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Bark depolymerization during submerged fermentation using monofloral honey, a natural mediator substitute, and integration between laccases vs. bark biopolymers, characterized by Py-GC-MS

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Due to increasing waste production and disposal problems arising from synthetic polymer production, there is a critical need to substitute materials with biodegradable and renewable resources. Attempts to use laccases as a catalyst to enhance the catalytic properties of enzymes have been shown to be a promising solution for bark depolymerization. In this study, eight different fungal strains were tested for laccase enzyme production during submerged fermentation (SF), and the *Pleurotus* species was shown to be the best producers among the competing fungal strains. *P. pulmonarius* mainly produces laccase enzyme in production medium (PM) at initial conditions of pH-5.5 and 30°C. Bark depolymerization was conducted in SF and we identified polyphenols/polyaromatic compounds after four weeks when the PM was induced with 50mg/100ml of each bark during the lag-phase. During SF where honey was used as a natural mediator substitute (NMS) in the PM, laccase activities were about 1.5 times higher than those found in comparable cultures without honey in the PM. These samples were analyzed by GC-MS/MS. The laccase enzyme was purified using UNO® sphere Q-1 anion exchange chromatography and the molecular weight was determined to be ~50kDa on 10% SDS-PAGE. The laccase kinetic parameters V_{max}, K_m, and turnover number (K_{cat}) were found to be 76.9µM min^{-1}, 909µM and 739min^{-1}, respectively, from a Lineweaver Burk plot. Furthermore, laccases are suitable for biotechnological applications that transform bark biomass into high value bark biopolymers / biochemicals. The differences observed among identified aromatic compound MS/MS profiles were due to the utilization of two different bark species. Py-GC-MS analysis of bark showed differing effects of fungal activity on bark composition. Polyphenolics were separated in reverse-phase mode using HPLC with a pinnacle DB Biphenyl, C18 column, and UV detector. Two recognition wavelengths of 290 and 340 nm were selected to improve the separation of each single compound in monofloral honey and bark-fermented samples. This study is novel because it replaces natural mediators (NM) with monofloral honey in PM and bark materials impregnated with honey, and studies the effects of fungi-derived laccases on bark biopolymers.

1. Introduction

The biobased economy relies on sustainable resources, and is likely to create an enormous impact comparable to the fossil-based economy. The biobased economy is not about simply

utilizing renewable resources and applying them to cutting-edge innovations; its principles can be used to modify procedures with far reaching effects on society.[1] Nations have set managed focuses to replace certain fuel and chemical commodities with biomass to help the biobased economy. Woody biomass provides lignocellulosic feedstock for the energy sector and chemical industry. Because of its inexhaustible accessibility, useful chemical composition, and reasonably low costs, woody biomass can improve products economically and sustainably. Global annual lignocellulosic biomass production was reported by Zhang[2] to be about 200 bnt. Haveren *et al.*[3] found that 0.3 bnt is differentiated to synthetic chemicals every year.

In Europe and North America, the *P. pulmonarius* is the most cultivated fungal species[4], and the most commonly found throughout the world, particularly in temperate and subtropical forest. This species is frequently found on hardwoods in eastern North American forests; however, it is also found on conifers in western United States.[5] The cultivation method, which is comparable to other *Pleurotus* species, is by spreading spores to grain and then dispersing the seeds to cellulosic biomass as substrate, like straw, coffee grounds, wood chips, sawdust and cardboard.

Pleurotus is considered an effective lignin degrader that can grow fairly fast on different types of lignocellulosic biomass. Laccase (EC 1.10.3.2) belongs to the multicopper oxidase family. Fungal growth conditions, media composition and cultivation method play an important role for the laccase production.[6] Laccase production in many fungi had certain effects on possibly in alteration of diverse polyphenolic compounds containing lignin and humic substances.[7]

The composition of honey, a natural bee product, depends mainly on the botanical source, topographical origin, and dispensing and atmospheric conditions. Due to similar antioxidant contents of many fruits and vegetables function as a natural food antioxidant.[8-10] In honey, the main antioxidant compounds are polyphenolics, flavonoids, enzymes (catalase, glucose oxidase), organic acids, ascorbic acid, carotenoid-like substances, amino acids and proteins. The antioxidant value contrasts significantly dependent on the floral source.[11] Several phenolic compounds including syringic acid (SA) and methyl syringate (MS) found in honey. Lately, a compound was found having substantial antibacterial activity beside *Staphylococcus aureus.*[12]

Mixed balsam fir (*Abies balsamea*) is monoecious and considered a valuable conifer from the boreal forest. It is mainly used in pulp, light frame construction, as well as, a food source and shelter for wildlife (http://www.borealforest.org/).

Mixed aspen (*Populus tremuloides*) is one of the fundamental source species and is essentially considered for managing biodiversity in the western and boreal zones in North America.[13] So as to better comprehend the fungal interaction and bark depolymerization, it is vital to investigate and think about the impacts of polyaromatics / polyphenolics reflected as natural mediators. Polyaromatics are hydrocarbon containing mainly C and H with multiple aromatic rings in which the electrons are delocalized. Primarily found in fossil fuels (oil, coal and tar deposits) and produced due to partial burning of organic matter.

Laccase alone does not polymerize, nor depolymerize or delignify pulp until it consolidates with a mediator like hydroxybenzotriazole (HBT). Poppius *et al.*[14] reported that the maximum degree of delignification up to 40% in pulp was examined using HBT as mediator. In laccases owing to high redox-potential in basidiomycetes from genus *Trametes* assists to lignin removal when its joined with HBT from complete[15] and preserved[16] lignocellulosic biomass, producing cellulose available to hydrolysis. Generally examined mediators are synthetic compounds based on nitrogen heterocycles. Because of high toxicity and price, it is hard to realize laccase-mediator systems (LMS) at an industrial scale.

The phenolic compounds as redox mediator's oxidation is a typical mechanism using laccases to enhance envision and switch enzymatic lignin-based biotransformation routes. The enzymatic oxidation of syringyl-type phenolics identified as natural mediators, i.e. methyl syringate (MS), acetosyringone (AS) and syringaldehyde (SA), directs to phenolics oxidation contingent on negatively charged residues similar to a substrate binding site of the enzyme.

Natural mediators in presence of laccase facilitates the oxidation of non-phenolics but it depends on the phenolic compound structure, as well as, the reactivity beside stability of phenoxy radicals produced ($MS^{\cdot} > AS^{\cdot} > SA^{\cdot}$) reported by Tania *et al.*[17] Due to high multiplicity, variable structure and composition in woody biomass, researchers are trying to profile radical-coupling routes that are involved in the formation of different phenolic species and identified as mediators. Its

structure and properties biochemically, phenotypically and through the exploration of its molecular properties depend on the capacity of plant cell wall to desist deconstruction because of fungal laccases.[18]

Molecular properties of bark/biomass combustion (BC) can be assessed by Pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS).[19-22] Heat-affected reaction and resulting changes produce structures that may be like the pyrolysis artifacts of BC.[23-24] Py-GC-MS are considered as a quick and inexpensive method for the bark characterization. The hydrolyzable bonds are cleaved and, subsequently, CO_2H and OH groups are changed in-situ amid Py-GC-MS directs to related methyl esters and methyl ethers, respectively, which are more acquiescent to GC than their underivatized complements.[25-26] It also provides supplementary data on structure over position of derivatized multifunctional rings.[26]

There is need to focus on bark depolymerization mechanisms and molecular changes as a function of carbonizing temperature aside from recognizing the effect of honey in production media where it can mainly be utilized as replacement of natural mediators.

2. Experimental methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich. Laccase Novozym®51003 (E.C.1.10.3.2) from *Myceliophthora thermophila* (1000 LAMU/g = 3.57 IU/ml/min) was obtained from Novozymes (Franklinton, NC, USA). Mixed balsam fir and aspen barks were supplied by FP Innovations, (Vancouver, BC, Canada) and milled to particle size less than 0.212 mm (US70 mesh size). All chemicals used in this study were reagent-grade and consumed just devoid of further purification.

2.2. Methods

2.2.1. Inoculum preparation

Pleurotus pulmonarius (UAMH-7992) and *Guignardia mangiferae* (UAMH 11209) cultures were purchased from Microfungus Collection and Herbarium, Faculty of Agricultural, Life, & Environmental Sciences, University of Alberta. A total of eight different fungal strains were used in our study included: *Pleurotus pulmonarius, Pleurotus cornucopiae, Pleurotus ostreatus, Phanerochaete chrysosporium, Bjerkandera adusta, Trichoderma harzianum, Trametes versicolor* and *Guignardia mangiferae*. Out of eight strains, six cultures were obtained from Applied Mycology Lab, Faculty of Forestry, University of Toronto. First, the cultures were plated on potato dextrose agar media from HiMedia® contains (Potatoes, infusion from 200g; dextrose 20g; agar 15g; water 1L; final pH-5.6) and stored at 4°C. A loop with fungal strains was transmitted into disinfected potato dextrose broth media pH-5.1 under antiseptic conditions and the flasks were incubated at 30^{0}C in orbital shaking incubator (150 rpm) aerated with continuous agitating.

2.2.2. Monofloral honey used as a NM-substitute in PM

Production media (PM) for *P. pulmonarius* contained: Monofloral Buckwheat Honey (Burke's Honey Ltd) mainly used as a substitute for natural mediators along with carbon, sugars, minerals, vitamin source in PM at three different concentrations 5%, 7% and 10% (w/v), L-Asparagine monohydrate 3g/L, $MgSO_4.7H_20$ 0.05g/L, KH_2PO_4 0.5g/L, K_2HPO_4 0.6g/L, $CuSO_4.5H_20$ 0.4mg/L, $MnCl_2.4H_20$ 0.09mg/L, $NaMoO_4.2H_20$ 0.02mg/L, $FeCl_3$ 1mg/L, Thiamine-HCl 0.1mg/L.[27] 50mL of PM were distributed into each 250 mL Erlenmeyer flasks and set pH to 5.5 with 1N NaOH, autoclaved at (121°C) for 15 minutes. Transfer 10% (v/v) inoculum into PM under sterile conditions and the flasks were incubated at 30^{0}C in a shaker (150 rpm) aerated with constant shaking. Bark containing lignophenols were induced in PM after three days during lag-phase of submerged fermentation and characterized bark polyaromatics/polyphenols using GC-MS/MS.

2.2.3. Time course study during SF

During submerged fermentation, time-courses were run for 24 days and, each day, samples were stored and kept at 4°C for laccase assays. For optimization, three samples were collected each

time from all experimental shake flasks containing different concentrations of honey in PM (e.g. 5%, 7%, and 10%). During SF, the collected samples were centrifuged in Heraeus Biofuge® Pico for about five minutes at speed 13,000 rpm and supernatant was used for laccase assays.

2.2.4. Assay of laccase activity

The oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (ABTS) at λ_{420nm} (ε = 3.6 x 10^4 $cm^{-1}M^{-1}$), which is symptomatic of laccase (Lcc) activity, was measured spectrophotometrically according to the method used by Machado *et al.*[28] The reaction mixture included 100µL of 0.3 mM-ABTS (substrate) in a 100 mM sodium acetate buffer (pH-3.5) and 100µL of crude enzyme from culture supernatant solution, which was incubated at 40°C for 1 min. The increase in absorbance at λ_{420}nm was observed due to oxidation of substrate (ABTS), including changes in blue colour intensity owing to Cu^{+2} ions in laccases. For all peroxidases, the unit activity is defined as the amount of enzyme involved to oxidize 1 µmol of substrate per minute. Total protein was quantified using BSA as a standard followed by Bradford method.[29]

2.2.5. Laccase purification by UNO® sphere Q-1 column

UNO® sphere Q-1 anion exchange column constructed for single-step polymerization. Q-1 anion exchange column specifications are: column bed volume (1.3mL), maximum protein loading (20 mg), flow rate (0.5ml/min), column dimension (7 x 35mm), and maximum operating pressure (4.5 MPa). The separation method was initiated by equilibrating the UNO Q-1 column with Buffer A (25mM Tris-HCl; pH-8.1). Crude laccase was injected onto a 50µl pre-column loop, after which, the sample in the loop was loaded onto the UNO Q-1 column using Buffer A.

The fluid flow in the system was incrementally changed to Buffer B (25mM Tris-HCl pH-8.1 + 0.5M NaCl) to elute the proteins captured in the column. At this phase, the laccase activity resembled to a peak of absorbance monitored at 280 nm and eluted as a single peak. Fractions were observed at 280 nm using ChromLab™ software**.** The FPLC system (Bio-Rad, USA) comprised a Biologic Duoflow pump system, BioFrac fraction collector, and UV detector. The purified and concentrated enzyme was preserved at -20^{0}C and did not show any significant loss

of enzymatic activity over several months. The molecular weight of laccase enzyme was determined by running 10% SDS-PAGE as earlier described by Höfer.[30] The protein gel was stained with Coomassie brilliant blue R-250 and commercially available prestained protein ladder (Fermentas, USA) was used as a standard.

2.2.6. Laccase kinetics

Laccase kinetic constants *(V_{max}, K_m, K_{cat} and K_{cat}/K_m)* were controlled by incubating the set quantity of purified laccase enzyme with different concentrations of substrate (ABTS) ranging from 200-1200 µM at 40^{o}C, pH-3.5 as depicted previously.[31-32]

2.3. Characterization techniques

2.3.1. Total polyphenolics estimation by Folin-Denis method

Total polyphenolic (TP) contents in the samples were estimated by spectrophotometric method using Folin-Denis reagent. 20 µl of sample or standard solution (catechol) was diluted with 1.58 ml of distilled water and mixed with 100 µl of Folin-Denis reagent (Fluka Analytical, Sigma-Aldrich, St Louis, MO). Entirely mixed the reaction mixture and left for 5 minutes at room temperature. Add 300 µl from 25% (w/v) Na_2CO_3 stock solution into the reaction mixture. The reaction mixture was incubated at 40°C in a water bath for 30 minute and after measured absorbance at λ_{765} nm by UV-160 UV/Vis spectrometer (Shimadzu, USA). The standard curve was plotted using 100, 200, 400, 600, 800 and 1000 ppm (mg/L) concentrations of catechol vs. absorbance at 765 nm. Results were expressed as (mg cat equiv/100ml of sample).[33-34]

2.3.2. Sample preparation for HPLC

Bark fermented samples were centrifuged for 10 min at 10,000 rpm. The supernatant was filtered through a 0.45 µm filter and analyzed by HPLC.

2.3.3. HPLC Analysis

The HPLC system comprised a Dionex BioLC 20 Series operational with a GP50 gradient pump, AS40 auto sampler, AD25 absorbance detector, and Chromeleon for information accumulation and examination (CMS); all of them from Dionex Technologies (USA). Ten μL of sample was injected in a column by autosampler. A Pinnacle DB Biphenyl C18 column (100 × 4.6 mm, particle size 3 μm) from Restek (USA) was used for chromatographic separation. Two mobile phase gradients were used for elution with a flow rate of 1 mL/min: 5% (v/v) HCOOH in aqueous solution as (eluent A), and CH_3OH as organic mobile phase (eluent B). Chromatograms were chronicled at two different wavelengths, 290 and 340 nm. [35-36]

The fermented samples were induced with 50 mg of each bark samples during lag-phase and polyphenolics were determined spectrophotometrically and characterized based on their reported retention time (t_r) values. Chromatographic separation was performed with gradient elution and the following steps were required:[35] 70% eluent A + 30% eluent B by an isocratic elution for 0–15 min, 60% eluent A + 40% eluent B by a linear increase for 16–20 min, 55% eluent A + 45% eluent B by a linear increase for 21– 30 min, 40% eluent A + 60% eluent B by a linear increase for 31–50 min, 20% eluent A + 80% eluent B by a linear increase for 51–52 min, 10% eluent A + 90% eluent B by a linear increase for 52–60 min, 10% eluent A + 90% eluent B by an isocratic elution for 61–63 min, 70% eluent A + 30% eluent B by a linear increase for 64–73 min and lastly 70% eluent A + 30% eluent B by an isocratic elution for 74–75 min, respectively.

2.3.4. Thermal Analysis

Thermogravimetric analysis (TGA) of fungal treated and untreated/control bark samples were performed on a computer controlled (TGA-Q500, TA Instruments, USA). Bark powder samples were loaded into alumina pan and samples were heated from room temperature to 800^oC at a rate of 10^oC min^{-1} under N_2 atmosphere.

2.3.5. GC-MS/MS

Fermented bark samples were centrifuged (10,000g for 15 min) to detach cell biomass. Supernatants were acidified to pH 2.5–3 with concentrated HCl and then completely separated with three volumes of $CH_3COOC_2H_5$. An organic layer was collected; remove moisture over

anhydrous Na_2SO_4, filtered, and then samples were dried over night to keep in the oven at 50°C. Using the Lundquist method[37], the ethyl acetate extracts residues were examined as trimethyl silyl (TMS) derivatives. Dioxane and pyridine (10:1 v/v) was added in the samples ensued by silylated with 5-ml of silylation derivatizing reagent trimethylchlorosilane (TMCS). To dissolve residual particles, the mixture was heated up to 60 °C for 15 min with regular shaking.

An aliquot of 1 ml of silylated compound was injected in Saturn 2100T GC/MS/MS (Varian, Inc. USA) equipped with Varian 3900 (GC oven) and Saturn® 200MS workstation software. PE–5MS capillary column (20m x 0.18mm i.d; 0.18 mm film thickness) and helium was utilized as a carrier gas with flow rate of 1ml min^{-1}. Column temperature was maintained (50 °C for 5 min), 50–300 °C (10 °C min^{-1}, hold time for 5 min). 3 min was chosen for solvent delay. The exchange line and particle source temperatures were sustained at 200 and 250°C.

In full-examine mode, electron ionization (EI) mass spectra within the scope of 30–550 (m/z) were recorded at electron energy of 70 eV.[38] Characterization of lignin related low molecular weight (LMW) compounds, which are isolated from fungal treatment, was interpreted by comparing their retention time (t_r) with an existing database of the original compounds.

2.3.6. MALDI-TOF-MS

Mass spectra of bark samples were analyzed using 4800 MALDI TOF/TOF Analyzer with 4000 Series Explorer Workflow software, by a fixed laser intensity, with evenly spontaneous spot pattern (Applied Biosystems/MDS Sciex). Instrumental calibration was done to redirect the laser beam on the rectified probe in an adjusted frame of diversion. Before analysis, the spot position was fixed carefully. The positive ion reflector mode for all MS measurements acquired for identical repetition whereas α-cyano-4-hydroxy cinnamic acid (Sigma-Aldrich) was used as the matrix compound.[27]

2.3.7. Sample preparation for Py-GC-MS

During time course study, the bark fermented samples were harvested in the late exponential-phase (after 18-20 days of inoculation) afterwards dried in an oven (Thelco GCA Precision

Scientific 18 Mechanical Convection Oven) at 60^{o}C for overnight. The oven-dried fermented samples were milled into a fine powder and further analyzed by Py-GC-MS.

2.3.8. Py-GC-MS

Pyrolysis-GC-MS analysis was conducted using a Pt-filament loop test Pyroprobe 5250 pyrolyzer (CDS Analytical, Oxford, USA). For that purpose, 1 mg of specimen was installed in quartz tubes applying glass wool, and pyrolyzed at 650 ^{o}C for 10 seconds (heating rate 10 ^{o}C/ms), with the pyrolysis valve oven and transfer line was set at 325 °C. Typically for thermostable materials such as charcoal or charred condensed tannins suggested 750^{O}C,[39] but 650^{O}C for uncharred biomass,[40] which does not need so much energy to pyrolyze adequately. The pyrolysis products were swept online into a 6890N GC instrument, with the inlet set at 325 °C, in splitless mode.

The GC instrument was fixed with non-polar 5% phenyl, 95% dimethylpolysiloxane (HP-5MS) column (30 m x 0.25 mm internal diameter; film thickness 0.25 µm) and helium was consumed as a carrier gas (flow rate 1 ml/min). The GC oven remained heated from 50 to 325 ^{0}C (held for 5 min) at 20 $^{\circ}$C/min. The transfer line of GC–MS was maintained at 325 ^{o}C. The ion source (electron impact mode, 70 eV) of the 5975 MSD (Agilent Technologies, Palo Alto, USA) was controlled at 230 ^{o}C and scanning of quadrupole detector at 150 ^{o}C, a range between *m*/*z* 50 and 550. Relative proportions of the pyrolysis products were estimated from their peak areas, built on one or two characteristic or major fragment ions. The aggregate of peak areas (total quantified peak area, TQPA) was fixed 100%.

3. Results and discussion

3.1. Extracellular laccases and screening of potential fungal strain

Extracellular laccase was produced during submerged growth conditions and screening of the best fungal strain was done based on the laccase production and growth conditions. These experiments were conducted both in solid as well as in liquid media. Among other competitor fungal strains, the *P. pulmonarius* was screened for further examination due to it possessing a strong laccase activity, which was determined on the malt extract agar media plates (malt extract

25g; agar 20g; distilled water 1L, and 0.05% of the dye Remazol Brilliant Blue R), and wrapped with aluminum foil to avoid with light and put in the incubator at 30^{o}C for two weeks. Ligninolytic activity was assessed on agar plates by observing decolorizing of polyaromatic anthraquinone dye and scoring attainable halos surrounding around the fungus-growing colonies as shown in Fig. 2 (K-M).

After the agar plate prescreening, all fungal strains were transferred into the production media and fungal growth was observed during submerged fermentation. Each bark contains mixed aspen and balsam fir (50mg per 100ml of each bark) and was induced during lag-phase, which appears five-days after when transfer inoculum into production media (PM). Maximum laccase activity was recorded after 25 days in submerged fermentation as 52U in *P. pulmonarius*, 46U in *P. cornucopiae*, 35U *P. ostreatus*, 28U in *P. chrysosporium*, 27U in *T. versicolor*, and 23U in *G. mangiferae*, respectively as shown in (Fig. 1).

Bark polyphenolics, might possibly seems lignin-like, polymeric substances with carbohydrates, celluloses, hemi-celluloses, and polyoses (non-cellulose).[41-42] By utilizing fungal degrading enzymes, the polyphenolic–carbohydrate complex compounds changed into smaller and more attainable moieties. As lignin retains high antioxidant activity,[43-44]therefore, hypothetically decrease in size of lignin-like, polyphenolics degraded through fungus is able to increased soluble bark phenolic contents. Instead of fungal activated phenolic moieties might reduce antioxidant activity of such bark phenolics.[45-47]

It was observed that fungal growth increased through mid (10–25 days) to late (20–30 days) phases, with improved soluble phenolic contents. *P. pulmonarius* had the maximum virtual laccase activity among other investigated fungal strains. In general, the phenolic compounds oxidized easily due to phenoxy radicals formations as compared to non-phenolic compounds.

Due to increase phenolic concentration and oxygen availability leads to assist polymerization. Degrading rate of lignin, via feedback control mechanism of laccases with polyphenolics, directs an enzyme into a latent catalytic state.[48] Therefore; it starts decline enzyme activity after certain time period owing to feedback inhibition due to accumulation of secondary metabolites and toxic compounds to a certain level. Morphological characterization of different fungal growth patterns

on each bark was observed using AmScope-WF25X/9 (magnification 0.5X) attached with cold-light source haloid lamp 150W (Fig. 2).

3.2. Laccase purification and kinetics

The extracellular laccases from *P. pulmonarius* cultivated under SF was purified almost to homogeneity level. The purification method has already explained in methodology section. The sequential purification steps were sum up in (Fig. 3), and its purification to visible on 10% SDS-PAGE stained with Comassie Blue R-250 (Fig. 4A). With the help of Lineweaver-Burk plot, the K_m and V_{max} values of laccase from *Pleurotus pulmonaris* were obtained 909µM and 76.9µMmin^{-1}, respectively (Fig. 4B). The turnover number (K_{cat}) and specificity constant (K_{cat}/km) was 739 min^{-1} and 0.81 µM^{-1}min^{-1} respectively. As compared to literature, the similar specific constant of laccase enzyme was investigated from *P. ostreatus*. As a result of high catalytic efficiency and binding affinity with substrate (ABTS), we were able to compare with other laccase producing microorganisms.[49]

3.3. Bark decomposition by TG/DTG

Thermal degradation of bark samples were estimated using TGA. All experiments were done under nitrogen atmosphere. Both control and fungal treated bark samples were examined (Fig. 5). Cellulose decomposition is subjugated by the main DTG peak, while the shoulder peak mainly to hemicellulose decomposition assigned at lower temperature (around 160 °C)[50] while lignin starts to decomposes from lower to wide temperature range between (200–400 °C).[51]

Mixed aspen and fungal treated aspen bark samples were started decomposed at about 190 ^{o}C and 140^{o}C, while mixed balsam fir and fungal treated fir bark samples were activated to decay at about 200 ^{o}C and 170^{o}C respectively. The solid-lines in both TG/DTG (thermograms- shown in Fig. 5) indicating to control or untreated barks whereas the dotted-lines specifying for fungal-treated bark samples. The weight loss of fungal degraded bark samples was faster between 140-400°C.[52]

3.4. Mass spectrometry (GC-MS/MS, MALDI-TOF/MS)

Mainly lower molecular weight compounds, including polyaromatics and polyphenolics, from each fungal-treated bark species were identified and analyzed by GC-MS/MS and MALDI-TOF-MS methods. In this study, GC–MS/MS has been considered to analyze LMW compounds liberated from fungal decayed bark. The total ion chromatograph (TIC) relating to ethyl acetate extracted compounds from acidified supernatants[53] attained from both control and fungal treated bark samples with *P. pulmonarius* are shown in (Fig. 6), and major identified peak (t_r) values were marked (in green) while all others (in black) are summarized in Table 1.

The ethyl acetate extracted compounds assigned to chemical oxidation of bark because of aeration during microbial fermentation (Fig. 6). Apparently, the fungal treated chromatographic profiles look different than the control, implying a strong biochemical ability of fungus on bark to alter bark composition. The previous studies have shown that the *Paenibacillus* sp. has minimal colour reduction as compared to *A. aneurinilyticus* and *Bacillus* sp.[54] Apart from an aldehyde and ketone-types, many acid-type complexes were also investigated due to microbial degradation of lignin.[55]

MALDI-TOF analyses were done in order to determine the molecular weight distribution (MWD) of each bark species treated by *P. pulmonarius* shown in (Fig. 7), and the main peak retention time (t_r) values were specified in (blue) enlisted in Table 1. It was observed that mostly bark comprised LMW compounds and their molecular weight less than 275 $gmol^{-1}$. The identical MWD pattern was very similar within each bark species that revealing correlated depolymerization reactions which indicate to separate identical compounds.[56]

3.5. Honey in PM as natural mediator substitute (NMS)

The effects of different honey concentrations on each bark species, extracellular laccase activity, and total polyphenolic contents were determined during a time course study in *P. pulmonarius.* Extracellular laccase activity by *P. pulmonarius* in response to different bark species were shown in (Fig. 8). A significant increase in the laccase activity was observed in aspen bark compared to fir and methyl syringate used as a natural mediator. The obtained results from fermented aspen bark species are comparable with MS but better than fir samples.

In presence of different applied honey concentrations and after 18-days of incubation, the laccase activity slightly decreased. In our study, we used three different (%, w/v) concentrations of honey but we observed the highest laccase activity 68 IU/ml/min at 7% (w/v) honey concentration after 18-days of growth (Fig. 8). During fermentation at a higher substrate concentration in production media precedes catabolite repression which ultimately affects enzyme productivity yield. In contrast to that, it has been suggested that during laccase production in presence of phenolic compounds caused toxicity brings about their oxidation to form quinones which considered as toxic for the fungal growth.[57-58]

Furthermore, induction with bark during lag-phase may decreases due to producing of any other extracellular proteolytic as well as ligninolytic enzymes including LiP, and MnP; therefore, it may upturn the laccase activity. Thus, we can propose that improved laccase activity owing to readily available of phenolics and aromatic compounds in fungal degraded aspen bark which might help in improve enzyme stability.[59]

In presence of ligninases, the oxidation mechanism of synthetic mediator likes hydroxybenzotriazole (HBT) similar to phenolic type mediators as methyl syringate (MS). During oxidation, the highly reactive phenoxy radicals were produced, which assists to remove one proton and one electron from the target substrate.[60] The ability of these phenolate ions mitigates oxidize intermediates, which may possibly control the efficacy of phenol as mediator equally modify through steric interferences.[61] The use of laccase-natural mediator system (Lcc-NMS) discussed here to offer for the bark degradation relating free radicals generated depends on both laccase and laccase mediator where honey used as a natural mediator substitute.

3.6. Polyphenolic chromatograms

HPLC detection mainly depends on the measurement of UV-Vis absorption at a specific wavelength. Qualitative identification of separated analytes directly approach to UV-Vis spectrum and its certain retention time (t_r). The performance of separation of analytes improves radically when different polyphenolic compounds are mixed in one sample. At selected suitable wavelength, it enhances separation of all groups with an utmost sensitivity. When the samples

have similar compounds is hard to detect by any regular method. Most flavonoids comprise phenolic hydroxyl groups having oxidation potential can be assessed by UV-Vis method.[62-65] Romani *et al.*[65] compared electrochemical detection methods with HPLC for polyphenolics in natural extract. Thus, the HPLC procedure found more precise as compared to differential pulse voltammetry method which was suitable for fast screening.

Polyphenolics separation was conducted in each fermented bark samples containing honey that mainly function as natural mediator. The chromatograms are shown in Fig. 9. Remarkably, it was noticed that the standard methyl syringate (MS) peak appeared at 290nm but vanished at 340nm. Similarly, the diluted honey sample appearing MS peak at 290 nm, but, when we ran the same sample at 340 nm, the peak was missing and new, different peaks formed. Our HPLC results confirmed that the wavelength at λ=290 nm was found more suitable for the separation of MS natural mediator compounds. All major peaks in the chromatograms were compared and characterized based on their t_r-values of honey polyphenolic compounds were earlier reported by Pyrzynska *et al.*[36]

Table 2 represents the total phenolic (TP) contents in a fermented sample when induced with 50 mg of each bark into 100 ml of PM during lag-phase. The minor variation observed during data collection possibly owing to some experimental errors. Total phenolic content (0.114 ± 0.61 mg cat equiv/g) was found in a buckwheat monofloral honey. Wood degrading fungi plays an important role by attacking on protein-polyphenolic complexes which possibly change substrate property.[66] Polyphenolics, mainly condensed tannin with protein complexes, assists to develop in fungal growth.[67] During fermentation in PM with honey, it was also noticed that total polyphenols in each bark species were significantly degraded as compared to glucose and natural mediator (MS) samples because of fungal biomass accumulation.

3.7. Py-GC-MS

Py–GC–MS total ion chromatograms (TIC) are represented in Fig. 10, while the major peaks in TIC-fingerprints are the pyrolysis products are reported in Table 3 with contrasting retention times (RT), characteristic ion fragments (m/z) utilized for the quantification and their relative proportions. Flags are used to organize the compounds and mainly divided into seven groups, i.e.

ALIPH = aliphatic compound, CARB = carbohydrate product, LIG = lignin, MAH = monocyclic aromatic hydrocarbon, NCOMP = nitrogen-containing compound, PHEN = phenol, SESQUI = sesquiterpenoid. Total quantified peak area (TQPA) values of each group are demonstrated in Table 4, and the values of each group were calculated based on Table 3.

3.7.1. Aspen bark

The pyrolysis fingerprints of untreated/control aspen bark is dominated by phenols (compounds 10, 13) and guaiacols (15-19, 21, 22), in addition to an unidentified aliphatic compound (14). The guaiacols originate predominantly from guaiacyl-based lignin, while syringyl-type markers were virtually absent. Such dominance of guaiacyl groups is typical of lignin in bark materials.[68]

The MS-treated bark sample from aspen produced a set of pyrolysis products that are rather similar to that of the control sample, even though there are minor differences such as a smaller relative proportion of phenols and higher proportion of guaiacols, especially guaiacol (15) and 4-vinylguaiacol (18). This might suggest that the material that is primarily affected by fermentation in MS is the phenol precursors while the lignin component is relatively unaffected.

The pyrolysis fingerprint of the aspen bark sample fermented in honey medium was very different from the control and MS-treated analogues. It is dominated by a set of furans, furaldehydes and cyclopentenones (2, 5, 7-9, 11), which are typical products of polysaccharides.[69] Guaiacol represents a lignin fraction that is relatively unaffected, but the intensities of the guaiacols are much lower than in the control and MS-treated samples.

Moreover, several N-containing pyrolysis products such as methyl pyridine (compound 3), in combination with the chitin marker acetamide (intensity too low for representation in Fig. 10, but identified unambiguously) are indicative of a major increase in the amount of microbial biomass in this sample. This makes it very likely that the carbohydrate products do not represent a recalcitrant polysaccharide component but rather that they originate from microbial sugars as well. Despite the small proportion of the lignin products, they can be traced from partial ion chromatograms (PIC) as shown in (Fig. 11).

3.7.2. Balsam fir bark

The pyrolysate of the untreated bark material from fir was strongly dominated by guaiacol products (15-19, 21-23) suggesting that the sample consists almost completely of guaiacyl-type lignin material. The MS-treated sample gave a similar pyrolysis fingerprint, even though minor increases of monocyclic aromatic hydrocarbons (MAHs, 1, 4, 6) can be witnessed. This suggests that fermentation in MS produced some degradation of the lignin backbone. The relative loss of 4-vinylguaiacol in comparison to other guaiacols might indicate that the sample contained a non-lignin polyphenolic material that was preferentially decomposed to a slight extent, as 4-vinylguaiacol is not only a product of guaiacyl-type lignin but also of cinnamic acids.[70]

The fir bark material submerged in honey medium produced a very different set of pyrolysis products, dominated by MAHs (1, 4) and furaldehydes (2, 8). Some remains of lignin can still be recognized from the total ion chromatograms (TIC), e.g. 4-methylguaiacol (compound 16) and 4-ethylguaiacol (17), and additional compounds can be traced from partial ion chromatograms (Fig. 11), but it is clear that this fermentation treatment almost completely degraded the original polyphenolic structures and the sample became dominated by microbial biomass. The lignin products are very abundant in control/untreated and MS-treated samples, and they can be identified directly from the TIC (with the peak labels).

There would be no added scientific value by presenting the partial ion chromatograms (PIC) of these compounds. By contrast to the honey-treated samples, they are not very abundant and, therefore, the PIC are useful. Possibly, the polyphenols in honey production media might be below the detection limit of Py-GC-MS or these polyphenols could have been degraded by pyrolysis, producing aromatics, such as toluene and benzene. Traces of catechol can be detected by Folin-Denis method but it should be below approximately ~1mg/100mg of catechol and then it would be imply that below the detection limit of the pyrolysis method.

Both control samples produced typical pyrolysis fingerprints of bark materials consisting primarily of lignin and polysaccharides. The MS-treatment seemed to preferentially degrade a non-lignin component in the aspen bark, causing the enrichment of guaiacol markers from lignin, while the opposite trend was observed for the Fir bark fermented in MS medium. This difference may be explained by the differences in original sample composition, with the fir bark being

composed almost purely of lignin while the aspen bark sample also contains polyphenolic precursors producing phenols rather that guaiacols and a more abundant aliphatic component. These additional components in fir bark materials are probably more heavily affected by fermentation in MS than the lignin component. Moreover, the effects of fermentation on the pyrolysis fingerprints of both bark samples were rather small.

By contrast, fermentation in honey medium eliminated most of the recognisable polyphenols (lignin) and both samples were almost completely converted into microbial tissue, composed mainly of carbohydrates as shown in (Fig. 12- Lignin/Carbohydrate index). Even though most of syringol products were found below the detection limit, the syringol was quantified at m/z 154 and 139 at 6.8 min to estimate the relative proportions of guaiacols and syringols using syringol/guaiacol (S/G) ratio also shown in Fig. 12. The lignin is strongly dominated by G-type lignin, and it can be concluded even though samples have a small proportion of S-type lignin (i.e. below 0.2 in all samples). MS-treated samples seem to slightly increase this ratio while the honey medium has opposite effects.

4. Conclusions

In the present study, we demonstrated that monofloral honey can be used as a natural mediator substitute in PM during submerged fermentation for the production of fungal laccases. We also found a methyl syringate in the diluted honey sample through HPLC analysis that separated at a wavelength of 290 nm. The time course study showed diverse laccase production among different fungal cultures. Laccase kinetics play an important role in biotechnological development when major constraints reduce catalytic efficiency to an inactive state. Moreover, GC-MS/MS and MALDI-TOF/MS analyses were found to be the most suitable methods to analyze LMW polyaromatics and polyphenolics due to fungal degradation of bark. Methyl syringate is considered to be the most effective natural mediator for the oxidation of non-phenolic lignin units, but our results during submerged fermentation with 7% honey in PM was comparable to the NM. Py-GC-MS of bark fermented samples in a honey medium indicated evidence of a profound effect on biotic degradation to an extent that a large proportion of the pyrolyzate can be traced back to microbial biomass. This microbial tissue may be related to the formation of the thermolabile component detected by thermogravimetry. The abundance of phenols and unidentified products combined with the polysaccharide fingerprint of degraded

material indicate significant fungal degradation and depolymerisation of bark biopolymers. So, finally, biotechnological procedures effectively used for the conversion of bark biomass into high value bark biopolymers/biochemicals.

Acknowledgements

The authors would like to acknowledge the financial support from ORF-RE (Bark Biorefinery Project), industry partners and Universidade de Santiago de Compostela (USC), Spain for access to the Pyrolysis-GC-MS facility and extremely grateful to *Cruz Ferro-Vazquez* from (Incipit-CSIC) and *Joeri Kaal* for his time, technical assistance and help in data acquisition.

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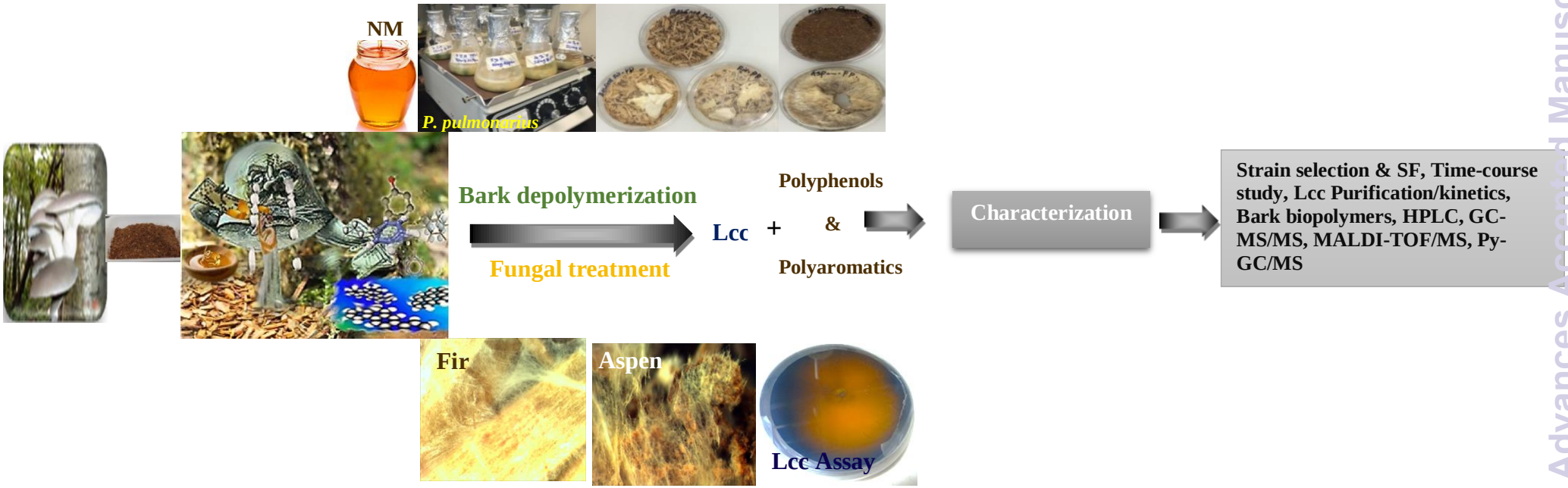
Graphical Abstract Image



Synopsis

This is a new green biochemical approach to edify that how we can replace synthetic, toxic and costly mediators into natural mediator using monofloral honey which accelerates oxidation mechanism with combination of fungal laccases at micro-scale for bark depolymerization into bark biopolymers.

Figs, Tables & Captions

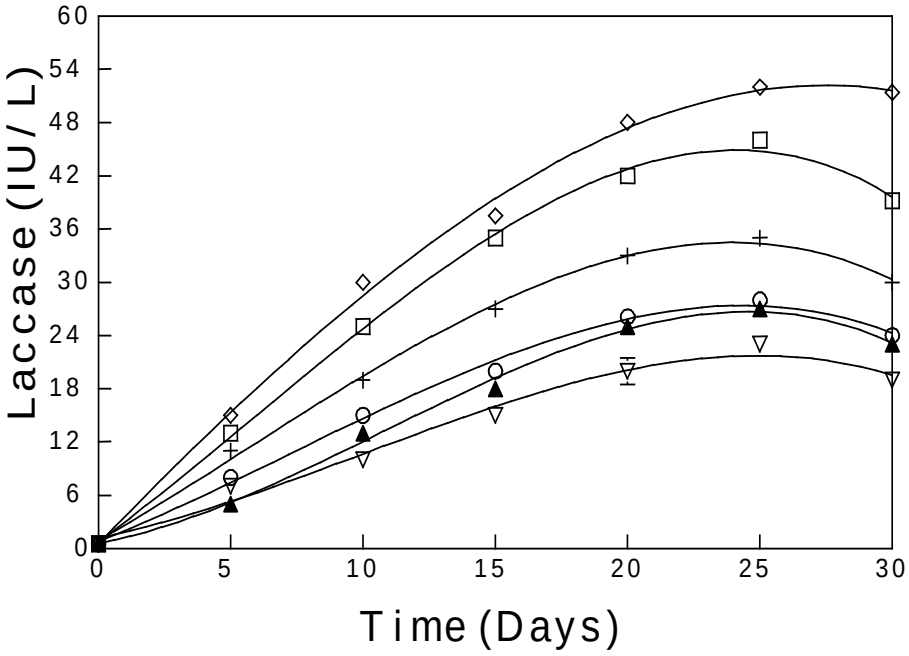
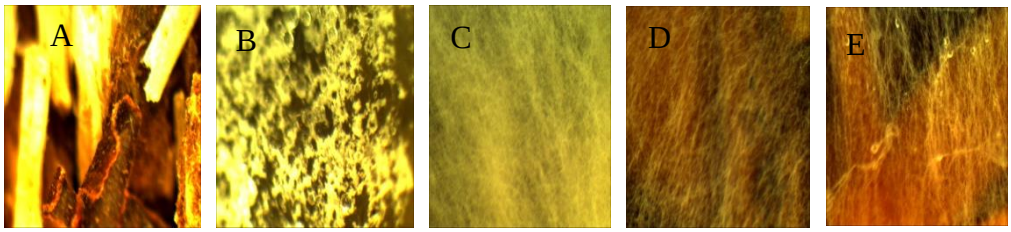
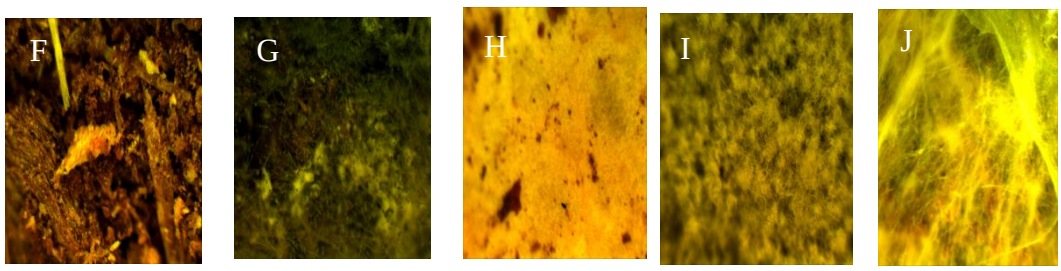


Fig. 1 During submerged fermentation laccase activity in PM containing 1% glucose studied in six different fungal strains induced with aspen bark during lag-phase including: (◊) *P. pulmonaris*, (□) *P. cornucopiae*, (+) *P. ostreatus*, (○) *P. chrysosporium*, (▲) *T. versicolor, and* (▽) *G. mangiferae.*



Mixed Balsam Fir bark *P. Chrysosporium* *P. cornucopiae* *P. ostreatus* *P. pulmonarius*



Mixed Aspen bark *P. chrysosporium* *P. cornucopiae* *G. mangiferae* *P. pulmonarius*

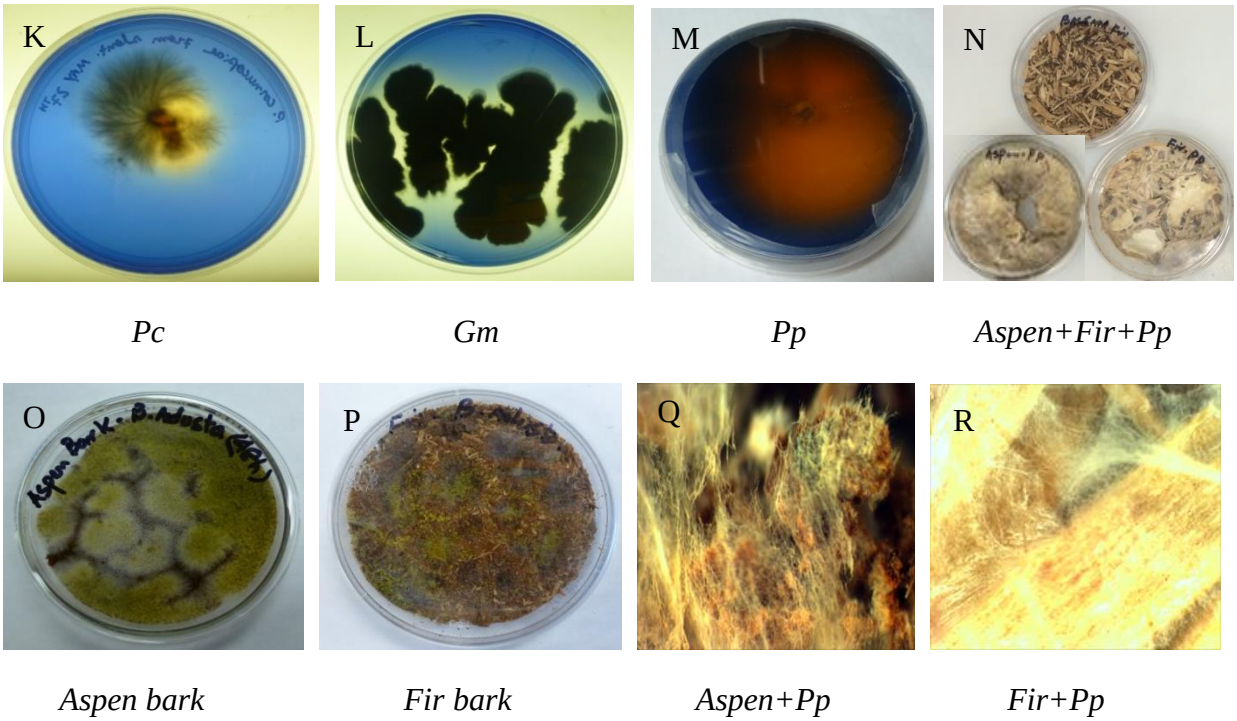
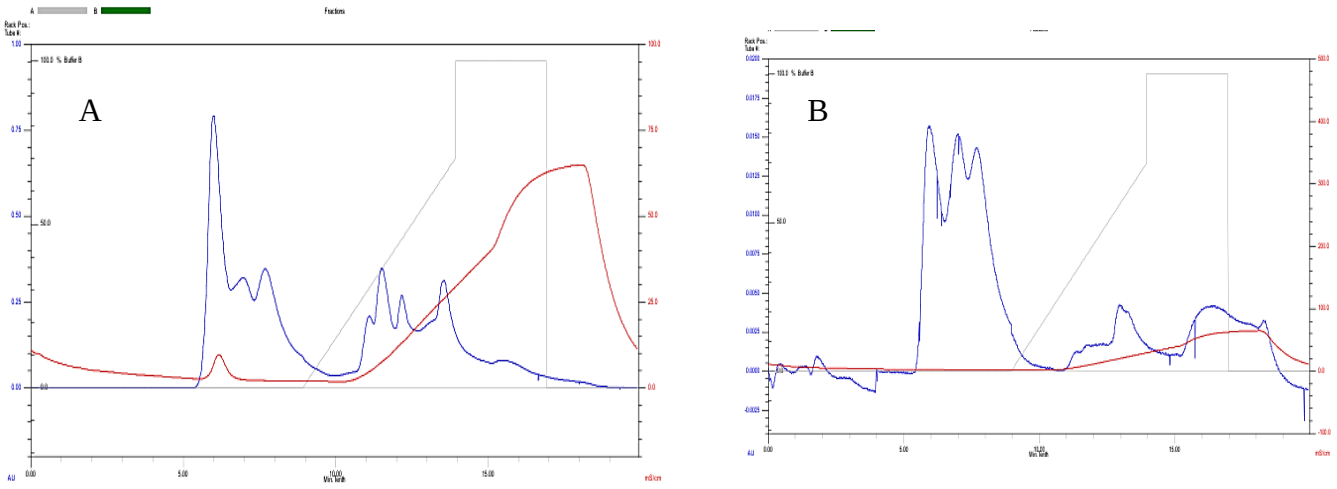


Fig. 2 Different fungal strains growth on mixed balsam fir (A-E) and mixed aspen bark (F-J) while, *P. cornucopiae*, *G. mangiferae*, *P. pulmonarius* (K-M) were exhibiting laccase activity on agar plates containing (2.5% malt extract +0.05%-RBBR). From (N-P) shows fungal growth on the bark surface and (Q-R) presenting bark and *P. pulmonarius* surface morphology was observed under microscope.



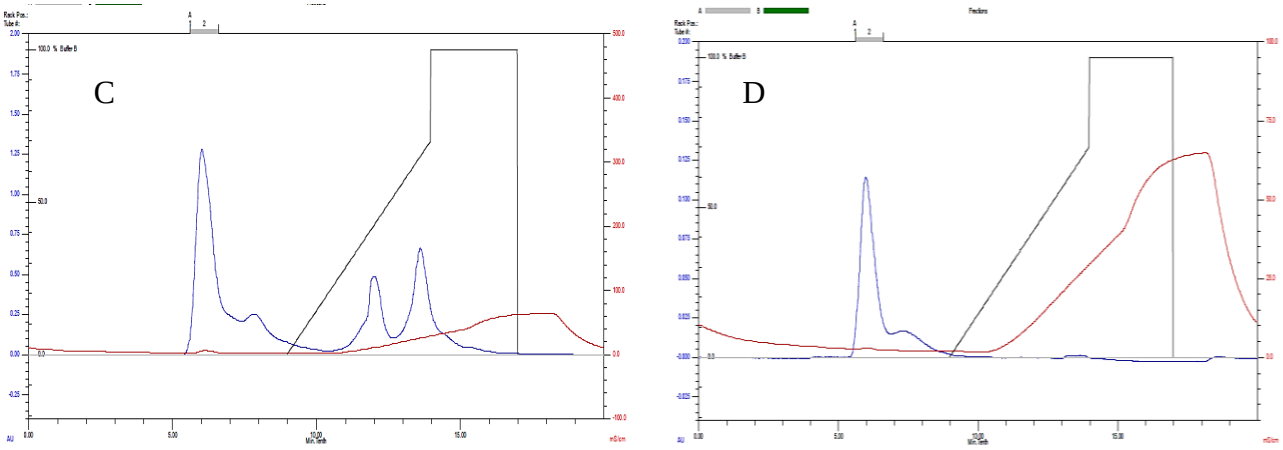
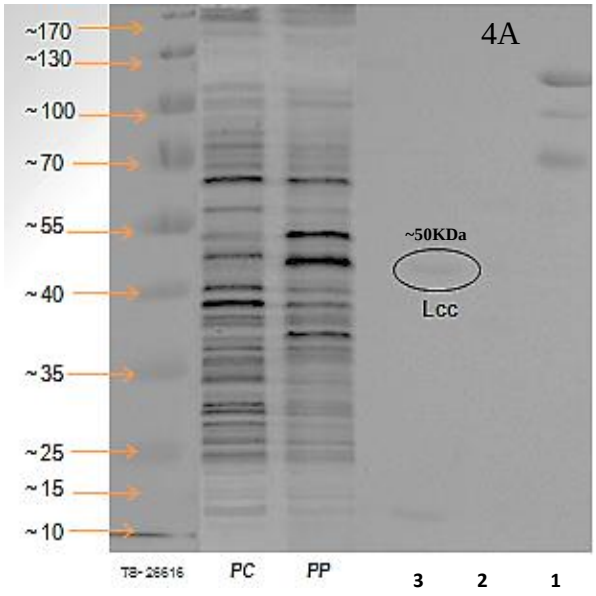


Fig. 3 Laccase purification using FPLC by UNO® sphere Q-1 anion exchange column where (A-D) representing sequential purification steps.



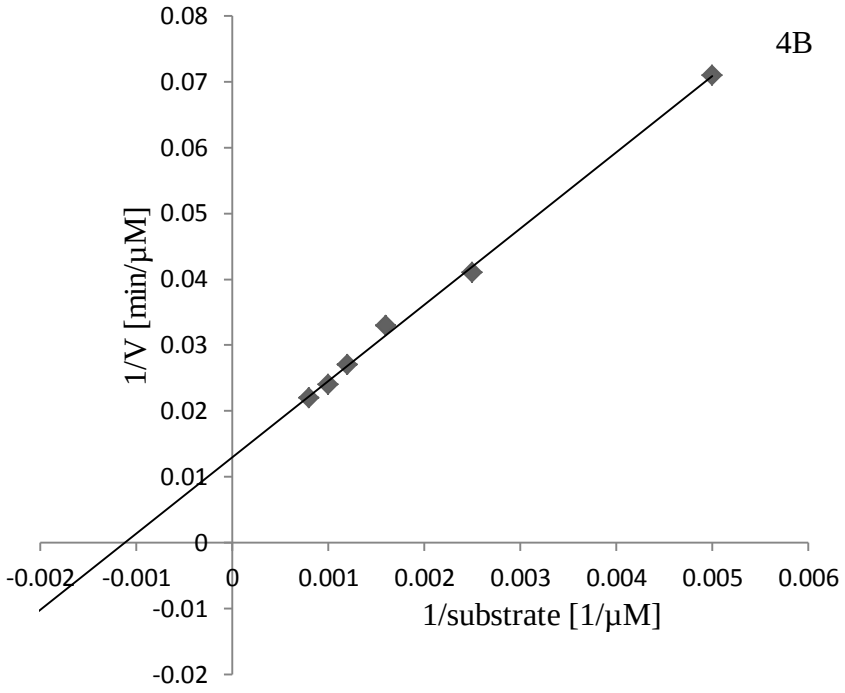
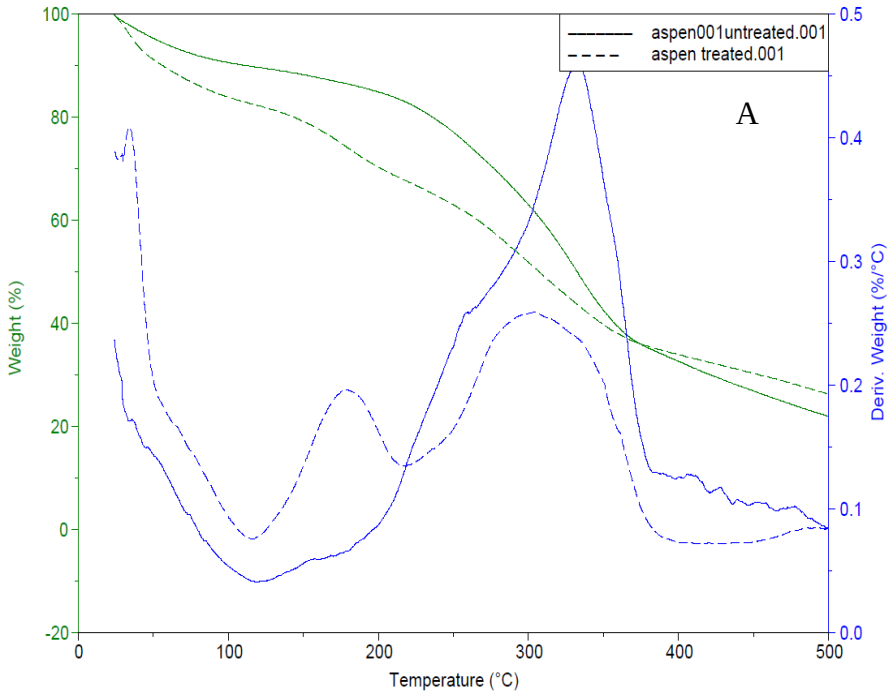


Fig. 4 Purified laccase molecular weight was determined on 10% SDS-PAGE from *P. pulmonarius* stained with Coomassie Blue R-250 showed Mw~50kDa. Lanes 1–2 are serially purified laccase enzyme, lane-3 shows purified laccase enzyme, Pp = total protein from *Pleurotus pulmonarius*, Pc = total protein from *Pleurotus cornucopiae*, and TS26616 for protein standard marker shown in (4A); and draw Lineweaver-Burk plot for the calculation of laccase kinetic parameters (4B).



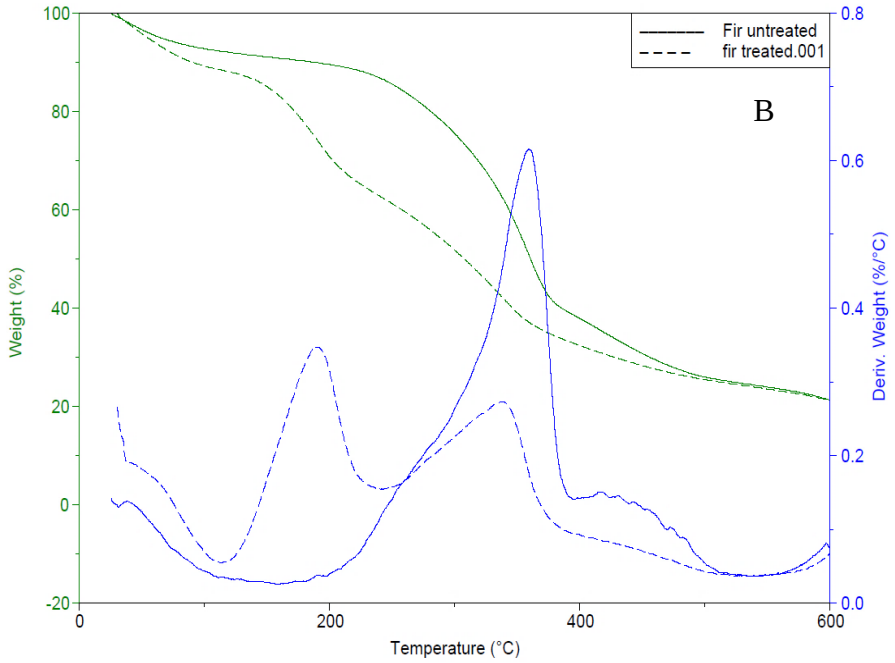


Fig. 5 TG/DTG curves for control/untreated (solid-lines) and fungal-treated (dotted- lines) bark samples (A): Mixed aspen, (B): Mixed balsam fir.

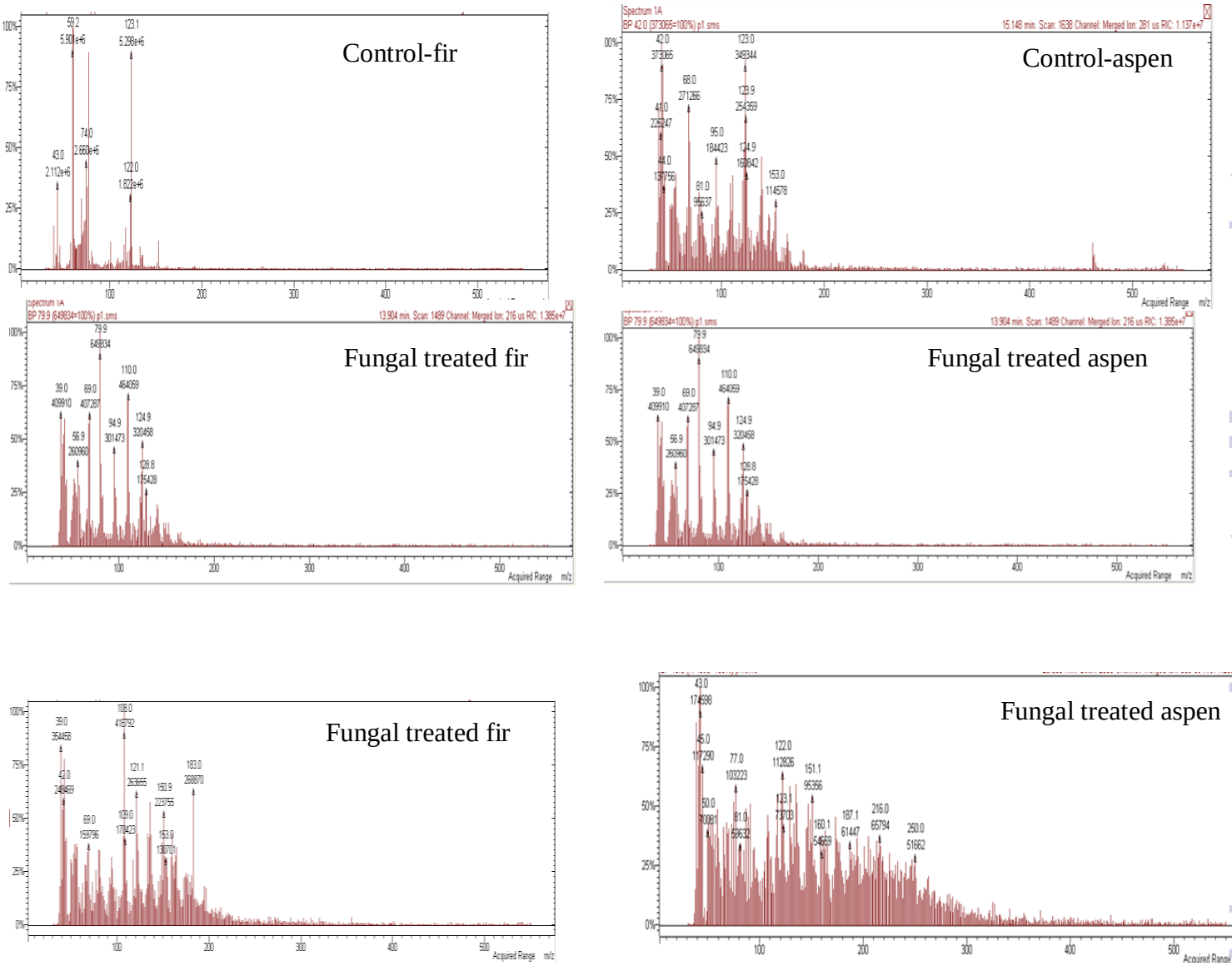


Fig. 6 TIC and MS of identified compounds w.r.t. their t_r-values characterized from both control and each fungal treated bark species are listed in Table 1.

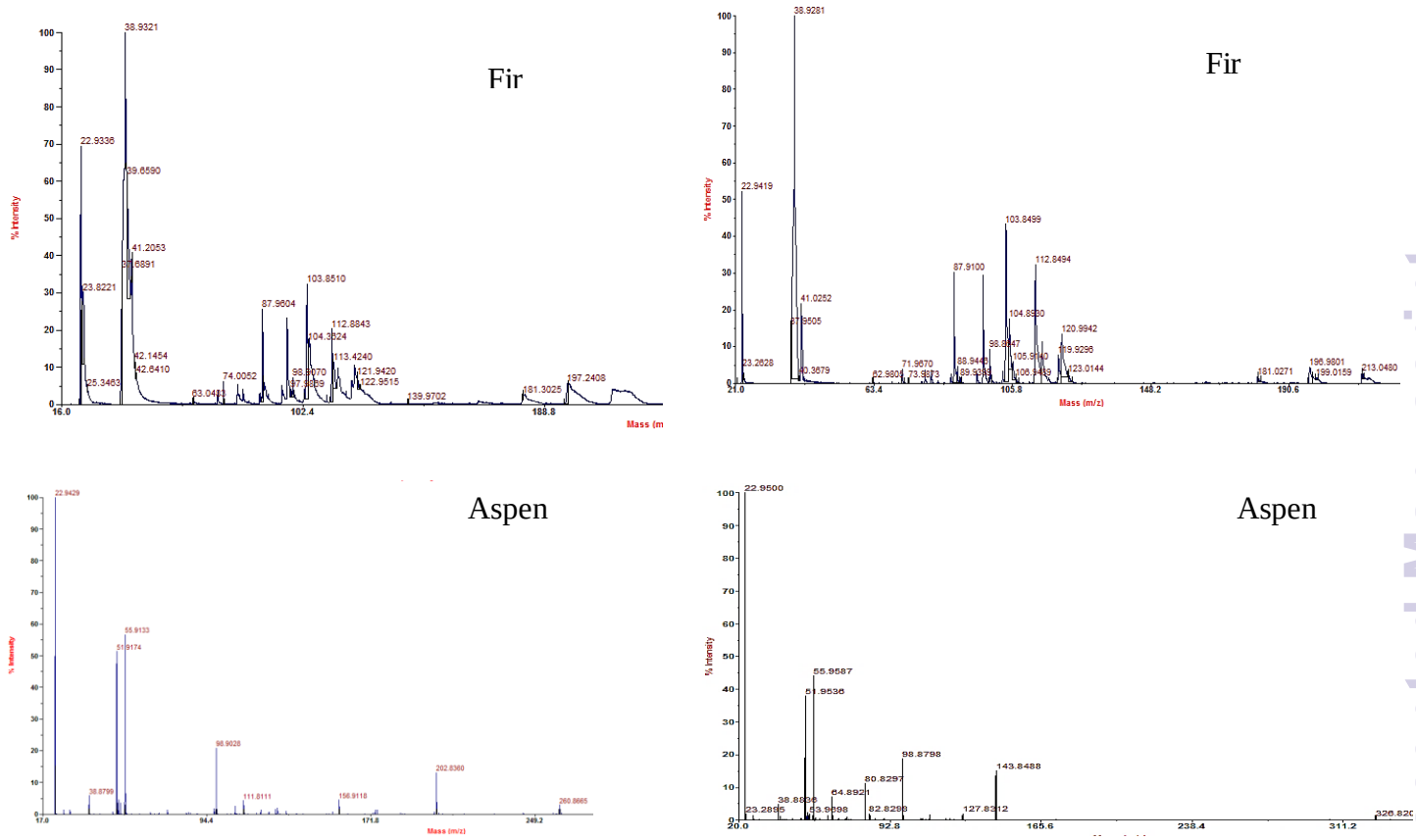
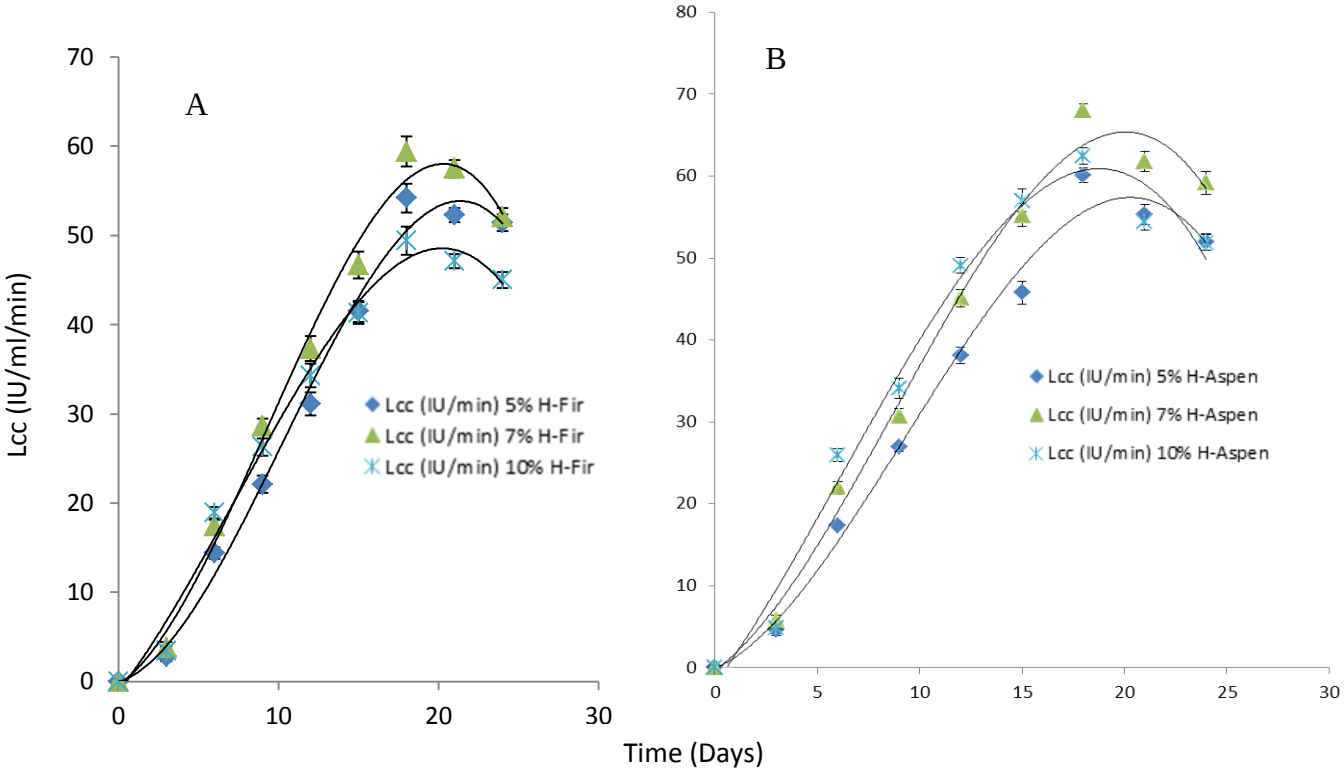


Fig. 7 MWD of each bark species treated by *P. pulmonarius* analyze from MALDI-TOF/MS and the values are enlisted in Table 1.

Table 1 Bark polyphenols/polyaromatics characterization

m/z	Polyaromatics/polyphenols	m/z	Polyaromatics/polyphenols
39, 39.6	CH_2=C=CH anion, Propyne	124.9	Guaiacol, 4-methoxy-1-oxide isocyanato-
41.2	Methyl Isocyanide	127.83	2-Propenoic acid, Oxiranylmethyl ester, 4-Pentenoic acid,
42.1	Propene	128.8	Isoquinoline, 2-propanoic acid
43.01, 44	Iso-cyanic acid	139.97	1-propanol, 3-phenoxy-
56.9	CH_2COCH_3	143.84	2-Butenedioic acid, dimethyl ester
59	CH_3COO^-, Glyoxal	150.9, 151	benzaldehyde, benzoic acid
68	1,3 butadiene, 2-methyl-	128.8	Isoquinoline, 2-propanoic acid
69	vinyl Isocyanate	153	Vanillin, Biphenylene protonated ethyl ester, 3,4-dimethoxy-
	CH_2=CHCH=CHO anion	128.8	Isoquinoline, 2-propanoic acid
74	Methyl propyl ether	160.1	Succinic acid, Isopropyl-Propanedioic acid, ethyl-dimethyl ester, 1,3 propanediol, tert-butyl Propanedioic acid,
77	Phenyl radical, Isopropyl methyl-d3-ether	182.1	Syringaldehyde (SA)
79.9	2-vinyl-1,3 butadiene	183	1,2-benzenediol
81	2-furanyl-CH_2 anion, C_6H_9	187.1	2-ethylhexyl ester
87.9	2-butene-1,4 diol	196.2	Acetosyringone (AS)
96	furaldehyde	199	Propyl ester
97	Isooxazole, 3,5 dimethyl-	202.83	Butanoic acid, Valeric acid, trimethyl silyl ester
98	1,3 butadiene-1-carboxylic acid	212.23	Methyl Syringate (MS), Phenylmethyl ester
104	Propanedioic acid	216	Benzoic acid, benzal-barbituric acid, Diethyl methyl isopropyl malonate, malonic acid, di-isobutyl ester, glutaric acid, ethyl isobutyl ester
108, 112.88, 113.42	Cresol, Quinone, Propanoic acid	250	Adipic acid, cinnamic acid, coniferyl aldehyde, trimethylsilyl ester, glyconic acid
110	Catechol, Resorcinol, HQ, 1,2 benzenediol, furan, 2,3,5-trimethyl-	260.86	L-Valine
121.1, 121.94	3,5-dimethyl-	326.82	Behenic alcohol, Isonipecotic acid, N-Isobutoxy carbonyl, heptyl ester
122, 123	carabamic acid	Weblink	http://webbook.nist.gov/chemistry/mw-ser.html



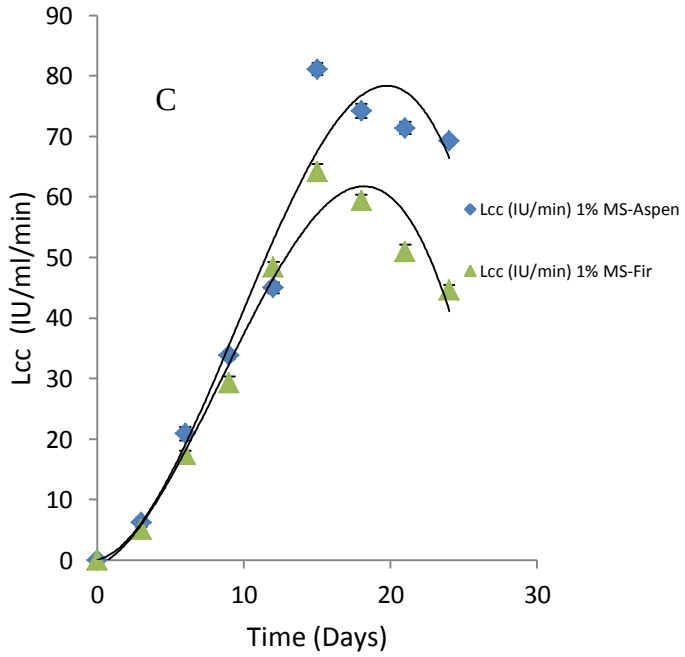
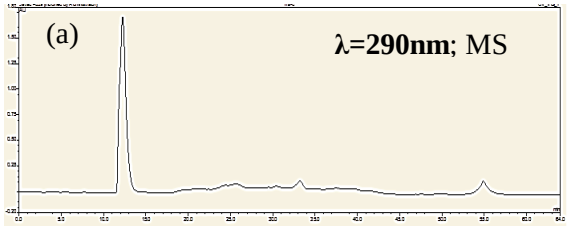
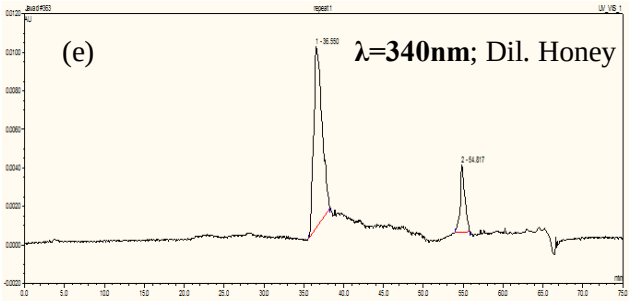
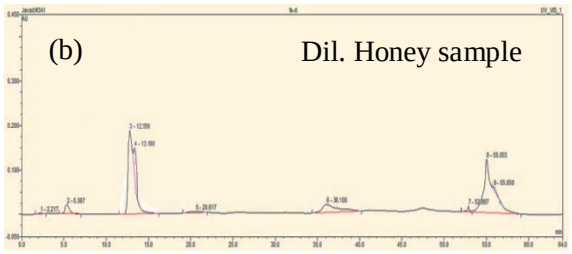
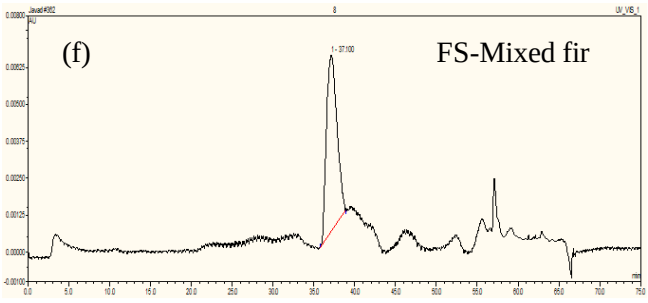


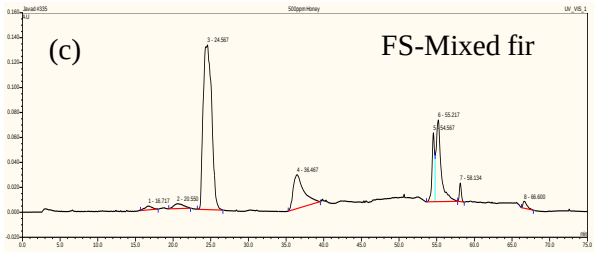
Fig. 8 Time course study for laccase activity in *P. pulmonarius* during SF containing 5, 7 and 10% (v/v) of monofloral honey in production media mainly used as a natural mediator substitute induced with 50mg of each bark in 100ml of PM during lag-phase (A)- Mixed balsam fir, (B)-Mixed aspen bark, and (C)-1% Methyl syringate as NM.

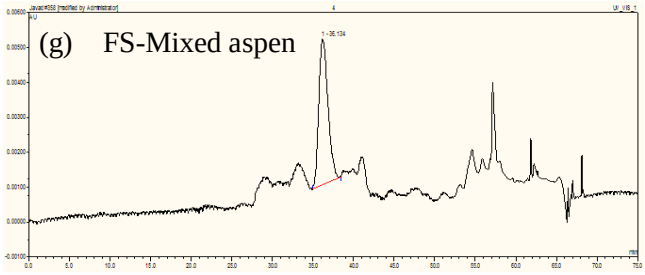


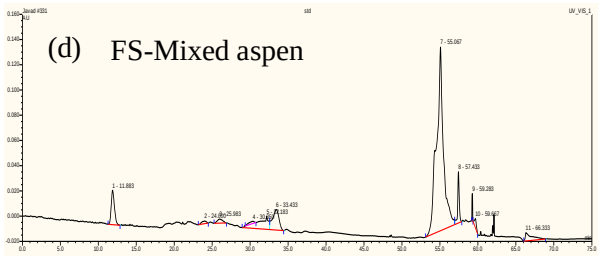












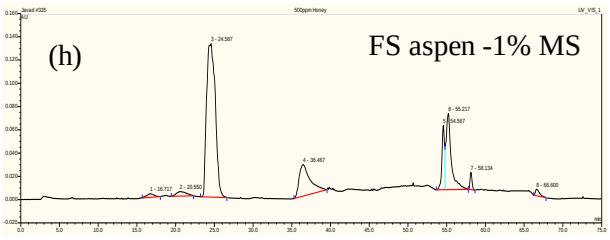


Fig. 9 HPLC chromatograms phenolic profiles of bark fermented samples at 7% honey in production media (a-d at λ_{290}) and (e-h at λ_{340}). Peak identification: methyl syringate (12.017), pinobanksin (13.100), 8-methoxykeampferol (24.567), pinocembrin (36.467), chrysin (39.62), pinocembrin 7-Me (55.217), tetochrysin (57.142). All major peaks were characterized based on their t_r values of honey polyphenolics was previously reported by Pyrzynska *et al.*[36]

Table 2 Total polyphenolics in fermented sample when induced with 50mg of each bark into 100ml of PM during lag-phase

	Samples	Mean total polyphenolics (mg cat equiv/100ml)
Aspen	PM-Gluc	18.4 ± 0.54
	PM-H	23.7 ± 0.61
	PM-MS	27.8 ± 0.48
Balsam Fir	PM-Gluc	20.1 ± 0.81
	PM-H	28.8 ± 0.36
	PM-MS	32.1 ± 0.74

Mean ± S.D (n = 5)

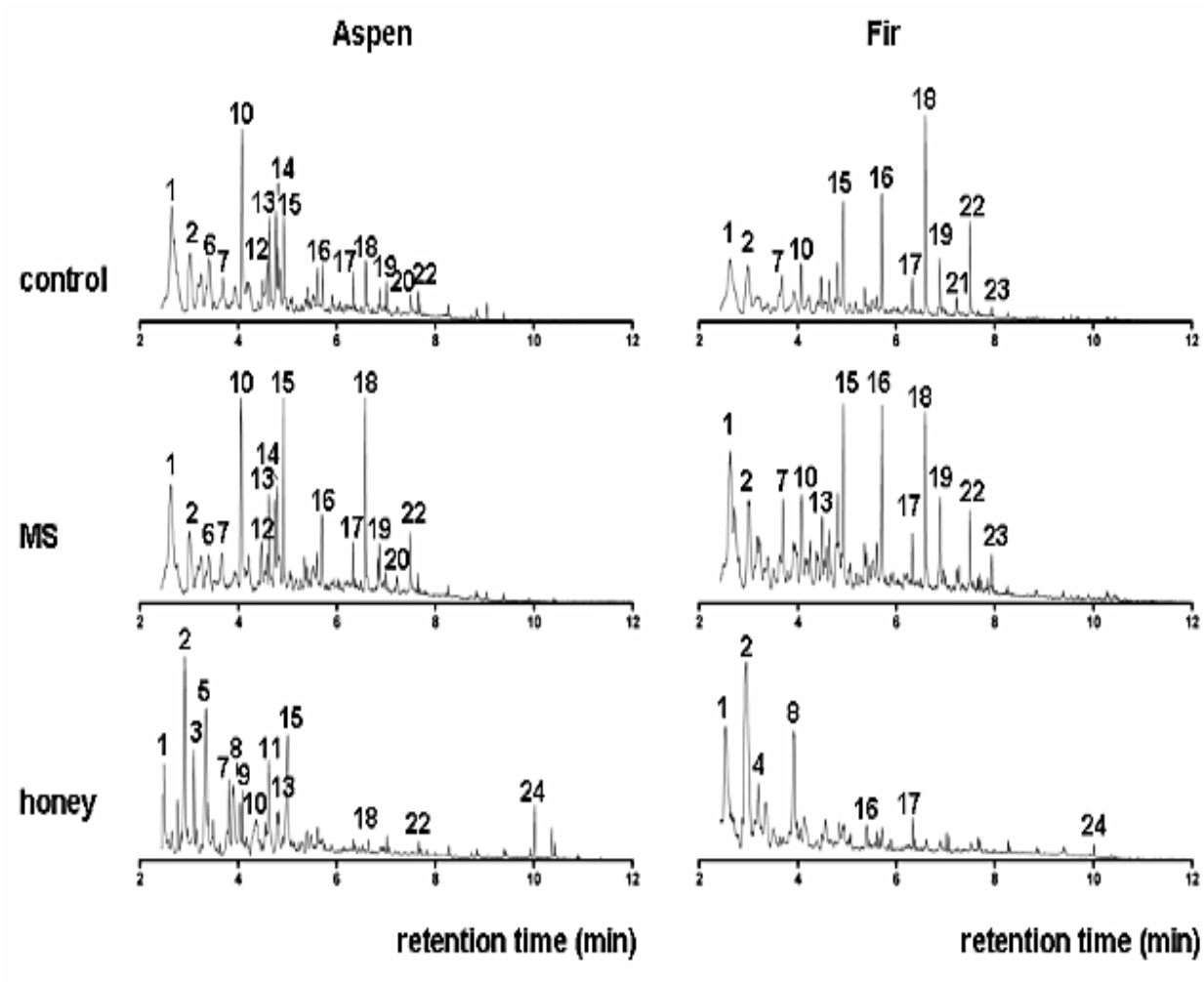


Fig. 10 Total ion chromatograms (TIC) of untreated (control) bark samples of aspen and fir, MS-treated and bark fermented samples in honey production medium. Peak labels refer to peak numbers in Table 3.

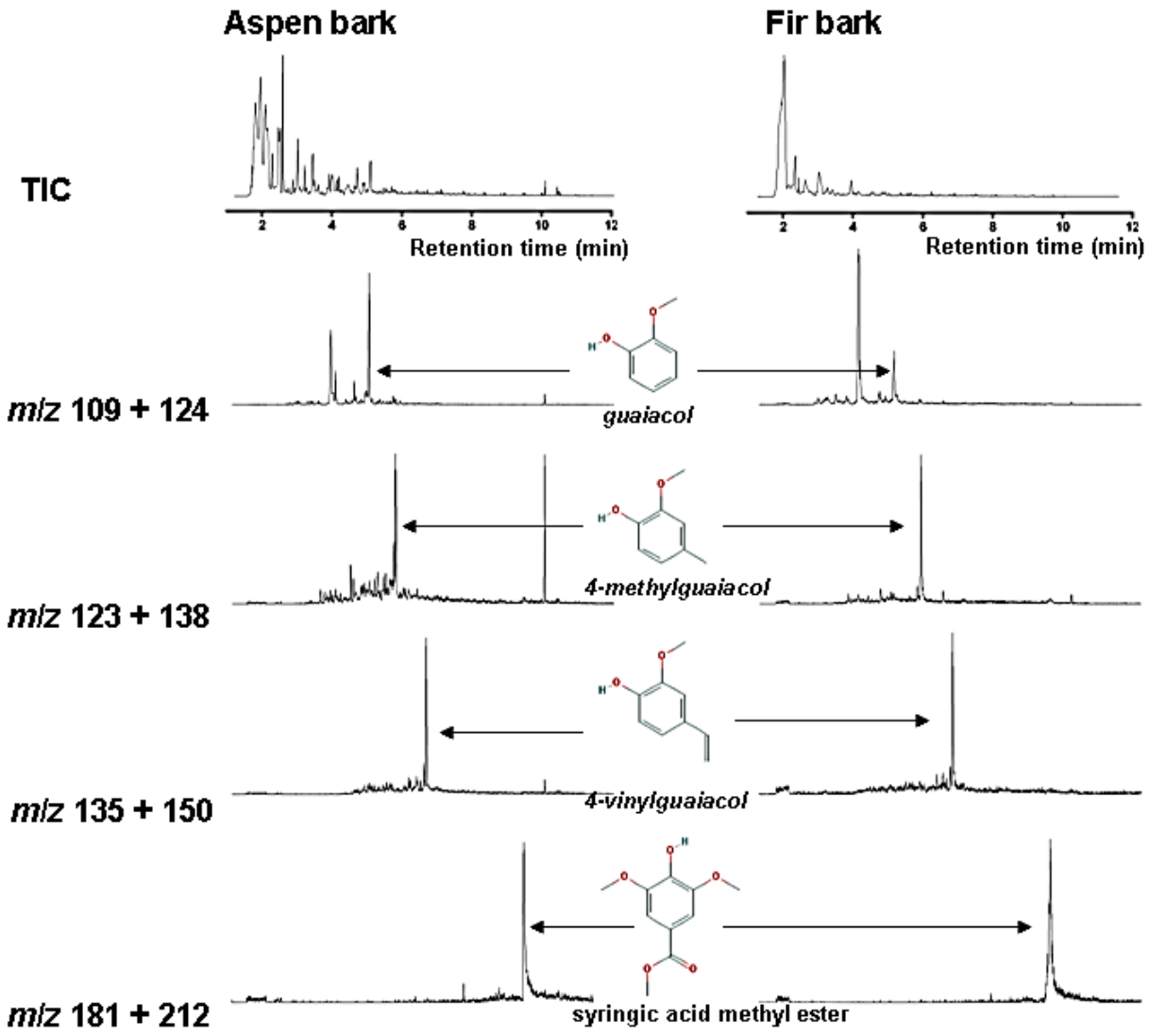


Fig. 11 Partial ion chromatograms (PIC) of selected compounds of polyphenolic origin from each bark fermented samples in PM with monofloral honey used as NMS analyzed by Py-GC-MS. Selected polyphenolic compounds are: guaiacol (m/z 109 +124), 4-methylguaiacol (m/z 123 + 138), 4-vinylguaiacol (m/z 135 + 150) and syringic acid methyl ester (m/z 181 + 212). Chromatograms were plotted between relative peak intensity vs. retention time (t_r).

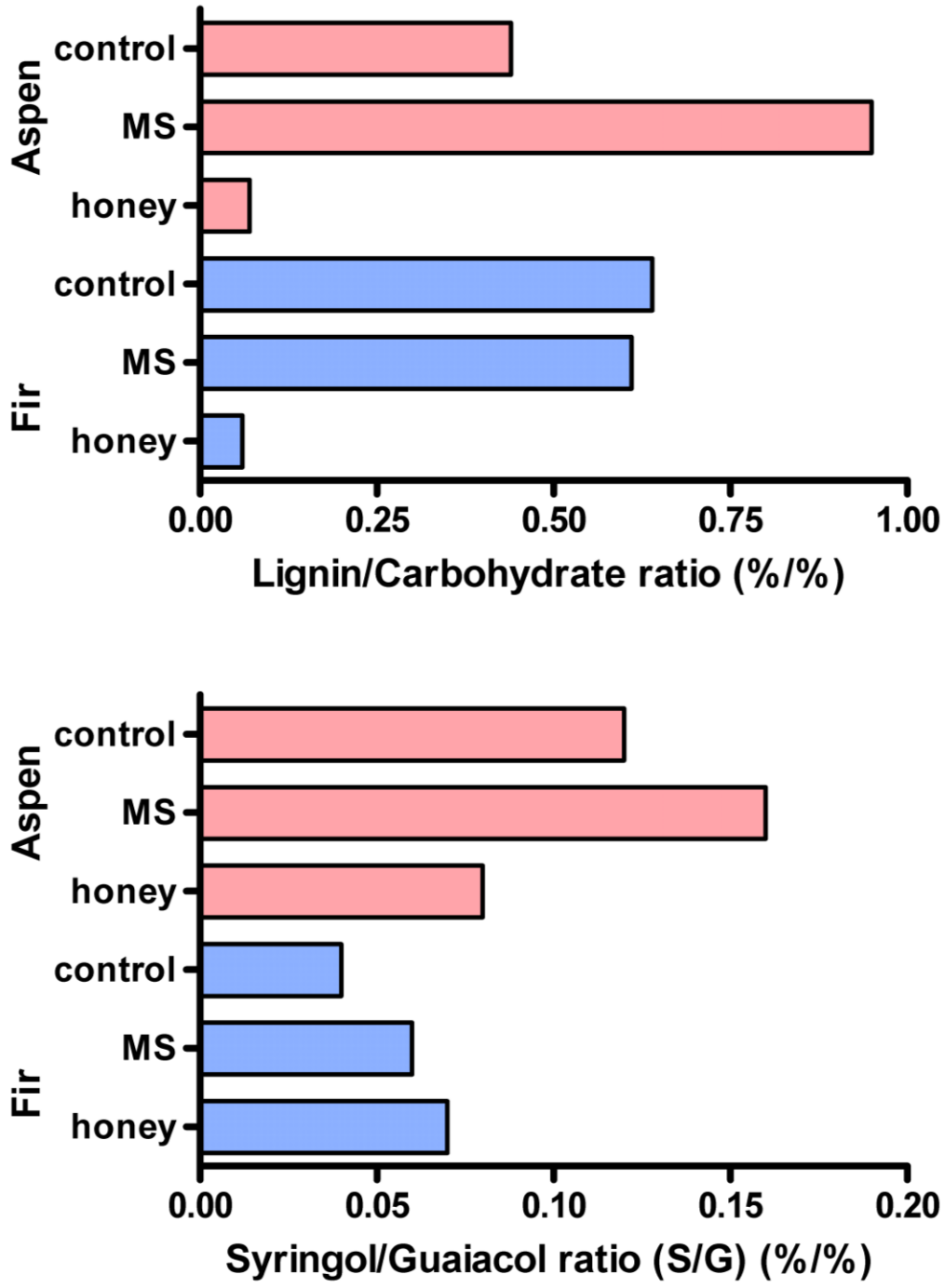


Fig. 12 Representation of the Lignin/Carbohydrate index and Syringol/Guaiacol ratio for the different samples analyzed.

Table 3 List of main pyrolysis products identified from the untreated/control, MS treated and honey fermented bark samples of Aspen and Fir. Percentages refer to the proportion of total quantified peak area (TQPA) accounted for by each compound (sum 100%). Peak numbers refer to labels in Fig.10. Retention time = (t_r), Dominant ion fragments = m/z. Flags are used to organise the compounds in main groups: ALIPH = aliphatic compound, CARB = carbohydrate product, LIG = lignin product, MAH = monocyclic aromatic hydrocarbon, NCOMP = Nitrogen-containing compound, PHEN = phenol, SESQUI = sesquiterpenoid

Peak No	Pyrolysis product	Retention time (t_r) min	m/z	Flag	Mixed Aspen Bark			Mixed Balsam Fir Bark		
					Control	MS treated samples	Honey treated samples	Control	MS treated samples	Honey treated samples
1	toluene	2.535	91, 92	MAH	23.2	19.7	31.4	13.0	20.2	25.1
2	3/2-furaldehyde	2.918	95, 96	CARB	15.0	11.0	24.9	18.5	18.1	46.9
3	C1-pyridine	3.107	93, 66	NCOMP	0.0	0.0	2.9	0.0	0.0	0.0
4	C2-benzene	3.107	91, 106	MAH	7.3	5.6	2.2	3.7	6.6	5.3
5	2-furanmethanol	3.147	98, 97	CARB	0.0	0.0	8.0	0.0	0.0	2.0
6	Styrene	3.404	104, 78	MAH	2.1	1.8	1.8	0.9	1.4	2.0
7	2,3 dihydro-5-methylfuran-2-one	3.896	98, 55	CARB	6.9	7.0	3.6	11.4	10.2	1.4
8	5-methyl-2-furaldehyde	3.939	110, 109	CARB	1.8	1.0	4.5	2.5	3.1	9.0
9	2-methyl-2-cyclopenten-1-one	4.091	96, 67	CARB	0.9	0.7	2.2	8.9	1.6	0.4
10	phenol	4.234	94, 66	PHEN	15.0	15.9	6.8	4.2	4.9	1.8
11	3-hydroxy-2-methyl-2-cyclopenten-1-one	4.486	112, 55	CARB	2.4	3.5	3.5	3.9	4.5	0.8
12	2-hydroxybenzaldehyde	4.583	121, 122	CARB	2.3	1.1	0.3	0.2	0.6	0.2
13	4-methylphenol	4.749	107, 108	PHEN	4.2	4.6	2.9	2.0	2.6	0.6
14	unidentified aliphatic compound	4.766	57, 70	ALIPH	6.5	5.7	0.5	1.4	2.6	0.2
15	guaiacol	5.046	109, 124	LIG	5.0	8.7	2.4	8.4	7.9	1.3
16	4-methylguaiacol	5.784	123, 138	LIG	1.7	2.6	0.2	6.1	5.5	0.9
17	4-ethylguaiacol	6.380	137, 152	LIG	0.9	1.3	0.1	1.7	1.7	1.7
18	4-vinylguaiacol	6.643	150, 135	LIG	2.6	6.5	0.3	8.1	4.6	0.4
19	C3-guaiacol	6.912	164, 149	LIG	0.5	0.9	0.1	1.4	1.1	0.1
20	alkene	7.015	55, 69	ALIPH	0.7	0.4	0.3	0.1	0.2	0.3
21	C3-guaiacol	7.244	164, 149	LIG	0.3	0.5	0.0	0.6	0.4	0.1
22	C3-guaiacol	7.530	164, 149	LIG	0.8	1.5	0.1	2.8	1.7	0.2
23	cf.α-muurolene (sesquiterpenoid)	7.833	105, 204	SESQUI	0.0	0.0	0.0	0.1	0.3	0.0
24	unidentified aliphatic compound	10.010	81, 95	ALIPH	0.0	0.0	1.2	0.1	0.1	0.3

Table 4 Main pyrolytic compound groups and their cumulative relative proportions (% TQPA). The calculated (% TQPA) values of each group were based on Table 3

	Aspen (% TQPA)			Fir (% TQPA)		
Flag	**control**	**MS**	**honey**	**control**	**MS**	**honey**
ALIPH	8.1	6.2	2.0	1.6	2.9	0.8
CARB	30.2	23.2	46.7	45.2	37.5	60.5
LIG	13.2	22.0	3.2	29.0	22.9	3.7
MAH	24.5	27.0	35.4	17.7	28.2	32.3
NCOMP	0.0	0.0	2.9	0.0	0.0	0.0
PHEN	24.0	21.6	9.9	6.4	8.1	2.6
SESQUI	0.0	0.0	0.0	0.1	0.3	0.0