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ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM SOIL ABLE TO LIVE ON LIGNIN AS A CARBON SOURCE AND TO PRODUCE ENZYMES WHICH CLEAVE THE β-O-4 BOND IN A LIGNIN MODEL COMPOUND

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Several strains of fungi were isolated and identified from Scandinavian soil using agar plates with lignin as a carbon source. The strains grew significantly faster on this medium than on control plates without lignin. Different types of technical lignins were used, some of which contained trace amounts of sugars, even if the increased growth rate seemed not related to the sugar content. Some strains were cultivated in shaking flask cultures with lignin as a carbon source, with lignin apparently consumed by microbes – while accumulation of the microorganism biomass occurred. The cell-free filtrates of these cultures could reduce the apparent molecular weights of lignosulphonates, while the culture filtrate of one strain could cleave the β-O-4 bond in a lignin model compound.

Keywords: lignin biodegradation, carbon source, soil microorganisms, extracellular enzymes, β-O-4 bond

INTRODUCTION

A considerable number of carbon atoms fixed through photosynthesis contribute to the cell walls of vascular plants. The most important components of these cell walls are the crystalline homoglucan cellulose, a group of amorphous and branched heteropolysaccharides, hemicelluloses/pectins, and the aromatic polymer lignin.1 These polymers belong to the most abundant materials of the biosphere, and their biodegradation is therefore central in the global circulation of carbon. There exist several classes of microorganisms, filamentous fungi and bacteria included, specialized in degrading the cell walls of the dead and, in some cases, of the living plants, as well. The mechanisms may vary but, in many cases, degradation is carried out by microorganisms excreting several classes of enzymes with diverse substrate specificities.

These degrade the wood polymers into water-soluble and energy-rich, low-molar mass compounds, such as sugars, that are imported into the microbial cells, where they are used as carbon and energy sources.2

Cellulose and hemicelluloses are mainly degraded by hydrolytic enzymes,3,4 although the oxidative enzymes may be involved at least in cellulose biodegradation.5,6 Pectin-degrading enzymes are somewhat different, lyases playing an important role in processes of addition to hydrolases.7 8 This might be due to the fact that the content of uronic acids in the polymer allows depolymerization by β–elimination. In many

cases, these enzymes, divided into endo-, exo- and (for hemicellulases/pectinases) debranching enzymes, have very high specificity. However, there are also individual enzymes that efficiently attack more than one wood polysaccharide. The products of degradation are well-defined mono- and disaccharides, energy-rich and easy to utilize for microorganisms. However, lignin represents a challenge for enzymatic degradations, for several reasons; first, it has a partially random structure that is racemic and contains several types of ethers and carbon-carbon bonds, which makes more difficult its degradation by specific enzymes. Secondly, lignin makes wood very compact by covalently crosslinking different polysaccharides, so that the molecules with sizes similar to proteins cannot diffuse into the cell wall. Therefore, the wood-degrading organisms have developed a strategy according to which a low molecular weight mediator which, by oxidation, is converted into a reactive form, can penetrate the cell wall, and therefore perform lignin oxidations, which lead to depolymerization (Fig. 1). The described extracted lignin-degrading systems are based on peroxidases, oxidases and dehydrogenases, the reactive species generated including Mn (III), hydroxyl and organic radicals.

![Figure 1: Principle of enzymatic lignin degradation by wood-degrading fungi: a) A lignin-modifying peroxidase oxidizes an inactive form of a redox mediator (i) to an active form (a); b) The active redox mediator can penetrate sterical barriers performing an unspecific lignin oxidation; c) The lignin gets fragmented in uncatalyzed reactions following oxidation. The redox mediator, now in inactive form, diffuses back to the lignin-modifying peroxidase for a new cycle.](image)

All these systems are, however, essentially unspecific and the degradation products are oxidized with relatively low enthalpy as, actually, when burned, native lignin generates more energy than the carbohydrate. Generally, it is believed that the wood-degrading organisms do not degrade lignin for utilizing the degradation products, but for uncovering the polysaccharides for degradation: also, it has been asserted that no microorganism lives solely on lignin.
However, there are differences in the severity of lignin degradation by the different functional classes of microorganisms; white rot fungi and certain types of wood-degrading bacteria degrade lignin totally to carbon dioxide and other low molecular weight compounds, whereas brown rot and soft rot fungi, depolymerize lignin only partially and chemically modify it, with concomitant formation of a brown color. The structural modifications observed in lignin after the attack of brown rot fungi are fundamental, yet the modified lignin is still polymeric and retains its aromatic structures. Brown rot fungi and probably also soft rot fungi produce therefore a polymeric residue lignin, which is believed to be the main source of the organic compound in the humus soil. A large amount of humus, which is relatively energy-rich, is therefore produced annually. Therefore, it is likely that a class of soil microorganisms that use humus as an energy and carbon source, especially since the lignin-type structures in humus appear degraded in nature, has been developed.

Lignolytic organisms from soil have been studied by several research groups. In many cases, oxidative enzymes similar to the ones occurring in wood-degrading fungi have been described. However, there have been also reported lignolytic enzymes that cleave the β-O-4 bonds by combinations of reduction and oxidation processes. Furthermore, reported an etherase able to hydrolyze the β-O-4 bond. This, however, is the first report on the cultivation of microbes under such conditions.

**EXPERIMENTAL**

**Materials**

Agar for microbiological use was purchased from MERCK Company. Lignosulphonate DP 401 was obtained from Boregaard Company, Sarpsborg, Norway, soda-pulp flax lignin – from Granit Company, Graz, Austria, and leonardite – from the International Humic Substances Society, St. Paul, Minnesota, USA. Lignoboost lignin was a kind gift of Professor Hans Theliander, Wallenberg Wood Science Center, Chalmers, Gothenburg, Sweden. Guaiacylglycerol β-O-guaiacyl was received from TCI Europe N.V. All other chemicals were of analytical grade.

** Cultivation media**

The cultivation media used were a modified version of the basic element medium, both the selection and cultivation media containing lignin as the only carbon source. Each liter of selected medium contained 10 g of lignosulphonate DP 401 or leonardite or soda-pulp flax lignin, 0.25 g KH₂PO₄, 1.0 g NH₄NO₃, 1.0 g CaCl₂, 0.25 g MgSO₄·7H₂O, 1.0 mg FeSO₄, and 15 g agar. Each liter of cultivation medium contained 10 g of soda-pulp flax lignin or lignosulphonate DP 401 or Lignoboost lignin, 0.25 g KH₂PO₄, 1.0 g NH₄NO₃, 1.0 g CaCl₂, 0.25 g MgSO₄·7H₂O, 1.0 mg FeSO₄. The pH of the medium was adjusted to 5.0. The medium was used for shaking flask experiments. Lignoboost lignin medium was performed as a suspension of insoluble carbon source medium.

**Soil samples**

Soil samples were collected from 5 different locations in the Lilljanskogen forest (59°20’60”E, 18°4’38”N), close to Central Stockholm, Sweden: “waterside”, i.e. from the sediment of a small water flow; “grassland”, i.e. from a meadow soil; “brown rotten stump”, i.e. from a stump of a Norway spruce (Picea abies) heavily degraded by brown rot; “hardwood forest”, i.e. soil from the forest area dominated by aspen (Populus tremula) and other hardwoods, and “spruce forest”, i.e. soil from the area dominated by Norway spruce (Picea abies). Soil samples were taken from approximately 1 dm depth.

**Isolation and identification of microorganisms**

Isolation and purification of microorganisms

1 g of each soil sample was suspended in 9 mL Milli-Q water. 1 mL 10⁻² or 10⁻³ dilutions of soil suspension of the 5 different soil samples were placed on different lignin agar plates. The plates were incubated for 4 to 7 days at room temperature until colonies appeared. Strains were purified by reinoculation of hyphen tips or cell colonies. When the microorganism appeared homogeneous, it was reinoculated for 3 times onto new plates. The strains were considered pure.

**Microorganism cultivation**

In a subsequent stage, the microbes were inoculated into 50 mL lignosulphonate DP 401 cultivation medium shaking flasks, and cultivated at 24 °C for 10 days, with continuous agitation of 150 rpm. Culture filtrates of five fast growing strains and control shaking flask media without strains inoculation were
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SEC analyzed, to confirm lignin consumption by the microbes. The same 5 strains were subsequently inoculated into a 50 mL soda-pulp flax lignin cultivation medium and a 50 mL lignoboost lignin cultivation medium.

**Strain identification**

Strain identification was performed by ribosome sequencing using Finnzymes’ Phire® Plant Direct PCR Kit. One cm² mycelium of each strain cultivated in lignosulphonate DP_401 medium was washed by Milli-Q water, 3 times, before PCR procedures, for amplifying the nuclear ribosomal DNA of the strains.

**PCR procedures**

ITS1 (5’-TCCGTAGGGTAAACCTTGCGG-3’) and ITS2 (5’-GCTGCGTTCTTCATCGATGC-3’) primers, targeting the conversed ribosomal regions of 18 s and 5.8 s, were used for PCR amplification. The reactions were performed in 50 µL. Parts of the washed mycelia (around 4-6 mm²) of the different above-mentioned strains were heated in 25 µL 2x Phire Plant PCR Buffer at 98 °C for 10 min, after which a mixture of 22 µL distilled water, 1 µL of each primer (10 µM) and 1 µL of the Phire Hot Start II DNA Polymerase was added to each sample, and PCR was performed using the following program: initial denaturation at 98 °C for 5 min; 30 cycles of 98 °C for 20 s, 54-60 °C for 30 s (every 2 cycles, the temperature being increased with 1 °C), 72 °C for 20 s; final incubation at 72 °C for 1 min. The PCR products were separated by agarose gel electrophoresis, purified with the QIAquick gel extraction kit (QIAGEN; Stockholm, Sweden) and sent for sequencing. The sequencing results were blasted in Genbank and the highest hits were selected for identification.

**Degradation of lignosulphonate with cell-free culture filtrate**

The five fastest growing strains were selected for lignin depolymerization assay. Microbes were inoculated into 50 mL soda-pulp flax lignin cultivation medium shaking flasks, and cultivated at 24 °C, with continuous agitation of 150 rpm. After 10 days of cultivation, the supernatant was collected by centrifuging down the cells at 4000 rpm for 15 min. The supernatant was considered as a cell-free culture filtrate. 500 µL cell-free culture filtrates were incubated with 400 µL 50 mM sodium acetate buffer, pH 5 and 100 µL lignosulphonate (50 g L⁻¹) overnight, after which the mixture was run on SEC to detect the depolymerization. The control experiment was done by incubating the same amount of lignosulphonate with 500 µL soda-pulp flax lignin cultivation medium.

**Size exclusion chromatography**

An alkaline SEC system was constructed with a Rheodyne 7725 Manual Injector equipped with 20 µL sample loop. Waters 515 HPLC pump, operated at a flow rate of 1 mL min⁻¹. The columns were 3 Tosoh TSKgel Columns (G3000PW-G4000PW-G3000PW) and a TSKgel Guard Column (PWL 7.5 cm x 7.5 mm), and the detectors were Waters 2487 dual λ Detector, operated at 254 and 280 nm, and Waters 410 Refractive Index Detector. 10 mM NaOH in Milli-Q H₂O was used as a mobile phase. A 20 µL injection volume was used for each sample analysis.

**Incubation of β-O-4 bond lignin model compound with cell-free culture filtrate**

The same five fastest growing strains were selected for a lignin degradation assay, by using a lignin model compound with β-O-4 ether linkage, namely guaiacylglycerol β-O-guaiacyl (Fig. 2). Microbes were inoculated into 50 mL lignoboost lignin cultivation medium shaking flasks, and cultivated at 24 °C, with continuous agitation of 150 rpm. The strain selected for the lignin degradation assay was later on identified as *Penicillium thomii*. The lignin-degrading assay was performed by incubating 500 µL cell-free culture filtrates of *Penicillium thomii* after 14 cultivation days with 400 µL 50 mM sodium acetate buffer, pH 5 and 100 µL lignin model compound solution (0.5 g L⁻¹ guaiacylglycerol β-O-guaiacyl in DMSO) at 24 °C, overnight. Control experiments were done by adding no culture filtrate as an enzyme blank and also by adding only the culture filtrate as a substrate blank.

**Gas chromatography-Mass spectrometry**

The degrading products were extracted three times with 200 µL ethyl acetate, and then evaporated to dryness. The residues were redissolved in 30 µL ethyl acetate. 10 µL of the product were treated with 10 µL pyridine and 50 µL of N,O-bis(trimethylsilyl), to
prepare trimethylsilyl derivatives. Further on, 2 μL of the derivatives solution was injected by gas chromatography and by a GC-MS device. GC analysis was carried out with a Hewlett-Packard 6890 instrument, equipped with a DB 5MS J&W Scientific column (30 m, 0.32 um I.D., 0.25 um film thickness). Helium was used as carrier gas at a flow rate of 0.9 mL min⁻¹. Oven temperature was raised at 5 °C min⁻¹ and from 50 to 300 °C. The eluent was detected by a flame ionization detector. MS analysis was carried out on a Thermoquest Finnigan Trace GC-MS device (San José, CA, USA), series 2000, operated at 70 Ev, with a flow of 1.5 mL min⁻¹ helium as carrier gas. A RTX-5MS column (Restek, Bellefonte, PA, USA) was used (30 m, 0.32 mm ID, 0.25 μm film thickness). The injector, the interface and the ion source (electric ionization) were maintained at 280 °C. Split injection with a flow of 30 mL min⁻¹ and a ratio of 20 was used for all samples. Oven temperature was raised from 50 to 300 °C, at a rate of 5 °C min⁻¹.

RESULTS AND DISCUSSION

Isolation and purification of lignin-utilizing microorganisms

Technical lignins are preferred to DHP as they are available in large quantity, which recommends them for subsequent industrial and technical objectives of enzyme production. To isolate organisms that might grow on lignin, 10⁻² and 10⁻³ dilutions of soil suspension, collected from 5 different locations in the Lilljanskogen forest (59°20'60"E, 18°4'38"N) near Stockholm, Sweden, were incubated on agar plates, the only carbon source being lignin of different qualities. Within 4 days, a visible growth of several strains of microbes, both filamentous and bacterial-like, was obtained from all 5 soil samples and 3 lignin qualities tested, which indicated the presence of microorganisms, which could utilize lignin as a carbon source; however, to exclude possible artifacts, for example, contaminations of carbohydrate in the lignin materials, some control experiments were performed. The purity of lignin is a critical factor and therefore carbohydrate analysis of the materials should be performed. The results obtained indicated that the sugar content was below 1% or insignificant in all lignin samples (Table 1). Microorganisms were purified by three subsequent re-inoculations, out of the total number of 21 purified strains, 11 of them being identified (Table 2).

<table>
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<tr>
<th>Lignin</th>
<th>Mass percent (%)</th>
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<td>Xylose</td>
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<td>0</td>
<td>0.01</td>
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<table>
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<tr>
<th>Name</th>
<th>Isolated from</th>
<th>Description</th>
<th>Classification</th>
<th>NCBI Taxonomy ID</th>
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<td>Phoma herbarum</td>
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<td>White, filamentous; no sporulation</td>
<td>Ascomycota</td>
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</tr>
<tr>
<td>Penicillium canescens</td>
<td>Hardwood forest</td>
<td>White, filamentous; white spores</td>
<td>Ascomycota</td>
<td>5083</td>
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<tr>
<td>Penicillium daleae</td>
<td>Hardwood forest</td>
<td>Heavy white filamentous; spores</td>
<td>Ascomycota</td>
<td>63821</td>
</tr>
<tr>
<td>Hypocrea pachybasioides</td>
<td>Spruce forest</td>
<td>Long, white, filamentous; spores</td>
<td>Ascomycota</td>
<td>40695</td>
</tr>
<tr>
<td>Penicillium thomii</td>
<td>Spruce forest</td>
<td>Transparent, filamentous; grey spores</td>
<td>Ascomycota</td>
<td>36647</td>
</tr>
<tr>
<td>Trichoderma asperellum</td>
<td>Spruce forest</td>
<td>Long, white, filamentous; green spores</td>
<td>Ascomycota</td>
<td>101201</td>
</tr>
<tr>
<td>Cylindrocarpon didymum</td>
<td>Bottom of a small stream</td>
<td>Heavy white, filamentous; spores</td>
<td>Ascomycota</td>
<td>109805</td>
</tr>
</tbody>
</table>
**Davidiella tassiana**  
Bottom of a small stream  
Green, filamentous; green spores  
Ascomycota  
29918

**Phoma macrostoma**  
Bottom of a small stream  
Transparent, filamentous  
Ascomycota  
73002

**Sphaerulina polyspora**  
Brown rotted wood from spruce  
Green, filamentous; green spores  
Ascomycota  
237180

**Cryptococcus podzolicus**  
Brown rotted wood from spruce  
Brown, bacterial-like  
Basidiomycota  
89927

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Figure 3: Comparison between lignin agar plate and control agar plates: *Cylindrocarpon didymium* after 4 day cultivation at room temperature on lignosulfonate and control plates

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Figure 4: SEC chromatogram of compared culture filtrates after 12 day cultivation and control shaking flask medium before strain inoculation (Solid line – control experiment of control shaking flask medium before strains inoculation; dash line – culture filtrates after 12 day cultivation). The latest perfect overlapping peaks on both the solid and dash line contribute to the exactly same amount of vanillin as the internal standard. The earlier peaks contribute to lignosulphonate. The chromatogram shows that the earlier solid peak has been consumed, which confirms that the lignosulphonate had been consumed by the growing strains. All 5 strains’ culture filtrates gave more or less similar lignosulphonate-consuming results.
The conclusion is that several strains could grow on lignin agar plates. To investigate whether the microbes utilize lignin or sugar contaminants in the material or agar, agar plates were prepared without any added carbon source, and with a sugar content similar to the trace contaminants found in lignosulphonates DP_401 (0.71% xylose of 10% carbon source, and a total amount of 0.071% xylose in the cultivation medium). Lignosulphonates DP_401 were also tested by size exclusion chromatography, which indicated the absence of low molecular mass aromatics. Some of the isolated strains could grow on these media, but significantly slower than on lignin agar plates (e.g., Fig. 3). For example, the strain *Cylindrocarpon didymum* grown on lignosulphonate plates had a visible colony color and a rhizoid form. Compared to the colony grown on control plates, where only a transparent mycelium appeared, the strain grown on the lignosulphonate plate was much larger. After 4 days of cultivation, the diameter of the colony grown on the lignosulphonate plate was 2.2 cm, the colony grown on the plate with 0.071% xylose, but no lignin, had a diameter of 1.5 cm, while the one grown on a blank agar plate had a diameter of only 1.3 cm.

It is very unlikely that a content of 0.071% total sugar is sufficient for microorganisms to grow on, as a carbon source. The faster growing of the strains on lignin plates, comparatively with those grown on the control plates used in the experiment, supports the idea that the isolated microorganisms can use lignin as a carbon source, while a subsequent shaking flask cultivation experiment excluded the possibility of using agar as a carbon source. The 5 strains with the fastest growth on lignin agar plates were cultivated in 50 mL shaking flask cultures using the same medium with lignosulphonate DP_401 as a sole carbon source as the one used in the agar plates (except for agar). After 12 days of cultivation, the cultures of the 5 strains were analyzed by SEC to see whether lignin had been consumed by microorganisms during growth. The results evidenced lignin consumption (Fig. 4). In time, creation of mycelia could be observed (Fig. 5). After 12 days of cultivation, the biomass of one of the five strains measured by dry weight was of 0.7 g, which further supports the hypothesis that these microorganisms could live on lignin as carbon sources.

**Identification of isolates**

Eleven strains were identified by ribosome sequencing using Finnzymes’ Phire Plant Direct PCR Kit (Table 2 and Appendix). Ten strains were ascomycetes, and one, the yeast, was a basidiomycete. This dominance for ascomycetes is interesting, since, up to now, efficient lignin degradation has been associated mainly with basidomycetic white rot fungi. To the best of our knowledge, none of the strains had been previously associated with lignin biodegradation, with the exception of *Penicillium thomii*. This organism has been reported as able to chemically modify kraft lignin.

**Lignin degradation by extracellular enzymes**

A question still to be answered is whether microorganisms use extracellular enzymes for degrading lignin into smaller molecules, to be subsequently introduced into the cell and catabolized, in a similar way as white rot and soft rot fungi degrade cellulose by extracellular enzymes. To investigate this possibility, cell-free culture filtrates of the isolated strains of *Phoma herbarum*, *Cryptococcus podzolicus*, *Sphaerulina polyspora*, *Penicillium thomii* and *Davidiella tassiana*, cultivated on soda-pulp flax lignin medium, were incubated with solutions of lignosulphonate. SEC characterization of the...
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reactions (Fig. 6) indicated that some depolymerization of the lignosulphonate had occurred, which agrees with the theory according to which extracellular enzymes capable of depolymerizing lignin were produced.

To investigate whether the β-O-4 bond was the most common intermonolignol bond in lignin cleaved by extracellular enzymes, a culture filtrate from Penicillium thomii was incubated with a model compound, guaiacylglycerol β-O-guaiacyl (Fig. 2). The reaction products were investigated with GC-MS, and two new peaks were created in the resulting spectrum (Fig. 7). The substrate and enzyme blank control products were run on GC. Only solvent peaks appeared for the substrate control reaction, and only solvent and substrate peaks appeared for the enzyme blank control reaction. This result showed that the two new peaks were neither the contamination of the module compound, nor the metabolism products of the strain culture, being rather similar to the degradation products. The silylated derivatives of the newly formed products and their secondary fragmentation products were represented on their respective mass spectrum (Fig. 7). Thus, it can be concluded that the β-O-4 ether was cleaved, as well as the methanol ether. Also, the aromatic rings seem to have been hydroxylated in paraposition (Fig. 7). The culture filtrate from Penicillium thomii was also tested for further enzyme activity assays, including laccase and Mn peroxidase activity. Nevertheless, none of these activities was detectable.

Figure 6: GPC data indicating Cryptococcus podzolicus cell-free culture filtrate was able to cause lignin depolymerization (solid line – control experiment; dash line – incubation reaction of 5 mg lignosulphonate with Cryptococcus podzolicus cell-free culture filtrate overnight). The first peak on the dash line shifted to a longer retention time and the later two dash peaks accumulated into the higher area show that the Cryptococcus podzolicus cell-free culture filtrate could depolymerize higher molecular weight lignosulphonate into a lower molecular weight one. All 5 strains culture filtrates showed more or less similar lignosulphonate depolymerizing activity

Figure 7: GC spectrum of lignin β-O-4 linkage model compound, guaiacylglycerol β-O-guaiacyl after incubation overnight with cell-free culture filtrates of Penicillium thomii

a) GC spectrum of lignin β-O-4 linkage model compound, guaiacylglycerol β-O-guaiacyl after incubation overnight with cell-free culture filtrates of Penicillium thomii
Figure 7: GC-MS analysis of TMS-derivatives of degradation products from guaiacylglycerol β-O-guaiacyl by Penicillium thomii cell-free culture filtrates: (a) GC chromatogram of reaction products of guaiacylglycerol β-O-guaiacyl after incubation overnight with cell-free culture filtrates of Penicillium thomii; (b), (c) and (d) mass spectra of peaks a, b and c in (a) respectively.

What types of reaction can produce such effects? Hydrolytic etherases similar to the one discovered by Otsuka et al. may explain both the cleaved bonds\(^\text{32}\) and the depolymerization of lignosulphonate, but not hydroxylation (Fig. 8). Hydroxyl radical generating enzymes, similar to cellobiose dehydrogenase, may explain the different reaction products (Fig. 8)\(^\text{40}\) while, on the other hand, hydroxyl radicals are expected to cause polymerization of lignosulphonate rather than depolymerization,\(^\text{41}\) and also to polymerize the model compound. On the other hand, the reaction products do not fit the pathways suggested by Reiter et al.\(^\text{31}\) and Masai et al.\(^\text{30}\). These reactions require glutathione and NAD\(^+\), and may be more important in intracellular lignin biodegradation. Of course, several different types of enzymes might be involved in degradation, and further work is necessary to fully understand such reactions. Lignin-degrading enzymes from wood-degrading fungi, e.g. white rot fungi – cellobiose dehydrogenase, manganese peroxidase, etc. –
generally need cosubstrates/cofactors, such as cellobiose, hydrogen peroxide, veratryl alcohol, Mn (III), etc. Since depolymerization and degradation of the module compound occurred without any addition of cofactors, and usually, oxidative enzymes, such as laccase, cause the polymerization of lignosulphonate under the same conditions as those applied in the present experiment,42 it appears more probable that the enzymes responsible for depolymerization and degradation are β-etherase or hydroxyl radical generating enzymes. Whichever the enzyme that performs the degradation of the reaction products, the different types of phenols are relatively energy-rich and may serve as a carbon source for microorganisms.43 Therefore, the microorganisms isolated in this study might degrade humus lignin to low molecular weight phenolic compounds, which are utilized as carbon and energy sources. Other works on the identification of the extracellular enzymes responsible for lignin degradation are in progress.

Figure 8: Hypothetical reaction schemes for enzymatic degradation of the guaiacylglycerol β-O-guaiacyl model compound30-32,40,41

CONCLUSIONS
Several strains of microorganisms were isolated from soil samples, including both filamentous fungi and yeast. To the best of our knowledge, this is the first demonstration that such microorganisms can live on lignin. The literature of the field mentions only Penicillium thomii as capable of contributing to the transformation of kraft lignin.37 Up to now, the other strains identified in the present study have not been associated with lignin degradation. Since many strains were isolated from different forest locations, these types of microorganisms are probably rather common in the soil, and their role in the carbon circulation may be to degrade residual lignin from brown rot and soft rot fungi. At least some of the microbes seem to use extracellular enzymes to cleave the lignin β-O-4 bond. These might be of similar type as the above-described β-etherase or the hydroxyl radical generating enzymes. Such types of enzymes might be very interesting for technical applications, since they do not need cofactors, and may also have high specificity, in contrast to lignin-modifying peroxidases from white rot fungi.
ACKNOWLEDGEMENTS: This work was supported by Wallenberg Wood Science Centre (WWSC). Thanks are addressed to Professor Lisbeth Ohlsson, University of Chalmers, Gothenburg, Sweden, for fruitful discussions.

REFERENCES
APPENDIX:
Sequencing and alignment result for each strain identification:
1. **dbj|AB470824.1| Phoma herbarum** genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, isolate: TS08-6-3
   Length = 542
   Score = 183 bits (99), Expect = 1e-43
   Identities = 101/102 (99%), Gaps = 0/102 (0%)
   Strand = Plus/Plus
   Query 1 TTTATGAGCACTTACGTTTCCTCGGGGGGTTCGCCCACCGATTGGACCACCTAAACCCTT 60
   Sbjct 72 TTTATGAGCACTTACGTTTCCTCGGTGGGTTCGCCCACCGATTGGACCACCTAAACCCTT 131
   Query 61 TGCAGTTTTGTAATCAGCGTCTGAAAAACTTAATAGTTACAA 102
   Sbjct 132 TGCAGTTTTGTAATCAGCGTCTGAAAAACTTAATAGTTACAA 173

2. **gb|FJ025212.1| Penicillium canescens** strain QLF83 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
   Length = 554
   Score = 368 bits (199), Expect = 6e-99
   Identities = 199/199 (100%), Gaps = 0/199 (0%)
   Strand = Plus/Plus
   Query 1 CACCTCCCACCCGTGTTTATTGTACCTTGTTGCTTCggcgggcccgcctcacggccgccg 60
   Sbjct 25 CACCTCCCACCCGTGTTTATTGTACCTTGTTGCTTCGGCGGGCCCGCCTACCGGCGCG 84
   Query 59 gggggcatctgceccgceccgggceccgggcAgagaACACCTTGAACCTGTGATGAAAATT 120
   Sbjct 85 GGGGGCATCTGCCCCCGGGCCCGCGCGCCCGGAGACACCTTGAACCTGTGATGAAAATT 144
   Query 121 GCAGTCTGAGTCTAAATATAAATTATTTAAAACTTTCAACAACGGGATCTCTTGGTCCGG 180
   Sbjct 145 GCAGTCTGAGTCTAAATATAAATTATTTAAAACTTTCAACAACGGGATCTCTTGGTCCGG 204
   Query 181 CATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAA 238
   Sbjct 205 CATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAA 223

3. **emb|AJ850133.1| Penicillium daleae** 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2, 26S rRNA gene (partial), type strain CBS211.28T
   Length = 568
   Score = 944 bits (511), Expect = 0.0
   Identities = 521/525 (99%), Gaps = 3/525 (0%)
   Strand = Plus/Plus
   Query 1 TCTGGGT-CACTCCCCACCGTGTTTATCGTACCTGTTGGTTTcgccggccggccgectca 58
   Sbjct 45 TCTGGGTCCAACCTCCCCACCGTGTTTATCGTACCTGTTGGTTTGGGGGCGGGCGGCGCTCA 104
   Query 59 ggggcatctgceccgceccgggceccgggcAgagaACACCTTGAACCTGTGATGAAAATT 120
   Sbjct 105 GGGGGCATCTGCCCCCGGGCCCGCGCGCCCGGAGACACCTTGAACCTGTGATGAAAATT 144
   Query 119 TCTGAAGATTTGGATCGTGACATCTTTAGCTAAATCGAGTTAAAAACTTTCAACAACGGGATCT 178
   Sbjct 165 TCTGAAGATTTGGATCGTGACATCTTTAGCTAAATCGAGTTAAAAACTTTCAACAACGGGATCT 224
   Query 179 TCTGGGTCCAACCTCCCCACCGTGTTTATCGTACCTGTTGGTTTGGGGGCGGGCGGCGCTCA 238
4. gb|GU067744.1| Hypocrea pachybasioides isolate F30 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length = 550
Score = 392 bits (212), Expect = 4e-106
Identities = 228/235 (98%), Gaps = 4/235 (1%)
Strand = Plus/Minus
Query 1 TTGA-TC-TTTTCGAAACGCCCGCTAGGGGCGCCGAGATGGTTCAGAATTATAAAAGTCC 58
||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 270 TTGATTCATTTTCGAAACGCCCGCTAGGGGCGCCGAGATGGTTCAGAATTATAAAAGTCC 211
Query 59 GCGA-GGGACATACAAAAGAGTTTTGGTTGGTCCTCCGGCGGGCGCCTTGGTCCGGGGCT 117
||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 210 GCGAGGGGACATACAAAAGAGTTTTGGTTGGTCCTCCGGCGGGCGCCTTGGTCCGGGGCT 151
Query 118 GCGACCGCGCCCGGGCATGAATTTTCCCGGCAAGCTTTGTTGTAACGTTCACATAGG 177
||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 150 GCGACCGCGCCCGGGCATGAATTTTCCCGGCAAGCTTTGTTGTAACGTTCACATAGG 91
Query 178 GTTTTGGAGATTGTTAAACTCGGTAATCCCTCAGCAAGGTTCTACCTACGGAGA 232
||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 90 GTTTTGGAGATTGTTAAACTCGGTAATCCCTCAGCAAGGTTCTACCTACGGAGA 37

5. gb|EU910586.1| Penicillium thomii isolate song-40 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length = 555
Score = 383 bits (207), Expect = 2e-103
Identities = 210/211 (99%), Gaps = 1/211 (0%)
Strand = Plus/Plus
Query 1 GGG-CTCTGGGTCACCTCCCACCCTGATTATTGTACCTTTGTTGCTTCGGTGCGCCCGCC 59
||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 16 GGGACTCTGGGTACCTCCCACCCTGATTATTGTACCTTTGTTGCTTCGGTGCGCCCGCC 75
Query 60 TCAAGCGCGCGGGGGCTTCTGCCCCC CGCGCACCGGAGACACCAATTTGAACT 119
||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 76 TCAAGCGCGCGGGGGCTTCTGCCCCC CGCGCACCGGAGACACCAATTTGAACT 135
6. **gb|HM246517.1|** *Trichoderma asperellum* strain A45-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length = 565
Score = 381 bits (206), Expect = 9e-103
Identities = 209/210 (99%), Gaps = 1/210 (0%)
Strand = Plus/Plus
Query 1
AATGTG-ACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCC
Sbjct 35
AATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCC

7. **gb|AY805554.1|** *Cylindrocarpon didymum* isolate olrim 522 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
Length = 474
Score = 307 bits (166), Expect = 1e-80
Identities = 171/173 (99%), Gaps = 2/173 (1%)
Strand = Plus/Minus
Query 1
CTCCC-AACCCCTGTG-ACATACCTATCGTTGCCTCGGCGGTGCCCGCTCCGGCGGCCCG
Sbjct 470
CTCCCAAACCCCTGTGAACATACCTATCGTTGCCTCGGCGGTGCCCGCTCCGGCGGCCCG

8. **emb|AM159622.1|** *Davidiella tassiana* partial ITS1, clone K17
Length = 384
Score = 372 bits (201), Expect = 5e-100
Identities = 201/201 (100%), Gaps = 0/201 (0%)
Strand = Plus/Plus
Query 2
ACGCCCGGGCTTCGCCTGGTTATTCTACAACCCCTTGTGCGACTCTGTGCGCTCCGG
Sbjct 86
ACGCCCGGGCTTCGCCTGGTTATTCTACAACCCCTTGTGCGACTCTGTGCGCTCCGG
Lignin biodegradation

Query 62 GGGCGACCGCTGCCTTCGCGGCGGGGCTCCGGGTGGACACTTCAAACCTTGGCAGTAACTTTG 121
Sbjct 146 GGGCGACCGCTGCCTTCGCGGCGGGGCTCCGGGTGGACACTTCAAACCTTGGCAGTAACTTTG 205

Query 122 CAGTGCTGAGTAACTTAATTAATAAAATTTTTTAACACGGATCTTCTGCTCTGG 181
Sbjct 206 CAGTGCTGAGTAACTTAATTAATAAAATTTTTTAACACGGATCTTCTGCTCTGG 265

Query 182 CATCGATGAAGAACGCAGCAA 202
Sbjct 266 CATCGATGAAGAACGCAGCAA 286

9. emb|FR668012.1| Phoma macrostoma genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, isolate 4477
Length = 432
Score = 322 bits (174), Expect = 4e-85
Identities = 179/181 (99%), Gaps = 1/181 (0%)
Strand = Plus/Plus
Query 1 AGTCGTGGGCTTTGCCTGCT-TCTCTTACCCATGTCTTTTGAGTACTTACGTTTCCTCGG 59
Sbjct 14 AGTTGTGGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACTTACGTTTCCTCGG 73
Query 60 TGGGTTCGCCCGCCGATTGGACAATTTAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAA 119
Sbjct 74 TGGGTTCGCCCGCCGATTGGACAATTTAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAA 133
Query 120 ACATAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA 179
Sbjct 134 ACATAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA 193
Query 180 G 180
Sbjct 194 G 194

10. gb|GU214651.1| Sphaerulina polyspora strain CBS 354.29 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length = 632
Score = 327 bits (177), Expect = 9e-87
Identities = 177/177 (100%), Gaps = 0/177 (0%)
Strand = Plus/Plus
Query 4 CGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGA 63
Sbjct 145 CGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGA 204
Query 64 CCCTGCCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGA 123
Sbjct 205 CCCTGCCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGA 264
Query 124 TGAGTAAACTTAATTAATAAATTTTAACACGGATCTCTTGGTTCTGGCAGTAACCTTGG 180
Sbjct 265 TGAGTAAACTTAATTAATAAATTTTAACACGGATCTCTTGGTTCTGGCAGTAACCTTGG 321

11. emb|FR716534.1| Cryptococcus podzolicus DNA fragment containing partial 5.8S rRNA gene, ITS2 and partial 26S rRNA gene, isolate k-1090
Length = 1091
Score = 285 bits (154), Expect = 5e-74
Identities = 156/157 (99%), Gaps = 0/157 (0%)
Strand = Plus/Plus
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Query 1 ACGCCCTCACGGGCTTATAACTATTCCAAACCTCTGTGACGGTGCCCTTCGGGGCTATT 60
Sbjct 35 ACGCCCTCACGGGCTTATAACTATTCCAAACCTCTGTGACGGTGCCCTTCGGGGCTATT 94

Query 61 TTACAAACATGGTGTAATGAACGTCATATATCATAACAAAACAAAACTTTCAACAACGGA 120
Sbjct 95 TTACAAACATGGTGTAATGAACGTCATATATCATAACAAAACAAAACTTTCAACAACGGA 154

Query 121 TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCCAAA 157
Sbjct 155 TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAA 191