UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

MARIANE PALUDETTI ZUBIETA

ASPERGILLUS NIDULANS COMO MODELO PARA MANIPULAÇÃO DE GENES ENVOLVIDOS NO PROCESSO DE *UNFOLDED PROTEIN RESPONSE*

ASPERGILLUS NIDULANS AS A MODEL TO MANIPULATE UNFOLDED PROTEIN RESPONSE-RELATED GENES

Campinas
2018

MARIANE PALUDETTI ZUBIETA

ASPERGILLUS NIDULANS COMO MODELO PARA MANIPULAÇÃO DE GENES ENVOLVIDOS NO PROCESSO DE *UNFOLDED PROTEIN RESPONSE*

ASPERGILLUS NIDULANS AS A MODEL TO MANIPULATE UNFOLDED PROTEIN RESPONSE-RELATED GENES

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Funcional e Molecular, na Área de Bioquímica.

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Functional and Molecular Biology, in the area of Biochemistry.

ESTE ARQUIVO DIGITAL CORRESPONDE A VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA MARIANE PALUDETTI ZUBIETA E ORIENTADA PELO ANDRÉ RICARDO DE LIMA DAMÁSIO

Orientador: Prof. Dr. André Ricardo de Lima Damásio

Coorientador: Prof. Dr. Fábio Marcio Squina

Campinas

2018

Agência(s) de fomento e nº(s) de processo(s): FAPESP, 2014/15403-6

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

Z81a

Zubieta, Mariane Paludetti, 1985-
Aspergillus nidulans as a model to manipulate unfolded protein response-related genes / Mariane Paludetti Zubieta. – Campinas, SP : [s.n.], 2018.

Orientador: André Ricardo de Lima Damásio.
Coorientador: Fábio Márcio Squina.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. *Aspergillus nidulans*. 2. Engenharia genética. 3. Resposta a proteínas não dobradas. 4. Proteínas recombinantes. I. Damásio, André Ricardo de Lima, 1983-. II. Squina, Fábio Márcio. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: *Aspergillus nidulans* como modelo para manipulação de genes envolvidos no processo de *unfolded protein response*
Palavras-chave em inglês:
Aspergillus nidulans
Genetic engineering
Unfolded protein response
Recombinant proteins
Área de concentração: Bioquímica
Titulação: Doutora em Biologia Funcional e Molecular
Banca examinadora:
André Ricardo de Lima Damásio [Orientador]
Iran Malavazi
Gabriela Felix Persinoti
Fernando Segato
Marcelo Mendes Brandão
Data de defesa: 13-07-2018
Programa de Pós-Graduação: Biologia Funcional e Molecular

Campinas, 13 de Julho de 2018

COMISSÃO EXAMINADORA

Prof. Dr. André Ricardo de Lima Damásio

Prof. Dr. Iran Malavazi

Prof. Dr. Fernando Segato

Prof. Dr. Marcelo Mendes Brandão

Dra. Gabriela Felix Persinoti

Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

AGRADECIMENTOS

A elaboração deste trabalho não teria sido possível sem a colaboração, estímulo e empenho de diversas pessoas. Gostaria de expressar toda a minha gratidão e apreço a todos aqueles que contribuíram para que este trabalho se tornasse uma realidade.

Em primeiro lugar agradeço minha mãe, minha vó, minhas irmãs, meus lindos sobrinhos e ao Nel que sempre me apoiaram e tenho certeza que continuarão me apoiando em todas as minhas escolhas.

Ao André Damásio por toda ajuda, atenção e confiança. Sou muito grata pela oportunidade de ter feito parte da sua equipe e por ser meu exemplo de liderança e de gestão de pessoas.

Ao pessoal do LEBIMO pelo apoio, aprendizado, companheirismo e pela ótima convivência diária que muitas vezes tornava o dia mais alegre diante das muitas dificuldades da biologia molecular.

Aos meus trutas Marcelo, Jaque e Cesar pela amizade, por sempre estarem dispostos a me ajudar, pelo bom humor, pelas conversas e pela paciência em me ensinar muitas coisas das quais eu mesmo não teria...rs. Jamais esquecerei tudo o que fizeram por mim!

À Thamyzita por ser uma amiga de longa data e por ter sido responsável pela minha vinda a Campinas e, consequentemente, tornando esse trabalho possível.

À Stillwater Family, especialmente Bia, Rolf e Ju, por serem tãos especiais e compartilharem a leveza da vida comigo pelos meses que morei em Stillwater, OK.

Ao pessoal do CTBE que me recebeu muito bem. Fiz muitos amigos por lá que levarei para sempre comigo.

E, finalmente, à CAPES e a Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela concessão da bolsa de Doutorado Regular (Processo 2014/15403-6).

Não te deixes destruir...
Ajuntando novas pedras
e construindo novos poemas.
Recria tua vida, sempre, sempre.
Remove pedras e planta roseiras e faz doces. Recomeça.

- Cora Coralina -

RESUMO

Em eucariotos, o *unfolded protein response* (UPR) regula positivamente genes responsáveis por restaurar a homeostase no retículo endoplasmático (RE) durante o acúmulo de proteínas enoveladas incorretamente. A homeostase é restaurada devido à ativação de genes relacionados à via secretória, como aqueles que codificam chaperonas e foldases, o que aumenta, por sua vez, a capacidade de enovelamento de proteínas pelo RE. Alguns sistemas de produção de proteínas heterólogas têm sido desenvolvidos com a super-expressão individual de chaperonas e foldases nas células. Entretanto, a taxa de sucesso com a aplicação dessa estratégia é baixa. Estudos têm mostrado que a manipulação de genes que respondem ao UPR em linhagens fúngicas podem levar ao aumento na produção de proteínas de interesse. Neste trabalho, inicialmente estudamos e identificamos o perfil de proteínas que são recrutadas para expressar e produzir proteínas heterólogas em *A. nidulans* por espectrometria de massas. Posteriormente, identificamos nas cepas de *A. nidulans* os genes que respondem ao tratamento com ditiotreitol e tunicamicina, drogas que induzem o UPR. Finalmente, selecionamos 12 genes associados à via de secreção em *A. nidulans*, os quais foram deletados em cepas recombinantes de *A. nidulans,* uma que secreta xilanase homóloga (*xlnE*, 5B3 strain) e outra xilanase heteróloga (*tpet_0854*, 854 strain). A deleção de uma ciclofilina e de uma chaperona molecular Hsp40 resultou no aumento de 125 e 170% vezes na secreção de *xlnE*, respectivamente. Da mesma forma, a deleção de uma tiorredoxina e uma manosiltransferase também aumentou, ainda que em níveis mais baixos, a secreção de *xlnE*. Os resultados ainda mostraram que a secreção de proteínas totais diminuiu nessas cepas delatadas. Considerando os resultados, essa abordagem demonstrou aumento expressivo na produção de enzimas-alvo, sugerindo que a manosiltransferase, a chaperona Hsp40, a ciclofilina e a tiorredoxina codificadas pelos genes deletados desempenham um papel importante na regulação da produção de proteína em *A. nidulans*. Entretanto, ainda não entendemos o mecanismo envolvido no aumento da secreção de *xlnE* após as deleções. Sugerimos que a maior produção de enzimas nas cepas deletadas possa estar relacionada à ativação do UPR e também a um "afrouxamento" no rigor do enovelamento de proteínas pela célula, resultando em um controle de qualidade mais brando e maior secreção de proteínas para o meio extracelular.

ABSTRACT

In eukaryotes, the unfolded protein response (UPR) positively regulates genes responsible for restoring homeostasis in the endoplasmic reticulum (ER) during accumulation of misfolded proteins. The homeostasis is restored due to the activation of genes related to proteins secretion such as those encoding for chaperones and foldases, which, in turn, increase protein folding capacity by RE. In fact, some systems for heterologous protein production have been developed by the individual overexpression of chaperones and foldases in the hosting cells. However, the success rate of this strategy usually is quite low. Studies on the manipulation of genes that respond to UPR in fungal strains are interesting aiming a higher production of proteins. In this work, we initially identified the profile of proteins that are recruited to express and produce heterologous proteins in *A. nidulans* by mass spectrometry. Subsequently, we proceeded the identification of genes that respond to UPR-activating chemicals such as dithiothreitol and tunicamycin. Finally, we selected 12 genes with a predicted function in the *A. nidulans* secretion pathway. These 12 genes were deleted in an *A. nidulans* recombinant strain producing an homologous xylanase (*xlnE*, 5B3 strain) and another recombinant strain producing an heterologous xylanase (*tpet_0854*, 854 strain). The deletion of cyclophilin and a molecular chaperone Hsp40 resulted in an increase around 125 and 170 % in the *xlnE* activity, respectively. Similarly, the deletion of thioredoxin and glycosyl phosphatidyl inositol-mannosyltransferase also increased the *xlnE* secretion even at lower levels. The results also showed a decreased production of total proteins production in these deleted strains. Thus, our results suggest that proteins such as glycosyl phosphatidyl inositol-mannosyltransferase, chaperone Hsp40, cyclophilin and thioredoxin play an important role in the regulation of proteins production by *A. nidulans*. However, we still do not understand the mechanism involved in increased secretion of 5B3 after the deletions. We suggest that the increased production of enzymes in the deleted strains is related to the activation of the UPR and a "less stringent" protein folding by the cell, resulting in a mild quality control and higher secretion of proteins into the extracellular medium.

SUMÁRIO

CAPÍTULO 1 - Introdução

Os ciclos do carbono e do nitrogênio têm sido afetados constantemente pelas atividades humanas. A exploração dos recursos da Terra tem aumentado a cada dia, dando origem a uma maior preocupação em relação ao esgotamento dos recursos naturais e a uma rápida geração de resíduos. A exploração de combustíveis fósseis é de especial relevância para o ciclo do carbono, uma vez que tem sido a principal fonte de energia e de produtos químicos em todo o mundo e, provavelmente, o principal responsável pelo aumento das emissões de CO_2 na atmosfera. Portanto, é necessário encontrar fontes alternativas de combustíveis e de produtos químicos para melhorar os processos que envolvem o uso dos recursos naturais, a fim de produzir mais a partir de menos, e diminuir a quantidade de resíduos através da conversão em produtos de valor agregado (FERREIRA et al., 2016; MOHAPATA et al., 2017). Como alternativa, a biomassa lignocelulósica, formada de celulose, hemicelulose e lignina, pode ser utilizada pois é o composto renovável mais abundante na Terra, além de ser relativamente barata. Os hidrolisados das paredes celulares vegetais são predominantemente utilizados para a produção de biocombustíveis. Como os biocombustíveis nem sempre são competitivos em termos de custos com os combustíveis fósseis, a glicose é cada vez mais utilizada como material de partida para a síntese de outros produtos químicos como butanol, acetoína, succinato, xilitol, lactato, dentre outros (GUPTA et al., 2016).

Para liberar os açúcares presentes na estrutura vegetal, é necessária a ação simultânea de várias enzimas para desconstruir os polissacarídeos em monômeros. Essas enzimas são chamadas enzima ativa em carboidratos (*Carbohydrate-Active Enzymes* - *CAZy*) e são classificadas com base na comparação de suas sequências de aminoácidos, estrutura tridimensional e mecanismos catalíticos em 5 famílias que estão depositadas em um banco de dados chamado de *CAZy* database (http://www.cazy.org/). As famílias são glicosil hidrolases (GHs), carboidrato esterases (CEs), polissacarídeo liases (PLs), glicosiltransferases (GTs) e enzimas com atividades auxiliares (AA) (LOMBARD et al., 2014).

Para desconstruir os polímeros de celulose presentes na biomassa vegetal são necessárias celulases clássicas, como endoglucanases, exoglucanases e β-glucosidases (BGLs). As β-1,4-endoglucanases (EGs) cliva as cadeias de celulose para liberar os oligossacarídeos. Exoglucanases ou celobiohidrolases (CBHs) liberam celobiose do final das cadeias de celulose. Os dois tipos de celobiohidrolases, CBHI e CBHII, degradam a celulose tanto da extremidade redutora como não redutora, respectivamente. As BGLs liberam os monômeros de glicose a partir dos oligossacarídeos menores. Existem outros dois grupos de enzimas que apresentam mecanismo oxidativo e que auxiliam as celulases na desconstrução

da biomassa, são as celobioses desidrogenases (CDHs) e as monooxigenases líticas de polissacarídeos (LPMOs). Para a desconstrução da hemicelulose, um conjunto específico de CAZyme é necessário pois a hemicelulose corresponde a um polissacarídeo ramificado e complexo formado principalmente por D-xilose e pequenas quantidades de L-arabinose, D-glicose, D-manose, D-galactose, ácido glucurônico e ácido manurônico. O esqueleto principal de xilose é clivado por β-1,4-endoxilanases (XLNs) em oligômeros mais curtos. As β-1,4-xilosidases (BXLs) liberam D-xilose da xilobiose e também do terminal não redutor de xilooligossacarídeos. O esqueleto de xiloglucano e de β-glucano, cuja estrutura é semelhante à da celulose pois são compostos de glicose, podem ser hidrolisados por EGs, CBHs e BGLs. Outras enzimas como β-1,4-endomannanase, β-1,4-manosidase, α-arabinofuranosidase, α-fucosidase, α-1,4-galactosidase, β-1,4-Galactosidase dentre outras, também atuam na hemicelulose. Outras CAZymes são necessárias para a completa degradação da parede celular vegetal como endopoligalacturonases (PGAs), exopoligalacturonases (PGXs), xilogalacturonana hidrolases (XGHs), exoramnogalacturonase (RHX), endoramnogalacturonase (RHG), ramnogalacturonano ramnohidrolase (RGXB), α-ramnosidase (RHA), pectina liase (PEL), pectato liase (PLY), ramnogalacturonano liase, dentre outras enzimas (RYTIOJA et al., 2014). Na **Figura 1** está representada uma visão geral das enzimas conhecidas que atuam na desconstrução da parede celular vegetal e os substratos em que atuam.

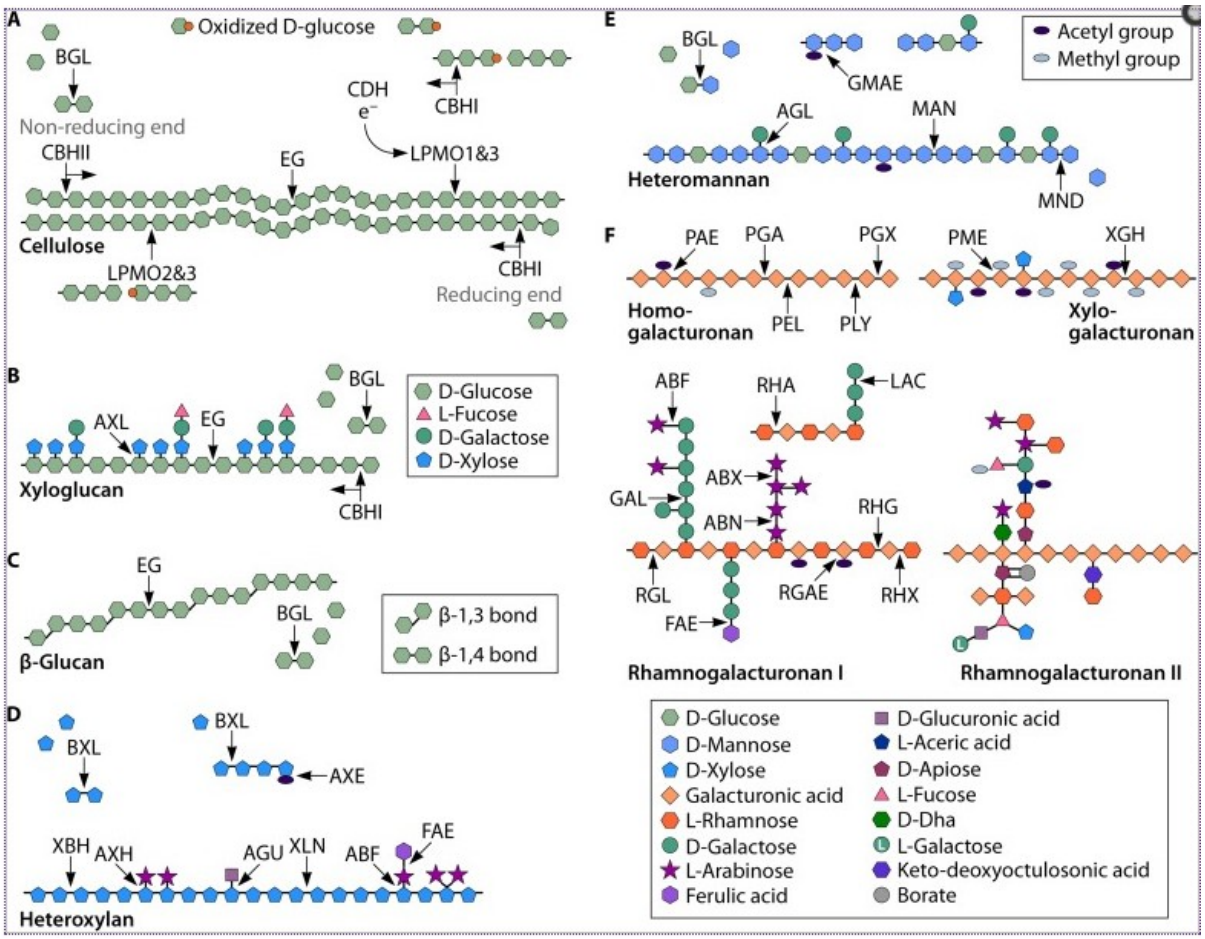


Figura 1. Representação esquemática dos polissacarídeos presentes na parede celular vegetal e enzimas que os degradam. (A) Celulose; (B) Xiloglucano; (C) β-Glucano; (D) Heteroxilano; (E) Heteromanana; (F) Pectina. As enzimas são: EG - β-1,4-Endoglucanase; CBHI – Celobiohidrolase; CBHII – Celobiohidrolase; BGL - β-Glucosidase, CDH - Celobiose desidrogenase; LPMO - Monooxigenase lítica de polissacarídeo; XLN - β-Endoxilanase; XBH – Xilobiohidrolase; BXL - β-Xilosidase; MAN - β-Endomananase; MND- β-Manosidase; LAC - β-Galactosidase; AGL - α-Galactosidase; ABF - α-Arabinofuranosidase; GMAE - Galactomanano acetil esterase; XEG - Xiloglucano β-endoglucanase; AXL - α-Xilosidase; AFC - α-Fucosidase; AXH - Arabinoxilano arabinofuranohidrolase / arabinofuranosidase ; AGU - α-Glucuronidase; AXE - Acetil xilano esterase; FAE - Feruloil esterase; PGA - Endopoligalacturonases; PGX – Exopoligalacturonases; XGH - Xilogalacturonano hidrolase; RHG – Endoramnogalacturonase; RHX – Exoramnogalacturonase; RGXB - Ramnogalacturonano ramnohidrolase; RHA - α-Ramnosidase; ABN – Endoarabinanase; ABX – Exoarabinanase; GAL - β-Endogalactanase; PEL - Pectina liase; PLY - Pectato liase; RGL - Ramnogalacturonano liase; PME - Pectina metil esterase; PAE - Pectina acetil esterase; RGAE - Ramnogalacturonano acetil esterase. Essa figura foi extraída de RYTIOJA et al., 2014.

Nesse sentido, os fungos filamentosos tem sido alvo de intenso estudo, pois são organismos capazes de secretar naturalmente uma grande quantidade e variedade de enzimas extracelulares. Os fungos vêm sendo utilizados, desde tempos mais antigos, em uma grande variedade de processos para obtenção de produtos como bebidas alcoólicas e pão. Mas

inicialmente, não se sabia que as enzimas eram responsáveis pela conversão e obtenção de produtos. Um dos trabalhos pioneiros publicado na área de enzimas fúngicas ocorreu em 1857, em que o químico francês Louis Pasteur demonstrou, pela primeira vez, que a fermentação alcoólica de bebidas ocorria apenas na presença de leveduras vivas (PASTEUR, 1857). E em 1877, o termo enzima foi cunhado por Wilhelm Friedrich Kiihne após descrever um "fermento sem forma e desorganizado" isolado do pâncreas e que digere proteínas (KUHNE, 1876). Esses trabalhos revolucionaram a ciência pois permitiu a exploração e obtenção de novos produtos.

Atualmente existe uma grande diversidade de enzimas fúngicas comercialmente disponíveis e suas aplicações se estendem aos mais diversos setores da indústria (SINGH et al., 2016). A partir da década de 80, a engenharia genética começou a ser aplicada na indústria de enzimas para melhorar sua produção e desempenho. Em 1988, Novozymes A/S ao observar a capacidade de fungos do gênero *Aspergillus* em apresentar naturalmente um bom sistema de produção de proteínas, desenvolveu cepas de *Aspergillus oryzae* que produziam proteinase aspártica oriunda de *Rhizomucor miehei* (CHRISTENSEN et al., 1988).

Aspergillus são classificados dentro do reino *Fungi*, um grande grupo dentro do super reino *Eukarya* que também incluem leveduras, basidiomicetos e demais fungos. Dentro desse super reino, *Aspergillus* se classifica dentro do sub-reino *Dikarya*, filo Ascomycota, e subfilo Pezizomycotina que incluem outros gêneros como *Penicillium*, *Fusarium*, *Cladosporium*, *Eurotium*, *Paecilomyces* e *Curvularia.* O gênero *Aspergillus* compreende cerca de 185 espécies e são amplamente distribuídos em todo o mundo, embora mais frequentemente encontrado em regiões mais quentes. Pertencente à família *Trichocomaceae*, e a ordem *Eurotiales*, são comumente isolados do solo, dos detritos vegetais e de outros ambientes (HIBBETT et al., 2007). Espécies de *Aspergillus* apresentam características ideais para serem utilizados na indústria como "*Generally Recognized as Safe*" (em inglês status "GRAS") e bom conhecimento a respeito do cultivo, o que permite a fácil separação da biomassa e altos rendimentos no crescimento (FLEIBNER; DERSCH, 2010).

Para expressar uma proteína de maneira eficiente, existem obstáculos que devem ser transpostos em fungos. Em geral, um processo com alto rendimento de produção de uma determinada proteína requer elevadas quantidades de transcrição e tradução do gene de interesse, direcionamento correto da proteína para a via de secreção (se for desejada secreção), enovelamento e modificações pós-traducionais corretas, secreção eficiente e pouca ou nenhuma degradação do produto no meio extracelular (DAVY; KILDEGAARD; ANDERSEN, 2017). Para esses passos serem alcançados, estratégias tem sido desenvolvidas

para otimizar tanto a quantidade quanto a qualidade dessas enzimas como, seleção ou engenharia genética de promotores que são vitais para o alto nível de transcrito, uso de códons preferenciais e remoção de íntrons, seleção ou engenharia genética do peptídeo sinal que irá garantir apropriado tráfego através do retículo endoplasmático e sua secreção e, deleção de proteases que poderiam degradar os produtos (BLOUNT et al., 2012; LANDOWSKI et al., 2015; ROONGSAWANG et al., 2016; VU et al., 2015). Algumas estratégias realizadas para obtenção de proteínas recombinantes estão descritas na **Tabela 1**.

Apesar da grande quantidade de trabalhos dedicados à esse tema e do desenvolvimento rápido de novas técnicas para obtenção de enzimas, não houveram grandes avanços publicados nas últimas décadas no sentido de impulsionar os rendimentos de produtos biotecnológicos produzidos por fungos. Como exemplo de sucesso, há 30 anos uma cepa hipersecretora foi desenvolvida por mutagênese aleatória, *Trichoderma reesei* RUT-C30 (MONTENECOURT; EVELEIGH, 1979). Para obter essa cepa, três rodadas de mutagênese foram realizadas, sendo duas delas de exposição à radiação UV e uma delas de tratamento com nitroguanidina. A exata constituição genética dessa cepa permaneceu desconhecida até que as sequências do seu genoma estivessem disponíveis, que revelaram uma série de mudanças na composição genética em comparação à linhagem parental, QM6a, e explicaram parcialmente algumas características fenotípicas e metabólicas observadas (SEIDL et al., 2008; PETERSON; NEVALAINEN, 2012). Contudo, os fatores responsáveis pela elevada secreção de proteína ainda permanecem desconhecidos.

Outras cepas hiperprodutoras têm sido desenvolvidas por empresas ao longo dos anos, entretanto as informações sobre a evolução genômica dessas cepas que passaram por várias melhorias racionais e não racionais, não são usualmente disponibilizadas à academia devido à confidencialidade empresarial. Assim, a indústria tornou-se um repositório de conhecimento, que tem potencial para transformar nossa compreensão fundamental do metabolismo, crescimento e desenvolvimento de fungos e que poderia acelerar o desenvolvimento de produtos (MEYER et al., 2016).

Tabela 1. Estratégias utilizadas para obter produção de proteínas recombinantes em fungos filamentosos e leveduras

HOSPEDEIRO	ENZIMA ALVO	MODIFICAÇÃO	AUMENTO DA PRODUÇÃO	TIPO DE EXPRESSÃO	REFERÊNCIA
A. oryzae	- Quimosina bovina - Lisozima humana	Deleção do gene *Aovps10* que codifica uma *vacuolar protein sorting*	- 3 vezes - 2,2 vezes	Heteróloga	(YOON et al., 2010)
A. oryzae	Quimosina bovina	Silenciamento de 3 α-amilases por RNA de interferência	20 a 57%	Heteróloga	(NEMOTO; MARUYAMA; KITAMOTO, 2009)
A. oryzae	Lisozima humana	Deleção do gene *aut1* que codifica uma proteína citoplasmática hipotética que contém um domínio folha-beta	2 vezes	Heteróloga	(JIN et al., 2016)
A. niger	Lacase	Modificação na região 5' flanqueadora do gene *glaA* que codifica uma glucoamilase	Até 500%	Homóloga	(TAMAYO-RAMOS et al., 2013)
A. oryzae	Quimosina bovina	Deleção dos genes *aoVip36* e *aoEmp47* que codificam receptores de lectinas,	2 vezes	Heteróloga	(HOANG; MARUYAMA; KITAMOTO, 2015)
A. oryzae	Lisozima humana	Deleção dos genes *tppA* e *pepE* que codificam proteases	1,9 vezes	Heteróloga	(NEMOTO et al., 2009)
Ogataea thermomethanolica	- Xilanase - Fitase	Super expressão dos genes *otBiP*, *otCNE1* e *otPDI*. que codificam chaperonas residentes no retículo endoplasmático	- 1,6, 1,3 e 1,7 vezes, respectivamente. - 1,2 vezes durante a super expressão do gene *otBiP*	Heteróloga	(ROONGSAWANG et al., 2016b)
Pichia pastoris	Endoxilanase	Otimização de códons e super expressão de hemoglobina de *Vitreoscilla*	35%	Heteróloga	(WANG; LI; LIU, 2016)
Pichia pastoris	α-Galactosidase	Substituição do peptídeo sinal, otimização do sítio de clivagem do peptídeo sinal e otimização de códon	12 vezes	Heteróloga	(ZHENG et al., 2016)
Saccharomyces cerevisiae	Glicoamilase	Otimização de códons e super expressão de *pdi1*	2 vezes	Heteróloga	(CRIPWELL; ROSE; VAN ZYL, 2017)
Saccharomyces cerevisiae	Amilase	- Deleção do gene *end3* que participa da endocitose - Deleção do gene *rvs161* que participa da endocitose	- 2 vezes - 12 vezes	Heteróloga	(RODRÍGUEZ-LIMAS; TANNENBAUM; TYO, 2015)
Saccharomyces	β-glicosidase	- Super expressão de componentes do complexo que	- 44% de β-glicosidase,	Heteróloga	(TANG et al., 2015)

cerevisiae	Endoglicanase α-amilase	reconhece a partícula sinal *srp14p* e *srp54p*	18% de endoglicanase e 38% de α-amilase - 56% de β-glicosidase, 16% de endoglicanase e 52% de α-amilase		
Saccharomyces cerevisiae	Endoglicanase	Super expressão dos genes *sec12*, *sec13*, *erv25* e *bos1* que participam do tráfego de vesículas	11, 17, 31 e 22%, respectivamente	Heteróloga	(TANG et al., 2017)
T. reesei	Endoglucanase	Deleção do gene *spw* que codifica uma protease alcalina	8%	Heteróloga	(ZHANG et al., 2014)
T. reesei	Endoglucanase I	- Uso de promotor e terminador de uma celobiohidrolase altamente expressa em *T. reesei* - Expressão de uma celobiohidrolase I mutada de *T. reesei* que é naturalmente bem secretada	- 1,9 vezes - 4 vezes	Homóloga	(HARKKI et al., 1991)
T. reesei	Cadeia pesada do anticorpo murine anti-2-phenyloxazolone IgGI	Uso de promotor e terminador de uma celobiohidrolase (*cbh1*) altamente expressa em *T. reesei*, e fusão com celobiohidrolase I de *T. reesei*	50 vezes	Heteróloga	(NYYSSÖNEN et al., 1993)
T. reesei	Xi~~y~~lanase	Uso de promotor dos genes que codificam piruvato descarboxilase e enolase	- 83% - 82%	Homóloga	(LI et al., 2012)
T. reesei	Glicose oxidase	Super expressão dos genes que codificam uma v-SNARE	2,2 vezes	Heteróloga	(WU et al., 2017)

Essas tecnologias bem-sucedidas de melhoramento de cepas resultaram em mais dúvidas que soluções, uma vez que podem resultar em aumento de uma determinada proteína, mas não são necessariamente aplicáveis a outras proteínas. Por exemplo, a produção de proteínas heterólogas em *T. reesei* RUT-C30 tem apresentado rendimentos consideravelmente menores em relação às proteínas nativas (CHERRY; FIDANTSEF, 2003).

Evidências experimentais têm sugerido que a maior parte das proteínas heterólogas produzidas por células fúngicas permanece aprisionada na via secretória devido a erros no processamento, na modificação ou no enovelamento, o que resulta na degradação das proteínas pelo sistema de controle de qualidade celular. Em cepas que produzem proteínas heterólogas foram realizadas análises e foi mostrado que a célula responde ativando diferentes conjuntos de genes de vias regulatórias relacionados ao controle de qualidade chamado de *unfolded protein response* (UPR) (PAKULA et al., 2003).

O UPR corresponde a uma resposta adaptativa que tem como objetivo restaurar a homeostase celular desencadeada por um acúmulo de proteínas mal enoveladas no retículo endoplasmático (RE). De acordo com múltiplos sinais ambientais, celulares e também superexpressão de genes de interesse, a quantidade de proteínas que passa pelo RE sofre flutuações. Nessas condições, proteínas mal enoveladas começam a se acumular no RE, prejudicando as funções celulares e podendo desencadear a morte celular precocemente. Então, a capacidade de enovelamento do RE é readaptada devido a reprogramação de genes responsáveis por ativar mecanismos que auxiliam a restaurar ou melhorar as funções do RE (MOORE; HOLLIEN, 2012; RON; WALTER, 2007).

O UPR tem três vias de sinalização que operam paralelamente. Cada via é desencadeada por uma proteína transmembrana residente no RE. A proteína transmembrana IRE1 (Inositol Requiring Enzyme 1) desencadeia uma via responsável pelo UPR em eucariotos inferiores, como os fungos. Em metazoários, existem outras duas proteínas transmembranas, além de IRE1, que foram adquiridas no decorrer da evolução e que atuam nas outras duas vias de UPR. As proteínas são: PERK (Protein Kinase RNA-like Endoplasmic Reticulum Kinase) e ATF6 (Activating Transcription Factor 6). A IRE1 é uma proteína monomérica e bifuncional (cinase/endorribonuclease) que possui porção luminal e citoplasmática). Na ausência de UPR, essa proteína permanece inativa devido à ligação com a chaperona KAR2 (BIP em mamíferos e em fungos filamentosos) no domínio luminal (GROOTJANS et al., 2016).

A ativação de IRE1 durante o UPR tem sido explicada por diferentes modelos propostos. De acordo com o modelo mais recente de ativação proposto, IRE1 encontra-se em equilíbrio dinâmico com KAR2 e algumas proteínas mal enoveladas que existem no lúmen do

RE. Quando há um acúmulo de proteínas mal enoveladas, o equilíbrio é afetado devido à competição pela ligação a IRE1 entre essas proteínas e a chaperona. A perturbação do equilíbrio faz com que a chaperona se dissocie, permitindo que IRE1 torne-se ativa em virtude da ligação com as proteínas mal enoveladas acumuladas. Portanto, na célula podem ser encontradas IRE1s em três estados: inativas ligadas a chaperona, inativas livres e ativas ligadas a proteínas mal enoveladas (PINCUS et al., 2010). Em *S. cerevisiae*, foi demonstrado que resíduos básicos e hidrofóbicos de peptídeos não enovelados se ligam diretamente a um sulco de IRE1α, e essa ligação é suficiente para induzir o UPR (GARDNER; WALTER, 2011). Atualmente, não está claro o que ativa a IRE1, se a dissociação da chaperona ou a ligação com as proteínas mal desenoveladas.

Após a ativação de IRE1, a dimerização é desencadeada e dois domínios quinases citoplasmáticos são justapostos, facilitando a trans-autofosforilação. Os domínios quinases fosforilados justapostos se ligam a nucleotídeos, o que resulta em uma configuração de dímero apto a atuar como uma ribonuclease no domínio citoplasmático (LEE et al., 2008). Posteriormente, a IRE1 reconhece uma estrutura em loop no transcrito do gene que codifica o fator transcricional zíper de leucina HAC1 (*hacA*^{u}, forma unduced ou non-spliced) (MULDER et al. 2004). Após o reconhecimento, o domínio de ribonuclease de IRE1 remove um íntron sem auxílio do spliceossomo. O tamanho do íntron pode variar de acordo com a espécie. Em *S. cerevisiae* e em *Caenorhabditis elegans*, um íntron de 252 e 26 nucleotídeos do transcrito do gene *hac1* e *xbp1* (*xbp1* é homólogo de *hac1*), respectivamente, é removido (CALFON et al., 2002; COX; WALTER, 1996). Nos transcritos dos genes *hac1* de *Trichoderma reesei* e *hacA* de *A. nidulans*, um íntron de 20 nucleotídeos é removido (MULDER et al., 2004). Após a remoção do íntron, os éxons são unidos por uma tRNA ligase e traduzidos, gerando o fator transcricional ativo (*hacA*^{i}, forma induced ou spliced). Esse fator se liga a UPREs, regiões encontradas nos promotores dos genes alvo do UPR, e ativa a transcrição desses genes (MULDER et al., 2004).

Evidências experimentais têm mostrado que o UPR tem papel fundamental em múltiplos processos fisiológicos que vão além do enovelamento de proteínas realizado por chaperonas e foldases. Durante a fase de infecção do fungo patogênico *Ustilago maydis* no hospedeiro, ocorre secreção de proteínas efetoras para suprimir a resposta da planta em defesa à infeção pelo fungo e redirecionamento do fluxo de nutrientes. Muitas dessas proteínas efetoras são glicosiladas, o que gera um grande estresse no RE e na maquinaria de secreção (HEIMEL et al., 2013). Outro processo regulado por UPR é a autofagia, que é um processo catabólico que fornece proteínas, componentes citoplasmáticos e organelas aos lisossomos

para degradação e reciclagem. Na presença de estresse de RE, o UPR é ativado e ativa genes de autofagia. Em eucariotos superiores foi descrito que a autofagia auxilia na recuperação da célula para a homeostase, pois auxilia na remoção de proteínas não enoveladas e remove regiões do RE que foram expandidas para alojar maior quantidade de proteínas durante o estresse (HØYER-HANSEN; JÄÄTTELÄ, 2007). Entretanto, a participação da autofagia para superar o estresse e recuperar a homeostase durante o UPR é controverso em fungos filamentosos. Em *A. niger*, a autofagia não auxiliou na remoção de proteínas desenoveladas no retículo durante o estresse (BURGGRAAF; RAM, 2016). Já em *A. oryzae*, a autofagia foi responsável pela entrega das proteínas desenoveladas acumuladas no retículo para vacúolos encaminhados à degradação (KIMURA et al., 2011).

Essas descrições refletem a complexa rede de interações entre o UPR e outras vias sinalizadoras presentes nas células. Dessa forma, a lista de genes conhecidos regulados pelo UPR tem aumentado a cada ano. Assim, novas pesquisas surgiram por meio de "ômicas" tentando descrever todos os genes e vias regulados por UPR na célula e propor genes-alvo que possam ser manipulados afim de aumentar a secreção de proteínas de interesse. Estas informações provêm de estudos de genoma, vias metabólicas e fluxos, dados de transcriptômica e proteômica (SIEBER et al., 2016; VOLMER et al., 2013; ZHOU et al., 2016). Entre estes, os perfis de transcrição e proteínas podem ser particularmente úteis em proporcionar uma visão geral de uma via não muito bem caracterizada em uma espécie que apresenta genes com funções desconhecidas, tais como *Aspergillus*. Dados de transcriptômica para cepas de *Aspergillus niger* expressando a forma ativa do fator de transcrição *hacA* mostraram genes ligados a processos de RE, tais como reconhecimento e clivagem de peptídeos sinal, translocação, enovelamento e controle de qualidade de proteína. Comparação entre transcriptomas de *A. niger* obtidos a partir de indução química (micélio tratado com ditiotreitol (DTT) e tunicamicina (TM)) e biológica (expressão heteróloga do ativador de plasminogênio tecidual (t-PA)) de UPR, além da expressão de *hacA* ativo, mostrou que 94 genes foram comumente induzidos, sendo estes relacionados principalmente ao enovelamento de proteínas, translocação/complexo da peptidase sinal, glicosilação, tráfico de vesículas e metabolismo de lipídeos (CARVALHO et al., 2012). Análises de transcriptômica em linhagens de *A. oryzae* com produção de amilase heteróloga e UPR ativo mostraram que os genes ativados estavam relacionados a glicosilação, biossíntese de glicosil fosfatidil inositol (GPI), biossíntese do dolicol, translocação, ERAD, COPI, COPII, enovelamento, parede celular, LDSV (low density secretory vesicle) e HDSV (high density secretory vesicle) (LIU et al., 2014). A maioria dos estudos com UPR foram realizados a partir do tratamento do

micélio com as drogas que ativam o UPR. Dentre os poucos trabalhos de proteômica descritos, foi realizado uma análise de proteômica de uma cepa de *Pichia pastoris* que produz uma xilanase de *Bacillus halodurans*. Muito processos celulares importantes foram alterados como metabolismo de carbono, resposta ao estresse e enovelamento de proteínas (LIN et al., 2013).

Como descrito, o UPR é ativado em cepas que produzem enzimas heterólogas para recuperar a homeostase. Assim, os genes dessa via se tornam um importante alvo para manipulação genética com o objetivo de melhorar a produção. A super-expressão do gene que codifica a chaperona BIP e da foldase dissulfito isomerase (PDI) em *S. cerevisiae*, ambos ativados durante o UPR, aumentou a produção de anticorpos humanos em cerca de 2 a 8 vezes, respectivamente (SHUSTA et al., 1998). Em *A. awamori*, a expressão constitutiva do gene *hacA* permitiu um aumento de 2,8 vezes na produção de quimosina bovina e de 7,6 vezes de lacase de *Trametes versicolor* (VALKONEN et al., 2003). A super expressão dos genes *bip1* e *hac1* em cepas de *T. reseei* produzindo glicose oxidase de *A. niger*, aumentou a produção da enzima em 1,5 vezes e 1,8 vezes, respectivamente (WU et al., 2017). Entretanto, efeitos não satisfatórios na produção de proteínas heterólogas também têm sido observados, como em *P. pastoris* em que a super-expressão de *kar2* levou a diminuição da secreção de lipase e a super-expressão do gene que codifica PDI em *A. niger* não aumentou a produção de lisozima de clara de ovo de galinha (NGIAM et al., 2000). Em *A. nidulans*, um gene que codifica uma proteína responsável pela glicosilação foi super-expresso, entretanto, não foi observado aumento da produção de duas enzimas alvo: glicoamilase e invertase (PERLIŃSKA-LENART et al., 2005).

O sucesso da obtenção de sistemas de produção de enzimas recombinantes em *A. nidulans* vai muito além da super-expressão de genes como as chaperonas e foldases, e requer um estudo com um número maior de genes controlados pela complexa regulação do UPR. Dessa forma, o objetivo deste trabalho foi inicialmente estudar e identificar o perfil de proteínas que são recrutadas para expressar e produzir proteínas heterólogas em *A. nidulans* utilizando dados de proteômica e, posteriormente, explorar os genes que respondem ao tratamento de drogas que induzem o UPR em cepas de *A. nidulans* utilizando dados de transcriptômica. Esses achados fornecerão um maior conhecimento dos mecanismos envolvidos na alta secreção de proteínas recombinantes em *A. nidulans*, bem como na manipulação racional de genes-alvo para a engenharia de fungos.

CAPÍTULO 2 - Perfil de proteínas em cepas recombinantes de *Aspergillus nidulans* super-produzindo proteínas heterólogas

Neste capítulo encontra-se o manuscrito referente aos estudos de proteômica intracelular e extracelular de Anid_A773, Anid_pEXPYR, Anid_AbfA e Anid_Cbhl compilados no manuscrito intitulado de: **"Protein profile in *Aspergillus nidulans* recombinant strains overproducing heterologous enzymes"** de autoria de Mariane Paludetti Zubieta, Fabiano Jares Contesini, Marcelo Ventura Rubio, Any Eliza de Souza Schmidt Gonçalves, Jaqueline Aline Gerhardt, Rolf Alexander Prade e André Ricardo de Lima Damásio. Esse manuscrito, em anexo abaixo, foi aceito para publicação na revista Microbial Biotechnology, fator de impacto 3,513, no dia 26/10/2017 (https://doi.org/10.1111/1751-7915.13027).

2.1 Introdução

Os fungos filamentosos funcionam como fábricas celulares e têm sido utilizados para a produção de grandes quantidades de enzimas sendo que *A. nidulans* se destaca por ser um modelo genético que tem sido estudado para a produção heteróloga de diferentes CAZymes. As CAZymes são biocatalizadores de relevância industrial capazes de degradar a biomassa das paredes celulares das plantas. Algumas destas enzimas incluem α-L-arabinofuranosidases (EC 3.2.1.55) que são enzimas acessórias capazes de hidrolisar as ligações α-L-arabinofuranosídicas. Outro grupo importante de enzimas corresponde às celobiohidrolases (EC 3.2.1.176), que hidrolisam as cadeias de celulose através da remoção da celobiose e desempenham um papel fundamental na degradação da celulose quando combinadas com endoglucanases e mono-oxigenases líticas de polissacarídeos (LPMOs).

Apesar dos fungos filamentosos apresentarem várias vantagens em comparação com outros microrganismos, a produção de proteínas heterólogas ainda está longe dos níveis ideais. Esse fato se deve à falta de um completo conhecimento da adaptação das células fúngicas à superexpressão de proteínas. Para este propósito, uma abordagem de proteômica intracelular pode ser eficiente para realizar uma análise global das proteínas envolvidas nesse processso.

Dessa forma, esse manuscrito teve como objetivo investigar o perfil global de proteínas em cepas recombinants de *A. nidulans* que produzem enzimas heterólogas. Cerca de 250, 441 e 424 proteínas intracelulares foram identificadas na cepa controle Anid_pEXPYR e nas cepas recombinantes Anid_AbfA e Anid_Cbhl, que produzem arabinofuranosidase e celobiohidrolase heterólogas, respectivamente. Neste contexto, os processos celulares

enriquecidos nas cepas recombinantes foram metabolismo energético, metabolismo de aminoácidos, biogênese de ribossomos, tradução, estresse de retículo endoplasmático e oxidativo, e "*repression under secretion stress"* (RESS). O perfil global de proteínas encontrado nas cepas recombinantes Anid_AbfA e Anid_Cbhl foram semelhantes, embora a cepa Anid_Cbhl produziu mais enzima recombinante que a cepa Anid_AbfA. Essas descobertas fornecem informações sobre os processos envolvidos na secreção de proteínas recombinantes em *A. nidulans*, bem como em relação à manipulação racional de genes alvo para engenharia de fungos.

2.2 Manuscrito em inglês

"Protein profile in *Aspergillus nidulans* recombinant strains overproducing heterologous enzymes"

Mariane Paludetti Zubieta[a,1], Fabiano Jares Contesini[a,2], Marcelo Ventura Rubio[a,3], Any Elisa de Souza Schmidt Gonçalves[b,4], Jaqueline Aline Gerhardt[a,5], Rolf Alexander Prade[c,6], André Ricardo de Lima Damasio[a*]

[a]Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas-SP, Brazil.

[b]Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas-SP, Brazil.

[c]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK, USA.

*Corresponding author:

E-mail: adamasio@unicamp.br, Telephone: +55 (19) 35211437

Authors' e-mails: [1]marianezubieta@gmail.com, [2]fabiano.contesini@gmail.com, [3]marcelov.rubio@gmail.com, [4]aelisee20@gmail.com, [5]jaquelinegerhardt@yahoo.com.br, [6]rolf.prade@okstate.edu

Running title: Overproduction of heterologous enzymes in *A. nidulans*

Abstract

Filamentous fungi are robust cell factories and have been used for the production of large quantities of industrially relevant enzymes. However, the production levels of heterologous proteins still need to be improved. Therefore, this article aims to investigate the global proteome profiling of *Aspergillus nidulans* recombinant strains in order to understand the bottlenecks of heterologous enzymes production. About 250, 441 and 424 intracellular proteins were identified in the control strain Anid_pEXPYR, and in the recombinant strains Anid_AbfA and Anid_Cbhl, respectively. In this context, the most enriched processes in recombinant strains were energy pathway, amino acid metabolism, ribosome biogenesis, translation, endoplasmic reticulum and oxidative stress, and repression under secretion stress (RESS). The global protein profile of the recombinant strains Anid_AbfA and Anid_Cbhl were similar, although the latter strain secreted more recombinant enzyme than the former. These findings provide insights into the bottlenecks involved in the secretion of recombinant proteins in *A. nidulans*, as well as in regards to the rational manipulation of target genes for engineering fungal strains as microbial cell factories.

Keywords: *Aspergillus nidulans*, cellobiohydrolase, arabinofuranosidase, heterologous expression, protein secretion

Introduction

Numerous efforts have been made to develop strategies that can supply the enzyme market, as well as those aimed at reducing its costs. Some of these include selecting an appropriate enzyme source and optimizing enzyme properties and secretion. Carbohydrate-Active Enzymes (CAZymes) are industrially relevant biocatalysts capable of degrading plant cell wall biomass. The most important secreted enzymes related to plant cell wall decomposition are cellulases, hemicellulases, and auxiliary enzymes (Levasseur et al., 2013; Lombard et al., 2014). These enzymes have been applied in plant biomass hydrolysis in order to produce second-generation ethanol and several other high-added value products (Goldbeck et al., 2016; Segato et al., 2014). Some of these enzymes include α-L-arabinofuranosidases (EC 3.2.1.55) that belong to glycoside hydrolase families 51, 54 and 62 (GH51, 54 and 62). These accessory enzymes hydrolyze α-L-arabinofuranosidic linkages and have different applications (Rémond et al., 2004). Another important group of enzymes corresponds to the cellobiohydrolases (EC 3.2.1.176), which hydrolyze cellulose chains by removing cellobiose and play a key role in cellulose degradation when combined with endoglucanases and lytic polysaccharide monooxygenases (LPMOs) (Segato et al., 2012; Vermaas et al., 2015).

Aspergillus spp. is an ascomycete that secretes remarkable quantities of proteins. *Aspergillus nidulans* is a genetic model that has been studied for the heterologous production of different CAZymes with promising results (Segato et al., 2012). In addition, *Aspergillus* spp. Is a suitable cell factory to produce heterologous enzymes from eukaryotic organisms recognizing and correctly processing introns (Jeenes et al., 1991) and have a tight regulation system for the N-glycosylation of proteins (Larkin and Imperiali 2011), including CAZymes (Rubio et al., 2016).

In spite of the fact that filamentous fungi present several advantages compared to other microorganisms due to the high level of protein production/secretion, heterologous protein production is far from optimal levels and there is still a need for improvements (Nevalainen and Peterson, 2014). Currently, heterologous production of certain proteins is considerably lower than the levels obtained for homologous proteins (Gouka et al., 1997). In this context, how fungal cells adapt to protein overexpression has not yet been significantly established, since protein secretion pathway involves more than 300 genes (Liu et al., 2014).

Many strategies have been studied to improve heterologous protein production by filamentous fungi, which include deleting genes that encode for proteases (Zhang et al., 2014; Landowski et al., 2015), deleting lectin-like ER-Golgi cargo receptors (Hoang et al., 2015) and co-expressing chaperones with the heterologous protein of interest (Conesa et al., 2002).

Therefore, more profound knowledge regarding the intracellular protein profile could shed light on the drawbacks and bottlenecks of heterologous protein production. For this purpose, an intracellular proteomic approach could be efficient to perform an overall analysis of the proteins involved in the complex regulatory circuits, which frequently result in different intracellular stress conditions.

Investigating individual genes and changes in the genome is not the best option to unveil the main bottlenecks in heterologous protein secretion (Nevalainen and Peterson, 2014). However, understanding the complex interactions of important proteins and genes, as well as how they are regulated, is a more promising option. For the purposes of our research, we applied mass spectrometry-based proteomic approaches to understand how *A. nidulans* adapts to the high expression and production of heterologous proteins by analyzing intracellular proteomes.

We compared three *A. nidulans* strains, along with an empty plasmid-transformed strain and two heterologous strains producing GH51 arabinofuranosidase (*abfA*) and GH7 cellobiohydrolase (*cbhI*), – both genes were isolated from *Aspergillus fumigatus*. Although there have already been several studies conducted on the proteomic analysis of different *Aspergillus* species, to the best of our knowledge there are no studies that have investigated the intracellular protein profile of *A. nidulans* strains overexpressing heterologous proteins.

Results and Discussion

The *abfA* and *cbhl* genes were highly expressed in recombinant strains

The aim of this research was to analyze the intra- and extracellular proteome of two recombinant strains Anid_AbfA and Anid_Cbhl, which produce heterologous arabinofuranosidase (GH51 - AbfA) and cellobiohydrolase (GH7 - Cbhl), respectively.

Initially, we evaluated the profile of secreted proteins following 72h of maltose induction. The strains Anid_AbfA and Anid_Cbhl secreted large quantities of proteins, although Anid_Cbhl accumulated a higher amount of recombinant protein than Anid_AbfA (**Figure 1A**). To evaluate these strains at the transcriptional level, the heterologous gene expression was quantified by qPCR. The *abfA* and *cbhl* genes were highly expressed in recombinant strains, Anid_AbfA and Anid_Cbhl, respectively (**Figure 1B).** This result indicates that the heterologous genes *abfA* and *cbhl* were efficiently transcribed, translated and secreted by *A. nidulans*.

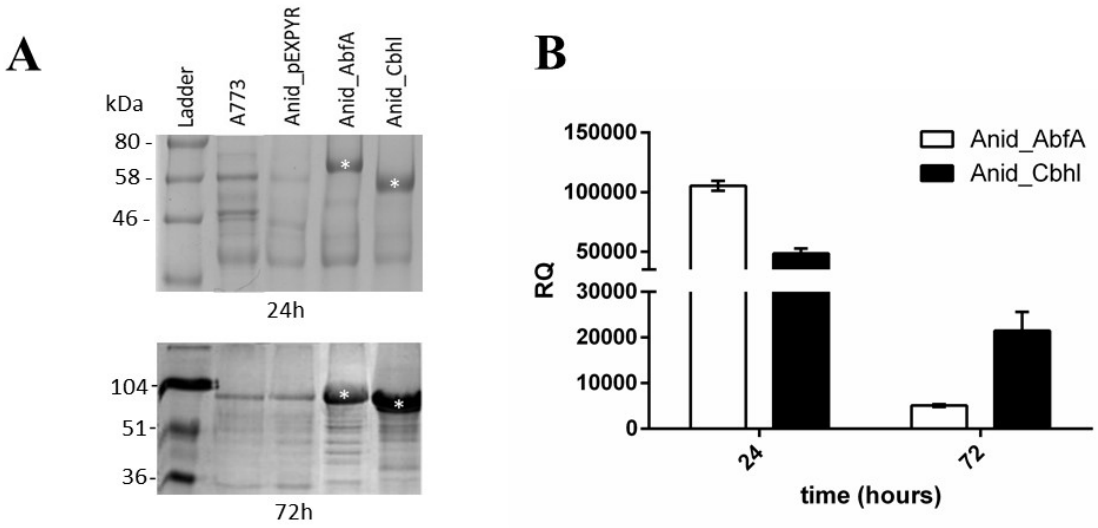


Figure 1. **Secretion of total proteins by *A. nidulans* strains.** The *A. nidulans* strains A773, Anid_pEXPYR (carrying an empty pEXPYR vector), Anid_AbfA and Anid_Cbhl expressing α-L-arabinofuranosidase and cellobiohydrolase, respectively, were grown on minimum media containing 2% maltose for 24 h and 72h at 37°C. (A) Ten micrograms of secreted proteins were resolved by Coomassie blue-staining SDS-PAGE gel. The strain A773 and Anid_pEXPYR were used as a control in this experiment. Asterisks (*) indicated the recombinant proteins. (B) qPCR of the recombinant genes was calculated by the relative standard curve method. The expression of genes *abfA* and *cbhl* were normalized using the gene *tubC* (tubulin) as reference. MM: molecular marker

To determine the point of time for intracellular proteomic profiling, the growth of all strains on 2% maltose was evaluated. A faster uptake of maltose was observed for the control strains, A773 and Anid_pEXPYR, compared with the recombinant strains. After 24 hours, the control strains consumed over 80% of the maltose, while only a small percentage of maltose was consumed by strains overexpressing heterologous genes (~ 23%). At 48 hours, no maltose was present in the medium of either control strains **(Figure 2A)**. The slower consumption of maltose in the recombinant strains reflects slower growth ratio (**Figure 2B)**.

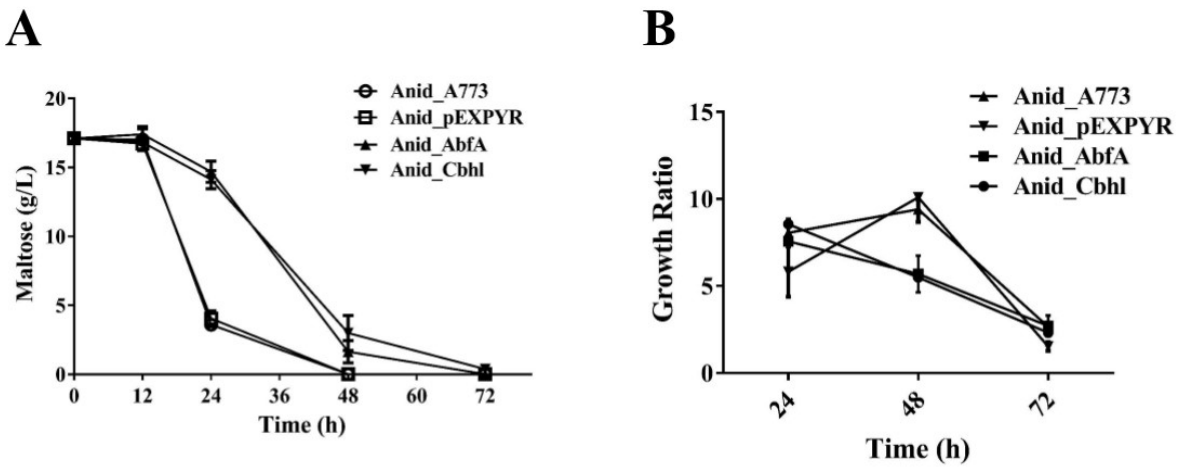


Figure 2. Analysis of *A. nidulans* growth. Spores solution was inoculated in 30 mL of minimum medium (MM) supplemented with 2% (m/v) maltose. (A) After various time points at 37°C, the supernatant was separated from the culture medium by gauze filtration and maltose content was measured by HPLC. (B) The mycelia of *A. nidulans* strains were dried overnight at 105°C for measure of the dry weight. Each bar represents the mean and the standard deviation of values from three independent experiments.

In several protein expression systems that use fungi as cell factories, slow growth conditions may ensure that cells allocate sufficient resources to recombinant protein production (Liu et al., 2014). Considering this, we established 24 hours as the point of time for our proteomic analysis, which is when there is some maltose remaining to support the growth of all strains.

The intracellular proteome of recombinant strains are closely related

Intracellular proteins were assessed by LC-MS/MS. The total number of proteins identified in the strains Anid_pEXPYR, Anid_AbfA and Anid_Cbhl were 250, 441 and 424, respectively (**Table S1**). Around 47.9% of the 480 proteins identified were common to all strains, 32.5% were exclusively found in the recombinant strains Anid_AbfA and Anid_Cbhl, and 0.2% was exclusively found in the control strain (Anid_pEXPYR) (**Figure 3A**). The results show that the protein profile of recombinant strains is especially closely related, and it is likely that this profile represents a pattern of cell response to heterologous proteins production in *A. nidulans*.

Additional analysis was performed using FunCat (The Functional Catalogue) annotations. Most of the proteins were annotated into the functional category of protein binding and C-compound and carbohydrate metabolism (**Figure 3B**).

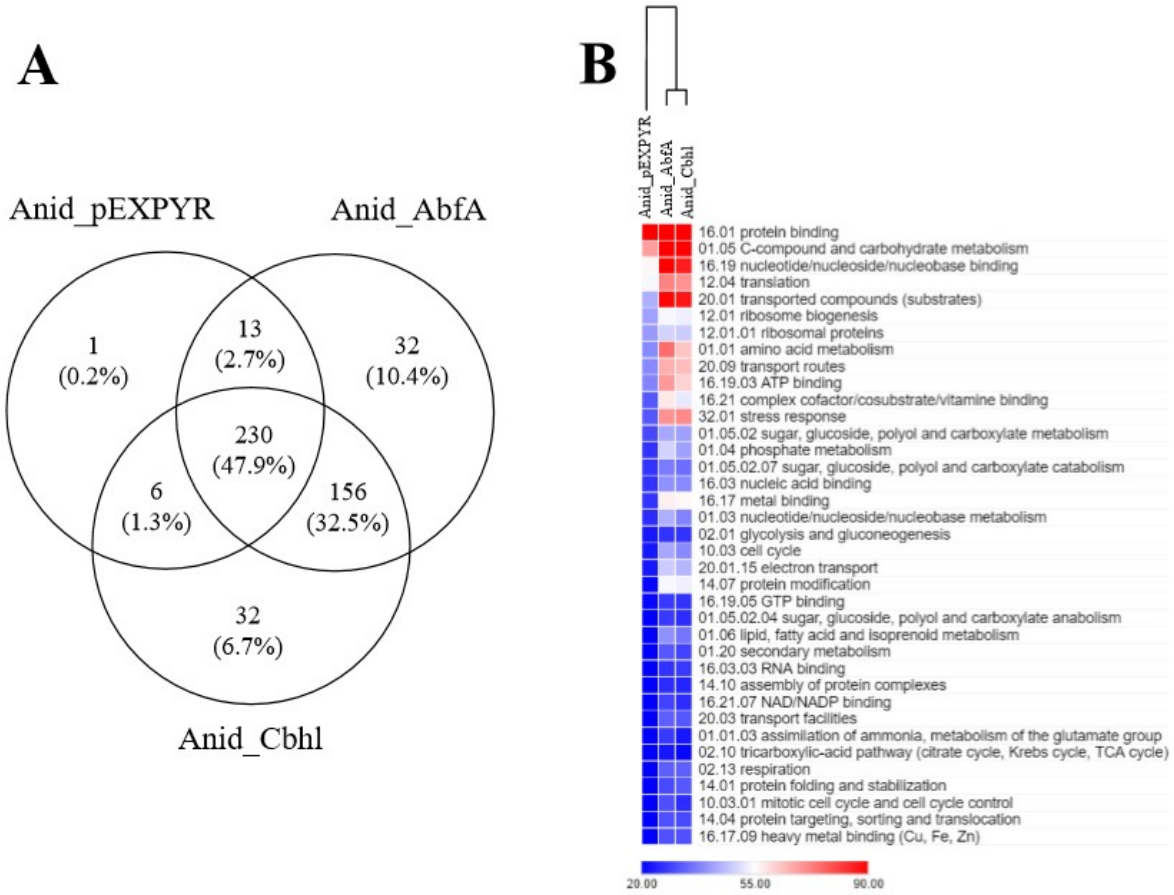


Figure 3. Abundance and functional analysis of intracellular proteins. The strains were grown on 2% maltose minimum medium for 24 h. (A) Venn diagrams represent the number of total proteins found in the intracellular proteome of each strain as well as the overlaps among groups. (B) A heat map of the 480 proteins categorized by MIPS FunCat (see Table S2) and the scale indicates the number of proteins found in each category. The intracellular proteomes were clustered based on their total spectra profiles.

Within the protein binding category, the elongation factor 1-α (AN4218) was present in higher quantities. This elongation factor, homologous to the *Saccharomyces cerevisiae* TEF-1 and TEF-2, delivers aminoacylated tRNA to the A-site of ribosomes to elongate nascent polypeptides during protein translation. Due to its role in the cell, TEF-1 is usually found at high levels in *S. cerevisiae*, which represents 5% of the total soluble protein pool (Thiele et al., 1985). Due to the strength of the promoter activity, several studies have reported the use of the *tef* promoter in protein production systems (Kitamoto et al., 1998; Magalhães et al., 2013).

The functional categories nucleotide/nucleoside/nucleobase binding, transported compounds, ATP binding, amino acid metabolism, translation, transport routes and stress response were further overrepresented, mainly in the recombinant strains. In Anid_AbfA and Anid_Cbhl, oxidoreductases were found more abundantly, namely 6-phosphogluconate

dehydrogenase (AN3954), aldehyde dehydrogenase (AN0554), mitochondrial malate dehydrogenase (AN6717), and NADP-specific glutamate dehydrogenase (AN4376). Cultivation on maltose was previously associated with the presence of large amounts of oxidoreductases synthesized by fungi. The secretome analysis of *Aspergillus niger* grown on maltose, xylose, and sorbitol, showed larger quantities of oxidoreductases on the maltose, such as superoxide dismutase and peroxiredoxin (Oliveira et al., 2011; Lu et al., 2010).

Biological processes altered in *A. nidulans* recombinant strains

In order to determine a biological response profile in regards to heterologous protein production, we performed a comprehensive analysis of the intracellular proteins by total spectra. The proteins were classified as more or less abundant according to the number of total spectra relative to the control strain.

Overall, 276 (84%) and 242 (74%) proteins were more abundant in Anid_AbfA and Anid_Cbhl, respectively. Almost all the proteins found in recombinant strains were classified as more abundant, while 6 (2%) and 14 (4%) proteins were classified as less abundant in Anid_AbfA and Anid_Cbhl, respectively. The protein profiles were similar between recombinant strains, especially within the more abundant proteins group **(Figure 4A and Table S2**). According to FunCat annotations, most of the more abundant proteins were related to protein binding (105 proteins in Anid_AbfA and 94 proteins in Anid_Cbhl). The second enriched functional category was C-compound and carbohydrate metabolism with 75 proteins in Anid_AbfA and 65 proteins in Anid_Cbhl. Other categories were enriched such as translation, nucleotide/nucleoside/nucleobase binding, amino acid metabolism and stress response. In the group of less abundant proteins, 19 proteins were annotated (**Figure 4B)**. Hereafter we described the main functional processes altered in the recombinant strains Anid_AbfA and Anid_Cbhl.

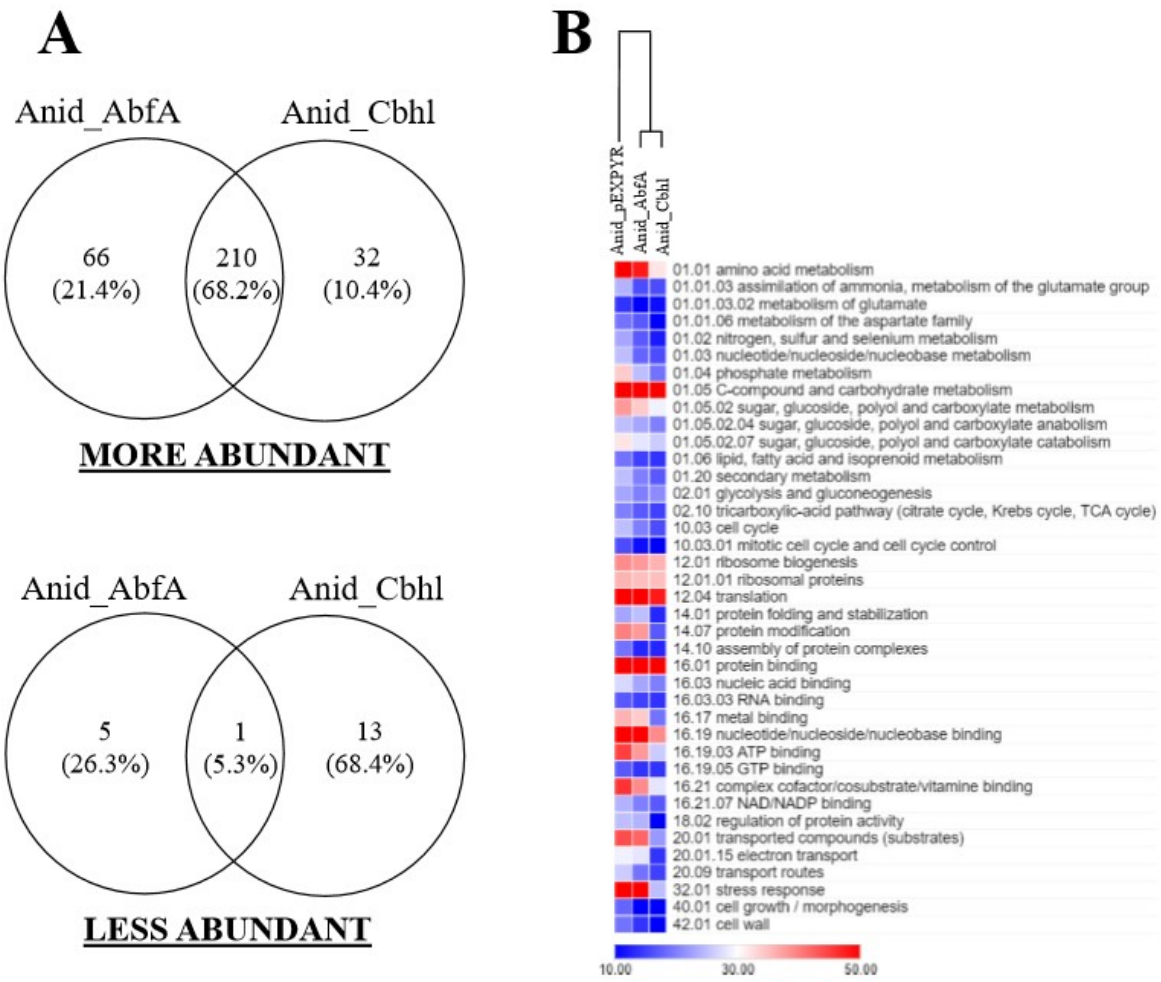


Figure 4. **Abundance and functional analysis of intracellular proteins**. The strains were grown on minimum medium and 2% maltose for 24 h. (A) Venn diagrams represent the number of more and less abundant proteins relative to *A. nidulans* Anid_pEXPYR strain. (B) A heat map of all proteins (see Table S3). MIPS FunCat categorization of the 308 more abundant proteins and 19 less abundant proteins common to Anid_AbfA and Anid_Cbhl strains. The scale indicates the number of proteins found in each category. The intracellular proteomes were clustered based on their total spectra profiles.

Energy pathway

About 190 proteins were annotated into the functional energy pathway category, comprising C-compound and carbohydrate metabolism and amino acid metabolism. At least seven proteins that are directly involved in glycolysis or tricarboxylic acid (TCA) cycle (AN2436, AN5525, AN5746, AN2875, AN8041, AN6717, AN6499, and AN1246) were more abundant in the recombinant strains. In agreement with our results, there was a reported increased in the TCA cycle flux during Fab-fragment antibody 3H6[12] production in *Pichia pastoris*, indicating an increased energy demand. In yeasts, this increased energetic cost in recombinant protein production can be related to protein refolding and secretion (Dragosits et al., 2009; Tyo et al., 2012).

Enzymes of the pentose phosphate pathway (PPP) were also more abundant such as transketolase (AN0688) and transaldolase (AN0240). PPP is responsible for generating NADPH and pentoses as well as ribose 5-phosphate, a precursor for nucleotides synthesis. NADPH is particularly necessary for biosynthesizing amino acids for use as building blocks for proteins. The demand for recombinant protein biosynthesis requires larger quantities of NADPH that become insufficient to support the normal *A. nidulans* growth, which may explain why the recombinant strains showed a lower growth rate than the control strains. An increased PPP flux was also observed for *A. niger* and *Aspergillus oryzae* producing fructofuranosidase and amylase, respectively (Pedersen et al., 1999; Driouch et al., 2012). Furthermore, the activity of PPP was highest in *S. cerevisiae* during the expression phase of heterologous protein, β-galactosidase, thereby resulting in an improvement of the foreign protein expression and cellular ATP yield (Jin et al., 1997).

The enrichment of TCA and PPP pathways confirms the higher energy requirement during heterologous protein production in filamentous fungi. This result shows that, among the various reactions of the central metabolism, these two pathways play a central role for recombinant protein production in fungal cells.

Amino acid metabolism, ribosome biogenesis and translation

Proteins related to amino acid metabolism were more abundant in Anid_AbfA and Anid_Cbhl, primarily enzymes with predicted role in methionine, alanine, aspartate, glutamine and glutamate metabolism such as methionine synthase (AN4443), NADP-linked glutamate dehydrogenase (AN4376), alanine transaminase (AN1923), adenosylhomocysteinase (AN1263) and S-adenosylmethionine synthetase (AN1222). Amino acid supplementation of the growth medium was shown to partially unburden cellular metabolism during recombinant protein production in yeast (Görgens et al., 2005; Heyland et al., 2011). In *S. cerevisiae*, adding a balanced mixture of the preferred amino acids, Ala, Arg, Asn, Gln, and Gly, improved recombinant xylanase production (Görgens et al., 2005).

Translation efficiency is usually the first concern when designing expression systems for heterologous protein production. We found that the synthesis of proteins involved in translation was significantly more abundant during recombinant protein production. Translation elongation factors AN1162, AN4218, AN6330, and AN6563 were found more abundantly as well as ribosome-structural proteins such as AN8176 and AN3413. During previous transcriptome studies, amino acid metabolism-related genes were downregulated in recombinant strains, which might be due to the slower growth during the sampling period or

the feedback inhibition of amino acid biosynthesis from ER stress overloaded (Liu et al., 2014). Transcription of genes encoding for translational and ribosomal proteins is also coordinated with and is essential for cell growth, proliferation, and differentiation. ER stress induction with DTT (dithiothreitol) reduced the growth rate of *A. nidulans* chemostat culture concomitantly with the downregulation of 34 (81%) ribosomal genes (Sims et al., 2005).

Maltose consumption was lower for recombinant strains (**Figure 2A**). Overall, this shows that Anid_AbfA and Anid_Cbhl are slow-growing strains due to ER stress as well as other *Aspergillus* recombinant strains (Liu et al., 2014). Moreover, the lower growth ratio along with upregulation of amino acid metabolism, ribosome biogenesis and translation process in Anid_AbfA and Anid_Cbhl may reflect the increased energy demand required for heterologous protein production.

Endoplasmic reticulum stress

The unusually high and non-physiological rates of recombinant protein production in filamentous fungi drive cells to an ER stress condition (Saloheimo et al., 1999). We detected proteins related to ER stress including chaperones (BipA, SgdE, Hsp104, Hsp70, Hsp90), foldases (Pdi, TigA, Cyp1 and AN3814), and calnexin (AN3592) in both recombinant strains, showing that ER stress was actively turned on. These proteins were classified into response to stress, response to chemical and protein folding categories. The overproduction of several homologous and heterologous proteins in *Aspergillus* strains results in a condition called *unfolded protein response* (UPR), which is characterized by the accumulation of incorrectly folded proteins or delayed folding during ER (Heimel, 2015). The basic leucine zipper (bZIP) transcription factor HacA is responsible for the transcriptional induction of UPR-target genes, which include ER-localized molecular chaperones and folding, components of the ER-associated degradation system, and other proteins acting at various stages of secretion, the purpose of which being disperse unfolded proteins accumulated during the ER (Sims et al., 2005; Pakula et al., 2003). The chaperones Hsp70, BipA and SgdE are useful to achieve an initial folding of nascent polypeptide in the ER (Mayer and Bukau, 2005). Hsp90 is responsible for folding and maintaining client proteins, transcriptional and posttranscriptional processes and activation of signal transducers (Zuehlke and Johnson, 2010), while Hsp104 is responsible for reactivating denatured and aggregated proteins (Grimminger-Marquardt and Lashuel, 2009). Calnexins are lectin chaperones that undergo releasing and re-binding cycles until the glycoprotein achieves its native conformation, after which the protein is released for secretion into the distal secretory pathway (Molinari et al., 2003).

Foldases, peptidyl-prolyl cis or trans isomerase (PPI) (cyclophilins), and protein-disulfide isomerase (PDI) facilitate the folding of several proteins by catalyzing the isomerization of prolyl peptide bonds between it's cis and trans forms and formation and isomerization of disulfide bonds for proper folding, respectively (Schönbrunner and Schmid, 1992). Induction of several UPR genes was detected in *A. niger* producing recombinant tissue plasminogen activator (t-PA), such as *bipA*, *pdiA,* and *pdiB* that are HSP70-family chaperones (Guillemette et al., 2007). During the transcriptome analysis of recombinant bovine chymosin and α-amylase production by *Aspergillus* strains, chaperones and foldases genes were also upregulated (Liu et al., 2014; Sims, 2005).

Several studies have reported that inducing the UPR genes in recombinant strains alleviates ER stress and may result in an improved secretion of a target protein. Thus, several strategies have been followed, such as the overexpression of protein disulfide isomerases or heat shock proteins acting as chaperones, but the results have been highly variable. The overexpression of chaperone BIPA and protein disulfide isomerase (PDIA) increased the secretion of a single-chain antibody fragment in *S. cerevisiae* by 2 and 8-fold, respectively (Shusta et al., 1998). In *S. cerevisiae*, the overexpression of *bipA* increased the amount of extracellular prochymosin more than 20-fold, but the secretion of thaumatin was not significantly stimulated (Harmsen et al., 1996). In *A. niger*, the expression of the activated form of the transcription factor *hacA* enhanced the production of *Trametes versicolor* laccase by 7-fold and bovine preprochymosin by 2.8-fold (Valkonen et al., 2003).

BIPA (AN2069) and PDIA (AN7436) were 2 and 3-fold more abundant in Anid_Cbhl than Anid_AbfA. These data can be related to different intensity levels of UPR in the recombinant strains, which has already been demonstrated for DTT-treated yeasts (Pincus et al., 2010). We suggest that UPR is more intense in Anid_Cbhl, resulting in the improvement of the cell protein-folding capacity due to a highest induction of chaperones and foldases.

Chaperonins (AN2918 and AN5713) were additionally more abundant at the 24h point of culture in both recombinant strains. Chaperonins are essential to mediate the ATP-dependent cellular protein folding in eukaryotes. It's interactome plays an important role in the folding or assembly of a range of proteins linked to the central and essential cellular processes, such as cytoskeleton assembly, cell-cycle regulation and chromatin remodeling (Yam et al., 2008). In humans, the chaperonin TRiC/CCT regulates HSF1, an evolutionarily conserved transcription factor that protects cells from protein-misfolding-induced stress and apoptosis (He et al., 2015). However, we found no reports in the literature linking UPR and

chaperonin-dependent transcription. In general, the presence of chaperonins in recombinant cultures may be involved in helping cells to restore protein-folding homeostasis.

In this study, our results suggest that producing heterologous proteins induced UPR in the recombinant strains due to an overload in the secretory pathway. This signaling network alleviates ER stress, promotes cell survival and adaptation, and restores cellular folding homeostasis (Hollien, 2013; Kozutsumi et al., 1988).

Oxidative Stress

The high secretion of recombinant proteins coordinately induced the production of proteins involved in oxidative stress in Anid_AbfA and Anid_Cbhl. We identified catalase (*catB*), thioredoxin (*trxA*), protein disulfide oxidoreductase activity (*ero1*) and aldehyde dehydrogenase (*aldA*) belonging to this group (**Table S2**). However, Anid_Cbhl showed a higher number of total spectra for *trxA* and *aldH* than Anid_AbfA. In the fungal platforms for protein production, protein folding is a crucial step of the secretory pathway, since a correct folding determines whether the newly synthesized protein will be targeted for secretion, otherwise, it will be assigned for ER-associated degradation (ERAD). In many cases, protein folding includes disulfide bond formation, which in eukaryotes is managed by the coordinated action of PDIs and Ero1, using molecular oxygen as the terminal electron acceptor, and generating reactive oxygen species (ROS) (Tu et al., 2000). Heterologous proteins require an overall ER folding capacity, resulting in misfolded endogenous proteins that can limit the efficiency of protein synthesis. Furthermore, non-native disulfide bonds are frequently formed during this process, which must then be broken down and subsequently rearranged to form the correct ones, thereby resulting in ROS accumulation and damage to biological macromolecules, such as DNA, lipids, and proteins (Tyo et al., 2012). Thus, an increased demand for protein folding and disulfide bonds activates oxidative stress defense, which includes the upregulation of catalases and thioredoxins. Oxidative stress was previously described in yeast, which supports an increase in the protein production capacity during batch fermentations (Tyo et al. 2012; Martínez et al. 2016). Here, the higher total spectra of oxidoreductases in the recombinant strains could alleviate the oxidative stress and improve protein production.

Repression under secretion stress (RESS) – the secretion of Carbohydrate-Active Enzymes is reduced in recombinant strains

RESS is a transcriptional feedback mechanism that has been shown in filamentous fungi (Pakula et al., 2003; Al-Sheikh et al., 2004). The expression of gene encoding to endogenous secreted proteins is downregulated by the UPR. Subsequently, the cargo load in ER decreases and accelerates the recovery of cell homeostasis (Pakula et al., 2003). This mechanism could occur in wild-type and heterologous expression systems that have a high target protein flux through ER (Guillemette et al., 2007), such as Anid_AbfA and Anid_Cbhl.

In order to investigate RESS mechanism in the recombinant strains, we performed a secretome analysis to verify whether this mechanism occurs in *A. nidulans* recombinant strains. The secretion of some CAZymes was reduced in the recombinant strains. Around 86% of the secreted proteins significantly less abundant in the recombinant strains were CAZymes, such as endoarabinanase (AN8007), feruloyl esterase (AN5267), pectate lyase (AN7646 and AN8453), pectin-methyl esterase (AN3390), β-1,4-endoxylanase and β-glucosidase (AN2828) (**Table S3**). Previous transcriptome profiling of *N. crassa* cultures on cellulose showed that the lignocellulase genes were downregulated, suggesting the presence of RESS that may be limiting lignocellulase synthesis (Fan et al., 2015).

We checked the level of transcripts of *amyR* and *xlnR*, two transcriptional regulators involved in the control of amylases and CAZymes-encoding genes, respectively, because these regulators are downregulated under RESS (Carvalho et al., 2012; Zhou et al., 2014). *amyR* and *xlnR* transcripts were detected at lower levels in the recombinant strains when compared to the control strain. It is likely to relate the downregulation of these transcription factors to the reduction of CAZymes and amylases secretion (**Figure 5**).

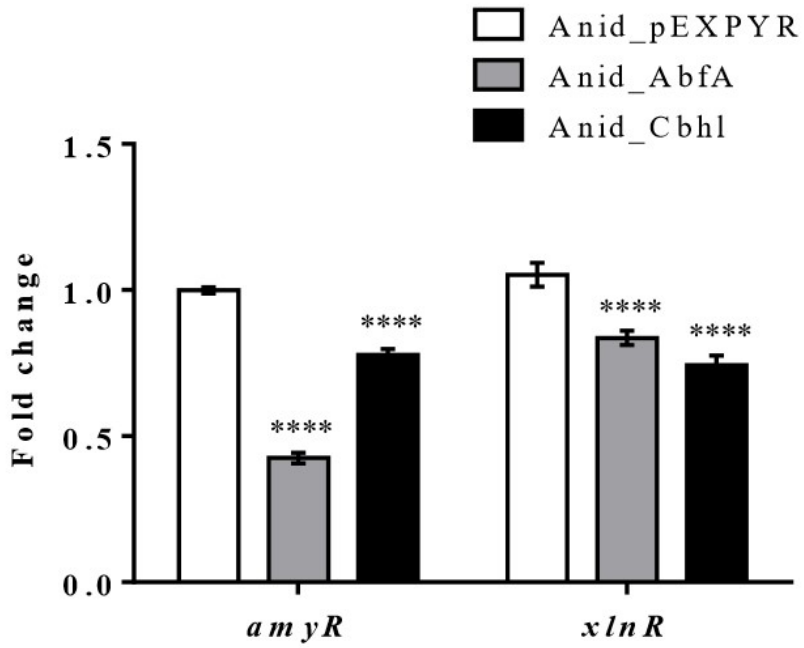


Figure 5. Evidence of *repression under secretion stress* (RESS) in *A. nidulans* recombinant strains. Transcriptional level of *amyR* and *xlnR* genes in Anid_AbfA and Anid_Cbhl was compared to the expression level in control strain (Anid_pEXPYR). Gene expression levels were normalized (ΔΔCT analysis) to the endogenous gene *tubC* (tubulin). Data was analysed using one-way-ANOVA with Bonferroni's post hoc test (****P<0.0001).

In addition, down-regulation of *amyR* may be related to lower growth of recombinant strains due to participation in maltose transport (**Figure 2B**). Down-regulation of *amyR* gene leads to lower secretion of glucoamylases, enzyme that cleaves maltose to glucose, resulting in down-regulation of hexose transporters and reduced carbon source uptake by recombinant strains (Vongsangnak et al., 2009).

However, RESS mechanism details are still unclear as well as its specific targets. Zhou et al., (2014) described the octamer sequence TCACGGGC (positions -307 to -300) in the *amyB* promoter, as essential for downregulation under RESS induced by DTT. Based on these findings, we suggest that RESS is activated in our recombinant strains, which is consistent with previous observations.

The protein sequence context influences the secretion levels

As described above, the Anid_Cbhl strain secretes more recombinant proteins than Anid_AbfA (**Figure 1A**). The overall response of both recombinant strains was quite similar; however, each strain showed an exclusive set of proteins more or less abundant (**Figure 4**). Therefore, we analyzed the sequences of AbfA and Cbhl enzymes. AbfA is 656 amino acids

in length, with three cysteines predicted forming disulfide bonds and nine possible N-glycosylation sites. Cbhl is 532 amino acids in length and has a predicted cellulose-binding module (CBM) corresponding to amino acid positions 500 to 528, twenty-two cysteines predicted forming disulfide bonds and one possible N-glycosylation site (**Figure S1**).

Protein glycosylation is one of the most common forms of the post-translational modification process, which are present across all kingdoms of life. N-glycosylation, involved in the process of protein folding in the ER, plays an important role in biological activity. In several studies, the production of enzymes increased when the glycosylation-related genes were introduced or its glycosylation sites changed (Han and Yu, 2015). However, the glycosylation role in recombinant protein production continues to be controversial. In *S. cerevisiae* N-glycosylation-deficient mutants, the expression and secretion of a *Bacillus licheniformis* thermostable α-amylase was improved. The authors suggested that the transfer of oligosaccharides may compete with α-amylase folding because of the slow transfer rates of the incomplete oligosaccharides. Folding preceded N-glycosylation and resulted in an underglycosylation of the recombinant enzymes, which could be preferentially folded and secreted (Hoshida et al., 2013).

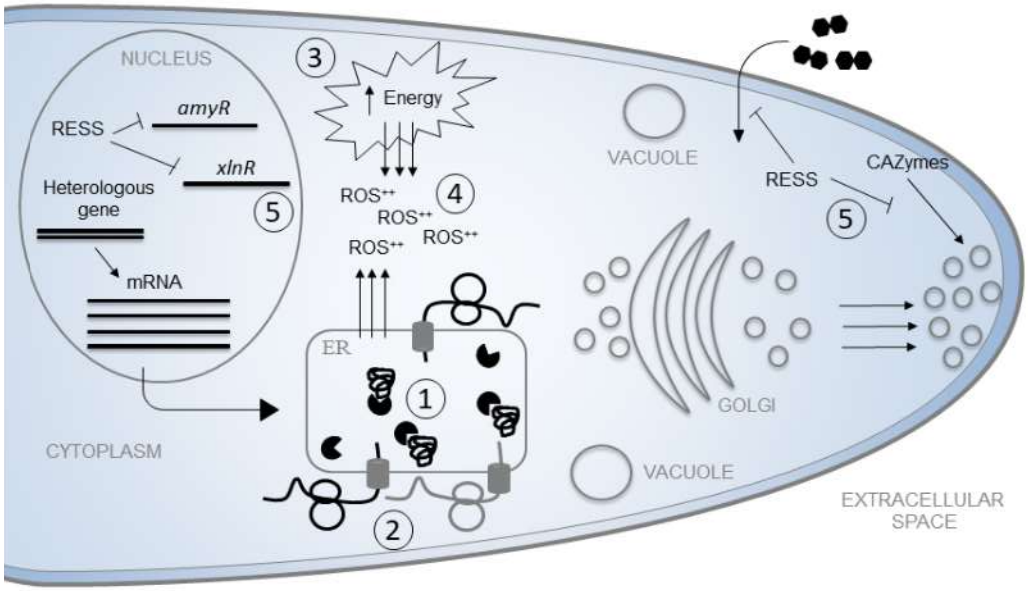
The secretion of human insulin precursor (IP), a small protein without glycosylation sites, was higher when compared to α-amylase, a larger protein that has one N-glycosylation site (Tyo et al., 2012). In *S. cerevisiae*, the disruption of genes involved in the N-glycosylation modification improved the production of recombinant enzymes and the transcription of key genes in the folding pathway such as chaperones *KAR2*, homologous to BipA, and other HSP70 chaperones (Tang et al., 2016). BipA and HSP70 were more abundant in the Anid_Cbhl strain, in which the recombinant protein production was higher than in the Anid_AbfA strain. This evidence suggests that the lower number of N-glycosylation sites in cellobiohydrolase enhances recombinant protein production due to the improvement in the secretory pathway capacity.

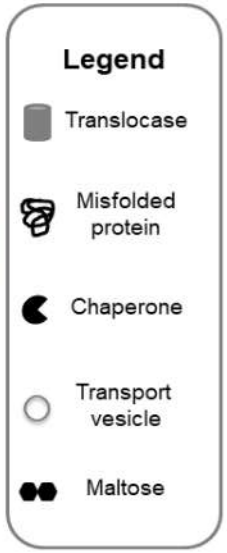
The other bottleneck in recombinant protein production could be the odd number of cysteines in the AbfA. The random disulfide isomerization process may incorporate the cysteine that should not be incorporated into a disulfide bond, thereby generating futile cycles of disulfide formation. In α-amylase and human insulin precursor production by yeast, the odd number of cysteines in α-amylase was one factor contributing to the six-fold fewer molecules secreted when compared to insulin (Tyo et al., 2012).

Conclusions

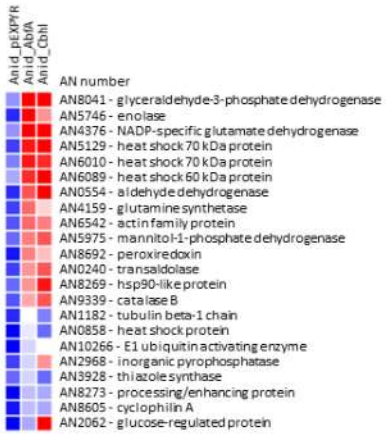
The primary goal of this study was to analyze the intra- and extracellular proteome profiles of *A. nidulans* recombinant strains and to describe the major bottlenecks involved in the production of two different heterologous proteins. In this study, we identified that the intracellular profile of the recombinant strains Anid_AbfA and Anid_Cbhl are similar, despite producing different heterologous proteins. The Anid_Cbhl strain secretes more recombinant enzyme than Anid_AbfA and we suggest that the higher amount of specific proteins such as PdiA, BipA, TrxA and AldA, which alleviate ER and oxidative stress, can contribute improving heterologous protein production. Moreover, we showed that the following processes – energy pathway, amino acid metabolism, ribosome biogenesis, translation, reticulum and oxidative stress were the main enriched mechanisms in the recombinant strains. The RESS phenomenon can be present in the recombinant strains, which probably prevents the high ER load with additional proteins during the high-level production of heterologous proteins in *A. nidulans.* All these findings in recombinant strains are represented in **Figure 6**.

Fig. 6

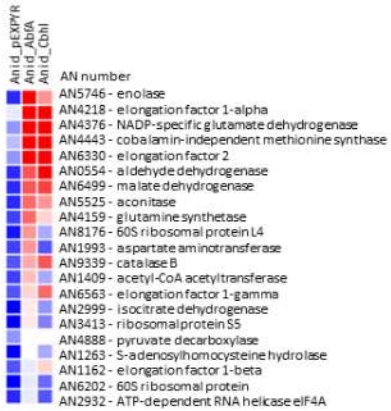




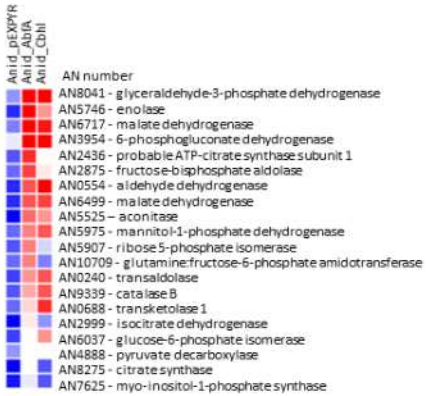
1. Endoplasmatic reticulum stress



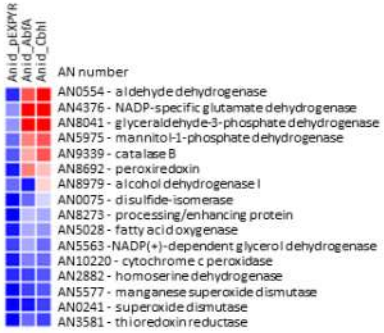
2. Amino acid metabolism, ribosome biogenesis and translation



3. Energy pathway



4. Oxidative Stress



5. RESS (Repression Under Secretion Stress)

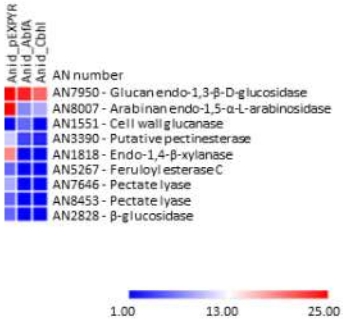


Figure 6. Overview of biological process overrepresented in *A. nidulans* recombinant strains. Heterologous protein production remains a complex process with some bottlenecks. Generally, the recombinant gene contains strong promoter for high levels expression of the target mRNA. Large quantities of mRNAs overload the translational pathway, which increase misfolded proteins amounts in ER, inducing an ER stress (1). The homeostasis maintenance is achieved by UPR that induces genes coding to chaperones, amino acid metabolism, ribosome biogenesis, translation, among others (2). Furthermore, energy demand required for heterologous protein production increased (3), resulting in high levels of reactive oxygen species in the cell (4). The secretome analysis of recombinant strains showed the downregulation of biomass-degrading enzymes and their genes, suggesting the presence of the RESS mechanism. Associated with the overload of misfolded proteins in the ER, this mechanism downregulates transcriptional activators, such as *amyR* and *xlnR* that regulates expression of several amylases and CAZymes, respectively (5). The heat maps (at the bottom) represent the protein abundance in the recombinant (Anid_AbfA; Anid_Cbhl) and control strains (Anid_pEXPYR) in each biological process categorized by MIPS FunCat. The scale indicates the number of proteins found in each category.

In addition, we suggest that the context of the protein sequence directly impacts the difference in the heterologous protein secretion levels, evidenced by an odd number of cysteines and the number of N-glycosylation sites. These findings helped us to comprehend the underlying mechanisms involved in the high secretion of recombinant proteins in *A. nidulans* and in the rational manipulation of target genes for the improvement of fungi strains as microbial cell factories.

Experimental Procedures

Aspergillus strains and growth conditions

A. nidulans A773 (pyrG89; wA3; pyroA4) was obtained from the Fungal Genetic Stock Center (FGSC). *A. nidulans* A773 recombinant strains secreting high levels of GH51 α-L-arabinofuranosidase (Anid_AbfA) and GH7 cellobiohydrolase (Anid_Cbhl), and *A. nidulans* A773 transformed with empty vector (Anid_pEXPYR) were obtained from our culture collection maintained at the Institute of Biology, UNICAMP.

The Anid_AbfA, Anid_Cbhl and Anid_pEXPYR strains were constructed using the pEXPYR vector as expression plasmid. The pEXPYR is a shuttle vector used for expression and secretion of client proteins in *Aspergillus* species. pEXPYR contains ampicillin resistance marker for propagation in *Escherichia coli*, and a phleomycin resistance eukaryotic selectable marker. For cloning and expression of proteins, target gene is overexpressed under control of the *A. niger* glucoamylase promoter and its N-terminal secretion peptide, and tryptophan synthase transcription terminator. Furthermore, the orotidine-5'-decarboxylase gene (*pyrG*)

from *A. niger* present in this vector is useful to complement *A. nidulans* pyrG89 mutation in strains such as *A. nidulans* A773 (Segato et al., 2012).

The heterologous genes (*abfA; cbhl*) were isolated from *Aspergillus fumigatus* by PCR, digested with *Not*I and *Xba*I and ligated onto *Not*I/*Xba*I digested pEXPYR plasmid. After cloning, the pEXPYR containing *A. fumigatus* genes and the empty pEXPYR was transformed into *A. nidulans* A773 (Damásio et al., 2012; Segato et al., 2012). The spores solution was inoculated in 15 mL of minimum medium (MM) supplemented with 2% (m/v) maltose as a gene expression inducer. The MM composition was 1X Clutterbuck's salts (20X Clutterbuck's salts stock: 1.4 M $NaNO_3$, 0.13 M KCl, 0.042 M $MgSO_4.7H_2O$ and 0.22 M KH_2PO_4), 1X trace elements (1000X Trace elements stock: 7.2 mM $ZnSO_4.7H_20$, 17.7 mM H_3BO_3, 2.52 mM $MnCl_2.4H_20$, 2.72 mM $FeSO_4.7H_20$, 0.95 mM $CoCl_2.5H_20$, 0.7 mM $CuS0_4.5H_20$, 0.21 mM $Na_2Mo0_4.4H_20$ and 17.11 mM EDTA), 11 mM maltose, pyridoxine (1 mg/L) and uracil/uridine (2.5 mg/L). All experiments were carried out in three biological replicates.

Maltose quantification by HPLC

Supernatant from *A. nidulans* cultures were collected after 12, 24, 48 and 72 hours of growth, and the maltose concentration was detected using high-performance liquid chromatography (HPLC) Agilent Infinity 1260 with a 50C IR detector, Aminex column HPX-87H 300 mm x 7.8 mm at 50°C and 0.5 mL/min of ultrapure Milli-Q water as eluent phase.

Samples preparation for liquid chromatography-tandem mass spectroscopy (LC-MS/MS)

Intracellular proteome

The fungal mycelium was harvested after 24 h of growth and ground into a fine powder in liquid nitrogen. The mycelial powder was suspended in 10 volumes of extraction buffer in an ice bath (20 mM Tris pH8, 0.05% Triton, 150 mM NaCl, and 2 mM PMSF), centrifuged at 7500 *x g* for 10 min at 4°C, and the supernatant was subsequently collected.

Extracellular proteome

Culture filtrates after 72 h of growth were washed with 2 mL ultrapure water and concentrated using 10,000 Da cutoff Amicon. The samples were quantified using the Bradford method (Bradford, 1976), and ten milligrams of intracellular and extracellular proteins were loaded onto SDS-PAGE. The band slices were destained with methanol and acetic acid, dehydrated

with acetonitrile, reduced with DTT, alkylated with iodoacetamide, and digested for 18 hours with 20 ng/µl trypsin using an ammonium bicarbonate buffer. After digestion at 37°C, the peptide extraction was carried out by methanol and acetic acid treatment (Shevchenko, 1996) with modifications.

LC-MS/MS analysis, protein identification and statistical analysis

The peptide mixture from the biological replicates was analyzed by LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with liquid chromatography-tandem mass spectrometry using an EASY-nLC system (Thermo Fisher Scientific, USA).

The LC-MS/MS raw files were used for the database search via the Mascot software application (Matrix Science, London, UK), comparing the *A. nidulans* peptides from *Aspergillus* Genome Database (*Asp*GD) using a zero false discovery rate (FDR) estimated by target/decoy searches. The Mascot was searched using a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 15 PPM. S-carbamoylmethylcysteine cyclization of the n-terminus, oxidation of methionine, n-formylation of the n-terminus, acetylation of the n-terminus, iodoacetamide derivative of cysteine and acrylamide adduct of cysteine were specified as variable modifications in Mascot.

Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate the MS/MS-based peptide and protein identification. Peptide identifications were accepted if they could be established with a probability higher than 95% by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established with a probability higher than 99.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, 2003). Proteome label-free quantification and individual protein abundance were obtained using the total spectrum count method.

Functional annotation of the proteomes was performed using Fisher's Exact test with a threshold of 95%. For the proteome data, we performed a statistical analysis (fold change; FC) and analyzed the data using a paired t-test with Bonferroni's posttest for multiple comparisons, where only proteins with p-values < 0.05 were selected. The resultant sequences were imported into FunCat for mapping the sequences into functional categories and a comparison was made between the proteomes from empty plasmid-transformed strain (Anid_pEXPYR) and the proteomes from the *A. nidulans* recombinant strains (Anid_AbfA and Anid_Cbhl). Finally, intracellular and extracellular proteins were evaluated for the presence of a signal peptide (SP) or a secretion signal to the non-classical pathway, which was

performed using the SecretomeP server. Proteins with SP or those with a "threshold" above 0.6 for non-classical pathways were classified as extracellular (Bendtsen, 2004).

RNA extraction, transcript analysis by qPCR (quantitative real-time PCR) and primer design

To measure the α-L-arabinofuranosidase and cellobiohydrolase gene transcripts, mycelia of Anid_A773, Anid_pEXPYR, Anid_AbfA, and Anid_Cbhl were harvested by filtration and used for RNA extraction. Harvested mycelia were ground into a fine powder in liquid nitrogen, and the total RNA extraction was performed using the RNAeasy mini kit (Qiagen) and then quantified using the Gen5 software Take3 Sessions from Biotek Synergy HT spectrophotometer (Thermo Fisher Scientific). Synthesis of cDNA from total RNA was carried out using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions.

All the PCR reactions were performed using the QuantStudio 6 Flex Real-Time PCR System (Solis BioDyne) and 5x HOT FIREPol Probe qPCR Mix Plus (ROX) (Applied Biosystems). Amplification reactions were performed in a final volume of 10 ul reaction mixtures containing 1× HOT FIREPol Probe qPCR Mix, 100-300 nM forward primer, 100-300 nM reverse primer, and 100 ng cDNA templates. Real-time PCR protocols were as follows: 12 min initial denaturation at 95°C, followed by 40 cycles of 5 s at 95°C, 20 s at 60°C. All analyses were conducted independently in triplicate with no amplification control (no added primers) and carried out in 96-well plates, which were covered with optical tape. The specificity of PCR amplifications was documented by melting curve analysis. Transcript levels of *abfA* and *cbhI* genes were normalized and the data analysis was performed using the ΔΔCT method and the relative standard curve method in according to the amplification efficiency of the targets. The primers used in real-time PCRs are listed in **Table S4.**

Acknowledgements

This work was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 2012/20549-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 441912/2014-1). We are grateful to the scholarships provided by the Coordination for the Improvement of Higher Education Personnel (CAPES) and FAPESP (2014/15403-6 to MPZ; 2013/24988-5 to MVR), and we would like to acknowledge the Mass Spectrometry Laboratory of the Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM) coordinated by Adriana Franco Paes Leme, PhD.

Conflict of Interest

None declared.

SUPPLEMENTARY MATERIALS (ANEXO 1)

CAPÍTULO 3 – A deleção de genes com função predita em secreção resultou em maior produção de uma xilanase GH10 homóloga em *Aspergillus nidulans*

Neste capítulo encontra-se o manuscrito referente aos estudos de deleção de genes envolvidos na via de secreção em cepas de *A. nidulans* secretando xilanases homóloga e heteróloga, sendo os resultados apresentados no manuscrito **"The deletion of genes with predicted function in the secretion pathway in *Aspergillus nidulans* improved the production of homologous GH10 xylanase"** de autoria de Mariane Paludetti Zubieta, Jaqueline Aline Gerhardt, Marcelo Ventura Rubio, Cesar Rafael Fanchini Terrasan, Fabiano Jares Contesini, Rolf Alexander Prade e André Ricardo de Lima Damásio. Esse manuscrito, em anexo abaixo, será submetido para o peródico Microbial Cell Factories, fator de impacto 4,17.

3.1 Introdução

A maior vantagem dos fungos filamentosos em relação a outros organismos utilizados como plataforma para produção de produtos biotecnológicos é seu grande potencial na produção de enzimas extracelulares. Para alcançar esse potencial produtivo, diferentes técnicas têm sido utilizadas para melhoramento de cepas. A mutagênese aleatória (irracional) é comumente utilizada para introduzir muitas mutações no DNA, sendo que, as mutações que resultam no aumento de produtividade, dificilmente são definidas. Por outro lado, a manipulação genética racional de genes-alvo é mais eficiente, entretanto mais difícil de realizar. Neste contexto, diferentes abordagens têm sido desenvolvidas para aumentar a produção de proteínas em sistemas fúngicos de expressão. Esses estudos incluem melhoramento de linhagens através da deleção de proteases extracelulares, deleção de genes associados a autofagia, superexpressão ou deleção de genes associados à via de secreção de proteínas e manipulação da via de "*unfolded protein response*" (UPR). Além disso, a melhoria na produção de algumas enzimas também tem sido obtida pela introdução de múltiplas cópias dos genes recombinantes, o uso de promotores fortes nativos ou artificiais, a fusão gênica a uma proteína endógena conhecidamente bem secretada, dentre outros.

O desafio para os programas atuais e futuros no desenvolvimento de cepas visando a exploração de *Aspergilli* como plataforma de expressão é o completo entendimento da biologia molecular desses hospedeiros e a identificação das limitações da produção de enzims de interesse. Assim, abordagens racionais para modificar células para produção eficiente e em larga escala de bioprodutos são de substancial interesse econômico e científico.

Neste trabalho, foi explorada a hipótese de que a maioria das proteínas-alvo expressas em fungos filamentosos pode ser perdida ou presa na via secretória devido a problemas no processamento, modificações pós-traducionais (PTMs), ou *misfolding*, induzindo uma condição denominada estresse de RE. O estresse do RE ativa uma rede de sinalização chamada UPR para aliviar o estresse e restaurar a homeostase, promovendo sobrevivência e adaptação celular. Com base no exposto acima, nossa lógica é que a manipulação de genes relacionados à via secretória, especialmente aqueles induzidos em condições de estresse de RE é uma estratégia possível para melhorar a secreção de enzimas em *A. nidulans*. Inicialmente, um banco de dados foi gerado a partir de dados de RNA-seq de *A. nidulans* exposto a DTT e TM (indutores de UPR). A partir desses dados, analisando os genes diferencialmente expressos (DE), foi realizada uma investigação sobre como alguns genes com funções preditas na via secretória, influenciam a produção de duas xilanases pertencentes à mesma família, mas de fontes homóloga e heteróloga. Após a deleção de genes-alvo, a produção da xilanase homóloga foi de até 1,7 vezes maior, enquanto a produção da xilanase heteróloga permaneceu inalterada.

3.2 Manuscrito em inglês

"**The deletion of genes with predicted function in the secretion pathway in *Aspergillus nidulans* improved the production of homologous GH10 xylanase**"

Mariane P. Zubieta[1,2], Jaqueline A. Gerhardt [1], Marcelo V. Rubio [1], Cesar R. F. Terrasan [1], Gabriela Felix Persinoti, Fabiano J. Contesini [1], Rolf A. Prade [2], André Damasio [1]*

[1]Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, SP, Brazil

[2]Microbiology and Molecular Genetics, Oklahoma State University, Stillwater-OK, USA

*To whom correspondence should be addressed:

André Damasio; Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas-SP, Brazil Email: adamasio@unicamp.br

Keywords: *Aspergillus nidulans*, recombinant enzymes, homologous, heterologous, cellular stress, transcriptome, gene deletion, secretion pathway

Abstract

Filamentous fungi are one of the most important fungal cell factories utilized in large-scale enzyme production, however, the production is usually low. The limitations in the production of enzymes has stimulated research into the genetic manipulation of genes with predicted function in the proteins secretory pathway in filamentous fungi. This pathway is the major route for synthesis, folding and delivery of proteins to the cell exterior. In addition, a positive relationship between the production of recombinant enzymes in *Aspergillus* strains, and the UPR pathway has been observed. In this study, mycelium of *A. nidulans* were exposed to UPR-inducing chemicals and genes differentially expressed were identified through transcriptome analysis by RNA-seq. Twelve genes were deleted in *A. nidulans* recombinant strains producing homologous and heterologous GH10 xylanase. The deletion of a cyclophilin, a Hsp40 co-chaperone, a glycosyltransferase and a thioredoxin resulted in 125 170, 77 and 104% increase in the homologous xylanase production, respectively. Interestingly the deletion of these four genes decreased the secretion of total proteins, that is, the homologous xylanase production was specifically increased. On the other hand, the production of the heterologous xylanase and secretion of total proteins did not change after the deletions of these four genes. Considering the results, this approach demonstrated expressive increase in homologous enzyme production, which suggests that glycosyltransferase, chaperone, cyclophilin and thioredoxin have played role in the regulation of protein production in *A. nidulans.* We suggest that the increased production of enzymes in the deleted strains is related to the activation of the UPR and a "less stringent" protein folding by the cell, resulting in a mild quality control and higher secretion of proteins into the extracellular medium.

Background

Aspergillus is one of the most studied genera of filamentous fungi, largely because of the medical, food spoilage, and industrial relevance of some species. In addition, several *Aspergilli* have a long history as producers of plant polysaccharide modifying and degrading enzymes what is associated to a generalist lifestyle that allows broad utilization of carbon sources from different substrates in different habitats. Among many other species, *Aspergillus nidulans* is a genetic model that has been extensively studied with respect to metabolism, cellular development, and regulation, broadly contributing to the understanding of eukaryotic cell biology and molecular processes [1]. The biggest competitive advantage of filamentous

fungi over other microbial cell factories is their potential to produce high quantities of extracellular enzymes [1,2].

Microbial enzymes find applications in many fields, including chemical, fermentation, agricultural, pharmaceuticals, and food production. Choosing a suitable expression system is critical for the enzyme production rate, and many bacteria, filamentous fungi, and yeasts have been commonly used to express recombinant enzymes [3]. Biotechnological applications have increased due to the advantages of these species. In the bioenergy field, having an optimized cell factory for large-scale enzyme production would help in reducing costs, which is much higher than generally considered, as revealed by techno-economic analysis [4].

Random mutagenesis generally used for strains breeding may introduce many mutations into genomic DNA, and the causative mutations leading to increased productivity are mostly unknown. On the other hand, the rational genetic manipulation of target genes is more efficient, but it is more difficult to apply [5]. In this context, different approaches have been assessed to increase protein production in fungal expression systems. These studies include strain improvement by the deletion of extracellular proteases [6], deletion of autophagic genes [7], overexpression or deletion of genes associated to the secretory pathway [8] and manipulation of unfolded protein response (UPR) pathway [9]. Furthermore, improvement of secretion yield has also been obtained by introducing multiple copies of the expressed genes [10], the use of native or artificial strong regulators [11], gene fusion to a well-secreted endogenous protein, the use of native signal sequences and others [9]. However, strain improvement programs aiming to use the highly efficient protein synthesis, folding and export machinery of fungi has mainly succeeded for endogenous proteins. In fact, the production of most heterologous proteins, especially non-fungal proteins, is considered problematic, generally remaining one to two orders of magnitude lower [5].

The challenge for current and future strains development programs aiming the exploitation of *Aspergilli* as multi-purpose expression platform is the full understanding of the molecular cell biology of these hosts, the identification of pathway limitations and the substantiate prediction of beneficial metabolic engineering strategies [12]. Thus, rational approaches to modifying cells for efficient large-scale production of native metabolites, expression of heterologous biosynthetic pathways, or protein expression, are of substantial economic and scientific interest. The economic footprint of these cell factories ranges in hundreds of billions dollars per year on the biotechnological industries global market, such as the enzymes market that reached $5.0 billion dollars in 2016 [13].

Here, we explored the hypothesis that most of the target proteins expressed in filamentous fungi may be lost or stuck in the secretory pathway because of issues in processing, post-translational modifications (PTMs), or misfolding, inducing a condition termed endoplasmic reticulum (ER) stress. ER stress activates UPR to alleviate stress and restore homeostasis, promoting cell survival and adaptation [14].

A relevant study published in 2005 showed that approximately 23% of the genes up-regulated in *A. nidulans* recombinant chymosin-producing strain also present increased expression levels in response to the addition of dithiothreitol (DTT), an UPR inducer [15]. Other study performed a genome-wide transcriptional analysis of yeast mutant strains with improved secretion of an heterologous α-amylase [16]. Another study aiming to identify unknown factors associated with protein trafficking and secretion in *N. crassa* screened ~ 600 strains carrying deletions in genes with predicted functions in the secretory pathway looking for alterations in the secretion of cellulolytic enzymes [17,18]. Based on the stated above, our rationale was that the manipulation of genes related to the secretory pathway, especially those highly expressed under ER stress conditions, e.g., chemically induced or during production of recombinant enzymes regulated by a strong promoter, is a possible strategy to improve enzymes secretion in *A. nidulans*. First, a data bank was generated by RNA-seq of *A. nidulans* exposed to DTT and tunicamycin (TM) (UPR inducers). Thus, by analyzing the differentially expressed (DE) genes, we performed a deep investigation on how some genes with predicted functions in the secretory pathway and overexpressed under ER stress conditions, influence the production of two xylanases belonging to the same family, but from homologous and heterologous sources. After deletion of target genes, production of the homologous xylanase was 170% higher, while production of the heterologous xylanase remained unaltered.

Results

The level of transcript is not a bottleneck for the production of heterologous enzymes in A. nidulans

To study the expression and production of recombinant enzymes in *Aspergillus*, different strains producing six different thermophilic archaeal and bacterial enzymes were constructed. The genes encoding for xylanase (*tpet_0854*), arabinanase (*tpet_0637*), and arabinofuranosidase were isolated from *Thermotoga petrophila*. Genes encoding for endoglucanases were isolated from *Pyrococcus furiosus* and *Caldivirga maquilingensis*, and the gene encoding for processive endoglucanase was isolated from *Pyrococcus abyssi.* Codon usage for the *P. furiosus* and *C. maquilingensis* endoglucanases, and *P. abyssi* processive

endoglucanase were optimized for rice (*Oryza sativa*) and corn (*Zea mays*), respectively, and renamed *tcel1*, *tcel6* and *tcel4*, respectively. All genes were cloned into the pEXPYR expression vector and transformed in *A. nidulans* [19]. The expression level of each heterologous gene was measured by quantitative real-time PCR (qPCR) and the enzymes secretion was evaluated by assaying activity on their respective substrate.

A comparison of codon usage by *A. nidulans* in relation to corn, rice and *Thermotoga petrophila* showed that the frequencies of codons correspond to 95, 90 and 45%, respectively (http://www.kazusa.or.jp/codon/). All bacterial and archaeal genes were expressed even with codon usage bias percentage, showing no transcriptional impairment in the *A. nidulans* recombinant strains. The transcript levels for *tpet_0631* were 2-fold higher and *tcel4* were 3-fold lower than *tpet_0854* transcript level. However, enzymatic activity was only detectable for the xylanase (**Figure 1**). In the next section, we chose to delete genes that encodes proteins predicted to play a role in secretion and concomitantly UPR induced chemical stress among current strategies to develop recombinant protein expression system.

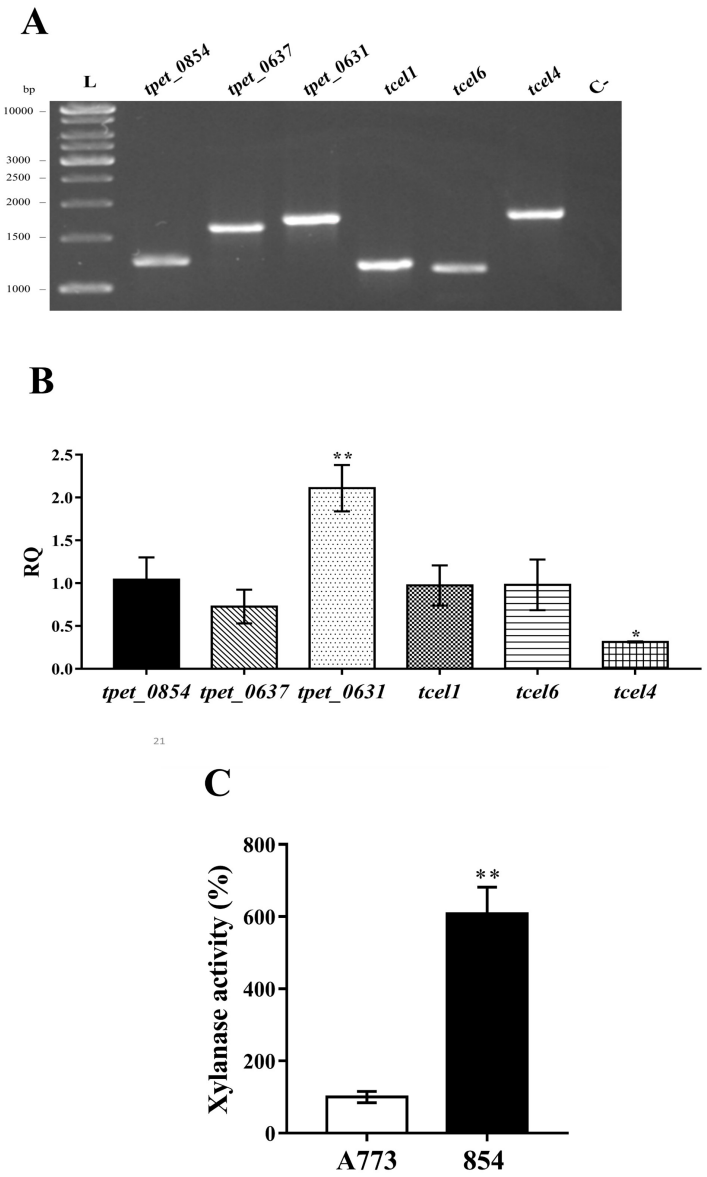


Fig. 1 – Overview of bacterial and archaeal enzymes production in *A. nidulans*. The *A. nidulans* strains expressing thermophilic archaeal and bacterial genes *tpet_0854*, *tpet_0637*, *tpet_0631*, *tcel1*, *tcel6* and *tcel4* were grown on minimal media containing 2% maltose for 36 h at 37°C. (A) Agarose gel electrophoresis analysis of PCR amplification fragments of heterologous genes expressed in *A. nidulans*. (B) qPCR of the recombinant genes was calculated by the Relative Standard Curve Method. The expression of each target gene was normalized using the gene *tubC* (tubulin) as reference and calibrated in relation to *tpet_0854* expression. (C) Xylanase activity in the crude extracellular produced by *A. nidulans* A773 (control strain), and *A. nidulans* 854 strain. One asterisk (*) identifies adjusted p-values between 0.01 and 0.05; two asterisks (**) identify adjusted p-values between 0.01 and 0.001.

Identification of DE genes under chemical-induced stress in A. nidulans

To generate a data bank of chemical-induced stress genes with predicted functions in the *A. nidulans* secretory pathway, the expression of classical UPR markers such as *bipA* and

hacA (*hacA*^{i} and *hacA*^{u}, induced and uninduced forms, respectively) were analyzed by quantitative PCR (qPCR). The expression levels of these genes were higher at early stages (2 h) of exposition of *A. nidulans* mycelia to DTT dropping at later periods (8 h) (**Additional file 1**). Thus, these times were set to perform the following experiments.

A. nidulans mycelia was exposed to DTT and TM [20] for 2 and 8 h, and analyzed by RNA-seq. A total of 294 million reads were generated and the average numbers of total filtered reads in the six groups were 38 million (2 h untreated control), 43 million (8 h untreated control), 40 million (2 h DTT), 40 million (8 h DTT), 38 million (2 h TM) and 41 million (8 h TM). These reads were mapped to the *A. nidulans* FGSC A4 reference genome. More than 94% of the filtered reads were mapped to the reference genome. A total of 10.774 genes were analyzed (**Additional file 2: Table S1**). For DTT, 1905 and 1172 genes were DE in 2 and 8 h, respectively, while for TM, 312 and 1862 genes were DE in 2 and 8 h, respectively (**Figure 2A and 2B, Additional file 2: Table S2**).

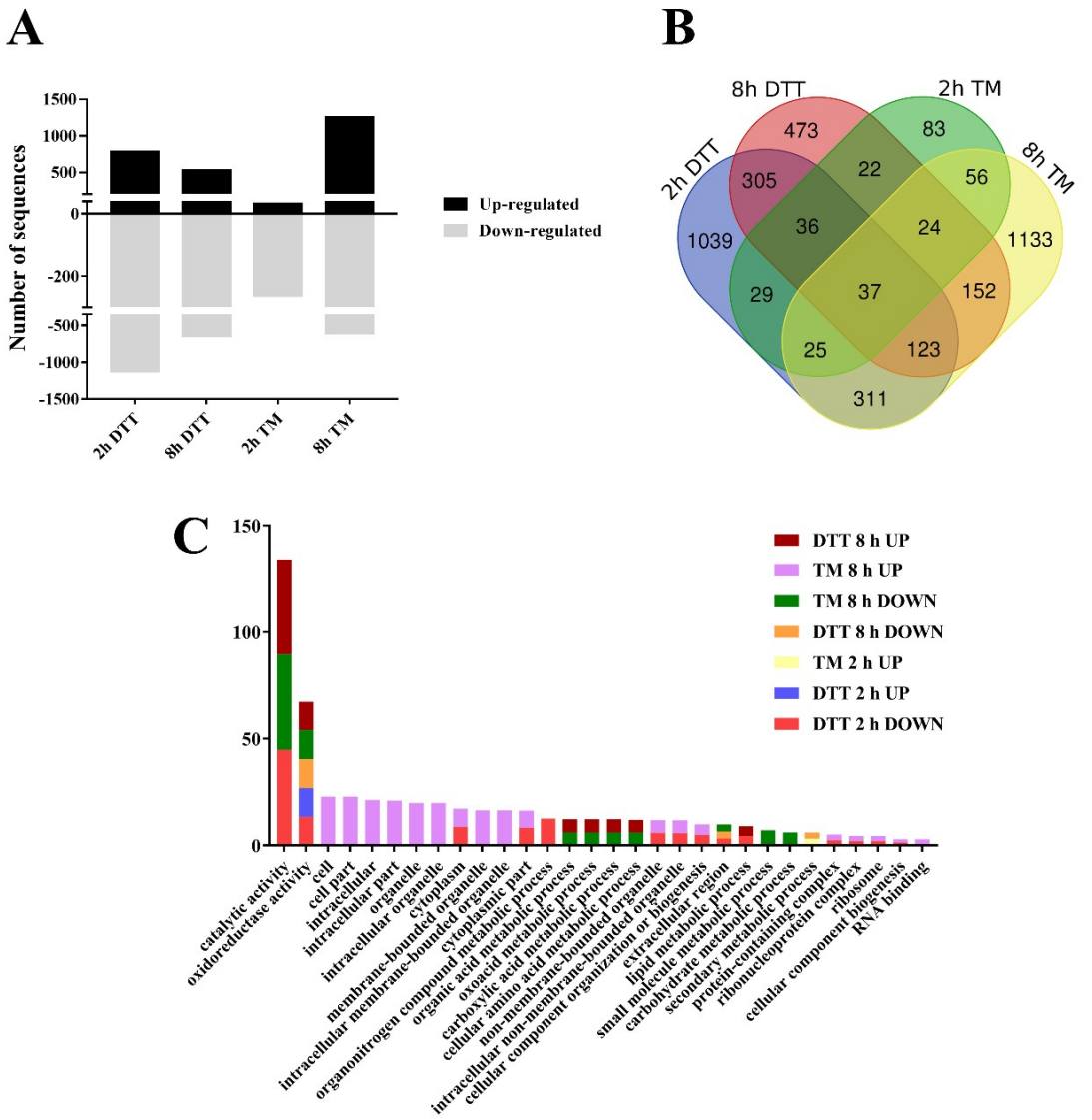


Fig. 2 – Analysis of DE genes after *A. nidulans* exposed to cellular stress-inducing chemicals. The *A. nidulans* A773 strains were grown on MM for 24 h, exposed to DTT and TM for 2 and 8 h, and then analyzed by RNA-seq. (A) Number of up- and down-regulated genes in each condition. (B) Venn diagram showing the DE genes. (C) Enrichment analysis of GO terms over-represented in the DE genes using the Blast2Go software. For the differential expression analysis, an adjusted p-value ≤ 0.01 was used as threshold, and a cutoff of log2 fold change ≥1 or ≤ −1 for TM treatment and log2 fold change ≥2 or ≤ −2 for DTT treatment.

Gene ontology (GO) enrichment of up-regulated genes was performed for functional analysis. The GO term "oxidoreductase activity" was enriched in the DTT 2 h treatment. “Catalytic activity” was found highly enriched and “lipid metabolic process”, “carboxylic acid metabolic process”, “organic acid metabolic process”, “cellular amino acid metabolic process”, “oxoacid metabolic process” were moderately enriched in the DTT 8 h treatment. Regarding the down-regulated genes, the GO terms “organonitrogen compound metabolic process”, “lipid metabolic process”, “cytoplasmic part”, “catalytic activity” and “cytoplasm”

were enriched in DTT 2 h, while “oxidoreductase activity” and “extracellular region” were enriched at DTT 8 h. Additionally, the GO term “secondary metabolic process” was enriched in the up-regulated genes in the TM 2 h treatment, while “intracellular” was enriched in 8 h (**Figure 2C**).

The genes encoding for proteins with predicted function in the secretory pathway were selected using a previously reported secretory model from *Aspergillus oryzae* as scaffold [10]. A total of 375 homologous genes were found in *A. nidulans* by performing a search in the *Aspergillus* Genome Database (*Asp*GD) (**Additional file 2: Table S3**). A set of 114 out 375 genes were up-regulated and 53 genes were down-regulated under DTT treatment, while 27 genes were up-regulated and 13 genes down-regulated with TM treatment (**Figure 3A**).

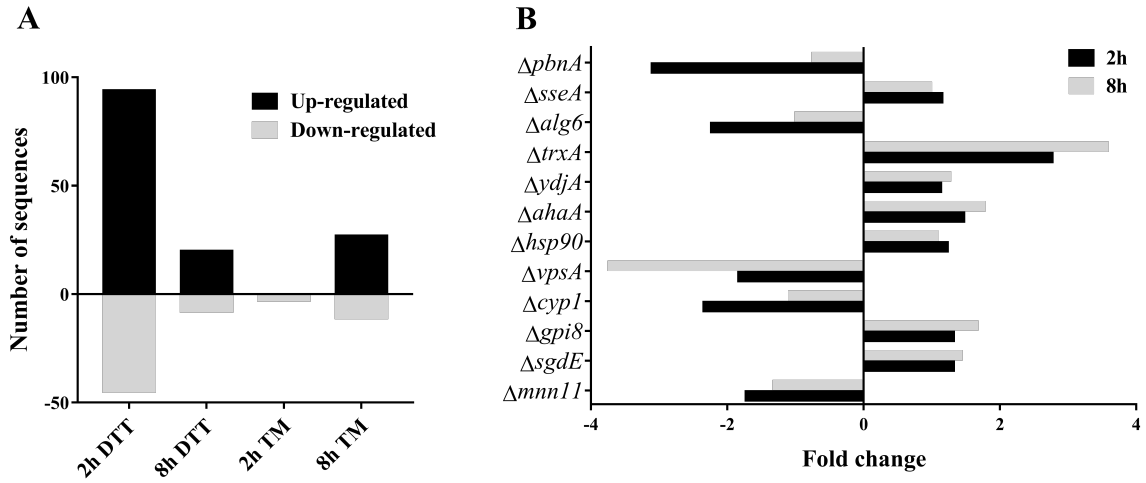


Fig. 3 – Genes associated to the secretory pathway in *A. nidulans* under stress conditions during different periods. Secretion genes identified in *A. nidulans* A773 after DTT and TM treatment after 2 and 8 h at 37°C. (A) A total of number of genes up- and down-regulated. (B) Expression profile of twelve genes chose to be deleted after chemical stress.

Over the 375 genes with predicted role in the secretion pathway, the target genes for deletion in *A. nidulans* were selected based on: (i) genes up- or downregulated in both treatment times, (ii) genes with highest and lowest fold-change, and (iii) genes with no redundant function in the secretory pathway, with predicted functions in different processes and not described as UPR marker. These approach resulted in the selection of 12 genes (Figure 3B and Additional file 3). Important secretion genes were selected based on the sequential criteria: (i) according to their expression profile at 2 and 8 h treatment in which, the expression profile was maintained up- or downregulated in both periods, (ii) ranking the top 25 genes with the highest and lowest fold-change, and (iii) the final list was composed by

genes with no redundant function in the secretory pathway, covering different processes and was not recognized as UPR marker. These ranking resulted in the selection of 12 secretion genes (**Figure 3B, Additional file 3 and 4**).

The deletion of four genes improved the production of the homologous xylanase in A. nidulans

The next step in our strategy was the deletion of the 12 previously selected genes in the *A. nidulans* recombinant strain producing the thermophilic xylanase (854) to improve the enzymatic production. For this purpose, in addition to this heterologous expression, we designed a recombinant strain producing a homologous xylanase (*xlnE*) allowing a comparison between the expression and production of homologous (5B3 strain; *xlnE*) and heterologous (854 strain) xylanases in the same biological system. These xylanases show 53% of amino acid sequence similarity and belong to the same glycoside hydrolase family (GH10) (**Figure 4A**). qPCR and enzymatic assays for the 5B3 and 854 strains showed no significant differences in the expression and activity of the homologous and heterologous xylanase (**Figure 4B and C**).

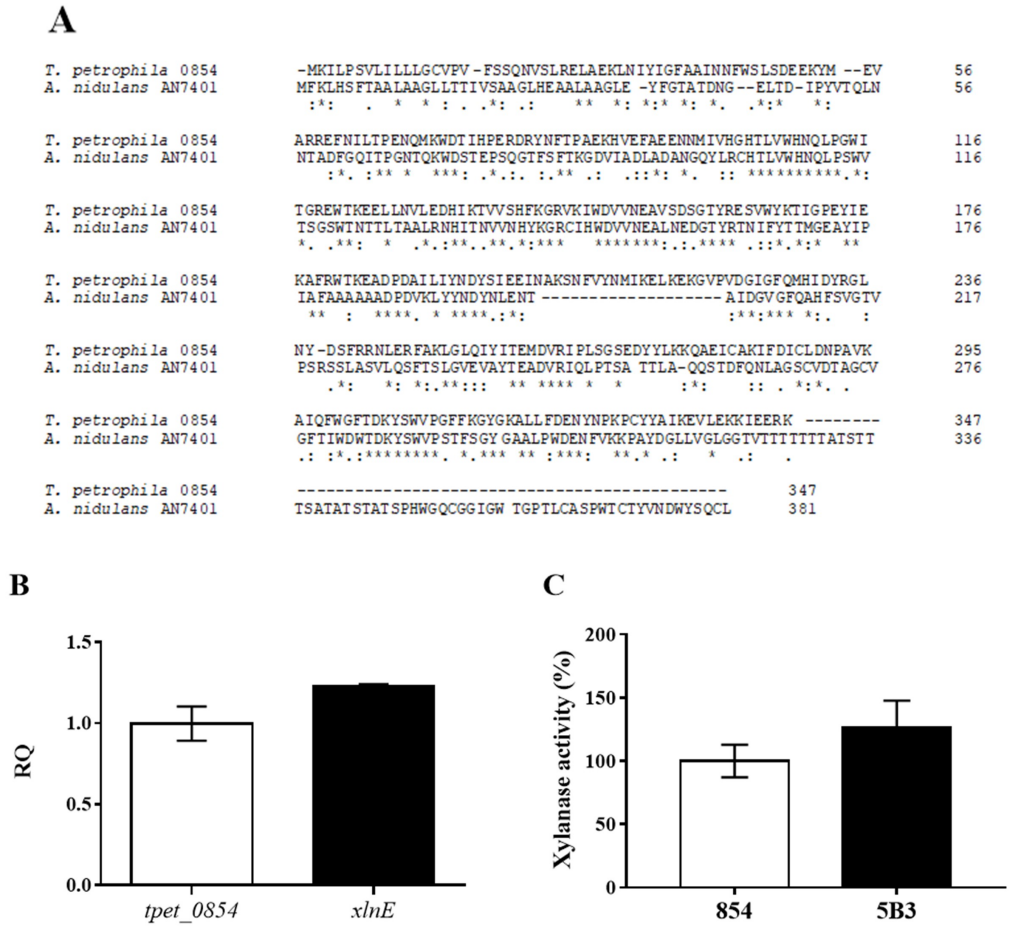


Fig. 4 – Overview of homologous and heterologous xylanase sequence, expression and production in *A. nidulans*. The *A. nidulans* 854 and 5B3 strains were grown in liquid MM supplemented with 2% maltose for 36 h at 37°C. (A) *In silico* protein alignment of xylanase sequences. (B) Expression of the genes calculated by qPCR using the Relative Standard Curve Method. The genes *tubC* (tubulin) and *tpet_0854* were used as reference and calibrator, respectively. (C) Xylanase activity in the crude extracellular filtrate produced by the *A. nidulans* 854 and 5B3 strains.

After successfully obtaining the recombinant strains 5B3 and 854, the auxotrophy for uridine and uracil was regenerated by selecting mutants growing in minimal medium (MM) with 5-fluorotic acid (5-FOA). A total of 20-30 5-FOA-resistant colonies appeared after three days of cultivation. After monosporic purification, more than 95% of the isolates showed uridine and uracil auxotrophy (*pyrG*$^{-}$). The strains 5B3.11 and 854.14 showed xylanase activity levels equivalent to their parental strains (data not shown) and were selected for further studies. We will continue to name these *pyrG*$^{-}$ strains as 5B3 and 854 during the manuscript. Transformation with the deletion cassettes was performed in the 5B3 and 854 strains, and deletions were confirmed by PCR and Southern blot (**Additional file 5**).

After obtaining the knocked-out mutants, protein secretion and xylanase activity were evaluated in the culture supernatant. 5B3 and 854 parental strains and the knock-out mutants were grown on MM containing 2% maltose for 36 h. In the 5B3 Δ*trxA*, Δ*cyp1*, Δ*ydjA* and Δ*pbnA* mutant strains, the xylanase activity was, respectively, 104, 125, 170, 77% higher relative to the control strain (**Figure 5A**). For the other 5B3 knock-out strains, no changes in the xylanase activity were observed (data not shown).

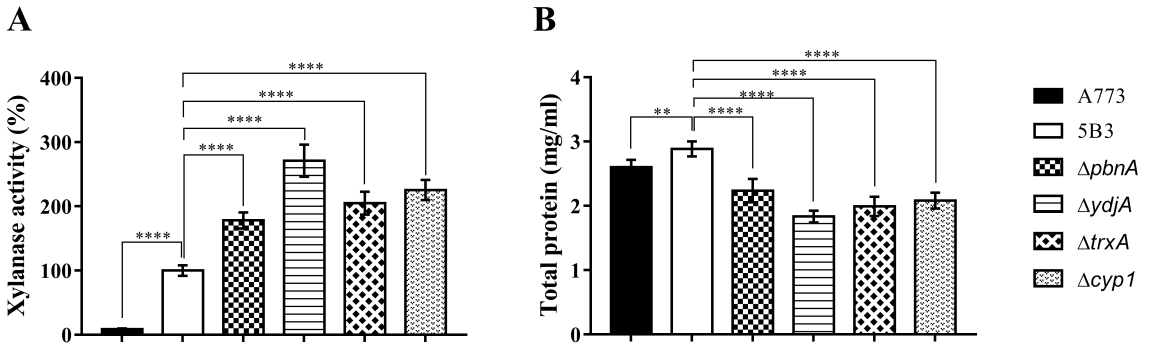


Fig. 5 – Extracellular xylanase and total proteins secretion by the *A. nidulans* 5B3 mutant strains. The *A. nidulans* 5B3 strain and its mutants were grown in liquid MM supplemented with 2% maltose for 36 h at 37°C. (A) Xylanase activity was assayed with Azo-Xylan as substrate, pH 5.5 at 50°C, and the data are shown relative to the 5B3 strain. (B) Protein quantification was assayed by the BCA method. Two asterisks (**) identify adjusted *p*-values between 0.01 and 0.001; four asterisks (****) identify adjusted p-values < 0.0001.

After analyzing the extracellular xylanase activity, we investigated whether the increased activity resulted from a specific increase in the xylanase production or from a general increase in protein secretion. Interestingly, lower level of protein secretion was observed for the 5B3 Δ*trxA*, Δ*cyp1*, Δ*ydjA* and Δ*pbnA* mutants, revealing that an increased xylanase secretion rather than a higher total protein secretion caused the activity increment (**Figure 5B**). On the other hand, the deletion of these four genes did not alter neither the xylanase production nor the level of secreted proteins in the 854 strain (**Additional file 6**).

Analysis of the mutant strains secreting homologous xylanase

To bring insights on why some deletions specifically increased the homologous xylanase secretion, we examined the unfolded protein response element (UPRE) in the promoters of *pbnA*, *cyp1*, *ydjA* and *trxA* genes using the JASPAR database [21]. The *hac1* matrix profile MA0310.1 from *S. cerevisiae* was used and the highest relative score was found for the following motifs: GACACGTC (0.969), AACACGTC (0.937), GCCACGTA (0.955), AACACATA (0.802), ACCACGTT (0.900), CCCACGTT (0.854), GACACGTA (0.984) in the promoters of *bipA*, *pdiA*, *hacA, pbnA*, *ydjA*, *trxA* and *cyp1*, respectively. The

CACGT motif was not found in the *pbnA* promoter, suggesting that probably this gene is UPR-independent (**Figure 6A**).

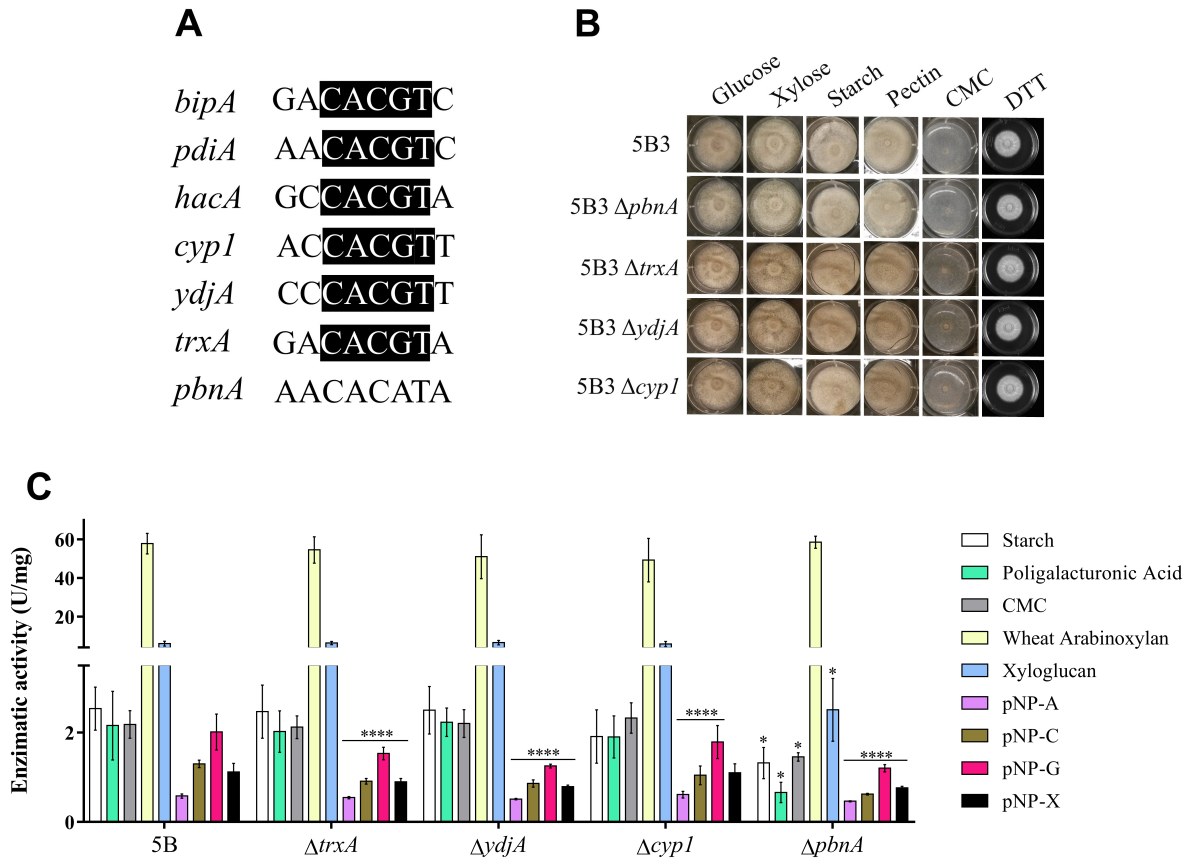


Fig. 6 - Genotypic and phenotypic analysis of *A. nidulans* 5B3 mutant strains. (A) Alignment of the putative promoters consensus sequences found in AN2062, AN7436, AN9397, *cyp1*, *ydjA*, *trxA* and *pbnA* genes. Black box: identical sequence. (B) Growth of the mutant strains on agar plates with different substrates and with DTT by 3 days at 37°C. (C) Enzyme activities in the culture supernatants of *A. nidulans* strains grown on 1% hydrothermal-pretreated sugarcane bagasse, pH 6.5 for 3 days at 37°C. Enzymatic activity assays were performed with different substrates at pH 5.5 and 50°C.

To investigate the phenotypes of the 5B3 mutant strains and if the genes deletion affected the cellular stress response, the strains were grown in the presence of DTT. Moreover, the mutant strains were cultivated on MM supplemented with glucose, CMC, xylose, starch or pectin to evaluate the growth. No changes in pigmentation or growth rate were found between the control and mutant strains (**Figure 6B**). In addition, the mutant strains were grown on hydrothermal-pretreated sugarcane bagasse and the secretion of different carbohydrate-active enzymes (CAZymes) was analyzed in the crude supernatants. Arabinofuranosidase, cellobiohydrolase, β-glucosidase and β-xylosidase activities were slightly lower in the 5B3 mutant strains, while amylase, pectinase, endoglucanase, xylanase and xyloglucanase activities were lower only in the Δ*pbnA* mutant strain (**Figure 6C**).

To further understand the functions of *pbnA*, *ydjA*, *trxA* and AN8506 in a secretory context, an interaction network was constructed using *S. cerevisiae* orthologues physical and genetic interactions data in the GeneMANIA software [22]. The networks revealed that the orthologues *cpr1* (*cyp1*), *pbn1* (*pbnA*), *ydj1* (*ydjA*) and *trx1* (*trxA*) have potential functional links with several genes/proteins involved in a wide range of processes related to protein secretion (**Figure 7A**).

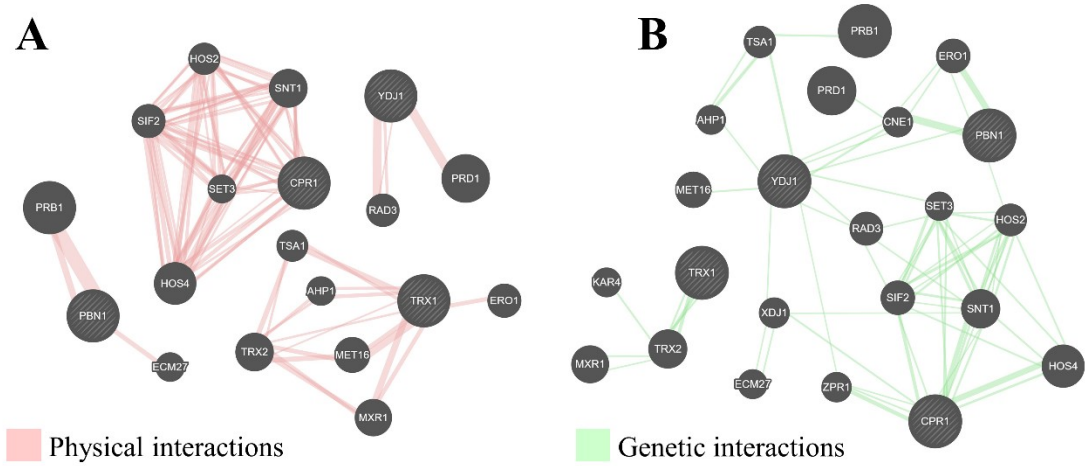


Fig. 7 – Physical and genetic interactions of *pbnA* (*pbn1*), *ydjA* (*ydj1*), *trxA* (*trx1*) and *cyp1* (*cpr1*) orthologous in *S. cerevisiae*. (A) Physical interactions (pink lines) and (B) genetic interactions (green lines) were analyzed using GeneMANIA online tool. Queries in scratched grey circles are the target genes and queries in smooth grey circles are the proteins/genes which directly interact with the target genes.

Notably, *cpr1* has both physical and genetic interactions with histone deacetylase genes complex such as *set3*, *hos4*, *hos2*, *sif2* and *snt1*. The *ydj1* interacts with *cne1* (calnexin), *prd1* (proteinase) and histone deacetylase complex. The *trx1* has a genetic and physical network but throughout these interactions it could influence networks of *ydj1*, *pbn1* and *cpr1*, and *pbn1* interacts with *prb1* (proteinase) and folding genes (*ero1* and *cne1*).

Discussion

Experimental evidences suggest that most target proteins expressed in filamentous fungi are usually lost or stuck in the secretory pathway due to errors in processing, modification or misfolding resulting in their elimination by the endoplasmic reticulum (ER) "quality control" [5]. Structural defects in proteins alter ER proper functioning and homeostasis, generating a state known as ER stress. This condition activates a conserved

signaling pathway known as UPR, which upregulates many genes responsible for restoring cell homeostasis by restructuring the secretory pathway [23]. Induction of the UPR pathway begins with the presence of unfolded proteins in the ER, which indicates to the ER-resident chaperone Kar2/BiP to dissociate from transmembrane protein IRE1. IRE1 subsequently oligomerizes for activation and splices the transcription factor *hacA*^{u} (uninduced form), creating a transcriptionally active form of *hacA*^{i} (induced form). HacA^{i} then moves to the nucleus where it recognizes and binds the UPREs in the promoter of regulated genes [23,24].

Given the usually low production of enzymes by *Aspergillus* strains, in-depth investigations are needed to improve the production and secretion of targets proteins in these microbial platforms. In *Aspergillus* strains, a positive relationship between the production of recombinant enzymes and the UPR pathway was previously reported [25]. Then, the manipulation of the UPR pathway and its components has been a common approach to overcome this limitation. But the question is: How does one decide which UPR genes are most appropriate to study? In *S. cerevisiae,* the UPR pathway results in activation of approximately 7-8% of the genome, *i. e*., more than 400 genes are activated [26]. The most used strategy to increase the production of recombinant proteins in several *Aspergillus* species has been the overexpression of key UPR genes encoding for foldases, chaperones and the transcription factor *hacA* (reviewed by 14). However, these strategies have not always led to satisfactory results [27]. In order to achieve more reliable results, some studies have been investigated other not so obvious ways and UPR-associated genes in the cell. In *A. oryzae*, the deletion of autophagy genes resulted in a higher production of bovine chymosin [7]. Thus, there is strong evidence of which a lot of candidate genes could be manipulated and there is no consensus on how these the genes must be manipulated to achieve improved enzyme secretion.

Based on these findings, we decide to perform RNA seq approaches to cover all genes and pathways regulated by UPR in a cell, indicating which genes can be targeted in *Aspergillus*. First, we evaluated the expression of the UPR genes, *hacA*^{i} and *bipA*, after treatment with two UPR inducers, DTT and TM to define the best time for transcriptome analysis. DTT is a strong reducing agent that prevents the formation of disulfide bonds and TM inhibits N- glycosylation by avoiding core oligosaccharide addition to newly synthesized proteins (**Additional file 1**) [25,26].

The RNA-seq and functional analysis performed for *A. nidulans* mycelia exposed to UPR-inducing chemicals revealed to be consistent with previous studies [15,28–30]. The presence of transcripts related to oxidoreductase activity, mainly 8 h after drugs treatment, is

partially explained by the role of DTT as reducing agent. In addition, sustained ER stress was reported to cause oxidative stress in yeast cells by the UPR-regulated oxidative folding machinery in the ER and mitochondria [31]. This seems to be a "side-effect" of cellular responses against ER stress, as oxidative protein folding intensified by loading of client proteins to the ER somehow results in the production of reactive oxygen species (ROS). Other processes enriched were lipid and cellular amino acid metabolic processes and can be explained by the ER expansion which accommodate, in turn, a higher influx of newly synthesized proteins [32] and supply the cell with amino acids to synthesize proteins involved in protection against reactive species, respectively [26,33]. The down-regulation of extracellular proteins can be associated to a process called *Repression Under Secretion Stress* (RESS) in which the addition of these drugs causes a reduction in protein secretion [34].

After transcriptome analysis, we developed *Aspergillus* strains that secretes heterologous enzymes. Some enzymes are very difficult to produce in a cost-effective manner such as the thermophilic enzymes. So far only a few of thermophilic enzymes produced in fungi or in bacteria were reported [35–37]. Differences in codon usage or improper folding of the proteins can result in reduced enzyme activity or low level of expression [38]. So, the codons from the archaeal *Pyrococcus furiosus*, *Caldivirga maquilingensis*, and *Pyrococcus abyssi* were optimized based on *A. nidulans* codon usage frequency. Even though seeing expression of these genes, no enzymatic activity was detected in these strains. Besides the differences in codon usage, many complex enzymes, like heterooligomers or those requiring covalently bound co-factors can be very difficult to produce in mesophilic host [37].

Even though all strains presented heterologous transcripts, the presence of extracellular enzymatic activity only in the 854 strain demonstrating that the bottleneck may be occurring during later cellular stages, *i. e.*, during translation or secretion (**Figure 1**). Thus, among a plethora of genes in our transcriptome which could undergo to manipulation, we focused on those with predicted function in the secretion pathway, the major route of enzyme production in filamentous fungi. The genes encoding for proteins with potential function in the secretory apparatus were selected according to Liu et al. 2014 [10]. These authors defined 369 genes of the *A. oryzae* secretory pathway by using a previously reported secretory model from *S. cerevisiae* as scaffold. Additional secretory components were obtained by performing a blast search with the functional components reported in other closely related fungal species such as *A. niger*. Components redundant in the different sources were excluded and finally 375 orthologues genes were included to the *A. nidulans* secretory list. Thus, we carefully

select twelve genes with different functions predicted in the secretory pathway (**Additional file 3 and 4**).

The deletion of the target genes *trxA*, *cyp1*, *ydjA* and *pbnA* markedly increased the production of homologous but not the heterologous xylanase (**Figure 5** and **Additional file 6**). Despite the above-mentioned progress, many challenges still lie ahead for viable expression of enzymes in fungal systems. Factors yet to be determined include the higher level of homologously produced proteins secretion when compared to heterologously, protein improvements for some proteins production are not necessarily transferable to other proteins and secretion of enzymes with high homology at significantly different levels (reviewed by [5,39,40]). Different molecular biological approaches to overcome this issue have been inconclusively discussed in the literature. A key issue is that our understanding of the different pathways of exocytosis is very limited, and we do not even know how many ways a cargo has to be secreted to the extracellular.

To further understanding how the deletion of the *ydjA*, *cyp1*, *trxA* and *pbnA* genes improved the production of *xlnE*, phenotype analysis and genetic network interactions was performed (**Figure 7**). UPREs is cis-acting element present in the promoters of UPR-target genes and were described in *A. niger* [41], but not in *A. nidulans.* However, it is difficult to find UPREs because are remarkably well conserved among homologous genes across the species boundary, whereas more variation occurs among the UPREs of the different UPR regulated genes within a single *Aspergillus* species [41]. After analysis of promoter of *A. nidulans* using the UPREs found in *A. niger* the results were inconclusive. Thus, we used the *hac1* upstream consensus sequence from *S. cerevisiae* as a model and five common nucleotides were identified in the promoter region of *ydjA*, *trxA* and *cyp1* suggesting these genes are regulated by UPR. The phenotype of the 5B3 mutant strains was primarily evaluated in MM with DTT, and the absence of the genes was not essential to metabolize this chemical compound (**Figure 6B**). In addition, deletion of the *pbnA* reduced secretion of the majority of the CAZymes quantified, suggesting that this gene is important for endogenous proteins processing (**Figure 6C**).

The *pbnA* gene is not characterized in *A. nidulans*, and its orthologous in *S. cerevisiae* (*pbn1*) encodes a chaperone-like protein, an essential component of the gycosylphosphatidylinositol-mannosyltransferase I complex. The network suggested physical interactions among *prb1* (vacuolar proteinase B) and *ecm27* (Na^{+}/Ca^{2+} exchanger). Then, the deletion of *pbnA* probably negatively affected the interactions with *prb1* and *ecm27*. *pbn1* is required for proper folding and/or the stability of a subset of proteins in the ER and its

depletion leads to a significant increase in the UPR pathway [42]. *prb1* contribute to eliminate toxic species of misfolded proteins by its essential role in autophagy and studies indicating that UPR genes is activated in autophagy-defective strains [43]. In addition, several *A. oryzae* autophagy-knockout strains present a three-fold increased production of bovine chymosin. These authors suggest that the improvement in protein productivity is due to an impairment in autophagic-induced degradation of the recombinant protein that normally occurs as part of the ER protein quality control [7]. Thus, we suggested that increase in the homologous xylanase production by *pbnA* strain may be result in UPR activation supported by the role of its homologous in proper folding and by loss of interaction with *prb1*.

The *trxA* gene encodes for thioredoxin (*trx1* orthologous in *S. cerevisiae*), an enzyme that participates in organism defense against reactive oxygen species (ROS) [44]. Organisms that use molecular oxygen as the final acceptor of electrons in the respiratory chain are exposed to ROS and high levels of these species can damage biological macromolecules. Detoxification mechanisms include the production of superoxide dismutases, catalases, peroxiredoxins, glutathione and the thioredoxin system, a dual system composed of thioredoxin and thioredoxin reductase (TrxR) [44]. *trx1* has physical interactions with *trx2* (thioredoxin), *ero1* (protein disulfide isomerase), *met16* (3'-phosphoadenylsulfate reductase), *ahp1* (thiol-specific peroxiredoxin), *tsa1* (thioredoxin peroxidase), and *mxr1* (methionine-S-sulfoxide reductase) (**Figure 7**). Excepting for *met16*, all these genes have a role of stress protection. *met16* participates in sulfate assimilation and methionine metabolism, and the peroxiredoxin Tsa1 plays dual functions, presenting peroxidase activity and acting as a molecular chaperone [45]. Analyzes of the genetic interactions showed a crosstalk between *trx1* and *pbn1* networks through *tsa1* and *prb1*. *tsa1* genetically interacts with *prb1* which, in turn, is physically linked to *pbn1*. After these analyses, we suggest that *trxA* deletion in *A. nidulans* causes the loss of interaction with *prb1* through *tsa1* orthologues, resulting in the same effect that deletion of *pbnA* in the *Aspergillus* cells.

The *yjdA* is a molecular chaperone Hsp40 and that have the *ydj1* as orthologous in *S. cerevisiae.* Hsp40 chaperones play critical roles in cell physiology, acting in conjunction with Hsp70 members to promote protein folding, assembly, translocation and degradation [46]. A group of chaperones that includes Ydj1 is induced in strains overexpressing *hac1* [47]. In our network analysis, *ydj1* physically interacts with *prd1* that encodes an intracellular proteinase involved in protein degradation [48]. In addition, *ydj1* genetically interacts with *pbn1* (*pbnA* orthologous), *tsa1* and *cne1* (calnexin). As a key ER chaperone, calnexin assists folding and subunit assembly of most Asn-linked glycoproteins that pass through the ER. Binding and

release cycles of molecular chaperones (calnexin/calreticulin) are controlled by the availability of terminal glucosyl residues on monoglucosylated Asn-linked oligosaccharides [49]. Thus, the deletion of *ydjA* in *A. nidulans* may have resulted in higher *xlnE* production due to sum of integrated factors such as less stringent quality control of unfolded proteins and UPR activation by loss interaction with *cne1* and *pbn1*, respectively.

Finally, *cyp1* (*cpr1* orthologous in *S. cerevisiae*) encodes a cyclophilin, which is a peptidyl-prolyl cis-trans isomerase (PPiases) and catalyzes *cis-trans* isomerization of peptide bonds in N-terminal proline residues. Proline is the only natural amino acid adopting both cis and trans conformations of its peptide bond, leading to distinct conformational changes in prolyl-containing polypeptide backbones [50]. Unlike regular peptide bonds, the process of cis-trans isomerization requires energy and it is catalyzed by PPiases [51]. The *cpr1* showed physical and genetic interactions with *set3*, *hos4*, *hos2*, *sif2* and *snt1.* These genes, including *cpr1*, forms the Set3p complex (SET3C) in which the subunits Hos2, Set3, Sif2, and Snt1 form the core complex, while Hst1p, Sum1p, and Cpr1p appear to be peripherally associated [52]. Many of these proteins are histone deacetylases (HDACs) that revert the reactions catalyzed by histone acetyltransferases (HATs). The acetylation of lysine residues in histones results in a less restrictive chromatin structure that is generally associated to transcriptional activation. On the other hand, deacetylation has been identified as a major regulator of eukaryotic gene transcription due to a repressive chromatin structure [53]. Then, the SET3C complex is responsible for transcriptional repression of some genes related to early/middle class of sporulation-specific genes including the key meiotic regulators in yeasts [52]. Based on the functional role of the *cyp1*/*cpr1* it is difficult to postulate a relationship between SET3C complex and higher secretion of homologous xylanase.

Our data do not allow explain why the thermophilic xylanase production did not increase after the deletions in *A. nidulans* even detecting mRNA levels equivalent to the homologous xylanase expression. mRNA stability, processing and translational efficiency are essential during heterologous expression, and at least to some extent depend on the coding sequence of an individual gene. The use of synonymous codons may vary widely between different genes and organisms. Sharp and Li observed that the variability in mRNA levels of different genes is related to their codon usage and the genome-wide codon usage is related to the number of tRNA genes copies [54]. Studies in *E. coli* have experimentally demonstrated that perturbation in the codon usage of a set of 40 proteins affected both the translation and the tRNA levels [55]. In addition, several studies have focused on the kinetics of translation.

Mehra and Hatzimanikatis studied the rates of initiation, elongation and termination and found that the response to mRNA levels is primarily dependent on the initiation step [56].

In conclusion, we successfully constructed *A. nidulans* strains capable of producing high levels of homologous xylanase after deleted four secretion genes induced by drugs that induces UPR. This analysis showed that the filamentous fungi can increase the productivity of recombinant proteins after rational engineering.

Experimental Procedure

Strains, primers, culture conditions and reagents

The strains used in this study are listed in the **Additional file 7**. *A. nidulans* A773 and its derivative strains were propagated on Minimal Medium (MM) (1X Clutterbucks salts (20X Clutterbucks salts: 1.4 M $NaNO_3$, 0.13 M KCl, 0.042 M $MgSO_4.7H_2O$ and 0.22 M KH_2PO_4), 1X trace elements (1000X Trace elements: 7.2 mM $ZnSO_4.7H_20$, 17.7 mM H_3BO_3, 2.52 mM $MnCl_2.4H_20$, 2.72 mM $FeSO_4.7H_20$, 0.95 mM $CoCl_2.5H_20$, 0.7 mM $CuS0_4.5H_20$, 0.21 mM $Na_2Mo0_4.4H_20$ and 17.11 mM EDTA)) pH 6.5 at 37°C. MM was supplemented with 2.5 mg/L uridine, 2.5 mg/L uracil, 1 mg/L pyridoxine, 1% (w/v) glucose, 2% (w/v) maltose, 1% (w/v) hydrothermal-pretreated sugarcane bagasse and 1.5% (w/v) agar when appropriate. Hydrothermal-pretreated sugarcane bagasse was submitted in a hammer-mill and carried out in a 350-L stainless steel-coated cast iron reactor, equipped with a stirrer and heated through a jacket with thermal oil located in Brazilian Bioethanol Science and Technology Laboratory (CTBE). The reaction conditions employed were as follows: 160°C, 60 min and 1:10 solid/liquid ratio (w/w). 5-Fluorotic acid (5-FOA) was purchased from Oakwood Chemical (CAS 207291-81-4) and all other chemicals were from Sigma-Aldrich or as indicated. The *E. coli* strain DH5α was used for cloning and plasmid propagation.

RNA preparation and RNA-seq analysis

Liquid batch cultures of *A. nidulans* A773 were inoculated with 10^6 spores/mL in 30 mL of MM at 37°C/150 RPM. After 48 h, the mycelium was treated with 20 mM DTT or 10 ug/mL TM and collected after 2 and 8 h-incubation at 37°C. Mycelium without drugs treatment was included as a control. Mycelia was separated from the culture supernatant by filtration through Miracloth (Merck), washed with ultrapure water and immediately frozen in liquid nitrogen. Frozen mycelia were ground into a fine powder and total RNA was isolated using TriZol reagent (Invitrogen) according to manufacturer's instructions. All RNA samples were treated with DNase I (Fermentas), purified with the RNeasy MinElute Cleanup Kit

(Qiagen), and RNA integrity was estimated using Agilent 2100 Bioanalyzer following the manufacturer's instructions.

The library construction was performed using the TruSeq Stranded mRNA Sample Preparation v2 kit (Illumina). Validation of the library was performed using an Agilent Bionalyzer followed by quantification using KAPA Library quantification kit for qPCR. Libraries were pooled in equimolar amounts and sequenced on the Illumina HiSeq 2500 system (paired-end 2x100 bp, ~15 Mill. reads/sample) at the CTBE NGS sequencing facility. Removal of low quality reads and Illumina adapters was performed using Trimmomatic [57]. The remaining reads were then aligned to the SILVA - high quality ribosomal RNA databases [58] using the SortMeRNA software to remove ribosomal RNA, and aligned to *A. nidulans* reference genomes available in the *Aspergillus* Genome Database (*Asp*GD) using the TopHat 2 software [59]. Differential gene expression analysis was performed using DESeq2 [60] with the raw counts obtained from subread function FeatureCounts [61]. Differentially expressed genes were identified by pairwise comparisons between treatments and analyzed by fold change ≥1 or ≤-1 for TM and log2 (fold change) ≥2 or ≤-2 for DTT treatment and an adjusted p-value <0.01 as threshold. Blast2GO v2.6.4 [62] was used for functional annotation of *A. nidulans* transcriptome. GO Enrichment analysis (Fisher's Exact Test) was used (p-value ≤ 0.05) to detect functional categories of biological processes and enriched molecular functions with statistical significance (FDR ≤ 0.05).

Construction of *Aspergillus* strains

The primers used in this study are summarized in **Additional file 8**. To construct heterologous and homologous strains, the genes *tcel1*, *tcel4*, *tcel6*, *tpet_0637*, *tpet_0631*, *tpet_0854* and *xlnE* were amplified separately by PCR, digested with *Nco*I and *Xba*I, and ligated onto *Not*I/*Xba*I digested pEXPYR plasmid [19] with T4 DNA ligase. The ligation product was then transformed into chemically competent *E.coli* and screened by colony PCR.

Before the construction of *Aspergillus* strains with a deleted gene, the *pyrG* selectable marker was recycled in the strains that secretes a homologous (*xlnE*, 5B3 strain) and heterologous (*tpet_0854*, 854 strain) xylanase. Freshly harvested conidia from these strains were suspended in water and plated on MM supplemented with uridine, uracil and 2 mg/ml 5-FOA. 5-FOA-resistant colonies were observed after incubation for 2 days at 37°C. Auxotrophic mutants was confirmed by plating replicas on MM agar plates supplemented with uridine, uracil in presence or absence of 5-FOA. The 5B3.11 and 854.14 mutants were selected based on the auxotrophic reversion and similar levels of recombinant xylanase. The

deletion cassettes used in this work were purchased from the Fungal Genetics Stock Center (FSGC) (Manhattan, Kansas, USA). The deletion cassettes from the target genes to be deleted from FGSC contain (from 5′ to 3′ position) 0.6-0.9 kb of the 5′ sequence flanking the target gene ORF, the selectable marker *pyrG* (orotidine-5′-decarboxylase gene from *A. fumigatus*), and 0.6-0.9 kb of the 3′ sequence flanking the target gene ORF.

All constructions were transformed in *A. nidulans* strains by previously described methods [63]. Conidia and mycelium of *A. nidulans* strains were inoculated into YG medium (5 g yeast extract, 2% glucose and 400 µL of 1000 X trace elements) supplemented with appropriate selectable marker and incubated at 130 RPM and 30°C. After 13 h, germinated conidia were washed in fresh YG, DSPS (1.1 M KCl, 0.1 M citric acid, 1 M KOH to correct pH to 5.8) and resuspended in 8 ml YG and 8 ml DSPS with 125 mg lysozyme from chicken egg white (Sigma L7651) and 1.02 g Vinotaste Pro (Novozymes). The slurry was incubated for 2 h, 100 RPM, 30°C, and the obtained protoplasts were centrifuged, washed twice with 30 ml 0.6 M KCl by centrifugation (2500 × g, 4°C, 10 min). The pellet was washed successively with 30 ml and 2 mL STC 50 (1.2 M sorbitol, 10 mM $CaCl_2$ and 50 mM Tris-HCl pH 7.5), and resuspended in 500 uL STC 50. In a new tube, 10 µL of the deletion cassette or the plasmid preparation was mixed with 100 µL protoplasts solution and 50 µL of filtered 25% (w/v) PEG in STC 50. After 20 min on ice, 1 ml of 25% PEG was added and the mixture was incubated at room temperature for 20 min. Then, 4-5 mL STC 50 was added and 1 ml of the suspension was poured onto protoplast-recovery medium (supplemented with 1.2 M sorbitol and appropriate selectable marker). Plates were incubated at 37°C until colonies growth.

Monosporic purification was performed by spreading conidia suspensions from the transformant strains on MM agar plates supplemented with appropriate selectable marker. Plates were incubated for 24 h at 37°C and colonies grown from a single nucleus were manually excised and transferred to new plates. Five rounds of this procedure were sequentially performed to obtain genetically pure mutants (homokaryotic).

Mutants were validated by spore PCR and Southern Blot. Spore PCR was performed by directly using conidia using a protocol based on Hervás-Aguilar and collaborators [64]. Conidia were taken from the colonies surface using a toothpick and suspended in 100 µL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl buffer pH 8) in tubes containing 150 mg of 1 mm glass beads. The tubes were vortexed for 30 s and incubated for 30 min at 65°C, vortexed every 10 min and then centrifuged top speed for 5 min. Two independent PCRs were performed, one using primers anchored outside the flanking targeting sequence and a second PCR using primers anchored within the cassette

(**Additional file 5**). For Southern Blot analysis, 80 µg of genomic DNA was cleaved with Fast Digest restriction enzymes (Thermo Fisher Scientific), according to the manufacturer's instructions. Southern blot was performed according to Southern [65], and the DNA fragments were detected using the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare), according to the manufacturer's instructions.

Protein evaluation: SDS-PAGE profile, enzymatic assays and protein determination

To quantify enzymatic activities, fresh conidia from *A. nidulans* A773 and mutants were inoculated into MM supplemented with maltose for promoter induction and cultivation was performed for 36 h at 37°C. Protein concentrations were determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. About 10 ug of supernatant proteins were analyzed by SDS-PAGE [66]. Xylanase activities were performed by 20 min/50°C for the homologous xylanase (*xlnE*, 5B3 and its mutants strains), and 30 min/85°C for the heterologous xylanase (*tpet*_0854, 854 and its mutants). Xylanase activitiy was assayed in a mixture consisting of 50 µl of 50 mM sodium acetate buffer pH 5.5, 250 uL of Azo-Xylan (Megazyme) and 200 uL of enzymatic extract. Endoglucanase (*tcel1* and *tcel6*), processive endoglucanase (*tcel4*), arabinanase (*tpet_0637*) and arabinofuranosidase (*tpet_0631*) activity assays were performed for 12 h in phosphoric acid swollen avicel (PASC), debranched arabinan and arabinan, respectively. The crude supernatants from the 5B3 strain and its mutants grown on 1% (w/v) hydrothermal-pretreated sugarcane bagasse were evaluated for enzymatic activities against starch, polygalacturonic acid, carboxymethyl cellulose (CMC), wheat arabinoxylan, xyloglucan, pNP-arabinofuranoside, pNP-cellobioside, pNP-glucopyranoside and pNP-xylopyranoside. The enzymatic reaction mixtures consisted of 50 µl of 50 mM sodium acetate buffer pH 5.5, 20 ul of enzymatic extracts and 50 ul of each substrate at 0.5%. Incubation was carried out for 30 min at 50°C. Absorbance was read at 405 to estimate pNP release [67], at 540 nm for reducing sugars release with the DNS method [68] and 590 nm with the Azo-Xylan method. All assays were performed in triplicate and specific activities were expressed as U/mg of protein.

RNA extraction and transcript analysis by qPCR (quantitative real-time PCR)

Total RNA was isolated from frozen mycelia with liquid nitrogen, followed by extraction using Direct-zol RNA MiniPrep (Zymo Research), according to the manufacturer's instructions. OD 260/280 were read by the DeNovix DS-11 spectrophotometer and ratios

were maintained close to 2.0 for all samples. Total RNA free DNA was subjected to reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific). cDNA samples were diluted to 50 ng/uL with RNA free sterile water. For each qPCR reaction was used 2 ul of cDNA (100 ng), 5 µL of 2X Hot FirePol EvaGreen qPCR Supermix (Solis Biodyne), forward and reverse primers (**Additional file 8**) and nuclease-free water to 10 uL final volume. The qPCR protocol consisted of 12 min of initial denaturation at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and it was performed using QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). All PCRs were carried out in biological and technical triplicates. Gene expression levels in the different samples were determined using a Relative Standard Curve Method and utilized beta-tubulin (*tubC*) as reference gene.

Statistical analysis and interaction network construction

Results are given as mean values and standard deviation from three independent experiments. The significance of differences in multigroup comparison was evaluated by one-way analysis of variance followed by unpaired Student's t-test using GraphPrism software. The genes *trx1*, *ydj1*, *cpr1* and *pbn1* encoding the *S. cerevisiae* orthologs of *A. nidulans trxA*, *ydjA*, *cyp1* and *pbnA* was used to construct the genetic and physically networks using the GeneMania software.

ADDITIONAL FILES (ANEXO 1)

CAPÍTULO 4 - Conclusão Geral

Os fungos filamentosos são amplamente utilizados como plataformas de expressão em biotecnologia com base no seu potencial para secretar grandes quantidades de proteínas. Neste trabalho, a via UPR foi investigada, pois consiste numa das principais barreiras na produção de proteínas de interesse.

Inicialmente, realizamos análises proteômicas intra- e extracelular em cepas recombinantes de *A. nidulans* que produzem enzimas heterólogas, com o objetivo de compreender e conhecer os perfis de proteínas diferencialmente produzidas. Analisamos o proteoma intracelular e extracelular de *A. nidulans* A773, uma cepa transformada com o vetor pEXPYR vazio (Anid_pEXPYR), e outras duas cepas de *A. nidulans* que secretam proteínas heterólogas de *A. fumigatus*, Anid_AbfA que produz arabinofuranosidase GH51 e Anid_Cbhl que produz celobiohidrolase GH7. Como resultado, o perfil intracelular das cepas recombinantes são semelhantes apesar de produzir proteínas heterólogas diferentes. A cepa Anid_Cbhl secreta mais enzima recombinante do que Anid_AbfA e pode estar relacionado ao maior número de espectros totais das proteínas PdiA, BipA, TrxA e AldH, que aliviam o estresse oxidativo e do RE, melhorando a produção de proteínas heterólogas. Além disso, os seguintes processos: metabolismo energético, metabolismo de aminoácidos, biogênese de ribossomos, tradução, estresse de retículo e oxidativo, e desenvolvimento foram os principais processos enriquecidos nas cepas recombinantes. O mecanismo de *Repression Under Secretion Stress* (RESS) pode estar presente nas cepas recombinantes, evitando, assim, a carga elevada de proteínas no RE durante a produção das proteínas heterólogas em *A. nidulans*. Além disso, a diferença nos níveis de secreção das proteínas heterólogas pode estar diretamente relacionada com características intrínsecas da sequência primária de aminoácidos, tal como número ímpar de cisteínas e o número preditos de sítios de N-glicosilação.

Posteriomente, foi realizada uma análise do transcriptoma do micélio de *A. nidulans* tratado com indutores de UPR, como DTT e TM, por 2 e 8 horas. Dos genes detectados no trancriptoma, selecionamos um total de 375 com função predita na secreção de proteínas em *A. nidulans*. Dentre esses, selecionamos 12 genes para serem deletados em 2 cepas recombinantes de *A. nidulans*, sendo que uma delas produz uma xilanase homóloga e a outra uma xilanase heteróloga termofílica. Os resultados mostraram que apenas a atividade enzimática da xilanase homóloga foi maior nos mutantes 5B3 Δ*trxA*, Δ*cyp1*, Δ*ydjA* e Δ*pbnA*. Além disso, essas cepas mutantes apresentaram menor quantidade de proteína total secretada, mostrando que o metabolismo do fungo direcionou para maior secreção específica da xilanase homóloga.

Referências

AMORES, G. et al. Recent progress on systems and synthetic biology approaches to engineer fungi as microbial cell factories. **Current Genomics**, v. 17, n. 2, p. 85–98, 2016.

BENDTSEN, J. D. et al. Improved prediction of signal peptides: SignalP 3.0. **Journal of Molecular Biology**, v. 340, n. 4, p. 783–795, 2004.

BLOUNT, B. A. et al. Rational diversification of a promoter providing fine-tuned expression and orthogonal regulation for synthetic biology. **PLoS ONE**, v. 7, n. 3, p. 1–11, 2012.

BOLGER, A. M.; LOHSE, M.; USADEL, B. Trimmomatic: a flexible trimmer for Illumina sequence data. **Bioinformatics**, v. 30, n. 15, p. 2114–2120, 2014.

BOWERS, G. N. et al. High-purity 4-nitrophenol: purification, characterization, and specifications for use as a spectrophotometric reference material. **Clinical Chemistry**, v. 26, n. 6, p. 724–729, 1980.

BRADFORD, M. M. A Rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976.

BRANDL, J.; ANDERSEN, M. R. Aspergilli: Models for systems biology in filamentous fungi. **Current Opinion in Systems Biology**, v. 6, p. 67–73, 2017.

BUCHLER, M.; TISLJAR, U.; WOLF, D. H. Proteinase yscD (oligopeptidase yscD). Structure, function and relationship of the yeast enzyme with mammalian thimet oligopeptidase. **European Journal of Biochemistry**, v. 219, p. 627–639, 1994.

BURGGRAAF, A. M.; RAM, A. F. J. Autophagy is dispensable to overcome ER stress in the filamentous fungus *Aspergillus niger*. **Microbiology Open**, v. 5, n. 4, p. 647-658, 2016.

CALFON, M. et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. **Nature**, v. 415, n. 6867, p. 92–96, 2002.

CARVALHO, N. D. et al. Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in *Aspergillus niger* reveals a coordinated cellular response to counteract ER stress. **BMC genomics**, v. 13, n. 1, p. 2–17, 2012.

CHERRY, J. R.; FIDANTSEF, A. L. Directed evolution of industrial enzymes: an update. **Current Opinion in Biotechnology**, v. 14, n. 4, p. 438-443, 2003.

CHRISTENSEN, T. et al. High level expression of recombinant genes in *Aspergillus oryzae*. **Nature Biotechnology**, v. 6, n. 12, p. 1419–1422, 1988.

CONESA, A. et al. Calnexin overexpression increases manganese peroxidase production in *Aspergillus niger*. **Applied and Environmental Microbiology**, v. 68, n. 2, p. 846–51, 2002.

CONESA, A. et al. The secretion pathway in filamentous fungi: A biotechnological view. **Fungal Genetics and Biology**, v. 33, n. 3, p. 155–171, 2001.

COX, J. S.; WALTER, P. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. **Cell**, v. 87, p. 391–404, 1996.

CRIPWELL, R. A.; ROSE, S. H.; VAN ZYL, W. H. Expression and comparison of codon optimised *Aspergillus* tubingensis amylase variants in *Saccharomyces cerevisiae*. **FEMS Yeast Research**, v. 17, n. 4, p. 1–12, 2017.

DAMÁSIO, A. R. D. L. et al. Improvement of fungal arabinofuranosidase thermal stability by reversible immobilization. **Process Biochemistry**, v. 47, n. 12, p. 2411–2417, 2012.

DAVY, A. M.; KILDEGAARD, H. F.; ANDERSEN, M. R. Cell factory engineering. **Cell Systems**, v. 4, n. 3, p. 262–275, 2017.

DE VRIES, R. P. et al. Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus Aspergillus. **Genome Biology**, v. 18, n. 1, p. 28, 2017.

DEMAIN, A. L.; VAISHNAV, P. Production of recombinant proteins by microbes and higher organisms. **Biotechnology Advances**, v. 27, n. 3, p. 297–306, 2009.

DEN HAAN, R. et al. Cellobiohydrolase secretion by yeast: Current state and prospects for improvement. **Process Biochemistry**, v. 48, n. 1, p. 1–12, 2013.

DEWAN, S. S. **Global Markets for Enzymes in Industrial Applications**. Disponível em: <https://www.bccresearch.com/market-research/biotechnology/enzymes-industrial-applications-report-bio030j.html>.

DRIOUCH, H.; MELZER, G.; WITTMANN, C. Integration of in vivo and in silico metabolic fluxes for improvement of recombinant protein production. **Metabolic Engineering**, v. 14, n. 1, p. 47–58, 2012.

DUFFNER, F. et al. A new thermoactive pullulanase from *Desulfurococcus mucosus*: cloning, sequencing, purification, and characterization of the recombinant enzyme after expression in Bacillus subtilis. **Journal of Bacteriology**, v. 182, n. 22, p. 6331–8, 2000.

EMRI, T. et al. Physiological and morphological changes in autolyzing *Aspergillus nidulans* cultures. **Folia Microbiologica**, v. 49, n. 3, p. 277–284, 2004.

FAN, F. et al. Genome-wide analysis of the endoplasmic reticulum stress response during lignocellulase production in *Neurospora crassa*. **Biotechnology for Biofuels**, v. 8, n. 1, p. 66, 2015a.

FERREIRA, J. A. et al. Waste biorefineries using filamentous ascomycetes fungi: Present status and future prospects. **Bioresource Technology**, v. 215, p. 334-45, 2016.

FIEDLER, M. R. M.; NITSCHE, B. M.; WANKA, F. Microbial enzyme technology in baked cereal foods. In: **Applications of Microbial Engineering**. CRC Press, 2013. p. 51.

FLEISSNE, A.; DERSCH, P. Expression and export: recombinant protein production systems for *Aspergillus*. **Applied Microbiology and Biotechnology**, v. 87, n. 4, p. 1255–1270, 2010.

GARDNER, B. M.; WALTER, P. Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. **Science**, v. 333, n. 6051, p. 1891–1894, 2011.

GOLDBECK, R. et al. Effect of hemicellulolytic enzymes to improve sugarcane bagasse saccharification and xylooligosaccharides production. **Journal of Molecular Catalysis B: Enzymatic**, v. 131, p. 36–46, 2016.

GOLDBECK, R.; DAMÁSIO, A. R. L.; GONÇALVES, T. A. Development of hemicellulolytic enzyme mixtures for plant biomass deconstruction on target biotechnological applications. **Applied Microbiology and Biotechnology**, v. 98, p. 8513–8525, 2014.

GÖRGENS, J. F. et al. Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. **Applied Microbiology and Biotechnology**, v. 67, n. 5, p. 684–691, 2005.

GOTHEL, S. F.; MARAHIEL, M. A. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. **Cellular and Molecular Life Sciences**, v. 55, n. 3, p. 423–436, 1999.

GÖTZ, S. et al. High-throughput functional annotation and data mining with the Blast2GO suite. **Nucleic Acids Research**, v. 36, n. 10, p. 3420–35, 2008.

GOUKA, R. J.; PUNT, P. J.; VAN DEN HONDEL, C. A. M. J. J. Efficient production of secreted proteins by *Aspergillus* : progress, limitations and prospects. **Applied Microbiology and Biotechnology**, v. 47, n. 1, p. 1–11, 1997.

GRAF, A. et al. Novel insights into the unfolded protein response using *Pichia pastoris* specific DNA microarrays. **BMC Genomics**, v. 390, n. 9, p. 1–13, 2008.

GRIMMINGER-MARQUARDT, V.; LASHUEL, H. A. L. Review structure and function of the molecular chaperone Hsp104 from yeast structure and function of the molecular chaperone Hsp104 from yeast. **Biopolymers**, v. 93, n. 3, p. 252–276, 2009.

GROOTJANS, J. et al. The unfolded protein response in immunity and inflammation. **Nature**, v. 16, n. 8, p. 469–484, 2016.

GUILLEMETTE, T. et al. Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. **BMC Genomics**, v. 8, p. 1–17, 2007.

GUILLEMETTE, T. et al. **Methods for investigating the UPR in filamentous fungi**. 1. ed. [s.l.] Elsevier Inc., 2011. v. 490

GUPTA, V. K. et al. Fungal enzymes for bio-products from sustainable and waste biomass. **Trends in Biochemical Sciences**, v. 41, n. 7, p. 633-645, 2016.

HAN, M.; YU, X. Enhanced expression of heterologous proteins in yeast cells via the modification of N-glycosylation sites. **Bioengineered**, p. 115–118, 2015.

HARDING, H. P. et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. **Molecular Cell**, v. 11, n. 3, p. 619–633, 2003.

HARKKI, A. et al. Genetic engineering of *Trichoderma* to produce strains with novel cellulase profiles. **Enzyme and Microbial Technology**, v. 13, p. 227–233, 1991.

HARMSEN, M. M. et al. Overexpression of binding protein and disruption of the PMR1 gene synergistically stimulate secretion of bovine prochymosin but not plant Thaumatin in yeast. **Applied Microbiology and Biotechnology**, v. 46, n. 4, p. 365–370, 1996.

HARRIS, S. D. Branching of fungal hyphae: regulation, mechanisms and comparison with other branching systems. **Mycologia**, v. 100, n. 6, p. 823–832, 2008.

HAYNES, C. M.; TITUS, E. A.; COOPER, A. A. Degradation of misfolded proteins prevents er-derived oxidative stress and cell death. **Molecular Cell**, v. 15, n. 5, p. 767–776, 2004.

HE, R. et al. Effect of highly branched hyphal morphology on the enhanced production of cellulase in *Trichoderma reesei* DES-15. **3 Biotech**, v. 6, n. 2, p. 214, 2016.

HEIMEL, K. et al. Crosstalk between the unfolded protein response and pathways that regulate pathogenic development in *Ustilago maydis*. **The Plant Cell**, v. 25, p. 4262–4277, 2013.

HEIMEL, K. Unfolded protein response in filamentous fungi - implications in biotechnology. **Applied Microbiology and Biotechnology**, v. 99, n. 1, p. 121–132, 2015.

HERNÁNDEZ-RODRÍGUEZ, Y.; HASTINGS, S.; MOMANY, M. The septin aspb in *Aspergillus nidulans* forms bars and filaments and plays roles in growth emergence and conidiation. **Eukaryotic Cell**, v. 11, n. 3, p. 311–323, 2012.

HERVÁS-AGUILAR, A. et al. Evidence for the direct involvement of the proteasome in the proteolytic processing of the *Aspergillus nidulans* zinc finger transcription factor PacC. **Journal of Biological Chemistry**, v. 282, n. 48, p. 34735–34747, 2007.

HERZOG, B. et al. Mutual cross talk between the regulators Hac1 of the unfolded protein response and Gcn4 of the general amino acid control of *Saccharomyces cerevisiae*. **Eukaryotic cell**, v. 12, n. 8, p. 1142–1154, 2013.

HETZ, C. The unfolded protein response : controlling cell fate decisions under ER stress and beyond. **Nature Publishing Group**, v. 13, n. 2, p. 89–102, 2012.

HEYLAND, J. et al. Carbon metabolism limits recombinant protein production in *Pichia*

pastoris. **Biotechnology and Bioengineering**, v. 108, n. 8, p. 1942–1953, 2011.

HIBBETT, D. S. et al. A higher-level phylogenetic classification of the fungi. **Mycological Research**, v. 111, n. 5, p. 509–547, 2007.

HOANG, H. D.; MARUYAMA, J.; KITAMOTO, K. Modulating endoplasmic reticulum-Golgi cargo receptors for improving secretion of carrier-fused heterologous proteins in the filamentous fungus *Aspergillus oryzae*. **Applied and Environmental Microbiology**, v. 81, n. 2, p. 533–43, 2015.

HOLLIEN, J. Evolution of the unfolded protein response. **Biochimica et Biophysica acta**, v. 1833, n. 11, p. 2458–63, 2013.

HOSHIDA, H. et al. N-glycosylation deficiency enhanced heterologous production of a *Bacillus licheniformis* thermostable α-amylase in *Saccharomyces cerevisiae*. **Applied Microbiology and Biotechnology**, v. 97, n. 12, p. 5473–5482, 2013.

HØYER-HANSEN, M.; JÄÄTTELÄ, M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. **Cell Death and Differentiation**, v. 14, n. 9, p. 1576–82, 2007.

HUANG, M. et al. Efficient protein production by yeast requires global tuning of metabolism. **Nature Communications**, v. 8, n. 1, p. 1131, 2017.

JANG, H. H. et al. Two enzymes in one: two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. **Cell**, v. 117, n. 5, p. 625–635, 2004.

JIN, F. J. et al. Comparative genomic analysis identified a mutation related to enhanced heterologous protein production in the filamentous fungus *Aspergillus oryzae*. **Applied Microbiology and Biotechnology**, v. 100, n. 21, p. 9163–9174, 2016.

JIN, S.; YE, K.; SHIMIZU, K. Metabolic flux distributions in recombinant Saccharomyces cerevisiae during foreign protein production. **Journal of Biotechnology**, v. 54, n. 3, p. 161–174, 1997.

KELLER, A. et al. Empirical statistical model to estimate the accuracy of peptide identifications made by MS / MS and database search. **Analytical Chemistry**, v. 74, n. 20, p. 5383–5392, 2002.

KHAN, A. et al. JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. **Nucleic Acids Research**, v. 46, p. 260–266, 2018.

KIM, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. **Genome Biology**, v. 14, n. 4, p. 36, 2013.

KIMURA, S. et al. Autophagy delivers misfolded secretory proteins accumulated in

endoplasmic reticulum to vacuoles in the filamentous fungus *Aspergillus oryzae*. **Biochemical and Biophysical Research Communications**, v. 406, n. 3, p. 464-470, 2011.

KISHISHITA, S.; FUJII, T.; ISHIKAWA, K. Heterologous expression of hyperthermophilic cellulases of archaea Pyrococcus sp. by fungus Talaromyces cellulolyticus. **The Journal of Industrial Microbiology and Biotechnology**, v. 42, p. 137–141, 2015.

KITAMOTO, N. et al. Utilization of the TEF1 gene (TEF1) promoter for expression of polygalacturonase genes, *pgaA* and *pgaB*, in *Aspergillus oryzae*. **Applied Microbiology and Biotechnology**, v. 50, n. 1, p. 85–92, 1998.

KLEIN-MARCUSCHAMER, D. et al. The challenge of enzyme cost in the production of lignocellulosic biofuels. **Biotechnology and Bioengineering**, v. 109, n. 4, p. 1083–1087, 2012.

KUHNE, W. Ueber das verhalten verschiedener organisirter und sog ungeformter fermente [On the behavior of different organized and so-called unformed ferments]. **Verhandlungen des Naturehistorisch-Medicinischen Vereins**, v. 1, p. 190, 1877.

KURDISTANI, S. K.; GRUNSTEIN, M. Histone acetylation and deacetylation in yeast. **Nature Reviews Molecular Cell Biology**, v. 4, n. 4, p. 276–284, 2003.

LAEMMLI, U. K. (1970): Cleavage of structural proteins during assembly of head of bacteriophage T4. **Nature**, v. 227, p. 680–685, 1970.

LANDOWSKI, C. P. et al. Enabling low cost biopharmaceuticals: A systematic approach to delete proteases from a well-known protein production host *Trichoderma reesei*. **PLoS ONE**, v. 10, n. 8, p. 1–28, 2015.

LARKIN, A.; IMPERIALI, B. The expanding horizons of asparagine-linked glycosylation. **Biochemistry**, v. 50, n. 21, p. 4411–4426, 2011.

LEACH, M. R.; WILLIAMS, D. B. Calnexin and Calreticulin, Molecular Chaperones of the Endoplasmic Reticulum. In: **Madame Curie Bioscience Database**. [s.l.] Landes Bioscience, 2013.

LEE, K. P. K. et al. Structure of the dual enzyme Ire1 reveals the basis for catalysis and regulation in nonconventional RNA splicing. **Cell**, v. 132, n. 1, p. 89–100, 2008.

LEVASSEUR, A. et al. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. **Biotechnology for biofuels**, v. 6, n. 1, p. 41, 2013.

LI, J. et al. Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters. **Microbial Cell Factories**, v. 11, n. 1, p. 84, 2012.

LI, J.; QIAN, X.; SHA, B. The crystal structure of the yeast Hsp40 Ydj1 complexed with its peptide substrate. **Structure**, v. 11, n. 12, p. 1475–1483, 2003.

LIAO, Y.; SMYTH, G. K.; SHI, W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. **Nucleic acids research**, v. 41, n. 10, p. 108, 2013.

LIN, X.-Q. et al. Quantitative iTRAQ LC–MS/MS proteomics reveals the cellular response to heterologous protein overexpression and the regulation of HAC1 in *Pichia pastoris*. **Journal of Proteomics**, v. 91, n. 2, p. 58–72, 2013.

LINDSEY, R. et al. Septins AspA and AspC are important for normal development and limit the emergence of new growth foci in the multicellular fungus *Aspergillus nidulans*. **Eukaryotic Cell**, v. 9, n. 1, p. 155–163, 2010.

LIU, L. et al. Genome-scale analysis of the high-efficient protein secretion system of *Aspergillus oryzae*. **BMC Systems Biology**, v. 8, p. 1–13, 2014.

LIU, Y. et al. Increased enzymatic hydrolysis of sugarcane bagasse by a novel glucose- and xylose-stimulated β-glucosidase from *Anoxybacillus flavithermus* subsp. *yunnanensis* E13T. **BMC biochemistry**, v. 18, n. 1, p. 4, 2017.

LOMBARD, V. et al. The carbohydrate-active enzymes database (CAZy) in 2013. **Nucleic Acids Research**, v. 42, n. D1, p. 490–495, 2014.

LOVE, M. I.; HUBER, W.; ANDERS, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. **Genome Biology**, v. 15, n. 12, p. 1–21, 2014.

LU, X. et al. The intra- and extracellular proteome of *Aspergillus niger* growing on defined medium with xylose or maltose as carbon substrate. **Microbial Cell Factories**, v. 9, p. 23, 2010.

MAGALHÃES, F.; AGUIAR, T. Q.; OLIVEIRA, C. High-level expression of *Aspergillus niger* β-galactosidase in *Ashbya gossypii*. **Biotechnology Progress**, v. 30, n. 2, p. 261–268, 2013.

MAYER, M. P.; BUKAU, B. Cellular and molecular life sciences Hsp70 chaperones: cellular functions and molecular mechanism. **Cellular and Molecular Life Sciences**, v. 62, p. 670–684, 2005.

MCCLUSKEY, K.; WIEST, A.; PLAMANN, M. The fungal genetics stock center: A repository for 50 years of fungal genetics research. **Journal of Biosciences**, v. 35, n. 1, p. 119–126, 2010.

MEHRA, A.; HATZIMANIKATIS, V. An algorithmic framework for genome-wide modeling and analysis of translation networks. **Biophysical Journal**, v. 90, n. 4, p. 1136–46, 2006.

MEYER, V. et al. Current challenges of research on filamentous fungi in relation to human welfare and a sustainable bio-economy: a white paper. **Fungal Biology and Biotechnology**,

v. 3, n. 1, p. 6, 2016.

MILLER, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**, v. 31, n. 3, p. 426–428, 1959.

MOHAPATRA, S. et al. Application of pretreatment, fermentation and molecular techniques for enhancing bioethanol production from grass biomass – A review. **Renewable and Sustainable Energy Reviews**, v. 78, p. 1007-1032, 2017.

MOLINARI, M. et al. Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. **Science**, v. 299, n. 5611, p. 1397–1400, 2003.

MONTENECOURT, B. S.; EVELEIGH, D. E. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. In: [s.l: s.n.]. p. 289–301, 1979.

MOORE, K. A.; HOLLIEN, J. The unfolded protein response in secretory cell function. **Annual Review of Genetics**, v. 46, p. 165–183, 2012.

MOUKHA, M. et al. In situ localization of the secretion of lignin peroxidases in colonies of Phanerochaete chrysosporium using a sandwiched mode of culture. **Journal of General Microbiology**, v. 139, p. 969–978, 1993.

MULDER, H. J. et al. The transcription factor HACA mediates the unfolded protein response in *Aspergillus niger*, and up-regulates its own transcription. **Molecular Genetics and Genomics**, v. 271, n. 2, p. 130–140, 2004.

MULDER, H. J.; NIKOLAEV, I.; MADRID, S. M. HACA, the transcriptional activator of the unfolded protein response (UPR) in *Aspergillus niger*, binds to partly palindromic UPR elements of the consensus sequence 5′-CAN(G/A)NTGT/GCCT-3′. **Fungal Genetics and Biology**, v. 43, n. 8, p. 560–572, 2006.

NEMOTO, T. et al. Isolation of *Aspergillus oryzae* mutants for heterologous protein production from a double proteinase gene disruptant. **Applied Microbiology and Biotechnology**, v. 82, n. 6, p. 1105–1114, 2009.

NEMOTO, T.; MARUYAMA, J.-I.; KITAMOTO, K. Improvement of heterologous protein production in *Aspergillus oryzae* by RNA interference with alpha-amylase genes. **Bioscience, Biotechnology, and Biochemistry**, v. 73, n. 11, p. 2370–2373, 2009.

NESVIZHSKII, A. I. et al. A statistical model for identifying proteins by tandem mass spectrometry abilities that proteins are present in a sample on the basis. **Analytical chemistry**, v. 75, n. 17, p. 4646–4658, 2003.

NEVALAINEN, H.; PETERSON, R. Making recombinant proteins in filamentous fungi- are we expecting too much? **Frontiers in microbiology**, v. 5, n. February, p. 75, 2014.

NGIAM, C. et al. Characterization of a foldase, protein disulfide isomerase A, in the protein

secretory pathway of *Aspergillus niger*. **Applied and Environmental Microbiology**,v. 66, n. 2, p. 775–782, 2000.

NITSCHE, B. M. et al. The carbon starvation response of *Aspergillus niger* during submerged cultivation: Insights from the transcriptome and secretome. **BMC Genomics**, v. 13, n. 1, p. 380, 2012.

NYYSSÖNEN, E. et al. Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. **Nature Biotechnology**, v. 11, n. 5, p. 591–595, 1993.

OLIVEIRA, M. P. F. DE et al. Proteomic analysis of the secretory response of *Aspergillus niger* to D-maltose and D-xylose. **PLoS ONE**, v. 6, n. 6, 2011.

PAKULA, T. M. et al. The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. **Journal of Biological Chemistry**, v. 278, n. 45, p. 45011–45020, 2003.

PASTEUR, L. M. Mémoire sur la fermentation alcoolique. **Compte-rendus l'Académie des Sciences**, v. 45, p. 1032–1036, 1857.

PEDERSEN, H.; CARLSEN, M.; NIELSEN, J. Identification of enzymes and quantification of metabolic fluxes in the wild type and in a recombinant *Aspergillus oryzae* strain. **Applied and Environmental Microbiology**, v. 65, n. 1, p. 11–19, 1999.

PERLIŃSKA-LENART, U. et al. Protein production and secretion in an *Aspergillus nidulans* mutant impaired in glycosylation. **Acta Biochimica Polonica**, v. 52, n. 1, p. 195–205, 2005.

PETERSON, R.; NEVALAINEN, H. *Trichoderma reesei* RUT-C30–thirty years of strain improvement. **Microbiology**, v. 158, n. 1, p. 58-68, 2012.

PIJNAPPEL, W. W. et al. The S. cerevisiae SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program. **Genes & Development**, v. 15, n. 22, p. 2991–3004, 2001.

PINCUS, D. et al. BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. **PLoS Biology**, v. 8, n. 7, p. 1–14, 2010.

PINEAU, L.; FERREIRA, T. Lipid-induced ER stress in yeast and β cells: parallel trails to a common fate. **FEMS Yeast Research**, v. 10, n. 8, p. 1035–1045, 2010.

QIN, L.; WU, V. W.; GLASS, N. L. Deciphering the regulatory network between the SREBP pathway and protein pecretion in *Neurospora crassa*. **mBio**, v. 8, n. 2, p. 1–16, 2017.

QUAST, C. et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. **Nucleic Acids Research**, v. 41, p. 590–596, 2013.

REILLY, M. C. et al. Deletion of homologs of the SREBP pathway results in hyper-

production of cellulases in *Neurospora crassa* and *Trichoderma reesei*. **Biotechnology for Biofuels**, v. 8, n. 1, p. 121, 2015.

RÉMOND, C. et al. Synthesis of pentose-containing disaccharides using a thermostable α-L-arabinofuranosidase. **Carbohydrate Research**, v. 339, n. 11, p. 2019–2025, 2004.

RODRÍGUEZ-LIMAS, W. A.; TANNENBAUM, V.; TYO, K. E. J. Blocking endocytotic mechanisms to improve heterologous protein titers in *Saccharomyces cerevisiae*. **Biotechnology and Bioengineering**, v. 112, n. 2, p. 376–385, 2015.

RON, D.; WALTER, P. Signal integration in the endoplasmic reticulum unfolded protein response. **Nature**, v. 8, p. 519–529, 2007.

ROONGSAWANG, N. et al. A novel potential signal peptide sequence and overexpression of er-resident chaperones enhance heterologous protein secretion in thermotolerant methylotrophic yeast *Ogataea thermomethanolica*. **Applied Biochemistry and Biotechnology**, v. 178, n. 4, p. 710–724, 2016.

RUBIO, M. V. et al. Mapping N - linked glycosylation of carbohydrate - active enzymes in the secretome of *Aspergillus nidulans* grown on lignocellulose. **Biotechnology for Biofuels**, v. 9, n. 168, p. 1–19, 2016.

RYTIOJA, J. et al. Plant-polysaccharide-degrading enzymes from basidiomycetes. **Microbiology and Molecular Biology Reviews**, v. 78, n. 4, p. 614-649, 2014.

SALOHEIMO, M.; LUND, M.; PENTTILÄ, M. E. The protein disulphide isomerase gene of the fungus *Trichoderma reesei* is induced by endoplasmic reticulum stress and regulated by the carbon source. **Molecular and General Genetics**, v. 262, n. 1, p. 35–45, 1999.

SCHALÉN, M. et al. Effect of secretory pathway gene overexpression on secretion of a fluorescent reporter protein in *Aspergillus nidulans*. **Fungal Biology and Biotechnology**, v. 3, n. 1, p. 1–14, 2016.

SCHÖNBRUNNER, E. R.; SCHMID, F. X. Peptidyl-prolyl cis-trans isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding. **Proceedings of the National Academy of Sciences**, v. 89, p. 4510–4513, 1992.

SEGATO, F. et al. Genomics review of holocellulose deconstruction by Aspergilli. **Microbiology and Molecular Biology Reviews,** v. 78, n. 4, p. 588–613, 2014.

SEGATO, F. et al. High-yield secretion of multiple client proteins in *Aspergillus*. **Enzyme and Microbial Technology**, v. 51, p. 100–106, 2012.

SEGATO, F. et al. Two structurally discrete GH7-cellobiohydrolases compete for the same cellulosic substrate fiber. **Biotechnology for Biofuels**, v. 5, n. 1, p. 21, 2012.

SEIDL, V. et al. The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT

C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. **BMC genomics**, v. 9, n. 1, p. 327, 2008.

SHARP, P. M.; LI, W. H. The codon adaptation index--a measure of directional synonymous codon usage bias, and its potential applications. **Nucleic Acids Research**, v. 15, n. 3, p. 1281–95, 1987.

SHEVCHENKO, A. et al. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. **Analytical Chemistry**, v. 68, n. 5, p. 850–858, 1996.

SHUSTA, E. V. et al. Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments. **Nature**, v. 16, n. 10, p. 773–777, 1998.

SIEBER, J. et al. Proteomic analysis reveals branch-specific regulation of the unfolded protein response by nonsense-mediated mRNA decay. **Molecular and Cellular Proteomics**, v. 15, p. 1584–1597, 2016.

SIMS, A. H. et al. Transcriptome analysis of recombinant protein secretion by *Aspergillus nidulans* and the unfolded-protein response in vivo. **Applied and Environmental Microbiology**, v. 71, n. 5, p. 2737–2747, 2005.

SINGH, R. et al. Microbial enzymes: industrial progress in 21st century. **3 Biotech**, v. 6, n. 2, p. 1–15, 2016.

SOUTHERN, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. **Journal of Molecular Biology**, v. 98, n. 3, p. 503–517, 1975.

SUBRAMANIAN, S. et al. Pbn1p: an essential endoplasmic reticulum membrane protein required for protein processing in the endoplasmic reticulum of budding yeast. **Proceedings of the National Academy of Sciences of the United States of America**, v. 103, n. 4, p. 939–944, 2006.

SZEWCZYK, E. et al. Fusion PCR and gene targeting in *Aspergillus nidulans*. **Nature protocols**, v. 1, n. 6, p. 3111–3120, 2006.

TAMAYO-RAMOS, J. A. et al. Enhanced production of *Aspergillus niger* laccase-like multicopper oxidases through mRNA optimization of the glucoamylase expression system. **Biotechnology and Bioengineering**, v. 110, n. 2, p. 543–551, 2013.

TANG, H. et al. Engineering protein folding and translocation improves heterologous protein secretion in *Saccharomyces cerevisiae*. **Biotechnology and Bioengineering**, v. 112, n. 9, p. 1872–1882, 2015.

TANG, H. et al. Engineering vesicle trafficking improves the extracellular activity and surface display efficiency of cellulases in *Saccharomyces cerevisiae*. **Biotechnology for Biofuels**, v. 10, n. 1, p. 53, 2017.

TANG, H. et al. N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in Saccharomyces cerevisiae. **Scientific Reports**, v. 6, p. 25654, 2016.

THIELE, D. et al. Elongation Factor la from *Saccharomyces cerevisiae*. **The Journal of Biological Chemistry**, v. 260, n. 5, p. 3084–3089, 1985.

THÖN, M. et al. The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: impact on development and oxidative stress response. **The Journal of Biological Chemistry**, v. 282, n. 37, p. 27259–27269, 2007.

TRAVERS, K. J. et al. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. **Cell**, v. 101, p. 249–258, 2000.

TURNER, P.; MAMO, G.; KARLSSON, E. N. Potential and utilization of thermophiles and thermostable enzymes in biorefining. **Microbial Cell Factories**, v. 6, p. 9, 2007.

TYO, K. E. J. et al. Imbalance of heterologous protein folding and disulfide bond formation rates yields runaway oxidative stress. **BMC Biology**, v. 10, n. 16, p. 1–14, 2012.

VALADARES, F. et al. Exploring glycoside hydrolases and accessory proteins from wood decay fungi to enhance sugarcane bagasse saccharification. **Biotechnology for Biofuels**, v. 9, n. 1, p. 110, 2016.

VALKONEN, M. et al. Improvement of foreign-protein production in *Aspergillus niger* var. awamori by constitutive induction of the unfolded-protein response. **Applied Microbiology and Biotechnology**, v. 69, n. 12, p. 6979–6986, 2003.

VERMAAS, J. V. et al. Effects of lytic polysaccharide monooxygenase oxidation on cellulose structure and binding of oxidized cellulose oligomers to cellulases. **Journal of Physical Chemistry B**, v. 119, n. 20, p. 6129–6143, 2015.

VOLMER, R. et al. Membrane lipid saturation activates endoplasmic reticulum unfolded protein response transducers through their transmembrane domains. **PNAS**, v. 110, n. 12, p. 4628–4633, 2013.

VU, T. et al. Enhance production of recombinant lumbrokinase by optimizing gene codon usage for expression in *Pichia pastoris* and its properties. **Journal of Biotech Research**, v. 6, p. 96–106, 2015.

WANG, J.; LI, Y.; LIU, D. Improved production of *Aspergillus usamii* endo-β -1,4-xylanase in *Pichia pastoris* via combined strategies. **BioMed Research International**, v. 2016, p. 1–9, 2016.

WARDE-FARLEY, D. et al. The GeneMANIA prediction server: biological network

integration for gene prioritization and predicting gene function. **Nucleic Acids Research**, v. 38, p. 214–220, 2010.

WEDEMEYER, W. J.; WELKER, E.; SCHERAGA, H. A. Proline cis-trans isomerization and protein folding. **Biochemistry**, v. 41, p. 14637–14644, 2002.

WU, Y. et al. Overexpressing key component genes of the secretion pathway for enhanced secretion of an *Aspergillus niger* glucose oxidase in *Trichoderma reesei*. **Enzyme and Microbial Technology**, v. 106, p. 83–87, 2017.

YAM, A. Y. et al. Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. **Nature Structural & Molecular Biology**, v. 15, n. 12, p. 1255–62, 2008.

YOON, J. et al. Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene Aovps10. **Applied and Environmental Microbiology**, v. 76, n. 17, p. 5718–5727, 2010.

YOON, J. et al. Enhanced production of bovine chymosin by autophagy deficiency in the filamentous fungus *Aspergillus oryzae*. **PloS One**, v. 8, n. 4, p. e62512, 2013.

YU, X. W. et al. Identification of novel factors enhancing recombinant protein production in multi-copy *Komagataella phaffii* based on transcriptomic analysis of overexpression effects. **Scientific Reports**, v. 7, n. 1, p. 16249, 2017.

ZHANG, G. et al. Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy. **Plasmid**, v. 71, n. 1, p. 16–22, 2014.

ZHANG, X. et al. Constitutive cellulase production from glucose using the recombinant *Trichoderma reesei* strain overexpressing an artificial transcription activator. **Bioresource Technology**, v. 223, p. 317–322, 2017.

ZHENG, X. et al. Improving the secretory expression of an α-galactosidase from *Aspergillus niger* in *Pichia pastoris*. **Plos One**, v. 11, n. 8, p. e0161529, 2016.

ZHOU, B. et al. Functional and transcriptomic analysis of the key unfolded protein response transcription factor HacA in *Aspergillus oryzae*. **Gene**, v. 593, p. 143–153, 2016.

ZHOU, B. et al. Identification of functional cis-elements required for repression of the Taka-amylase A gene under secretion stress in *Aspergillus oryzae*. **Biotechnology Letters**, v. 37, n. 2, p. 333–341, 2014.

ZOURIDIS, H.; HATZIMANIKATIS, V. A model for protein translation: polysome self-organization leads to maximum protein synthesis rates. **Biophysical Journal**, v. 92, n. 3, p. 717–730, 2007.

ZUEHLKE, A.; JOHNSON, J. L. Hsp90 and co-chaperones twist the functions of diverse client proteins. **Biopolymers**, v. 93, n. 3, p. 211–217, 2010.

Anexo 1. Material suplementar

- Capítulo 2

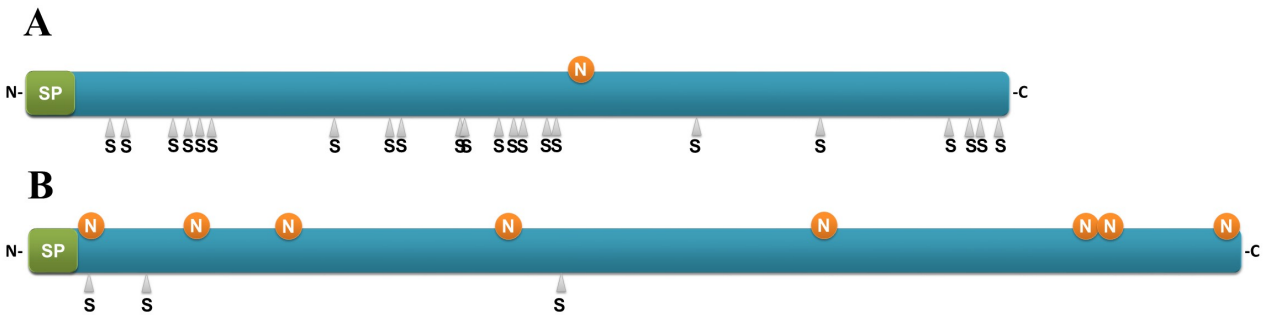


Figure S1. Overview of target proteins structure. Cbhl (532 aa; 56.4 kDa) and AbfA (656 aa; 71.7 kDa) structures were analyzed according to the number of cysteines residue and predicted N-linked glycosylation sites. One N-glycosylated site and 22 cysteines were predicted in the Cbhl (A), while AbfA showed eight potential N-glycosylation sites and three cysteines (B). Orange circles and gray triangle: N-linked glycosylation sites and cysteines, respectively. SP: signal peptide.

Table S1. List of proteins identified by LC-MS/MS and spectrum counts on the replicates. [a]AN numbers are from *Aspergillus* genome database (*Asp*GD) at http://www.*Aspergillus*genome.org. [b]Proteins were identified based on NCBI and *Asp*GD databanks. [c]Predicted molecular weight was obtained from National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov. [d]Total spectra method was applied to quantify protein abundance in the samples.

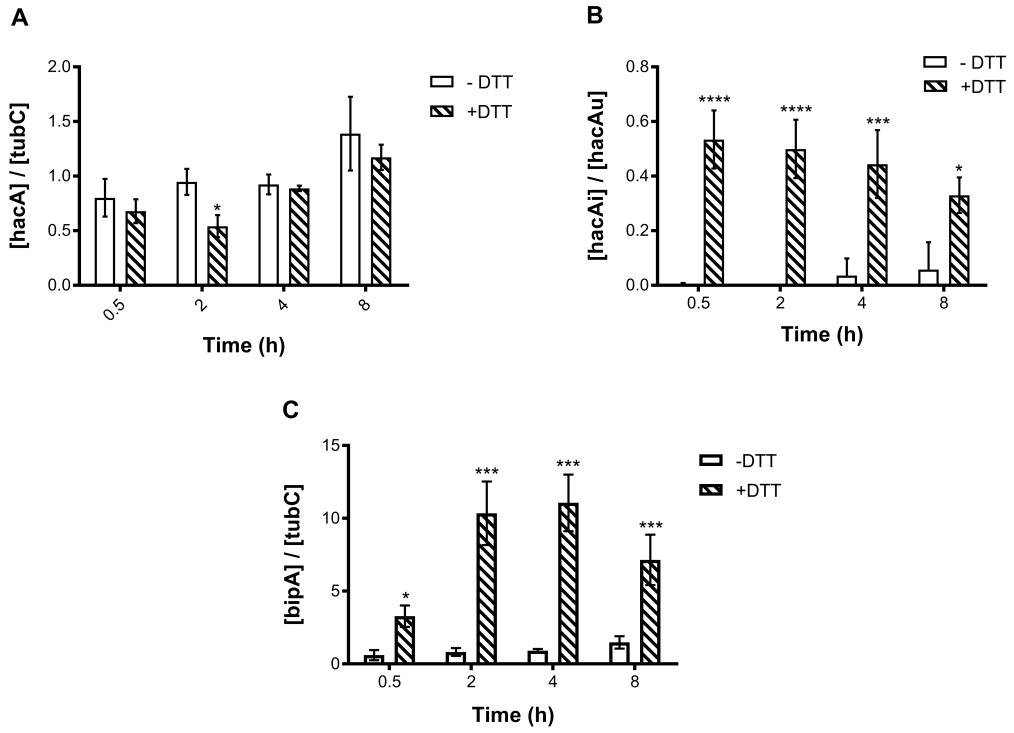
Table S2. Comparative analysis of proteins abundance in the recombinant strains. Only proteins with Fisher's exact test p values <0.05, and fold change ≥ 2.0 and ≤ 0.5 were included. Proteins were classified as "more abundant" or "less abundant" according to the total spectra compared to control strains. [a]AN numbers are from *Aspergillus* genome database (*Asp*GD) at http://www.*Aspergillus*genome.org. [b]Identified proteins were obtained from NCBI, *Asp*GD and CADRE. [c]The quantification of proteins abundance in the samples was based on total spectra method. [d]Signal P was used to predict signal peptides (http://www.cbs.dtu.dk/services/SignalP/). [e]More or Less relating to control Anid_pEXPYR.

Table S3. Total spectra of proteins identified in the *Aspergillus nidulans* secretomes. Proteins were classified as "more abundant" or "less abundant" according to the total spectra compared to control strains. [a]AN numbers are from *Aspergillus* genome database (*Asp*GD) at http://www.*Aspergillus*genome.org. [b]Identified proteins were obtained from NCBI, *Asp*GD and CADRE. [c]Difference in the proteins abundance was calculated by the total spectra method. [d]Signal P was used to predict signal peptides (http://www.cbs.dtu.dk/services/SignalP/). [e]More or Less relating to control Anid_pEXPYR.

Table S4. Oligonucleotides used in this study for qPCR analysis.

Gene	Forward (5´to 3´)	Reverse (5´to 3´)
abfA (Afu2g15160)	TTCTGGGTGCCGAAAGAAAC	TGAGCATGGGTGCGTAGGT
cbhB (Afu6g11610)	GCATGACCGTCGACACCAAG	GGATCACCTTGCCGTTCTGC
tubC (AN6838)	ACTGCTCTGTGCTCTATG	TGTGAACTGGTTGGAGAC
xlnR (AN7610)	TCGGTGGTTTTGGTGTCTCG	GGCGACATCATCCACAGCTC
amyR (AN2016)	CCTCCCGTTCCATCTTCCTG	TGTTCCATCCCAATCCCATGC

- Capítulo 3



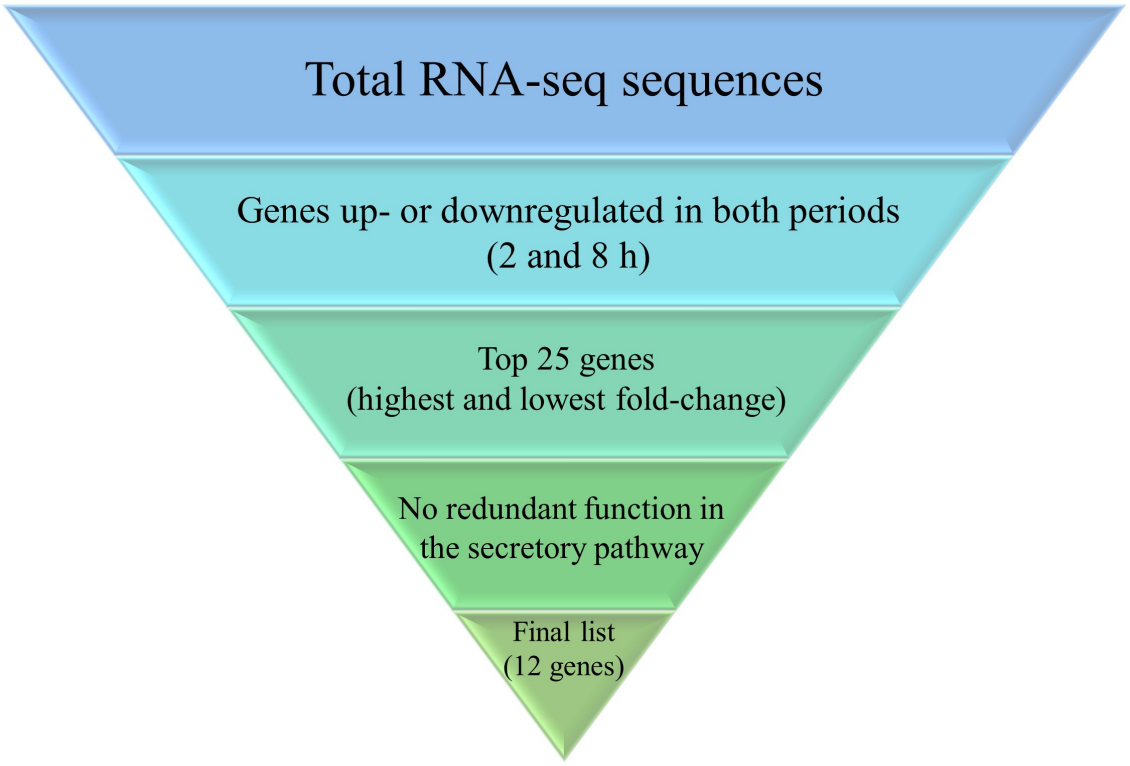
Additional file 1. qPCR analysis of the expression of UPR genes in *A. nidulans* A773 during exposure to DTT (20 mM) for 0.5, 2, 4 and 8 h. A) *hacA* total; B) *hacA*^{i}; C) *bipA*.

Additional file 2. Transcriptome overview, DE genes and secretion genes.

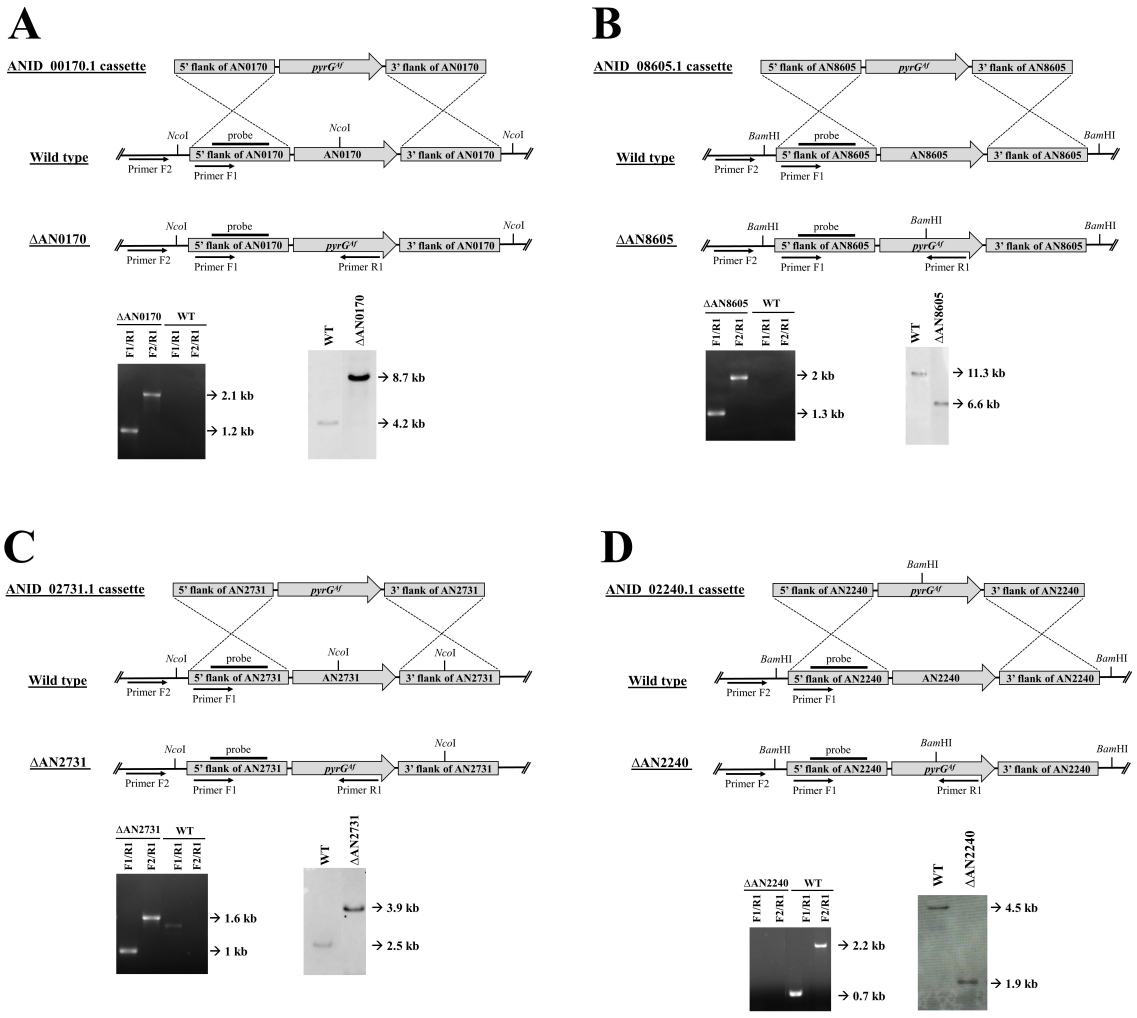
Additional file 3. List of selected genes deleted from *A. nidulans* in this study.

AN number[a]	Standard name	*Saccharomyces cerevisiae* orthologue	*Asp*GD description[a]	Sequence flank homology (bp)[b]	
				5' flank	3' flank
AN12492	*vpsA*	*vps1*	Has domains with predicted GTP binding, GTPase activity	801	959
AN8605	*cyp1*	*cpr1*	Putative peptidyl-prolyl cis-trans isomerase PPIase; cyclophilin	950	758
AN4864	*alg6*	*alg6*	Putative glucosyltransferase; locus contains the conserved upstream open reading frame uORF AN4864-uORF	705	791
AN1969	*mnn11*	*mnn11*	Orthologs have alpha-1,6-mannosyltransferase activity, role in protein N-linked glycosylation and alpha-1,6-mannosyltransferase complex, endoplasmic reticulum localization	784	981
AN0871	*gpi8*	*gpi8*	Orthologs have GPI-anchor transamidase activity, role in attachment of GPI anchor to protein and GPI-anchor transamidase complex localization	717	891
AN0170	*trxA*	*trx2*	Thioredoxin; predicted role in cell redox homeostasis; required for conidiation; expression upregulated after exposure to farnesol	977	852
AN6010	*sgdE*	*ssc1*	Hsp70-family protein; required for conidial germination; protein expressed at increased levels during osmoadaptation	862	807
AN1047	*sseA*	*sse2*	Putative heat shock protein, Hsp110	872	729
AN8269	*hsp90*	*hsc82*	90 kilodalton heat shock protein; physically associates with importin-alpha, KapA; palA-dependent expression independent of pH	750	643
AN5602	*ahaA*	*aha1*	Ortholog(s) have ATPase activator activity, chaperone binding activity, role in cellular response to heat, protein folding and cytoplasm localization	673	908
AN2240	*pbnA*	*pbn1*	Ortholog(s) have mannosyltransferase activity, role in GPI anchor biosynthetic process, protein processing, ubiquitin-dependent ERAD pathway and glycosylphosphatidylinositol-mannosyltransferase I complex localization	855	725
AN2731	*ydjA*	*ydj1*	Orthologs have ATPase activator activity, unfolded protein binding activity	697	704

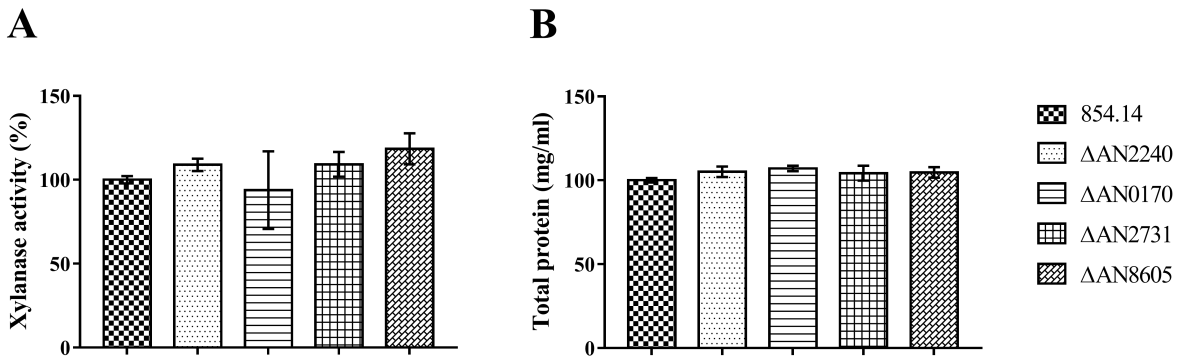
[a] *Aspergillus* Genome Database (http://www.aspgd.org/).
[b] The length of 5′ and 3′ sequence flanking the target gene found in each knock-out cassette provided by Fungal Genetics Stock Center (http://www.fgsc.net/).



Additional file 4. Diagram showing the rationale for target genes selected from the RNA-seq data and deletion in *A. nidulans*. The *A. nidulans* A773 was grown on MM for 24 h, exposed to DTT and TM for 2 and 8 h, and then analyzed by RNA-seq.



Additional file 5. Strategy used for construction and confirmation of the *A. nidulans* mutants. The represented mutants were A) 5B3 Δ*trxA*, B) 5B3 Δ*cyp1*, C) 5B3 Δ*ydjA* and D) 5B3 Δ*pbnA*. The *pyrG* gene from *Aspergillus fumigatus* (*pyrG*^{Af}) was used as a selectable marker. The transformants were purified by five rounds of monosporic purification and colonies containing genetic identical nuclei was submitted to two independent PCRs, one using primers anchored outside the flanking targeting sequence (F2/R1) and a second PCR using primers anchored within the cassette (F1/R1). After PCR, Southern Blot analysis was performed to confirm deletion. Mutants gDNA were digested with *Nco*I and *Bam*HI and 5′ flanking regions of each gene was used as probe. Differences among the size of the detected fragment allowed differentiation of mutant and wild-type strain. This strategy was used for all deleted genes in the 5B3 strains.



Additional file 6. Xylanase activity and protein secretion by the *A. nidulans* 854 mutant strains. A) Enzyme activity measured with Azo-xylan. The assay was performed using 1 ug of protein at pH 5.5 and 50°C and expressed in relation to the activity of the control strain 854. B) Evaluation of protein secretion yield.

Additional file 7. Strains used in this work

Strains	Description	Sources
Aspergillus nidulans A773	Wild-type strain (pyrG89, wA3, pyroA4)	[69]
Anid_pEXPYR	Wild-type strain transformed with empty vector pEXPYR (wA3, pyroA4)	[19]
Tcel1	Wild strain transformed with pEXPYR vector containing synthetic endoglucanase gene (*tcel1*, JF715060) (wA3, pyroA4)	Our stock
Tcel4	Wild strain transformed with pEXPYR vector containing synthetic endoglucanase processive gene (*tcel4,* JF715061) (wA3, pyroA4)	Our stock
Tcel6	Wild strain transformed with pEXPYR vector containing synthetic endoglucanase gene (*tcel6,* JF715062) (wA3, pyroA4)	Our stock
Them4	Wild strain transformed with pEXPYR vector containing arabinanase gene from *Thermotoga petrophila* (*tpet_0637,* WP_011943247) (wA3, pyroA4)	Our stock
Them5	Wild strain transformed with pEXPYR vector containing arabinofuranosidase gene from *Thermotoga petrophila* (*tpet_0631,* WP_011943241) (wA3, pyroA4)	Our stock
854	Wild strain transformed with pEXPYR vector containing xylanase gene from *Thermotoga petrophila* (*tpet_0854,* WP_011943438) (wA3, pyroA4)	Our stock
5B3	Wild strain transformed with pEXPYR vector containing xylanase gene from *A. nidulans* (AN7401, *xlnE*) (wA3, pyroA4)	This study
854.14	854 *pyrG*-deficient strain (pyrG$^-$, wA3, pyroA4)	This study
5B3.11	5B3 *pyrG*-deficient strain (pyrG$^-$, wA3, pyroA4)	This study
5B3.11 Δ*alg6*	5B3.11 strain (wA3, pyroA4); Δ*alg6*::*A. fumigatus pyrG*	This study
5B3.11 Δ*mnn11*	5B3.11 strain (wA3, pyroA4); Δ*mnn11*::*A. fumigatus pyrG*	This study
5B3.11 Δ*gpi8*	5B3.11 strain (wA3, pyroA4); Δ*gpi8*::*A. fumigatus pyrG*	This study
5B3.11 Δ*trxA*	5B3.11 strain (wA3, pyroA4); Δ*trxA*::*A. fumigatus pyrG*	This study
5B3.11 Δ*sgdE*	5B3.11 strain (wA3, pyroA4); Δ*sgdE*::*A. fumigatus pyrG*	This study
5B3.11 Δ*sseA*	5B3.11 strain (wA3, pyroA4); Δ*sseA*::*A. fumigatus pyrG*	This study
5B3.11 Δ*hsp90*	5B3.11 strain (wA3, pyroA4); Δ*hsp90*::*A. fumigatus pyrG*	This study
5B3.11 Δ*ahaA*	5B3.11 strain (wA3, pyroA4); Δ*ahaA*::*A. fumigatus pyrG*	This study
5B3.11 Δ*pbnA*	5B3.11 strain (wA3, pyroA4); Δ*pbnA*::*A. fumigatus pyrG*	This study
5B3.11 Δ*ydjA*	5B3.11 strain (wA3, pyroA4); Δ*ydjA*::*A. fumigatus pyrG*	This study
5B3.11 Δ*cyp1*	5B3.11 strain (wA3, pyroA4); Δ*cyp1*::*A. fumigatus pyrG*	This study
5B3.11 Δ*vpsA*	5B3.11 strain (wA3, pyroA4); Δ*vpsA*::*A. fumigatus pyrG*	This study
5B3.11 Δ*cyp1*/Δ*ydjA*	5B3.11 strain (wA3, pyroA4); Δ*ydjA*::*A. fumigatus pyrG*; Δ*cyp1*::*A. fumigatus pyroA*	This study
854.14 Δ*alg6*	854.14 strain (wA3, pyroA4); Δ*alg6*::*A. fumigatus pyrG*	This study
854.14 Δ*mnn11*	854.14 strain (wA3, pyroA4); Δ*mnn11*::*A. fumigatus pyrG*	This study
854.14 Δ*gpi8*	854.14 strain (wA3, pyroA4); Δ*gpi8*::*A. fumigatus pyrG*	This study
854.14 Δ*trxA*	854.14 strain (wA3, pyroA4); Δ*trxA*::*A. fumigatus pyrG*	This study
854.14 Δ*sgdE*	854.14 strain (wA3, pyroA4); Δ*sgdE*::*A. fumigatus pyrG*	This study
854.14 Δ*sseA*	854.14 strain (wA3, pyroA4); Δ*sseA*::*A. fumigatus pyrG*	This study
854.14 Δ*hsp90*	854.14 strain (wA3, pyroA4); Δ*hsp90*::*A. fumigatus pyrG*	This study
854.14 Δ*ahaA*	854.14 strain (wA3, pyroA4); Δ*ahaA*::*A. fumigatus pyrG*	This study
854.14 Δ*pbnA*	854.14 strain (wA3, pyroA4); Δ*pbnA*::*A. fumigatus pyrG*	This study
854.14 Δ*ydjA*	854.14 strain (wA3, pyroA4); Δ*ydjA*::*A. fumigatus pyrG*	This study
854.14 Δ*cyp1*	854.14 strain (wA3, pyroA4); Δ*cyp1*::*A. fumigatus pyrG*	This study
854.14 Δ*vpsA*	854.14 strain (wA3, pyroA4); Δ*vpsA*::*A. fumigatus pyrG*	This study

Additional file 8. Primers used in this study

Primer name	**Primer sequence (5' - 3')**
Primers internal to the gene of interest	
10691 FWD	CCAAGGGCCATTGCCCATCTCGTT
0170 FWD	CCCAGGCGCGCAATGGATGATGATG
8605 FWD	TGCGGTCGTCCTTTGCAATCCCCTTG
1969 FWD	TTTGGGACTGCCAGAACCGCCGTCA
4864 FWD	ACAGCTTGCGCTCCCACGTCAGTAGAAG
6010 FWD	CGGCAAGCCTCGGTCGGAAGCTCGTAAT
0871 FWD	TGCAAAAGAACCCCGAGCACCCAAC
2731 FWD	AATTCAGTGGGCAGCCTGCGTGGT
8111 FWD	GCGAGGTGCTTGAGGGTAAGAAGG
2240 FWD	CTTCTCATCCGTCAGCCAGCCA
5602 FWD	TTTGCGGTTATCTGAAGGCGGTTAC
8269 FWD	GGAGGACGACTTGTGAGCGAGAG
1047 FWD	TGCGTTGCCAACCACCTCACC
Primers external to the gene of interest	
10691RP FWD	AGCGGTGGGCCGATGTCCA
0170RP FWD	AAGCCCACCGCCCGAGAACAA
8605RP FWD	GAGTTTGCTCCGAACGAACGAGTCT
1969RP FWD	ATTGCCCTTGACCTCGCTGATTTGGA
4864RP FWD	CCTTGGGAGACTGGGTAGCCTTG
6010RP FWD	GCGAAGCGACAAGGCGACAACGA
0871RP FWD	TGCGACGCCGTCTGGCAATGAA
2731RP FWD	CGTGCTGAAAATGCTATACAAAGGCGA
8111RP FWD	ACACAGACACGCAGACGCTCATC
2240RP FWD	GGCTGTGGATGGGCGGACTA
5602RP FWD	CAGGAAGGGAGGGTGGATTGCT
8269RP FWD	CAGGCTGCTTAAATCTTCCTTGGACA
1047RP FWD	CCACCGCACATGGGGAGG
Reverse primer	
pyrG REV	TGCCAGAGGATTGGGGTGCTTGCTG
Primers to amplify deletion cassettes from FGSC	

FGSC FWD	GTAACGCCAGGGTTTTCCCA
FGSC REV	GCGGATAACAATTTCACACA
Primers for qPCR	
SB AN7401 FWD	GATGGTGTAGGCTTTCAGGCA
RT AN7401 REV	GGACATCAGCCTCCGTGTAC
Tpet0854 FWD	TGAGCGATTCTGGAACCTACAGG
Tpet0854 REV	TCGCATCTGGATCGGCTTCT
Tpet0637 FWD	TGTGGACGGTCCGAGTGAA
Tpet0637 REV	AACGCCAGGATCGAGTGC
Tpet0631 FWD	TGTTGCCAGGCGAGATCG
Tpet0631 REV	AACTTTGTGTCGAACTACCACTGG
Tcel1 FWD	GGCGACGGAAATCCTGAGTTC
Tcel1 REV	GGTCATCTCTGCGAATCCAGTC
Tcel6 FWD	GGCAGACTCATCGGGGAAC
Tcel6 REV	CTATTCCTGTGTCAGCCACCCAT
Tcel4 FWD	CGAACCCCGAGTTTGAGGAAC
Tcel4 REV	ACCCACGCCCAGGATAGAT
Primers for Southern Blot	
SB_AN2240 fwd	TCGGCGACATCCTGTGCATTG
SB_AN2240 rev	CCGCATTTGGCACCGGCTTA
8605 FWD	TGCGGTCGTCCTTTGCAATCCCCTTG
SB_AN8605 rev	GCGCTGGGGACGGAGCTC
SB_AN2731 fwd	TGCTCGAATCACGGCACTGGA
SB_AN2731 rev	TGCGGCGGAGGGTTTGAATG
0170 FWD	CCCAGGCGCGCAATGGATGATGATG
SB_AN0170 rev	CGTTGGGTTCGGCTGCGGCAT

*Overlapping complementary sequences are uppercase letters

Anexo 2. Termo de biossegurança

Uso exclusivo da CIBio:

Número de projeto / processo: 2015-35

Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança do CNPEM – Centro Nacional de Pesquisa em Energia e Materiais

Título do projeto: *Aspergillus nidulans* como modelo para manipulação de genes envolvidos no processo de "*unfolded protein response*"

Pesquisador responsável: André Ricardo de Lima Damásio

Experimentador: Mariane Paludetti Zubieta

Classe de risco do OGM: [x] Risco I [ ] Risco II

Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [x]-doutorado, [ ]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro, especifique:_____

Resumo do projeto:

Em eucariotos, *unfolded protein response* (UPR) regula positivamente genes responsáveis por restaurar a homeostase no retículo endoplasmático (RE) durante o acúmulo de proteínas enoveladas incorretamente. A homeostase é restaurada devido a ativação de genes relacionados à via secretória como os que codificam chaperonas e foldases, o que aumenta a capacidade de enovelamento de proteínas pelo RE. Alguns sistemas de produção de proteínas heterólogas têm sido desenvolvidos com a superexpressão individual de chaperonas e foldases nas células. Entretanto, a taxa de sucesso com essa estratégia é baixa. Estudos têm mostrado que a indução constitutiva do sistema UPR em linhagens fúngicas têm aumentado a produção de proteínas de interesse. Em *Aspergillus*, o fator de transcrição HACA é o gene central responsável pela ativação do UPR. Ainda, sabe-se que existe uma comunicação entre a via de UPR e a via de metabolismo de aminoácidos, sendo esta última regulada pelo fator de transcrição CPCA. Recentemente, uma hipótese sugeriu que esta comunicação se dá pela formação de um heterodímero estável entre HACA e CPCA que se liga nas regiões promotoras de genes envolvidos em UPR, ativando a transcrição. Diante disso, o objetivo desse projeto é, em um primeiro momento, obter linhagens de *A. nidulans* expressando constitutivamente os genes *hacA* e *cpcA* e avaliá-las quanto a expressão heteróloga de proteínas alvo. Além disso, serão obtidos resultados de transcriptômica global através de RNA-seq de linhagens selvagens após indução de UPR por drogas tradicionais, como tunicamicina e ditiotreitol, e de linhagens com alta produção de proteínas, gerando assim um banco de dados importante para a prospecção de novos alvos a serem manipulados com o objetivo de obter maior secreção de proteínas de interesse.

OGMs utilizados neste projeto:

1) *Aspergillus nidulans*, denominada A773 (pyrG89; pyroA4; wA3): cepa auxotrófica adquirida do Fungal Genetics Stock Center, Department of Plant Pathology, Kansas State University, manhattan, USA. Os genes introduzidos nesta cepa são genes que codificam para "carbohydrate active enzymes" (www.cazy.org), provenientes de diferentes microrganismos classe I;

A CIBio analisou este projeto em reunião realizada no dia: 01/06/2015 .

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, comentários anexo.

Presidente da CIBio – CNPEM-LNBio
Marcio Chaim Bajgelman

Membro da CIBio da CNPEM -CTBE
Fabio Márcio Squina

Membro da CIBio – CNPEM -LNBio
Celso Eduardo Benedetti

Membro da CIBio da CNPEM -CTBE
Sindélia Freitas Azzoni

Membro da CIBio – CNPEM -LNBio
Carolina Borsoi Moraes H. Freitas

Membro da CIBio da CNPEM -CTBE
Roberto Ruller

Anexo 3. Termo de direitos autorais

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **ASPERGILLUS NIDULANS COMO MODELO PARA MANIPULAÇÃO DE GENES ENVOLVIDOS NO PROCESSO DE UNFOLDED PROTEIN RESPONSE**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 13 de Julho de 2018

Assinatura : Mariane P. Zubieta

Nome do(a) autor(a): **Mariane Paludetti Zubieta**

RG n.° 43566007X

Assinatura : André R. L. Damásio

Nome do(a) orientador(a): **André Ricardo de Lima Damásio**

RG n.° 595124227