mannose D(+) (no.M-4625 Sigma)
in water)	0.2 g galactose D(+) (art.nr.4058 Merck)
	2.0 g glucose D(+) (art.nr.8342 Merck)
	1.5 g inositol (myo) (art.nr.4731 Merck)
100 ml volume flask :	1.5 g inositol (myo) (art.nr.4731 Merck)
(internal standard solution)	

Method

Reduction:

1 Prepare a new solution of sodiumborohydrid in 3M NH_3.
2 Add 1 ml hydrolysate and 0.2 ml to 0.4 ml of 25% NH_3 solution in a 10 ml glass tube and stir.
3 Check the pH of the solution (with pH indicator paper) and add extra 25% NH_3 solution if pH<7.
4 Add 0.1 ml solution of 150 g/l sodiumborohydrid in 3M NH_3 and stir.
5 Put all samples in a stove at 30°C for one hour.
6 Cool down in an icebath.
7 Add 0.05 ml to 0.1 ml of concentrated acetic acid and stir thoroughly.
8 If the solution keeps on boiling during stirring, repeat the addition of acetic acid.

Acetylation:

1 Put 0.3 ml of the solution in a glass tube.
2 Cool down in icebath.
3 Add 0.45 ml 1-methylimidazole and 3.0 ml acetic anhydride and stir.
4 Put the tubes in a stove at 30°C for 30 minutes.
5 Cool down in icebath.
6 Add 5 ml of water and 4 ml of dichloromethane.
7 Close tube (with teflon coated cap) and stir well (by hand).
8 Wait until phase separation is completed.
9 Remove upper layer with use of a Pasteur pipette and a vacuum pump.
10 Three times: Add 3 ml of water, stir, phase separate and remove upper layer.
11 Add 3 ml of water, stir, shortly centrifuge carefully (max. 1500 rpm).
12 Remove upper layer once more.
13 Evaporate the solvent (dichloromethane) by flushing air into the tubes.
14 Three times: add 1 ml of acetone, stir and evaporate.
15 Close tubes for preserving the samples (preferably at –10°C).
16 Before measurements on GLC: Dissolve samples in 0.4 ml of acetone.

GLC system:

GC apparatus

- Gaschromatograph: Hewlett-Packard HP8590
- Injector: Split / splitless injector
- Capillary column: CP-SIL 88 WCOT
  25 m x 0.25 mm ID X 0.2 µm df
- Carrier gas: Helium 5.0
- Detector: Flame ionisation detector
- Software: HP Chem Station (Rev.A.04.02.)

GC conditions

- Injection temperature: 300°C
- Injection volume: 1 µl
- Column pressure: 150 kPa (about 1.25 ml/min He)
- Splitflow: 60 ml/min
- Oven temperature: 220°C isocratic
- Runtime: 20 minutes
- Detector temperature: 300°C