UNIVERSIDADE FEDERAL DO PARANÁ

GEISON EDUARDO CAMBRI

UTILIZAÇÃO DE FUNGOS LIGNOLÍTICOS NA DEGRADAÇÃO DE CORANTES TÊXTEIS E PRODUÇÃO DE ENZIMAS COM APLICAÇÃO BIOTECNOLÓGICA

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Tese apresentada ao Programa de Pós-Graduação em Ciências – Bioquímica, Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial para a obtenção do título de Doutor.

Orientadora: Profa. Joana Lea Meira Silveira

Co-orientador: Prof. Jaime Paba

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Utilização de Fungos Lignolíticos na Degradação de Corantes Têxteis e Produção de Enzimas com Aplicação Biotecnológica

Tese aprovada como requisito parcial para obtenção do grau de Doutor no curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, pela seguinte banca examinadora:

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Resumo

Fungos da podridão da madeira compreendem organismos com a habilidade de degradar substratos lignocelulósicos. Eles secretam uma variedade de enzimas com potencial aplicação industrial na produção de papel, indústria textil, bioremediação e síntese orgânica. Explorando-se o potencial biotecnológico dos fungos, este trabalho apresenta a descrição do perfil de secreção proteica do fungo *Lentinus crinitus* e a utilização de uma lacase de *Lepista sordida* para descoloração de corantes têxteis, bem como a descrição parcial do gene que codifica para esta enzima. A fim de se obter um perfil de proteínas de secreção de *L. crinitus*, o fungo foi cultivado em meio contendo concentração variável de fontes de carbono, nitrogênio e teor de água. O perfil de proteinas secretadas mudou drasticamente em complexidade e intensidade de acordo às condições de cultivo, sendo que as culturas líquidas usando altas concentrações de maltose e ureia resultaram no perfil com maior número e intensidade de bandas proteicas na eletroforese unidimensional. Uma mistura de extratos de secreção provenientes de culturas com maltose e ureia em diferentes condições foi analisada via cromatografia líquida acoplada a espectrometria de massas (LC-MS) e eletroforese bidimensional (2D-SDS-PAGE). O espectro de proteínas identificadas (98 proteínas) inclui vários tipos de CAZymes (*carbohydrate-active enzymes*), oxidoreductases, proteases, esterases, proteínas com funções não relacionadas, classificadas como proteínas "miscelânea" e finalmente proteínas hipotéticas ou desconhecidas. Embora a prévia separação dos extratos solúveis por eletroforese bidimensional tenha melhorado o número de proteínas identificadas (150 spots correspondendo a 171 identificações), as duas estratégias revelaram uma distribuição semelhante de proteínas em cada grupo funcional de classificação proteica. A análise de bandas proteicas expressas particularmente em culturas com baixo teor de água mostra que o cultivo em estado sólido favorece a expressão de oxidases tais como lacases, manganês peroxidases, glucose-metanol-colina oxidoreductases e glioxal oxidases. A diversidade de proteínas observadas nos extratos de secreção de *L. crinitus* revela neste fungo um poderoso arsenal de enzimas envolvidas na quebra e consumo de lignocelulose e com possíveis aplicações biotecnológicas. O segundo basidiomiceto analisado foi um isolado de *L. sordida*. Os extratos de secreção derivados do fungo apresentaram atividade descorante contra diversos corantes têxteis e esta capacidade foi associada à produção de um par de polipeptídeos com atividade de lacase. Os extratos enzimáticos brutos demonstraram boa atividade e estabilidade em temperaturas entre 20 a 50 °C e em valores de pH entre 3,0 a 5,0. As culturas contendo maltose e nitrato de sódio, como fontes de carbono e nitrogênio, resultaram em uma melhor produção da enzima, e esta produção permaneceu inalterada quando houve suplementação com íons metálicos. Por fim, a introdução de mediadores redox (siringaldeído e acetosiringona) nos ensaios enzimáticos permitiu uma descoloração eficiente de 12 substratos. Com o intuito de obter informações sobre a estrutura primária do gene da lacase, sequências de nucleotídeos pertencentes a domínios conservados da enzima foram usadas em diferentes estratégias de amplificação via PCR e sequenciamento. Como resultado, uma sequência parcial do gene correspondendo a 1.519 nucleotídeos foi obtida. Esta sequência codifica para 386 aminoácidos, possui identidade com várias multicobre oxidases e contém 6 introns e 7 exons, assim como 4 potenciais sítios de glicosilação.

Palavras chave: lacase, secretoma, biodegradação, tratamento de efluentes, enzimas lignocelulósicas, *Lepista sordida*, *Lentinus crinitus*.

Abstract

Wood-rotting fungi are organisms with the ability to degrade lignocellulolytic substrates. They secrete a variety of enzymes with several industrial aplications in the production of biofuels, paper, textil industry, bioremediation and organic synthesis. In the present work the biotechnological potential of two local lignolytic basidiomycetes, *Lentinus crinitus* and *Lepista sordida* was assessed. Thus, the profile of secreted proteins produced by *L. crinitus* was determined and the catalytic properties of a laccase derived from *Lepista sordida* as well as the partial primary structure of the corresponding gene are presented. To study the protein secretion profile of *L. crinitus*, the fungus was grown in diferent culture media with variable carbon, nitrogen and water content and the resulting soluble extracts analysed by gel electrophoresis. The secretion profile changed drastically in complexity and intensity according with culture conditions. Liquid cultures with high concentration of maltose and urea resulted in secretion extracts with the higher number of protein bands and intensity in SDS-PAGE. A mixture of secretion extracts derived from different maltose and urea culture conditions was analysed by liquid chromatography coupled to mass spetrometry (LC-MS) and by two-dimensional electrophoresis (2D SDS-PAGE). The identified proteins (98) included several CAZymes (carbohydrate-active enzymes), oxidoreductases, proteases, esterases, proteins with unrelated functions (classified as miscellany) and a group of hypothetical proteins. Separation of soluble extracts by 2D electrophoresis improved the number of identified proteins (150 spots corresponding to 171 IDs), both strategies revealed a similar protein distribution in functional classes. Additionally, the analysis of particularly expressed protein bands in cultures with low water content showed that solid state cultivation favores the expression of oxidases such as laccases, manganese peroxidases, glucose-methanol-choline oxidoreductases and glyoxal oxidases. The observed protein diversity in the soluble extracts of *L. crinitus* reveals a powerful arsenal of enzymes involved in the breakdown and consumption of lignocellulose, with large biotechnological applications. *L. sordida* secretion extracts displayed high destaining activity against several colored substrates and this capacity was related to a pair of polypeptides with laccase activity. Crude enzymatic extracts demonstrated good activity and stability at pH and temperatures between 3.0 to 5.0 and 20 to 50 °C, respectively. Cultures containing maltose and sodium nitrate as carbon and nitrogen sources resulted in the best enzyme production. Finally, the introduction of redox mediators, syringaldehyde and acetosyringone, in enzyme assays allowed efficient destaining of 12 dye substrates. In order to obtain primary structure information of the laccase gene, nucleotide sequences belonging to enzyme conserved domains were used in several amplification and sequencing strategies. As a result a partial gene sequence corresponding to 1,519 nucleotides was obtained. This sequence encodes 386 amino acids, it has identity with several multi-copper oxidases and contains 6 exons and 7 introns, as well as 4 potential glycosylation sites.

Keywords: laccase, secretome, biodegradation, waste-water treatment, lignocellulolytic enzymes, *Lepista sordida*, *Lentinus crinitus*.

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1D: unidimensional.

2D: bidimensional.

ABTS: 2,2′-azino-bis(3-etiltiazolina-6-sulfonato).

azoR: Azoreductase.

CAZymes: *carbohydrate-active enzymes*.

CDH: Cellobiose Desidrogenase.

DCIP: 2,6-diclorophenol-indophenol.

DyP: Peroxidase de corantes, do inglês *Dye Peroxidase*.

EDTA: Ácido etileno-diamino-tetracético, do inglês, *EthyleneDiamine Tetraacetic Acid.*

FPase: atividade de celulase em papel filtro, do inglês *Filter paper activity*.

GMC oxidoreductase: glucose-metanol-colina oxidoreductase.

IDs: identificações, do ingês: *identifications*.

Lac: Lacase.

LC-MS: Cromatografia líquida associada à espectrometria de massas, do inglês *Liquid chromatography associated with tandem mass spectrometry*.

LiP: Lignina Peroxidase.

LME: Enzimas modificadoras de lignina, do inglês: *lignin modifying enzymes*.

LMM: Meio mínimo líqudio, do inglês *liquid minimal medium*.

MnP: Manganês Peroxidase.

MS: Espectrometria de massas, do inglês *Mass spectrometry*.

PAGE: Eletroforese em gel de poliacrilamida, do inglês *Polyacrylamide Gel Electrophoresis*.

PMF: *Peptide Mass Fingerprint*.

RB220: corante têxtil reactive blue 220

SDS: Dodecil sulfato de sódio, do inglês *sodium dodecyl sulfate*.

SF: Fermentação submersa, do inglês, *submerged fermentation*.

SMM: meio mínimo sólido, do inglês, *solid minimal medium*

SSF: Fermentação em estado sólido, do inglês *solid state fermentation.*

TBE: Tris borato EDTA.

VO: Álcool veratril oxidase, do inglês *veratryl alcohol oxidase*.

Sumário

1 - Introdução

1.1 - Fungos lignolíticos secretam um leque variado de enzimas

Os fungos lignolíticos compreendem fungos saprofíticos cicladores de carbono capazes de mineralizar polímeros componentes da madeira, tais como celulose, hemicelulose e lignina (LUNDELL; MAKELA; HIDDEN, 2010). Estes fungos, sendo naturalmente digestores extracelulares, necessitam secretar um amplo arsenal de enzimas para captação de nutrientes. Uma vez que lignina, hemicelulose e celulose são polímeros recalcitrantes e com estruturas químicas altamente diferentes entre si os fungos secretam enzimas especializadas na quebra destes componentes (GIRARD et al., 2013).

As enzimas modificadoras de lignina (LME, *lignin modifying enzymes*) são capazes de degradar lignina (Figura 1), um polímero formado por fenilpropanóides que confere resistência física a plantas e proteção contra ataques microbiológicos. Este grupo enzimático compreende oxidoreductases como manganês peroxidase (MnP) (EC 1.11.1.13), lignina peroxidase (LiP), (EC 1.11.1.14), e lacases (EC 1.10.3.2) (WESENBERG; KRYIAKIDES; AGATHOS, 2003). O modo de ação das LMEs baseia-se na oxidação de lignina com transferência de elétrons do substrato a enzima. Um carbono do polímero de lignina é oxidado e elétrons são transferidos à enzima. Consequentemente, há formação de radicais na estrutura de lignina, o que leva a sua desestabilização estrutural seguido de decomposição. Pontos distintos da lignina são oxidados, sendo liberados diferentes sub-produtos dependendo dos pontos de oxidação e enzimas que atuam sobre a lignina (BARR; AUST, 1994; WONG, 2009). Para que a lignina seja oxidada há participação de grupos alostéricos enzimáticos, onde o grupo heme compõe LiP e MnP, enquanto que 4 átomos de cobre estão presentes nas lacases. Esses grupos alostéricos participam da catálise enzimática com transferência de elétrons entre enzima e substrato (WESENBERG; KRYIAKIDES; AGATHOS, 2003; WONG, 2009). Essas enzimas, além de degradar lignina são capazes de quebrar uma grande gama de compostos com estrutura similar à mesma, como hidrocarbonetos aromáticos policíclicos (STEFFEN; HATAKKA; HOFRICHTER, 2003), compostos orgânicos clorados e corantes sintéticos (POINTING, 2001).

As hemicelulases são responsáveis pela quebra de hemicelulose (Figura 1), um grupo heterogêneo de polissacarídeos lineares ou ramificados que interagem com a

celulose por pontes de hidrogênio. Hemiceluloses também estão ligadas covalentemente à lignina, formando uma estrutura complexa entre os três polímeros. As hemicelulases são responsáveis por quebras específicas de ligações glicosídicas ou de cadeias laterais que compõem a hemicelulose. Este grupo enzimático é composto por enzimas como xilanases (EC 3.2.1.8), β-mananases (EC 3.2.1.78) e α-L-arabinofuranosidases (EC 3.2.1.55) (SHALLOM; SHOHAM 2003).

Por fim, grupos enzimáticos de celulases quebram o polímero de celulose (Figura 1), composto por monômeros de D-glucose unidos por ligações glucosídicas β-1,4. O primeiro passo para a quebra da estrutura de celulose envolve o fracionamento de ligações cruzadas de glucanas por endoglucanases (EC 3.2.1.4). O segundo passo é catalisado por celobiohidrolases (EC 3.2.1.91) por meio da hidrólise da cadeia de glucanas, gerando celobioses livres e por fim as β-glucosidases (EC 3.2.1.21) convertem as celobioses em glucose (WILSON, 2008).

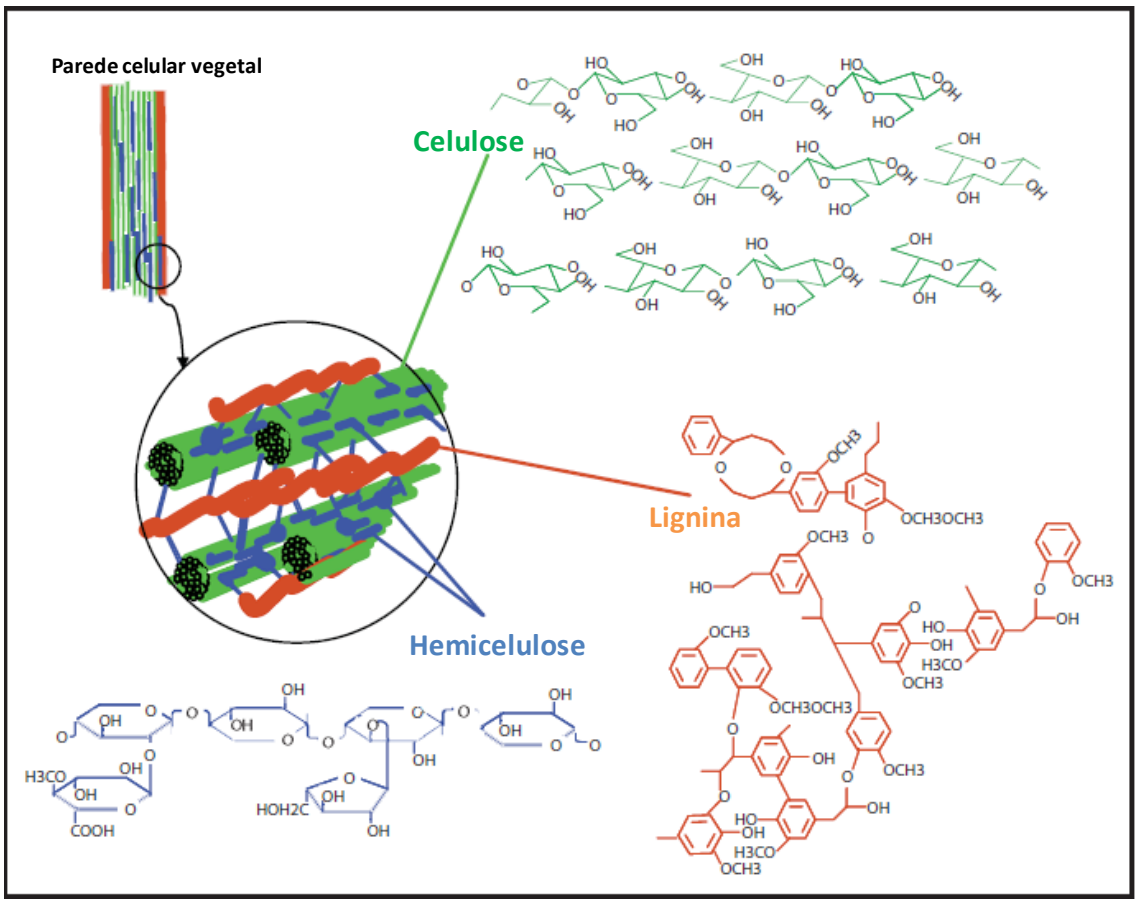

Figura 1. Imagem representativa da organização e estrutura química de lignocelulose.
Modificado de https://microbewiki.kenyon.edu (Acessado em outubro de 2015)

1.2 - Enzimas secretadas por fungos lignoliticos possuem aplicações industriais

Os grupos enzimáticos acima citados possuem um alto potencial industrial podendo ser utilizados em diversos processos biotecnológicos. As enzimas modificadoras de lignina são utilizadas em processos de branqueamento de papel e tecidos, tratamento de fibras têxteis, remoção de poluentes em efluentes e solos e modificação das qualidades organolépticas de produtos da indústria alimentícia (DWIVEDI et al., 2011). A ampla utilização destas enzimas se deve à similaridade estrutural entre lignina e os diferentes substratos em que a enzima age, normalmente compostos fenólicos. Desta maneira, LMEs são capazes de quebrar compostos xenobióticos como corantes têxteis (NIEBISCH et al., 2010). Os corantes têxteis são compostos químicos não abrasivos (SARON; FELISBERTI, 2006) descritos como tóxicos, mutagênicos e carcinogênicos (CHUNG et al., 1992), que dificilmente são retirados de efluentes por métodos físico-químicos convencionais de tratamento de água (BANAT et al., 1996). Assim, a biodegradação destes compostos por meio da utilização de LMEs é uma alternativa viável e ecosustentável, já que permitiria a introdução de resíduos industriais na natureza, através do ciclo de carbono e nitrogênio, e além disto, reduziria o uso de insumos químicos convencionalmente usados e a quantidade de subprodutos resultantes da aplicação de estratégias não biológicas (RAUF; SALMAN, 2012).

Outra aplicação para enzimas modificadoras de lignina é o biorefinamento, ou seja, a utilização de biomassa para geração de produtos ou energia através de métodos enzimáticos (RAGAUSKAS et al., 2006). A lignina está presente em resíduos agroindustriais, considerados produtos de baixo valor agregado, sendo destinados majoritariamente para queima em caldeiras, incorporação de ração animal ou compostagem (ZENG; SABRA 2011). Entretanto, através do biorefinamento esses resíduos podem ser direcionados à obtenção de biocompósitos e compostos orgânicos, utilizáveis na indústria farmacêutica e química, além de gerar os chamados "*building blocks*" para a obtenção de polímeros (SCHORR; DIOUF; STEVANOVIC, 2014; WITAYAKRAN; ZETTILI; RAGAUSKAS, 2007; KUSUMOTO; NOZAKI, 2015).

O conceito de biorefinamento ainda pode ser extendido para celulases e hemicelulases. Estas enzimas são utilizadas para obtenção de oligosacarídeos (SHALLOM; SHOLAM 2003), melhora nutricional de rações animais, melhora na

fermentação de cervejas e vinhos e produção de papel (KUHAD; GUPTA; SINGH, 2011). Outro ramo promissor para utilização destas enzimas é a produção de biocombustíveis de primeira e segunda geração, uma vez que os fungos são capazes de degradar toda a estrutura lignocelulósica de plantas e consequentemente gerar açúcares fermentáveis (SUKUMARAN; SINGHANIA; PANDEY, 2005).

Comercialmente, as LMEs, celulases e hemicelulases são produzidas a partir de organismos modelo, como por exemplo: lacase produzida por *Trametes versicolor* e celulase produzida por *Aspergillus niger* e *Trichoderma reesei* (SIGMA ALDRICH®). Algumas enzimas, produzidas industrialmente, também são geradas a partir de expressão heteróloga, onde as enzimas são oriundas de organismos pouco estudados, como a lacase produzida pelo fungo termófilo *Myceliophthora thermophila* e expressada em *Aspergillus oryzae* (BERKA et al, 1997). As LMEs e hidrolases são produzidas para utilização industrial sendo divulgadas em brochuras comerciais de empresas como a NOVOZYMES® (www.novozymes.com), onde há uma breve descrição da produção destas enzimas através de fermentação submersa e fermentação em estado sólido por fungos filamentosos do gênero *Aspergillus*. Além disto, há descrições das aplicações destas enzimas em diversos ramos industriais, dentros os quais os já citados acima.

Sabendo-se da ampla gama de possibilidades de utilização de enzimas secretadas por fungos, a otimização da produção destas enzimas é desejada. Desta maneira são realizados estudos para regulação da produção enzimática através de formas e meios de cultivo variados.

1.3 - A produção enzimática é alterada por fatores externos

A síntese e a secreção de enzimas são reguladas por fatores externos como concentração e tipo de fontes de carbono e nitrogênio e quantidade de água livre presentes no meio de cultivo.

Em basidiomicetos lignolíticos a produção de lacases, peroxidases, celulases e xilanases é alterada significativamente quando os organismos são cultivados em meio líquido ou em meio sólido (ELISASHVILI et al., 2009). Assim, a atividade enzimática produzida pode variar de 15 a 45 vezes para xilanases e celulases produzidas por *Lentinus edodes* quando cultivado em meio submerso (ELISASHVILI et al., 2008). Além disto, a produção enzimática também é afetada pelo tipo de substrato utilizado

para cultivo. A atividade de celulase de *Pleurotus ostreatus* é variada em até 7,5 vezes quando o fungo é cultivado em meio sólido variando-se a fonte de carbono entre folhas de árvore e palha de trigo (ELISASHVILI et al., 2008). Efeito semelhante também é verificado na produção de enzimas extracelulares em *Phanerochaete chrysosporium*, onde, por exemplo, peroxidases são detectadas preferencialmente em cultivos submersos contendo glucose, enquanto que glicohidrolases são produzidas similarmente em cultivos líquido ou sólido (SATO et al., 2007). Para oxidases, as fontes de nitrogênio desempenham um papel importante na regulação da expressão proteica, com efeitos dependentes da concentração e natureza (orgânica ou inorgânica) da fonte de nitrogênio (EGGERT et al., 1996). Em isolados fúngicos dos gêneros *Phlebia* e *Trametes*, fontes inorgânicas de nitrogênio levaram a baixos níveis de lacase, enquanto fontes orgânicas aumentaram a produção de lacases. (ARORA; RAMPAL, 2002; COLAO et al., 2003). Entretanto, vale ressaltar que a influência da concentração de nitrogênio na produção de oxidases é um fator controverso, uma vez que há descrições de aumento de atividade de lacases em concentrações limitantes e não-limitantes de nitrogênio (PISCITELI et al., 2011). Em um isolado fúngico, nomeado I-62 (CECT 20197) há descrição de aumento da transcrição de lacases em até 100 vezes em cultivo em condições não-limitantes (10 mM) de nitrogênio (MANSUR; SUÁREZ; GONZÁLEZ, 1998). Da mesma maneira, em *Phlebia* sp. e em *Cerrena unicolor*, a suplementação de culturas com nitrogênio leva a um aumento na produção de lacases (JANUSZ; ROGALSKI; SZCZODRAK, 2007; ARORA; RAMPAL, 2002). Efeito semelhante ocorre em relação a fontes de carbono, onde a suplementação com glucose resulta em maior atividade de lacase para *P. ostreatus* (PERIASAMY; PALVANNAN, 2010), porém, em concentrações limitantes de carbono os níveis de expressão de lacase aumentaram em *Cryptococcus neoformans* (ZHU; WILLIAMSON, 2004). Desta maneira, verifica-se que quando condições de cultivo são alteradas não há um padrão de regulação da produção enzimática entre cepas ou espécies de fungos.

A modulação de produção enzimática decorrente das formas de cultivo torna justificável o estudo destas variações para a otimização da produção de uma enzima específica ou para elucidar o conjunto completo de proteínas secretadas pelo organismo, o secretoma (NIEBISCH et al., 2014; POIDEVIN et al., 2014).

1.4 Secretoma de fungos

O termo secretoma foi utilizado pela primeira vez como definição para todas as proteínas secretadas e a maquinaria celular envolvida no processo de secreção (TJALSMA; BOLHUIS, 2000). Posteriormente, o termo foi aprimorado e definido como o grupo global de proteínas secretadas por uma célula, órgão ou organismo, em qualquer tempo e condição, por mecanismos conhecidos ou desconhecidos, envolvendo organelas reguladas (AGRAWAL; JWA; LEBRUN, 2010). Apesar das primeiras utilizações do termo terem sido para bactérias e plantas, os fungos vem ganhando espaço no estudo de secretomas, pois produzem um complexo arsenal de enzimas secretadas para sua interação com o ambiente (GIRARD et al., 2013).

O estudo de secretomas proteicos pode ser considerado um ramo da proteômica, beneficiando-se assim de avanços nas metodologias cromatográficas, eletroforéticas e técnicas de espectrometria de massas, bem como da disponibilidade de genomas fúngicos anotados e desenvolvimento de ferramentas de bioinformática (BOUWS; WATTENBERG; ZORN, 2008). Como metodologia para separação e isolamento de proteínas, a eletroforese bi-dimensional (O'FARREL, 1975; GORG; POSTEL; GÜNTHER, 1988) foi por anos a primeira escolha. Aliada à eletroforese, métodos de espectrometria de massas contribuem para identificação de proteínas através de PMF (*Peptide Mass Fingerprint*) ou homologia de sequências obtidas (MS/MS). Recentemente, a utilização de métodos livre de géis, como a cromatografia líquida associada à espectrometria de massas (LC-MS) oferece uma alternativa mais rápida e sensível para a identificação de proteínas, sendo utilizada em estudos proteômicos de larga escala ou *shotgun proteomics* (ALFARO et al., 2014).

Outra possibilidade para a determinação de secretomas é a utilização de métodos bioinformáticos para predição de proteínas que contenham peptídeo sinal característico de secreção, por meio de sequências genômicas ou transcriptômicas de organismos (CACCIA et al., 2013). Entretanto, não há garantia de que proteínas preditas existam de fato e a regulação de expressão proteica por meio de fatores externos torna divergente resultados obtidos por métodos bioinformáticos e proteômicos (BIANCO; PERROTTA, 2015). Ainda que mais laborioso, a identificação de enzimas secretadas por métodos proteômicos ainda permite a identificação de proteínas, não preditas como secretadas, por sequências gênicas (ANTELMANN et al., 2001).

Os estudos de secretomas fúngicos demonstram uma alta flutuação no número de proteínas identificadas, variando de dezenas (14) a centenas de proteínas (413) , o que é resultado tanto dos métodos proteômicos utilizados para identificação proteica, como pela produção enzimática de cada organismo (ALFARO et al., 2014). Em estudos utilizando-se LC-MS, como metodologia para identificação de proteínas, há demonstração de diferenças no padrão de produção de grupos enzimáticos conforme o organismo estudado e as condições de crescimento. A descrição do secretoma de *Phanerochaete chrysosporium* revelou 329 proteínas secretadas, das quais 10, 20 e 52% compreendem enzimas lignolíticas, proteases e hidrolases, respectivamente (ADAV; RAVINDRAN; SZE, 2012). O secretoma de *Trichoderma harzianum*, por outro lado, revelou produção de aproximadamente 17% de hemicelulases e 5% de celulases e a expressão de enzimas lignolíticas não chega a 1% e só é verificada quando o fungo é cultivado em meio contendo glucose (GOMEZ-MENDOZA et al., 2014). Essas variações de expressão proteica também ocorrem com *Irpex lacteus* e *Pleurotus ostreatus* onde o primeiro secreta hidrolases e oxidases em proporções parecidas, enquanto que *P. ostreatus* produz uma maior gama de oxidases e poucas hidrolases (BIANCO; PERROTTA, 2015). Esses resultados sugerem padrões diferenciais na degradação e uso de substratos, justificando assim o estudo de proteínas secretadas por diferentes espécies ou linhagens fûngicas a fim de se identificar proteínas com possível aplicação industrial. Além disto, há a possibilidade de identificação de complexos multienzimáticos que permitem uma ação mais efetiva sobre o substrato alvo (BOUWS; WATTENBERG; ZORN, 2008; DA SILVA et al., 2012)

1.5 - *Lepista sordida*

Lepista sordida é um fungo basidiomiceto saprofítico de coloração lilás (Figura 2) que habita serapilheira (SULZBACHER et al., 2006). Além de ser um fungo comestível, este é relatado como produtor de polissacarídeos com atividade imunorreguladora (LUO et al., 2012) e atividade apoptótica em células de câncer de laringe (MIAO et al., 2013), além de produzir metabólitos secundários (diterpenóides) com atividade antibacteriana e antifúngica (MAZUR et al., 1996) e tocoferóis com atividade antioxidante (HELENO et al., 2010).

Espécies do gênero *Lepista* foram descritas como produtoras de LME com atividade descorante e quebra de compostos poluentes (OKINO et al., 2000; STEFFEN;

HATAKKA; HOFRICHTER, 2003), enquanto que a única descrição existente para *L. sordida* é a de produção de MnP e lacase (CAVALLAZZI; OLIVEIRA; KASUYA, 2004), porém sem informações sobre a caracterização e otimização da produção enzimática.

Figura 2. Imagem de *Lepista sordida*.
(FONTE: Disponível em: http://www.first-nature.com. Acessado em setembro de 2015).

1.6 - *Lentinus crinitus*

O gênero *Lentinus* pertence à família de fungos basidiomicetos lignolíticos associados à podridão da madeira. Diversas espécies do gênero são comestíveis e possuem potencial aplicação na bioremediação de poluentes, como *L. subnudus* na degradação de petróleo em solos contaminados (ADENIPEKUN, 2005) e *L. lepideus* na degradação de trinitrotolueno (SAMSON et al., 1998). As espécies mais estudadas do gênero são *L. edodes* e *L. tigrinus*, pela produção de enzimas celulolíticas (ELISASHVILI et al., 2008; FENICE; FEDERICI; ANNIBALE, 2003), oxidoredutases (CHUM et al., 2011; ZHONG et al., 2013) e atividade antinflamatória (CARBONERO et al., 2008). *L. crinitus* (Figura 3) é um organismo pouco estudado, porém com potencial de utilização biotecnológica, devido às características do seu gênero e família, conforme citado acima. Entretanto, as únicas informações disponíveis na literatura são a produção de lacase (NIEBISCH et al., 2010; VALLE et al., 2014) e manganês peroxidase (ARBOLEDA et al., 2008).

Figura 3. Imagem de *Lentinus crinitus*.
(FONTE: Disponível em: http://mushroaming.com. Acessado em setembro de 2015).

2 - Justificativa

A utilização de métodos biológicos ou enzimáticos para produção industrial é uma estratégia em ascenção, uma vez que possibilita a utilização de reagentes renováveis e emprega metodologias menos poluentes. Os fungos lignolíticos desempenham um papel crucial neste panorama, pois são produtores de uma gama de enzimas capazes de agir sobre toda a estrutura lignocelulósica de substratos de origem vegetal. Desta maneira, resíduos agroindustriais como bagaço de cana, palha de arroz e casca de laranja podem ser utilizados como substratos para produção enzimática, além de possibilitarem a geração de produtos de alto valor agregado a partir destes substratos. A lignina presente nos substratos pode ser utilizada na síntese de compostos aromáticos de baixa massa molar, aplicáveis na indústrica química e farmacêutica. A partir da degradação da hemicelulose e celulose são gerados açúcares fermentáveis para produção de biocombustíveis de primeira e segunda geração. As enzimas modificadoras de lignina, além de possuírem aplicação na indústria de papel, madeira, tecidos e bebidas, podem ser utilizadas no tratamento de compostos químicos poluentes descartados em efluentes industriais. Os compostos xenobióticos são degradados por oxidases fúngicas possibilitando o emprego destes organismos para tratamento de rejeitos industriais poluentes.

Desta maneira, este trabalho tenta contribuir na prospecção de enzimas fúngicas capazes de degradar substratos lignocelulósicos ou compostos xenobióticos mais eficientemente. Para tal os seguintes objetivos foram traçados:

- Caracterizar a atividade de lacase de *Lepista sordida* na degradação de corantes têxteis, bem como determinar a estrutura primária do gene correspondente. Este objetivo compreende os resultados presentes no artigo I deste documento.

- Identificar as proteínas secretadas por *Lentinus crinitus* e avaliar a atividade enzimática das proteínas identificadas. Este objetivo é apresentado no artigo II deste documento.

3 – Artigo I

Partial characterization of the primary structure of a laccase gene in the litter-degrading fungi *Lepista sordida* and its application in the destaining of textile dyes

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Abstract

Basidiomycetes are usual sources of laccase, a copper-containing oxidase with promising potential in the treatment of colored effluents and several other industrial applications. Here we describe the gene structure and catalytic properties of the enzyme in the litter-degrading fungus *Lepista sordida*. Fungal secretion extracts containing a pair of polypeptides with laccase activity destained several textile dyes. The production of the enzyme doublet was improved several times in cultures containing maltose and sodium nitrate. Crude enzyme extracts showed good activity and stability in temperatures ranging from 20 to 50^{o}C and pH values between 3.0 and 5.0. Enzyme expression was not induced by the introduction of metal ions in culture media and enzyme activity was negatively affected by their presence. Finally, the introduction of redox mediators in enzyme assays resulted in the efficient destaining of 12 dye substrates, suggesting a potential application in the remediation of dye containing effluents. The partial structure of the *L. sordida* Lac gene was obtained by PCR using nucleotide sequences of the conserved copper binding domains. The resulting 1,519 nt fragment displayed high identity to several multicopper oxidases, it codes for 386 aminoacid residues and contains 6 introns, 7 exons as well as four potential glycosylation sites.

Keywords

biodegradation, waste-water treatment, enzymes, lignin, *Lepista*, laccase

Nomenclature

Lac	laccase
SMM	solid minimal medium
LMM	liquid minimal medium
MnP	manganese dependent peroxidase
LiP	lignin peroxidase
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
RB220	reactive blue 220
LMEs	lignin modifying enzymes

1. Introduction

The estimated annual production of dyes is of 0.7 million tons worldwide, with approximately 10,000 different dyes available for multiple industrial applications [**1**]. During fabric processing in textile industries up to 15% of the dye is lost to wastewaters resulting in highly colored effluents also containing a complex mixture of several organic pollutants and salts. The release of these residues into water streams and reservoirs results in local modification of pH, temperature and ionic strength, affecting water quality and the dynamics of the indigenous biota [**2**]. Currently, the removal of dyes from effluents is carried out by a variable combination of physical, chemical and biological techniques which main constraints are related to the efficiency, cost, and generation of sludge and toxic by-products when applied to large volumes of water [**3**]. In the last years, due to the increasing demand for more sustainable approaches and in order to overcome the limitations of conventional treatment strategies, several studies focused on the application of a relatively less explored area, the biological consumption of pollutants, namely biodegradation. Biodegradation is a environmental friendly and potentially low-cost alternative for the treatment of pollutants that involves the use of microorganisms, plants and algae, or enzymes derived from them, to break up and mineralize pollutants. Lignolytic basidiomycetes have been the most frequently employed organisms in biodegradation studies. They exhibit an exceptional ability to degrade pollutants such as pesticides, drugs, polycyclic aromatic hydrocarbons (PHAs) and textile dyes, associated to the production of lignin-modifiying enzymes (LMEs) [**4**]. Although earlier studies focused on two model organisms, *Phanerochaete chrysosporium* and *Trametes versicolor,* some authors have highlighted the degradation potential of other groups of basidiomycetes, underlining the importance to shift from the current model organisms. At this point, the eco-physiological group of litter-decomposing fungi is particularly interesting, since its members secrete a similar spectrum of oxidative enzymes [**5**]. Within this group it is found the *Lepista* genus. It comprises edible fungi with important medicinal and biotechnological applications. Some *Lepista* species have been assessed for the biodegradation of pollutants [**6, 7**] and the presence of LMEs [**6, 8, 9**]. In the present work the dye destaining ability of a secreted laccase from *L. sordida* was characterized, its catalytic properties as well as the partial primary structure of the corresponding gene.

2. Materials and methods

2.1. Organisms and culture conditions

The fungus *L. sordida* IOC 4580 [**10**] here studied is currently deposited in the Fungal Collection of the Instituto Oswaldo Cruz (Rio de Janeiro, Brazil). It was maintained by serial cultivation on solid minimal medium (SMM) and liquid minimal medium (LMM) containing (per liter): $NaNO_3$ 6.0 g; KH_2PO_4 1.5 g; KCl 0.5 g; $MgSO_4$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $ZnSO_4 \cdot 7H_2O$ 0.02 g; glucose 10.0 g; bacteriological agar 10.0 g; pH 6.8. LMM did not contain agar. After 7–10 days of growth on SMM, 4-mm diameter plugs from the active borders of cultures were used to inoculate 5 mL replicates of different liquid and solid culture media. Cultures were always incubated at 28^{o}C in the subsequent assays.

2.2. Dyes and chemicals

All the dyes tested in the present study were kindly supplied by Dystar (São Paulo, Brazil) and Siderquímica (Curitiba, Brazil). 2,2-Azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS), guaiacol and the reagents used as redox mediators were obtained from Sigma Chemical Company (St. Louis, MO, USA). All chemicals used were of the highest available purity and of analytical grade.

2.3. Preparation of fungal secretion extracts

Liquid cultures were centrifuged at 209 xg for 5 min and supernatants collected for direct analysis or stored at −10^{o}C for further analysis. Mycelia (pellets) were used for the determination of biomass. Thus, mycelia were filtered through previously dried and tared Whatman No. 1 filter papers, washed with distilled water and dried at 50^{o}C to constant weight. For solid cultures, 2 mL of salt solution (LMM without carbon or nitrogen) were added and left for agitation for 30 min. The soluble extracts were collected and the extraction repeated. After centrifugation at 209 xg for 5 min the resulting supernatants were stored at -10^{o}C.

2.4. Decolorization assays

Two kinds of tests were performed: the in-culture decolorization assay and the destaining activity assay, both using the diazo dye reactive blue 220 (RB220) as substrate. The former reflects the consumption of the dye during cultivation (15-30

days), by both the fungal biomass and secreted enzymes, while the latter reflects only the activity of secreted enzymes (90 min). In the in-culture decolorization assay, the fungus was grown in media already containing the RB220 dye (final concentration 0.1g L^{-1}) and the absorbance was monitored at 280 and 600 nm on a Shimadzu UV-160A UV–Vis spectrophotometer after 10–30 days of fungal growth. The percentage of in-culture decolorization was calculated as follows:

$$\text{in-culture decolorization}(\%) = \frac{(A_{CONTROL} - A_{SAMPLE}) \times 100}{A_{CONTROL}} \quad (1)$$

Where $A_{CONTROL}$ is the absorbance (at either 280 or 600 nm) of a non-inoculated culture containing the dye and A_{SAMPLE} is the absorbance of an inoculated culture containing RB220, after 10-30 days. In the destaining activity assay supernatants (derived from cultures grown for 15 days) were added to a solution containing RB220 (final concentration 0.1g L^{-1}) and the consumption of the dye measured over a 90-min period. The destaining activity was calculated as follows:

$$\text{destaining activity}(\%) = \frac{(A_0 - A_{90}) x 100}{A_0} \quad (2)$$

where A_0 is the initial absorbance (at 600 or at 280 nm) at time zero and A_{90} is the absorbance after 90 minutes of incubation at 28^{o}C.

2.5. Effect of carbon and nitrogen content on enzyme production

To improve enzyme production, a traditional stepwise strategy was adopted varying one factor at time and maintaining the previously optimized conditions. Six carbon sources (glucose, fructose, starch, maltose, sucrose and glycerol) were used to substitute the original carbon source in LMM or SMM. Subsequently there were tested different nitrogen containing compounds (sodium nitrate, ammonium tartrate, ammonium carbonate, ammonium oxalate, peptone and urea). The evaluated concentrations were 5, 10 and 15 g L^{-1}. All inoculated media were incubated for 15 days at 28°C in the dark and the supernatants or soluble extracts collected for further analysis.

2.6. Effect of pH, temperature, agitation and supplementation of cultures in the production of the enzyme

Culture medium was supplemented with copper sulfate (0.1 to 5 mM) or manganese sulfate (5 and 10 mM) and incubated as previously described. To assess the effect of agitation, the cultures were incubated in an orbital shaker (150 rpm) and in stationary

conditions for 15 days. Additionally, different temperatures (28, 35 and 40°C) and pH values from 5.5 to 7.0 were tested. In all cases culture supernatants were collected and the destaining activity assessed.

2.7. Lignin modifying enzymes, LMEs

The activity of LMEs was determined on culture supernatants. Total oxidase and laccase (Lac) activities were assayed by monitoring the oxidation of ABTS at 420 nm (ε_{420} = 36,000 $M^{-1}cm^{-1}$) [**11**]. Lignin peroxidase (LiP) activity was determined by the oxidation of veratryl alcohol at 310 nm (ε_{310} = 9,300 $M^{-1}.cm^{-1}$) [**12**] and the manganese-dependent peroxidase (MnP) assay was based on the oxidation of phenol red at 440nm [**13**]. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate per minute.

2.8. Effect of pH, temperature and salt concentration on destaining activity

Soluble extracts, derived from 15 days liquid cultures carried out under optimized conditions, were tested for destaining activity (see subsection 2.4) on different pH values, temperatures and salt concentrations. The pH values ranged from 3.0 to 8.0 using 50 mM citrate-phosphate buffer. The assessed temperatures were 4, 20, 30, 40, 50, 60 and 70°C. Destaining activity was also determined in the presence of different NaCl concentrations (0.05, 0.1, 0.2 and 0.6 M). In all experiments UV–Vis absorbance was monitored in each condition after 90 min at 28°C. The stability was assessed by pre-incubation of the soluble extracts in the conditions described above at intervals of 0, 3, 6, 9 and 24 hours. At the end of the incubation the destaining activity was determined and the residual activity calculated by assuming that the initial value (time zero) corresponded to 100%.

2.9. Effect of metal ions and enzyme inhibitors on destaining activity

The effect of several metal ions normally found in colored effluents such as Cu^{2+} ($CuSO_4$); Mg^{2+} ($MgSO_4$); Mn^{2+} ($MnSO_4$); Fe^{2+} ($FeSO_4$); Zn^{2+} ($ZnSO_4$) and Al^{3+} ($Al_2(SO_4)_3$) was assessed. Soluble extracts were incubated with salts of the above-mentioned ions at concentrations of 2, 10 and 50 mM and the destaining activity determined. The effect of potential enzyme inhibitors EDTA and sodium azide, at

concentrations of 0.1, 1 and 5 mM, was also evaluated. In all experiments, the absorbance was monitored in each condition after 90 min at 28^{o}C.

2.10. Destaining of different dyes and effect of redox mediators

The ability of the soluble extracts to destain different colored substrates (final concentration 0.1g.L^{-1}) was verified using 15 textile (RY84, RY135, RR141, R-Smax, RR195, C-HEXL, RR120, B-SMax, RB198, R-PF3B, RY107, RB220, RR198, AB194 and AB193) and 4 laboratory dyes (eosin, bromophenol blue, methylene blue and malachite green) (**Table S.1 Suppl Information**) in the presence and absence of 1mM p-coumaric acid, vanillin, acetosyringone or syringaldehyde as redox mediators. The corresponding dyes were reactive blue 220 (RB220), reactive Blue 198 (RB198), blue S-max (B-Smax), acid Blue 193 (AB193), acid black 194 (AB194), reactive red 195 (RR195), reactive red 198 (RR198), red S-max (R-Smax), reactive red 120 (RR120), reactive red 141 (RR141), red PF3B (R-PF3B), reactive yellow 84 (RY84), reactive yellow 135 (RY135), reactive yellow 107 (RY107), crimson HEXL, bromophenol blue (BB), eosin, methylene blue (MB) and malachite green (MG). The destaining activity was measured after 90 minutes and 24 hours by monitoring the decrease in the absorbance at the maximum absorption visible wavelength (λ_{max}) of each dye.

2.11. Zymograms

Soluble extracts derived from *L. sordida* liquid cultures were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without previous heating or reducing agent. The system consisted of a homogeneous 12% (w/v) acrylamide resolving gel and a 4% (w/v) acrylamide stacking gel. After electrophoresis, gels were washed in distillated water (2x 20 min) and soaked in renaturation buffer (50 mM sodium phosphate pH 7.4; 50 mM sodium chloride, 10% glycerol; 1% nonidet P-40; 1 mM beta-mercaptoethanol) overnight. Gels were then rinsed with water (3 x 20 min) and immersed in a solution containing 10 g L^{-1} RB220. The excess of liquid was removed, the gels were incubated at 28°C and the appearance of destained bands was monitored during a period of 24h. Alternatively, after washing out renaturation buffer, gels were soaked in 50 mM acetate buffer pH 5.0 containing either 0.05 mM ABTS or 0.01% guaiacol and incubated under the same conditions. Replicate gels were

developed by silver staining [**14**] to search for protein bands with same molecular weight of proteins reavealed in zymogram.

2.12. Nucleic acid extraction

L. sordida genomic DNA was purified as described by Moller et al. [**15**]. Total RNA was extracted using SV Total RNA isolation System kit (Promega, Madison, USA). Nucleic acid purity was verified by agarose 1% (w/v) gel electrophoresis in TBE buffer and quantified by NANODROP 2000 (Thermo Scientific) spectrophotometer.

2.13. Gene Cloning

In order to isolate the *L. sordida* Lac gene several pairs of primers corresponding to L1, L2, L3 and L4 copper binding sites (**Table 1**) were used in PCR assays. The PCR mixture was 50 mM KCl; 1.5 mM $MgCl_2$; 0.2mM dNTPs mix; 0.5 µM of each primer, 1.25 U of JumpStart Taq DNA Polymerase (Sigma, St Louis, USA) and 50-100 ng of template DNA. PCR was performed as follows: initial denaturation at 94 ºC for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. The product of amplification was submitted to 1% agarose gel electrophoresis, extracted and purified with GenElute PCR Clean-Up Kit (Sigma, St Louis, USA). The purified product was cloned into the pGEM® T easy vector (Promega, Madison, USA), transformed into chimiocompetent *Escherichia coli* DH10B by and sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, São Paulo, Brazil)

2.14. Bioinformatics

Nucleotide sequence alignments were made with BLASTX software using the nr NCBI database (http://blast.ncbi.nlm.nih.gov/). DNA sequences were assembled with Codon Code Aligner software (http://www.codoncode.com/aligner) and the prediction of exons and introns in assembled sequences was made with Augustus software (http://bioinf.uni-greifswald.de/augustus/) [**16**]. Analysis of post-translational modifications (PTM) was performed using the ExPASy ProSite tools (http://www.expasy.org).

2.15. Statistical analysis

Experimental data were evaluated by one-way analysis of variance (ANOVA) followed by the Tukey test, through the ASSISTAT 7.5 software (assistat.com). Differences were considered significant when *p* values were <0.01.

Table 1 Primer sequences used in this work

Primer	Sequence	Location
L1F	**5´ACN WSN ATH CAY TGG CAY GGN TTY TTY CA 3´**	**L1**
L1R	**5' CAG TTC GTG CCC TTC TGA AA 3'**	**L1**
L2R	**5´G RCT GTG GTA CCA GAA NGT NCC 3´**	**L2**
L3F	**5'CGG TCT ACA ACT ACG ACA ACC C 3 '**	**L3**
L4R	**5´RTG RAA RAC DAT RAG RCA RTG NAR RAA CCA 3´**	**L4**

L1 to L4 correspond to the conserved copper binding domains in the laccase family

3. Results and discussion

3.1. Screening and activity of lignin modifying enzymes (LMEs)

Biodegradation potential of *L. sordida* was assessed by using RB220 textile dye and measuring the percentage of in-culture decolorization and destaining activity in samples from 15 days old cultures. UV-Vis spectra of the dye displayed a significant reduction of the original absorbance peaks at 600 and 280 nm, suggesting the reduction of azo-linkages and loss of aromatic rings respectively [**17, 18**] (**Fig 1**). Tests for lignin modifying enzymes in culture supernatants revealed the presence of a laccase, since neither Mn^{2+} nor H_2O_2 were required for ABTS oxidation (data not shown). Zymograms revealed that two polypeptides of 48.5 and 36.5 kDa were responsible for both dye destaining and Lac activity (**Fig 2**). Cavallazi et al. [**8**] working with another *L. sordida* isolate reported the concomitant production of Lac and MnP. In the present work, neither LiP nor MnP activities were detected in specific assays. These results associate textile dye biodegradation of *L. sordida* with a lacase enzyme. Reported molecular mass for most of the fungal laccases is within the range of 60 to 100 kDa [**19**], but monomers with lower values have also been described [**20, 21**].

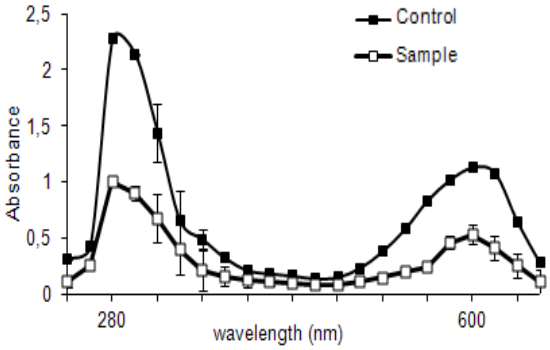

Fig. 1. UV–Vis spectrum of a solution of RB220 (0.1 g.L^{-1}) before and after incubation with a *L. sordida* soluble extract for 90 minutes. Results are mean of triplicate experiments.

In order to determine if the fungal components responsible for dye-decolorization were also associated to the mycelium, supernatants and biomass from 15-day old liquid cultures and eluted extracts from solid cultures were subjected to the 90 min destaining activity assay. Supernatants from liquid media displayed the highest destaining activity (88% and 71% for 280 and 600 nm, respectively) with relatively low activity being detected in mycelia (21%) (**Fig. S1 suppl information**). In solid media, although high in-culture decolorization was observed, the active component was not given to elution resulting in the absence of destaining activity in soluble extracts (not shown).

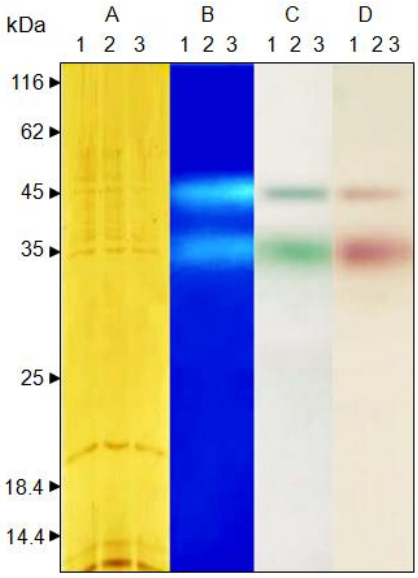

Fig. 2. 12% SDS PAGE of *L. sordida* liquid culture replicates (1-3) after silver staining (**A**) and in-gel activity using RB220 (**B**), ABTS (**C**) and guaicol (**D**) as substrates. Results are mean of three independent experiments.

3.3. Effect of carbon and nitrogen culture content on the production of the enzyme

The production of LMEs in fungi is usually affected by the type and concentration of carbon and nitrogen sources in culture media. *L. sordida* was grown on liquid media containing glucose, fructose, maltose, starch, sucrose or glycerol as the sole carbon source. Three concentrations (5, 10 and 15 g L^{-1}) of each substrate were tested and after 15 days of growth the destaining activity assay was performed using culture supernatants. Drastic differences in the production of destaining activity were observed. The highest rates were obtained when cultures were carried out in maltose, yielding a destaining activity ranging from 50 to 65%. Values obtained in the remaining carbon sources were below 20%, excepting glucose (10 g L^{-1}) with 41% of activity (**Fig 3A**).

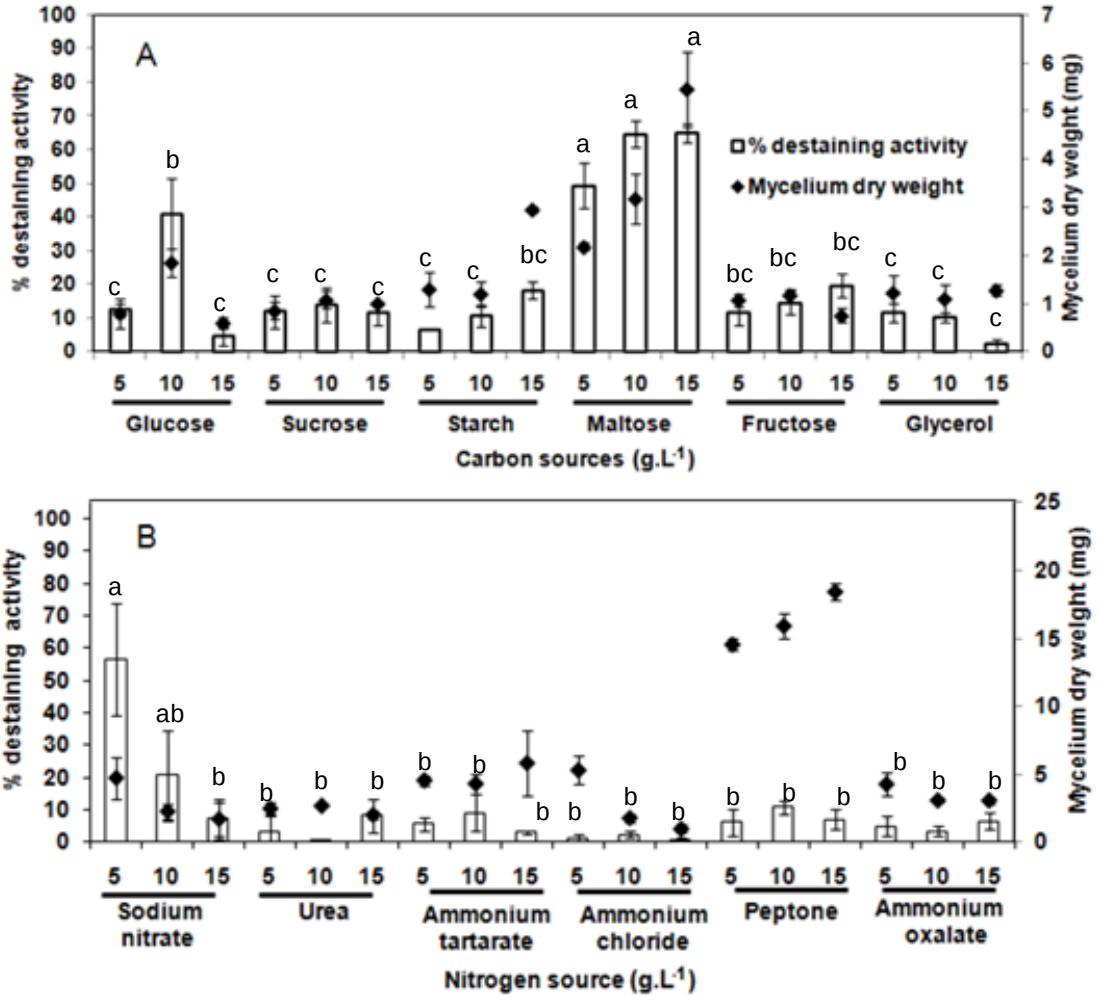

Fig. 3. Effect of carbon (**A**) and nitrogen (**B**) culture content on the production of destaining activity in liquid medium. Cultures in upper panel used sodium nitrate (5 g L^{-1}) as nitrogen source. Cultures in lower panel used maltose (5 g L^{-1}) as carbon source. Lower case letters correspond to statistic analysis. $p < 0.001$. Results are mean of three independent experiments.

When nitrogen sources were tested (using maltose 5 g L^{-1} as sole carbon source), the best production of destaining activity (57%) was promoted in cultures containing sodium nitrate (5 g L^{-1}) (**Fig 3B**). Laccases are often expressed in different isoforms and rates according to growing conditions. Although early reports suggested that their synthesis and secretion are frequently induced by limited levels of carbon and nitrogen sources [**22**], subsequent studies revealed this is not a general feature since enzyme production may change substantially (10 to 100 fold variation) depending on the carbon/nitrogen source and type of fungal isolate [**23, 24**]. In the case of carbon content, it is clear that high Lac production was frequently accompanied by high fungal growth. Interestingly, for nitrogen sources, sometimes the best biomass yield resulted in a poor Lac secretion (**Fig 3B**).

3.4. Effect of pH, temperature, agitation and copper/manganese supplementation of cultures on the production of the enzyme

Cultures were carried out in liquid medium with optimized carbon and nitrogen content (5 g L^{-1} maltose and 5 g L^{-1} sodium nitrate*)* and serially tested for different agitation conditions, pH, temperature and copper/manganese supplementation. Destaining activity was assessed in supernatants derived from 15-days-old cultures. Cultures incubated at 28°C and with initial pH values ranging from 6.0 to 6.5 yielded the best destaining activity (**Table 2**). Moreover, temperatures above 28^{o}C, agitation and supplementation with copper or manganese arrested fungal growth and undermined the production of the enzyme. Culture supplementation with metal ions usually stimulate

the production of laccases [**25**] since they are part of the functional structure of the enzyme and they can also interact with response elements inducing gene expression, but that effect was not observed in this work. Conversely, ion supplementation drastically arrested fungal growth and led to a diminished enzyme production. These toxic effects have been described before [**26**].

Monitoring the production of laccase and dye-destaining activity in cultures along time revealed that both follow the same behavior with the highest levels being obtained among days 15 to 20 and a peak production of 3,721 units of laccase per liter (**Fig 4**). This corresponds to 6.8 fold (not shown) the yield of the enzyme on the initial non-optimized media and 24 fold the value reported by Cavallazi et al.[**8**] in low nitrogen

media after 42 days of culture. Thus, optimized culture conditions demanded a higher content of carbon and nitrogen but resulted in a better enzyme production in a shorter period of time (20 days).

Table 2 Effect of agitation, temperature, pH, and metal ion culture supplementation on the production of destaining activity

Agitation	**(+)**	**(—)**		
% d.a	45.78 ± 0.7	55.5 ± 3.0		
m.d.w(mg)	1 ± 0.3	0.4 ± 0.02		
T(°C)	**28**	**35**	**40**	
% d.a.	56.88 ± 0.8	7.24 ±1.2	2.85 ± 0.2	
m.d.w(mg)	2.1 ± 0.6	1.93 ± 0.28	0.4 ± 0.13	
pH	**5.5**	**6**	**6.5**	**7**
% d.a	55.47 ± 14.1	70.46 ± 1.0	75.83 ± 2.1	56.88 ± 0.8
m.d.w.(mg)	2.8 ± 0	2.9 ± 1.6	2.97 ± 0.03	0.21 ± 0.06
Mn^{2+} (mM)	**0**	**5**	**10**	
% d.a.	63.17 ± 9.7	1.31 ± 0.3	0.745 ± 0.3	
m.d.w.(mg)	6.07 ± 0.2	3.2 ± 0.14	4.32 ± 0.24	
Cu^{2+} (mM)	**0**	**0.1**	**1**	**5**
% d.a.	53.54 ± 9.7	25.27 ± 3.6	3.94 ± 1	2.17 ± 0.4
m.d.w.(mg)	5.05 ± 1.95	4.02 ± 1.5	0.82 ± 0.2	0.77 ± 0.09

note: destaining activity (d.a); mycelial dry weight (m.d.w). Values are mean of three experiments ± SD (standard deviation). Assays were realized with supernatant cultured in LMM with 5 g L^{-1} maltose and 5 g L^{-1} sodium nitrate. Conditions for the assays were: 28°C, pH: 6.8 and absence of agitation and metallic ions with respective modifications for each variable tested.

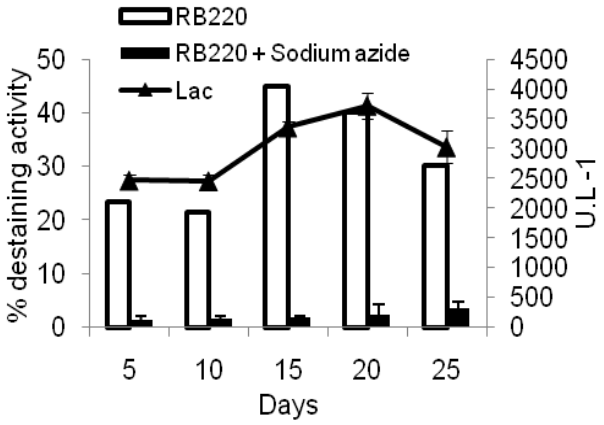

Fig. 4. Production of RB220 destaining activity and Lac along time in optimized liquid cultures. The effect of 0.1mM sodium azide on RB220 destaining is also depicted. Results are mean of triplicate experiments.

3.5. Effect of temperature, pH, and salt concentration on enzyme activity

Destaining assays were performed at several pH values, temperatures and salt concentrations. Little variation was observed in temperatures ranging from 20 to 70°C (**Fig 5A**). When thermal stability was assessed, all the samples pre-incubated in

temperatures from 4 to 40°C and periods among 3 to 24 hours, displayed a high residual destaining activity, between 95% (3h) and 65% (24h) (**Fig 5B**). Assessing the effect of

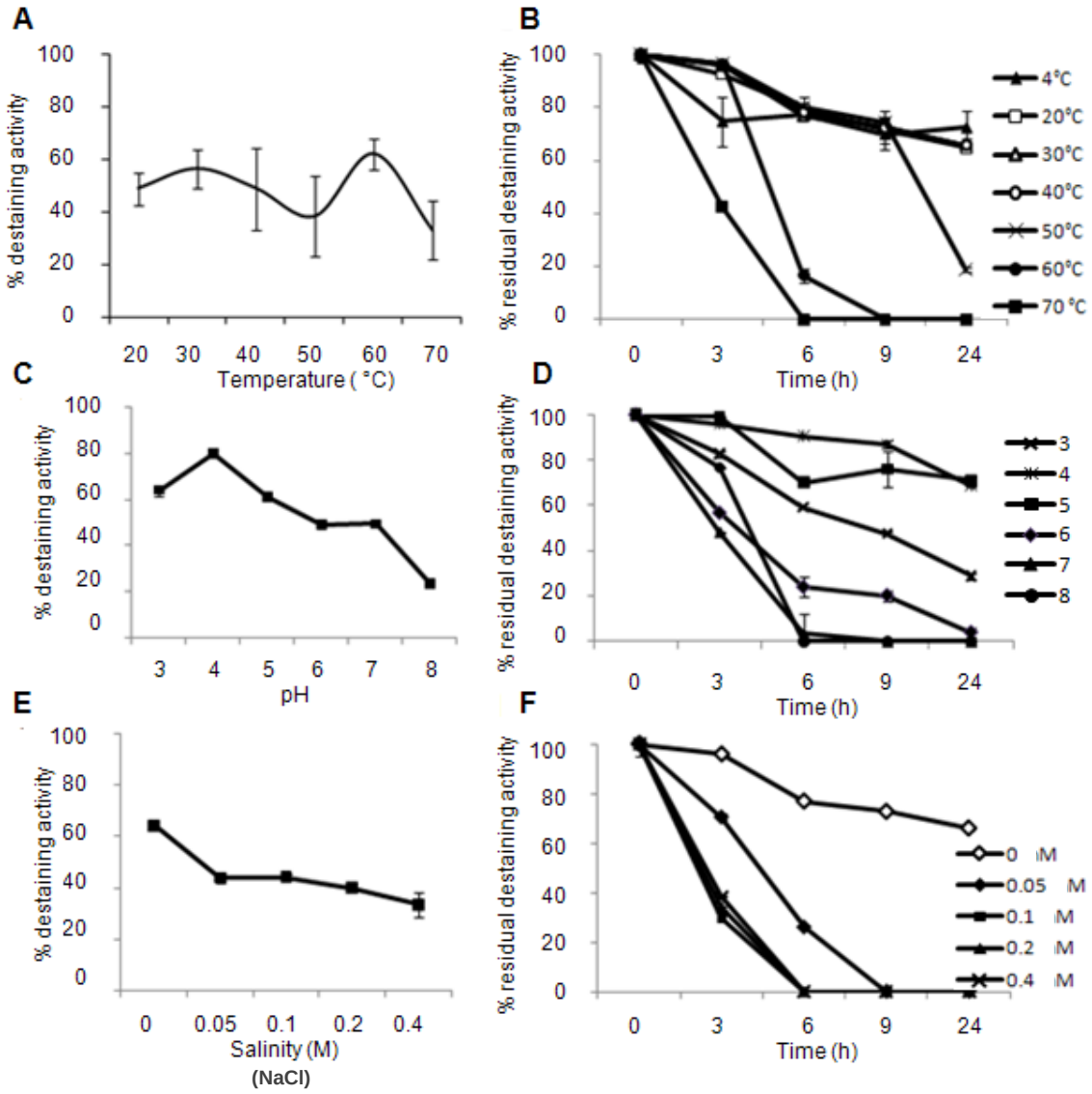

Fig. 5. Effect of temperature, pH and salt concentration on the destaining activity and stability of *L. sordida* soluble extracts: (**A**) effect of temperature, (**B**) thermal stability, (**C**) effect of pH, (**D**) stability at different pH values, (**E**) effect of salinity and (**F**) stability at different salt concentrations. For each variable tested initial conditions of assays were maintained but the modified parameter. Results are mean of three independent experiments ± SD (standard deviation).

pH, the best destaining activity was observed in pH values between 3.0 and 5.0 with a peak around pH 4.0 (**Fig 5C**). The activity after 24 hours of pre-incubation on pH 4.0 and 5.0 remained very close to the maxima, with residual activity values higher than

70%. Sample pre-incubation in pH values below or above the latter range resulted in a rapid loss of activity in the first hours (**Fig 5D**). In the presence of different salt concentrations (0.05 to 0.4M) the enzyme extract displayed good destaining activity although accompanied by a relatively low half life (2-3h) (**Fig 5E, F**). The typical reaction conditions for fungal laccases cover temperatures between 50^{o}C and 70^{o}C [**19**] and pH values around 4.0 [**19, 27**]. Temperature stability of laccases varies considerably: the half life at 50^{o}C ranges from minutes in *Galerina sp*.[**28**] to over 4h in *Fomes fomentarius* [**29**], to up to 50–70 h in *Coriolus sp* [**30**]. Furthermore, the stability of fungal laccases is generally better at acidic pH [**27**]. Since the laccase doublet here described displayed optimal destaining activity and stability in temperatures among 20 to 50^{o}C and pH values ranging from 4.0 to 5.0, it can be included in the group of mesophilic laccases. The absence of bimodal curves in the graphics depicting the behavior of the extract with changes in pH, temperature and salt concentrations, suggests that both polypeptides behave in a similar way, although it cannot be affirmed if they correspond to post-translational modification or to the expression of isoforms as have been described in other fungi.

3.6. Effect of metal ions and enzyme inhibitors on destaining activity

At concentrations of 2 mM, none of the tested ions excepting iron and aluminum ions exerted major changes on enzyme activity. Higher concentrations resulted in partial inhibition of enzyme activity with a reduction range among 38 to 94% (**Fig. 6**). In contrast, the increase in manganese ions content resulted in up to 22% improvement in destaining activity. EDTA (0.1-5mM) partially inhibited the enzyme activity while sodium azide (0.1 mM) totally removed the destaining activity (**Fig. 6**). The effect of metal ions on laccase activity varies from one enzyme to another, according to type of ion and concentration. Guo et al. [**31**] described the promotion of the enzyme by Mg^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+} ions while the addition of Co^{2+}, Al^{3+}, Cu^{2+}, or Fe^{2+} resulted in inhibition of the activity. In contrast, the absence of effect by a similar group of cations has been also reported [**29**]. The strong inhibitory effect displayed by copper and iron ions in this work may partly be due to the metal catalyzed oxidation of some important amino acid residues in the enzyme [**28**]. On the other side, the better catalysis observed in high concentration of manganese ions may be related to the redox activity of this ion promoting the autoxidation of intermediate metabolites of enzyme substrates [**32**] or due

to the enzymatic generation of the strong oxidant Mn^{3+} ions that can act directly on the final substrate of the enzyme [**33**].

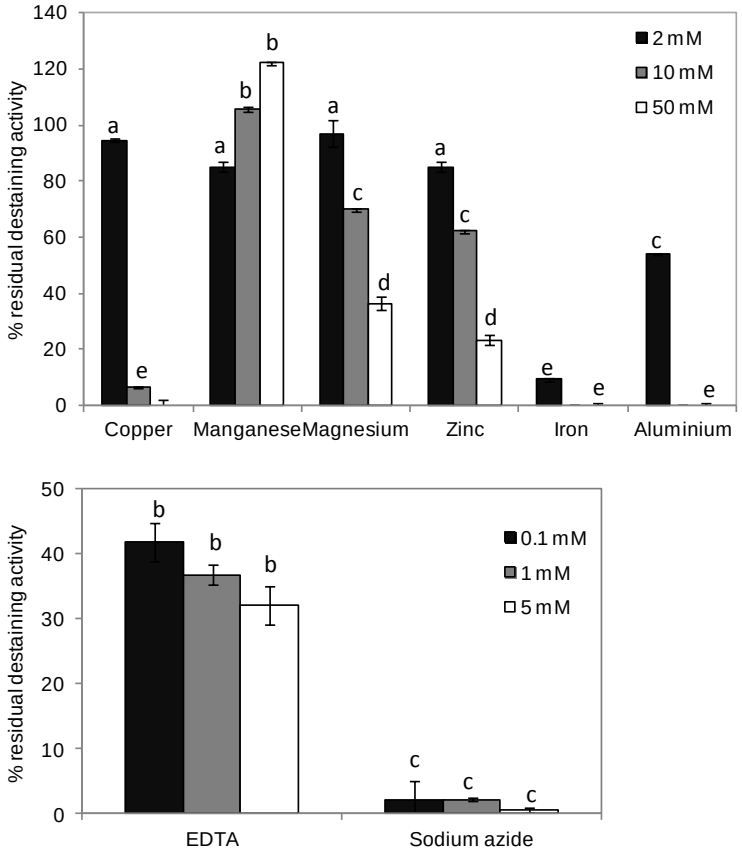

Fig. 6. Effect of metal ions (**A**) and enzyme inhibitors (**B**) in the destaining of RB220 by *L. sordida* soluble extract. Lower case letters correspond to statistic analysis. p <0.001. Results are mean of three independent experiments.

3.7. Destaining of different dye substrates and effect of redox mediators

Twenty dyes were incubated with filtrates of *L. sordida* liquid cultures, with or without the addition of redox mediators (p-coumaric acid, vanillin, syringaldehyde and acetosyringone). In a 90 minutes interval and in the absence of mediators, significant destaining (among 26-60%) was obtained for four dyes (RB220, AB194, RB198, AB193). Lengthening the incubation time to a 24 h period increased the number of destained substrates to six (**Fig 7A**). The dyes RR195 and B-Smax were the new additions and their destaining ranged among 14-28%. The introduction of acetosyringone in the experiments resulted in the destaining of six additional dyes: RR198, R-Smax, RR120, R-VPF3B, bromophenol blue and malachite green with destaining efficiencies among 16 to 87% (24h) (**Fig 7B**). Similar results were obtained

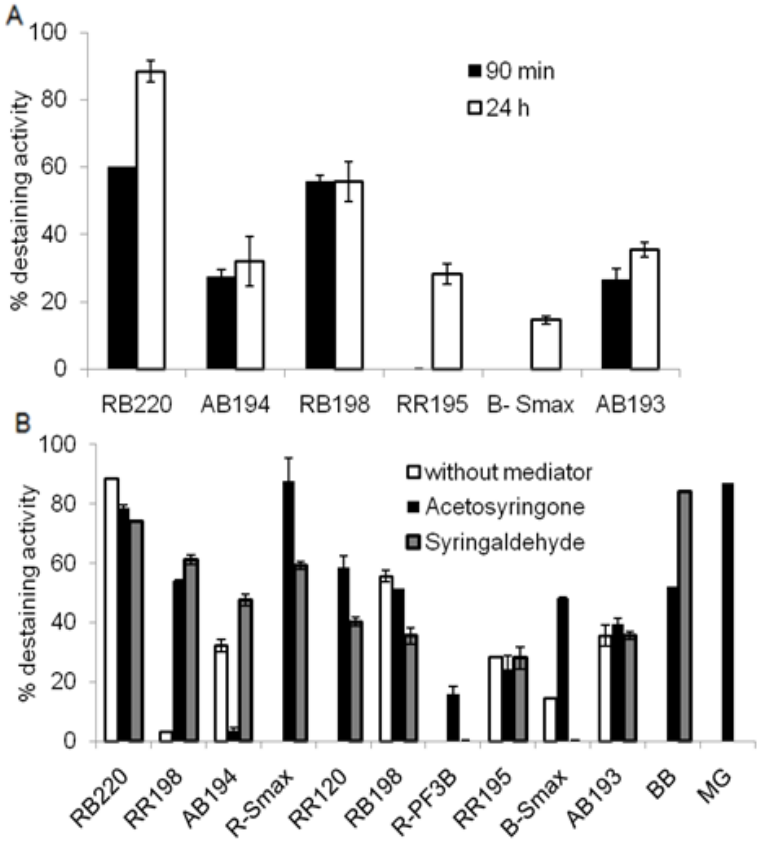

Fig. 7. Destaining activity of *L. sordida* soluble extract on different dyes after 90 min and 24 h of incubation (**A**) and after 24 h in the presence of redox mediators (**B**). Reactive blue 220 (RB220); acid black 194 (AB194); reactive Blue 198 (RB198); reactive red 195 (RR195); blue S-max (B-Smax); acid Blue 193 (AB193); reactive red 198 (RR198); red S-max (R-Smax); reactive red 120 (RR120); red PF3B (R-PF3B); bromophenol blue (BB) and malachite green (MG). Results are mean of three independent experiments

when syringaldehyde was introduced: four new dyes (RR198, R-Smax, RR120 and BB) turned into laccase substrates, with destaining effiencies among 40% (RR120) to 85% (BB) in 24h, although all of them were already included in the acetosyringone-dependent destaining group. Thus, lengthening the incubation time and introducing redox mediators in the assay increased the number of putative Lac substrates from 4 to 12 dyes. Neither p-coumaric acid nor vanillin improved laccase mediated destaining (data not shown). A deletereous effect in the destaining assay was observed for three dyes when redox mediators were used: AB194 (for acetosyringone), RB198 and B-Smax (for syringaldehyde). The use of redox mediators without the addition of the enzyme extract did not result in any destaining of substrates (not shown). One of the most important constraints for the application of laccases in the treatment of real-life textile effluents is the ability of the enzyme to act onto several different substrates, since

the quantity and kind of dyes routinely used by a medium sized industry suffer a great variation according to the season and textile fiber in use. One strategy employed to achieve this goal is the introduction of redox mediators that allow the oxidation of substrates that cannot be directly used by the enzyme due to sterical hindrance or a kinetic barrier. In the present work acetosiryngone and syringaldehyde were the only useful mediators as they extended the number of substrates used by the enzyme. These mediators have already been described as the most efficient with laccases from other sources [**34**]. Though it has been shown that the type of substitutions in the aromatic dye structure of the substrate influences the susceptibility to enzyme oxidation [**35**], in this work it was not observed a particular preference of the enzyme nor mediator for a specific class of dyes.

3.8. Laccase gene structure

PCR assays using primers belonging to the conserved laccase copper binding domains resulted in two amplification products of 200 and 1,500bp respectively (**Fig 8**). Assembled sequences revealed a 1,519 nt amplicon with 57-74% identity with several other multicopper oxidases, specially with *Trametes versicolor* laccases (not shown).

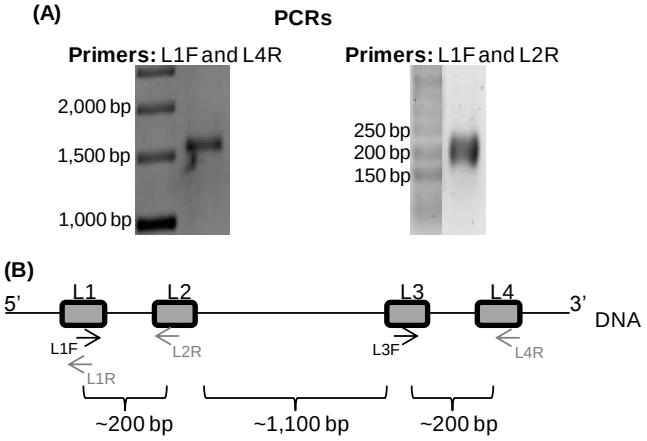

Fig. 8. PCR products obtained with primers for copper binding sites L1 and L4 and L1 and L2 (**A**). Schematic illustration of the conserved copper binding domains (L1-L4) and location of primers used in the cloning of *L. sordida* Lac gene (**B**).

The partial laccase gene of *L. sordida* is composed of 6 introns and 7 exons. Protein prediction analysis resulted in an ORF coding for 386 amino acids residues (**Fig. 9**) comprising consensus regions from laccases, such as four copper binding sites, four

substrate binding loops and cysteines residues potentially involved in essential disulfide bonds [**36**]. BlastP aligment of the 1,519 nt amplicon resulted in a sequence coverage of 99-100% and an identity of 74-93% with other laccases. Two potential N-glycosylation sites are present in the *L. sordida* Lac ORF (**Fig. 9**). These sites have been already

```
  1    TWIHWHGFFQKGTNWADGPAFINQCPISSGHSFLYDFQVPDQAGTFWYHS   50
 51    HLSTQYCDGLRGPFVVYDPNDPSADLYDVDNDDTVITLADCLGADATINN  100
101    GKGRSPSTTTVDLSVISVTQGKRYRFRLVSLSCDPNFTFSIDGHNMTIIE  150
151    TDSINTAPLVVDSIQIFAAQRYSFVLEANQAVDNYWIRANPSFGNVGFTG  200
201    GINSAILRYDGAAAIEPTTTQTTSTEPLNEVNLHPLVATAVPGSPVAGGV  250
251    DLAINMAFNFNGTNFFINGASFTPPTVPVLLQIISGAQNAQDLLPSGSVY  300
301    SLPSNADIEISFPATTAAPGAPHPFHLRGHAFAVVRSAGSPVYNYDNPIF  350
351    RDVVSTGTPAAGDNVTIRFRTDNPGPWFLQCLIVFH               386
```

Fig. 9. Predicted amino acids sequence for the *L. sordida* laccase gene. Underlined letters correspond to amino acid residues from copper binding sites. Italic bold letters are the substrate binding loops. Light gray shading are cysteine residues which potentially participate in disulfide bridges. Dark gray shading are potential sites for N-glycosylation predicted with ProSite (ExPASy). Black shading with white letters are N-glycosylation sites described in literature.

described in other laccases [**37**]. Additionally, two new potential glycosylation sites were found when ProSite database was used for the search of PTM (**Fig. 9**). The consistent appearance of only one amplicon in the PCR assays suggests that the two bands observed in zymograms represent the same polypeptide although with different post-translational modifications. Nevertheless, different genes with minor differences in sequence may generate a single PCR product due to the low resolution power of agarose gel electrophoresis. Determination of the 5' and 3' regions of the laccase gene was attempted by using a single primer walking amplification protocol, SPW [**38**] and a modified 5´, 3´RACE strategy [**39**]. PCR products in a wide range of sizes were obtained but neither cloning nor sequencing gave consistent results. Attempts to obtain the sequence of the Lac transcript were equally unsuccessful.

4. Conclusions

To date, more than a hundred of laccases have been characterized, many of them derived from common representatives of the lignolytic fungi group. Here it is described the partial primary structure of a laccase gene and the catalytic properties of the enzyme in the litter degrading fungi *L. sordida*. Lac gene structure displayed high identity with laccases from other basidiomycetes and the enzyme showed a good tolerance to pH and temperature and also the ability to degrade several colored substrates in the presence of redox mediators, implying its potential use for the remediation of colored effluents.

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Role of the funding source.

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5. Supplementary information

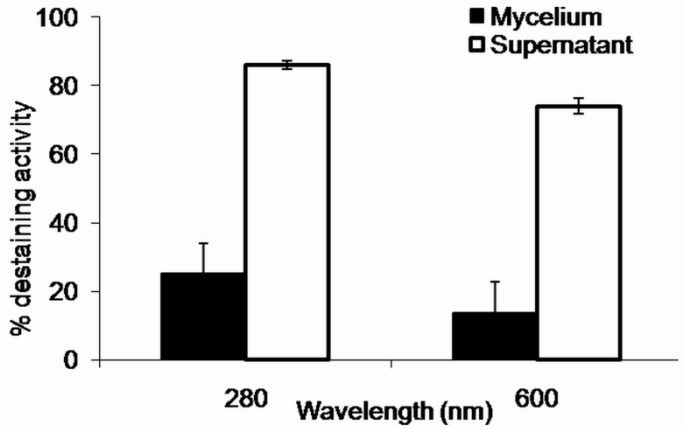
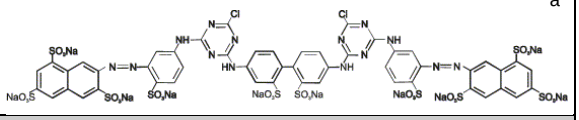
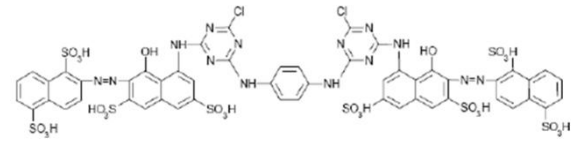
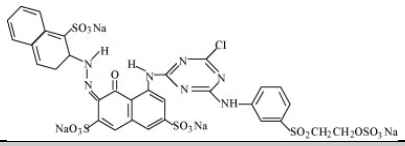
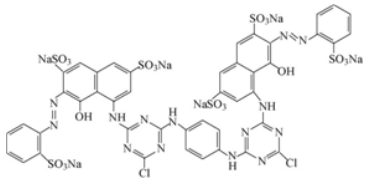
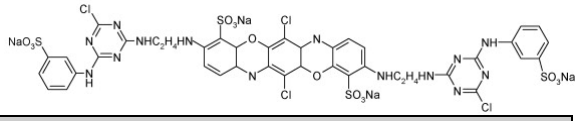
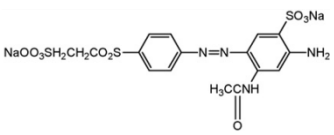
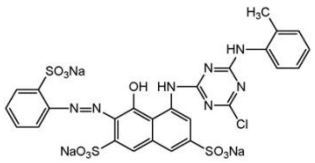
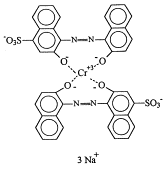
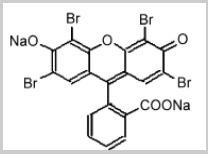
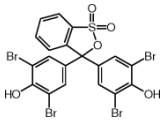
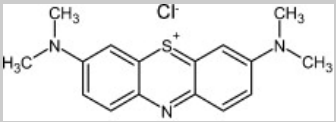
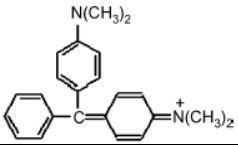


Fig. S1. RB220 destaining activity (90 min.) of mycelia and culture supernatants derived from 15 days old cultures. Culture conditions: LMM at 28°C.

Table S1. Structures and maximum absorption visible wavelength of textile and laboratory dyes used for destaining activity assays.

	Name	abbreviation	λ_{max} (nm)	Structure
Textile dyes	Reactive Yellow 84	RY84	420	a 
	Reactive Yellow 135	RY135	400	n.a.
	Reactive Red 141	RR141	520	b 
	Red SMax	R-SMax	520	n.a.
	Reactive Red 195	RR195	540	c 
	Crimson HEXL	C- HEXL	540	n.a.
	Reactive Red 120	RR120	540	c 
	Blue SMax	B-SMax	600	n.a.
	Reactive Blue 198	RB198	620	d 
	Red PF3B	R-PF3B	520	n.a.
	Reactive Yellow 107	RY107	420	e 

	Reactive Blue 220	RB220	600	n.a.
	Reactive Red 198	RR198	520	e 
	Acid Black 194	AB194	560	n.a.
	Acid Blue 193	AB193	580	f 
Lab. Dyes	eosin	EO	500	g 
	Bromophenol blue	BB	600	h 
	Methylene blue	MB	660	i 
	Malachite green	MG	600	g 

n.a. not avaliable.

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4 - Artigo II

Essential Title Page Information

Title

Analysis of the biotechnological potential of a *Lentinus crinitus* isolate in the light of its secretome

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Abstract

Due to their great potential for biotechnology and large-scale industrial applications, enzymes derived from microbial sources have been the focus of research projects worldwide. The analysis of fungi secreted proteins has been increasingly employed as a powerful strategy for the screening of new enzymes. Since enzyme production is strongly modulated by several factors such as pH, available nutrients, water content, oxygen levels and temperature, the evaluation of growth conditions is of utmost importance to achieve optimal enzyme production. Here, a non-sequenced fungus, *L. crinitus*, was selected as a model organism for secretome analysis by means of *in vitro* enzymatic assays and in-depth characterization via proteomics. For enzyme production, the fungus was cultured with several types and concentrations of carbon and nitrogen sources and variable water content. Seven carbon substrates (glucose, maltose, starch, sucrose, carboxymethylcellulose (CMC), glycerol and fructose) and three nitrogen containing compounds (urea, sodium nitrate and ammonium chloride) were used as substitutes of the original carbon and nitrogen sources in culture media. Interestingly, the biomass yield as well as the array of secreted proteins differed drastically under different growing conditions. For instance, reduced water content stimulated the expression of oxidases/reductases such as Lac, MnP, GMC oxidoreductases and glyoxal oxidase. A mixture of soluble secreted extracts derived from different culture conditions was analyzed both by shotgun mass spectrometry and through protein separation by two-dimensional gel electrophoresis (2DE) prior to identification via LC-MS/MS. Proteins were identified by sequence homology searches using MS-driven BLAST. The spectrum of secreted enzymes comprised various types of CAZymes (carbohydrate-active enzymes), oxidase/reductases, proteases, lipase/esterases, proteins with non-related functions thereby classified as miscellany proteins and hypothetical or unknown predicted proteins. Although pre-separation by 2DE improved the number of identifications (protein map of 150 spots corresponding to 171 identifications) compared to the shotgun approach (98 identifications) both strategies revealed similar distribution of proteins within the functional categories described above. The diversity of proteins observed within both the CAZyme and oxidoreductase groups revealed in this fungus a powerful arsenal of enzymes dedicated to the breakdown and consumption of lignocellulose. Moreover, further secretome characterization after sequencing and analysis of the *L. crinitus* genome can potentially lead to the discovery of novel enzymes of industrial and biotechnological interest.

1 - Introduction

Lignocellulose is the major reservoir of organic carbon in our planet. It is highly recalcitrant to physical and biological turnover due to the crystalline structure of cellulose and the protective action of lignin. Few organisms can degrade lignocellulose, most of them are wood rotting basidiomycetes. They release into the environment a complex set of enzymes that break down the lignin polymer gaining access to polysaccharides that are then used as a source of carbon and energy (GIRARD et al., 2013). Enzymatic consortia derived from wood rotting fungi include carbohydrate active enzymes (CAZymes), oxidases and peroxidases (lignin degradation) and also proteases and lipases (ALFARO et al., 2014). Lignin-degrading enzymes and CAZymes display a wide range of industrial applications such as the modification of the final properties of fabric in textile industry (WESENBERG et al., 2003); biodegradation of recalcitrant pollutants (COUTO; HERRERA, 2006); saccharification of agroindustrial wastes in the production of second generation biofuels (KHARE et al, 2015) and generation of building blocks to supply chemical synthesis (CHERUBINI; STROMMAN 2011).

Lentinus genus comprises a group of edible fungi with around 40 different species and worldwide distribution mainly in subtropical regions. The most well known species is *L. edodes* (shitake mushroom), one of the most popular cultivated mushroom in the world. Several species and strains have been also tested for other biotechnological applications associated to the production of exopolysaccharides (JUNG et al., 2008), terpenes and antioxidant/anti inflammatory properties (WANG et al., 2013), as well as the repertoir of lignolytic enzymes (SARNTHIMA et al., 2009).

In the present work we approached the characterization of the secretome of a local lignolytic *Lentinus crinitus* isolate. To favor the expression of the repertory of potential secreted enzymes, the fungus was grown in media with variable content of carbon, nitrogen and water, and the resulting soluble extracts analyzed through two-dimensional gel electrophoresis, LC-MS and conventional enzyme activity assays.

2 - Materials and methods

2.1 - Organism and culture conditions

L. crinitus IOC 4579 was originally collected in Campina Grande do Sul (Paraná, Brazil) and maintained by serial cultivation on potato dextrose agar at 28 ^{o}C in the dark. It is currently deposited in the Fungal Collection of the Oswaldo Cruz Institute (Rio de Janeiro, Brazil). For each assay, it was grown on plates of solid minimal medium (SMM), containing per liter: $NaNO_3$ 6.0 g; KH_2PO_4 1.5 g; KCl 0.5 g; $MgSO_4$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $ZnSO_4 \cdot 7H_2O$ 0.02 g; glucose 10.0 g; bacteriological agar 10.0 g; pH 6.8. Liquid minimal medium (LMM) had an identical composition as SMM excluding agar. After 7–10 days of growth on SMM, three mycelial plugs collected using a 4-mm diameter biopsy punch were used to inoculate 5mL replicates of different sterilized culture media. Cultures were incubated at 28±0.2 ◦C for subsequent assays. Seven different carbon substrates (glucose, maltose, starch, sucrose, carboxymethilcellulose (CMC), glycerol and fructose) and nitrogen containing compounds (urea, sodium nitrate and ammonium chloride) were used as substitutes of the original carbon and nitrogen sources in culture media. The assessed concentrations were 1, 5 and 20 g L^{-1} (for carbon sources) and 4, 20 and 100 mM (for nitrogen sources). Low water content cultures were obtained by adding vermiculite (0.12 g mL^{-1}) to liquid media.

2.2 - Sample preparation

Culture supernatants were separated from the mycelium with filter paper and then sieved through 0.45 µm nitrocellulose membranes. For cultures on solid media, two extractions with 2 mL of minimum media (carbon and nitrogen free) were performed in an orbital shaker (150 rpm) for 30 min. Samples were stored at -20°C until utilization. When required, protein precipitation was carried out by the addition of trichloroacetic acid (20% final conc.) followed by an overnight incubation at -20^{o}C. Samples were then centrifuged (15,000 xg) for 10 min and washed twice with cold ethanol. Proteins were resuspended in water prior to measurement of protein concentration by modified Bradford assay (ERNST; ZOR, 2010).

2.3 Protein electrophoresis

2.3.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

Culture supernatants (80 µL) were solubilized in equal volume of Laemmli buffer 2X, boiled and subjected to electrophoresis on 10% SDS-PAGE gels (LAEMMLI, 1970) in a vertical cube Hoeffer SE 600 Ruby (GE Healthcare) with 30 mA of constant current. Protein profiles were revealed by silver staining (BLUM; BEIER; GROSS, 1987).

2.3.2 2D-SDS-PAGE

For two-dimensional gel electrophoresis, 13 cm IPG strips pH 4-7 (GE Healthcare, São Paulo, Brazil) were loaded with 80 µg of protein and then submitted to isoeletric focusing (IEF) in an Ettan IPGphor II system (GE Healthcare, São Paulo, Brazil) with running conditions of 20°C and 50 µA of current per strip for total 20 kVh according to following protocol: step and hold 0.5 kVh, gradient 0.8 kVh, gradient 11.3 kVh, step and hold 7.4 kVh. Reduction and alkylation of IPG gels were performed in 3 mL of equilibrium buffer (50 mM Tris pH 8.0, 6 M Urea, 30% glycerol and 2% SDS) containing 125 mM DTT for 30 min followed by incubation in the same buffer containing 300 mM acrylamide instead of DTT for 30 minutes (MINEKI et al., 2002). The second dimension run was performed in 10% SDS-PAGE gels, as described previously, and proteins were visualized after silver staining (BLUM; BEIER; GROSS, 1987).

2.4 LC-MS analysis

2.4.1 Sample preparation

Lyophilized fungi secretion was resuspended in 100 µL of lysis buffer consisting of 8 M urea, 0.5 % Triton, 100 mM DTT, 2x phosphatase inhibitor cocktails II and III (Sigma Aldrich, St Louis, USA), 2x Complete-EDTA free protease inhibitor cocktail (Roche, Oslo, Norway) and protein concentration was determined using the Bio-Rad Protein assay. The lysate (50 µg protein) was submitted to methanol-chloroform precipitation as described (BATTH et al., 2012) followed by resuspension in 50 mM NH_4HCO_3 containing 5 mM tris (2-carboxyethyl phosphine (TCEP) and incubation for 30 min prior to alkylation with 1 µmol/mg protein of iodoacetamide for 30 min in the dark. Overnight protein digestion was performed using LysC-Trypsin mix (Promega, Madison, WI, US) at 1:50 ratio (w/w, enzyme:protein) at 37◦C. Tryptic digests were

diluted to final concentration 0.5 µg/µL in 0.1% formic acid prior to mass spectrometry analysis.

2.4.2 In-gel tryptic digestion

Protein bands excised from a 1D gel were reduced and alkylated with iodoacetamide as described above while protein spots excised from 2D gels were alkylated with acrylamide prior to in-gel tryptic digestion as described (SHEVCHENKO et al., 1996). Tryptic digests were dried out followed by resuspension in 0.1 % formic acid for mass spectrometry analysis.

2.4.3 Mass spectrometry analysis

Tryptic digests were analyzed on a Thermo Scientific Q Exactive mass spectrometer operating in FullMS-ddMS2 mode coupled to an EASY-nLC 1000 UHPLC system (Thermo Scientific, Oslo, Norway). Peptides (2 µg) were injected onto a Acclaim PepMap100 C-18 column (75 µm i.d. × 2cm, C18, 3 µm, 100 Å) (Thermo Scientific, Oslo, Norway) and further separated on a Acclaim PepMap100 C-18 analytical column (75 µm i.d. × 50 cm, C18, 2 µm, 100 Å) (Thermo Scientific, Oslo, Norway). For the fungi secretion peptides, a 240 min method was employed consisting of a 250 nL/min flow rate, starting with 100 % buffer A (0.1 % formic acid) with an increase to 5% buffer B (100 % acetonitrile, 0.1 % formic acid) in 5 min, followed by an increase to 40 % buffer B over 220 min and an increase to 100 % buffer B in 8 min, where it was held for 10 min. Peptides extracted from gel bands and protein spots were processed using a 60 min method starting with 100 % buffer A with an increase to 5% buffer B in 2 min, followed by an increase to 35 % buffer B over 46 min and a rapid increase to 100 % buffer B in 3 min, where it was held for 9 min. The peptides eluting from the column were ionized by a Nanospray Flex Ion Source (Thermo Scientific, Oslo, Norway) and analyzed on the Q Exactive operating in positive-ion mode using electrospray voltage 1.9 kV and HCD fragmentation. Each MS scan (m/z 300–1600) was acquired at a resolution of 70 000 FWHM, automatic gain control (AGC) target value of 3×10^6, maximum injection time of 200 ms. TopN 10 MS/MS scans were acquired at a resolution of 17 500 FWHM, maximum injection time of 100 ms, normalized collision energy (NCE) 30, AGC target value of 1×10^5, and isolation window 2 m/z, dynamic exclusion 30 s. MS and MS/MS scans were acquired with similar parameters for gel bands and protein spots, however, AGC of 1×10^6 and maximum injection time of 250

ms were applied for MS scans and AGC target value of 1×10^5 with maximum injection time of 100 ms were employed for MS/MS scans.

2.4.4 Data analysis

Prior to protein identification, MS spectra were analysed using Preview software (KIL et al., 2011) revealing high degree of deamidation (about 40 %) on data acquired from the fungi secretion extract. Therefore, deamidation was included as variable modification in database searches for this sample. Protein identification was performed via analysis of acquired MS spectra using Thermo Proteome Discoverer version 1.4.0.288 software running Mascot and the Sequest HT search algorithms. Spectra were searched against a Polyporales database with the following parameters: Max. missed cleavage = 2, precursor mass tolerance = 20 ppm, fragment mass tolerance = 0.5 Da. For data acquired from gel bands, static modification: carbamidomethyl (C: +57.021 Da) and dynamic modifications: oxidation (M: +15.995 Da), were included. Deamidation (N,Q: 0.984 Da) was also included as dynamic modification for searches using data collected from fungi secretion. For protein spots data, static modifications were not assigned, instead, propionamide (C: +71.037 Da) was added as variable modification. Proteome Discoverer assign peptides identified with high degree of confidence as having False Discovery Rate (FDR) ≤ 0.01, medium confidence FDR ≤ 0.05, and low peptide confidence for FDR lower than 5%.

MS-BLAST searches were performed as described by Shevchenko et al. (2001). Briefly, peptides generated from each protein spot were spaced with minus symbols (-), merged into a single string and edited according to the rules described. Searching was carried out at http://genetics.bwh.harvard.edu/msblast/, using default settings and the PAM30MS matrix against nrdb95 database. Matches were considered significant only if matched fungal proteins and displayed and a high-scoring segment pairs value of 85 or above.

2.5 Enzyme activities determination

2.5.1 Hydrolases

Glucoside hydrolase activity was determined by measuring the amount of reducing sugar released after incubation of secretion extracts with a particular substrate using the 2,4-dinitrosalicylic acid (DNS) (MILLER, 1959). Spectrophotometric assays were performed in Epoch Microplate Spectrophotometer (Biotek, Winooski, VT, US). The

amount of reducing sugar released in the reactions was subtracted from the control replicates of each sample. An enzyme unit was defined as the amount of sample which releases 1 µmol of reducing sugar per minute.

2.5.1.1 Cellulase

Cellulase activity was measured using the filter paper assay, FPase (ZIA et al., 2014). Briefly, the reaction mixture consisted of 1 mL of culture secretion extract and 1 mL of acetate buffer 200 mM pH 5.0, added to 50 mg of filter paper (4 pieces of 0.5x1.5 cm). After incubation for 50 min at 45ºC, 3mL of DNS reagent were added and the mixture incubated in a boiling water bath for 20 min. Absorbance was then measured at 570 nm and reducing sugar concentration was determined by a standard curve using 0-5 µmol glucose.

2.5.1.2 Xylanase

Xylanase activity was measured according to Kim et al. (2014). Sample volumes of 20 µL were added to 20 µL of 1% xylan solution and 10 µL of phosphate buffer 100 mM pH 5 with subsequent incubation for 1 hour at 40°C. Next, 200 µL of DNS solution were added followed by incubation in boiling water for 20 min and the absorbance determined at 570 nm. Controls were carried out by replacing substrate by water. The standard curve was prepared using 0-1 µmol xylose.

2.5.1.3 Pectinase

Pectinase assays were carried according to Biz et al. (2014)**.** A 0.25 mL pectin solution (0.5% in acetate buffer 200 mM pH 4.5) was added to 0.25 mL fungal secretion extracts and incubated for 20 min at 30°C. Next, 0.5 mL of DNS was added and the test tube transferred to a boiling water bath for 20 min. Absorbance was measured at 545 nm and the amount of released reducing sugar was defined using a galacturonic acid standard curve (0-250 mmol).

2.5.2. Oxidases/reductases

2.5.2.1 Laccase

Total oxidase/peroxidase activity was followed by the oxidation of 0.3 mM 2,2′-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS) to its cation radical (ε_{420}= 36,000 cm^{-1} M^{-1}) in 50 mM acetate buffer pH 5.0, 20 mM $MnSO_4$ and 0.05 mM H_2O_2 at 5 min intervals

during 20 min at 28°C (JORDAAN; LEUKES, 2003). Laccase activity was determined in the same conditions without the addition of neither manganese ions nor peroxide. One enzyme unit was defined as the amount of enzyme which oxidizes 1 µmol of ABTS per minute.

2.5.2.2 Dye Peroxidase (DyP)

DyP activity was monitored by the decolorization of an anthraquinone dye, 50 µM Reactive Blue 19 (RB19) (ε_{595}= 10,000 cm^{-1} M^{-1}) in the presence and absence of 0.1 mM of H_2O_2 in 100 mM acetate buffer pH 4.0. The absorbance was monitored during 90 min at 28°C. One enzyme unit was defined as the amount of enzyme which destained 1 µmol of RB19 per minute (SALVACHUA et al., 2013).

2.5.2.3 Azoreductase

Azoreductase activity was measured by the decrease in absorbance of the reduced methyl red form (ε_{430}: 23,360 M^{-1} cm^{-1}) (CHEN et al., 2005). The reaction mixture contained 150 µL of sample supernatants in presence of 4.45 µM methyl red, 20 µM NADH and 50 mM phosphate buffer pH 5.5. Absorbance was monitored during 90 min at 28°C. One enzyme unit was defined as the amount of enzyme which reduced 1 µmol of methyl red per minute

2.5.2.4 Alcohol veratryl oxidase (VO)

The enzyme activity was tested in a reaction mixture containing 1 mM of veratryl alcohol and 100 mM tartarate buffer pH 3.0. Absorbance was measured at 310 nm after 0 and 90 min incubation at 28°C (JADHAV et al., 2009).

2.5.2.5 Lignin peroxidase (LiP)

LiP activity was determined in a reaction mixture containing 100 mM n-propanol, 250 mM tartaric acid and 10 mM H_2O_2. Absorbance was measured at 300 nm after 0 and 90 min incubation at 28°C (JADHAV et al., 2009).

2.5.2.6 Cellobiose Dehydrogenase (CDH)

CDH activity was determined by following the decrease in absorbance of 2,6-diclorophenol-indophenol (DCIP) at 520 nm. The reaction mixture contained sample supernatants diluted in 30 mM lactose, 0.3 mM DCIP, 30 mM sodium azide and 50 mM

phosphate buffer pH 4.0. Absorbance was measured after 0 and 90 min incubation at 28°C (ENAYATZAMIR, 2009).

2.5.2.7 NADH-DCIP reductase

Enzyme activity was measured in a 5.0 mL reaction mixture containing 25mM DCIP (2,6-dichlorophenol indophenol) and 0.1mL enzyme solution in potassium phosphate buffer (50 mM, pH 7.4). 2.0 mL of reaction mixture was assayed at 590 nm after adding 250mM NADH. The DCIP reduction was calculated using the extinction coefficient of 0.019 mM^{-1} cm^{-1} (LADE et al., 2012).

Results and discussion

L. crinitus IOC 4581 was grown in seven types of carbon sources (maltose, glycerol, glucose, fructose, starch, sucrose and CMC) in concentrations ranging from1 to 20 g L^{-1}. In general, the increase in carbon concentration stimulated fungus growth as well as the yield of secreted proteins (**Fig 1**). The highest production of biomass was observed in maltose, fructose and glycerol (20 g L^{-1}) based cultures. However, the amount of secreted proteins was markedly higher in maltose (20 g L^{-1}) cultures in relation to other growth conditions. The protein profile also displayed qualitative changes according to the carbon source (**Fig 2**). Maltose, sucrose and fructose based media induced the

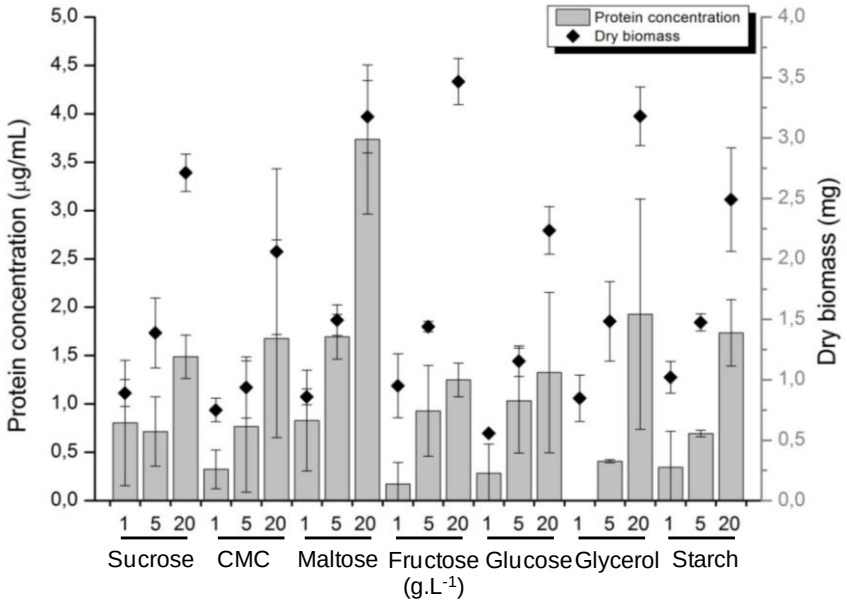

Figure 1. Biomass and protein concentration from cultures of *Lentinus crinitus* in media with variable carbon content. Carboxymethylcellulose (CMC). Results are mean of three independent samples.

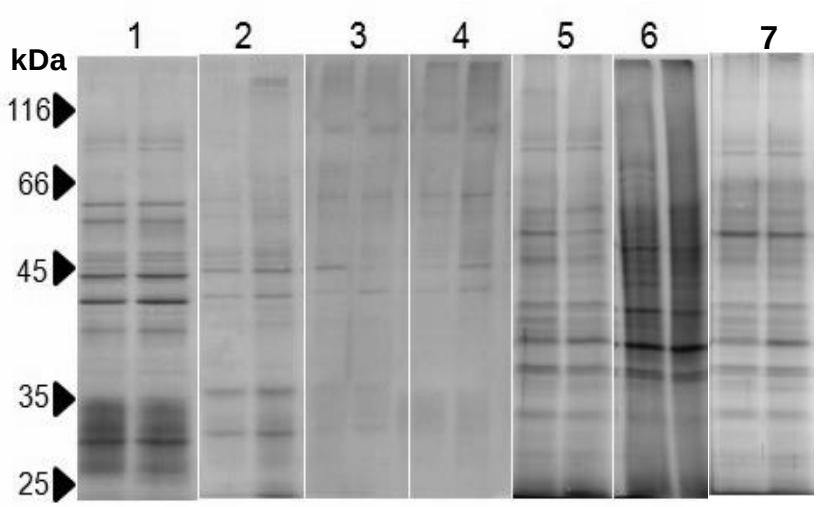

Figure 2. Effect of the culture media carbon-content on the protein secretion profile of *Lentinus crinitus*. Equal volumes of each culture supernatant were solubilized in Laemmli buffer 2X, boiled, submitted to 10% SDS-PAGE and the resulting profile revealed by silver staining. (1) maltose 20 $g.L^{-1}$, (2) glycerol 20 $g.L^{-1}$, (3) glucose 20 $g.L^{-1}$, (4) carboxymethylcellulose 1 $g.L^{-1}$, (5) fructose 20 $g.L^{-1}$, (6) starch 20 $g.L^{-1}$, (7) sucrose 20 $g.L^{-1}$.

expression of polypeptides in a wide range of molecular masses while growth on glycerol, glucose and starch based media resulted in rather poor protein profiles. Analysis of CMC derived samples was unfeasible due to excessive production of exopolysaccharide, hindering protein separation. In order to check the effect of nitrogen culture content on the protein secretion profile, three previously used carbon sources (maltose, starch and glycerol 20 g L^{-1}) were combined with one of three different nitrogen compounds (urea, sodium nitrate and ammonium chloride) in concentrations ranging between 4 and 100 mM. Maltose, starch and glycerol cultures were selected since they display different patterns of expression between them. Furthermore, glycerol is a by-product of bioethanol production, enabling cheaper costs of production for potential biotechnology applications. Notably, nitrogen content resulted in distinct protein profiles according to the carbon source (**Fig 3 and 4**). Thus, in glycerol or maltose based-medium, different nitrogen sources and alteraration of its concentration affected fungal growth only a slightly. However, drastic changes were observed in starch based media in each condition, with the highest amount of biomass obtained in medium containing urea (4 and 20 mM) (**Fig 3**). Protein secretion was also affected by the addition of nitrogen compounds. Here the highest levels of protein secretion were induced by the mixtures urea (100mM)/ maltose (20gL^{-1}) and nitrate (20mM)/maltose (20 g L^{-1}). The most complex protein profiles were observed when the fungus was

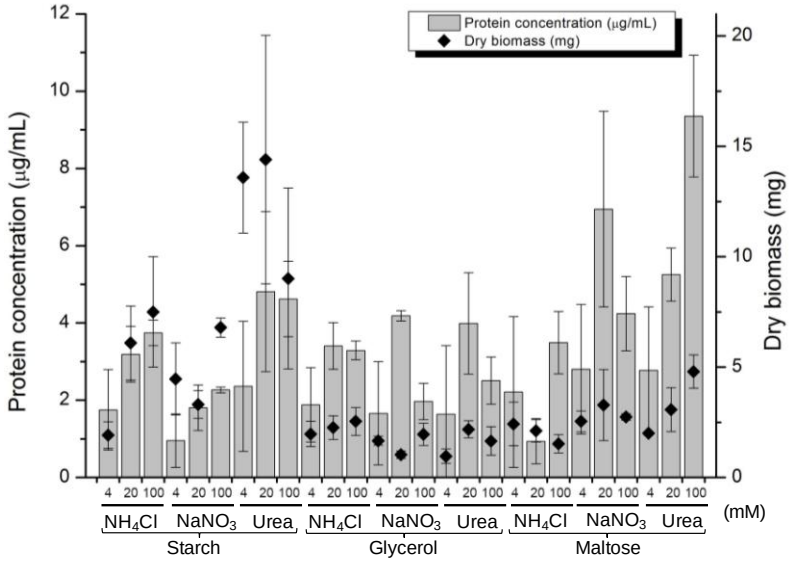

Figure 3. Effect of nitrogen content on culture media in the growth and protein secretion of *Lentinus crinitus.* Starch, glycerol and maltose concentration was 20 g L^{-1}. Results are mean of triplicate experiments.

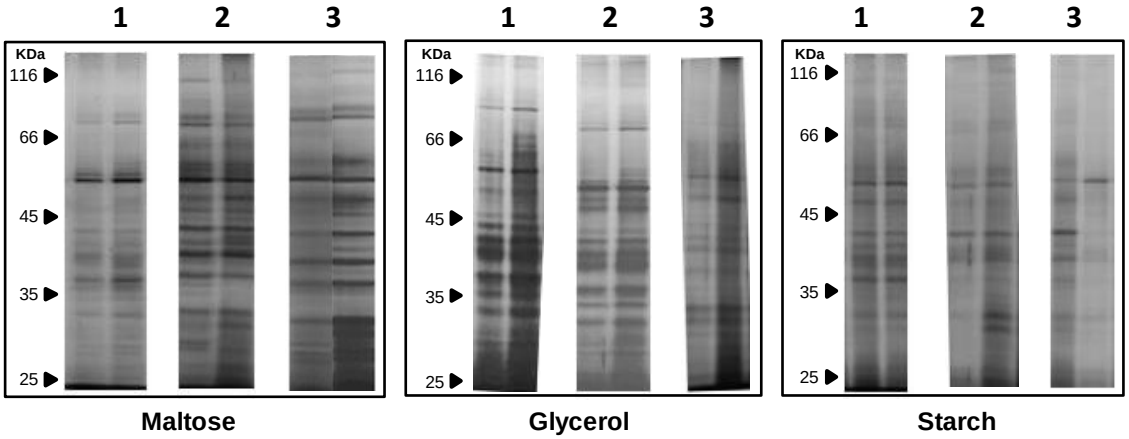

Figure 4. Effect of the culture media nitrogen-content on the protein secretion profile of *L. crinitus*. Equal volumes of each culture supernatant were submitted to 10% SDS-PAGE and the resulting protein profiles revealed by silver staining. 100 mM NH_4Cl (**1**), 20mM $NaNO_3$ (**2**) and 100 mM urea (**3**)

grown in medium containing maltose or glycerol (20 g L^{-1}) with 20mM urea and 20mM ammonium chloride or sodium nitrate, respectively. Importantly, carbon and nitrogen content in culture media has largely been associated to the control of several genes in wood rotting fungi as well as several other microorganisms (KENKEBASHVILI et al., 2009; MIKIASHVILI et al., 2005). Oxidative enzymes (Lac, MnP, LiP), CAZymes and peptidases are the main groups of enzymes produced by white rot fungi. These enzymes have been shown to be differentially regulated in response to a wide variety of environmental signals such as the availability of carbon and nitrogen in the culture media. Ligninolytic enzymes are mainly produced during secondary metabolism, as a

response to nitrogen and/or carbon depletion (FARACO et al., 2013; GOLD; ALIC 1993; PRIBNOW et al., 1989; RONNE, 1995; KIRK; FARRELL; 1987; JEFFRIES; CHOI; KIRK, 1981). Apparently, the carbon-regulated expression of ligninolytic enzymes occurs via a cAMP mechanism, since cAMP-responsive elements (creA) have been described in Lac, LiP and MnP gene promoters (GALHAUP et al., 2002; XIAO et al., 2006). The nitrogen repression response element (NIT2), which activates the expression of many structural genes in nitrogen-limited conditions as well as other regulatory elements such as Mig (carbon response element) have been described in fungi (MARZLUF, 1981), although only in *P. sajor-caju* (SODEN; DOBSON 2003) among the white rot fungi. In addition, controlled expression of cellulases and xylanases by creA sites in filamentous fungi (MACH et al., 1996; ILMEN et al., 1996; STRAUSS et al., 1999; SUTO; TOMITA, 2001) suggests that the production of enzymes involved in wood degradation is also regulated by creA and Mig proteins.

To assess the effect of water content on protein secretion, *L. crinitus* was cultured in two different medias with high and low water content. The first media containing maltose (20 g L^{-1}) and urea (100 mM) and the second glycerol (20 g L^{-1}) and sodium nitrate (20 mM). Maltose and urea based culture was chosen since it displays a intense and complex protein profile. Besides, this culture condition exhibits the highest amount of secreted protein among tested samples (**Fig 3**). Glycerol based media was chosen since presents differents pattern of protein profile and intensity in comparison with maltose based media. Protein profiles were analyzed by 1D-SDS PAGE. Interestingly, alterations in water content lead to drastic differences in the pattern of secreted proteins (**Fig 5, 6**). While a large number of proteins with molecular weight varying between 20 and 100 kDa was satisfactorily detected in the secretion from fungi cultivated in liquid medium, solid media resulted in extremely simple protein profiles, exhibiting only two intense protein bands around 42 kDa. Notably, most of the polypeptides particularly expressed in liquid media were strongly suppressed in solid media based cultures. Tryptic digestion and MS analysis of the 42 KDa doublet resulted in 11 matches to known proteins with ten corresponding exclusively to enzymes involved in lignin degradation such as laccase, glyoxal oxidase and MnP and only one match to a CAZyme (see **Table 1**). The activity of some CAZymes and oxidases was assessed in secretion extracts derived from cultures in media with variable content of water, carbon

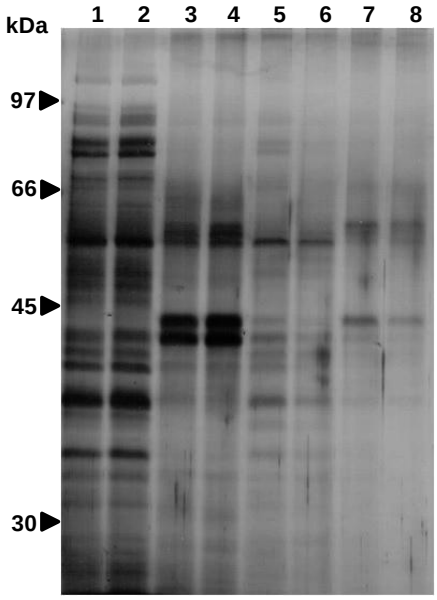

Figure 5. Effect of water content on the secretion of proteins by *Lentinus crinitus***.** Supernatants derived from liquid (**1, 2, 5, 6**) and solid cultures (**3, 4, 7, 8**) containing 20 g L^{-1} maltose and 100 mM urea (**1-4**) or 20 g L-1 glycerol and 20 mM sodium nitrate (**5-8**) were submitted to 10% SDS-PAGE and silver staining.

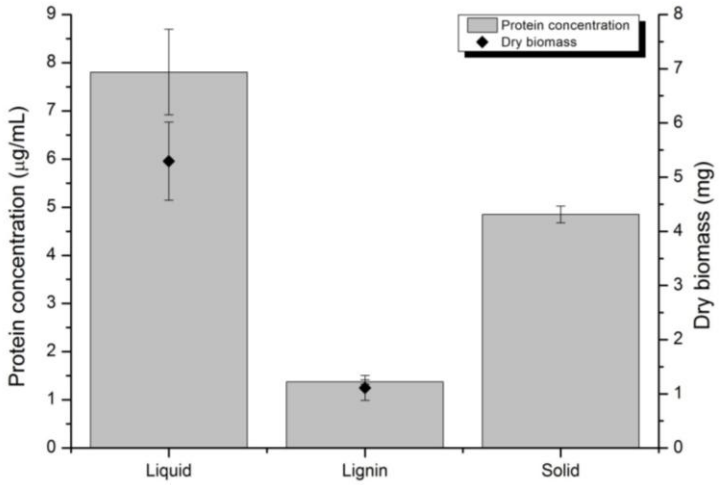

Figure 6 . Dry biomass and concentration of secreted proteins in solid and liquid cultures in the presence and absence of lignin as substrate . Assessed conditions were 20 g L^{-1}maltose and 100 mM urea in liquid media (**liquid**); 1 g L^{-1} maltose, 100 mM Urea and 1 g L^{-1} lignin in liquid medium (**lignin**) and 20 g L^{-1}maltose and 100 mM urea in solid medium (**solid**). Results are mean of three independent samples.

and lignin. Lignin was chosen as potential inducer of these enzymes. The evaluated CAZymes were xylanase, pectinase and cellulase (filter paper activity, FPase) each one displaying a distinct activity profile. FPase activity and pectinase were induced by low water content since higher titers were obtained when the fungus was grown in solid media (**Fig 7 and 8**). Xylanase activity was not detected in any of the assessed

TABLE 1. Results of the MS analysis of the 42 kDa protein doublet exclusively expressed by *L. crinitus* in solid cultures

Description **Name/ organism/ accesion code**	**Group**	**Sequence aligned**	**MW [kDa]**	**calc. pI**
Laccase/ *Pycnoporus cinnabarinu* / [LAC1_PYCCI]	Oxidoreductase	STSTPTADLAVISVTK, ANPNFGTTGFADGVNSAILR, YSFVLNANQDVDNYWIR	50.1	5.00
Laccase/ *Pycnoporus cinnabarinus*/ [LAC1_PYCCI]	Oxidoreductase	FPLGADATLINGLGR	56.0	5.12
Ligninase/ *Phanerochaete chrysosporium*/ [Q01775_PHACH]	Oxidoreductase	TACEWQSFVANQSK	39.2	4.49
Manganese peroxidase/ *Lenzites gibbosa*/ [G9I531_9APHY]	Oxidoreductase	GTLFPGTGGNQGEVESPLHGEIR, LQSDSELAR	38.0	4.63
Melanin-decolorizing enzyme/ *Ceriporiopsis* sp./ [B3IWB3_9APHY]	Oxidoreductase	LQSDHDLAR	38.3	5.10
Mn peroxidase MNP3/ *Polyporus brumalis*/ [G0Z9F2_9APHY]	Oxidoreductase	LTFHDAIGISPAIASR, LQSAFAAAFR, LQSDSELAR	38.1	4.58
Manganese peroxidase 2/ *Lenzites gibbosa*/ [G9I531_9APHY]	Oxidoreductase	GTLFPGTGGNQGEVESPLHGEIR, LQSDSELAR	38.0	4.63
Mn peroxidase MNP3/ *Polyporus brumalis*/ [G0Z9F2_9APHY]	Oxidoreductase	LTFHDAIGISPAIASR, LQSAFAAAFR, LQSDSELAR	38.1	4.58
Mn peroxidase MNP6/ *Polyporus brumalis*/ [G0Z9F5_9APHY]	Oxidoreductase	LQSAFAAAFR, LQSDSELAR, LTFHDAIGISPAIAAR	38.0	4.42
Laccase (Fragment)/ *Lentinus tigrinus*/ [Q5EBY5_9APHY]	Oxidoreductase	ANPNFGTTGFADGVNSAILR, STSTPTADLAVISVTK, YSFVLNANQDVDNYWIR	50.1	5.00
Glucoamylase/ *Phanerochaete carnosa* (strain HHB-10118-sp)/ [K5WMZ0_PHACS]	CAZyme	SLIDEFVSAEATLQQVTNPSGSVT TGGLGEPK	61.0	4.68

conditions. Cultivation in liquid media with low carbon content and supplemented with 1% lignin resulted in a poor yield of all tested enzymes. Thus, the MS data asserts for a preferred expression of oxidative enzymes in low water content cultures with minimal expression of CAZymes. The activity level of the latter was not corroborated by enzymatic assays, where low water content media samples promoted the expression of hydrolytic enzymes. This suggests a possible event of substrate inhibition during testing since samples derived from liquid medium contained higher amount of free monossacharides than those derived from solid culture assays. Indeed, substrate inhibition of glucosidases by low mM glucose concentrations has been extensively described (GUEGUEN et al., 1995; TEUGJAS et al., 2013).

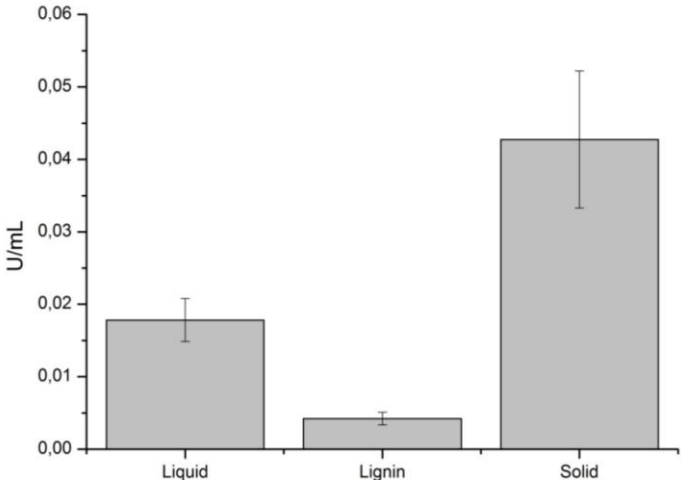

Figure 7. *L. crinitus* secreted FPase activity in solid and liquid cultures in the presence and absence of lignin as substrate. Assessed conditions were 20 g L^{-1}maltose and 100 mM urea in liquid media (**liquid**); 1 g L^{-1} maltose, 100 mM Urea and 1 g L^{-1} lignin in liquid medium (**lignin**) and 20 g L^{-1}maltose and 100 mM urea in solid medium (**solid**). Results are mean of triplicate experiments.

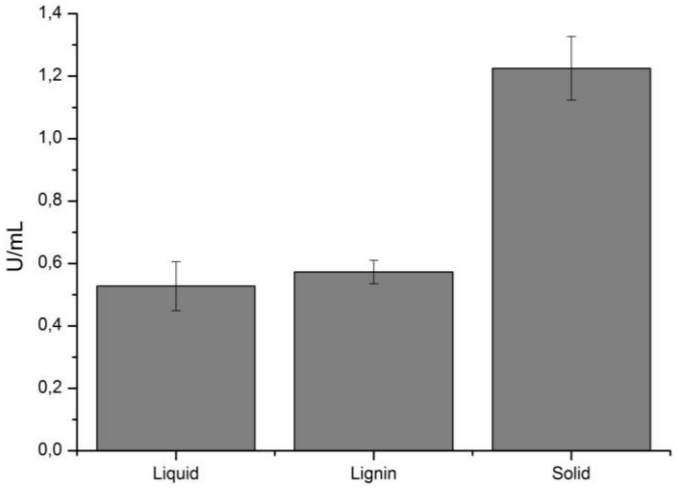

Figure 8. *L. crinitus* secreted pectinase activity in solid and liquid cultures in the presence and absence of lignin as substrate. **.** Assessed conditions were 20 g L^{-1}maltose and 100 mM urea in liquid media (**liquid**); 1 g L^{-1} maltose, 100 mM Urea and 1 g L^{-1} lignin in liquid medium (**lignin**) and 20 g L^{-1}maltose and 100 mM urea in solid medium (**solid**). Results are mean of three independent samples.

In wood rotting fungi the expression of hydrolytic enzymes is usually improved by submerged fermentation. Elisashvili et al. (2008), using three *L. edodes* strains and six *Pleurotus* isolates observed that submerged fermentation resulted in strong induction of CMCase, xylanase and FPAse activities. In SSF low levels of hydrolytic enzymes were similarly secreted by all strains of *L. Edodes* showing a 15-45 fold increase in the production of xylanase and cellulase respectively when cultured by SF in comparison

with SSF (ELISASHVILI et al., 2008). Additionally culture medium with a rich content of easily metabolizable sugars led to a similar effect. The same authors working with several genera of wood rotting fungi (*Pseudotremella*, *Fomes*, *Trametes*, *Trichaptum*, *Ganoderma* and *Pleurotus*) showed significant differences in the production of hydrolytic enzymes using several substrates in liquid and solid fermentation (ELISASHVILI et al., 2009), reinforcing the hypothesis that each fungal isolate requires a particular condition to achieve the best yield of a particular group of enzymes.

To investigate the contribution of oxidases and accessory proteins namely Lac, DyP, azoR, MnP, VO, LiP, NADH-DCIP-R (2,6 dichlorophenol indophenol reductase) and CDH (cellobiose dehydrogenase) *in vitro* activity assays were performed in secretions collected from fungus cultured in liquid or solid media. Only Lac, MnP and DyP activities were detected in one or more soluble extracts. Moreover, Lac, DyP and azoR (**Fig 9 and 10**) were mostly detected in solid medium. Thus, the overall higher yield of total oxidase activity was observed in solid cultures with high carbon content. No oxidase activity was detected in liquid cultures with low carbon content and supplemented with 1% lignin.

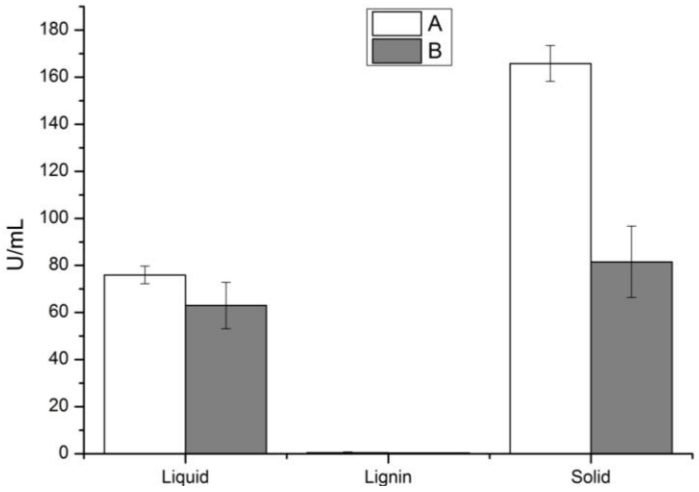

Figure 9. Secreted oxidase/peroxidase activity in liquid and solid cultures of *L. crinitus* in the presence and absence of lignin as substrate. Assessed conditions were 20 g L^{-1}maltose and 100 mM urea in liquid media (**liquid**); 1 g L^{-1} maltose, 100 mM urea and 1 g L^{-1} lignin in liquid medium (**lignin**) and 20 g L^{-1}maltose and 100 mM urea in solid medium (**solid**). Total oxidase/peroxidase assays (**A**) used ABTS as substrate in the presence of $MnSO_4$ and H_2O_2. Laccase assays (**B**) used only ABTS. Results are mean of three independent samples.

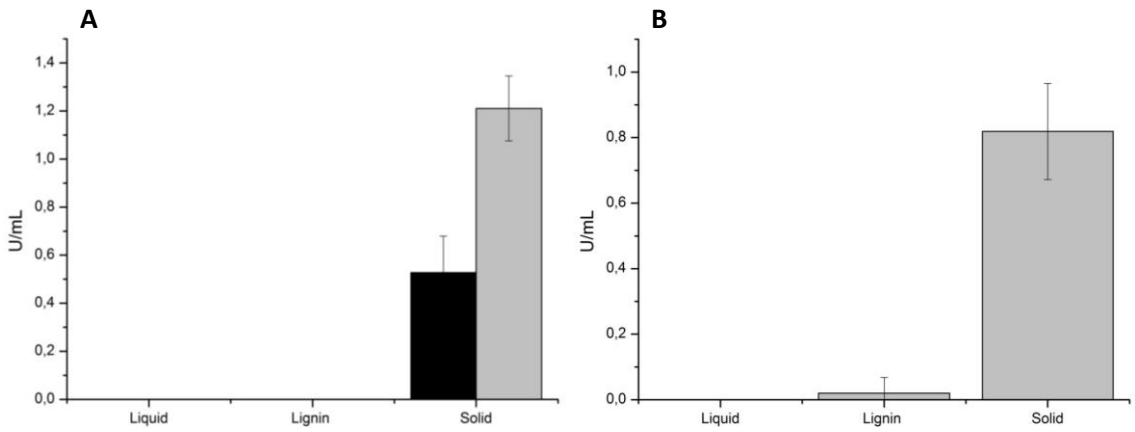

Figure 10. Secreted DyP and azoR activities in solid and liquid cultures of *L. crinitus* in the presence and absence of lignin . Assessed conditions were 20 g L^{-1}maltose and 100 mM urea in liquid media (**liquid**); 1 g L^{-1} maltose, 100 mM urea and 1 g L^{-1} lignin in liquid medium (**lignin**) and 20 g L^{-1}maltose and 100 mM urea in solid medium (**solid**). DyP activity in the presence (black) and absence (gray) of H_2O_2 (**A**). Azoreductase activity (**B**). Results are mean of three independent samples.

In *Lentinus* as well as in most wood rotting fungi, the expression of lignin degrading enzymes can be modulated by water availability and the presence of lignocellulosic material, among several other variables such as carbon and nitrogen content, supplementation with metal ions (SHUTOVA et al., 2008), aromatic compounds (QUARATINO et al., 2008) and alcohol (KADIMALIEV et al., 2008). In *L. edodes* isolates Lac expression is usually enhanced in solid state fermentation while lower yields are obtained in liquid cultures (KENKEBASHVILI et al., 2009; ELISASHVILI et al., 2008). The same behavior was observed here. However when growth media is supplemented with lignin different outcomes have been described. By studying several white rot fungi, including a *L.edodes* strain, Kenkebashvili et al. (2009) observed a fivefold improvement in Lac production due to lignin supplementation. However, huge variations were observed in response to different types of lignocellulosic substrate used. Notably, no substrate produced the same response for every strain. This great variability was also described by Elisashvili et al. (2008) using several *Lentinus* and *Pleurotus* isolates where SSF and supplementation with raw plant material favored the secretion of oxidative enzymes.

The degradation of RR19 (anthraquinone dye) and methyl red, the current substrates of DyP and azoR respectively, was described in *Lentinus sp*. (HSU et al., 2012), *L. tigrinus* (VALENTIN et al., 2006), *L. polychrous* (WANGPRADIT et al., 2014; SARNTHIMA et al., 2009; PHETSOM et al., 2009) and *L. edodes* (MINUSSI et al., 2001), although these activities were assigned to the presence of Lac, MnP and LiP. Our data show

different activity patterns for each substrate suggesting that either Dyp and azoR are present, or alternatively, that the pool of oxidase/reductase enzymes in the secretion extract displays wider substrate specificity. DNA response elements necessary for controlling expression through metal ions, xenobiotics (XIAO et al., 2006) and carbohydrates (ZHUO et al., 2011; GALHAUP et al., 2002) have been described in Lac and MnP genes but none of them were associated to water content effects on culture medium.

A major goal of this work was to induce the expression of the maximum number of potential secreted proteins encoded on the *L. crinitus* genome through the modulation of growth conditions. According to our data, highest levels of secreted protein were induced by the combination urea-maltose in culture medium. Therefore, secretion extracts derived from solid and liquid cultures containing maltose (20 g L^{-1}) and urea (100mM) were pooled and submitted to two strategies: (1) shotgun LC-MS/MS proteome analysis and (2) protein separation by two-dimensional gel electrophoresis prior to protein identification by mass spectrometry analysis. LC-MS/MS analysis of the whole secretion extract resulted in **98** protein identifications using the non-redundant NCBI database (nrdb95) (**Table S1 suppl information**). These corresponded to CAZymes (carbohydrate-active enzymes) (**25**), oxidase/reductases (**20**), proteases (**3**); lipase/esterases (**5**) and proteins with non-related functions we called miscellany proteins (**9**). Several matches corresponded to hypothetical or unknown predicted proteins (**36**). Within the CAZymes group, glycoside hydrolases from different families, glucanases, alfa/beta glycosidases, amylases and galacturonases were detected revealing a powerful arsenal of enzymes dedicated to the consumption of polyssacharides. Also, several oxidases associated to lignin depolymerization were identified such as laccases, peroxidases, GMC oxidoreductases, MnP and glyoxal oxidases. By using the 2D gel approach a protein map containing 150 spots was resolved (**Fig. 11**). Most of the detected polypeptides displayed an acidic p*I* and relative molecular mass among 30 and 100 kDa. MS analysis of the resolved spots resulted in 171 protein identifications (**Table S2 suppl information**) corresponding to CAZymes (**34**); oxido/reductases (**25**), proteases (**37**); lipase/esterases (**4**); miscellany (**42**) and hypothetical/uncharacterized proteins (**29**). The group of identified enzymes classified as CAZymes in this study is quite heterogeneous. It comprises alfa/beta hydrolases, cellulases, cellobiohydrolases, xylanases, polygalacturonases and glycoside hydrolases from at least 11 CAZyme

families suggesting that *L. crinitus* growth can be potentially enhanced by a wide variety of saccharide substrates. Furthermore, a wide range of oxidase/reductases was identified including 2 alcohol-oxidases, 13 multicopper oxidases, 4 glyoxal oxidases, 5 GMC reductases, 8 MnPs and 3 LiPs. The overall distribution of proteins identified in each experimental strategy regarding protease family groups was quite similar (**Fig. 12**). However, a slightly larger number of proteins was identified via 2D-SDS PAGE combined with downstream MS analysis compared to the shotgun proteomics approach. Moreover, a larger number of proteases was identified via the 2D gel LC-MS/MS strategy (3 proteases by shotgun proteomics vs 37 proteases using 2D gel LC-MS/MS).

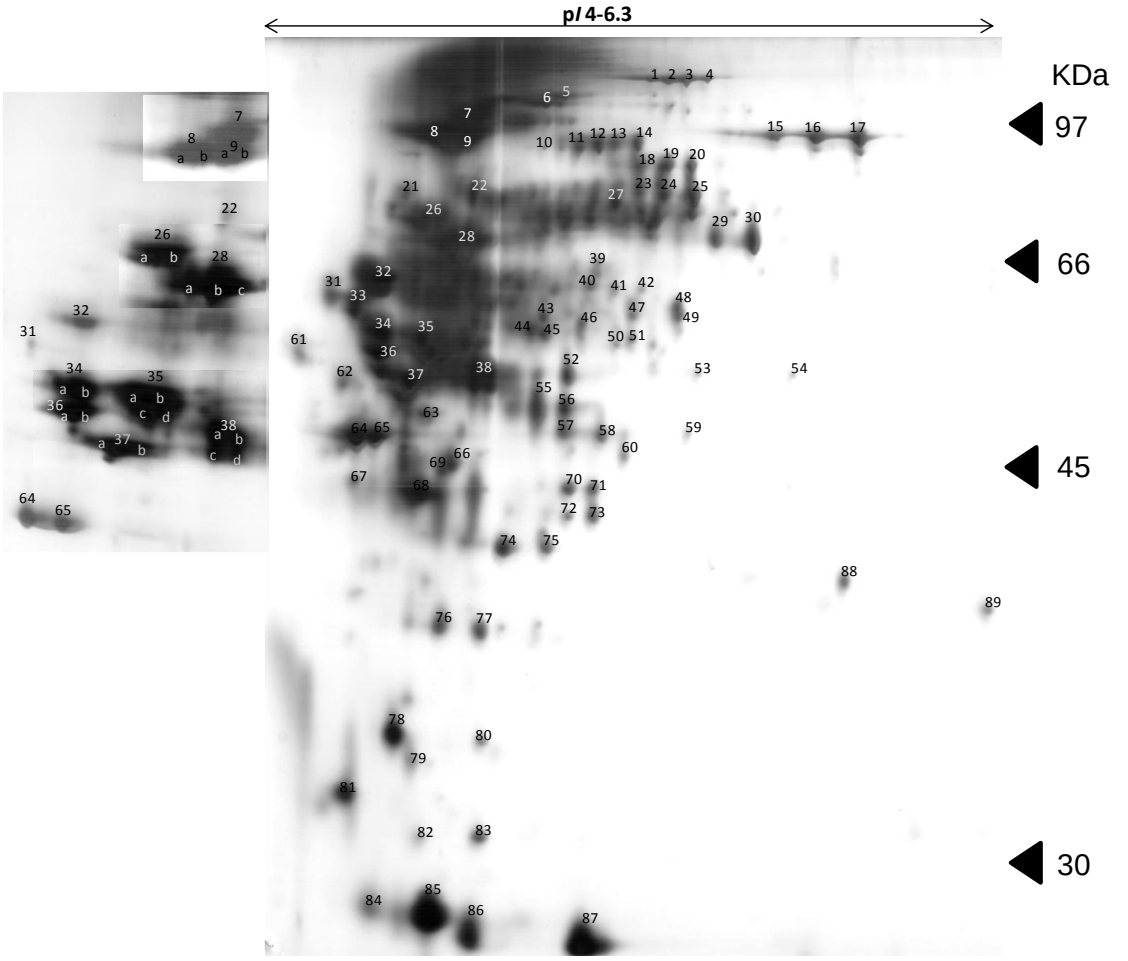

Figure 11. Two dimensional gel eletrophoresis of *L. crinitus* secreted proteins derived from a mixture of liquid and solid cultures containing 20 g L^{-1} maltose and 100 mM urea. Numbered spots were submitted to protein identification. Gels presented left are different replicates of 2D-SDS-PAGE with different intensity of silver staining for better visualization of protein spots.

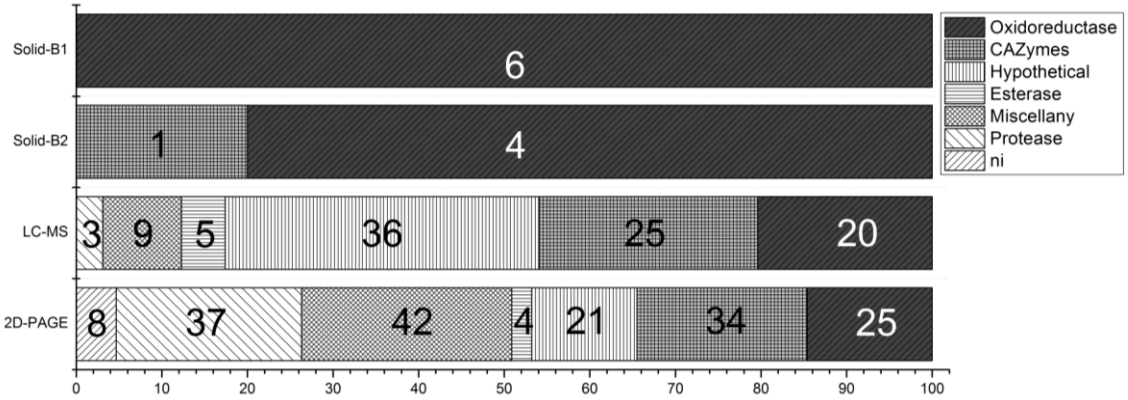

Figure 12. Functional classification of proteins identified from *L. crinitus*. Proteins were identified from four different sources: two intense protein bands (~42 kDa) from supernatant of solid cultures submitted to 1D-SDS-PAGE (named solid B1 and solid B2), LC-MS analysis and spots from 2D-SDS-PAGE. LC-MS and 2D-SDS-PAGE results are derived from a pool of proteins from solid and liquid cultures containing 20 g L^{-1} maltose and 100 mM urea. Percentages are given according to total numbers of proteins from each experiment. ni: non identified proteins.

The good performance obtained with the 2D-SDS PAGE approach was quite surprising since several limitations and constrains have been related when compared to high throughput LC-MS strategies, such as low load ability and poor separation of hydrophobic, acidic and alkaline proteins as well as loss of proteins with molecular mass above 100 kDa or below 20 kDa (BUNAI; YAMANE, 2005). It is feasible that when analyzing a pool of proteins characterized by a limited number of functional classes and also with a high content of isoforms or multiple post translational variants in each protein class, the shotgun approach has a higher difficulty in discerning pretty similar protein species. Pre-fractionation, using 2D PAGE, drastically decreases the complexity of the analysis due to a smaller number of peptides and a higher possibility that they belong to a fewer number of proteins (1-5) (GYGI et al., 2000).

In summary the spectrum of detected proteins in the secretion extract of *L.crinitus* resembles the expected profile of a good candidate for the delignification and saccharification of lignocellulose (ALFARO et al., 2014). Currently genomic (genome.jgi.doe.gov; kwanlab.bio.cuhk.edu.hk) and transcriptomic information (genome.jgi.doe.gov/Lentignscriptome) have been obtained from two related *Lentinus* species, *L. tigrinus* and *L. edodes*, the most common species in the literature. However, these data are not readily available and lacks thorough annotation, which is paramount for the prediction or comparison of the number of potential secreted proteins by the

fungus through bioinformatic resources. When contrasting the data from the *L. crinitus* secretome with other basidiomycetes according to their lifestyle (white rot, brown rot, symbionts or parasites), the abundance of lignin oxidases and glycoside hydrolases fits well with the profile observed in the majority of analyzed white rot fungi (ALFARO et al., 2014). Additionally the total number of identified proteins is quite comparable with the observed in other wood rotting fungi secretomes such as *Phlebia brevispora* (178 proteins) (HORI et al., 2013), *Bjerkandera adusta* (157) (REINA et al., 2014), *Phanerochaete chrysosporium* (190) (WYMELENBERG et al., 2009) and *Ganoderma sp*. (71) (MANAVALAN et al., 2012) among others (ALFARO et al., 2014). Several of the enzymes here described have been reported in other *Lentinus* species but none of them using a high throughput approach. Thus, in *L. edodes* it has been demonstrated the control of enzyme secretion according to the fungus growth phase. Lac and MnP activities are high during substrate colonization while cellulases and xylanases are predominant during fruit body development (ELISASHVILI et al., 2015), (VETCHINKINA et al., 2015). Additionally, oxidative enzymes are preferentially secreted in solid media while liquid media provides better production of hydrolytic enzymes (ELISASHVILI et al., 2008). Several hydrolytic enzymes such as endoglucanase, cellobiohydrolase, beta-glucosidase, xylanase, polygalacturonase were described in *L. edodes* with most of them being induced in media with high sugar content (CHICATTO et al., 2014; LEE et al., 2007) as it was observed in the present work. There are only a few reports on *L. crinitus* describing the production of laccase and MnP (NIEBISCH et al., 2010) and its general lignolytic activity (HOSSAIN et al., 2007). Importantly, there is currently a lack of information on secreted enzymes or the influence of different growth conditions on the *L. crinitus* secretome.

The study of fungal secretomes reveals the extraordinary variability among species as well as a wide range of potential applications. In the present study we gained insight into the secretome of a poorly studied *Lentinus* species. One hundred seventy-one (171) proteins were identified in a mixed soluble extract containing polypeptides being produced in solid and liquid cultures and displaying a diverse array of lignocellulolytic and proteolytic enzymes with promising applications on several branches of industry. The relatively high number of hypothetical or uncharacterized proteins matched in database search indicates higher complexity of fungal secretomes and highlights the

need for genome information in order to better understand the physiology of these organisms.

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Role of the funding source.

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Supplementary Information

TABLE S1. Functional classification of protein matches derived from the LC-MS analysis of the *L. crinitus* soluble secretion extract

Description/organism	Group	Sequence aligned	MW [kDa]	calc. pI
Acid protease/ *Dichomitus squalens*	Protease	KLINPPVFTVK, LINPPVFTVK	44.9	5.86
Family S53 protease-like protein/ *Dichomitus squalens*	Protease	AGWDPVTGLGTPNFAK, AGWDPVTGLGTPNFAK	58.4	4.75
Peptide hydrolase/ *Pycnoporus cinnabarinus*	Protease	VTDLMNFTDDAPGADDDASGVAVVLELLR, VTVDQSKDNVVFGVR, VNGAALWSLAQAPGTPK, FLRGGDHEPFLER, VYVITGHYDSR, VTDLMNFTDDAPGADDDASGVAVVLELLR, FTEPHENFAHQHQDVR	52.0	5.33
Alcohol oxidase/ *Dichomitus squalens*	Oxidoreductase	YVTMFQYLEYPASR, AVGVAYVPSR, MVVLSSGTLGTPQILER, LSSNAIDAGFK, KMVVLSSGTLGTPQILER, MVVLSSGTLGTPQILER, GADLIAEDLGLK, SGVGNAELLNK, ATFYTDTMK	72.4	6.54
Alcohol oxidase/ *Pycnoporus cinnabarinus*	Oxidoreductase	AVGVAYVPSR, MVVLSSGTLGTPQILER, VSNETQTLDDFLR, LSSNAIDAGFK, KMVVLSSGTLGTPQILER, MVVLSSGTLGTPQILER, SGVGNAELLNK	72.4	6.73
Catalase/ *Pycnoporus cinnabarinus*	Oxidoreductase	QLSVFAAVDQTLSDR, IGPLLLQDFHHIDLLAHFDR, NVENYFAEIEQAAFSPSHLVPGIEPSADPVLQSR, LFSYPDTHR, IGPLLLQDFHHIDLLAHFDRER, IGPLLLQDFHHIDLLAHFDR, YNILDLTK, FPHFIHTQK	57.0	7.01
Copper radical oxidase/ *Pycnoporus cinnabarinus*	Oxidoreductase	ILNPVTFDTIK, TDPNNLYPFLHMLPSGNIFVGYYNEAR	106.4	4.48
Glyoxal oxidase/ *Dichomitus squalens* (strain LYAD-421)]	Oxidoreductase	ITLTPEGIAKGWEVEHMLEGR, ITLTPEGIAKGWEVEHMLEGR, GWEVEHMLEGR, SLPANLFPR, GWEVEHMLEGR, ITLTPEGIAK	59.3	5.06
Glyoxal oxidase/ *Dichomitus squalens* (strain LYAD-421)	Oxidoreductase	TLSFVTPPNGR, ITVTPEGIAK	59.6	4.88
Glyoxal oxidase/ *Phanerochaete chrysosporium*	Oxidoreductase	VQVALMDLGFSSHAFHSSAR, ITLTPEGIKAGWQVEHMLEAR, ITLTPEGIKAGWQVEHMLEAR, SLPANLFPRVQVALMDLGFSSHAFHSSAR	59.1	5.22
Glyoxal oxidase/ *Pycnoporus cinnabarinus*	Oxidoreductase	VQTLDPPFMFVERPK, VQTLDPPFMFVERPK, LVFMDATISR, SLPANLFPR, LVFMDATISR, IFALPDGK, VQVSLMDLGFSSHAFHSSAR	64.2	6.25
GMC Oxidoreductase/ *Dichomitus squalens* (strain LYAD-421)	Oxidoreductase	GGVVDTHLK, SPHILELSGIGNR	66.4	5.71
Laccase (Fragment)/ *Lentinus tigrinus*	Oxidoreductase	ANPNFGTTGFADGVNSAILR, YSFVLNANQDVDNYWIR, ANPNFGTTGFADGVNSAILR, STSTPTADLAVISVTK	50.1	5.00
Laccase/ *Ganoderma lucidum* - [B5G551_GANLU]	Oxidoreductase	FPLGSDSTLINGLGR, SAGSTVYNYDNPVWR, SAGSTVYNYDNPVWR	56.2	5.16

Laccase/ *Lentinus* sp. WR2	Oxidoreductase	LGPRFPLGADATLINGLGR, LGPRFPLGADATLINGLGR, FPLGADATLINGLGR, FPLGADATLINGLGR, SAGSTVYNYDNPIWR	56.2	5.08
Manganese peroxidase 2/ *Lenzites gibbosa*	Oxidoreductase	GTLFPGTGGNQGEVESPLHGEIR, LQSDSELAR, GTLFPGTGGNQGEVESPLHGEIR	38.0	4.63
Manganese peroxidase isozyme/ *Dichomitus squalens* (strain LYAD-421)	Oxidoreductase	ALTDCSEVIPVPK, LFSDANIAR	37.6	5.11
Melanin-decolorizing enzyme/ *Ceriporiopsis* sp. MD-1	Oxidoreductase	TACEWQSFVNNQAK, LQSDHDLAR, LQSDHDLAR	38.3	5.10
Mn peroxidase MNP3/ *Polyporus brumalis*	Oxidoreductase	GQFGGGGADGSIALFEDIETNFHANNGVDEIIDEQRPLIQR, GQFGGGGADGSIALFEDIETNFHANNGVDEIIDEQRPLIQR, LTFHDAIGISPAIASR, TACEWQSFVNNQAK, LQSDSELAR, LQSAFAAAFR, LQSAFAAAFRK, LAVFVGRPDATQPAPDLTVPEPFDTVDSILQR, LQSDSELARDSR	38.1	4.58
Mn peroxidase MNP5/ *Polyporus brumalis*	Oxidoreductase	AAPDGLVPEPFDTIDDILAR, LQSDHLLAR	39.0	4.82
Mn peroxidase MNP6/ *Polyporus brumalis*	Oxidoreductase	GQFGGGGADGSIALFEDIETNFHANLGVDEIIDEQRPLLQR, GQFGGGGADGSIALFEDIETNFHANLGVDEIIDEQRPLLQR, TACEWQSFVNNQAK, LQSDSELAR, LQSAFAAAFR, LQSAFAAAFRK, LQSDSELARDSR, LAVFVGR	38.0	4.42
MnP-short short manganese peroxidase/ *Ceriporiopsis subvermispora* (strain B)	Oxidoreductase	LQSDHNLAR, LQSDHNLAR	37.9	4.70
Peroxidase MNP2/ *Polyporus brumalis*	Oxidoreductase	AAPDGLVPEPFDSVDKILAR, LQSDHLLAR	38.5	5.10
Actin (Fragment)/ *Phanerochaete chrysosporium*	Miscellany	AVFPSIVGRPR, AGFAGDDAPR, VAPEEHPVLLTEAPLNPK, DSYVGDEAQSKR, IWHHTFYNELR, SYELPDGQVITIGNER	39.5	5.69
Adenosylhomocysteinase/ *Pycnoporus cinnabarinus*	Miscellany	GLSEETTTGVHHLYK, ANVFVTTTGNR, VAVVAGFGDVGK, ATDVMLAGK, VPAINVNDSVTK	47.1	6.20
Argininosuccinate synthetase/ *Dichomitus squalens* (strain LYAD-421)	Miscellany	QALLAYAAEK, AAHVDLEGLTLDR	46.2	5.71
Glyceraldehyde-3-phosphate dehydrogenase/ *Pycnoporus cinnabarinus*	Miscellany	VPTIDVSVVDLVVR, AVGNNIIPSSTGAAK, EAAAGNMQGILDYTEEK, VINDKFGIVEGLMTTVHATTATQR	40.9	7.56
Glyceraldehyde-3-phosphate dehydrogenase/ *Pycnoporus coccineus*	Miscellany	VPTIDVSVVDLVVR, AVGNNIIPSSTGAAK, LISWYDNEWGYSKR	36.3	7.44
Hypothetical histidine kinase/ *Postia placenta* (strain ATCC 44394/ Madison 698-R)	Miscellany	IVEQLGGQLRVDSK	113.9	6.01
Nucleoside diphosphate kinase/ *Fomitopsis pinicola* (strain FP-58527)]	Miscellany	AMLGATNPLASAPGTIR, AMLGATNPLASAPGTIR, TYIMVKPDGVQR, TYIMVKPDGVQR, GDFALAVGR	16.7	8.16
Nucleoside diphosphate kinase/ *Pycnoporus cinnabarinus*	Miscellany	AMLGATNPLASAPGTIR, AMLGATNPLASAPGTIR, GDFALAVGR, GLVGNIISR	17.6	8.15
Sphingomyelin phosphodiesterase/	Miscellany	FLAMAAPEQGPALAVR, FLAMAAPEQGPALAVR	74.6	4.54

Fomitopsis pinicola (strain FP-58527)				
Carboxylic ester hydrolase/ *Ceriporiopsis subvermispora* (strain B)	Esterase	AAIFDSSTGPFK, AEAWYTDNMFLAPR	58.9	4.65
Carboxylic ester hydrolase/ *Ceriporiopsis subvermispora* (strain B)	Esterase	HIGAFGGDPTK	53.3	5.43
Carboxylic ester hydrolase/ *Dichomitus squalens* (strain LYAD-421)	Esterase	SIELGQPVIFVSMNYR, SIELGQPVIFVSMNYR	58.0	4.87
Carboxylic ester hydrolase/ *Dichomitus squalens* (strain LYAD-421)	Esterase	AGDPNTFKLDR	59.2	5.49
Carboxylic ester hydrolase/ *Pycnoporus cinnabarinus*	Esterase	WLGIPFAEPPVGSLR	54.4	5.11
Alpha/beta-hydrolase/ *Dichomitus squalens* (strain LYAD-421)]	CAZyme	VGNPAWATLFDSK	31.9	4.60
Beta-galactosidase/ *Dichomitus squalens* (strain LYAD-421)]	CAZyme	EGWHLPGFDTSSWTAR, GVMNEGGLFGER, VGGYTNFPDKVR, GVMNEGGLFGER, LPVPDLWLDIFQK, GVMNEGGLFGEREGWHLPGFDTSSWTAR, GVMNEGGLFGEREGWHLPGFDTSSWTAR, GVMNEGGLFGEREGWHLPGFDTSSWTAR	108.5	5.10
Beta-galactosidase/ *Postia placenta* (strain ATCC 44394/ Madison 698-R)	CAZyme	LPVPDLWLDIFQK, GILNEGGLYGER	109.9	4.50
Beta-xylanase/ *Dichomitus squalens* (strain LYAD-421)	CAZyme	SWDVVNEIFNEDGTLR, SSVFSNVLGQSFVNIAFQAAR, SSVFSNVLGQSFVNIAFQAAR, WDATEPSR	39.7	6.54
Candidate glucoamylase/ *Postia placenta* (strain ATCC 44394/ Madison 698-R)]	CAZyme	FNINETAFTGAWGRPQR, TLIDEFVSAEATLQQVTNPSGSVSTGGLGEPK	60.9	4.23
Carbohydrate Esterase Family 4 protein/ *Pycnoporus cinnabarinus*	CAZyme	DVLGVTPTYWRPPYGDVDNR	48.9	4.92
Glucanase (Fragment)/ *Ganoderma* sp. E CFW-2011	CAZyme	FVTHGPYSTNIGSR	18.6	4.59
Glucanase/ *Pycnoporus cinnabarinus*	CAZyme	QLVQIVVYDLPDRDCAAK, VVAVIEPDSLANLVTNLNVQK, ASNGEFSIADGGQQKYYNYIDQIVAQIK, ASNGEFSIADGGQQKYYNYIDQIVAQIK, ASNGEFSIADGGQQK, QLVQIVVYDLPDR, YYNYIDQIVAQIK	47.7	5.11
Glucoamylase/ *Phanerochaete carnosa* (strain HHB-10118-sp)	CAZyme	SLIDEFVSAEATLQQVTNPSGSVTTGGLGEPK, FNIDETAFTGAWGRPQR	61.0	4.68
Glycoside hydrolase family 16 protein/ *Phlebiopsis gigantea*11061_1 CR5-6	CAZyme	ADDTTVLSASGPGR, VNYVDQATALAK	33.5	5.10
Glycoside hydrolase family 18 protein/ *Ceriporiopsis subvermispora* (strain B)	CAZyme	TLVSSAHNSGHGTK	61.4	5.33

Glycoside hydrolase family 18 protein/ *Phanerochaete carnosa* (strain HHB-10118-sp)	CAZyme	AQYSSFGGVMLWDASQAYANGR, AQYSSFGGVMLWDASQAYANGR	48.9	4.89
Glycoside Hydrolase Family 3 protein/ *Pycnoporus cinnabarinus*	CAZyme	YDLSVWDAVAQGWR, HFDANNIEPR	78.8	5.17
Glycoside Hydrolase Family 30 protein (Fragment)/ *Pycnoporus cinnabarinus*	CAZyme	APSYLFSVINDIR, IHILPWSPPGWMK	61.6	5.05
Glycoside hydrolase family 30 protein/ *Phlebiopsis gigantea*11061_1 CR5-6	CAZyme	VHLLPWSPPGWMK, SLMDANGFSSTR, VHLLPWSPPGWMK	57.8	6.27
Glycoside Hydrolase Family 31 protein/ *Pycnoporus cinnabarinus*	CAZyme	DAYGVPQGTNLYGNHPIYFEHR, NHNADTSISQEFYR, IFTVDPDYFPLNR, STFAGAGTR	98.4	6.07
Glycoside Hydrolase Family 5 protein/ *Pycnoporus cinnabarinus*	CAZyme	AAGINTVR	49.4	5.29
Glycoside Hydrolase Family 5 protein/ *Pycnoporus cinnabarinus*	CAZyme	VIVDLHGAPGSQNGFDNSGHR, VIVDLHGAPGSQNGFDNSGHR	23.1	6.24
Glycoside Hydrolase Family 55 protein/ *Pycnoporus cinnabarinus*	CAZyme	NVKDFGAKGDGVTDDTAAINAAISSGNR, GDGVTDDTAAINAAISSGNR, DFGAKGDGVTDDTAAINAAISSGNR, NVKDFGAKGDGVTDDTAAINAAISSGNR	91.8	6.27
Glycoside Hydrolase Family 76 protein/ *Pycnoporus cinnabarinus*	CAZyme	AQCAATLSTAESVANR, IGQLALQQDR	24.2	4.97
Glycoside Hydrolase Family 76 protein/ *Pycnoporus cinnabarinus*	CAZyme	HLQFYLDNAPDR	18.9	4.91
Glycoside hydrolase family 92 protein/ *Dichomitus squalens* (strain LYAD-421)	CAZyme	GMINIQQHEGWLPECR, VELTATKR	87.9	4.74
Glycoside Hydrolase Family 92 protein/ *Pycnoporus cinnabarinus*	CAZyme	TLYPLMSLHDPENFAR, TLYPLMSLHDPENFAR, GMINIQQHEGWLPECR	79.1	4.88
Glycoside hydrolase family 92 protein/ *Trametes versicolor* (strain FP-101664)	CAZyme	ALLADAELQPPNWDLQGR, GMINIQQHEGWLPECR	90.4	5.36
Transaldolase/ *Trametes versicolor* (strain FP-101664)]	CAZyme	IASTWEGIQAAR, VTYVDNEAEFR, LSFDKEATK	28.0	7.83
Predicted protein (Fragment)/ *Postia placenta* (strain ATCC 44394/ Madison 698-R)	hypothetical	TVPFDVAFIGAPFDTGTSYRPGAR	15.6	5.45
Putative uncharacterized protein/ *Postia placenta* (strain ATCC 44394/ Madison 698-R)	hypothetical	INMGIPLYGR, INMGIPLYGR	56.4	4.39
Uncharacterized protein (Fragment)/ *Pycnoporus cinnabarinus*	hypothetical	VTDTFPNLWSVLGFPR, VAIVHGLADFILVAEGTR, AVGVRPECR	56.0	5.29
Uncharacterized protein (Fragment)/ *Pycnoporus cinnabarinus*	hypothetical	NRSPSAYLANPAEER, SPSAYLANPAEER, QTADAIVAGTVLAVQR, YKYDQDKDLTLLR, SDSHPSTTYQWTR	80.9	5.68

Uncharacterized protein/ *Ceriporiopsis subvermispora* (strain B)	hypothetical	QFNSLNDVK	37.8	6.40
Uncharacterized protein/ *Ceriporiopsis subvermispora* (strain B)	hypothetical	TFDFQPGDTAVFPISYGHYIK, EQTITDLPPSKDIAAAEIR	53.8	5.05
Uncharacterized protein/ *Ceriporiopsis subvermispora*	hypothetical	IINEPTAAAIAYGLDKK, TLSSATQTSIEIDSLFEGVDFYTSLTR, VEIIANDQGNR	70.7	5.21
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	QLLLPLFEYQATGQYPNK, EISSDGNVNTVDVVFPSWPIFLYTNPVIGK	74.2	5.90
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	GGQENDAVIANAQDGSGYNNANFMTPPDGQNGR, MYLWNTANPYR, DGDLEAGIVIHELSHGLSTR, MYLWNTANPYRDGDLEAGIVIHELSHGLSTR, GGQENDAVIANAQDGSGYNNANFMTPPDGQNGR, MYLWNTANPYRDGDLEAGIVIHELSHGLSTR, MYLWNTANPYRDGDLEAGIVIHELSHGLSTR	91.5	5.07
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	SFLVTDVYGR, QDGGPLPPPVITPPDAEAGGR	36.8	4.65
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	QLLLPLFEYQATGQYPNK, ISSDYADIVALSIR, DLLISSVR	75.9	5.38
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	FGNYPAMGAVYDIASR, FGNYPAMGAVYDIASR, GGWTTHAMNMGQR, GGWTTHAMNMGQR	82.5	5.39
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	VNSVFSSYR, TANLKDDPLAPPDLLVNADFR	72.1	4.83
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	ALFFEFPDEPELFAVDR, SEPDAQPLFDTR	97.6	5.14
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	TLAEEVIVMSCGVAWMASLEK	85.2	6.32
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	TGALLAKPATGK, MDEFAQRR, MDEFAQRR	58.7	4.93
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	SHPYSTSATTNPLR, DAWIQADANR	65.3	4.93
Uncharacterized protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	GTLPANLQGK	89.9	4.92
Uncharacterized protein/ *Fibroporia radiculosa* (strain TFFH 294)	hypothetical	FLSDSSADVR	144.5	6.84
Uncharacterized protein/ *Fibroporia radiculosa* (strain TFFH 294)	hypothetical	ASVPSGASTGVHEAVELR	46.8	5.78
Uncharacterized protein/ *Fibroporia radiculosa* (strain TFFH 294)	hypothetical	QFTDFYYSTFDTNR	13.8	4.65
Uncharacterized protein/ *Phanerochaete carnosa* (strain HHB-10118-sp)	hypothetical	NPLGGGNGMVPHYSGTTLDAQAR, GLVNEELLK, GAWLVNTAR	39.5	6.71
Uncharacterized protein/ *Phlebiopsis gigantea*11061_1	hypothetical	NPLGGGNGMVPHYSGTTLDAQAR, NFVPAHEMIER, GAWLVNTAR	39.3	6.61
Uncharacterized protein/ *Phlebiopsis gigantea*11061_1	hypothetical	QTADAIVAGTVLAVQR, LTYYGDSK	77.0	4.97
Uncharacterized protein/ *Phlebiopsis gigantea*11061_1	hypothetical	ITLTPEGIKAGWQVEHMLEAR, ITLTPEGIKAGWQVEHMLEAR, ALPANLFPR	59.1	5.20
Uncharacterized protein/ *Phlebiopsis gigantea*11061_1	hypothetical	LYHSEATLLPDGR	107.2	4.51
Uncharacterized protein/ *Phlebiopsis gigantea*11061_1	hypothetical	GIWIADPSLSWGLVQQEIPALR	51.3	4.79
Uncharacterized protein/ *Phlebiopsis gigantea*11061_1	hypothetical	SVTATFTVPTPK	26.6	5.05

Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	LFEGVLVYGTPIK, RGLGPVPPQDPLIYR	26.2	5.64
Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	IFLSGRPVSIGGTSAAAPTFSGIVSLLNSAR, GWDPVTGVGTPNFGK, AIPDVAAQADLFR, AIPDVAAQADLFR, GLFNPNGR, GLFNPNGR	65.8	5.52
Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	FVDFSATQWLALMPR, NLSPTEPLIYLELFK	48.3	5.36
Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	LGANAILGVSIAVAQAGAAEK, GNPTVEVDLYTAK	47.0	6.10
Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	TGALLAKPATGK, MDEFAERR, MDEFAERR	55.6	5.00
Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	GLGVNAANHNDDSTVPAGC	64.9	5.31
Uncharacterized protein/ *Pycnoporus cinnabarinus*	hypothetical	FADVLEQFESQFYQQALQK	44.9	4.25
WSC-domain-containing protein/ *Dichomitus squalens* (strain LYAD-421)	hypothetical	AQVFFPSCWDGVNLDSADHK	80.0	5.85

TABLE S2. Functional classification of the proteins matches derived from the MS analysis of spots from 2D gels

Spot No	ID	GROUP	Sequence aligned	Organism
13	DUF1793-domain-containing protein	?	BSSZPLGDWYETTDGSVEGFR, BAMAELASLVGDTTR	*Trametes versicolor* FP-101664 SS1
23	Frt2p	?	BFLZSMEPSSSGZSK	*Saccharomyces cerevisiae* S288c
20	alfa-L-arabinofuranosidase precursor	CAZyme	DZLNFVLGDPATSAPAALR, BFPASSSFSGTLTVGLR, FVELGNEDFLGSAPSTY, BSFTYNAPAFSVSVLTV, TYPYR	*Pleurotus* sp. 'Florida'
23	alpha/beta-hydrolase	CAZyme	BTNDLTLSGSNAEZEPALESSR, BFZVATGESDVLCGR, VYPASDFDSDAZR	*Dichomitus squalens* LYAD-421 SS1
39	alpha/beta-hydrolase	CAZyme	LVHGLADFLLVAEGTR, YLDEF, BALPDV	*Fomitiporia mediterranea* MF3/22
73	alpha/beta-hydrolase	CAZyme	BVALVHGLADFLLVAEGTR, BAVGLLNPVFDVYR, FPFNET, FNETLR	*Trametes versicolor* FP-101664 SS1
26a	alpha/beta-hydrolase	CAZyme	BLTLAGZSAGANMVR, BCATWTFAR, GFADGVNSAL	*Dichomitus squalens* LYAD-421
19	alpha-l-arabinofuranosidase a	CAZyme	DZLNFVLGDPATSAPAALR, FVELGNEDF, BFPASSSFSG, PSTYPYR, TLTVG,	*Moniliophthora roreri* MCA 2997
6	beta-glucosidase	CAZyme	GFDAVWLNPGESK, HFDANNLEPR, TLGAASAVLLK, ZHELYLHPFLR, ARGEAMGAEFR, TVTLSLSR,	*Dichomitus squalens* LYAD-421
70	carbohydrate esterase family 16 protein	CAZyme	BSFLFLTVPPTNR, BLFSZVELLYEDGAR	*Phanerochaete carnosa* HHB-10118-sp
63	carbohydrate-binding module family 1 protein	CAZyme	BVANNLAASTSTNSEDFHVGVLR, TCEAL	*Phanerochaete carnosa* HHB-10118-sp
15	carboxylesterase from carbohydrate esterase	CAZyme	BLTLFGZSAGAASVR, BLSTLGFLALDDGK, BAVDPNTLVNLATVAR, BTFFANDLVP	*Trametes versicolor* FP-101664 SS1
38a	cellobiohydrolase I	CAZyme	NCALDGADYSGTYGLTTSGNALTLK, MLWLDSDYPLDZDASZPGVSR, BVYLMDTSDSK, BTAFGDNNSFE, BMGDZTFLGTGK, PASCSENYA, VEMD	*Dichomitus squalens* LYAD-421 SS1
43	cellobiohydrolaseI	CAZyme	BFVTHGPYSTNLGSR, BLGGLNVMGZSFDK, BMGDZTFLGTGK, BTAFGDNNSFE, GSTYA,	*Dichomitus squalens* LYAD-421 SS1
44	cellobiohydrolaseI	CAZyme	BNCALDGADYSGTYGLTTSGNALTLK, BFTVVTZFLTNNNZTSGTLSELRR, BMLWLDSDYPLDZDASZPGVSR, BYGGLCDZDGCDFNSWR, BGMVLVMSLWDDHAAR, BMGDZTFLGTGK, BTAFGDNNSFE, BLGGLNVMGZSFDK, BVYLMDTSDSK	*Trametes versicolor* FP-101664 SS1
35b	cellobiohydrolaseI	CAZyme	BMGDZTFLGPGLTVDT, BTAFGDNNSFE, BFVTZGPYS	*Dichomitus squalens* LYAD-421
44	cellulase	CAZyme	NCALDGADYSGTYGLTTSGNALTLK, FTVVTZFLTNNNZTSGTLSELRR, MLWLDSDYPLDZDASZPGVSR, GMVLVMSLWDDHAA, GLCDZDGCDFNSWR, BMGDZTFLGTG, BTAFGDNNSF, BVYLM	*Phanerochaete chrysosporium*
35b	cellulose -beta-cellobiosidase precursor	CAZyme	BMGDZTFLGPGLTVDT, BFVTZGPYS, AFGDNNSFE	*Moniliophthora roreri* MCA 2997
70	cellulose-binding GDSL lipase/acylhydrolase-like protein	CAZyme	BSFLFLTVPPTNR, BLFSZVELLYEDGAR	*Heterobasidion annosum*

Spot No	ID	GROUP	Sequence aligned	Organism
52	endo-1,4-beta-xylanase C precursor	CAZyme	BGTFTFSGGDZLANLAK, BLYLNDYNLEZSGAK, BTTTPASZSALAZZZK, BWDATEPTR, CVGLTVWGLSDK,	*Trametes versicolor* FP-101664 SS1
56	endo-1,4-beta-xylanase C precursor	CAZyme	BGTFTFSGGDZLANLAK, BLYLNDYNLEZSGAK, BWDATEPTR, DYETVL	*Trametes versicolor* FP-101664 SS1
52	endo-1,4-B-xylanase A	CAZyme	BGTFTFSGGDZLANLAK, BLYLNDYNLEZSGAK, BWDATEPTR, BWDATEPTR, TPASZSALAZZZK	*Dichomitus squalens* LYAD-421 SS1
73	endo-beta-glucanase	CAZyme	BVNYVDZGTALAZNLTYASGDTFLLR, BADHTTVLSPSGPGR, HVAVFNLR	*Gloeophyllum trabeum* ATCC 11539
63	endo-polygalacturonase PG1	CAZyme	BNWDGPLFZVSGK, BGSNLVFZR	*Trametes versicolor* FP-101664 SS1
8a	exo-beta-1,3-glucanase	CAZyme	BTHPZYADVAVDZLVSVR, HZGLAAFNSAPSSY	*Fomitiporia mediterranea* MF3/22
69	Galactosyltransferase domain-containing protein	CAZyme	ATLVLATGDGNVGZFNEDGFLGCVR, LRWLT, SLRYL	*Rhizoctonia solani* AG-1 IA
15	glucan 1,3-beta-glucosidase	CAZyme	GDGVTDDTAALNAALSSGNR, BGDGVTDDTAALNA, ASWS	*Trichoderma atroviride*
22	glucoamylase	CAZyme	BAGVLANLGPSGSK, AAAYA	*Punctularia strigosozonata* HHB-11173 SS5
36	glucoamylase	CAZyme	BAGVIANIGPSGSK, BAIIDZFTTGEDDSIR, BVYVDAFR	*Trametes versicolor* FP-101664 SS1
39	glucoamylase	CAZyme	BAGVLANLGPSGSK, BALLDZFTTGEDDSLR	*Trametes versicolor* FP-101664 SS1
55	glucoamylase	CAZyme	BAGVLANLGPSGSK, BALLDZFTTGEDDSLR, BVYVDAFR, AAAYA, BGSNLVFZ	*Punctularia strigosozonata* HHB-11173 SS5
26a	glucoamylase	CAZyme	BAGVLANLGPSGSK, BSLWSLNSGLAANAAAAVGR, BYTPSNGGLAEZFSK, BALLDZFTTGEDDSLR, AAAYA, SGDGGVSGS	*Punctularia strigosozonata* HHB-11173 SS5
8a	glucoamylase	CAZyme	BAGVLANLGPSGSK, BALLDZFTTGEDDSLR	*Trametes versicolor* FP-101664 SS1
9a	glucoamylase	CAZyme	BAGVLANLGPSGSK, BALLDZFTTGEDDSLR, BVYVDAFR, BDSSLVFK, WSDAGFD	*Trametes versicolor* FP-101664 SS1
59	glycoside hydrolase	CAZyme	BLVLGLPLYGYVSZSSK, VLHR	*Auricularia delicata* TFB-10046 SS5
52	glycoside hydrolase family 10 protein	CAZyme	BGTFTFSGGDZLANLAK, BLYLNDYNLEZSGAK, CVGLTVWGLSDK, BWDATEPTR, TTPAS	*Ceriporiopsis subvermispora* B
56	glycoside hydrolase family 10 protein	CAZyme	GTFTFSGGDZLANLA, BLYLNDYNLEZSGAK, BWDATEP, BADYETVL	*Ceriporiopsis subvermispora* B
36	glycoside hydrolase family 15 protein	CAZyme	BAIIDZFTTGEDDSIR, BAGVIANIGPSGSK, BVYVDAFR	*Ceriporiopsis subvermispora* B
39	glycoside hydrolase family 15 protein	CAZyme	BALLDZFTTGEDDSLR, , BAGVLANLGPSGSK	*Ceriporiopsis subvermispora* B
55	glycoside hydrolase family 15 protein	CAZyme	BALLDZFTTGEDDSLR, BAGVLANLGPSGSK, BVYVDAFR, BGSNLVFZ	*Ceriporiopsis subvermispora* B
84	glycoside hydrolase family 15 protein	CAZyme	BALLDZFTTGEDDSLR	*Ceriporiopsis subvermispora* B
26a	glycoside hydrolase family 15 protein	CAZyme	BALLDZFTTGEDDSLR, BSLWSLNSGLAANAAAAVGR, BYTPSNGGLAEZFS, BAGVLANLGPSGSK	*Ceriporiopsis subvermispora* B
8a	glycoside hydrolase family 15 protein	CAZyme	BALLDZFTTGEDDSLR, BAGVLANLGPSGSK	*Ceriporiopsis subvermispora* B

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9a	glycoside hydrolase family 15 protein	CAZyme	BALLDZFTTGEDDSLR, BAGVLANLGPSGSK, BVYVDAFR, BDSSLVFK, SSGAM	*Ceriporiopsis subvermispora* B
73	glycoside hydrolase family 16 protein	CAZyme	BVNYVDZGTALAZNLTYASGDTFLLR, BADHTTVLSPSGPGR, HVAVFNLR	*Phanerochaete carnosa* HHB-10118-sp
88	glycoside hydrolase family 18 protein	CAZyme	BATPAAPHFAVYADZYZSGVTGPPAVSAV, BAYEWTTLSASDR	*Dichomitus squalens* LYAD-421 SS1
38d	glycoside hydrolase family 35 protein	CAZyme	BVGGYTNFPDZVR, BGVMNEGGLFGER, VZDSAGLDNAD, DAEAG, ATYSZ, SPLFYK	*Moniliophthora roreri* MCA 2997
19	glycoside hydrolase family 51 protein	CAZyme	BFVELGNEDFLGSAPSTYPYR, DZLNFVLGDPATSAPAALR, BFPASSSFSGTLTVGL	*Trametes versicolor* FP-101664 SS1
20	glycoside hydrolase family 51 protein	CAZyme	BFVELGNEDFLGSAPSTYPYR, BSFTYNAPAFSVSVLTVTTH, DZLNFVLGDPATSAPAALR, BFPASSSFSGTLTVGL	*Trametes versicolor* FP-101664 SS1
15	glycoside hydrolase family 55 protein	CAZyme	GDGVTDDTAALNAALSSGNR, BGDGVTDDTAALNA, AVDPNTLV	*Grosmannia clavigera* kw1407
16	Glycoside Hydrolase Family 55 protein OS=Pycnoporus cinnabarinus	CAZyme	BNVZDFGAZGDGVTDDTAALNAALSSGNR, AGTAGSTGVLELTDMLFSTR, GAZGDGVTDDTAAL	*Dichomitus squalens LYAD-421 SS1*
17	Glycoside Hydrolase Family 55 protein OS=Pycnoporus cinnabarinus]	CAZyme	BNVZDFGAZGDGVTDDTAALNAALSSGNR, BTHPZYZDYTPDZFLSVK, BGDGVTDDTAAL, LVLNNLK	*Dichomitus squalens LYAD-421 SS1*
74	glycoside hydrolase family 61 protein	CAZyme	BLYVNGVDZGELTGLR	*Trametes versicolor* FP-101664 SS1
44	glycoside hydrolase family 7 protein	CAZyme	NCALDGADYSGTYGLTTSGNALTLK, NCALDGADYSGTYGLTTSGNALTLK, BYGGLCDZDGCDFNSWR, GMVLVMSLWDDHAA, TVVTZFLTNNNZTSGTLSELRR, BMGDZTFLGTGK, BVYLMDT, GGLNVMGZSFD	*Schizophyllum commune* H4-8
38a	glycoside hydrolase family 7 protein	CAZyme	NCALDGADYSGTYGLTTSGNALTLK, MLWLDSDYPLDZDASZPGVSR, BMGDZTFLGTG, BTAFGDNNSF, BVYLM	*Phanerochaete carnosa* HHB-10118-sp
36a	Glycoside Hydrolase Family 76 protein OS=Pycnoporus cinnabarinus	CAZyme	identication by LC-MS	identication by LC-MS
8b	Glycoside Hydrolase Family 76 protein OS=Pycnoporus cinnabarinus	CAZyme	BTLYPLMSLHDPENFAR, BVTAGSTYAASFGPGZYR, BLVVDVTNDGZZSNSL, EFGTYLGNSPLR, ANPNFGTTGF, SGAMGS	*Dichomitus squalens* LYAD-421
2	glycoside hydrolase family 92 protein	CAZyme	LVVDVTNDGZZSNSL	*Dichomitus squalens* LYAD-421
7	glycoside hydrolase family 92 protein	CAZyme	BGMLNLZZHEGWLPECR, BTLYPLMSLHDPENFAR, BVTAGSTYAASFGPGZYR, BSLGYVPZDLLEPGGANTK, BLVVDVTNDGZZSNSL, BFEVCPCDLNSR, EFGTYLGNSPLR, BVYTCMDFK, BYTFPANSTTPR, BADVPLPDPAS, BATWNELLGR, BGVGFDLGZP, ANPNFGTTGF, BLLNNSADAEZYATR, BVGVSFLSVEZA, SGAMGS, NPVFD, AEAEL,	*Dichomitus squalens* LYAD-421
43	glycoside hydrolase family 92 protein	CAZyme	BVTAGSTYAASFGPGZYR, BLVVDVTNDGZZSNSL, WDATEP	*Dichomitus squalens* LYAD-421 SS1
9a	glycoside hydrolase family 92 protein	CAZyme	BGMLNLZZHEGWLPECR, BGMLNLZZHEGWLPECRVTAGSTYAASFGPGZYR, BGMLNLZZHEGWLPECRLVVDVTNDGZZSNSL, BATWNELLGR, ANPNFGTTGF, SGAMGS	*Dichomitus squalens* LYAD-421
9b	glycoside hydrolase family 92 protein	CAZyme	BVTAGSTYAASFGPGZYR, BSLGYVPZDLLEPGGANTK, BLVVDVTNDGZZSNSL, EFGTYLGNSPLR, BATWNELLGR, BGVGFDLGZP, ANPNFGTTGF, SGAMGS, DGSPPDV, NPVFD	*Dichomitus squalens* LYAD-421 SS1

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56	mannose-6-phosphatase	CAZyme	PVHFTYLNTHLDDZSDDZRR, BLDFVYGGSNGZWTADAY, BDDGVAAGEFSPLFYZK, GEZPWSTR, BFPGAGSFR, BLATYNLR, DDGVAAGEFSPLF, BLCTATSLLLK, BRLAASMMLVR	*Phanerochaete chrysosporium*
57	mannose-6-phosphatase	CAZyme	PAAPVHFTYLNTHLDDZSDDZR, BDDGVAAGEFSPLFYZK, BLDFVYGGSNGZW, BLAASMMLVR	*Punctularia strigosozonata* HHB-11173 SS5
15	Pectin lyase fold/virulence factor	CAZyme	GDGVTDDTAALNAALSSGNR, BGDGVTDDTAALNAAL, FANDLV, BAVDPNTLV	*Macrophomina phaseolina* MS6
74	polysaccharide lyase family 14	CAZyme	CNSDGFGTSLDR, NGEVLLYYNNV	*Ceriporiopsis subvermispora* B
50	putative isomerase YbhE	CAZyme	GSEVFVPDLGADZVWR, FRVZGZLDZPTGSGPR, LLGHVFTGLDZLR, BLVEDGAPGZF	*Dichomitus squalens* LYAD-421 SS1
73	putative laminarinase	CAZyme	BVNYVDZGTALAZNLTYASGDTFLLR, BADHTTVLSPSGPGR, HVAVFNLR	*Phanerochaete chrysosporium*
55	esterase 1	esterase/lipase	BGTPVVYVSLNYR, BVGPFGFPZGTEADTR	*Dichomitus squalens* LYAD-421 SS1
55	extracellular triacylglycerol lipase precursor	esterase/lipase	BVGPFGFPZGTEA, BGTPVVYVSLNYR, LSAFG	*Dichomitus squalens* LYAD-421 SS1
56	GDSL lipase/acylhydrolase	esterase/lipase	BNFLFZNMVPLETTPLY, NLVTFGDSYTDL, BELTTSGNELAK, LYLNDY, AAAYALA	*Stereum hirsutum* FP-91666 SS1
62	GDSL-like lipase/Acylhydrolase domain-containing protein	esterase/lipase	ZTLVAFGDSYTDGGRZDGGPLPPPVLTPPDAEAGGR, BSFLVTDVYGR, BTDGEANLZAAAZVLLAZL	*Rhizoctonia solani* AG-1 IA
68	PLC-like phosphodiesterase	esterase/lipase	BRVVVFLDAGADTDR, BATVCNGHAELCDR	*Trametes versicolor* FP-101664 SS1
5	hypothetical protein TRV_05213	Hypothetical	SMZMGDSVZVNRMFR	*Trichophyton verrucosum* HKI 517
11	hypothetical protein FOMPIDRAFT_1022279	Hypothetical	BLLLVLWZTLLTCCGGLRELASVK	*Fomitopsis pinicola* FP-58527 SS1
13	hypothetical protein DICSQDRAFT_106181	Hypothetical	BSSZPLGDWYETTDGSVEGFR, AMAELASLVGDTTR	*Dichomitus squalens* LYAD-421 SS1
15	predicted protein	Hypothetical	BLTLFGZSAGAASVR, BLSTLGFLALDDGK, TFFANDLVPLL, LVNLATVAR	*Fibroporia radiculosa*
21	hypothetical protein E5Q_04952	Hypothetical	BMTZLMFETFNAPAFYVALZAVLSLYASGR, BYSVWLGGSLLASLSTFZZMWLSK, BLWHHTFYNELR, LDAGADTDR, EZLAZLEAZL	*Mixia osmundae* IAM 14324
23	predicted protein	Hypothetical	FZVATGESDVLCGR, SGSNAEZEPALESSR	*Fibroporia radiculosa*
29	hypothetical protein DICSQDRAFT_158045	Hypothetical	BHSHCWAYVZGLCHVZDCZYLHPVAVNMFSAHTPC LAWPNCR	*Dichomitus squalens* LYAD-421
30	hypothetical protein SS1G_04065	Hypothetical	BRVFFMTPZTVENDLK, LVFMDAT, GFLGGR	*Sclerotinia sclerotiorum* 1980
32	hypothetical protein MELLADRAFT_115685	Hypothetical	BSLPPPVNPTGDAFDPEEDEPVLELAWPHLZLVYEFFL R, LFASGDGGV	*Melampsora larici-populina* 98AG31
33	hypothetical protein PHACADRAFT_263851	Hypothetical	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGV, AGVLA	*Phanerochaete carnosa* HHB-10118-sp
36	hypothetical protein CERSUDRAFT_156000	Hypothetical	BGTSIIFASGDGGVSGSZS, NSIGVAGYIDEFAN, BAFPDVSAZSVNFI	*Ceriporiopsis subvermispora* B
42	hypothetical protein G647_08260	Hypothetical	DDGEGEEDDDDDEGDDEDEE, DDDDGEGEEDDDDDEGDDE, DDDGEGEEDDDDDEGDDED, BVTVDESZDNVV, LTSRPG, ASDTPF	*Cladophialophora carrionii* CBS 160.54
54	hypothetical protein E5Q_04952	Hypothetical	BTTGLVMDSGDGVTHTVPLYEGFSLPHALLR, BSYELPDGZVLTLGNER, BAVFPSLVGRPR,	*Mixia osmundae* IAM 14324]/

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			BAGFAGDDAPR, BZEYDESGP, FDNSGHR, ZLWDT, SGPSLVHR	
71	hypothetical protein TRAVEDRAFT_154838	Hypothetical	BVFDFYTFEDYYLK, BSPPPFZDSTVNLR, BZFVPPADSLACLK, BVNSVFSSYR, LEGZDPYAYA, BZNVLTVVSR	*Trametes versicolor* FP-101664 SS1
79	hypothetical protein CERSUDRAFT_132940	Hypothetical	BLASZLZGZVGLFFTDSPPZEVLDWFADFZZPDFAR	*Ceriporiopsis subvermispora* B
35a	hypothetical protein PHACADRAFT_263851	Hypothetical	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGV, ADLZTFF, DNENDP	*Phanerochaete carnosa* HHB-10118-sp
35b	hypothetical protein PHACADRAFT_263851	Hypothetical	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGV, ADLZTFF	*Phanerochaete carnosa* HHB-10118-sp
35c	hypothetical protein PHACADRAFT_263851	Hypothetical	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGV, ADLZTFF	*Phanerochaete carnosa* HHB-10118-sp
35d	hypothetical protein PHACADRAFT_263851	Hypothetical	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGV, ADLZTFF	*Phanerochaete carnosa* HHB-10118-sp
38 c	hypothetical protein DICSQDRAFT_61994	Hypothetical	BVTLSVZDSAGLDNADFATPPDGZSGR, BGLGVNAANHNDDSTVPAGC, BFGGANFCTLWTAFASR, BSHPYSTSATTNPLR, BDAWLZADANR,	*Dichomitus squalens* LYAD-421 SS1
9a	hypothetical protein FOMPIDRAFT_1133514	Hypothetical	BVADASVFPLZFAAHLZAPTYGLAEZAA, BSAYLDPLPPR, BEVLLSSGAMGSP, BVYGLANVR	*Fomitopsis pinicola* FP-58527 SS1
5	mitochondrial hypoxia responsive domain-containing protein	Miscellany	SMZMGDSVZVNRMFR	*Arthroderma otae* CBS 113480
11	N1221-domain-containing protein	Miscellany	BLLLVLWZTLLTCCGGLRELASVK	*Dichomitus squalens* LYAD-421 SS1
55	putative gpi anchored protein	Miscellany	WLLYDDGTGZVZESGR, DAAAAYALALR	*Neofusicoccum parvum* UCRNP2
	sulfite reductase	Miscellany	ZAZGVEPAYSFMLRVRMPAGVCLPEZWLLMDZLADE HGCGTFK, AASPFSATAAA, TMFTE	*Cryptococcus neoformans* var. neoformans JEC21
21	actin actin-like protein	miscellany/ citoesqueleton	BMTZLMFETFNAPAFYVALZAVLSLYASGR, BMTZLMFETFNAPAFYVSLZAVLSLYASGR, BYSVWLGGSLLASLSTFZZMWLSK, BLWHHTFYNELR	*Coniophora puteana* RWD-64-598 SS2
54	actin	miscellany/ citoesqueleton	BTTGLVMDSGDGVTHTVPLYEGFSLPHALLR, BSYELPDGZVLTLGNER, BZEYDESGPSLVHR, BAVFPSLVGRPR, BAGFAGDDAPR, LRPALNV, LTNDWED	*Aspergillus oryzae* RIB40
54	actin-2-like protein	miscellany/ citoesqueleton	BTTGLVMDSGDGVTHTVPLYEGFSLPHALLR, BSYELPDGZVLTLGNER, BZEYDESGPSLVHR, BAGFAGDDAPR, BAVFPSLVGRP, LVLVGGST, MVGLDAAG, DFDN	*Ceriporiopsis subvermispora* B
18	helicase of the DEAD superfamily	miscellany/ DNA	BAYZESSGSDANR	*Aspergillus oryzae* 3.042
23	RFC related checkpoint protein Rad17	miscellany/ DNA	BMLNVDZCFTSZR	*Schizosaccharomyces pombe* 972h-
30	Full=ATP-dependent DNA helicase	miscellany/ DNA	BRVFFMTPZTVENDLK, LVFMDAT	*Botryotinia fuckeliana* T4
30	helicase C-terminal domain containing proein	miscellany/ DNA	BRVFFMTPZTVENDLK, BYNF	*Pyrenophora tritici-repentis*
30	P-loop containing nucleoside triphosphate hydrolase protein	miscellany/ DNA	BRVFFMTPZTVENDLK, LVFMDAT	*Sphaerulina musiva* SO2202
53	Catalytic subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation	miscellany/ DNA	LTZZPSLLVGGTLZEYZLK, ELRK	*Komagataella pastoris* GS115
53	KLTH0G09196p (helicase)	miscellany/ DNA	ZZPSLLVGGTLZEYZ, ADELRK	*Lachancea thermotolerans*

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53	P-loop containing nucleoside triphosphate hydrolase	miscellany/ DNA	ZPSLLVGGTLZEYZLK, ADEL	*Glarea lozoyensis* ATCC 20868
53	putative Tetratricopeptide (helicase)	miscellany/ DNA	ZZPSLLVGGTLZEYZLK, YERADELR	*Sclerotinia borealis* F-4157
53	SNF2-like protein	miscellany/ DNA	BLTZZPSLLVGGTLZEYZL, ERAD	*Saccharomyces cerevisiae* YJM789
56	DNase I-like protein	miscellany/ DNA	FTYLNTHLDDZSDDZRR, BLDFVYGGSNGZWTADAY, BRLAASMMLVR, BDDGVAAGEFSPLFYZK, BFPGAGSFR, DDGVAAGEFSPLF	*Auricularia delicata* TFB-10046 SS5
57	DNase I-like protein	miscellany/ DNA	FTYLNTHLDDZSDDZRR, BLDFVYGGSNGZWTADAY, BDDGVAAGEFSPLFYZK, BLAASMMLVR, DDGVAAGEFSPLF	*Auricularia delicata* TFB-10046 SS5
58	Piso0_000299	miscellany/ DNA	NVLLFDLGGGTFDVSLLTLEDGLFEVK, EVAGNF	*Millerozyma farinosa* CBS 7064
64	Leptomycin B resistance protein pmd1	miscellany/ DNA	BDLSLTFPAGZTLALVGASGSGK, AAAAGGAT, ACEEG, AGGATP	*Rhizoctonia solani* AG-1 IB
64	P-loop containing nucleoside triphosphate hydrolase protein	miscellany/ DNA	BDLSLTFPAGZTLALVGASGSGK, FSPALT, LVTLAA, GZLDACEE	*Trametes versicolor* FP-101664 SS1
67	histone acetyltransferase complex protein	miscellany/ DNA	BLZCADPACELGDGVDLCPSCFCAGZEFGNHK	*Laccaria bicolor* S238N-H82
67	SWIRM-domain-containing protein	miscellany/ DNA	GDGVDLCPSCFCAGZEFGNHK, BLZCADPACELGDG	*Punctularia strigosozonata* HHB-11173 SS5
67	transcriptional adapter 2	miscellany/ DNA	BLZCADPACELGDGVDLCPSCFCAGZEFGNHK	*Moniliophthora roreri* MCA 2997
68	ribonuclease T2	miscellany/ DNA	BGAEAVAFFZTVVK, BGSVLDGDFVALDAP, GFSPAL	*Punctularia strigosozonata* HHB-11173 SS5
69	WD40 repeat-like protein	miscellany/ DNA	MESLRYLGSMAGHZGWVTALATSSENPDMLLTASR	*Gloeophyllum trabeum* ATCC 11539
75	ref2p	miscellany/ DNA	BLSZMDGLVDAVLNLCVLDTSVAENVRSYMK	*Saccharomyces arboricola* H-6
75	RNA-binding protein	miscellany/ DNA	BLSZMDGLVDAVLNLCVLDTSVAENVRSYMK	*Saccharomyces cerevisiae* YJM789]
75	thaumatin-like protein	miscellany/ DNA	BGPFDSTGFPVGCK	*Moniliophthora roreri* MCA 2997
75	U3 snoRNP protein Utp20 (predicted)	miscellany/ DNA	BFLESLTMNDASNZFZLDVFVZFLFPFLK	*Schizosaccharomyces pombe* 972h-
78	RecName: Full=Ribosomal protein VAR1	miscellany/ DNA	BDNLPMNNSLSLZNNYMTNLLNNNNLK	*Williopsis saturnus* var. mrakii
79	mRNA turnover protein 4-like protein	miscellany/ DNA	BLASZLZGZVGLFFTDSPPZEVLDWFADFZZPDFAR	*Trametes versicolor* FP-101664 SS1
84	ATP-dependent DNA helicase Snf21	miscellany/ DNA	BMSDLESYPAVMR	*Schizosaccharomyces pombe* 972h-
64	ATP-binding cassette (ABC) transporter	miscellany/ membrane transport	BDLSLTFPAGZTLALVGASGSGK, GZLDACEE, FSPALT	*Trichosporon asahii* var. asahii CBS 2479
69	G-protein complex beta subunit	miscellany/ membrane transport	MESLRYLGSMAGHZGWVTALATSSENPDMLLTASR	*Ganoderma lucidum*
69	guanine nucleotide binding protein beta subunit	miscellany/ membrane transport	MESLRYLGSMAGHZGWVTALATSSENPDMLLTASR	*Schizophyllum commune* H4-8
38b	putative gpi anchored protein	miscellany/ membrane transport	WLLYDDGTGZVZESGR, WLLYDDGTGZVZESGR, DAAAAYALALR, BGSPASCSENY,	*Neofusicoccum parvum* UCRNP2
13	glutaminase GtaA	miscellany/ metabolism	BSSZPLGDWYETTDGSVEGFR, AMAELASLVGDTTR	*Gloeophyllum trabeum* ATCC 11539
32	protein phosphatase PP2A0 B	miscellany/	BSLPPPVNPTGDAFDPEEDEPVLELAWPHLZLVYEFFL	*Trametes*

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	subunit gamma isoform	metabolism	R	*versicolor* FP-101664 SS1
42	fatty acid desaturase	miscellany/ metabolism	DDDDGEGEEDDDDDEGDDEDE, DDDGEGEEDDDDDEGDDEDEE, DVDDDDGEGEEDDDDDEGDDE, SDLTSR	*Colletotrichum graminicola* M1.001
48	YALI0D21670p	miscellany/ metabolism	BYFVAFVMTGGLCGLYLVGNSLAHVLCYK	*Yarrowia lipolytica*
55	feruloyl esterase	miscellany/ metabolism	BGTPVVYVSLNYR, BVGPFGFPZGTEADTR, BADLZTF	*Pleurotus eryngii*
79	3-hydroxy-3-methylglutaryl-coenzyme A reductase	miscellany/ metabolism	BVGGLZEFCSVAAVALAMDCLMTFTLYTAVLTLMVEVR, VVHPPS	*Trametes versicolor* FP-101664 SS1
87	sulphite reductase hemo protein beta subunit	miscellany/ metabolism	BZAZGVEPAYSFMLRVRMPAGVCLPEZWLLMDZLADEHGCGTFK, SPFSATAAASTPTTP, PAASPFSATAAAS, TMFTE	*Gloeophyllum trabeum* ATCC 11539
9a	Choline dehydrogenase	miscellany/ metabolism	BVADASVFPLZFAAHLZAPTYGLAEZ, BEVLLSSGAMGSP, BELGGVVDEDLK, BVYGLANVR, DAGFDTSNL	*Wallemia ichthyophaga EXF-994*
78	YALI0D17204p	miscellany/ proteasome	BTMLELLNZLDGFEPTZNLZVLMATNR, ZDELH	*Yarrowia lipolytica* CLIB122
78	26S proteasome subunit P45	miscellany/ proteasome	BTMLELLNZLDGFEPTZNLZVLMATNR, BRVEF	*Dichomitus squalens* LYAD-421
61	vacuolar protein sorting-associated protein 13	miscellany/ secretory	BFRELMPAAZHERNZVMLLFTMSGG	*Dichomitus squalens* LYAD-421 SS1
62	thermolabile hemolysin	miscellany/ secretory	BVZTLVAFGDSYTDGGRZDGGPLPPPVLTPPDAEAGGR, BSFLVTDVYGR	*Moniliophthora roreri* MCA 2997
38d	protein kinase c	miscellany/ signal transduction	BCTECDLTCHANCAHLVPDFCGMSMETANALL, ELVSYDTPHL, ENYANL, MLEEFR, RLAEPZMH, DDVY	*Talaromyces stipitatus* ATCC 10500
23	LEA domain protein	miscellany/ stress response	BETAAZEYDALTSK	*Schizosaccharomyces pombe* 972h-
66	heat shock cognate 70	miscellany/ stress response	BNVLLFDLGGGTFDVSLLTLEDGLFEVK, DSPEVEA	*Punctularia strigosozonata* HHB-11173 SS5
74	thaumatin-like protein	miscellany/ stress response	BGPFDSTGFPVGCK	*Moniliophthora roreri* MCA 2997
37a	ZYBA0S05-06964g1_1 (heat shock 70 kDa protein - HSP70 interaction site)	miscellany/ stress response	BTLSSSAZTSLELDSLYEGLDFYTSLTR, NVLLFDLGGGTFDVSLLTLEDGLFEVK, SVLDAYVNVLR	*Zygosaccharomyces bailii* CLIB 213
38a	Heat shock 70 kDa protein	miscellany/ stress response	BNVLLFDLGGGTFDVSLLTLEDGLFEVK, BTLSSSAZTSLELDSLYEGLDFYTSLTR, STSATT	*Wickerhamomyces ciferrii*
4	non-fungi aligment	nd	-	-
12	non-fungi aligment	nd	-	-
14	non-fungi aligment	nd	-	-
49	no significant results	nd	-	-
60	no significant matches	nd	-	-
77	no significant matches	nd	-	-
83	no significant matches	nd	-	-
86	no significant matches	nd	-	-
19	alcohol oxidase	oxidoreductase	ESGNYFNLPVGAVSPASR, BSNLDVLVGAZV	*Punctularia strigosozonata* HHB-11173 SS5
9a	alcohol oxidase	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZA, BSAYLDPLPPR, BEVLLSSGAMGSP, BVYGLANVR, GGVVDEDLK, FADGVNSAL	*Stereum hirsutum* FP-91666 SS1
9a	aryl-alcohol dehydrogenase	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZA, BSAYLDPLPPR, BVYGLANVR, BEVLLSSGAMGSP, TAGSTYA,	*Exophiala dermatitidis* NIH/UT8656
22	bilirubin oxidase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR	*Ganoderma tsunodae*
72	bilirubin oxidase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BYSFVLNANZDVDNYWLR, BSTSTPTADLAVLSVT	*Ganoderma tsunodae*

Spot No	ID	GROUP	Sequence aligned	Organism
26a	bilirubin oxidase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BSTSTPTADLAVLSVT	*Ganoderma tsunodae*
9b	bilirubin oxidase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BSTSTPTADLAVLSVT, WLZADAN	*Ganoderma tsunodae*
89	cytochrome P450	oxidoreductase	BLTDDELLAZVSTVMVAGLDTTSNGMSRMLYLLAZN ZDAZERLR, SVLRGP	*Dichomitus squalens* LYAD-421 SS1
82	d-lactaldehyde dehydrogenase	oxidoreductase	BRLVVTSSCASVLTPSTEPRLFSEENWNDASLAE	*Moniliophthora roreri* MCA 2997
8a	DUF1929-domain-containing protein	oxidoreductase/ glyoxal oxidase	BVYLLDZSENNAAK, BWSDAGFDTSNLAR	*Trametes versicolor* FP-101664 SS1
65	fungal class II heme-containing peroxidase	oxidoreductase	BLTFHDALGFSPALTA, BLZSDFALAR	*Ceriporiopsis subvermispora* B
9a	gmc oxidoreductase	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZAA, BVYGLANVR, BEVLLSSGAMGSP, BSAYLDPLP, BELGGVVDEDL, FDTSN	*Byssochlamys spectabilis* No. 5
24	Glyoxal oxidase	oxidoreductase	BVZVSLMDLGFSSHAFHSGAR, BVZTLDPPFMFVERPK, BLVFMDATLSRD, BSLTFVTPPNGR, BSLPANLFPR, FALPDGK, BFPAEFR	*Trametes versicolor* FP-101664 SS1
25	Glyoxal oxidase	oxidoreductase	BVZVSLMDLGFSSHAFHSGAR, BLVFMDATLSR, BSLTFVTPPNGR, BSLPANLFPR, BLTLTPEGLAK, BLLNTPEZLGFGK, FALPDGK, BFPSELR,	*Trametes versicolor* FP-101664 SS1
27	glyoxal oxidase precursor	oxidoreductase	BVZVALMDLGFSSHAFHSSAR, BGFPGNPALZPGNZALR, BEETVRPSAFLER, BSLPANLFPR, VPFPVT	*Dichomitus squalens* LYAD-421
9a	GMC oxidoreductase	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZAAK, BSAYLDPLPPR, BVYGLANVR, BEVLLSSGAMGSP, GGVVDEDLK, FDTSN	*Trametes versicolor* FP-101664 SS1
9a	gmc oxidoreductase	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZAA, BSAYLDPLPPR, BEVLLSSGAMGSP, BVYGLANVR, LZEETE	*Moniliophthora roreri* MCA 2997
72	LAC1	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BYSFVLNANZDVDNYWLR, BSTSTPTADLAVLSVTK, DDTTVL	*Polyporus brumalis*
22	laccase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, AAAAYA	*Dichomitus squalens* LYAD-421 SS1
65	laccase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, TDTVTV	*Dichomitus squalens* LYAD-421 SS1
9b	laccase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BSTSTPTADLAVLSVTK	*Lentinus tigrinus*
28b	Laccase (Fragment) OS=Lentinus tigrinus GN=lac1 PE=2 SV=1 - [A5ACB1_9APHY]	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BYSFVLNANZDVDNYWLR, BSTSTPTADLAVLSVTZGKR, BDVVSTGTPZAGDNVTLR	*Lentinus tigrinus*
28c	Laccase (Fragment) OS=Lentinus tigrinus GN=lcc1 oPE=1 SV=1 - [Q5EBY5_9APHY]	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BDVVSTGTPZAGDNVTLR, BLGPRFPLGADATLLNGLGR, BSTSTPTADLAVLSVTK	*Lentinus tigrinus*
28a	Laccase (Fragment) OS=Lentinus tigrinus GN=lcc1 PE=1 SV=1 - [Q5EBY5_9APHY]	oxidoreductase/ multicopper	BLGPRFPLGADATLLNGLGR, BYSFVLNANZDVDNYWLR, BDVVSTGTPZAGDNVTLR, BANPNFGTTGFADGVNSALLR, BSTSTPTADLAVLSVTZGK, TPTAD, TTSPTMT, AAAYA	*Lentinus sp. WR2*
50	Laccase Chain A, Crystal Structure Determination Of A Blue Laccase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BSTSTPTADLAVLSVTK	*Lentinus tigrinus*
26a	Laccase Chain A, Crystal Structure Determination Of A Blue Laccase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BSTSTPTADLAVLSVTK,	*Lentinus tigrinus*
88	Laccase, Chain A, Crystal	oxidoreductase/	BSTSTPTADLAVLSVTK	*Lentinus tigrinus*

Spot No	ID	GROUP	Sequence aligned	Organism
	Structure Determination Of A Blue Laccase	multicopper		
72	LaccaseChain A, Crystal Structure Determination Of A Blue Laccase	oxidoreductase/ multicopper	BANPNFGTTGFADGVNSALLR, BYSFVLNANZDVDNYWLR, BSTSTPTADLAVLSVTK	*Lentinus tigrinus*
64	**lignin peroxidase**	oxidoreductase	BLTFHDALGFSPALTA, FPAGZT, TLALVG	*Bjerkandera adusta*
68	lignin peroxidase	oxidoreductase	BLTFHDALGFSPALTA, BLZSDHLLAR, PFDST	*Bjerkandera adusta*
65	lignin peroxidase-like protein	oxidoreductase	BLTFHDALGFSPALTA, BLZSDFALAR, NDLDAT	*Phlebia chrysocreas*
9a	GMC oxidoreductase (mala s 12 allergen)	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZA, BSAYLDPLPPR, BVYGLANVR, BEVLLSSGAMGSP, ATYNL	*Coprinopsis cinerea* okayama7#130
65	manganese peroxidase	oxidoreductase	BLTFHDALGFSPALTA, BLZSDFALAR	*Ganoderma lucidum*
85	manganese peroxidase	oxidoreductase	BTACEWZSFVNNZAK, BLZSDHDLAR, BLTFHDALG, BLZSAFAAAFRK, FHDALGFSPA	*Schizophyllum* sp. F17
34a	Manganese peroxidase 2 OS=Lenzites gibbosa GN=mnp2 PE=2 SV=1 - [G9I531_9APHY]	oxidoreductase	id by LC-MS	*Lenzites gibbosa*
34b	Mn peroxidase MNP6	oxidoreductase	BLTFHDALGLSPALAAR, BLZSAFAAAFRK, BLZSDFALAR, FHDALGFSPA	*Polyporus brumalis*
36	Mn peroxidase MNP6	oxidoreductase	BITFHDAIGFSPAITA, BIZSAFAAAFR, BIZSDHDIAR	*Polyporus brumalis*
68	Mn peroxidase MNP6	oxidoreductase	BLTFHDALGFSPALTA, BLZSAFAAAFR, PFDST, BLZSDHLLAR	*Polyporus brumalis*
65	peroxidase	oxidoreductase	BLTFHDALGFSPALTA, BLZSDFALAR, TTGFAD	*Coprinellus disseminatus*
9a	GMC oxidoreductase - putative glucose-methanol-choline oxidoreductase	oxidoreductase	BVADASVFPLZFAAHLZAPTYGLAEZA, BSAYLDPLPPR, BVYGLANVR, BEVLLSSGAMGSP, ELGGVVDEDL, TAGSTYA, ADVAV,	*Auricularia delicata* TFB-10046 SS5
32	family S53 protease	protease	BGTSLLFASGDGGVSGSZSZ, NSLGVAGYLDEFAN, CZAYAZLGAR, ADLZTFFZR	*Phanerochaete chrysosporium*
32	subtilisin-like protein	protease	BNSLGVAGYLDEFANR, BGTSLLFASGDGGVSG, BADLZTFFZR, CZAYAZLGAR	*Dichomitus squalens* LYAD-421
33	family S53 protease	protease	BGTSLLFASGDGGVSGSZ, BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, AGVLANL	*Trametes versicolor* FP-101664 SS1
33	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, FASGDGGVSG	*Leucoagaricus gongylophorus*
33	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BGTSLLFASGDGGV, AGVLA	*Trametes versicolor* FP-101664 SS1
33	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG	*Moniliophthora roreri* MCA 2997
36	family S53 protease-like protein	protease	BGTSIIFASGDGGVSGSZ, NSIGVAGYIDEFAN, BAFPDVSAZSVNF, YSGNDDAI	*Postia placenta* Mad-698-R
36	protease s8 tripeptidyl peptidase	protease	BGTSIIFASGDGGVSGSZSZ, NSIGVAGYIDEFAN, BAFPDVSAZ	*Moniliophthora roreri* MCA 2997
36	subtilisin-like protein	protease	BGTSIIFASGDGGVSGSZS, NSIGVAGYIDEFAN, BAFPDVSAZSVNFI	*Trametes versicolor* FP-101664 SS1
39	peptidase M28	protease	BVTVDESZDNVVFGVR, BGGDHEPFLER, BVALVHGLADF	*Punctularia strigosozonata* HHB-11173 SS5
39	subtilisin-like protein	protease	NSLGVAGYLDEFAN, BALPDVAAZ, NPFLFDDGTA	*Punctularia strigosozonata* HHB-11173 SS5
39	tripeptidyl peptidase A	protease	BALPDVAAZADLFR, NSLGVAGYLDEFAN	*Trametes versicolor* FP-101664 SS1
39	Zn-dependent exopeptidase	protease	BVTVDESZDNVVFGVR, BGGDHEPFLER	*Trametes versicolor* FP-101664 SS1

Spot No	ID	GROUP	Sequence aligned	Organism
40	peptidase M28	protease	BFTEPHENFAHZHZDVR, BVTVDESZDNVVFGVR, BFLRGGDHEPFLER, GPGSALVPZAPTAELR	*Punctularia strigosozonata* HHB-11173 SS5
40	Zn-dependent exopeptidase	protease	BFTEPHENFAHZHZDVR, BFLRGGDHEPFLER, BGPGSALVPZAPTAELR, BVTVDESZDNVVFGVR, BLVSFGTR, BAMLAZLDP	*Trametes versicolor* FP-101664 SS1
41	peptidase M28	protease	BFTEPHENFAHZHZDVR, BVTVDESZDNVVFGVR, BFLRGGDHEPFLER, GPGSALVPZAPTAELR	*Punctularia strigosozonata* HHB-11173 SS5
41	Zn-dependent exopeptidase	protease	BFTEPHENFAHZHZDVR, BFLRGGDHEPFLER, BGPGSALVPZAPTAELR, BVTVDESZDNVVFGVR, BDWLAAEMR	*Trametes versicolor* FP-101664 SS1
42	Zn-dependent exopeptidase	protease	BLPTPTNLSDVVATLHGASDANR, BFLRGGDHEPFLER, BVTVDESZDNVVFGVR	*Trametes versicolor* FP-101664 SS1
45	tripeptidyl peptidase A	protease	BALPDVAAZADLFR, HVPGL	*Trametes versicolor* FP-101664 SS1
46	family S53 protease	protease	BGWDPVTGVGTPNF, NSLGVAGYLDEFAN, BAYPDVAA, TAFGDNNS	*Phanerochaete chrysosporium*
46	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, GWDPVTGVGTPNFGK	*Leucoagaricus gongylophorus*
46	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, GWDPVTGVGTPNFGK	*Trametes versicolor* FP-101664 SS1
46	tripeptidyl peptidase A	protease	BGWDPVTGVGTPNFGK, NSLGVAGYLDEFAN, BAYPDVAAZG, GMTH, TAFGDN	*Coniophora puteana* RWD-64-598 SS2
47	tripeptidyl peptidase A	protease	BALPDVAAZADLFR	*Dichomitus squalens* LYAD-421 SS1
50	family S53 protease	protease	BGWDPVTGVGTPNF, NSLGVAGYLDEFAN, BALPDVAA, ADLZTFFZR	*Phanerochaete chrysosporium*
50	serine protease 2	protease	BNSLGVAGYLDEFANR, GWDPVTGVGTPNFGK, BADLZTFFZR, BALPDVAAZ	*Leucoagaricus gongylophorus*
50	subtilisin-like protein	protease	GWDPVTGVGTPNF, BNSLGVAGYLDEFAN, PDVAAZADLF, ADLZTF, GTTGFADG, FGTTGFA	*Stereum hirsutum*
50	tripeptidyl peptidase A	protease	GWDPVTGVGTPNFGK, BALPDVAAZADLFR, NSLGVAGYLDEFAN, DLZTFF	*Dichomitus squalens* LYAD-421 SS1
52	family S53 protease	protease	BAGWDPVTGLGTPNFA, BGTSLLFASGDGGVSGSZSZ, NSLGVAGYLDEFAN, BAFPDVSAZ, TLDFATGV	*Trametes versicolor* FP-101664 SS1
52	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, GWDPVTGLGTPNFAK, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSGS, FATGVGVDPV, NPFLFDDGTA	*Punctularia strigosozonata* HHB-11173 SS5
52	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, BAGWDPVTGLGTPNFAK, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG,	*Moniliophthora roreri* MCA 2997
55	subtilisin-like protein	protease	BGTSLLFASGDGGVSGSZS, DAAAAY	*Trametes versicolor* FP-101664 SS1
61	family S53 protease-like protein	protease	BGTSLLFASGDGGVSGSZS, BAFPDVSAZSVNFL	*Postia placenta* Mad-698-R
61	protease s8 tripeptidyl peptidase	protease	BGTSLLFASGDGGVSGSZSZ, BAYPDVAAZGZGFZVVLGGR	*Moniliophthora roreri* MCA 2997
61	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, BGTSLLFASGDGGVSGS	*Punctularia strigosozonata* HHB-11173 SS5
61	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, BGTSLLFASGDGGVSG	*Moniliophthora roreri* MCA 2997
62	family S53 protease	protease	BGTSLLFASGDGGVSGSZSZ, GWDPVTGLGTPNFA, TSLDLVVD	*Phanerochaete chrysosporium*

Spot No	ID	GROUP	Sequence aligned	Organism
62	Metallo-hydrolase/oxidoreductase	protease	BTNATGSWLYLAGDTAHDMZLLTGERDVAFSFDDA GNMVCAHTD	*Dichomitus squalens* LYAD-421 SS1
62	subtilisin-like protein	protease	BGTSLLFASGDGGVSGSZS, GWDPVTGLGTPNFAK, SSLPCDGA, DAAAAY	*Trametes versicolor* FP-101664 SS1
63	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BADLZTFFZR	*Leucoagaricus gongylophorus*
63	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BADLZTFFZR	*Trametes versicolor* FP-101664 SS1
63	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, ADLZTF	*Moniliophthora roreri* MCA 2997
64	aspartic protease	protease	TSPASEFWGLNZSLR, AGGAT, BALNSTLGG	*Pholiota nameko*
65	acid protease	protease	BSATDTVTVGGVAAK, TLPSAE, SLLFASG	*Trametes versicolor* FP-101664 SS1
65	family S53 protease	protease	BGTSLLFASGDGGVSGSZ, BAYPDVAAZGZGFZVVLGGR	*Trametes versicolor* FP-101664 SS1
65	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, BGTSLLFASGDGGVSGS, GTTGFADGV	*Punctularia strigosozonata* HHB-11173 SS5
68	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, BADLZTFFZR	*Dichomitus squalens* LYAD-421 SS1
70	extracellular elastinolytic metalloproteinase precursor	protease	BDGDLEAGLVLHELSHGLSTR, VLSER	*Cryptococcus gattii* WM276
70	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, GDFVAL	*Trametes versicolor* FP-101664 SS1
71	elastinolytic metalloproteinase mep	protease	BDGDLEAGLVLHELSHGLSTR, BYGFTEVAGNFZZHNFGR, VFDF	*Moniliophthora roreri* MCA 2997
72	peptidase s41 family protein	protease	YATDYDFNHDLYEFTNZLNDGHTR, BVFDFYTFEDYYL, SPPPFZDSTVNLR, BSFPFNETLR, BVNSVFSSYR, BZFVPPADSLAC, LEGZDPYAY, BZNVLTVVSR, BLSGTDYSZR	*Moniliophthora roreri* MCA 2997
73	carboxypeptidase cpds	protease	LVHGLADFLLVAEGTR, BAVGLLNPVFDVYR, FPFN	*Moniliophthora roreri* MCA 2997
73	peptidase family s41 domain-containing protein	protease	TDYDFNHDLYEFTNZLNDGHTR, ZFVPPADSLACLK, FYTFED, PPPFZDSTVNL, ZNVLTVV	*Rhizoctonia solani* AG-1 IA
76	family S53 protease	protease	GTSLLFASGDGGVSGSZS, BAYPDVAAZGZGFZVVLGG, GLSSL	*Auricularia delicata* TFB-10046 SS5
76	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, FASGDGGVSG	*Leucoagaricus gongylophorus*
76	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, BGTSLLFASGDGGVSGS	*Punctularia strigosozonata* HHB-11173 SS5
76	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, BGTSLLFASGDGGVSG	*Moniliophthora roreri* MCA 2997
80	metalloprotease	protease	BYTTWFGSYTSSR, LYLCDVFWETTTTGTDSR, BGGTLLHESSHFT, VYGGSSAK, AALLLLA	*Dichomitus squalens* LYAD-421 SS1
80	peptidyl-Lys metalloendopeptidase	protease	BLYLCDVFWETTTTGTDSR, BYTTWFGSYTSSR, BGGTLLHESSHFT, VYGGSSAK	*Punctularia strigosozonata* HHB-11173 SS5
81	aspergillopepsin	protease	BSVTGTFTVPTPR	*Trametes versicolor* FP-101664 SS1
35a	family S53 protease-like protein	protease	BGTSLLFASGDGGVSGSZ, NSLGVAGYLDEFAN, BAYPDVAAZGZGFZVVLGG, NENDP, ADLZTF, NLCNA	*Postia placenta* Mad-698-R

Spot No	ID	GROUP	Sequence aligned	Organism
35a	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BGTSLLFASGDGGV, BADLZTFFZR	*Trametes versicolor* FP-101664 SS1
35a	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG, DNENDP, ADLZTF	*Moniliophthora roreri* MCA 2997
35b	family S53 protease	protease	BGTSLLFASGDGGVSGSZSZ, NSLGVAGYLDEFAN, TAFGDNNS, ADLZTFFZR	*Phanerochaete chrysosporium*
35b	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BADLZTFFZR, FASGDGGVSG	*Leucoagaricus gongylophorus*
35b	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BGTSLLFASGDGGV, BADLZTFFZR	*Trametes versicolor* FP-101664 SS1
35b	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG, ADLZTF	*Moniliophthora roreri* MCA 2997
35c	family S53 protease-like protein	protease	BAGWDPVTGLGTPNFAK, BGTSLLFASGDGGVSGSQSQR, BAYPDVAAQGQGFQVVLGG, LGVAGYLDEFAN, ADLQTF	*Fomitiporia mediterranea* MF3/22
35c	serine protease 2	protease	BAYPDVAAQGQGFQVVLGGR, BNSLGVAGYLDEFANR, GWDPVTGLGTPNFAK, BADLQTFFQR, FASGDGGVSG	*Leucoagaricus gongylophorus*
35c	subtilisin-like protein	protease	BAYPDVAAQGQGFQVVLGGR, GWDPVTGLGTPNFAK, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSGS, ADLQTFFQR, GTTGFADGV	*Punctularia strigosozonata* HHB-11173 SS5
35c	tripeptidyl peptidase a	protease	BAYPDVAAQGQGFQVVLGGR, BAGWDPVTGLGTPNFAK, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG, ADLQTFFQR	*Moniliophthora roreri* MCA 2997
35d	family S53 protease	protease	BGTSLLFASGDGGVSGSZ, NSLGVAGYLDEFAN, BAYPDVAAZG, ADLZTF, GGLRP	*Stereum hirsutum* FP-91666 SS1
35d	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BADLZTFFZR, FASGDGGVSG	*Leucoagaricus gongylophorus*
35d	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSGS, ADLZTFFZR, GTTGFADGV	*Punctularia strigosozonata* HHB-11173 SS5
35d	tripeptidyl peptidase a	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG, ADLZTF	*Moniliophthora roreri* MCA 2997
37b	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, GWDPVTGLGTPNFAK, NSLGVAGYLDEFAN, BGTSLLFASGDGGVSGS, ADLZTFFZR, GTTGFADGV	*Punctularia strigosozonata* HHB-11173 SS5
38a	metalloprotease MEP2	protease	BVTLSVZDSAGLDNADFATPPDGZSGR, BGLGVNAANHNDDSTVPAGC, BSHPYSTSATTNPL, BNAWLZADANR	*Auricularia delicata* TFB-10046 SS5
38a	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, NSLGVAGYLDEFAN, BGTSLLFASGD, ADLZTFFZR, TVPAGC, GDSYTD, YSGTYGLT	*Punctularia strigosozonata* HHB-11173 SS5
38b	family S53 protease	protease	BGTSLLFASGDGGVSGSZ, BAFPDVSAZSVN, NSLGVAGYLDEFAN, ADLZTF	*Stereum hirsutum* FP-91666 SS1
38b	serine protease 2	protease	BNSLGVAGYLDEFANR, BADLZTFFZR, BAFPDVSAZ, FASGDGGVSG, FSVLNDLR	*Leucoagaricus gongylophorus*
38b	subtilisin-like protein	protease	NSLGVAGYLDEFAN, BGTSLLFASGDGGVSGS, ADLZTFFZR, BAFPDVSAZ, PASCS	*Punctularia strigosozonata* HHB-11173 SS5
38b	tripeptidyl peptidase a	protease	NSLGVAGYLDEFAN, BGTSLLFASGDGGVSG, BAFPDVSAZSVNF, ADLZTF	*Moniliophthora roreri* MCA 2997
38d	serine protease 2	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BADLZTFFZR, PPVLT	*Leucoagaricus gongylophorus*
38d	subtilisin-like protein, partial	protease	BAYPDVAAZGZGFZVVLGGR, BNSLGVAGYLDEFANR, BADLZTFFZR, DDGVAAGE	*Trametes versicolor* FP-101664 SS1
39 c	metalloprotease MEP2	protease	BVTLSVZDSAGLDNADFATPPDGZSGR, BGLGVNAANHNDDSTVPAGC, BSHPYSTSATTNPL, BFGGANFCTLWTAFASR, AWLZADANR,	*Auricularia delicata* TFB-10046 SS5

Spot No	ID	GROUP	Sequence aligned	Organism
			VAFGDSYTDG,	
9a	subtilisin-like protein	protease	BAYPDVAAZGZGFZVVLGGR, BGTSLLFASGDGGVSGS, ADLZTFFZR, GTTGFADGV, ATDLHVL,	*Punctularia strigosozonata HHB-11173 SS5*
63	sure-like protein	secretion nucleotidase	LNYVNSFPVDSVR, BSDVSASVZAZVLZR	*Dichomitus squalens* LYAD-421 SS1
48	HCP-like protein	secretion system effector	BSYYDSPELEGSZDZAEGZGTLEEVEEFDD, DDGTWYMGK	*Dichomitus squalens* LYAD-421 SS1
82	NAD(P)-binding protein		BRLVVTSSCASVLTPSTEPRLFSEENWNDASLAEVK	*Dichomitus squalens* LYAD-421 SS1

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5 - Considerações finais/conclusões

Os fungos são imprescindíveis no desenvolvimento e estabelecimento de processos químicos renováveis para a indústria. A exploração da biodiversidade fúngica tende a contribuir para descobrimento e elucidação de cepas ou enzimas úteis para melhoramento de processos biotecnológicos proporcionando redução de custos e diminuição da poluição gerada pela indústria. Neste panorama, no presente trabalho utilizam-se dois fungos poucos explorados, *L. sordida* e *L. crinitus*, a fim de colaborar no desenvolvimento destas tecnologias.

A lacase de *L. sordida* aqui descrita apresenta potencial para utilização em tratamentos de efluentes da indústria têxtil, minimizando impactos ambientais ocasionados por este ramo industrial. A enzima aqui descrita apresenta uma boa atividade descorante e estabilidade frente a diferentes pHs (3,0 a 5,0) e temperaturas (20 a 50 °C). A atividade descorante de lacase evidencia a quebra não só de ligações azo, responsáveis pela cor, como dos anéis aromáticos estruturais do corante testado. Além disto, a sequência parcial do gene que codifica para lacase do fungo é descrita a fim de caracterizar a enzima para melhorias quanto a sua produção, atividade e estabilidade. Ainda que não tenha sido possível a descrição das regiões reguladoras de transcrição do gene de lacase, o conhecimento destas regiões poderia contribuir para o entendimento da regulação de expressão proteica para o aumento da produção enzimática. Ainda neste ramo, o conhecimento de aminoácidos específicos da sequência proteica permite a possibilidade de mutações pontuais para aumento de atividade e estabilidade enzimáticas.

Acerca dos secretomas fúngicos, o secretoma de *L. crinitus* evidencia uma ampla gama de grupos enzimáticos com diferentes funções. As proteínas identificadas compreendem CAZymes, oxidases, proteases e lipases, todas com aplicação biotecnológica. A identificação de bandas proteicas altamente expressas em extratos solúveis de condições de cultivo com baixo teor de água, além dos ensaios enzimáticos, demonstram uma forte associação da expressão proteica com estímulos ambientais e fontes de energia disponíveis.

Ainda que *L. crinitus* não possua seu genoma descrito, foi possível a identificação de 98 proteínas utilizando-se banco de dados com genomas de organismos relacionados, *L. edodes* e *L. tigrinus*. Ademais, as sequências peptídicas geradas por espectrometria de massas possibilitou a identificação de 171 proteínas por busca de homologia de

sequência entre grupos proteicos de outros fungos, utilizando-se o banco de dados do NCBI. Estes resultados demonstram a possibilidade de caracterização de secretomas de fungos que não possuam genoma descrito.

Por fim, atividades de azoreductase e dye-peroxidase foram detectadas, especialmente, em sobrenadantes de cultivo em fermentação em estado sólido. Embora os métodos de identificação de proteínas, aqui utilizados, não tenham identificado nenhuma dessas enzimas, a presença de atividades específicas para estas sugerem duas possibilidades: (i) essas enzimas são secretadass em baixa quantidade ou (ii) as oxidases secretadas por *L. crinitus* possuem capacidade de ação sobre substratos específicos de azoR e DyP, ou seja, corantes azo e corantes antraquinônicos, respectivamente.

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