Trends in Comparative Biochem & Physiol
Vol. 17, 2013

Short Communication

Production of lignocellulose-related hydrolases by filamentous fungi

Isabelle Benoit[1,2], Ad Wiebenga[1,2] and Ronald P. de Vries[1,2,*]

[1]CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands,
[2]Microbiology and Kluyver Centre for Genomics of Industrial Fermentation, Utrecht University, Utrecht, The Netherlands

ABSTRACT

Filamentous fungi naturally produce a broad range of plant cell wall degrading enzymes that are widely used in several industrial sectors such as pulp and paper, biofuels or textiles. In this study, the enzymatic profiles of twelve fungal species after being grown on three plant polysaccharides were compared with one another. Our results demonstrate significant differences in the set of enzymes the filamentous fungi produce during growth on plant polymers and provide leads for improved biotechnology applications.

KEYWORDS: filamentous fungi, lignocellulases, plant polymers

INTRODUCTION

Plant biomass is the most important renewable material on earth and its conversion to monomers has become a key step in the transition to the use of sustainable energies. Plant cell walls are made of polysaccharides, lignin and proteins. The polysaccharide components are cellulose, hemicelluloses and pectins and the respective amounts of each polysaccharide vary with the plant origin. Cellulose is a linear chain of β-1,4-glucose. Hemicelluloses are chemically and physically more complex and their monomeric composition vary between species and between tissues and cell types within an individual plant [1]. They are classified according to the main chain of the polymer: for xylan this is β-1,4-D-xylose, for mannan β-1,4-D-mannose and for xyloglucan β-1,4-D-glucose. Each backbone is branched by monomers such as D-galactose, D-xylose, L-arabinose, D-glucuronic acid or L-fucose. Saprobic and phytopathogenic fungi produce a broad range of extracellular enzymes to degrade the polysaccharides of the plant cell wall. For decades fungal enzymes have been largely used to improve industrial processes related to those from food and health to pulp and biofuels. Enzymatic degradation of cellulose requires at least cellobiohydrolases (CBH), β-1,4-glucosidases (BGL) and β-1,4-endoglucanases (EGL). Many more enzymes are needed to completely degrade hemicelluloses [2], such as α-arabinofuranosidase (ABF), and α- and β-1,4-galactosidases (AGL, LAC).

In this study, 12 fungi were chosen from three phyla, Ascomycetes, Basidiomycetes and Zygomycetes, some of which are plant pathogens while others are saprobes (Table 1). *Aspergillus nidulans* is a well-known model organism for fungal biology, while *Aspergillus niger* and *Aspergillus oryzae* are industrially used fungi for metabolite and enzyme production. *Trichoderma reesei* and *Penicillium chrysogenum* are also industrial fungi, used in particular for the production of cellulases [3] and penicillin [4], respectively. *Trichoderma harzianum* is the most frequently found *Trichoderma* sp. worldwide and is the principal component in several commercial bio-fungicide formulations [5]. *Botrytis cinerea* and *Ustilago maydis* are important plant pathogens. *B. cinerea* can infect at least 235 plant species [6], while *U. maydis* has a very narrow

*Corresponding author: r.devries@cbs.knaw.nl

Table 1. Fungal species used in this study.

Fungus	Type	Phylum	Reference
Aspergillus niger (N402)	Saprobe	Ascomycete	[26]
Aspergillus nidulans (WG096)	Saprobe	Ascomycete	[27]
Aspergillus oryzae (NRRL 3488)	Saprobe	Ascomycete	[28]
Botrytis cinerea (SAS 56)	Plant pathogen	Ascomycete	[29]
Penicillium chrysogenum (CBS 906.70)	Saprobe	Ascomycete	[30]
Trichoderma harzianum (CBS 466.94)	Saprobe	Ascomycete	This study
Trichoderma reesei (QM9414-A1)	Saprobe	Ascomycete	[31]
Pycnoporus cinnabarinus (BRFM 44)	Saprobe	Basidiomycete	[32]
Schizophyllum commune (4.39)	Saprobe	Basidiomycete	[33]
Ustilago maydis (FB1)	Plant pathogen	Basidiomycete	[34]
Rhizomucor miehei (CBS182.67)	Saprobe	Zygomycete	This study
Rhizopus microspores var. *oligosporus* (CBS 338.62)	Saprobe	Zygomycete	This study

host range and infects mainly maize and its probable ancestor, teozintle [7]. *U. maydis* has become a model system for studying host-pathogen interactions [8]. *Pycnoporus cinnabarinus* and *Schizophyllum commune* are two white rot fungi commonly found in the Northern hemisphere [9]. *P. cinnabarinus* is known for producing high-redox potential laccases suitable for the industrial biotechnology [10], while *S. commune* is a model system for mushroom development [11]. *Rhizopus oligosporus* and *Mucor miehei* are two Zygomycetes, both classified within the order Mucorales. *M. miehei* produces enzymes of industrial importance, in particular aspartic proteases used as substitute for chymosin in cheese making and lipases [12]. *R. oligosporus* produces lipases as well and together with *A. oryzae*, *R. oligosporus* is also one of the main agents in Tempeh preparation [13]. This set of fungi covers a broad range of life styles and is distributed over several fungal taxa. In this study, the production of plant biomass degrading enzymes by these fungi has been compared to determine their potential as sources of industrial enzymes.

MATERIAL AND METHODS

Fungal strains

The fungal strains used in this study are listed in Table 1.

Fungal growth and enzymatic assays

The 12 fungal strains were pre-grown overnight at 25 °C in 50 ml liquid shaken cultures containing complete medium [14] with 1% glucose. 5g of wet-weight mycelium of each strain was transferred to duplicate flasks containing minimal medium [14] with 1% crude wheat arabinoxylan (LAX), 1% birchwood xylan or 1% cellulose (CMC) and incubated for 8 h. After 8 h, culture filtrate samples were taken for enzymatic activity assays. Exo-acting enzyme activities used 25 mM sodium acetate (pH 5), 0.01% substrate and suitably diluted culture filtrate. The mixture was incubated at 30 °C for 2 h and the reaction was terminated by the addition of 100 µL 250 mM sodium carbonate. Enzyme activities (α-arabinofuranosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and β-xylosidase) were determined spectrophotometrically at 405 nm by measuring the release of *p*-nitrophenol (*p*NP) from their appropriate *p*NP-substrates and standardized against a known concentration of *p*-nitrophenol (*p*NP). Activities were expressed as U/ml of sample. 1 unit is the amount of enzymes necessary for releasing 1 micromol *p*NP per minute. Endo-1,4-β-glucanase and endo-1,4-β-xylanase activity were measured using 20 mg/mL Azo-CM-cellulose (S-ACMC) and 10 mg/mL Azo-wheat arabinoxylan (S-AWAXP), respectively, and assayed according to the suppliers (Megazyme) recommendations.

RESULTS AND DISCUSSION

Seven enzymatic activities (Table 2) involved in plant cell wall polysaccharide degradation were selected to evaluate the ability of the fungi to degrade

Table 2. Enzymatic activities measured in this study.

Enzymatic activity	Substrate	CAZy family[1]
α-arabinofuranosidase (ABF)	Xyloglucan, xylan, pectin	GH51, 54
β-1,4-galactosidase (AGL)	Xylan, galactomannan	GH27, 36
β-1,4-galactosidase (LAC)	Xyloglucan, xylan, galactomannan	GH2, 35
β-1,4-glucosidase (BGL)	Cellulose	GH1, 3
β-1,4-xylosidase (BXL)	Xylan, pectin	GH3, 43
β-1,4-endoglucanase (EGL)	Cellulose	GH5, 7, 12, 45
β-1,4-endoxylanase (XLN)	Xylan	GH10, 11

[1]http://www.cazy.org/

lignocellulose. These hydrolase activities were measured extracellularly for each fungus grown on three carbon sources: birchwood xylan, crude wheat arabinoxylan and carboxymethyl cellulose (Figure 1). This approach excluded any cell wall bound enzymes from the analysis, but provided a good overview of the produced enzymatic activities. The highest enzyme activity on all three carbon sources was for β-1,4-glucosidase (BGL) followed by α-arabinofuranosidase (ABF) and β-1,4-galactosidase (LAC). Eleven fungi showed a BGL activity of at least 5 mU/ml of culture filtrate. In contrast, the two lowest activities were for β-1,4-endoglucanase (EGL) and β-1,4-endoxylanase (XLN). Among the twelve fungi tested in this study, eight appeared to be generalists and showed a broad enzymatic activity range while the other four were more specialized (*M. miehei*, *R. oligosporus*, *P. cinnabarinus*, *S. commune*). *M. miehei* had no endo-activities on any of the three carbon sources tested, and only low exo-activities were measured. The highest activities for this fungus were measured on arabinoxylan and xylan for BGL and β-1,4-xylosidase (BXL). *R. oligosporus* has a similar profile as *M. miehei*. Its highest activity was for BGL on all three carbon sources. Zygomycetes are fast growing filamentous fungi and they are known as primary or secondary colonizer [15]. A high production of BGL with the complementary action of BXL and EGL but no XLN, fits well with a lifestyle that uses accessible and easily digestible substrates such as fruits or vegetables. Only three Zygomycetes genomes have been sequenced and annotated so far, *M. miehei*, *Rhizopus oryzae* and *Phycomyces blakesleeanus*. Interestingly, *R. oryzae* has 6 GH3 and 5 GH7 (most are cellobiohydrolases), and 7 GH45 members, but no GH10 or GH11 members in its genome [16]. Considering that *R. oryzae* and *R. oligosporus* are closely related species, it is likely that their genomes are similar in CAZy content. This would mean that the *R. oligosporus* enzyme production correlates well with its genome content.

Although the highest activity produced by *S. commune* grown on all three substrates was for BGL, it was lower than the BGL activity measured in the Zygomycete culture filtrates. Moreover, *S. commune* produced EGL and XLN which provide a full range of plant cell wall degrading enzymes. BGL activity like many other CAZyme activities can be the result of more than one enzyme. The *S. commune* genome contains 11 putative BGLs (3 GH1, 8 GH3), 2 putative ABFs (GH51), 1 putative α-galactosidase (AGL, GH27), 8 putative LACs (4 GH2, 4 GH35), 6 putative XLNs (5 GH10, 1 GH11) and 18 putative EGLs (17GH5, 1GH45) [11], which correlates well with the activity measurements. In contrast, *P. cinnabarinus* produced high levels of AGL and BGL mainly on xylan and cellulose, but showed no endo-activity. Both *S. commune* and *P. cinnabarinus* grow on dead hardwood such as birch and beech wood, which are rich in xylan [17, 18]. Instead of producing endo-activities, *P. cinnabarinus* may use AGL, acting on xylan and galactomannan, to efficiently degrade its natural substrate.

The basidiomycete *U. maydis* produced the lowest hydrolase levels among all twelve fungi, but all the seven activities could be measured. The highest enzymatic activities measured were for ABF and AGL and were induced by arabinoxylan and xylan. In a recent study these two activities were also the highest measured when *U. maydis* was grown on

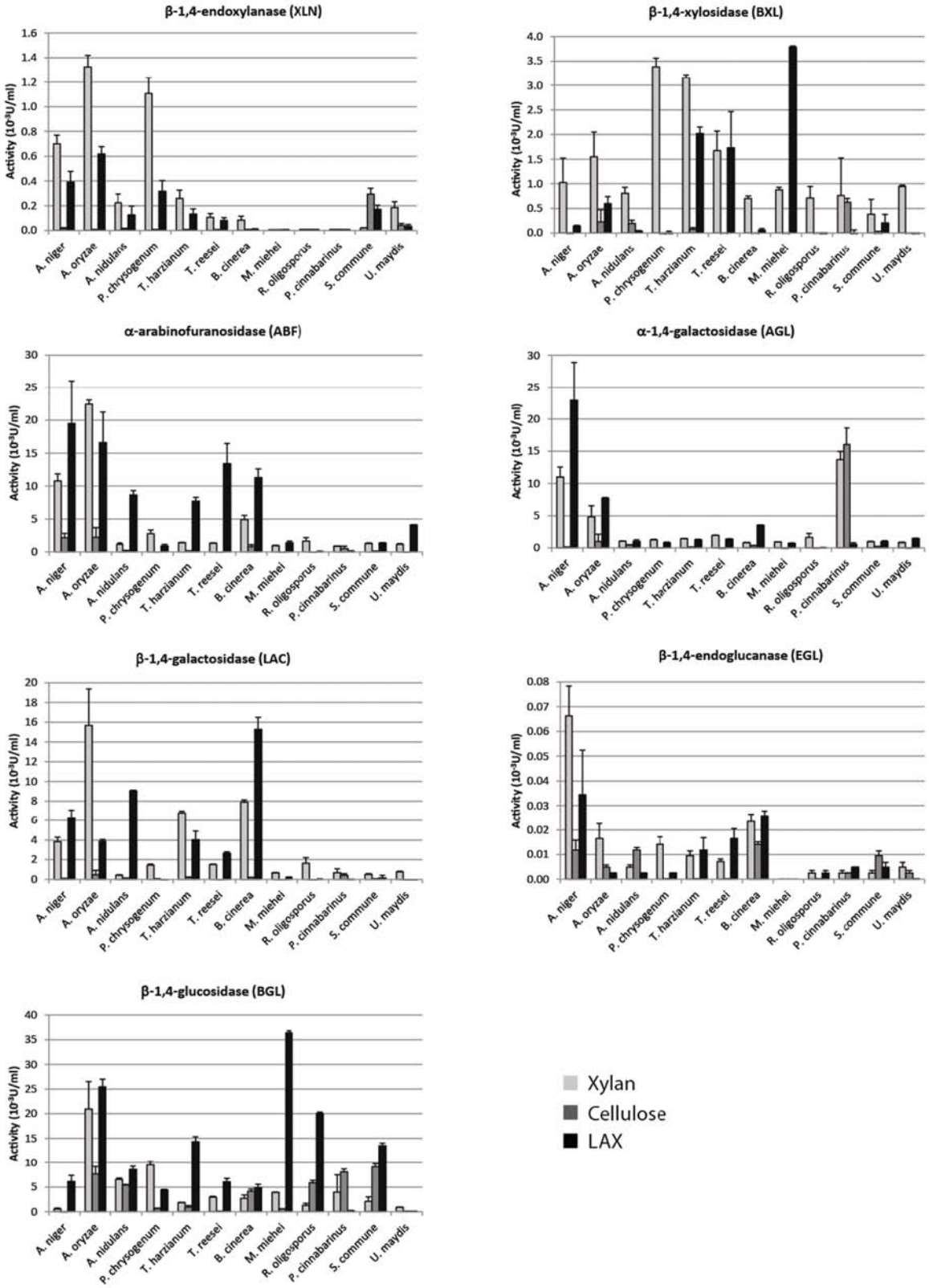

Figure 1. Enzymatic activities of twelve fungi after growth on birchwood xylan, crude arabinoxylan and cellulose. Error bars display the standard deviation between the two biological replicates.

maize bran [19]. *U. maydis* is a biotrophic plant pathogen that establishes an intimate interaction with its living host. Together with the mechanisms involved in suppressing host defense, exo- and endo-activities allow penetration of the maize tissues [20]. The other plant pathogen in this study, the ascomycete *B. cinerea*, has a necrotrophic and polyphagous lifestyle. The level of exo-activities, including the EGL activity, measured for *B. cinerea* was higher than that measured for *U. maydis*, but the level of BXL was lower. In contrast to *U. maydis*, *B. cinerea* produced these enzymes when grown on cellulose. *U. maydis* and *B. cinerea* both secreted a broad range of plant cell wall degrading enzymes fitting well with their ability to penetrate plant cell walls. Differences in the induction and in the level of production of these enzymes may be related to their pathogenic mechanisms. *B. cinerea* has a broad host range while *U. maydis* mainly infects maize. It was shown in previous studies that during infection steps, *U. maydis* expresses many genes encoding secreted proteins which are not detected during saprophytic fungal growth [8] which could explain the low level of hydrolase activity measured in this study. Moreover, Couturier and coworkers [19] have highlighted the presence of putative oxido-reductases in *U. maydis* genome that could play a crucial role in the hydrolysis of plant cell walls.

The CAZyme analysis of the three Aspergilli revealed relatively few differences among the three species. *A. nidulans* has a higher number of cellulose and galactomannan related Open Reading Frames (ORFs) and a lower number of xyloglucan related ORFs than the other two species [21]. *A. nidulans* had in general the lowest level of activity compared to the two other Aspergilli. Also during growth on maize bran *A. niger* produced higher hydrolase levels than *A. nidulans* [19]. Nonetheless, for *A. nidulans*, the highest activity induced by all three carbon sources was for BGL. On arabinoxylan, ABF and LAC activities were as high as the BGL activity. Conversely, the lowest BGL activity was measured for *A. niger* which had the highest AGL activity, induced by xylan and arabinoxylan and had high endo-activities as well. *A. oryzae* showed a low AGL activity but resembled *A. nidulans* with a high BGL activity induced by the three carbon sources. Thus, despite a similar plant polysaccharide degrading enzyme potential, the three Aspergilli clearly showed differences in the level and the type of enzymes they produce. *P. chrysogenum* had an enzymatic profile similar to the Aspergilli, particularly *A. nidulans* with overall low activities while the highest activity was measured for BGL on all three carbon sources. This is in accordance with a close taxonomic relationship between *Aspergillus* and *Penicillium* [22].

T. harzanium and *T. reesei* have received great attention from biofuel industries due to their potential for cellulase production. Interestingly, the hydrolytic activities measured from these two fungi when grown in cellulose medium were very low although the highest activity measured was for BGL. Recently, the proteome of *T. harzanium* grown in cellulose medium was described, revealing that even though some cellulases were found to be produced, the major secreted hydrolytic enzymes were chitinases and endochitinases, which may reflect the mycoparasitic behavior of this fungus [24].

To our knowledge, few studies so far compared the hydrolytic potential of several fungi on crude carbon sources. Van Gool *et al.* recently screened for the activities of endoxylanase, β-xylosidase and α-arabinofuranosidase from *A. niger*, *A. oryzae* and *Trichoderma spp.* grown on wheat arabinoxylan [24], while Thygesen *et al*. compared endoxylanase, β-xylosidase, endoglucanase and β-glucosidase from *A. niger, T. reesei, S. commune* and *B. cinerea* grown on wet oxidized wheat straw [25]. Couturier *et al.* measured β-xylosidase, α-arabinofuranosidase, β-glucosidase and α-1,4-galactosidase from *T. reesei, R. oryzae, U. maydis, A. niger* and *A. nidulans* grown in maize bran [19]. A comparison of our results with these three studies suggests that the induction of hydrolytic enzymes is highly dependent on the composition of the crude substrate. However, differences in culture and assay conditions may contribute to the variation in results between the studies.

This study shows the diverse strategies used by filamentous fungi to degrade the plant biomass. Differences in enzyme production are not only obvious between generalist and specialized fungi such as *A. niger* and *U. maydis* or between fungi from different biotopes like *B. cinerea* and *P. cinnabarinus*, but also within closely related species such as the Aspergilli. This study therefore provides a good starting point for more detailed studies into the mechanism that underlies plant biomass degradation in different fungi. This will require a

combination of post-genomic approaches, detailed biochemical analysis and a detailed evaluation of the regulatory systems governing the expression of genes encoding plant cell wall degrading enzymes.

ACKNOWLEDGEMENTS

IB was supported by a grant from the Kluyver Centre for Genomics of Industrial Fermentation (KCII 2.2.27) to RPdV.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

REFERENCES

1. Scheller, H. V. and Ulvskov, P. 2010, Annual Review of Plant Biology, 61(1), 263-289.
2. van den Brink, J. and de Vries, R. P. 2011, Appl. Microbiol. Biotechnol., 91(6), 1477-1492.
3. Kubicek, C. P., Messner, R., Gruber, F., Mandels, M. and Kubicek-Pranz, E. M. 1993, J. Biol. Chem., 268, 19364-8
4. van den Berg, M. A., Albang, R., Albermann, K., Badger, J. H., Daran, J.-M., Driessen, A. J., Garcia-Estrada, C., Fedorova, N. D., Harris, D. M., Heijne, W. H. M., Joardar, V., Kiel, J. A. K., Kovalchuk, A., Martin, J. F., Nierman, W. C., Nijland, J. G., Pronk, J. T., Roubos, J. A., van der Klei, I. J., van Peij, N. N. M. E., Veenhuis, M., von Dohren, H., Wagner, C., Wortman, J. and Bovenberg, R. A. L. 2008, Nat. Biotechnol., 26(10), 1161-1168.
5. Druzhinina, I. S., Kubicek, C. P., Komon-Zelazowska, M., Mulaw, T. B. and Bissett, J. 2010, BMC Evol. Biol., 10, 94.
6. Jarvis, W. R. 1977, Botryotinia and Botrytis species: taxonomy, physiology, and pathogenicity Monographs, Canada Department of Agriculture, Ottawa. 15.
7. León-Ramírez, C. G., Cabrera-Ponce, J. L., Martínez-Espinoza, A. D., Herrera-Estrella, L., Méndez, L., Reynaga-Peña, C. G. and Ruiz-Herrera, J. 2004, New Phytologist, 164(2), 337-346.
8. Kamper, J., Kahmann, R., Bölker, M., Ma, L.-J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Müller, O., Perlin, M. H., Wösten, H. A., de Vries, R. P., Ruiz-Herrera, J., Reynaga-Pena, C. G., Snetselaar, K., McCann, M., Pérez-Martín, J., Feldbrügge, M., Basse, C. W., Steinberg, G., Ibeas, J. I., Holloman, W., Guzman, P., Farman, M. L., Stajich, J. E., Sentandreu, R., González-Prieto, J. M., Kennell, J. C., Molina, L., Schirawski, J., Mendoza-Mendoza, A., Greilinger, D., Münch, K., Rössel, N., Scherer, M., Vranes, M., Ladendorf, O., Vincon, V., Fuchs, U., Sandrock, B., Meng, S., Ho, E. C. H., Cahill, M. J., Boyce, K. J., Klose, J., Klosterman, S. J., Deelstra, H. J., Ortiz-Castellanos, L., Li, W., Sanchez-Alonso, P., Schreier, P. H., Häuser-Hahn, I., Vaupel, M., Koopmann, E., Friedrich, G., Voss, H., Schlüter, T., Margolis, J., Platt, D., Swimmer, C., Gnirke, A., Chen, F., Vysotskaia, V., Mannhaupt, G., Güldener, U., Münsterkötter, M., Haase, D., Oesterheld, M., Mewes, H.-W., Mauceli, E. W., DeCaprio, D., Wade, C. M., Butler, J., Young, S., Jaffe, D. B., Calvo, S., Nusbaum, C., Galagan, J. and Birren, B. W. 2006, Nature, 444(7115), 97-101.
9. Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henrissat, B., Martinez, A. T., Otillar, R., Spatafora, J. W., Yadav, J. S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P. M., de Vries, R. P., Ferreira, P., Findley, K., Foster, B., Gaskell, J., Glotzer, D., Gorecki, P., Heitman, J., Hesse, C., Hori, C., Igarashi, K., Jurgens, J. A., Kallen, N., Kersten, P., Kohler, A., Kues, U., Kumar, T. K., Kuo, A., LaButti, K., Larrondo, L. F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D. J., Morgenstern, I., Morin, E., Murat, C., Nagy, L. G., Nolan, M., Ohm, R. A., Patyshakuliyeva, A., Rokas, A., Ruiz-Duenas, F. J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J. C., St John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D. C., Martin, F., Kubicek, C. P., Komon-Zelazowska, M., Mulaw, T. B., Bissett, J., Cullen, D., Grigoriev, I. V. and Hibbett, D. S. 2012, Science, 336(6089), 1715-1719.
10. Lesage-Meessen, L., Haon, M., Uzan, E., Levasseur, A., Piumi, F., Navarro, D., Taussac, S., Favel, A. and Lomascolo, A. 2011, FEMS Microbiol. Lett., 325(1), 37-48.

11. Ohm, R. A., de Jong, J. F., Lugones, L. G., Aerts, A., Kothe, E., Stajich, J. E., de Vries, R. P., Record, E., Levasseur, A., Baker, S. E., Bartholomew, K. A., Coutinho, P. M., Erdmann, S., Fowler, T. J., Gathman, A. C., Lombard, V., Henrissat, B., Knabe, N., Kues, U., Lilly, W. W., Lindquist, E., Lucas, S., Magnuson, J. K., Piumi, F., Raudaskoski, M., Salamov, A., Schmutz, J., Schwarze, F. W., Vankuyk, P. A., Horton, J. S., Grigoriev, I. V. and Wosten, H. A. 2010, Nat. Biotechnol., 28(9), 957-963.
12. Foltman, B. 1987, Elsevier Applied Science, London, New York, 1, 33-62.
13. Starzynska-Janiszewska, A., Stodolak, B., Dulinski, R. and Mickowska, B. 2012, Food Sci. Technol. Int., 18(2), 113-122.
14. de Vries, R. P., Burgers, K, van de Vondervoort, P. J. I., Frisvad, J. C., Samson, R. A. and Visser, J. 2004, Appl. Environ. Microbiol., 70(7), 3954-3959.
15. Richardson, M. 2009, Clin. Microbiol. Infect., 15(Suppl. 5), 2-9.
16. Battaglia, E., Benoit, I., van den Brink, J., Wiebenga, A., Coutinho, P. M., Henrissat, B. and de Vries, R. P. 2011, BMC Genomics, 12, 38.
17. Breitenbach, J. and Kränzlin, F. 1991, Fungi of Switzerland. Mykologia, Luzern, Switzerland, 3, 318.
18. Cooke, W. B. 1961, Genus Schizophyllum. Mycologia, 53, 575-599 .
19. Couturier, M., Navarro, D., Olivé, C., Chevret, D., Haon, M., Favel, A., Lesage-Meessen, L., Henrissat, B., Coutinho, P. M. and Berrin, J-G. 2012, BMC Genomics, 13, 57.
20. Skibbe, D. S., Doehlemann, G., Fernandes, J. and Walbot, V. 2010, Science, 328(5974), 89-92.
21. Coutinho, P. M., Andersen, M. R., Kolenova K., vanKuyk, P. A., Benoit, I., Gruben, B. S., Trejo-Aguilar, B., Visser, H., van Solingen, P. and Pakula, T. 2009, Fungal Genet Biol., 46(Suppl. 1), S161-S169.
22. Houbraken, J. and Samson, R. A. 2011, Stud. Mycol., 70(1), 1-51.
23. Do Vale, L. H. F., Gómez-Mendoza, D. P., Kim, M.-S., Pandey, A., Ricart, C. A. O., Edivaldo, X. F. F. and Sousa, M. V. 2012, Proteomics, 12(17), 2716-2728.
24. Van Gool, M. P., Vancsó, I., Schols, H. A., Toth, K., Szakacs, G. and Gruppen, H. 2011, Bioresource Technology 102(10), 6039-6047.
25. Thygesen, A., Thomsen, A. B., Schmidt, A. S., Jørgensen, H., Ahring, B. K. and Olsson, L. 2003, Enzyme and Microbial Technology, 32(5), 606-615.
26. Bos, C. J., Debets, A. J., Swart, K., Huybers, A., Kobus, G. and Slakhorst, S. M. 1988, Curr, Genet., 14(5), 437-443.
27. de Vries, R., Flipphi, M. J. A., Witteveen, C. F. B. and Visser, J. 1994, FEMS Microbiology Letter, 123(1-2), 83-90.
28. Hashem, A. M., Ismail, A. M., El-Refai, M. A. and Abdel-Fattah, A. F. 2001, Cytobios, 105(409), 115-30.
29. Wubben, J. P., Mulder, W., ten Have, A., van Kan, J. A. and Visser, J. 1999, Appl. Environ. Microbiol., 65(4), 1596-602.
30. Samson, R. A., Hadlok, R. and Stolk, A. C. 1977, Antonie van Leeuwenhoek, 43(2), 169-175.
31. Montenecourt, B. S. and Eveleigh, D. E. 1977, Appl. Environ. Microbiol., 34(6), 777–782.
32. Alves, A. M., Record, E., Lomascolo, A., Scholtmeijer, K., Asther, M., Wessels, J. G. and Wösten, H. A. 2004, Appl. Environ. Microbiol., 70(11), 6379-84.
33. van Wetter, M. A., Wösten, H. A. and Wessels, J. G. 2000, Mol. Microbiol., 36(1), 201-10.
34. Banuett, F. and Herskowitz, I. 1989. Proc. Natl. Acad. Sci. USA, 86(15), 5878–5882.