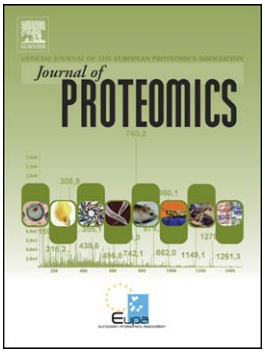


Investigating Aspergillus nidulans secretome during colonisation of cork cell walls

Isabel Martins, Helga Garcia, Adélia Varela, Oscar Núñez, Sébastien Planchon, Maria Teresa Galceran, Jenny Renaut, Luís P.N. Rebelo, Cristina Silva Pereira

PII: S1874-3919(13)00630-1
DOI: doi: 10.1016/j.jprot.2013.11.023
Reference: JPROT 1637

To appear in: *Journal of Proteomics*

Received date: 27 September 2013
Accepted date: 23 November 2013

Please cite this article as: Martins Isabel, Garcia Helga, Varela Adélia, Núñez Oscar, Planchon Sébastien, Galceran Maria Teresa, Renaut Jenny, Rebelo Luís P.N., Pereira Cristina Silva, Investigating Aspergillus nidulans secretome during colonisation of cork cell walls, *Journal of Proteomics* (2013), doi: 10.1016/j.jprot.2013.11.023

Investigating Aspergillus nidulans secretome during colonisation of cork cell walls

Isabel Martins,[a,b] Helga Garcia,[a] Adélia Varela,[a,c] Oscar Núñez,[d] Sébastien Planchon,[e] Maria Teresa Galceran,[d] Jenny Renaut,[e] Luís P.N. Rebelo[a] and Cristina Silva Pereira,[a,b*]

[a] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

[b] Instituto de Biologia Experimental e Tecnológica (IBET), Apartado 12, 2781-901, Oeiras, Portugal

[c] Instituto Nacional de Investigação Agrária e Veterinária, (INIAV), Av. da República, Quinta do Marquês, 2784-505 Oeiras, Portugal

[d] Department of Analytical Chemistry, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain

[e.] Proteomics Platform, Centre de Recherche Public - Gabriel Lippmann, Belvaux, Luxembourg

Corresponding author *

Cristina Silva Pereira

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, EAN, 2781-901, Oeiras Portugal

Tel.+351 211157786

Fax. +351 214411277

E-mail: spereira@itqb.unl.pt

Abstract

Cork, the outer bark of *Quercus suber*, shows a unique compositional structure, a set of remarkable properties, including high recalcitrance. Cork colonisation by Ascomycota remains largely overlooked. Herein, *Aspergillus nidulans* secretome on cork was analysed (2DE). Proteomic data were further complemented by microscopic (SEM) and spectroscopic (ATR-FTIR) evaluation of the colonised substrate and by targeted analysis of lignin degradation compounds (UPLC-HRMS). Data showed that the fungus formed an intricate network of hyphae around the cork cell walls, which enabled polysaccharides and lignin superficial degradation, but probably not of suberin. The degradation of polysaccharides was suggested by the identification of few polysaccharide degrading enzymes (β-glucosidases and endo-1,5-α-L-arabinosidase). Lignin degradation, which likely evolved throughout a Fenton-*like* mechanism relying on the activity of alcohol oxidases, was supported by the identification of small aromatic compounds (*e.g.* cinnamic acid and veratrylaldehyde) and of several putative high molecular weight lignin degradation products. In addition, cork recalcitrance was corroborated by the identification of several protein species which are associated with autolysis. Finally, stringent comparative proteomics revealed that *A. nidulans* colonisation of cork and wood share a common set of enzymatic mechanisms. However the higher polysaccharide accessibility in cork might explain the increase of β-glucosidase in cork secretome.

Key words

Secretome, *Aspergillus nidulans*, cork, Fenton-*like* reactions, lignin degradation, alcohol oxidase

Introduction

Cork, the outer bark of *Quercus suber* L., is a lightweight plant composite displaying remarkable properties, such as elasticity, compressibility, low density and permeability, and significant chemical and microbial resistance [1]. Wine stoppers are still the major industrial application of cork, notwithstanding the significance of other applications, *e.g.* boards for thermal and sound insulation [1]. One striking example of its historic use can be seen in the Convent of the Friars Minor Capuchin (founded in 1560, Sintra, Portugal) where extensive indoor surfaces are coated with cork. Globally, ≥ 300000 tonnes of cork are processed *per annum*, half of which forms the basis of the Portuguese cork manufacturing industries [2].

Despite meaningful differences, cork and wood share a set of similarities, including, among others, comparable composition (Table 1), microbial recalcitrance and potential add-value [3]. Both composites, as well as the wastes produced by the corresponding manufacturing industries, are important from ecological, environmental and economical perspectives [1, 4]. Remarkable progress has been done in the understanding of wood degradation by fungi, recently reaching the omics *era* with several studies reporting the associated transcriptome (*e.g.* [5, 6]) or proteome (*e.g.* [7, 8]).

Cork microbial degradation has been up to now only seldom investigated. Ascomycota have been often recognised as cork main colonisers [9-11] and also associated with its degradation [12-14]. Their ability to perforate the full thickness of the cork cell wall has been previously reported, suggesting that fungi can degrade even the most recalcitrant cork components, namely suberin and lignin [13]. These polymers constitute 40% and 20% of the cork cell wall dry weight, respectively [15, 16]. Suberin is generally accepted to be composed by two domains, one polyaliphatic and one polyphenolic [3, 17]. Accordingly, their degradation by fungi involves esterases [3, 18, 19] and also likely lignin degrading enzymes.

Studies on cork colonising fungi have essentially focus on the appearance of cork taint in bottled wines, in general associated with fungal metabolites which might contaminate cork during manufacturing, transport or storage [20]. Consequently, while significant information has been reported on the species diversity found in these environments, those occurring in field conditions have been often neglected [21]. For long, the Ascomycota *Chrysonilia sitophila* has been considered the dominant colonising fungus during the cork stoppers manufacturing process [20]. However, it is now well established that a more complex colonising community is usually present, which includes, among others, *Penicillium* spp., *Cladosporium* spp., *Aspergillus* spp. and *Mucor* spp. [9-11, 14]. Early studies suggested *Aspergillus* spp. as pioneer species in cork [22] and, more recently, several other Ascomycota (*e.g. Trichoderma* spp., *Penicillium* spp. and *Cladosporium* spp.) were isolated from cork oak tissues [21].

Aspergillus nidulans was herein selected for investigating the fungal secretome after cork colonisation. After twenty one days of incubation, the fungal secretome showed a major accumulation of proteins species associated with the remodelling of the fungal cell wall. However, few polysaccharide degrading enzymes, namely β-glucosidases and endo-1,5-α-L-arabinosidase C, were herein identified. In addition, the identification of alcohol oxidase

(AN9348, annotated as a putative aryl alcohol oxidase) and of several putative lignin degradation products (UPLC-HRMS analyses) suggest that degradation of lignin was mediated by Fenton-type reactions. Overall the data, which included spectroscopic and microscopic analysis of the colonised cork cell walls, suggest the capacity of this fungus to degrade partially polysaccharides and lignin, leaving suberin intact. Better understanding of the enzymatic toolbox used by fungi during cork degradation is essential to promote the use of this renewable resource in bio-refinery.

Materials and Methods

Chemicals: Ammonium persulfate, N,N,N',N'-tetramethylethylene-diamine (TEMED), Remazol Brilliant R (RBBR), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid (ABTS), Rhodamine B (RhodB), trichloroacetic acid (TCA), α-Cyano-4-hydroxycinnamic acid (CHCA) and all the compounds used in the preparation of minimal media (see below) with the exception of NaCl (Panreac, Spain), were purchased from Sigma Aldrich (USA); Coomassie brilliant blue G 250 dye from Fluka (Switzerland); Dithiothreitol (DTT), Triton X-100, Sodium dodecyl sulfate (SDS), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), Iodoacetamide, Bromophenol blue, Glycine, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), IPG buffer (pH 3.5-5.0), Thiourea and Urea from GE Healthcare (Sweden); Trypsin Gold (MS grade) from Promega; Ammonium sulphate from Riedel-de Haën (Germany), Polyvinylpolypyrrolidone (PVPP) from Merck (Germany) and finally, Bradford reagent, Flamingo stain and 30% Acrylamide:Bisacrylamide solution (3.3 %C) from BioRad (USA). All HPLC and MS solvents used were of the highest analytical grade and water was obtained from a Milli-Q system (Millipore).

Materials: Cork and wood powders were sequentially extracted in a soxhlet apparatus, sequentially using dichloromethane (8 hours); ethanol (8 hours) and water (24 hours); then lyophilized and finally sterilised in an excess of water (121 °C, 30 min.). The soxhlet treatment eliminates any possible contribution of plant proteins in the secretome analysis.

Culture conditions: Aspergillus nidulans (FGSC A4) was obtained from the Fungal Genetics Stock Centre and conserved as a suspension of conidia in saline solution (NaCl, 0.85 % w/v) containing glycerol (10 % v/v) at -80 °C. The minimal media (MM), which contains (w/v) 0.1 % K_2HPO_4; 0.03 % $NaNO_3$; 0.0001 % $ZnSO_4•7H_2O$; 0.0005 % $CuSO_4•5H_2O$; 0.005 % $MgSO_4•7H_2O$; 0.0001 % $FeSO_4•7H_2O$; 0.005 % KCl, was prepared as previously described [23].

Fungal cultures (200 mL) in MM supplemented with cork or wood powder (0.1 % w/v) were inoculated with two-day old mycelia previously grown from conidia (10^5 *per* mL) in MM containing 1 % w/v of glucose, and incubated under controlled conditions (27 ºC, dark, 90 rpm). At the end of the incubation (21 days), the solids (*viz.* mycelia and the insoluble substrate) and the extracellular fraction of the fungal cultures were separated by vacuum assisted filtration [23] and conserved at -80 °C (triplicate cultures).

The ability of *A. nidulans* to modify standard substrates, namely RBBR (1 % w/v), ABTS (0.1 % w/v) and RhodB (0.1 % w/v), during growth on solid media was tested (triplicates) as previously described [24]. The cultures were inoculated from conidia suspension with a loop, and the plates incubated at 27 ºC, in the dark, for 7 days. The formation around the colony of a clearing halo in the RBBR media or a green halo in the ABTS media, and of orange halo upon UV illumination (256 nm) in the RhodB media, were taken as an indication of oxidoreductase and esterase activity, respectively.

Morphological analysis: At the end of the incubation the recovered solid fractions of the fungal cultures, composed of mycelia and either cork or wood, were processed for Scanning Electron Microscopy (SEM) [25] and for Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) [26] analyses as previously described. An analytical field emission gun scanning electron microscope (FEG-SEM: JEOL 7001F with Oxford light elements EDS detector) operated at 5-10 kV was used. The FTIR spectrometer (Bruker IFS66/S FTIR), equipped with single reflection ATR cell (DuraDisk) and a Parker Balston 75-52 FTIR Purge Gas Generator, was operated at room temperature, in the range of 4000–600 cm^{-1}, by accumulating 128 scans with a resolution of 4 cm^{-1}. The selected micrographs and spectra should be regarded as representatives (ten replicates).

Extracellular protein extraction: The extracellular proteins were extracted from the culture filtrates (*i.e.* extracellular fraction of the fungal cultures) as previously described [23]. Briefly, the filtrates were first concentrated *c.a.* 100 times using centrifugal concentration devices (Startorius, cut-off 10 kDa), the protein recovered by precipitation (6 volumes of ice cold acetone with 60 mM of DTT, for 1 h at -20 °C), the pellet washed 3 times with 60 mM of DTT in acetone, then dried under soft nitrogen flow and finally resuspended in 1 % (v/v) IPG buffer, containing 4 % (w/v) CHAPS, 2 M thiourea, 7 M urea, 1 % (w/v) Triton X-100 and 60 mM

DTT. Protein quantification was done using the Bradford protocol modified by Ramagli [27], using bovine serum albumin (BioRad) as a standard.

Electrophoresis and image acquisition: Extracellular protein samples (65 µg BSA equivalents) were loaded in precast 13 cm nonlinear IPG strips pH 3-5.6 (GE Healthcare). Protein isoelectric focusing and electrophoresis were performed as previously described [23]. The gels were stained with flamingo dye accordingly to the manufacturer's instructions. Three biological replicates were analysed for each sample. For spot excision, gels were loaded with 100 µg (BSA equivalents) of proteins and stained with colloidal coomassie blue [28].

Image and statistical analyses: The stained gels were digitalized in a Fuji TLA-5100 to generate 64bit images. These were analysed using Progenesis SameSpots v2.0 (Nonlinear Dynamics) and included gel alignment to a reference image. Geometric alignment of the pixel levels, spot measurement, background subtraction and subsequent normalisation were automatically generated; however, when necessary spot detection was refined by manual edition. Protein spots with areas lower than 1500 were excluded from the analysis. Aiming at a stringent statistical analysis as previously described the normalised spot volumes were used for calculating mean values, SD and CV across the two gels sets (cork and wood secretome) (Supplementary File S3) [29]. Only those spots showing high consistency between replicate gels (CV below 25%) were used to generate the list of *p*-values for the individual spots (ANOVA, X-Stat). Those showing *p*-values < 0.05 were considered to constitute significant alterations between the two secretomes and were further analysed using a PCA (X-Stat) (Supplementary File S3).

Protein identification: The protein spots were excised from gels manually, and processed using the Ettan Digester robot of the Ettan Spot Handling Workstation (GE Healthcare) as previously reported [23]. Samples (0.7 µL) were then spotted on MALDI-TOF target plates (Applied Biosystems), before the deposit of 0.7 µL CHCA (7 mg mL^{-1} in ACN 50%, TFA 0.1%). Peptide mass determinations were carried out using the Applied Biosystems 5800 Proteomics Analyzer (Applied Biosystems). Both peptide mass fingerprinting and tandem MS in reflectron mode analyses were performed. The ten most intense peaks were selected automatically and used for MS/MS. Calibration was carried out with the peptide mass calibration kit for 4700 (Applied Biosystems). Proteins were identified, with ProteinPilot, by searching against the NCBInr database (restricted to fungi taxonomy, 1291260 sequences) with Mascot v2.3 (Matrix Science). All searches were executed allowing for a mass window of 150 ppm for the precursor mass and

0.75 Da for fragment ion masses (Supplementary File S4). Search parameters allowed for carboxyamidomethylation of cysteine as fixed modification. Oxidation of methionine and tryptophan (single oxidation, double oxidation and kynurenin) were set as variable modifications, as previously described [30, 31]. Homology identification was retained with probability set at 95 %. All identifications were confirmed manually. Presence of a signal peptide was predicted using signalP (http:// cbs.dtu.dk/services/SignalP/).

Chromatographic and spectrometry analyses of extracellular metabolites: Ethyl acetate / water partition was used to recover extracellular compounds from the filtrates (*i.e.* extracellular fraction of the fungal cultures) (5 mL) [32]. Negative controls, i.e. non-inoculated cork media incubated under the same conditions as the fungal cultures, were tested as well. These extracts were dried under soft nitrogen flow, resuspended in methanol and analysed by ultra-high-performance liquid chromatography-electrospray-high resolution mass spectrometry (UHPLC-ESI-HRMS) using a Q-Exactive Orbitrap MS system (ThermoFisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source (HESI-II). Chromatographic separation was carried out in an UHPLC system (Accela; ThermoFisher Scientific) using an Ascentix Express C18 (150x2.1 mm, 2.7 µm particle size) column from Supelco (USA). The mobile phase, at a flow rate of 300 µL/min, consisted of a solution of 0.1% formic acid (solvent A) and a solution of acetonitrile containing 0.1% formic acid (solvent B), set as follows: 10% B in 1 min, followed by a liner gradient of 10-95% B in 4.7 min, 1.3 min to reach 100% B, 3 min of 100% B, 0.5 min to return to the initial conditions, and 5.5 min to re-equilibrate the column. HESI-II was operated in negative ionization mode. Nitrogen was used as a sheath gas, sweep gas and auxiliary gas at flow rates of 60, 0 and 10 a.u. (arbitrary units), respectively. Heater temperature was set at 350 ^{o}C. Capillary temperature was set at 320 ^{o}C and electrospray voltage at -2.5 kV. A S-Lens RF level of 50 V was used. Q-Exactive Orbitrap MS system was tuned and calibrated using ThermoFisher calibration solution once a week. The HRMS instrument was operated in full MS scan with a *m/z* range from 50 to 600, and the mass resolution tuned into 70,000 full width half maximum (FWHM) at *m/z* 200, with an automatic gain control (AGC) target (the number of ions to fill C-Trap) of 5.0E5 with a maximum injection time (IT) of 200 ms. The full MS scan was followed by a data-dependent scan operated in All Ion Fragmentation (AIF) mode with a fragmentation energy applied of 30 eV into the high-energy collision dissociation (HCD) cell. At this stage, mass resolution was set at 17,500 FWHM at *m/z* 200,

AGC target at 5.0E5, maximum IT at 200 ms, and the scan range also from *m/z* 50 to 600. MS data was processed by ExactFinder^{TM} 2.0 software (ThermoFisher) by applying a user target database list. Several parameters such as retention time, accurate mass errors and isotopic pattern matches were used to confirm the identity of compounds. When available, standards compounds were also analysed by UHPLC-ESI-MS to better confirm the identity.

Results and Discussion

The mechanisms used by *Aspergillus* spp. during cork colonisation remain largely overlooked, despite early evidence of the species significance in cork colonisation [22]. In the present study, *A. nidulans* showed oxidoreductase and esterase activity when grown in RBBR or ABTS media and RhodB media, respectively (Supplementary Fig. S1).

Analysis of cork after colonisation with Aspergillus nidulans

A suspension of cork powder (0.1 % w/v) in minimal media was inoculated with two-day old *A. nidulans* mycelia and recovered after twenty-one days of incubation for SEM analysis. The mycelia formed an intricate network of hyphae that colonised the surface of the cork cell walls (Fig. 1A and 1B), where numerous conidia (Fig. 1C and 1D) and ascospores (Fig. 1E and 1F) were deposited. Similar observations were attained in the wood control (data not shown). A comparable hyphal colonisation profile on cork has been reported before in Ascomycota [14], even though in some cases hyphae perforating the cork cell walls have been detected [13]. The heterothallic *Penicillium* spp. has also formed conidia during the colonisation of cork [14]. In the present study, it remains unclear if *A. nidulans* sexual spores were produced by the submerged mycelia (rarely observed [33]) or in the floating mycelial mat.

ATR-FTIR has been used for assessing the degradation provoked by microbial attack of numerous plant composites [34]. The spectral profiles of the untreated cork and of *A. nidulans* mycelia showed that several peaks cannot be exclusively assigned to either constituents of cork or mycelia (Fig. 2, Supplementary Table S2). Aiming to normalise the interference provoked by the mycelia, the ATR-FTIR spectral profiles of cork recovered after two- or twenty-one days of fungal colonisation were compared (Fig. 2). In general, the peaks mostly attributed to the long aliphatic chains (2923 and 2851 cm^{-1}) and ester moieties (1738 cm^{-1}) of suberin (which can be almost exclusively associated with cork) showed a minor increase along the colonisation period. The likely enrichment of suberin suggests that the remaining cork constituents were

preferentially degraded by the fungus. The stretching of alkene bonds (1509 cm^{-1}) typical of aromatic compounds in lignin showed no major alterations. In addition, the polysaccharides spectral region was dominated by peaks assigned to the mycelia masking those assigned to cork (Supplementary Table S2).

Overview analysis of Aspergillus nidulans cork and wood secretomes

Aspergillus nidulans extracellular proteins (secretome) after cork colonisation were analysed using a 2DE approach. In addition, as a control, *A. nidulans* secretome after growth in wood media was also analysed. IPG strips with a 3-5.6 pH range were used for the first dimension, since preliminary tests showed that, in general, the bulk of the polypeptides were detected at pH values lower than 6 (data not shown). The protein yield of the method employed for protein extraction was reasonably similar in both secretomes. These data, as well as the maximal and minimal number of spots detected after the 2DE separation of the protein extracts is depicted in Table 2.

Even though fungi are known to fine tune their secretome in function of the substrate [35], *A. nidulans* secretome in cork and in wood displayed very similar 2DE maps (Fig 3. and Supplementary File S3); consequently few differentially accumulated protein species (see below). A global analysis of the cork secretome will be first presented, followed by a comparative analysis of this secretome with that of wood. Amongst the proteins herein identified in the cork and wood secretome, no plant proteins existed. Probably due to the fact that both plant substrates were submitted to solvent extraction prior to their use as substrates for fungal growth.

Analysis of Aspergillus nidulans cork secretome

Aspergillus nidulans secretome in the cork media showed 171 spots, from which 156 showed to be statistically representative, *i.e.* highly consistent and reporting CV values below 15% (Fig. 3, Supplementary File S3). From these spots, 155 were manually excised from the gel. Protein identification retrieved 28 unique protein species which are listed in Table 3 (Supplementary File S4) (in a small set of spots, two protein species were identified). As frequently reported in fungal secretomes, multiple isoforms of the same protein species were herein detected. This is generally related to posttranslational modifications, of which glycosylation is the most frequently reported [36] and in some cases also to proteolysis [37]. The identified proteins were grouped according to their biological functions, namely fungal cell wall remodelling enzymes, plant cell

wall degrading enzymes, defence/stress response, proteases/nitrogen and miscellaneous (Table 3). More than 60% of the protein spots in the secretome were identified as cell wall remodelling enzymes (49%) or plant cell wall degrading enzymes (12%). In a sub-set of the excised spots (24%) no protein identification could be obtained, probably, as previously suggested due to the presence of posttranslational modifications that hampered protein identification [38]. In addition, some protein spots retrieved low Mascot scores and were not considered for the analysis (4%).

Fungal cell wall remodelling enzymes

The fungal cell wall accounts for a great percentage of the cell dry weight and plays an essential role in maintaining its shape, counteracting the turgor pressure and protecting the plasma membrane against mechanical damage [39]. The cell wall of *A. nidulans* is composed of an inner layer of cross-linked polysaccharides, mainly chitin (polymer of β-1,4-*N*-acetylglucosamide) and β-1,3- and α-1,3- glucans, with minor amounts of β-1,6- and β-1,4-glucans and an outer layer composed of highly glycosylated proteins and mannans [40]. The mannans (polymers of mannose linked by α-1,6, α-1,2, α-1,3 or β-1,2 linkages) and the highly glycosylated proteins can be either cross-linked or covalently bounded to glucans [39].

Chitinase B (ChiB, AN4871), which catalyses hydrolysis of β-1,4-glycosidic linkages in chitin, was identified in 65 protein spots(Table 3), accounting for 40% of the total volume of protein spots in gel. Eighteen genes putatively encoding chitinases have been annotated in the genome of *A. nidulans* [41, 42], some of which have been shown to be differentially regulated along the fungal growth cycle. In particular, ChiB mRNA levels have been shown to increase when the fungus reaches the stationary phase of growth [43]. More than 90% of extracellular chitinase activity measured in *A. nidulans* autolysing cultures was attributed to ChiB [43-45]. This protein, apparently induced by carbon-starvation conditions and conidiation, constitutes a strong evidence that *A. nidulans* was undergoing autolysis at the twenty-first day of growth on cork. High spectral counts of ChiB peptides have been reported in *A. nidulans* secretomes after prolonged growth on different lignocellulosic substrates (sorghum stover [46], leaf litter [47] and maize bran [48]). Similar to our finding, ChiB high abundance in the sorghum stover secretome was related to autolysis [46]. The β-1,3-endoglucanase EngA (AN0472) plays also a major role during autolysis and its expression is tightly correlated with that of ChiB [49]. EngA was herein

detected in one spot in the cork secretome, in agreement with the aforementioned studies [46-48].

Amongst the protein species identified in the cork secretome, some hydrolases, namely *N*-acetylglucosaminidase (NagA, AN1502) and alkaline protease (prtA, AN5558), further corroborate that *A. nidulans* was probably undergoing autolysis. The coordinated expression of these hydrolases and of ChiB has been reported before in *A. nidulans* [43, 50]. They are thought to be associated with the degradation of empty hyphae supporting fungal growth under carbon starvation conditions [51, 52]. In particular, NagA have been suggested to mediate the breakdown of chito-oligomers [45, 53]. PrtA role was inferred since deletion of the encoding gene has significantly decreased proteolysis during autolysis [51]. Additional proteases were found in the cork secretome (AN2366, AN2903 and AN3918), similar to that reported in the secretome of *A. nidulans* grown on sorghum stover or on leaf litter [46, 47]. Their involvement in the hydrolysis of secreted proteins has been suggested in the secretome of fungi other than *A. nidulans* [37]. Some proteins species in the cork secretome were probably induced by carbon starvation conditions, such as glutaminase A (AN4809) [52] and acid phosphatase (AN4055) [54]. The last is also known to be induced by an inorganic source of nitrogen [55].

Several β-1,3-glucanases, namely two exo-β-1,3-glucanases (AN4825 and AN7950), one α-1,3-glucanase (AN9042) and one glucanase hydrolysing β-1,3- and β-1,4- glucans (AN0245) were identified in the cork secretome. Their involvement in the degradation of cell wall glucans during autolysis has been suggested as levels increased in a autolysing *engA* deleted *A. nidulans* mutant [49]. The identification of the β-1,3-glucosidase EglC (AN7950) might be related to sexual development (Fig. 1E and 1F), since the corresponding mRNA levels have been shown to increase during early sexual developmental [56].

The presence of mutanase (AN7349) might imply that the α-1,3-glucans accumulated during vegetative growth were used as glucose reserves during sexual development [57]. It is well-known that ascospores, which are more resistant than conidia, are usually formed in adverse growth conditions [58]. Thus, most likely, sexual development was triggered in *A. nidulans* due to the increasing recalcitrance of cork along the twenty-one days of colonisation. This protein species has been also detected in the *A. nidulans* leaf litter secretome after seventeen days of growth [47] and, although at lower abundance, also in that of sorghum stover after fourteen days of growth [46]. The colonisation profiles of the substrates at these time points were not

evaluated; consequently in these studies mutanase accumulation was not associated to sexual development.

An extensive hyphal network was still detected on cork at the twenty-first day of incubation (Fig. 1A and 1B). Catalase B (AN9339) identification in fungal secretomes (*e.g.* [46]) has been implicated in the detoxification of hydrogen peroxide, thus strongly associated with growing and developing hyphae [59]. Its identification in the cork secretome (8 protein spots) supports the idea that *A. nidulans* was actively growing on cork until the end of the incubation.

Fungal enzymes involved in the degradation of the plant cell wall

To better understand cork degradation, its composition (Table 1) needs to be revised in greater detail. The polysaccharides constitute approximately 20% of the cork cell wall dry weight, which in turn are essentially composed of glucose (50-70%) and xylose (20-35%), with minor amounts of arabinose, mannose and galactose [16]. Cork contains cellulose (*ca.* 9-10%), hemicellulose (*ca.* 15%) and pectin (*ca.* 1.5%) [16].

The degradation of polysaccharides by fungi involves the synergistic action of several glycosyl hydrolases that first release oligomers from the polymer backbones, which are in turn cleaved to their corresponding reducing sugars [35]. In the cork secretome three β-glucosidases (AN4102, AN7396 and AN2828) were identified (10 protein spots). This class of enzymes specifically breaks the β-1,4-linkage between two glucose residues. Their presence in the cork secretome clearly emphasises that *A. nidulans* degraded cellulose during cork colonisation.

The hemicellulose fraction of cork is mainly composed by xylans, namely 4-*O*-methyl-glucuronoxylan, arabino-4-*O*-methylglucuronoxylan and 4-*O*-methylglucurono-arabinogalactoglucoxylan [16]. Typically, these are polymers of β-1,4-xylose, ramified to β-1,4-, β-1,3-, β-1,6- or α-1,2- sugar chains containing xylose, arabinose, glucose, galactose, 4-*O*-methylglucuronic acid or rhamnose. The protein encoded by AN9380 has been putatively identified as bifunctional, displaying both chitin deacetylase and xylanase activity [46]. Its role in the cork secretome remains unclear. Though likely associated with autolysis [60], it can be involved in the degradation of xylans as suggested before in an *A. nidulans* autolysing culture growing on sorghum stover [46]. The identification of ferulic acid in the extracellular media after cork colonisation (Table 4) can be also related with the degradation of xylans. Diferulic bridges can mediate the linkage between xylans and lignin or between xylan chains [35].

Despite that cork only contains minor amounts of pectin, its degradation by *A. nidulans* was highlighted by endo-1,5-α-L-arabinosidase C (AN8007) (5 protein spots). The degradation of pectins in the cork cell walls by the fungus *Armillaria mellea* lead to the separation of the cell wall at the middle lamella level [61]. In the present study, pectin degradation was not sufficient to thin the middle lamella of the cork cell walls (Fig. 1).

Taken together, the polysaccharide degrading enzymes herein identified emphasise the capacity of *A. nidulans* to degrade cork polysaccharides, even pectins which are only present in minor amounts. All these enzymes have been previously identified in *A. nidulans* secretomes during colonisation of sorghum stover [46], leaf litter [47] and maize bran [48]. Even if not all the polysaccharide degrading enzymes identified in the previous studies (gel free proteomics) were herein detected in the cork secretome. Most likely the high abundant proteins masked the low abundant ones in the gel. Protein low abundance in the gels was taken as the main reason for the lack of cellulases and hemicellulases in the secretome of *Gloeophyllum trabeum* after prolonged growth on wood [8].

The accumulation of a set of *A. nidulans* substrate-specific proteins becomes apparent when different secretomes are compared (172, 1 and 19 in sorghum stover [46], leaf litter [47] and maize bran [48], respectively). Most of the dissimilarities in the corresponding secretomes have been associated with differences in substrate composition and incubation conditions. Despite the higher polysaccharide content in leaf litter [62], when compared to that of cork, the incubation conditions were similar to those of the present study. In general, the polysaccharide degrading enzymes identified in the cork secretome, increased along the incubation in the leaf litter secretome, reaching very high spectral counts at the seventeenth day [47]. The lack of some, namely β-1-4-endoglucanase (AN1285) and pectin lyase (AN2569), might be due to their p*I*, which was out of the range analysed here. Unobserved in the cork secretome were any β-1-4-endoglucanases and β-1-4-xylosidases, which have been identified in the secretome (gel-dependent) of the Ascomycota *Fusarium graminearum* grown on barley and wheat flour when a shorter incubation period was used [63]. In addition, the few lipases identified in the *A. nidulans* secretome grown on leaf litter reported very low spectral counts at the seventeenth day [47]. This agrees with the high recalcitrance of cutin, which shares high similarity with the polyaliphatic suberin [3]. It seems reasonable to hypothesise that *A. nidulans* failed to degrade the polyaliphatic suberin in cork, as suggested above by the spectroscopic analysis (Fig. 2).

However, one cannot disregard that the release of ferulic acid (Table 4), which is also present in suberin [16], was related to its degradation.

Lignin accounts for approximately 20% of the weight of the cork cell walls. Lignin is a branched polyaromatic polymer constituted of variable proportions of phenylpropanoid units [15] predominantly linked by β-*O*-4-linked ether bonds that are not easily hydrolysable. This polymer is known to impact on the cellulose digestibility mainly due to their cross-linking in the cell wall [64]. In cork cell walls, lignin is especially abundant in the primary wall, while the tertiary wall (outer cell wall layer) is essentially composed by polysaccharides [16]. Overall, the main phenylpropanoid units identified in cork are guaiacyl (95 %), followed by syringyl (3 %) and *p*-hydroxyphenyl (2 %) [15, 16]. Lignin composition in softwoods is very similar, with the exception of syringyl units which are lacking [15].

Lignin degradation by *A. nidulans* has been suggested since during growth on sorghum stover, at least one tyrosinase (AN5311) and two feruloyl esterases (AN1772 and AN5267) were identified. Even though these enzymes were undetected in the cork secretome several oxidoreductases were identified, agreeing with the ability of this fungus to oxidise RBBR and ABTS (Supplementary Fig. S1). These were three FAD-linked oxidoreductases (AN7269, AN3351 and AN9348) (8 protein spots), including AN9348, an alcohol oxidase which has been annotated as a putative aryl-alcohol oxidase in *A. nidulans* genome database (http://www.aspergillusgenome.org/). Alcohol oxidases, as well as aryl-alcohol oxidases, have be associated with the generation of hydrogen peroxide required either in the catalytic cycle of lignolytic peroxidases (white rot fungi, *e.g. Phanerochaete chrysosporium*) or in Fenton-type reactions (brown rot fungi, *e.g. Pleurotus eryngii* and *Postia placenta*) [65, 66]. The genome of *A. nidulans* contains genes putatively encoding for peroxidases [42, 67]. However, none of these protein species were identified in the cork secretome, in agreement with that observed on comparable secretomes [46-48].

It is well-known that Fenton-type reactions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + {}^{-}OH$) require a mechanism for extracellular hydrogen peroxide generation and for reduction of Fe^{3+} to Fe^{2+} [68]. The generation of extracellular hydrogen peroxide during cork colonisation by *A. nidulans* was probably ensured by the activity of the alcohol oxidase. The reduction of Fe^{3+} might be accomplished by extracellular enzymes such as cellobiose dehydrogenase or by extracellular low molecular weight compounds (*e.g.* ferulic acid or hydroquinones [69, 70]). Cellobiose

dehydrogenase (AN7230) accumulated in the secretome during *A. nidulans* growth of sorghum stover [46]. This enzyme was however undetected when *A. nidulans* was grown on cork. Oxygen is an inhibitor of cellobiose dehydrogenase activity [71] and its availability was probably significantly higher in the cork media, when compared to that of sorghum stover (submerged culture with agitation and solid state culture, respectively).

Aiming to corroborate that lignin degradation occurred during cork colonisation by *A. nidulans* the diversity of aromatic compounds in the culture filtrates (*viz.* extracellular media of the fungal cultures) were analysed by UHPLC-ESI-HRMS (Table 4). Non-inoculated controls of the cork media were also analysed. The identification of ferulic acid, veratraldehyde and cinnamic acid (as well as of ellagic acid, which is extremely abundant in cork), by ExactFinder^{TM} software was confirmed using pure standard compounds. Veratraldehyde formation, as well as that of cinnamic acid, might be related to lignin degradation [69]. Ellagic acid and ferulic acid were also found in the control. However, ferulic acid concentration in the culture filtrates was *ca.* ten fold higher (Table 4). Veratraldehyde can, as previously suggested in studies focussing lignin degradation by white rot fungi, be also formed due to the oxidation of the fungal metabolite veratryl alcohol [69]. Finally, several lignin degradation products were also putatively identified (Compound 1-5, Table 4) [72], despite the lack of standard compounds for confirming their identification.

Comparison of the secretomes of A. nidulans grown in cork or in wood

The composition of softwood is comparable to that of cork, with the exception of suberin content, lacking in wood and abundant in cork (Table 1). A comparative analysis of cork and wood secretome could lead to the identification of suberin putative degrading enzymes, if any. As aforementioned, the produced 2DE maps displayed very similar patterns. Nevertheless, 40 protein spots were differentially accumulated; of which 38 could be identified retrieving 13 unique protein species (Table 5, Supplementary File S3 and S4). Amongst the 19 protein spots here identified as ChiB (AN4871), 13 decreased in the cork secretome. This suggests reduced autolysis during growth on cork, yet their total normalised volume was nearly the same in both secretomes. A more in deep statistical analysis of spot variation within the differentially accumulated spots was performed using principal component analysis (Supplementary File S3). Total variance explained by the first three principal components (PCs) was of 97.85%. ChiB, as well as some uncharacterised proteins species, were still amongst some of the protein species

which contributed more significantly to the total variation in PC1 and PC2 (30% and 5%, respectively). Other protein spots (retrieving single protein identifications) that had significantly contributed for the total variation in PC1 and PC2 were β-glucosidase (AN2828, 6% and 8%, respectively) and catalase B (AN9339, 10% and 6%, respectively), while in PC3 was only catalase B (AN9339, 8%). Taken together, these proteins species which increased in the cork secretome, suggest that cork degradation, when compared to that of wood, was more pronounced at the twenty one day of colonisation. Cork tertiary wall (outer layer) is essentially enriched in polysaccharides [16]. On the contrary, in wood this layer contains also lignin [15], which hampers the degradation of the polysaccharides [64]. Higher polysaccharide accessibility might thus partially explain the observed increase of β-glucosidase (AN2828) in the cork secretome. In addition, catalase B (AN9339) major contribution to the first three PCs reinforced the idea that mycelial development was more active in cork. Amongst those proteins spots retrieving multiple protein identifications, spots 22 and 21 identified as oxidoreductase (AN3351), contributed to 6% and 17% of the total variation in PC2 and PC3. Though, their multiple identities raise ambiguity, one possible interpretation is that oxidoreductase activity was higher in cork than in wood. Such interpretation is coherent with catalase B major contribution to the observed variability.

Conclusions

Ascomycota play a prominent role as cork colonisers, however the enzymatic toolbox used during its degradation remains largely unanswered. *Aspergillus nidulans* was able to form an intricate network of hyphae on the surface of the cork cell walls, while also undergoing both sexual and asexual development. The ability of the fungus to partially degrade some cork constituents, with the exception of polyaliphatic suberin, was suggested after spectroscopic analysis of the substrate and further validated by the proteins species identified in the cork secretome. The polysaccharide degrading enzymes herein identified constitute sufficient evidence of polysaccharide degradation. They included enzymes involved in the last steps of the degradation of cellulose and of pectin. Direct comparison with other *A. nidulans* secretomes grown in lignocellulosic substrates [46-48], suggests that autolysis related proteins might have hampered the identification of less abundant proteins in the gels. Nevertheless, major findings are consistent with the previous reported data. One valid hypothesis is that *A. nidulans* used

Fenton-type chemistry during the degradation of the cork cell walls. Most probably this also led to cleavage of lignin as suggested by the accumulation of veratraldehyde and cinnamic acid, and of other putative lignin degradation products during fungal growth. The identification of small aromatic compounds which might act as iron reducers and the identification of alcohol oxidases, which might release extracellular hydrogen peroxide, further substantiate this hypothesis [69, 73]. Lignin degradation by Fenton-type reactions has been previously associated with the activity of extracellular cellobiose dehydrogenase [46]. Herein, the obtained data showed that the fungus likely uses a different Fenton-chemistry in high oxygen availability conditions. Comparison of cork and wood secretomes supported the idea that substrate accessibility plays a major role during fungal colonisation of lignocellulosic substrates. During the superficial colonisation of the wood pieces by fungi, accessibility to polysaccharides in the outer cell wall is hampered by lignin. Accordingly, *A. nidulans* produced higher levels of polysaccharide degrading enzymes during growth in cork, than in wood. Overall this study emphasises the high recalcitrance of cork to fungal degradation, particularly of the suberin barrier in the cork cell walls which remained virtually unaltered. In general, the obtained data revealed that *A. nidulans* superficial colonisation of cork and wood share a common set of enzymatic mechanisms.

Acknowledgements

I.M. and H.G. are grateful to Fundação para a Ciência e a Tecnologia (FCT) and Fundação Calouste Gulbenkian, Portugal, for the fellowships SFRH/BD/38378/2007 and 21-95587-B, respectively. The work was partially supported by a grant from Iceland, Liechtenstein and Norway through the EEA financial mechanism (Project PT015), FCT through the grants PEst-OE/EQB/LA0004/2011, PTDC/QUI/71331/2006, and PTDC/QUI-QUI/120982/2010 and by Ministerio de Ciencia e Innovación of the Spanish Government under the project UNBA10-4E-441. The authors wish to thank I. Nogueira from Instituto Superior Técnico, Portugal for the acquisition of SEM images and to M.C. Leitão from Instituto de Tecnologia Química e Biológica for the help with chromatographic analyses.

Captions Tables and Figures

Table 1. Average composition of the cell walls of cork and wood

Table 2. Protein extraction yield and number of detected spots in the 2DE gels.

Table 3. Extracellular proteins identified in the spots of the 2DE gels of *Aspergillus nidulans* cork secretome. Protein species are grouped by their functional classification.

Table 4. Extracellular metabolites identified by UHPLC-ESI-HRMS in the organic extracts of *Aspergillus nidulans* cultures after cork colonization.

Table 5. Identification of the differentially accumulated extracellular proteins in the 2DE gels of *Aspergillus nidulans* grown on cork when compared to that of wood.

Figure 1. SEM images of cork after *Aspergillus nidulans* colonization. Formation of a hyphal network growing on the surface of the cork pieces (A, B) and the deposition of conidia (C, D) and ascospores (E, F). Magnifications of 200x (Scale bar:100 μm; A), 1100x (Scale bar:10 μm; B, C and E) and 3000x (Scale bar: 1 μm; D and F).

Figure 2. ATR-FTIR spectra of **a)** cork, **b)** cork colonised with *Aspergillus nidulans* after two (−) or twenty-one days (--), and **c)** *Aspergillus nidulans* mycelia. S, L and P stands for suberin, lignin and polysaccharides, respectively

Figure 3. Two dimensional gels obtained for the secretome of *Aspergillus nidulans* grown on cork (A) and wood (B). All of the analysed spots are labelled accordingly to Table 3. Unnumbered spots were analysed but their identification was not attained.

References

[1] Silva SP, Sabino MA, Fernandes EM, Correlo VM, Boesel LF, Reis RL. Cork: properties, capabilities and applications. Intl Mater Rev 2005;50:345-65.
[2] Maga JA, Puech JL. Cork and alcoholic beverages. Food Rev Int 2005;21:53-68.
[3] Kolattukudy PE. Polyesters in higher plants. In: Scheper T, Babel W, Steinbuchel A, editors. Biopolyesters. Berlin: Springer-Verlag Berlin; 2001. p. 1-49.
[4] Paliwal R, Rawat AP, Rawat M, Rai JPN. Bioligninolysis: Recent Updates for Biotechnological Solution. Appl Biochem Biotech 2012;167:1865-89.
[5] Wymelenberg AV, Gaskell J, Mozuch M, Sabat G, Ralph J, Skyba O, et al. Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. Appl Environ Microb 2010;76:3599-610.
[6] MacDonald J, Doering M, Canam T, Gong Y, Guttman DS, Campbell MM, Master ER. Transcriptomic responses of the softwood-degrading white-rot fungus *Phanerochaete carnosa* during growth on coniferous and deciduous wood. Appl Environ Microb 2011;77:3211-8.
[7] Manavalan A, Adav SS, Sze SK. iTRAQ-based quantitative secretome analysis of *Phanerochaete chrysosporium*. J Proteomics 2011;75:642-54.
[8] Kang Y-M, Prewitt ML, Diehl SV. Proteomics for biodeterioration of wood (*Pinus taeda* L.): Challenging analysis by 2-D PAGE and MALDI-TOF/TOF/MS. Int Biodeter Biodegr 2009;63:1036-44.
[9] Barreto MC, Houbraken J, Samson RA, Brito D, Gadanho M, San Romão MV. Unveiling the fungal mycobiota present throughout the cork stopper manufacturing process. Fems Microbiol Ecol 2012;82:202-14.
[10] Danesh P, Caldas FMV, Marques JJF, San Romão MV. Mycobiota in Portuguese 'normal' and 'green' cork throughout the manufacturing process of stoppers. J Appl Microbiol 1997;82:689-94.
[11] Oliveira AC, Peres CM, Pires JMC, Silva Pereira C, Vitorino S, Marques JJF, Crespo MTB, San Romão MV. Cork stoppers industry: defining appropriate mould colonization. Microbiol Res 2003;158:117-24.
[12] Riu H, Roig G, Sancho J. Production of carpophores of *Lentinus edodes* and *Ganoderma lucidum* grown on cork residues. Microbiologia 1997;13:185-92.
[13] Silva Pereira C, Soares GAM, Oliveira AC, Rosa ME, Pereira H, Moreno N, Romão MVS. Effect of fungal colonization on mechanical performance of cork. Int Biodeter Biodegr 2006;57:244-50.
[14] Serra R, Peterson S, CTCOR, Venancio A. Multilocus sequence identification of *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers. Res Microbiol 2008;159:178-86.
[15] Plomion C, Leprovost G, Stokes A. Wood formation in trees. Plant Physiol 2001;127:1513-23.
[16] Pereira H. Cork: Biology, production and uses. Amsterdam: Elsevier; 2007.
[17] Bernards MA, Lewis NG. The macromolecular aromatic domain in suberized tissue: A changing paradigm. Phytochemistry 1998;47:915-33.
[18] Sweigard JA, Chumley FG, Valent B. Cloning and analysis of cut1, a cutinase gene from *Magnaporthe grisea*. Mol Gen Genet 1992;232:174-82.
[19] Kontkanen H, Westerholm-Parvinen A, Saloheimo M, Bailey M, Ratto M, Mattila I, Mohsina M, Kalkkinen N, Nakari-Setala T, Buchert J. Novel *Coprinopsis cinerea* polyesterase that hydrolyzes cutin and suberin. Appl Environ Microb 2009;75:2148-57.
[20] Silva Pereira C, Marques JJF, San Romão MV. Cork taint in wine: Scientific knowledge and public perception - A critical review. Crit Rev Microbiol 2000;26:147-62.
[21] Linaldeddu BT, Sirca C, Spano D, Franceschini A. Variation of endophytic cork oak-associated fungal communities in relation to plant health and water stress. Forest Pathol 2011;41:193-201.
[22] Moreau. La mycoplore des bouchons de liège. Son évolution au contact du vin. Consequences possibles du métabolism des moisissures. Revue de Mycology 1978;42:155-89.

[23] Carvalho MB, Martins I, Medeiros J, Tavares S, Planchon S, Renaut J, Núñez O, Gallart-Ayala H, Galceran MT, Hursthouse A, Silva Pereira C. The response of *Mucor plumbeus* to pentachlorophenol: A toxicoproteomics study. J Proteomics 2013;78:159-71.
[24] Peterson RA, Bradner JR, Roberts TH, Nevalainen KMH. Fungi from koala (*Phascolarctos cinereus*) faeces exhibit a broad range of enzyme activities against recalcitrant substrates. Lett Appl Microbiol 2009;48:218-25.
[25] Petkovic M, Hartmann DO, Adamova G, Seddon KR, Rebelo LPN, Silva Pereira C. Unravelling the mechanism of toxicity of alkyltributylphosphonium chlorides in *Aspergillus nidulans* conidia. New J Chem 2012;36:56-63.
[26] Garcia H, Ferreira R, Petkovic M, Ferguson JL, Leitão MC, Gunaratne HQN, Seddon KR, Rebelo LPN, Silva Pereira C. Dissolution of cork biopolymers in biocompatible ionic liquids. Green Chem 2010;12:367-9.
[27] Ramagli LS. Quantifying protein in 2-D PAGE solubilization buffers. 2-D Proteome Analysis Protocols 1999;112:99-103.
[28] Neuhoff V, Arold N, Taube D, Ehrhardt W. Improved staining of proteins in polyacrylamide gels including isoelectric-focusing gels with clear background at nanogram sensitivity using coomassie brilliant blue G-250 and R-250. Electrophoresis 1988;9:255-62.
[29] Valledor L, Jorrin JV, Luis Rodriguez J, Lenz C, Meijon M, Rodriguez R, Jesus Canal M. Combined proteomic and transcriptomic analysis identifies differentially expressed pathways associated to *Pinus radiata* needle maturation. J Proteome Res 2010;9:3954-79.
[30] Condell O, Sheridan A, Power KA, Bonilla-Santiago R, Sergeant K, Renaut J, Burgess C, Fanning S, Nally JE. Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. J Proteomics 2012;75:4505-19.
[31] Sergeant K, Spiess N, Renaut J, Wilhelm E, Hausman JF. One dry summer: A leaf proteome study on the response of oak to drought exposure. J Proteomics 2011;74:1385-95.
[32] Petkovic M, Ferguson JL, Bohn A, Trindade J, Martins I, Carvalho MB, et al. Exploring fungal activity in the presence of ionic liquids. Green Chem 2009;11:889-94.
[33] R. K.Saxena, U. Sinha. Sexual differentiation under submerged conditions in *Aspergillus nidulans*. J Gen Microbiol 1977;102:195-8.
[34] Naumann A, Navarro-Gonzalez M, Peddireddi S, Kues U, Polle A. Fourier transform infrared microscopy and imaging: Detection of fungi in wood. Fungal Genet Biol 2005;42:829-35.
[35] de Vries RP, Visser J. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Mol Biol R 2001;65:497-522.
[36] Wang L, Aryal UK, Dai ZY, Mason AC, Monroe ME, Tian ZX, et al. Mapping N-linked glycosylation sites in the secretome and whole cells of *Aspergillus niger* using hydrazide chemistry and mass spectrometry. J Proteome Res 2012;11:143-56.
[37] Espino JJ, Gutierrez-Sanchez G, Brito N, Shah P, Orlando R, Gonzalez C. The *Botrytis cinerea* early secretome. Proteomics 2010;10:3020-34.
[38] Packer NH, Harrison MJ. Glycobiology and proteomics: Is mass spectrometry the Holy Grail? Electrophoresis 1998;19:1872-82.
[39] Osherov N, Yarden O. The cell wall of filamentous fungi. In: Borkovich K, Ebbole D, editors. Cellular and molecular biology of filamentous fungi. Washington: AES press; 2010. p. 224-37.
[40] de Groot PWJ, Brandt BW, Horiuchi H, Ram AFJ, de Koster CG, Klis FM. Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. Fungal Genet Biol 2009;46:S72-S81.
[41] Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, et al. Sequencing of Aspergillus nidulans and comparative analysis with A-fumigatus and A-oryzae. Nature 2005;438:1105-15.

[42] Arnaud M, Chibucos M, Costanzo M, Crabtree J, Inglis D, Lotia A, et al. The *Aspergillus* Genome Database, a curated comparative genomics resource for gene, protein and sequence information for the *Aspergillus* research community. Nucleic Acids Res 2010;38:D420-7.
[43] Pusztahelyi T, Molnar Z, Emri T, Klement E, Miskei M, Kerekgyarto J, Balla J, Pocsi I. Comparative studies of differential expression of chitinolytic enzymes encoded by chiA, chiB, chiC and nagA genes in *Aspergillus nidulans*. Folia Microbiol 2006;51:547-54.
[44] Yamazaki H, Yamazaki D, Takaya N, Takagi M, Ohta A, Horiuchi H. A chitinase gene, chiB, involved in the autolytic process of *Aspergillus nidulans*. Curr Genet 2007;51:89-98.
[45] Pocsi I, Leiter E, Kwon NJ, Shin KS, Kwon GS, Pusztahelyi T, Emri T, Abuknesha RA, Price RG, Yu JH. Asexual sporulation signalling regulates autolysis of *Aspergillus nidulans* via modulating the chitinase ChiB production. J Appl Microbiol 2009;107:514-23.
[46] Saykhedkar S, Ray A, Ayoubi-Canaan P, Hartson SD, Prade R, Mort AJ. A time course analysis of the extracellular proteome of *Aspergillus nidulans* growing on sorghum stover. Biotechnol Biofuels 2012;5:52.
[47] Schneider T, Gerrits B, Gassmann R, Schmid E, Gessner MO, Richter A, Battin T, Eberl L, Riedel K. Proteome analysis of fungal and bacterial involvement in leaf litter decomposition. Proteomics 2010;10:1819-30.
[48] Couturier M, Navarro D, Olive C, Chevret D, Haon M, Favel A, Lesage-Meessen L, Henrissat B, Coutinho PM, Berrin J-G. Post-genomic analyses of fungal lignocellulosic biomass degradation reveal the unexpected potential of the plant pathogen *Ustilago maydis*. Bmc Genomics 2012;13:57.
[49] Szilagyi M, Kwon NJ, Dorogi C, Pocsi I, Yu JH, Emri T. The extracellular beta-1,3-endoglucanase EngA is involved in autolysis of *Aspergillus nidulans*. J Appl Microbiol 2010;109:1498-508.
[50] Szilagyi M, Anton F, Forgacs K, Yu JH, Pocsi I, Emri T. Antifungal activity of extracellular hydrolases produced by autolysing *Aspergillus nidulans* cultures. J Microbiol 2012;50:849-54.
[51] Szilagyi M, Kwon NJ, Bakti F, M-Hamvas M, Jambrik K, Park H, Pocsi I, Yu JH, Emri T. Extracellular proteinase formation in carbon starving *Aspergillus nidulans* cultures - physiological function and regulation. J Basic Microbiol 2011;51:625-34.
[52] Szilagyi M, Miskei M, Karanyi Z, Lenkey B, Pocsi I, Emri T. Transcriptome changes initiated by carbon starvation in *Aspergillus nidulans*. Microbiology+ 2013;159:176-90.
[53] Shin KS, Kwon NJ, Kim YH, Park HS, Kwon GS, Yu JH. Differential Roles of the ChiB chitinase in autolysis and cell death of *Aspergillus nidulans*. Eukaryot Cell 2009;8:738-46.
[54] Nitsche BM, Jorgensen TR, Akeroyd M, Meyer V, Ram AFJ. The carbon starvation response of *Aspergillus niger* during submerged cultivation: Insights from the transcriptome and secretome. Bmc Genomics 2012;13:380.
[55] Paietta JN. Sulfur, phosphorus, and iron metabolism. In: Borkovich K, Ebbole D, editors. Cellular and molecular biology of filamentous fungi. Washington: AES press; 2010. p. 359-76.
[56] Choi CJ, Ju HJ, Park BH, Qin R, Jahng KY, Han DM, Chae KS. Isolation and characterization of the *Aspergillus nidulans* eglC gene encoding a putative beta-1,3-endoglucanase. Fungal Genet Biol 2005;42:590-600.
[57] Wei HJ, Scherer M, Singh A, Liese R, Fischer R. *Aspergillus nidulans* alpha-1,3 glucanase (mutanase), mutA, is expressed during sexual development and mobilizes mutan. Fungal Genet Biol 2001;34:217-27.
[58] Dyer PS, O'Gorman CM. Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. Fems Microbiol Rev 2012;36:165-92.
[59] Kawasaki L, Aguirre J. Multiple catalase genes are differentially regulated in *Aspergillus nidulans*. J Bacteriol 2001;183:1434-40.
[60] Alfonso C, Nuero OM, Santamaria F, Reyes F. Purification of a heat-stable chitin deacetylase from *Aspergillus nidulans* and its role in cell-wall degradation. Curr Microbiol 1995;30:49-54.

[61] Rocha SM, Coimbra MA, Delgadillo I. Demonstration of pectic polysaccharides in cork cell wall from *Quercus suber* L. J Agr Food Chem 2000;48:2003-7.
[62] Yadav V, Malanson G. Progress in soil organic matter research: litter decomposition, modelling, monitoring and sequestration. Prog Phys Geog 2007;31:131-54.
[63] Yang F, Jensen JD, Svensson B, Jorgensen HJL, Collinge DB, Finnie C. Secretomics identifies *Fusarium graminearum* proteins involved in the interaction with barley and wheat. Mol Plant Pathol 2012;13:445-53.
[64] Laureano-Perez L, Teymouri F, Alizadeh H, Dale BE. Understanding factors that limit enzymatic hydrolysis of biomass. Appl Biochem Biotech 2005;121:1081-99.
[65] Hernandez-Ortega A, Ferreira P, Martinez AT. Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation. Applied Microbiology and Biotechnology 2012;93:1395-410.
[66] Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, et al. Genome, transcriptome, and secretome analysis of wood decay fungus Postia placenta supports unique mechanisms of lignocellulose conversion. P Natl Acad Sci USA 2009;106:1954-9.
[67] Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, et al. Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature 2005;438:1105-15.
[68] Dashtban M, Schraft H, Syed TA, Qin W. Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 2010;1:36-50.
[69] Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, Martinez MJ, Gutierrez A, del Rio JC. Biodegradation of lignocellulosics: microbial chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 2005;8:195-204.
[70] Aguiar A, Ferraz A. Fe3+- and Cu2+-reduction by phenol derivatives associated with Azure B degradation in Fenton-like reactions. Chemosphere 2007;66:947-54.
[71] Ludwig R, Harreither W, Tasca F, Gorton L. Cellobiose Dehydrogenase: A Versatile Catalyst for Electrochemical Applications. ChemPhysChem 2010;11:2674-97.
[72] Thompson DN, Hames BR, Reddy CA, Grethlein HE. *In vitro* degradation of natural insoluble lignin in aqueous media by the extracellular peroxidases of Phanerochaete chrysosporium. Biotechnol Bioeng 1998;57:704-17.
[73] Guillen F, Evans CS. Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*. Appl Environ Microb 1994;60:2811-7.

FIGURES

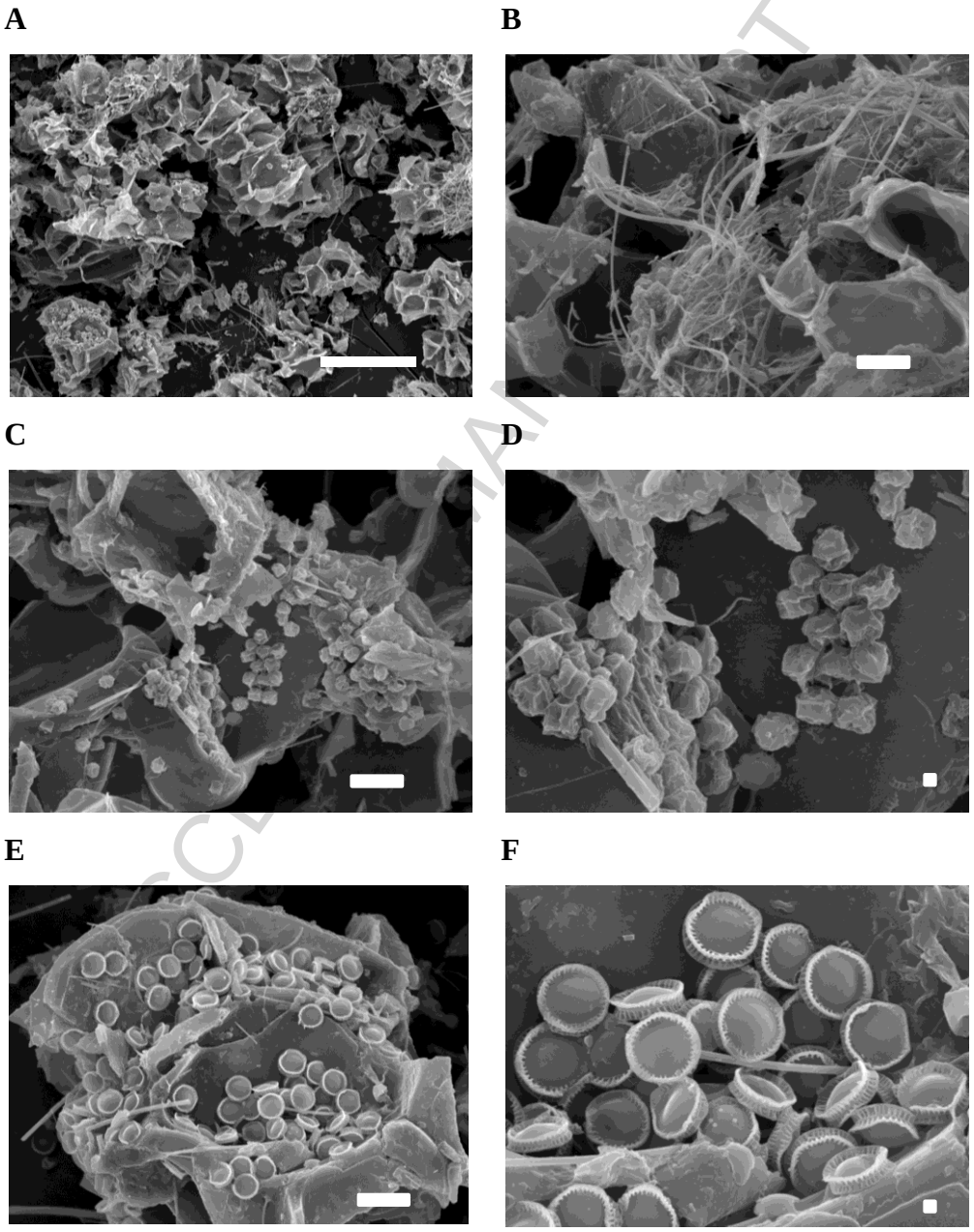


Figure 1.

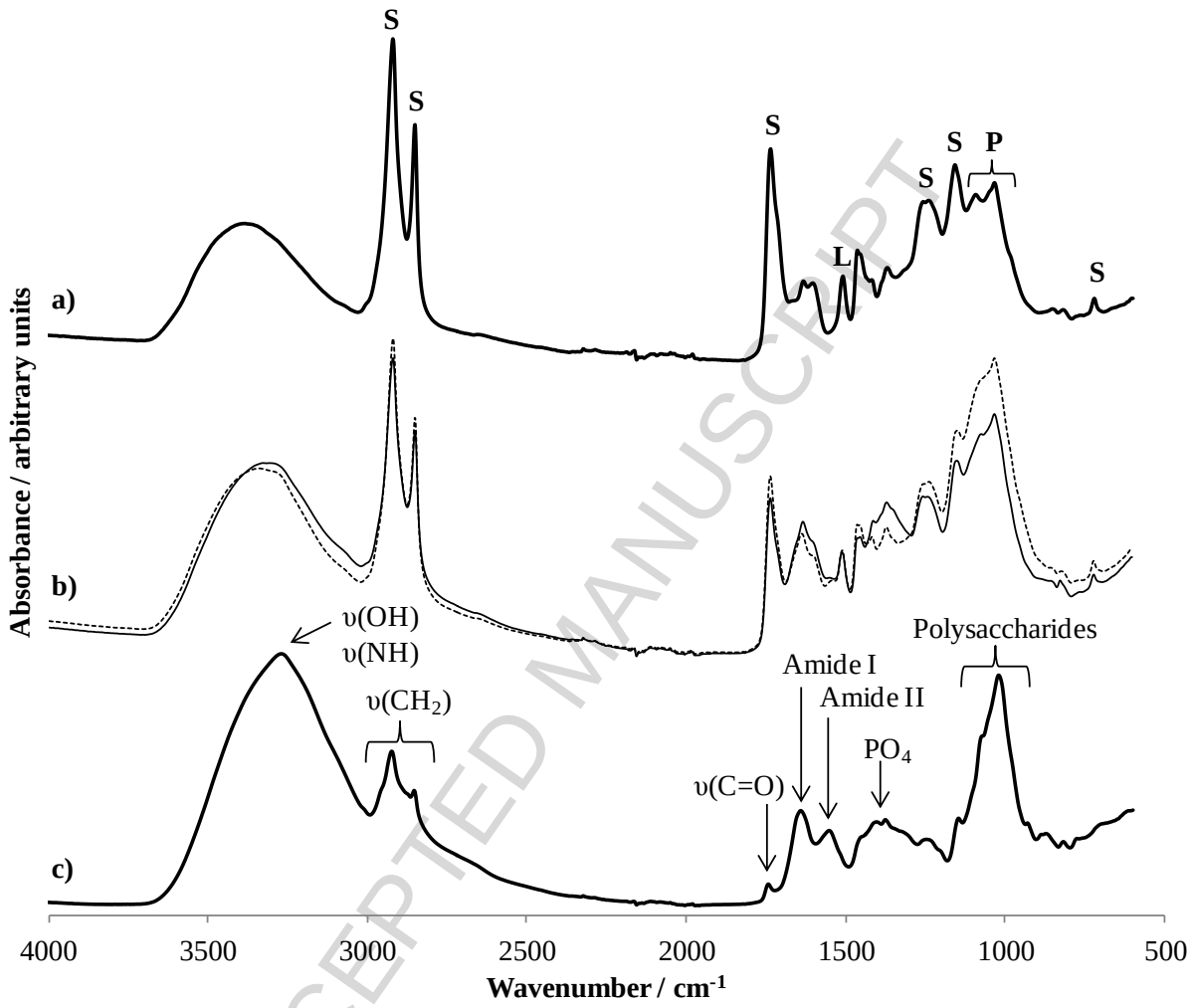
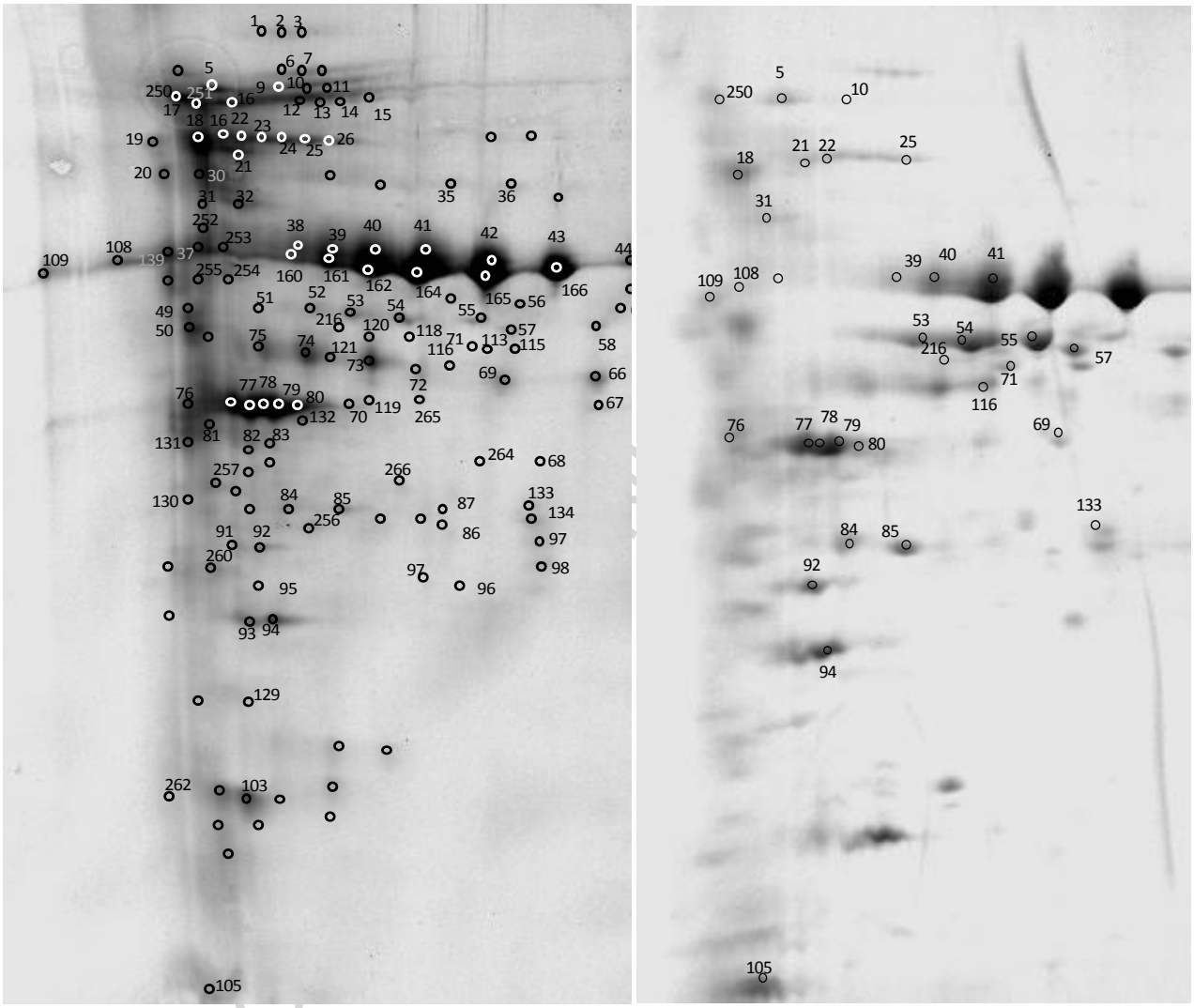
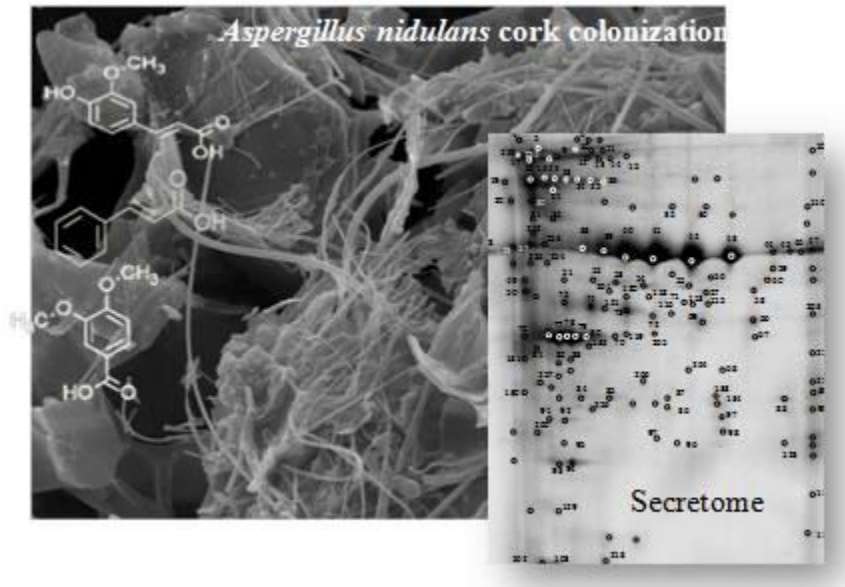


Figure 2.



A B

Figure 3.



Graphical abstract

TABLES

Table 1.

Composite	**Wood** [15] (% of weight)	**Cork** [16] (% of weight)
Lignin	25-35	20-30
Suberin	0	40-50
Cellulose	35-40	9
Hemicellulose	35-40	16
Pectin	3-9	1.5
Extractives	10	8-24

Table 2.

	Protein yield (mg/mL)	**Number of spots**	
		Total number	**Differentially accumulated spots**
Cork secretome	0.98 ± 0.19	149 ± 9	18
Wood secretome	1.23 ± 0.35	144 ± 7	22

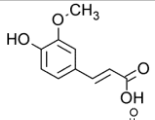
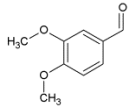
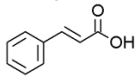
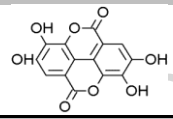
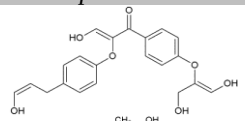
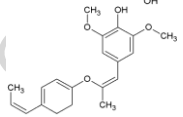
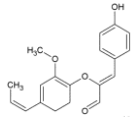
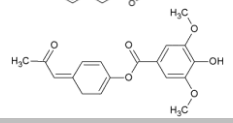
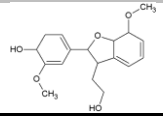
Table 3.

GenBank accession #	*Aspergillus nidulans* gene #	Protein name (EC number)	Spot number [1]
		Plant cell wall degrading enzymes	
gi|67524741	AN2828	β-glucosidase (3.2.1.21)	5; 30; 76; 77; 107
gi|67900818	AN7396	β-glucosidase (3.2.1.21)	6; 7
gi|259481308	AN4102	β-glucosidase (3.2.1.21)	1; 2; 3
gi|67902040	AN8007	Endo α-1,5-arabinosidase C (3.2.1.99)	73; 74; 75; 119; 121
gi|67904722	AN9348	Alcohol oxidase	14
gi|259483385	AN7269	Oxidoreductase (activity FAD)	30
gi|67525787	AN3351	Oxidoreductase (activity FAD)	21; 22; 23; 24; 25; 26
		Fungal cell wall remodeling	
gi|67537402	AN4871	Chitinase (3.2.1.14)	37-48; 51-57;59-64; 66; 68-70; 77; 78; 80; 82-84; 86; 88; 89;91; 94; 103; 108-110; 113; 115-118; 120; 124; 125; 132; 133; 142; 160-162; 164-168; 253; 256; 258; 259; 263-266
gi|67515927	AN0245	β-1,3- glucanase, engA (3.2.1.59)	257
gi|67522090	AN1502	*N*-acetylglucosaminidase (3.2.1.52/3.2.1.96)	21
gi|67537310	AN4825	1,3-β-glucosidase	36; 52
gi|67900724	AN7349	α-1,3-glucanase (mutanase; 3.2.1.59)	167
gi|67901926	AN7950	β-1,3-glucosidase eglC (3.2.1.39)	76; 81; 257
gi|259486505	AN9042	α-1,3-glucanase (3.2.1.59)	50
gi|67517318	AN0933	Transglycosidase (2.4.2.29)	19; 20
		Defense/stress response	
gi|1737449	AN9339	Catalase B (1.11.1.6)	10; 11; 85; 90; 92; 97; 216; 250
		Protease/nitrogen	

gi|255941434	AN2366	Serine protease	81; 260
gi|67524891	AN2903	Vacuolar aspartyl protease (Proteinase A)	254; 255
gi|67526921	AN3918	Aminopeptidase	163
gi|67538776	AN5558	Alkaline protease 1 (3.4.21.63)	77; 78; 79; 80; 129
gi|6456480	AN4809	Glutaminase A (3.5.1.2)	12-15; 22-25; 33
gi|67527351	AN4055	Acid phosphatase (PHO)	18
		Miscellaneous	
gi|67904786	AN9380	Xylanase/chitin deacetylase (3.5.1.41)	260
gi|67524993	AN2954	Extracellular serine rich protein	18; 257
gi|67900640	AN7307	DUF1237 domain protein	31; 32
gi|67904134	AN9054	Uncharacterized	49; 50; 139
gi|6754203	AN7181	Uncharacterized	31; 252; 257
gi|259481436	AN3983	Uncharacterized	105

[1] Numbered accordingly to the 2DE map of *Aspergillus nidulans* grown on cork (Fig. 4).

Table 4.

Name	Compound Structure [a]	Formula	MM	Delta (ppm)	RT [c]	Isotopic pattern Nr	Isotopic pattern %	Area [d]
LMW aromatic compounds [b]								
Ferulic acid		$C_{10}H_{10}O_4$	194.05790	2.04	4.55	3 of 4	100	6.9 e^{+7}
Veratraldehyde		$C_9H_{10}O_4$	182.057907	2.34	4.74	4 of 4	100	6.6 e^{+7}
Cinnanic acid		$C_9H_8O_2$	148.052429	0.85	5.72	2 of 2	100	5.6 e^{+5}
Cork extractive (plant metabolite)								
Ellagic acid		$C_{14}H_6O_8$	300.9990	1.22	4.04	3 of 3	100	2.6 e^{+5}
Compounds retaining an β-aryl ether linkage [c]								
Compound 1		$C_{21}H_{18}O_7$	381.0980	0.44	7.53	3 of 3	100	1.3 e^{+6}
Compound 2		$C_{20}H_{24}O_4$	373.1657	0.05	5.72	2 of 3	100	2.3 e^{+6}
Compound 3		$C_{19}H_{20}O_5$	373.1293	1.30	5.13	3 of 4	100	1.5 e^{+7}
Compound 4		$C_{18}H_{16}O_6$	373.0929	1.32	5.15	3 of 4	100	6.1 e^{+6}
Pinoresinol derivative								
Compound 5		$C_{19}H_{26}O_5$	333.1707	0.45	6.56	3 of 3	100	2.3 e^{+6}

a. Structure drawn using ChemSketch software based on [74]; b. The identification was validated using commercially available standards. Other standards tested were veratric acid; vanillin, phenyl acetic acid; 4-hydroxyphenylacetic acid 2,5-dimethoxy-1,4-benzoquinone and coumaric acids. c. putative

identification based on the molecular formulas obtained using ExactFinder^{TM} 2.0 software (ThermoFisher) and based on the structures proposed in [74]. d. Areas obtained for the analyses of the cork media after fungal colonization, only ellagic and ferulic acids were found in the control with areas of 1.2 e^{+9} and 8.9 e^{+6}, respectively.

Table 5.

Anova (*p*-value)	FC [2]	Spot no.[1]	Gel[3]	Protein name (EC number)	*Aspergillus nidulans* gene	GenBank accession #
				Spots showing increased levels in cork secretome		
0.0310	7.2	5	C	β-glucosidase (3.2.1.21)	AN2828	gi|67524741
0.0030	7.9	31	C	Uncharacterized	AN7181	gi|6754203
				DUF1237 domain protein	AN7307	gi|6700640
0.0006	13.6	18	C	Acid phosphatase (PHO)	AN4055	gi|67527351
				Extracellular serine rich protein	AN2954	gi|67524993
0.0113	7.2	250	C	Catalase B (1.11.1.6)	AN9339	gi|1737449
0.0011	5.0	22	C	Oxidoreductase (activity FAD)	AN3351	gi|67525787
				Glutaminase A (3.5.1.2)	AN4809	gi|6456480
0.0070	2.8	10	C	Catalase B (1.11.1.6)	AN9339	gi|1737449
0.0358	3.9	21	C	Oxidoreductase (activity FAD)	AN3351	gi|67525787
				N-acetylglucosaminidase (3.2.1.52/3.2.1.96)	AN1502	gi|67522090
0.0087	4.7	39	C/W	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0019	5.2	40	C/W	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0166	3.2	139	C	Uncharacterized	AN9054	gi|67904134
0.0382	3.0	80	C	Alkaline protease 1 (3.4.21.63)	AN5558	gi|67538776
				Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0166	2.7	79	C	Alkaline protease 1 (3.4.21.63)	AN5558	gi|67538776
0.0034	2.5	109	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0032	2.6	108	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0104	2.9	78	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0307	2.8	41	C/W	Chitinase (3.2.1.14)	AN4871	gi|67537402
				β-glucosidase (3.2.1.21)	AN2828	gi|67524741
0.0052	2.5	77	C	Alkaline protease 1 (3.4.21.63)	AN5558	gi|67538776
				Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0178	2.1	76	C	β-1,3-glucosidase eglC (3.2.1.39)	AN7950	gi|67901926
				Spots showing decreased levels in cork secretome		
0.0182	0.7	25	C	Oxidoreductase (activity FAD)	AN3351	gi|67525787
0.0382	0.5	94	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0228	0.4	85	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0082	0.4	116	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0263	0.4	51	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0204	0.4	84	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0392	0.3	216	C	Catalase B (1.11.1.6)	AN9339	gi|1737449
0.0440	0.3	133	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0180	0.2	92	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0077	0.2	53	C/W	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0008	0.2	54	C	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0020	0.4	57	C/W	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.000311	0.2	71	C	Unidentified	-	-
			W	Chitinase (3.2.1.14)	AN4871	gi|67537402
0.0005	0.2	55	C	Chitinase (3.2.1.14)	AN4871	gi|67537402

Anova (*p*-value)	FC [2]	Spot no.[1]	Gel[3]	Protein name (EC number)	*Aspergillus nidulans* gene	GenBank accession #
0.0340	0.3	105	C	Uncharacterized	AN3983	gi|259481436
0.0011	0.1	69	C	Chitinase (3.2.1.14)	AN4871	gi|67537402

[1] Numbered accordingly to the 2DE map of *A. nidulans* grown on cork (Fig. 3); [2] Ratio between the normalized volumes of spots in gels obtained for *A. nidulans* growth on cork or on wood; [3] Indicates the gel from which the corresponding spot was manually excised: W and C stand for wood and cork, respectively.

Highlights

- *Aspergillus nidulans* degraded cork superficially not reaching suberin barrier
- Degradation of cork constituents involved enzymatic and Fenton-*like* reactions
- Several β-glucosidases accumulated in the cork secretome
- Lignin degradation products were released during cork degradation
- Differences in cork and wood secretomes reflect polysaccharide accessibility