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 micro-organisms 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.[5] However, there are also individual enzymes that efficiently attack more than one wood polysaccharide.[9] 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.[10-12] 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.[13,14] 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).[5] The described extracted lignin-degrading systems are based on peroxidases, oxidases and dehydrogenases, the reactive species generated including Mn (III), hydroxyl and organic radicals.[15-18]

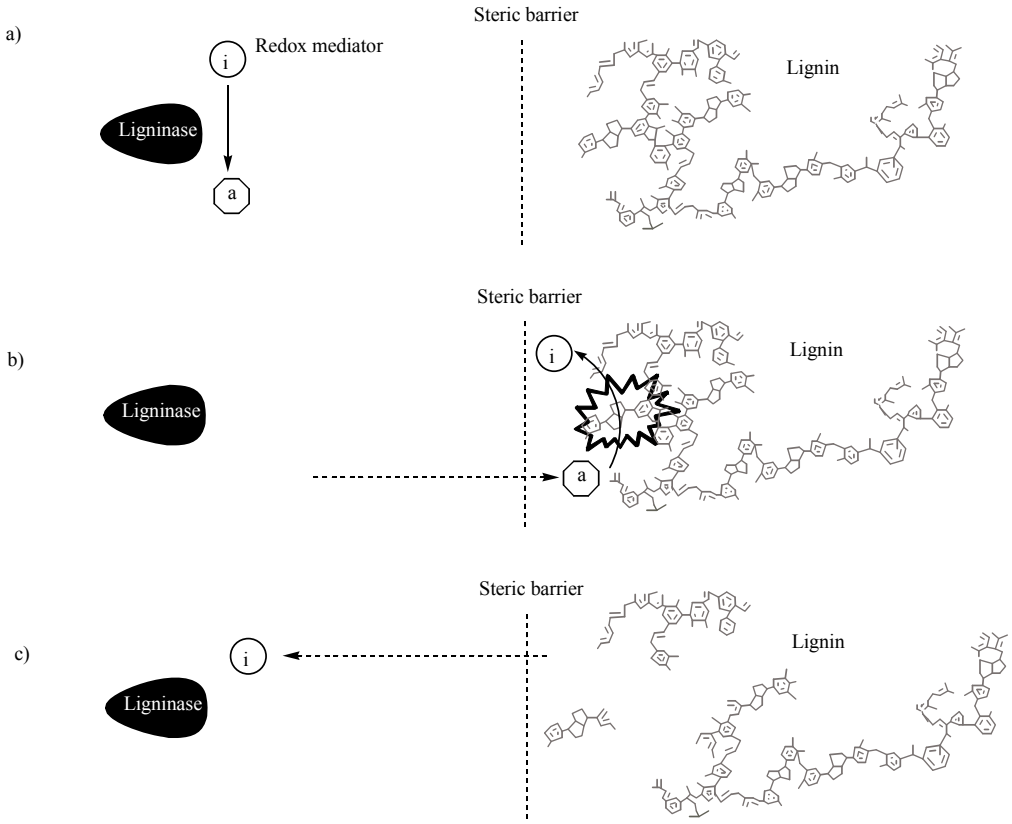


Figure 1: Principle of enzymatic lignin degradation by wood-degrading fungi: a) A lignin-modifying peroxidase oxidizes an inactive form of a redox mediator (i) to an active form (a); b) The active redox mediator can penetrate sterical barriers performing an unspecific lignin oxidation; c) The lignin 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

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.[19,20]

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,[2,21] whereas brown rot and soft rot fungi, depolymerize lignin only partially and chemically modify it, with concomitant formation of a brown color.[19]
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.[22] The content of the β-O-4 bond, which is the most common inter-monomer bond in lignin,[10] is lowered but not depleted, and novel covalent bonds seem to have been formed.[22] 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.[23] 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.[24,25]

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.[23,26-29] However, there have been also reported lignolytic enzymes that cleave the β-O-4 bonds by combinations of reduction and oxidation processes.[30,31] Furthermore, Otsuka *et al*. reported an etherase able to hydrolyze the β-O-4 bond.[32] However, cultivations of lignolytic soil organisms have generally been performed on similar types of medium as for the wood-degrading organisms, in some cases even with glucose as a carbon and energy source. In the present work, organisms from soil, living on lignin as a sole carbon source, were isolated and identified. To our knowledge, this is the first report on the cultivation of microbes under such conditions.

EXPERIMENTAL

Materials

Chemicals

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[33] was a kind gift of Professor Hans Theliander, Wallenberg Wood Science Center, Chalmers, Gothernburg, 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,[34] 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 KH_2PO_4, 1.0 g NH_3NO_3, 1.0 g $CaCl_2$, 0.25 g $MgSO_4 \cdot 7H_2O$, 1.0 mg $FeSO_4$, 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 KH_2PO_4, 1.0 g NH_3NO_3, 1.0 g $CaCl_2$, 0.25 g $MgSO_4 \cdot 7H_2O$, 1.0 mg $FeSO_4$. 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 re-inoculation of hyphen tips or cell colonies. When the microorganism appeared homogeneous, it was re-inoculated 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

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.[35] One cm^2 mycelium of each strain cultivated in lignosulphonate DP_401 medium was washed by Mili-Q water, 3 times, before PCR procedures, for amplifying the nuclear ribosomal DNA of the strains.

PCR procedures

ITS1 (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') primers, targeting the conversed ribosomal regions of 18 s and 5.8 s, were used[36,37] for PCR amplification. The reactions were performed in 50 µL. Parts of the washed mycelia (around 4-6 mm^2) 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 H_2O 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.

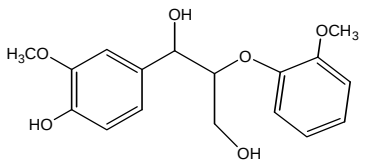


Figure 2: Structure of lignin model compound (guaiacylglycerol β-O-guaiacyl) used for degrading assay

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[32] 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).

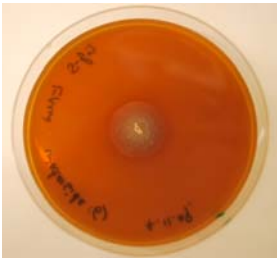
Table 1
Data on lignins used

Lignin	Mass percent (%)				
	Arabinose	Mannose	Xylose	Galactose	Glucose
Lignosulphonate DP_401	0	0	0.71	0	0
Leonardite	0.59	0	0.29	0	0
Granit lignin	0.35	0	0	0	0
Lignoboost lignin	0	0	0	0.01	0

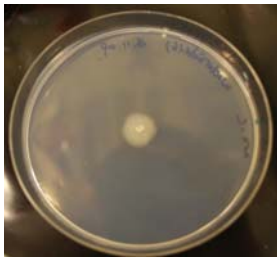
Table 2
Data on microorganisms purified

Name	Isolated from	Description	Classification	*NCBI Taxonomy ID*
Phoma herbarum	Hardwood forest	White, filamentous; no sporulation	Ascomycota	73001
Penicillium canescens	Hardwood forest	White, filamentous; white spores	Ascomycota	5083
Penicillium daleae	Hardwood forest	Heavy white filamentous; spores	Ascomycota	63821
Hypocrea pachybasioides	Spruce forest	Long, white, filamentous; spores	Ascomycota	40695
Penicillium thomii	Spruce forest	Transparent, filamentous; grey spores	Ascomycota	36647
Trichoderma asperellum	Spruce forest	Long, white, filamentous; green spores	Ascomycota	101201
Cylindrocarpon didymum	Bottom of a small stream	Heavy white, filamentous; spores	Ascomycota	109805

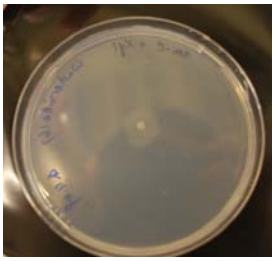
Davidiella tassiana	Bottom of a small stream	Green, filamentous; green spores	Ascomycota	29918
Phoma macrostoma	Bottom of a small stream	Transparent, filamentous	Ascomycota	73002
Sphaerulina polyspora	Brown rotted wood from spruce	Green, filamentous; green spores	Ascomycota	237180
Cryptococcus podzolicus	Brown rotted wood from spruce	Brown, bacterial-like	Basidiomycota	89927



Lignosulphonate agar plate



Blank agar plate



Same sugar concentration plate as the lignosulphonate

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

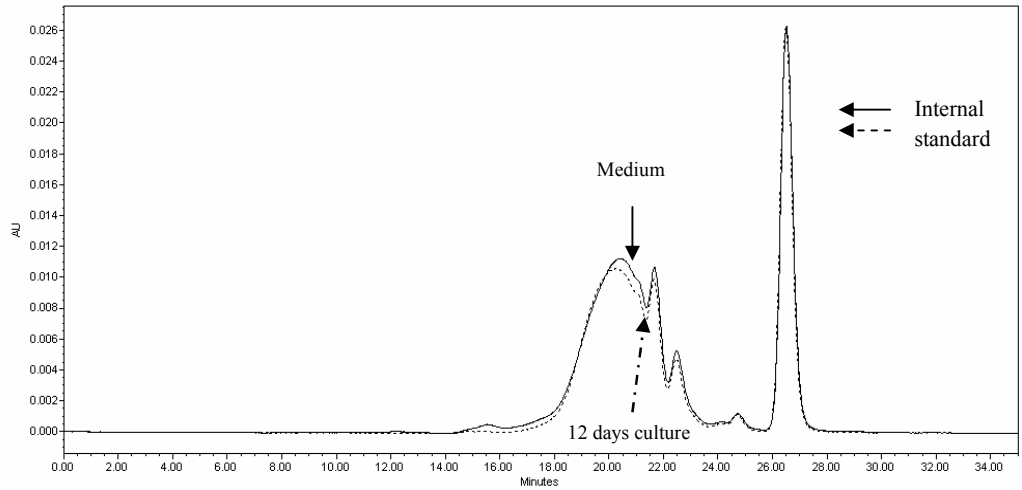
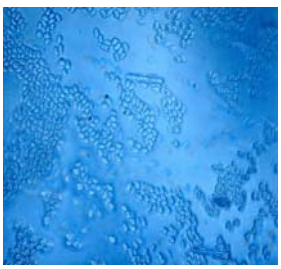
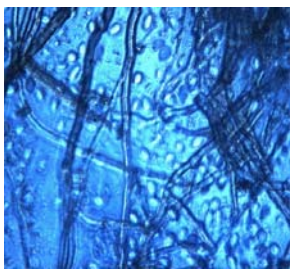


Figure 4: SEC chromatogram of compared culture filtrates after 12 day cultivation and control shaking flask medium before strain inoculation (Solid line – control experiment of control shaking flask medium before strains inoculation; dash line – culture filtrates after 12 day cultivation). The latest 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



(a)



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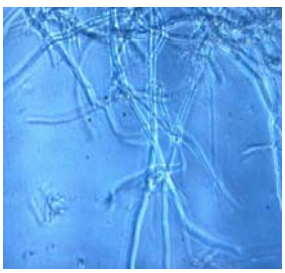


(c)


(d)

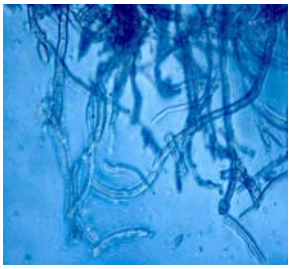

(e)

Figure 5: Hyphae generated by 5 fastest growing strains: (a) *Cryptococcus podzolicus*; (b) *Sphaerulina polyspora*; (c) *Phoma herbarum*; (d) *Penicillium thomii*; (e) *Davidiella tassiana*; (cultivated in lignosulfonate DP_401 shaking flask medium at 24 °C for 12 days)

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 of 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.[8,19] To the best of our knowledge, none of the strains had been previously associated with lignin biodegradation, with the exception of *Penicillum thomii*. This organism has been reported as able to chemically modify kraft lignin.[39]

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

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.

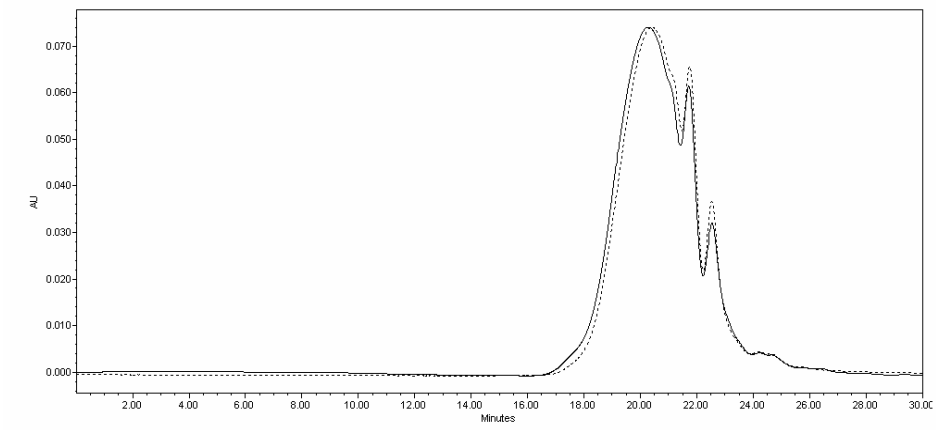
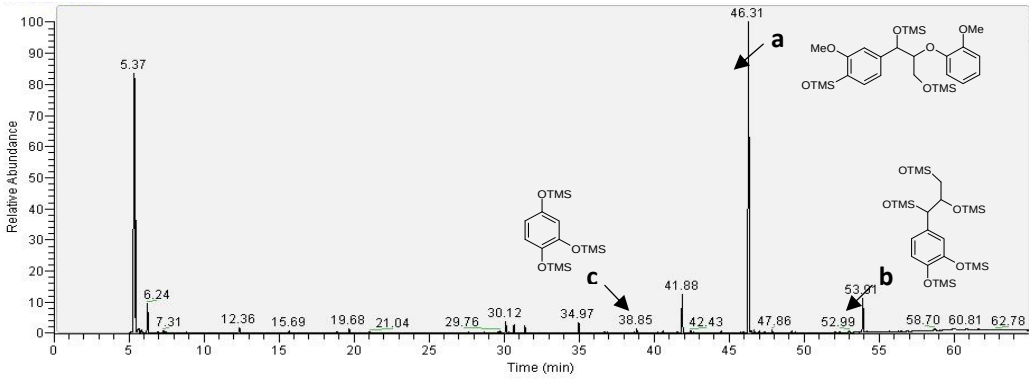
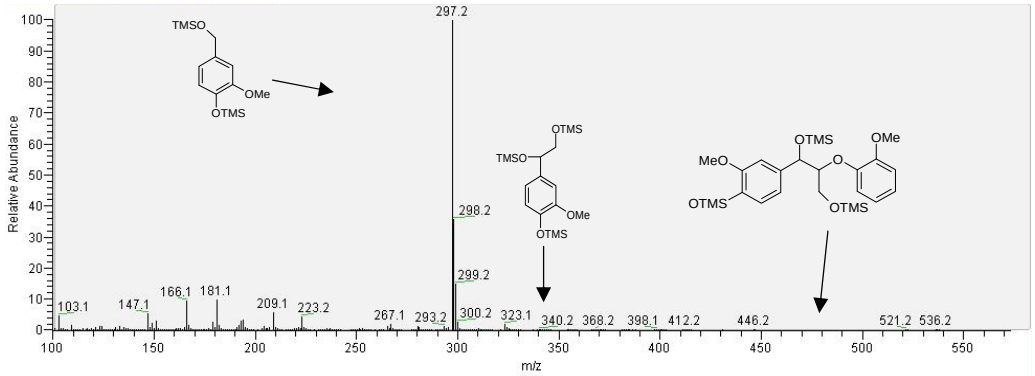


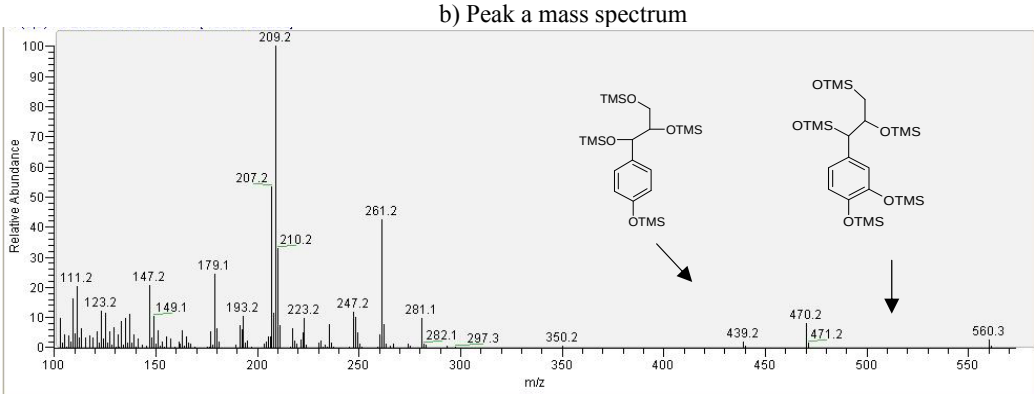
Figure 6: GPC data indicating that *Cryptococcus podzolicus* cell-free culture filtrate was able to cause lignin depolymerization (solid line –control experiment; dash line – incubation reaction of 5 mg lignosulphonate with *Cryptococcus podzolicus* cell-free culture filtrate overnight). The first peak 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



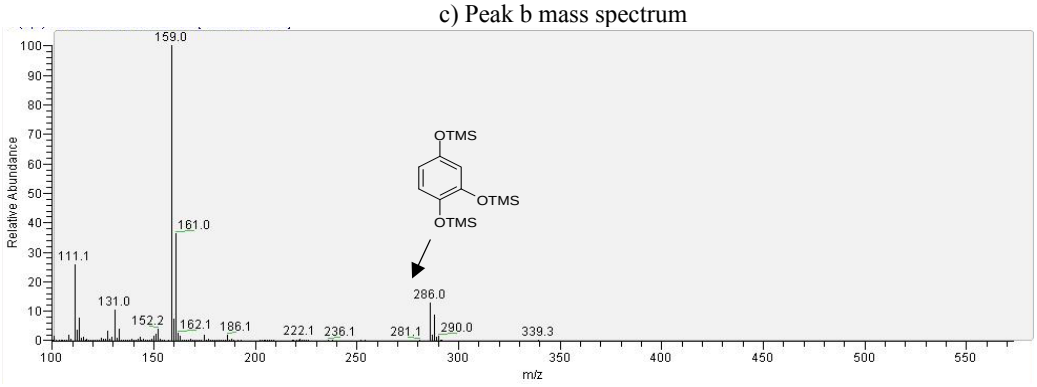
a) GC spectrum of lignin β-O-4 linkage model compound, guaiacylglycerol β-O-guaiacyl after incubation overnight with cell-free culture filtrates of *Penicillium thomii*



b) Peak a mass spectrum



c) Peak b mass spectrum



d) Peak c mass spectrum

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[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)[40] while, on the other hand, hydroxyl radicals are expected to cause polymerization of lignosulphonate rather than depolymerization,[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*.[31] and Masai *et al*.[30] These reactions require gluthathione 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.

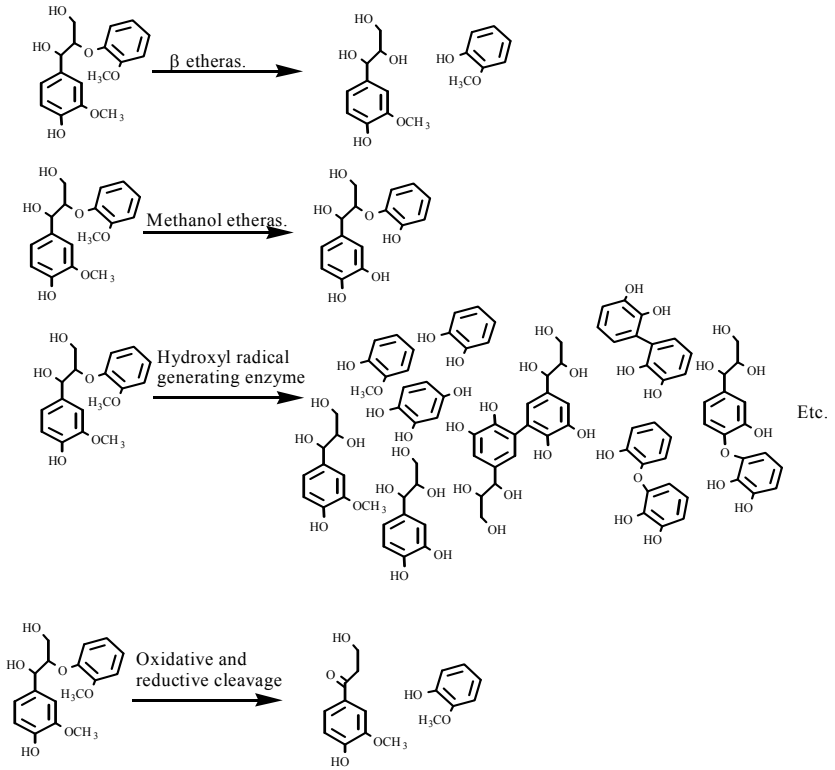


Figure 8: Hypothetical reaction schemes for enzymatic degradation of the guaiacylglycerol β-O-guaiacyl model compound[30-32,40,41]

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.

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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 TTTATGAGCACTTACGTTTCCTCGGGGGGTTCGCCCACCGATTGGACCACTTAAACCCTT 60
|||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct 72 TTTATGAGCACTTACGTTTCCTCGGTGGGTTCGCCCACCGATTGGACCACTTAAACCCTT 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 CACCTCCCACCCGTGTTTATTGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCG 84

Query 61 gggggcatctgccccgggcccgcgcccgccgAAGACACCTTGAACTCTGTATGAAAATT 120
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 85 GGGGGCATCTGCCCCCGGGCCCGCGCCCGCCGAAGACACCTTGAACTCTGTATGAAAATT 144

Query 121 GCAGTCTGAGTCTAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGG 180
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 145 GCAGTCTGAGTCTAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGG 204

Query 181 CATCGATGAAGAACGCAGC 199
|||||||||||||||||||
Sbjct 205 CATCGATGAAGAACGCAGC 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-C-ACCTCCCACCCGTGTTTATCGTACCTTGTTGCTTcggcgggcccgcctca 58
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Sbjct 45 TCTGGGTCCAACCTCCCACCCGTGTTTATCGTACCTTGTTGCTTCGGCGGGCCCGCCTCA 104

Query 59 cggccgccgggggggcacccgccccgggcccgcgcccgccgAAGACACCATTGAACGCTG 118
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Sbjct 105 CGGCCGCCGGGGGGGCACCCGCCCCCGGGCCCGCGCCCGCCGAAGACACCATTGAACGCTG 164

Query 119 TCTGAAGATTGCAGTCTGAGCATCTTAGCTAAATCAGTTAAAACTTTCAACAACGGATCT 178
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Sbjct 165 TCTGAAGATTGCAGTCTGAGCATCTTAGCTAAATCAGTTAAAACTTTCAACAACGGATCT 224

Query 179 CTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAA 238
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Sbjct 225 CTTG-TTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAA 283

Query 239 TTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATG 298
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Sbjct 284 TTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATG 343

Query 299 CCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGccccccgcccccG 358
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Sbjct 344 CCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGCCCCCCG 403

Query 359 GCTCCCGGGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATG 418
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Sbjct 404 GCTCCCGGGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATG 463

Query 419 GGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGAccccccTCAATCT 478
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 464 GGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGACCCCCCCTCAATCT 523

Query 479 TTTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA 523
|| ||||||||||||||||||||||||||||||||||||||||
Sbjct 524 TTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA 568

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 GCGACGCGCCCGGGGCATGAATTTCCCGCCGAGGCAACAGTTTGGTAACGTTCACATAGG 177
|||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 150 GCGACGCGCCCGGGGCATGAAATTCCCGCCGAGGCAACAGTTTGGTAACGTTCACATAGG 91

Query 178 GGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAAGGTTCACCTACGGAGA 232
||||||||||||||||||||||||||||||| |||||||| |||||||
Sbjct 90 GGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCT-GGTTCACCAACGGAGA 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-CTCTGGGTCACCTCCCACCCGTGTTTATTGTACCTTGTTGCTTCGGTGCGCCCGCC 59
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Sbjct 16 GGGACTCTGGGTCACCTCCCACCCGTGTTTATTGTACCTTGTTGCTTCGGTGCGCCCGCC 75

Query 60 TCACGGCCGCCGGGGGGCTTCTGCCCCCGGGTCCGCGCGCACCGGAGACACCATTGAACT 119
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 76 TCACGGCCGCCGGGGGGCTTCTGCCCCCGGGTCCGCGCGCACCGGAGACACCATTGAACT 135

Query 120 CTGTCTGAAGATTGCAGTCTGAGCATAAACTAAATAAGTTAAAACTTTCAACAACGGATC 179
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Sbjct 136 CTGTCTGAAGATTGCAGTCTGAGCATAAACTAAATAAGTTAAAACTTTCAACAACGGATC 195

Query 180 TCTTGGTTCCGGCATCGATGAAGAACGCAGC 210
|||||||||||||||||||||||||||||||
Sbjct 196 TCTTGGTTCCGGCATCGATGAAGAACGCAGC 226

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
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Sbjct 35 AATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCC 94

Query 60 CGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACG 119
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Sbjct 95 CGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACG 154

Query 120 TTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCT 179
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 155 TTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCT 214

Query 180 CTTGGTTCTGGCATCGATGAAGAACGCAGC 209
|||||||||||||||||||||||||||||
Sbjct 215 CTTGGTTCTGGCATCGATGAAGAACGCAGC 244

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 58
||||| ||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 470 CTCCCAAACCCCTGTGAACATACCTATCGTTGCCTCGGCGGTGCCCGCTCCGGCGGCCCG 411

Query 59 CCAGAGGACCCCCAAACTCTTGTTTTATACAGTATCTTCTGAGTAACACGATTAAATAAA 118
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Sbjct 410 CCAGAGGACCCCCAAACTCTTGTTTTATACAGTATCTTCTGAGTAACACGATTAAATAAA 351

Query 119 TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC 171
|||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 350 TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC 298

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 ACGCCCGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGG 61
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 86 ACGCCCGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGG 145

Query 62 GGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTG 121
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Sbjct 146 GGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTG 205

Query 122 CAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGG 181
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 206 CAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGG 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
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Sbjct 145 CGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGA 204

Query 64 CCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTC 123
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 205 CCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTC 264

Query 124 TGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCA 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 265 TGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCA 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 ACGCCCTCACGGGCTTATAACTATTCCAAACCTCTGTGAACCGTGCCCTTCGGGGCTATT 60
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 35 ACGCCCTCACGGGCTTATAACTATTCCAAACCTCTGTGAACCGTGCCCTTCGGGGCTATT 94

Query 61 TTACAAACATGGTGTAATGAACGTCATATATCATAACAAAACAAAACTTTCAACAACGGA 120
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 95 TTACAAACATGGTGTAATGAACGTCATATATCATAACAAAACAAAACTTTCAACAACGGA 154

Query 121 TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCCAAA 157
          |||||||||||||||||||||||||||| |||
Sbjct 155 TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAA 191
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