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Fungal secretomics of ascomycete fungi for biotechnological applications

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Abstract

The enzymatic degradation of plant biomass is of growing interest for the development of a sustainable bio-based industry. Ascomycete fungi, which degrade complex and recalcitrant plant polymers, secrete enzymes acting on the different components of plant cell wall (cellulose, hemicellulose and lignin). In this study, we present proteomic analyses of enzyme cocktails (secretomes) produced by five strains of *Ascomycota* (*Aspergillus wentii, Aspergillus niger, Aspergillus niger, Neocosmospora haematococca, Penicillium variabile*) from different geographical origins. Expert annotation of enzymes secreted revealed a large array of carbohydrate-active enzymes targeting plant cell wall polysaccharides. This study reveals that systematic proteomic analyses of fungal secretomes can contribute to the phenotyping of fungal strains from different geographical origins.

Key words – Ascomycete – biomass degradation – cellulase – proteomics – secretomes

Introduction

The conversion of plant cell wall (i.e. lignocellulose) to monomers or platform polysaccharides has attracted lots of research efforts in the past decades. However, the enzymatic hydrolysis (i.e. saccharification) of lignocellulose still remains a major bottleneck in the biorefinery process due to the recalcitrance of the tight complex formed by cellulose, hemicellulose and lignin within the plant cell wall (Sánchez 2009, Chundawat et al. 2011).

In Nature, filamentous fungi are potent degraders of lignocellulosic biomass as they produce a broad variety of enzymes with different and complementary catalytic activities. Many ascomycete species have been identified as good candidates for the release of monosaccharides. The most extensively used species is *Trichoderma reesei*, whose wild strain QM6a was originally selected for

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its ability to degrade cotton cellulose. The release of *T. reesei* QM6a genome revealed a relatively small number and poor diversity of carbohydrate-active enzymes (CAZymes) (Martinez et al. 2008). Other ascomycete genomes have been sequenced such as *Neurospora crassa* (Galagan et al. 2003), *Neocosmospora haematococca (as Nectria haematococca)* (Coleman et al. 2009) and many Aspergilli, among which *Aspergillus nidulans* (Galagan et al. 2005), *Aspergillus niger* (Pel et al. 2007), *Aspergillus oryzae* (Machida et al., 2005) and others (see CAZy database; Lombard et al. 2014). Computational analyses revealed large number of genes coding for potential CAZymes, of which numerous have been biochemically characterized and applied in biorefinery processes (van den Brink et al. 2011, Vasu et al. 2012, Yamada et al. 2015, Johansen et al. 2016). Although *in silico* annotations of fungal genomes provide large amounts of information about the genes that encode putative lignocellulose-degrading enzymes, experimental analyses are necessary to better understand complex enzyme mixtures that are secreted (i.e., the secretome) in response to inducers. In this study, we produced secretomes from five ascomycete species and analysed the enzymes secreted by means of proteomic analyses. Expert annotation of proteins secreted revealed a large array of CAZymes targeting plant cell wall polysaccharides.

Materials & Methods

Fungal strains

Identity of fungal strains used in this study (Table 1) was verified by ITS (Internal Transcribed Spacer) sequencing and maintained in the fungal culture collection of the International Centre of Microbial Resources at the French National Institute for Agricultural Research (INRA; Marseille, France). The strains were maintained on MYA2 medium slants (malt extract at 2% w/v and yeast extract at 0.1 % w/v).

Culture conditions

Cultures were carried out as described in (Couturier et al. 2012). Briefly, the fungal cultures were grown in a liquid medium containing 15 $g.l^{-1}$ autoclaved maize bran (provided by ARD, Pomacle, France) as a carbon source, 2.5 $g.l^{-1}$ maltose as a starter, 1.842 $g.l^{-1}$ diammonium tartrate, 0.5 $g.l^{-1}$ yeast extract, 0.2 $g.l^{-1}$ KH_2PO_4, 0.0132 $g.l^{-1}$ $CaCl_2.2H_2O$ and 0.5 $g.l^{-1}$ $MgSO_4.7H_2O$. The sugar content (w/w) of the autoclaved maize bran fraction was 16.10% arabinose, 28.73% xylose, 0.17% mannose, 5.65% galactose, and 22.06% glucose as determined by acidic hydrolysis and liquid chromatography (Bonnin et al. 2001). Fungal cultures were carried out in baffled flasks as described in (Navarro et al. 2014). The cultures were inoculated with 2×10^5 $spores.ml^{-1}$ prior to incubation at 30°C with orbital shaking at 140 rpm (Infors HT, Switzerland).

Secretome preparation

Cultures were stopped seven days after inoculation. Supernatants (i.e. secretome) were harvested (total volume of 20 to 30 ml), filtered using 0.2 µm polyethersulfone membrane, (Vivaspin, Sartorius), diafiltered with a 10 kDa cut-off polyethersulfone membrane in 50 mM acetate solution buffer, pH 5 (Vivaspin, Sartorius), and concentrated to a final volume of 3 ml. Samples were stored at -20°C until use. The total amount of protein was assessed using Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Ivry, France) with a BSA standard ranging from 0.2 to 1 $mg.ml^{-1}$.

Proteomic analysis

Proteins of each secretome (25 µg) were separated by one-dimensional (1D) electrophoresis (Precast Tris-Glycine 12% SDS-PAGE gels, BioRad) and stained with Coomassie blue (BioRad). Each 1D electrophoresis lane was cut into 24 gel fragments (2 mm in width) and protein identification was performed using the PAPPSO platform facilities as described in (Couturier et al. 2012). Briefly, in-gel digestion was carried out with the Progest system (Genomic Solution) according to a standard trypsinolysis protocol. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

analysis was subsequently performed on an Ultimate 3000 LC system (Dionex) connected to an LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher, USA) using a nanoelectrospray ion source.

Protein identification

The raw mass data were first converted to the mzXML format with the ReAdW software (http://tools.proteomecenter.org/software.php). Protein identification was performed by querying the MS/MS data against the corresponding protein databases along with an in-house contaminant database, using the X!Tandem software (X!Tandem Tornado 2008.02.01.3, http://www.thegpm.org) as described in (Couturier et al. 2012). All the peptides that matched with an E-value lower than 0.05 were parsed with an in-house programme (http:/PAPPSO.inra.fr/bioinformatique.html). Proteins identified by at least two unique peptides and a log (E-value) lower than 1.E-8 were considered to be validated.

Functional annotation of CAZymes

Annotation of the set of CAZymes for each secretome was performed by comparing the predicted proteins to the CAZy database. CAZymes were listed based on the number of representatives of each of the glycoside hydrolases (GH), carbohydrate esterases (CE), polysaccharide lyases (PL), auxiliary activities (AA) and carbohydrate binding modules (CBM) families. For GH5 family, the annotation was performed at the subfamily level (Asperborg et al. 2012).

Results and discussion

Cultures of Ascomycete strains on maize bran

Several ascomycete strains (Table 1) were selected in order to identify new promising CAZymes for biomass degradation applications. In particular, the genus *Aspergillus* has been studied to identify new enzymes of interest and allowed the discovery of novel plant cell wall-active enzymes (Benoit et al. 2015). Accordingly, we selected five ascomycetes strains, including three *Aspergilli* (*A. wentii* BRFM 279, *A. niger* BRFM 131 and *A. niger* BRFM 442) one *Penicillium* (*P. variabile* BRFM 110) and one *Neocosmospora* (*N. haematococca* BRFM 1286) from different geographical origins (Table 1).

All strains were able to grow on maize bran with a satisfactory yield of secreted proteins and cultures were harvested at a single time point, i.e. 7 days of growth. Maize bran is a complex and recalcitrant hemicellulosic substrate containing high amount of arabinose and ferulic acid substitutions (Lesage-Meessen et al., 2002). It was previously shown to be a powerful inducer for the secretion of a broad range of CAZymes targeting the plant cell wall, e.g. endo-xylanase, endo-mannanase, arabinofuranosidase and carbohydrate esterases (Bonnin et al. 2001, Lesage-Meessen et al. 2002, Couturier et al. 2011, Couturier et al. 2012).

Secretion of carbohydrate-active enzymes

Proteomic analysis using LC-MS/MS is the leading tool for the profiling of fungal secreted proteins (Couturier et al. 2012, Navarro et al. 2014, Poidevin et al. 2014). Here, LC-MS/MS analysis allowed the identification of varying numbers of proteins, ranging from 46 to 159, in the five strains selected (Table 2).

A. niger BRFM 442 contained the largest set of enzymes, with 159 proteins identified, among which more than half (54%) were CAZymes. Although *P. variabile* BRFM 110 secretome contained the smallest set of proteins identified, most of them were CAZymes (76% of the secreted proteins) thus confirming that maize bran was an inducer adapted to ascomycetes strains for the secretion of CAZymes. Detailed comparison of secreted CAZymes revealed the striking differences between fungal secretomes. Overall, secreted CAZymes were targeting all polysaccharide

Table 1 Description of the fungal strains used in this study

Species	Order	Country of origin	Strain CIRM number	Equivalent collection strain number
Aspergillus wentii (Wehmer)	*Eurotiales*	Belgium	BRFM 279	MUCL 1049
Aspergillus niger (van Tieghem)	*Eurotiales*	Sudan	BRFM 131	MUCL 28698
Aspergillus niger (van Tieghem)	*Eurotiales*	Bahamas	BRFM 442	-
*Neocosmospora haematococca (*Berkeley & Broome; Samuels, Nalim & Geiser)	*Hypocreales*	French Guiana	BRFM 1286	CLL 8012
Penicillium variabile (Sopp)	*Eurotiales*	France	BRFM 110	-

Table 2 Distribution of secreted CAZymes identified by means of proteomic analysis. AA. Auxiliary Activity. GH: Glycoside Hydrolase. CE. Carbohydrate Esterase. PL. Polysaccharide Lyase. CBM. Carbohydrate binding Module.

	A_went BRFM 279	*A_nig BRFM 131*	*A_nig BRFM 442*	*N_ haem BRFM 1286*	*P_var BRFM 110*
Non CAZy	25	35	72	53	11
CAZy	46	54	86	36	35
AA	1	1	3	4	0
GH	42	50	77	30	33
CE	2	2	5	0	0
PL	1	1	1	2	2
CBM*	9	9	14	5	4
Total	71	89	159	89	46

* CBMs are not included in the total of CAZymes

components of the plant cell wall except the P. variabile BRFM 110 secretome that does not contain any CAZyme targeting hemicellulose (Fig. 1). The secretome of *A. niger* BRFM 442 displayed a complete set of cellulolytic enzymes comprising GH5 and GH12 endoglucanases, GH6 and GH7 putative cellobiohydrolases (CBH), GH1 and GH3 β-glucosidases. This rich secretome was the only one to contain AA9 lytic polysaccharide monooxygenase (LPMO) that that were recently identified as boosting factors of biomass degradation (Harris et al. 2010). To a minor extent, the *A. niger* 131 secretome displayed the major enzymatic components for efficient cellulose conversion, while the other strains, *A. wentii* BRFM 279, *N. haematococca* BRFM 1286 and *P. variabile* BRFM 110, all lacked putative CBH from GH6 and GH7 families.

The *A. niger* BRFM 442 displayed the highest diversity of hemicellulose-targeting enzymes with at least one member of (i) xylanases from family GH10 and GH11, (ii) putative arabinofuranosidases from family GH43, GH51 and GH62 and (iii) acetyl esterases from family CE16. The latter were recently shown to be essential for complete xylan depolymerization (Puchart et al. 2015). *A. wentii* 279 and *N. haematococca* BRFM 1286 produced more representatives of some GH families. For example, five and three members of the xylan-active GH43 and GH10 families were respectively identified in *A. wentii* BRFM 279 (Figure 1), illustrating the efficiency of maize bran as inducer of hemicellulases.

Interestingly, the only two secretomes containing pectinases were from the two *Aspergillus niger* strains BRFM 131 and BRFM 442. This is in line with previous analyses that have reported the potential of *Aspergillus* for pectin degradation (deVries et al. 2002).

Target	CAZy family	*A. wentii*	*A. niger 131*	*A. niger 442*	*N. haema*	*P. variabilis*
	Sum of all	46	54	86	36	35
Cellulose	AA7	1	1	1	3	0
	AA9	0	0	2	0	0
	GH1	0	0	1	1	1
	GH3	4	7	5	4	7
	GH5	1	2	4	0	1
	GH6	0	0	2	0	0
	GH7	0	1	1	0	0
	GH12	1	2	3	0	0
	GH45	0	0	0	2	0
	GH74	0	1	1	0	0
Hemicellulose	GH10	3	2	1	1	0
	GH11	1	3	1	0	0
	GH5	0	1	1	0	0
	GH26	0	0	0	0	0
	GH43	5	3	2	4	0
	GH51	0	0	1	1	0
	GH62	0	1	1	0	0
	CE1	1	0	0	0	0
	CE16	0	1	2	0	0
Pectin	GH28	0	2	6	0	0
	GH31	0	3	3	2	3
	CE8	0	0	2	0	0
	PL4	0	0	1	0	0
	GH5_16	0	1	1	1	0

Fig. 1 – Number of representatives for selected CAZy families identified in the studied secretomes. AA. Auxiliary Activity. GH. Glycoside Hydrolase. CE. Carbohydrate Esterase. PL. Polysaccharide Lyase.

The present study illustrates that ascomycetes degrade lignocellulose using very diverse strategies. The number of CAZymes identified highlights the interest in a systematic assessment of fungal biodiversity to improve the enzymatic conversion of lignocellulosic biomass. It enabled the identification of new fungal strains issued from biodiversity with high biotechnological potential. This basic characterization of fungal secretomes will guide the selection of novel CAZymes for further investigation of the substrate specificities. Systematic proteomic analyses of secretomes could contribute to the phenotyping of fungal strains from different geographical origins.

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CAZy; Carbohydrate-active enzymes database; www.cazy.org

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