Investigation of the indigenous fungal community populating barley grains: Secretomes and xylanolytic potential

Sultan, Abida; Frisvad, Jens Christian; Andersen, Birgit; Svensson, Birte; Finnie, Christine

Published in:
Journal of Proteomics

Link to article, DOI:
10.1016/j.jprot.2017.03.009

Publication date:
2017

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Title: Investigation of the indigenous fungal community populating barley grains: secretomes and xylanolytic potential

Article Type: SI:INPPO 2016

Section/Category: Original Article

Keywords: Environmental proteomics; fungal community; grain proteome; secretome; xylanase; barley

Abstract: The indigenous fungal species populating cereal grains produce numerous plant cell wall-degrading enzymes including xylanases, which could play important role in plant-pathogen interactions and in adaptation of the fungi to varying carbon sources. To gain more insight into the grain surface-associated enzyme activity, members of the populating fungal community were isolated, and their secretomes and xylanolytic activities assessed. Twenty-seven different fungal species were isolated from grains of six barley cultivars over different harvest years and growing sites. The isolated fungi were grown on medium containing barley flour or wheat arabinoxylan as sole carbon source. Their secretomes and xylanase activities were analysed using SDS-PAGE and enzyme assays and were found to vary according to species and carbon source. Secretomes were dominated by cell wall degrading enzymes with xylanases and xylanolytic enzymes being the most abundant. A 2-DE-based secretome analysis of Aspergillus niger and the less-studied pathogenic fungus Fusarium poae grown on barley flour and wheat arabinoxylan resulted in identification of 82 A. niger and 31 F. poae proteins many of which were hydrolytic enzymes, including xylanases.
Significance

The microorganisms that inhabit the surface of cereal grains are specialized in production of enzymes such as xylanases, which depolymerize plant cell walls. Integration of gel-based proteomics approach with activity assays is a powerful tool for analysis and characterization of fungal secretomes and xylanolytic activities which can lead to identification of new enzymes with interesting properties, as well as provide insight into plant-fungal interactions, fungal pathogenicity and adaptation. Understanding the fungal response to host niche is of importance to uncover novel targets for potential symbionts, anti-fungal agents and biotechnical applications.
Identification of cell wall degrading enzymes including xylanases

Barley grains

Qualitative and quantitative xylanase assays

1D secretomes

31 fungal isolates

2-DE based secretomics analysis

Identification of cell wall degrading enzymes including xylanases
Highlights

- Profiling the fungal community populating barley grains
- Xylanase production by the indigenous fungi present on barley grains
- Expanding the *Asperigllus niger* secretome
- Initial secretome maps of *Fusarium poae* grown on barley flour and wheat arabinoxylan
Investigation of the indigenous fungal community populating barley grains: secretomes and xylanolytic potential

Abida Sultan\textsuperscript{a,b}, Jens C. Frisvad\textsuperscript{c}, Birgit Andersen\textsuperscript{d,e}, Birte Svensson\textsuperscript{a}, Christine Finnie\textsuperscript{d,f}\textsuperscript{*}

a. Enzyme and Protein Chemistry, Department of Biotechnology and Biomedicine, Technical University of Denmark, Elektrovej, Building 375, DK-2800 Kgs. Lyngby, Denmark.

b. Present address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, DK-2800 Kgs. Lyngby, Denmark.

c. Fungal Chemodiversity, Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark.

d. Agricultural and Environmental Proteomics, Department of Systems Biology, Technical University of Denmark, Søltofts Plads, Building 224, DK-2800 Kgs. Lyngby, Denmark.

e. Present address: Plant and Soil Science Section, Department of Plant & Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

f. Present address: Carlsberg Research Laboratory, J.C. Jacobsens Gade 4, DK-1799 Copenhagen, Denmark.

\textsuperscript{*}Manuscript Click here to download Manuscript: Manu_plant-fungus2_Chris030317_changes marked.docx Click here to view linked References
Abstract

The indigenous fungal species populating cereal grains produce numerous plant cell wall-degrading enzymes including xylanases, which could play important role in plant-pathogen interactions and in adaptation of the fungi to varying carbon sources. To gain more insight into the grain surface-associated enzyme activity, members of the populating fungal community were isolated, and their secretomes and xylanolytic activities assessed. Twenty-seven different fungal species were isolated from grains of six barley cultivars over different harvest years and growing sites. The isolated fungi were grown on medium containing barley flour or wheat arabinoxylan as sole carbon source. Their secretomes and xylanase activities were analysed using SDS-PAGE and enzyme assays and were found to vary according to species and carbon source. Secretomes were dominated by cell wall degrading enzymes with xylanases and xylanolytic enzymes being the most abundant. A 2-DE-based secretome analysis of *Aspergillus niger* and the less-studied pathogenic fungus *Fusarium poae* grown on barley flour and wheat arabinoxylan resulted in identification of 82 *A. niger* and 31 *F. poae* proteins many of which were hydrolytic enzymes, including xylanases.

Keywords: Environmental proteomics, fungal community, grain proteome, xylanase, barley.
1. Introduction

Over 200 species of microorganisms populate the surface of barley grains, including fungi, yeast and actinomycetes [1]. The composition of the microbial population varies significantly according to developmental stage of the grains, environmental factors, as well as post-harvest storage conditions. The initial colonizers of cereal grains after ear emergence are bacteria, which are replaced by yeasts and eventually by fungi after anthesis [2]. Fungi can have severe effects on the quality of the grains, due to e.g. discoloration, reduced germination, and the production of mycotoxins [3,4]. The fungi colonizing the grains can be categorized as field or storage fungi. The field fungi are those colonizing the developing and mature grains on the plant, with major genera comprising Alternaria, Cladosporium and Fusarium, which typically require high moisture content [3,4]. The storage fungi become more abundant on and within the stored grains, where the moisture level has decreased, these are primarily Aspergillus and Penicillium. Some of the fungi are known pathogens, e.g. Fusarium.

Contamination of grains by fungal mycotoxins e.g. aflatoxin and ochratoxin, poses a critical hazard to food safety, human and animal health [5]. A characteristic of the invading fungi is the secretion of a collection of enzymes, including xylanases, polygalacturonases, pectate lyases and lipases, which play important roles in nutrient acquisition, host colonization, virulence and ecological interaction [6,7]. The availability of complete fungal genome sequences and advances in -omics techniques have significantly contributed to a better understanding of plant-fungus interactions, fungal pathogenicity and defense mechanisms in plants. Gel-based proteomics has enabled detailed analysis of several fungal secretomes [8–11]. Aspergillus oryzae, traditionally used in production of fermented foods, was found to produce a combination of cell wall degrading enzymes when grown on wheat bran, including β-
glucosidases, α-mannosidases, cellulases and xylanases [12]. The secretome of A. niger grown with xylose and maltose was characterized with identification of about 200 proteins and reported to be strongly influenced by the culture conditions and available nutrient source [13]. Secretomes of F. graminearum grown on a variety of media, including isolated plant cell walls, wheat and barley flour, contain numerous secreted enzymes such as xylanases, cellulases, proteinases and lipases, depending on the nutrient source [11,14,15]. An obvious key challenge, however, is the further analysis of the function and regulation of the identified fungal proteins, including xylanases.

Although plant responses to fungal attack have been studied mainly in the context of single plant-fungus interactions, plants in the field are exposed to a diverse community of microorganisms, and rely for general protection on proteins and defense molecules produced at the interface with the environment. For example, wheat bran tissues contain numerous oxidative stress and defense-related proteins and inhibitors, as well as proteins that improve tissue strength to hinder pathogen entry [16]. Previously, we investigated the plant-microbe interface by analysis of the surface-associated proteome of barley grains [17] and found this to be dominated by plant proteins with roles in defense and stress-response. However, numerous proteins from the populating microbiota were also identified including fungal and bacterial proteins involved in polysaccharide degradation [17]. Grain-surface-associated xylanase activity was of microbial origin, and xylanases were identified from the fungi Verticillium dahlia, Cochliobolus sativus (the teleomorph of Bipolaris sorokiniana) and Pyrenophora tritici-repentis (anamorph of Drechslera tritici-repentis) [17], all of which are known to be present on grasses or cereals. To gain more insight into the ability of the fungi to produce xylanases, we isolated and identified fungi from the surface of barley grains.
and analysed their secreted proteins and xylanolytic activities. Moreover, to better understand adaptation of the fungi to varying carbon sources, the secretomes of two fungi (Aspergillus niger and Fusarium poae) grown on barley flour and wheat arabinoxylan to mimic the natural growth substrates, were characterized.

2. Materials and Methods

2.1 Barley grains and growing sites

Barley grains from six cultivars (Barke, Cabaret, Frederik, Quench, Scarlett, Simba) harvested in 2009–2011 were obtained from Sejet Plant Breeding, Horsens, Denmark (9° 50’ 51.32” E, 55° 51’ 29.27” N, 34 m). Each cultivar was grown in three plots in the same field in a fully randomized block design and grains from different plots were mixed to eliminate location effects. Quench and Simba were also grown at another site in Denmark, Koldkørgård (10°04’ 40.3” E, 56° 18’ 28.1” N, 39.99 m) in 2010 and 2011. The cultivar Himalaya harvested in 2003 in Pullman, WA, USA was also analyzed. Danish spring (March–May) and summer (June–August) of 2009 were characterized with mean temperature of 8.3°C and 16.2°C with 652 and 700 hours of sunshine, and 119 and 217 mm of precipitation. Spring and summer of 2011 had mean temperature of 8.1°C and 15.9°C with 301 and 573 hours of sunshine, and 172 and 321 mm of precipitation, respectively (Danish Meteorological Institute). According to the Danish soil classification system, soil at both growing sites Sejet and Koldkørgård were assigned a JB number of five, which denotes a coarse sandy clay texture.

2.2 Isolation of fungi from barley grains

Fungi were isolated and identified by direct plating of 20–35 grains on (i) potato
dextrose agar (PDA [18]), (ii) malt extract agar (MEA) and (iii) MEA Oxoid [19]. For species identification, the isolated fungi were cultivated on a range of different media, including dichloran glycerol (18%) agar (DG18 [20]), dichloran rose bengal yeast extract sucrose agar (DRYES [21]), vegetable juice water (V8 [22]) and Czapek Dox oprodione dichloran agar (CZID [23]). DG18 and DRYES were incubated at 25°C in the dark, while V8 and CZID plates were incubated in alternating light and dark cycles at 20–23°C. For black fungi such as Alternaria species, V8, DRYES and potato carrot agar (PCA) were used. For Fusarium species, PDA, YES (yeast extract sucrose agar [18]), and SNA (Synthetischer Nährstofffarmer agar) were used. For Penicillium species, MEA, YES, CYA (Czapek yeast extract agar [19]), and CREA (Creatine sucrose agar; [18]) were used, while for Aspergillus section Aspergillus species, CYA, CYA20S (CYA with 20% (w/v) sucrose [19]), CZ (Czapek Dox agar [18]), DG18 and YES, were used. These cultures were incubated for 7 days at different temperatures and alternating dark and light cycles. Fungi were identified based on typical colony form under a stereomicroscope (lower magnification and perception of depth) and conidia morphology with light microscope (higher magnification). The percentage of kernels infected with each identified fungal species was calculated.

2.3 Cultivation of fungi on solid medium

The fungi were cultivated in medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) agar and 1% (w/v) wheat arabinoseylan (WAX) as carbon source [24]. The fungi were grown at 25°C for 7 days.

2.4 Liquid medium
Fungi from densely covered agar plates were used to inoculate 8 mL liquid medium composed of 0.67% (w/v) yeast nitrogen base, 0.2% (w/v) asparagine and 0.5% (w/v) KH$_2$PO$_4$ supplemented with either 1% (w/v) WAX or finely ground barley flour as carbon source into 50 mL tubes [25]. The fungi grew on the surface of the medium, and the proteins were secreted into the medium to break down nutrients. Negative controls composed of medium and WAX or barley flour were included. The samples were incubated for 7 days at 25°C. Subsequently, culture supernatants were collected by centrifugation at 3200 g for 30 min at 4°C.

2.5 Washing procedure to extract the surface-associated grain proteins

A washing procedure was implemented that effectively extracts the surface-associated proteins from grains [26] in 25 mM sodium acetate pH 5.0 containing 0.02% (w/v) sodium azide under agitation for 8 h at room temperature. The washing liquids containing extracted proteins were filtered through MN 615 filter paper (Macherey-Nagel, Dueren, Germany) and assayed for xylanase activity.

2.6 Agarose plate assay for detection of xylanase activity

Agarose gels containing dyed substrate (0.1% (w/v) Remazol Brilliant Blue-dyed WAX (Megazyme), 1% (w/v) agarose, 0.2 M sodium citrate-HCl pH 4.8) were prepared in petri dishes. Five microliters of supernatant from fungal liquid cultures were added to 2 mm diameter wells punched into the plates and incubated overnight at room temperature. Xylanase activity appeared as clearing zones around the wells. The assay was used for an initial screen of all fungal isolates grown on barley flour and
WAX, and was subsequently repeated for fungal strains grown on WAX, with similar results (not shown).

2.7 Xylanase activity assay

Xylanase activity was determined in supernatants from fungal liquid cultures using the colorimetric Xylazine-AX method (Megazyme, Ireland) based on quantification of released products from the azurine-cross linked wheat arabinoxylan (AZCL-AX).

Culture supernatants (0.5 mL) were pre-incubated for 10 min at 40°C prior to addition of an AZCL-AX tablet (30% w/v). The mixture was incubated for 30 min at 40°C and 5 mL stop solution (2% (w/v) Tris base pH 9.0) was added and mixed vigorously. After 10 min at room temperature, the reaction mixtures were filtered and the absorbance was measured at 590 nm (Ultrospec II, Amersham Biosciences, Uppsala, Sweden) against a blank prepared by adding 5 mL stop solution to samples prior to addition of substrate. Correction was made for non-enzymatic color release from the AZCL-AX tablets. Duplicate measurements were performed for each of two independent growth experiments.

2.8 Protein content determination and SDS-PAGE

The protein content of the fungal culture supernatants was estimated using the amido black method with bovine serum albumin as standard [27]. Twenty micrograms of protein was precipitated by adding 4 volumes of ice-cold acetone and separated by SDS-PAGE using 4–12% BisTris NuPAGE gels and a vertical slab mini gel unit (NuPAGE Novex system, Invitrogen) according to the manufacturer’s instructions. The gels were stained with colloidal Coomassie Blue [28]. A broad-range molecular mass protein ladder (Mark 12™, Invitrogen) was used.
2.9 2D-gel electrophoresis

Fungal culture supernatants were desalted on a NAP-5 column (GE Healthcare) and 50 μg protein was precipitated by adding four volumes of ice-cold acetone and dissolved in 125 μL rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 200 mM destreak reagent (bis (2-hydroxyethyl) disulfide; GE Healthcare), 0.5% (v/v) pharmalytes pH range 3–10 (GE Healthcare), trace of bromophenol blue). The samples were applied to 7 cm pH 3–10 IPG strips (GE Healthcare) for isoelectric focusing (IEF) (Ettan™ IPGphor; GE Healthcare) after rehydration (12 h at 50 mA/strip at 20°C), performed to reach a total of 20 kVh (1 h at 150 V, 1 h at 300 V, 1 h at 1000 V, gradient to 8000 V, held at 8000 V until a total of 20 kVh). The strips were equilibrated (2 × 15 min) in 5 mL equilibration buffer (6 M urea, 30% (v/v) glycerol, 50 mM Tris HCl, pH 8.8, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) supplemented with 1% (w/v) dithiothreitol and 2.5% (w/v) iodoacetamide in the first and second equilibration steps, respectively. The strip and molecular weight marker (Mark 12, Invitrogen) were placed on NuPAGE Novex 4–12% Bis-Tris Zoom gels (Invitrogen) and run according the manufacturer’s instructions. Gels were stained with colloidal Coomassie Blue (G-250). 2D-gel electrophoresis was performed in duplicate (2 biological replicates, Supplementary Figure S1).

2.10 In-gel digestion and MALDI-TOF/TOF mass spectrometry

Spots or bands were manually excised and subjected to in-gel tryptic digestion [29]. Briefly, gel pieces were washed (100 μL 40% ethanol, 10 min), shrunk (50 μL 100% ACN) and soaked in 2 μL 12.5 ng/μL trypsin (Promega, porcine sequencing grade) in
25 mM NH$_4$HCO$_3$ on ice for 45 min. The gel pieces were rehydrated by addition of 10 μL 25 mM NH$_4$HCO$_3$ followed by incubation at 37°C overnight. Tryptic peptides (1 μL) were loaded onto an AnchorChip™ target plate (Bruker-Daltonics, Bremen, Germany), covered by 1 μL matrix solution (0.5 μg/μL CHCA in 90% ACN, 0.1% TFA) and washed in 0.5% (v/v) TFA [30]. Tryptic peptides were analyzed on an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) using Flex Control v3.0 and processed by Flex Analysis v3.0 (Bruker-Daltonics, Bremen, Germany). **MS analysis** was performed in positive ion reflector mode with 500 laser shots per spectrum. MS/MS data were acquired with an average of 1000–2000 laser shots for each spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/μL). Internal calibration was performed using trypsin autolysis products (m/z 842.5090, m/z 1045.5637 and m/z 2211.1040). Filtering of spectra was performed for known keratin peaks. Acquired MS and MS/MS spectra were analyzed using Biotools v3.1 (Bruker-Daltonics, Bremen, Germany). MASCOT 2.0 software (http://www.matrixscience.com) was used for database searches in the NCBInr (National Center for Biotechnology Information) fungi, and green plants (2555264 and 1749148 entries, respectively) and Broad Institute for *Fusarium graminearum* gene index (http://www.broad.mit.edu/annotation/genre/fusarium_graminearum, 13313 entries). The following search parameters were applied: monoisotopic peptide mass accuracy of 50 ppm; fragment mass accuracy to ± 0.7 Da; maximum of one missed cleavage; carbamidomethylation of cysteine (fixed) and oxidation of methionine (partial). The signal to noise threshold ratio (S/N) was set to 6. Probability-based MOWSE scores above the calculated threshold value (p < 0.05) with a minimum of two matched unique peptides were considered for protein identification.
3. Results

3.1. Screening fungal isolates for xylanolytic activity on barley flour and wheat arabinoxylans

To obtain an insight into the plant-fungus relationship, fungal proteins and their enzymatic activities, grain-associated fungi were isolated and identified by direct plating of 20–35 grains on various media and substrates and microscopic examination for subspecies determination. Thirty-one fungal isolates (Table 1) were grown in liquid cultures containing barley flour or WAX as the sole carbon source and the culture supernatants were analyzed for xylanase production.

Table 1. Fungal isolates used for xylanase activity assays.

<table>
<thead>
<tr>
<th>Fungal Isolate</th>
<th>Species</th>
<th>Barley cultivar</th>
<th>Harvest Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fusarium avenaceum</td>
<td>Quench</td>
<td>2010</td>
</tr>
<tr>
<td>2</td>
<td>Fusarium avenaceum</td>
<td>Quench</td>
<td>2011</td>
</tr>
<tr>
<td>3</td>
<td>Fusarium avenaceum</td>
<td>Quench</td>
<td>2010</td>
</tr>
<tr>
<td>4</td>
<td>Fusarium culmorum</td>
<td>Quench</td>
<td>2011</td>
</tr>
<tr>
<td>5</td>
<td>Fusarium graminearum</td>
<td>Simba</td>
<td>2010</td>
</tr>
<tr>
<td>6</td>
<td>Acremoniella verrucosa</td>
<td>Cabaret</td>
<td>2011</td>
</tr>
<tr>
<td>7</td>
<td>Epicoccum nigrum</td>
<td>Scarlett</td>
<td>2009</td>
</tr>
<tr>
<td>8</td>
<td>Fusarium poae</td>
<td>Frederik</td>
<td>2009</td>
</tr>
<tr>
<td>9</td>
<td>Fusarium equiseti</td>
<td>Quench</td>
<td>2010</td>
</tr>
<tr>
<td>10</td>
<td>Drechslera sp.</td>
<td>Scarlett</td>
<td>2011</td>
</tr>
<tr>
<td>11</td>
<td>Alternaria infectoria</td>
<td>Simba</td>
<td>2011</td>
</tr>
<tr>
<td>12</td>
<td>Epicoccum nigrum</td>
<td>Simba</td>
<td>2010</td>
</tr>
<tr>
<td>13</td>
<td>Alternaria tenuissima</td>
<td>Scarlett</td>
<td>2009</td>
</tr>
<tr>
<td>14</td>
<td>Alternaria infectoria</td>
<td>Frederik</td>
<td>2009</td>
</tr>
<tr>
<td>15</td>
<td>Cladosporium sp.</td>
<td>Scarlett</td>
<td>2009</td>
</tr>
<tr>
<td>16</td>
<td>Drechslera sp.</td>
<td>Quench</td>
<td>2010</td>
</tr>
<tr>
<td>17</td>
<td>Cladosporium sp.</td>
<td>Quench</td>
<td>2010</td>
</tr>
<tr>
<td>18</td>
<td>Cladosporium sp.</td>
<td>Frederik</td>
<td>2009</td>
</tr>
<tr>
<td>19</td>
<td>Penicillium brevicompactum</td>
<td>Simba</td>
<td>2011</td>
</tr>
<tr>
<td>20</td>
<td>Penicillium brevicompactum</td>
<td>Quench</td>
<td>2010</td>
</tr>
<tr>
<td>21</td>
<td>Penicillium brevicompactum</td>
<td>Cabaret</td>
<td>2011</td>
</tr>
<tr>
<td>22</td>
<td>Penicillium verrucosum</td>
<td>Cabaret</td>
<td>2011</td>
</tr>
<tr>
<td>23</td>
<td>Aspergillus niger</td>
<td>Simba</td>
<td>2010</td>
</tr>
<tr>
<td>24</td>
<td>Penicillium chrysogenum</td>
<td>Scarlett</td>
<td>2011</td>
</tr>
<tr>
<td>25</td>
<td>Aspergillus pseudoglaucus</td>
<td>Simba</td>
<td>2010</td>
</tr>
<tr>
<td>26</td>
<td>Aspergillus pseudoglaucus</td>
<td>Scarlett</td>
<td>2011</td>
</tr>
</tbody>
</table>
Phoma sp.         Cabaret   2009
Fusarium tricinctum  Cabaret  2009
Fusarium tricinctum  Cabaret  2009
Penicillium cyclopium  Himalaya  2003
Penicillium freii       Himalaya  2003

WAX was used to induce the production of xylan degrading enzymes, while barley flour was used to resemble a natural substrate for the fungi. Plate zymograms prepared with 0.1% dyed substrate (RBB WAX) were used as a screen for xylanolytic activity (Fig.1). Xylanases produced by the cultivated fungi could be assessed qualitatively by the clarity and size of the degradation zones surrounding the punched wells.

The culture supernatants from WAX showed more prominent clearing zones compared to barley flour. Noticeably, the storage fungi *Penicillium* and *Aspergillus* gave rise to larger and more opaque degradation zones, while field fungi such as *Fusarium, Alternaria, Epicoccum* and *Drechslera* spp. generally gave rise to smaller and clearer zones. The different qualitative character of the clearing zones will depend on the amount and activity of the xylanase(s) present in the samples and be influenced by different growth rates of the fungi in the liquid cultures, but may also reflect production of xylanases with different functional characteristics. Quantitative xylanase activity assays were performed for five selected fungi of three genera (*Fusarium, Phoma* and *Penicillium*, Table 2) isolated from the two barley cultivars Cabaret 2009 and Himalaya 2003, which exhibited high (0.127 U/g) and low (0.043 U/g) surface-associated xylanase activity levels. The field fungus species *F. tricinctum* and *Phoma* spp. were predominantly found on Cabaret 2009, while storage fungus species *P. cyclopium* and *P. freii* essentially found on Himalaya 2003.
Table 2. Xylanase activity measured in culture supernatants from five selected fungi grown in medium with wheat arabinoxylan and ground barley flour as substrate.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Species</th>
<th>Specific xylanase activity (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Replicate 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WAX</td>
</tr>
<tr>
<td>27</td>
<td><em>Phoma</em> sp.</td>
<td>0.01</td>
</tr>
<tr>
<td>28</td>
<td><em>Fusarium tricinctum</em></td>
<td>0.18</td>
</tr>
<tr>
<td>29</td>
<td><em>Fusarium tricinctum</em></td>
<td>0.40</td>
</tr>
<tr>
<td>30</td>
<td><em>Penicillium cyclopium</em></td>
<td>1.52</td>
</tr>
<tr>
<td>31</td>
<td><em>Penicillium freii</em></td>
<td>3.25</td>
</tr>
<tr>
<td>32</td>
<td>Negative control</td>
<td>0.01</td>
</tr>
</tbody>
</table>

3.2. Secreted protein profiles of five selected fungi grown on barley flour and wheat arabinoxylans

SDS-PAGE was used to screen the secreted protein profiles of the fungal isolates grown on WAX and barley flour (Fig. 2). Protein patterns of the cultivated fungi are species-specific, reflecting secretion of different proteins. The fungal supernatants grown with barley flour as carbon source displayed a prominent band of molecular size of 10 kDa, which is probably a barley protein originating from the medium. The fungal strains grown with WAX gave rise to faint bands after Coomassie Blue staining and an accumulation of high molecular weight material was visible in the wells. Twenty bands (Fig. 2) of five selected fungi were excised, tryptic digested and analyzed by MALDI-TOF/TOF, which resulted in ten confident identifications, of which four (bands 3, 8, 14 and 15) were identified as endo-1,4-β-xylanases (Table 3). Other bands contained cell wall-degrading enzymes and hypothetical proteins.
Table 3. Identification of proteins in SDS-PAGE bands of culture supernatants from five selected fungi grown on WAX and barley flour\(^a\).

<table>
<thead>
<tr>
<th>no</th>
<th>Accession no.</th>
<th>Organism</th>
<th>Protein (GH family)</th>
<th>Mw theor/meas</th>
<th>PMF score</th>
<th>E-value</th>
<th>Sequence coverage %</th>
<th>Unique peptides</th>
<th>MS/MS Precursors</th>
<th>MS/MS Peptide sequences</th>
<th>Ion score</th>
<th>Expect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>gi</td>
<td>255931857</td>
<td><em>Penicillium chrysogenum</em></td>
<td>Alpha-amylase GH13</td>
<td>51032/68700</td>
<td>179</td>
<td>1.9E-12</td>
<td>14</td>
<td>5</td>
<td>1112.5729</td>
<td>R.NIYFALTD.R.I</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1830.8534</td>
<td>R.DLYSINENYGTADDL.K.S</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2312.0883</td>
<td>R.GIPIVYYGTEQFYAGGNDPANR.E</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R.VIGEDFVR.I</td>
<td>53</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R.IAFETAR.A</td>
<td>40</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>169159203</td>
<td><em>Penicillium citrinum</em></td>
<td>Endo-1,4-beta-xylanase GH11</td>
<td>35338/38000</td>
<td>134</td>
<td>4.9E-08</td>
<td>24</td>
<td>7</td>
<td>934.5074</td>
<td>R.VIGEDFVR.I</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>807.4367</td>
<td>R.IAFETAR.A</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>70996610</td>
<td><em>Aspergillus fumigatus</em></td>
<td>Beta-xylosidase XylA GH3</td>
<td>86731/121600</td>
<td>250</td>
<td>1.2E-19</td>
<td>13</td>
<td>9</td>
<td>1612.8372</td>
<td>R.YGLDVYAPNINA.F.R.S</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1778.9684</td>
<td>R.VLYPGYELALN.NER.S</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>gi</td>
<td>255930951</td>
<td><em>Penicillium chrysogenum</em></td>
<td>Endo-arabinase GH43</td>
<td>36188/33600</td>
<td>118</td>
<td>1.80E-06</td>
<td>15</td>
<td>3</td>
<td>892.4683</td>
<td>K.WLVGFDR.L</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1795.958</td>
<td>K.VGADGVTPIGDAVQILD.R.D</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1676.7563</td>
<td>K.TGLISPGGGNGVCCGD.R.M</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>gi</td>
<td>3915310</td>
<td><em>Aspergillus aculeatus</em></td>
<td>Endo-1,4-beta-xylanase GH10</td>
<td>35423/38000</td>
<td>156</td>
<td>3.10E-10</td>
<td>15</td>
<td>4</td>
<td>807.434</td>
<td>R.IAFETAR.A</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1775.9111</td>
<td>K.LYINDYNLDSASYP.K.L</td>
<td>90</td>
</tr>
<tr>
<td>9</td>
<td>gi</td>
<td>344228869</td>
<td><em>Candida tenuis</em></td>
<td>Hypothetical protein</td>
<td>76355/113600</td>
<td>76</td>
<td>0.031</td>
<td>16</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>gi</td>
<td>310699603</td>
<td><em>Fusarium oxysporum</em></td>
<td>Alpha-D-galactopranosidase GH31</td>
<td>45222/46600</td>
<td>94s</td>
<td>0.00055</td>
<td>14</td>
<td>5</td>
<td>984.4848</td>
<td>K.FGLYGDGGGAK.T</td>
<td>59</td>
</tr>
<tr>
<td>14</td>
<td>gi</td>
<td>374253734</td>
<td><em>Fusarium oxysporum</em></td>
<td>Endo-1,4-beta-xylanase A GH10</td>
<td>36403/48600</td>
<td>95</td>
<td>0.00038</td>
<td>13</td>
<td>4</td>
<td>935.541</td>
<td>R.LVKSYGL.R.I</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>gi</td>
<td>302913666</td>
<td><em>Nectria haematococca</em></td>
<td>Hypothetical protein</td>
<td>24103/329900</td>
<td>65</td>
<td>0.35</td>
<td>17</td>
<td>3</td>
<td>1989.9925</td>
<td>K.GEVTVDGSVYDIYSTR.T</td>
<td>46</td>
</tr>
<tr>
<td>20</td>
<td>gi</td>
<td>119481903</td>
<td><em>Neosartorya fischeri</em></td>
<td>Alpha-L-arabinofuranosidase A GH43</td>
<td>70046/75100</td>
<td>100</td>
<td>0.00014</td>
<td>3</td>
<td>2</td>
<td>1561.7308</td>
<td>R.FPGGNLEGTIDGR.W</td>
<td>93</td>
</tr>
</tbody>
</table>

\(^a\) theor: theroretical; meas.: measured; GH: glycoside hydrolase

\(^b\) refers to Fig. 2.
3.3 Profiling the fungal communities on barley grains

Barley harvested from two different sites in years 2009–2011 was used to profile the fungal communities. Surface xylanase activity [17] of the grains was measured in parallel (Table 4). The barley samples could be divided into two groups with low (<0.06 U/g) and high (>0.10 U/g) activity, respectively. The two most predominant fungal species, identified on the majority of grain samples were *Alternaria infectoria* and *Fusarium culmorum*. The cultivars (cvs.) showed varying levels of grain colonization by different fungal species. The predominant species harboured by the low xylanase group comprising cvs. Frederik, Simba, Barke, Cabaret, Quench (all 2011), Cabaret (2010) and Himalaya (2003), were *Aspergillus pseudoglaucus*, *Epicoccum nigrum*, *Penicillium spathulatum* and *Chalastospora gossypii* (Table 4).

The high xylanase group comprising Cabaret, Frederik and Simba (all 2009), Quench and Simba (2011) contained *F. tricinctum*, *Cochiobolus sativus* (*Drechslera*), *Gonatobotrys simplex* and *Phoma sp.*. However, there was no clear correlation between fungal species, growth location or year, and grain surface xylanase activity.

3.4 Proteome analysis of *Aspergillus niger* and *Fusarium poae* secretomes on barley flour and wheat arabinoxylan

To access the secretomes of *A. niger* (isolate 23) and *F. poae* (isolate 8), 2-DE was performed to map and identify the proteins in the culture medium containing WAX and barley flour. Representative 2D-gels (pH 4–8.5) of the secretomes of *A. niger* and *F. poae* grown on WAX and barley flour are shown (Fig. 3). The protein patterns of *A. niger* grown either on WAX or barley flour were similar and contained approximately 105 resolved spots, while the *F. poae* secretomes were less well resolved and with
only 54 spots. Visible spots were excised from the 2D gels for identification by MALDI-MS and MS/MS, which resulted in 82 and 30 confident protein identifications from the *A. niger* and *F. poae* gels, respectively (Table 5, Supplementary Table S1). The identified proteins included glycoside hydrolases, proteases, oxidoreductases, esterases, nuclease, lyases, housekeeping enzymes, hypothetical proteins, and proteins with unknown function.
Table 4. The isolated fungi listed in order of incidence (number of samples from which the fungus was isolated) followed by severity (percentage of grains containing fungus).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>S</th>
<th>S</th>
<th>U</th>
<th>S</th>
<th>S</th>
<th>S</th>
<th>S</th>
<th>S</th>
<th>S</th>
<th>K</th>
<th>K</th>
<th>S</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth location</td>
<td>Frederik</td>
<td>Simba</td>
<td>Himalaya</td>
<td>Barke</td>
<td>Cabaret</td>
<td>Cabaret</td>
<td>Quench</td>
<td>Cabaret</td>
<td>Frederik</td>
<td>Quench</td>
<td>Simba</td>
<td>Simba</td>
<td></td>
</tr>
<tr>
<td>Surface xylanase (U/g)</td>
<td>0.031</td>
<td>0.041</td>
<td>0.043</td>
<td>0.057</td>
<td>0.058</td>
<td>0.059</td>
<td>0.060</td>
<td>0.127</td>
<td>0.168</td>
<td>0.171</td>
<td>0.171</td>
<td>0.172</td>
<td></td>
</tr>
<tr>
<td>Percentage of grains with fungus* (incidence)</td>
<td>40</td>
<td>5</td>
<td>-</td>
<td>2</td>
<td>10</td>
<td>7</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>5</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Alternaria infectoria (10)</td>
<td>-</td>
<td>20</td>
<td>14</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fusarium culmorum (8)</td>
<td>7</td>
<td>4</td>
<td>-</td>
<td>15</td>
<td>7</td>
<td>17</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Epicoccum nigrum(7)</td>
<td>30</td>
<td>-</td>
<td>5</td>
<td>4</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Aspergillus pseudoglaucus (6)</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Nigrospora spp.(5)</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gotatobryts simplex (5)</td>
<td>-</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cochliobolus sativus (Drechslera) (5)</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Penicillium saphulatum (3)</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chalastospora gossypii (Alt. malorum) (2)</td>
<td>15</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cladosporium cladosporoides (complex)(2)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Aspergillus spinulosporus (2)</td>
<td>-</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harzia vernicosa (2)</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tricoderma hamatum(2)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Phoma sp.(1)</td>
<td>20</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium tricinatum (1)</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claetomium globosum (1)</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillium scabrosum (1)</td>
<td>20</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium poae (1)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Penicillium verrucosum (1)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acremoniella verrucosa (1)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Penicillium brevicompactum (1)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Penicillium verrucosum(1)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rhizopus nigricans (1)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Penicillium freii (1)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Walleria (1)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulocladium atrium (2)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Penicillium sp.(1)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Barley samples listed in order of increasing surface xylanase activity
1 S: Sejet, Denmark; U: Pullman, WA, USA; K: Koldkærgaard, Denmark
2 Fungi listed in order of incidence (number of samples from which the fungus was isolated) followed by severity (percentage of grains containing fungus)
Table 5. MALDI-MS and MS/MS identification of proteins from culture supernatants of *Aspergillus niger* and *Fusarium poae* grown with wheat arabinoxylan (WAX) or barley flour as carbon source. Spot numbers correspond to Fig.3. For identification details refer to Supplementary Table S1.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession</th>
<th>Locus</th>
<th>Organism</th>
<th>Protein name</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1</td>
<td>gi</td>
<td>358375153</td>
<td>GAA91739</td>
<td><em>Aspergillus kawachii</em> IFO 4308</td>
</tr>
<tr>
<td>a2</td>
<td>gi</td>
<td>224027</td>
<td>1008149A</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a4</td>
<td>gi</td>
<td>3913152</td>
<td>AXHA_ASPTU</td>
<td><em>Aspergillus tubingensis</em></td>
</tr>
<tr>
<td>a5</td>
<td>gi</td>
<td>3913152</td>
<td>AXHA_ASPTU</td>
<td><em>Aspergillus tubingensis</em></td>
</tr>
<tr>
<td>a6</td>
<td>gi</td>
<td>317028138</td>
<td>XP_001389996</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a8</td>
<td>gi</td>
<td>358375979</td>
<td>GAA92552</td>
<td><em>Aspergillus kawachii</em> IFO 4308</td>
</tr>
<tr>
<td>a10</td>
<td>gi</td>
<td>317028138</td>
<td>XP_001389996</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a11</td>
<td>gi</td>
<td>1362263</td>
<td>S55931</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a12</td>
<td>gi</td>
<td>145228427</td>
<td>XP_001388522</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a13</td>
<td>gi</td>
<td>1362263</td>
<td>S55931</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a14</td>
<td>gi</td>
<td>1362263</td>
<td>S55931</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a15</td>
<td>gi</td>
<td>1362263</td>
<td>S55931</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a16</td>
<td>gi</td>
<td>19919756</td>
<td>AF490982_1</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a19</td>
<td>gi</td>
<td>145228427</td>
<td>XP_001388522</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a20</td>
<td>gi</td>
<td>19919756</td>
<td>AF490982_1</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a21</td>
<td>gi</td>
<td>145228427</td>
<td>XP_001388522</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a22</td>
<td>gi</td>
<td>9858848</td>
<td>AAG01166</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a23</td>
<td>gi</td>
<td>19919756</td>
<td>AF490982_1</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>a24</td>
<td>gi</td>
<td>145228427</td>
<td>XP_001388522</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a25</td>
<td>gi</td>
<td>145228427</td>
<td>XP_001388522</td>
<td><em>Aspergillus niger</em> CBS 513.88</td>
</tr>
<tr>
<td>a30</td>
<td>gi</td>
<td>358369412</td>
<td>GAA86026</td>
<td><em>Aspergillus kawachii</em> IFO 4308</td>
</tr>
<tr>
<td>a31</td>
<td>gi</td>
<td>328864038</td>
<td>EGG13137</td>
<td><em>Melampsora larici-populina</em> (strain 9BAG31)</td>
</tr>
<tr>
<td>a32</td>
<td>gi</td>
<td>328864038</td>
<td>EGG13137</td>
<td><em>Melampsora larici-populina</em> (strain 9BAG31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Putative uncharacterized protein</td>
</tr>
<tr>
<td>a64</td>
<td>gi</td>
<td>317025187</td>
<td>XP_001388626</td>
<td>Aspergillus niger CBS 513.88</td>
</tr>
<tr>
<td>a65</td>
<td>gi</td>
<td>358369379</td>
<td>GAA85994</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a68</td>
<td>gi</td>
<td>358370052</td>
<td>GAA8666</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a69</td>
<td>gi</td>
<td>461623</td>
<td>BGAL_ASPNG</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>a70</td>
<td>gi</td>
<td>134077473</td>
<td>P29853</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>a71</td>
<td>gi</td>
<td>350633946</td>
<td>EHA22310</td>
<td>Aspergillus niger ATCC 1015</td>
</tr>
<tr>
<td>a72</td>
<td>gi</td>
<td>358373696</td>
<td>AGALC_ASPNG Q9UUZ4</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a74</td>
<td>gi</td>
<td>358375153</td>
<td>GAA85748</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a75</td>
<td>gi</td>
<td>358375222</td>
<td>GAA91807</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a76</td>
<td>gi</td>
<td>358375153</td>
<td>GAA91739</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a77</td>
<td>gi</td>
<td>358370756</td>
<td>GAA91739</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a79</td>
<td>gi</td>
<td>350633910</td>
<td>EHA22274</td>
<td>Aspergillus niger ATCC 1015</td>
</tr>
<tr>
<td>a80</td>
<td>gi</td>
<td>350633910</td>
<td>EHA22274</td>
<td>Aspergillus niger ATCC 1015</td>
</tr>
<tr>
<td>a81</td>
<td>gi</td>
<td>145231236</td>
<td>XP_001389882</td>
<td>Aspergillus niger CBS 513.88</td>
</tr>
<tr>
<td>a82</td>
<td>gi</td>
<td>358367957</td>
<td>GAA85748</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a83</td>
<td>gi</td>
<td>317036371</td>
<td>XP_001398198</td>
<td>Aspergillus niger CBS 513.88</td>
</tr>
<tr>
<td>a84</td>
<td>gi</td>
<td>358365618</td>
<td>GAA85748</td>
<td>Aspergillus kawachii IFO 4308</td>
</tr>
<tr>
<td>a85</td>
<td>gi</td>
<td>308212489</td>
<td>ADG21450</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>a86</td>
<td>gi</td>
<td>157829865</td>
<td>EHA19519</td>
<td>Aspergillus niger ATCC 1015</td>
</tr>
<tr>
<td>a87</td>
<td>gi</td>
<td>189484494</td>
<td>ACE00420</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>a88</td>
<td>gi</td>
<td>358368862</td>
<td>EHA22274</td>
<td>Aspergillus niger ATCC 1015</td>
</tr>
</tbody>
</table>

### Aspergillus niger on WAX

| a32 | gi|40313280 | BAD06004 | Aspergillus awamori | Glucoamylase, GH15 |
| a33 | gi|358375978 | GAA92551 | Aspergillus kawachii IFO 4308 | Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68 |
| a34 | gi|259016351 | FAEA_ASPAW | Aspergillus awamori | Feruloyl esterase A |
| a35 | gi|3913152 | AXHA_ASPTU | Aspergillus tubingensis | Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68 |
| a36 | gi|3913152 | AXHA_ASPTU | Aspergillus tubingensis | Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68 |
| a37 | gi|300706143 | XP_002995371 | Nosema ceranae BRL01 | Hypothetical protein NCER_101765 |
| a38 | gi|254571817  | XP_002493018  | Komagataella pastoris GS115 | Hypothetical protein |
| a39 | gi|317028138  | XP_001389996  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase F GH10 |
| a40 | gi|358375979  | GAA92552  | Aspergillus kawachii IFO 4308 | Endo-1,4-β-xylanase A GH11 |
| a41 | gi|317028138  | XP_001389996  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase F1 GH10 |
| a42 | gi|13626263  | SS5931  | Aspergillus niger | Cellulase GH12 |
| a43 | gi|19919756  | AF490982_1  | Aspergillus niger | Endo-1,4-β-xylanase GH11 |
| a44 | gi|380865431  | XYNB_ASPKW  | Aspergillus kawachii IFO 4308 | Endo-1,4-β-xylanase B GH11 |
| a45 | gi|145228427  | XP_001388522  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase A GH11 |
| a46 | gi|145228427  | XP_001388522  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase A GH11 |
| a47 | gi|13242071  | AAK16546  | Aspergillus niger | Xylanase |
| a48 | gi|13242071  | AAK16546  | Aspergillus niger | Xylanase |
| a49 | gi|145228427  | XP_001388522  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase A GH11 |
| a50 | gi|145228427  | XP_001388522  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase A GH11 |
| a51 | gi|19919756  | AF490982_1  | Aspergillus niger | Endo-1,4-β-xylanase, GH11 |
| a52 | gi|9858848  | AAG01166  | Aspergillus niger | xylanase GH11 |
| a53 | gi|145228427  | XP_001388522  | Aspergillus niger CBS 513.88 | Endo-1,4-β-xylanase A GH11 |
| a54 | gi|138948494  | ACE00420  | Aspergillus niger | Alpha-L-arabinofuranosidase E GH43 |
| a55 | gi|138948494  | ACE00420  | Aspergillus niger | Alpha-L-arabinofuranosidase E GH43 |
| a56 | gi|145324270  | XP_001400506  | Aspergillus niger CBS 513.88 | Glutaminase GtA |
| a57 | gi|358370298  | GAA86910  | Aspergillus kawachii IFO 4308 | Six-hairpin glycosidase |
| a58 | gi|1340838763  | CAK47097  | Aspergillus niger | Peptidase_S10 |
| a59 | gi|358370493  | GAA87104  | Aspergillus kawachii IFO 4308 | Melibiose D (GH27 or 13) |
| a60 | gi|4235093  | AAD13106  | Aspergillus niger | Beta-xylosidase GH3 |
| a61 | gi|7009581  | CAB75696  | Aspergillus niger | Beta-glucosidase |
| a62 | gi|118582212  | ABL07484  | Aspergillus niger | Lactase, partial |
| a63 | gi|358367698  | GAA84316  | Aspergillus kawachii IFO 4308 | Hypothetical protein AKAW_02431 |
| a64 | gi|358376345  | GAA92905  | Aspergillus kawachii IFO 4308 | Beta-glucosidase |
| a65 | gi|358373696  | GAA90293  | Aspergillus kawachii IFO 4308 | Alpha-galactosidase C |
| a66 | gi|358375222  | GAA91807  | Aspergillus kawachii IFO 4308 | Mycelial catalase Cat1 |
| a67 | gi|3912991  | AGUA_ASPTU  | Aspergillus tubingensis | Alpha-glucuronidase A GH67 |
| a68 | gi|358375006  | GAA91593  | Aspergillus kawachii IFO 4308 | Alpha-xylosidase GH31 |
| a69 | gi|358370259  | GAA86871  | Aspergillus kawachii IFO 4308 | EstA precursor |
| a70 | gi|358370442  | GAA87053  | Aspergillus kawachii IFO 4308 | Tripeptidyl-peptidase |
| a71 | gi|55670667  | 1WD3_A  | Aspergillus kawachii | Chain A, Crystal structure of arabinofuranosidase |
| a72 | gi|358367805  | GAA84423  | Aspergillus kawachii IFO 4308 | Beta-glucuronidase |

**Fusarium poae on barley flour**

<p>| f2 | gi|1310677  | CAA66232  | Hordeum vulgare subsp. vulgare | Protein z-type serpin |
| f3 | gi|1310677  | CAA66232  | Hordeum vulgare subsp. vulgare | Protein z-type serpin |</p>
<table>
<thead>
<tr>
<th>Accession</th>
<th>gi</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>f4</td>
<td>gi</td>
<td>1310677</td>
</tr>
<tr>
<td>f5</td>
<td>gi</td>
<td>1310677</td>
</tr>
<tr>
<td>f11</td>
<td>gi</td>
<td>146120810</td>
</tr>
<tr>
<td>f12</td>
<td>gi</td>
<td>358375979</td>
</tr>
<tr>
<td>f23</td>
<td>gi</td>
<td>225102</td>
</tr>
<tr>
<td>f24</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f25</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f26</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f27</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f28</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f29</td>
<td>gi</td>
<td>585290</td>
</tr>
<tr>
<td>f30</td>
<td>gi</td>
<td>225102</td>
</tr>
<tr>
<td>f31</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f32</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f33</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f34</td>
<td>gi</td>
<td>149237516</td>
</tr>
<tr>
<td>f36</td>
<td>gi</td>
<td>1405736</td>
</tr>
<tr>
<td>f37</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f38</td>
<td>gi</td>
<td>123970</td>
</tr>
<tr>
<td>f39</td>
<td>gi</td>
<td>326503930</td>
</tr>
<tr>
<td>f40</td>
<td>gi</td>
<td>1405736</td>
</tr>
<tr>
<td>f41</td>
<td>gi</td>
<td>68305063</td>
</tr>
<tr>
<td>f42</td>
<td>gi</td>
<td>1588926</td>
</tr>
<tr>
<td>f43</td>
<td>gi</td>
<td>1405736</td>
</tr>
<tr>
<td>f44</td>
<td>gi</td>
<td>326499596</td>
</tr>
<tr>
<td>f45</td>
<td>gi</td>
<td>225465030</td>
</tr>
<tr>
<td>f46</td>
<td>gi</td>
<td>326503930</td>
</tr>
</tbody>
</table>

**Fusarium poae on WAX**

<table>
<thead>
<tr>
<th>Accession</th>
<th>gi</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>f48</td>
<td>gi</td>
<td>46115498</td>
</tr>
<tr>
<td>f50</td>
<td>gi</td>
<td>46138441</td>
</tr>
<tr>
<td>f52</td>
<td>gi</td>
<td>46138969</td>
</tr>
</tbody>
</table>

---

21
4. Discussion

4.1. Profiling the populating fungal community on barley grains and their secretomes

Several proteomics studies have analyzed the effect of fungal infections on cereal grain proteomes [31,32]. Metagenomics and metaproteomics analyses of the populating microbial communities and proteomes have to date mainly been applied to the rhizosphere and phyllosphere [33]. Little is yet known about the dynamic composition of the microbial populations on barley grains, their interactions with the host, proteomes and enzymatic activities. In this study, barley cvs. over different harvest years and growing sites were cultivated in a range of different media to compare the composition of the fungal populations, and to investigate their secretomes and xylanolytic activities. In general the grains were colonized by field fungal genera *Alternaria*, *Fusarium*, *Dreschlera* (*Cochliobolus sativus*) and *Nigrospora* and *Epicoccum*, which are all known to be part of the natural mycobiota on cereals. The universal presence of *Alt. infectoria* on barley grains has also been reported elsewhere [4]. Identification of high numbers of field fungi on stored grains from the 2009 harvest indicates that field fungal spores survived the storage period and still provide a snapshot of conditions in the field. In a previous study of the surface-associated proteome of barley grains, among the identified microbial proteins, numerous fungal proteins were found [17]. The matched database sequences originated from the fungal genera *Dreschlera*, *Fusarium* and *Penicillium*, all of which were isolated and identified in the present study. The identified proteins included xylanases of glycoside hydrolase families GH10 and GH11 from *Cochliobolus sativus* (*Dreschlera sorokiniana = Bipolaris sorokiniana*) and *Pyrenophora tritici-repentis* (telemorph of...
Dreschslera tritici-repentis) and enzymes involved in primary metabolism such as
glyceraldehyde 3-phosphate dehydrogenase from Fusarium and RNA processing
protein IPI3 from Penicillium [17]. The correlation between the inferred origin of
identified fungal proteins in the washing liquids of barley grains with the current set of
isolated fungi further validates the approach.

The presence of fungal species on barley grains is highly dependent on environmental
factors. In samples from 2009, where there was a warm spring and summer in
Denmark (see 2.1), high numbers of the genera Alternaria, Fusarium, Gonatobotrys
and Phoma are seen (Table 4). In contrast, spring and summer of 2011 had fewer
hours of sunshine and more precipitation (see 2.1) and samples are dominated by
Alternaria, Fusarium and Aspergillus section Aspergillus. In addition, the barley
cultivar Himalaya harvested in the US in 2003 was analyzed and exhibited a distinct
profile with Chalastospora gossypii (earlier name Alt. malorum), Ulocladium atrum
and Epicoccum nigrum. Barley cvs. harvested in UK were reported to be invaded by
large numbers of Alt. alternate, Cladosporium Cladosporioides, Aureobasidium
pullulans, Epicoccum nigrum, and several Penicillium species [2]. Most of the barley-
associated fungi are regarded as non-toxigenic including Cladosporium, Aspergillus
pseudoglaucus and Epicoccum. Both Alternaria and Cladosporium have been reported
to cause various degrees of grey discoloration, which in part can be due to pigments in
their mycelium or from melamins produced by plant cells. However, some fungi not
only cause staining to the grains, but also produce mycotoxins, which are harmful to
humans and animals [4,34]. High levels of Fusarium can be harmful, as some species
are capable of producing an array of mycotoxins [4,18]. Other commonly known
toxigenic species include Alternaria (except Alt. infectoria species-group), Aspergillus
and Penicillium verrucosum (ochratoxin A and citrinin) and P. freii (xanthomegnin
and viomellein).

No clear correlation was observed between any of the isolated fungal species and the surface xylanase activity measured for the corresponding samples (Table 1 and 4). This probably reflects the contribution of multiple organisms to the total xylanolytic capacity of the population, many of which may not be culturable and are therefore not taken into account using the present methodology.

4.2 Profiling the grain-associated fungal secretomes and xylanolytic activities

Fungi colonizing the grains must be able to proliferate and become established rapidly, as well as to produce necessary enzymes for nutrient acquisition. Fungal growth is strongly influenced by abiotic factors such as temperature, water activity and pH. In this study, fungal isolates were grown in WAX and barley flour and culture supernatants analyzed for xylanolytic activities. It might be expected that the fungi would produce higher xylanase activity levels when grown on WAX compared to barley flour, since the amount of arabinose (36%) and xylose (51%) is much higher in WAX than in barley flour, which is a complex mixture of nutrients, including starch, non-starch polysaccharides (e.g. β-glucans), proteins and lipids. Arabinoxylans constitute about 7.1–8.0% of barley grains [35]. Moreover, barley grains contain xylanase inhibitors targeting bacterial and fungal xylanases [36], which would decrease the amount of measureable xylanase activity in flour-containing culture supernatants. While this is suggested by smaller or weaker clearing zones produced by supernatants from flour-grown cultures (Fig. 1), it is not the case for all and the higher xylanase for isolates 27–31 is for flour cultures (Table 2), probably reflecting superior fungal growth supported by the more complex flour medium.
The storage fungi genera *Penicillium* and *Aspergillus* are known to produce higher xylanase levels than the field fungi [37–39]. In the field, invading fungi can infect the grain and further invade the plant, while storage fungi only have access to the grain as a source of nutrition. The storage fungi are therefore more specific in nutrient acquisition [37,40], which might explain their higher xylanase production.

Analysis of SDS-PAGE bands from culture supernatants enabled identification of proteins with roles in degradation of complex polysaccharides. Synergistic actions of the identified arabinases, xylanases and xylosidases can efficiently depolymerize the arabinoxylan found in high abundance in plant cell walls, thus drastically changing the mass and solubility of the substrate enhancing nutrient availability. Four of the ten identified proteins were xylanases, validating the approach for identification of xylanolytic activities from grain-associated fungi. Notably, none were identical to the xylanases identified directly from the grain surface proteome [17], indicating the complementary of the approaches applied. Two xylanases from family GH10 and two from GH11 were identified (Table 3). Based on these identifications it was decided to undertake a 2-DE-based analysis of secretomes from two selected fungal isolates.

### 4.3 Profiling the 2D-secretome of *Aspergillus niger* grown on barley and WAX

Proteomes are dynamic, and a large amount of information about the functional responses of an organism can be obtained by characterizing the proteome under different physiological conditions. Hitherto, only a few proteomics studies have been performed on grain-associated fungi with substrates present in the host plant [11,14,41,42]. Secretome analysis of *A. niger* grown on WAX and barley flour as sole
carbon source resulted in identification of a battery of proteins targeted towards plant cell wall degradation and carbohydrate catabolism. The majority of the identifications were of xylanolytic enzymes, namely 1,4-β-arabinoxylan arabinofuranohydrolase AxhA (GH43, spots 4–5, 33, 35–36 and 62), α-L-arabinofuranosidase A and E (GH51, spots 51, 86 and GH43, spot 89), α-glucuronidase A (GH67, spot 101), β-glucuronidase (GH2, spot 105), endo-1,4-β-xylanases F1 (GH10, spots 6, 10, 39, 43), A (GH11, spots 8, 12, 19, 21, 24–25, 42, 47–48, 51 and 63) and B (GH11, spot 46), xyloglucanase (GH16, spot 64), and α- and β-xidosidase (GH31, spot 102 and GH3, spot 94). α-Galactosidase C and D (melibiase, GH36, spots 72, 99 and GH27, spot 93), and β-glactosidase (LacA, GH35, spot 68 and 69) found from A. niger grown on barley flour are specialized in hydrolysing α- and β-linked galactosides from oligo- (e.g. melibiose and raffinose) and polysaccharides (e.g. xylan and galactomannan) [42,43]. Feruloyl esterase A (spot 34) removes ferulic acid from plant cell wall polysaccharides, and is known to act synergistically with xylanolytic enzymes and facilitate access to the backbone of cell wall polymers. Endo-1,4-β-xylanases cleave glycosidic bonds in the xylan backbone generating substituted or unsubstituted xylo-oligosaccharides (XOS), while β-xidosidases cleave these products from the non-reducing end, liberating xylose [44,45]. It has been reported that these key enzymes are regulated at the transcriptional level by the activator XlnR and the genes encoding the xylanolytic enzymes are induced upon growth on XOS [46]. We also found α-glucosidase AgIU (GH31, spots 1, 74–76), which hydrolys α-1-6 bonds found in oligosaccharides such as melibiose and raffinose produced by α- and β-amylases. Cellulolytic enzymes constituted another major group, including α-1,3-glucanase (mutanase, spot 78), β-1,4-glucanase (cellulase, GH12, spots 11–15, 44), β-1,4-glucan cellobiohydrolase B (GH7, spot 86), exoglucanase CBHII (GH7, spot 84) and β-
The polysaccharides, cellulose and hemicellulose xylan, are the major structural components of plant cell walls, and both xylanolytic and cellulolytic enzymes work in concert in degradation of cellulose to glucose. Starch degrading enzymes, i.e. glucoamylase (GH15, spots 2 and 32) and α-amylase (GH13, spot 85) as well as polysaccharide hydrolyzing α-mannosidase (GH92, spot 70), β-mannosidase MndA (GH2, spot 65) and α-galactosidase C and D (melibiase, GH36, spots 72, 99 and GH27 spot 93), were identified in the culture medium of A. niger grown on WAX or barley flour. In addition, β-galactosidase LacA (GH35, spot 68) that hydrolyzes lactose to galactose and glucose, was also found in both media (Fig. 3, Table 5). The most abundant proteins identified on the 2D-gels were arabinofuranosidases and xylanases present in multiple spots with varying pI values, which could be due to post translational modifications or existence of closely related gene products/isoforms, illustrating the strengths of 2DE-based studies. It was however not possible on the basis of the MS data obtained to determine the nature of the modification. Collectively, plant cell wall degrading enzymes, also termed pathogenicity/virulence factors, have been predicted to function in the penetration and maceration of plant tissues for nutrient acquisition [14]. The enzymes α/β-xylosidase, α/β-glucuronidase and feruloyl esterase, were only identified from cultures grown on WAX. Notably, cellulases and α-amylases were produced in much higher amounts by Aspergillus grown on barley flour, of which almost 90% is starchy endosperm [47]. Furthermore, peptides derived from spot 87 (barley flour) matched glucoamylase catalyzing breakdown of α-(1,4)-linked malto-oligosaccharides to glucose.

Growth on barley flour resulted in identification of several peptidases including tripeptidyl-peptidase, carboxypeptidases S1 and CpdS, probably involved in nutrient acquisition as well as in enhancement of fungal pathogenicity [48,49]. Moreover,
proteases have been reported to be involved in infection processes in fungi, such as *Aspergillus fumigatus* and *Candida albicans*, in plants as well as in animal hosts [49,50]. Mycelial catalase Cat1 (oxidoreductase, spots 73 and 100) can remove reactive oxygen species and protect the cells from oxidative damage. Glutaminase (GtaA, spot 90) that catalyzes hydrolysis of glutamine to glutamate and ammonia, was also identified for *A. niger* growing on barley flour.

*A. niger* is a well-studied filamentous fungus due to its high secretory capacity and value for biotechnology. Only a few proteome studies are reported of secreted fungal proteins on different substrates and no studies have been performed of *A. niger* on WAX and barley flour mimicking the natural hosts of cereal fungi. **Comparison and analysis** of the intra- and extra-cellular proteins produced by *A. niger* grown on xylose or maltose showed considerable similarities in the intracellular proteomes, while the secretomes were strongly influenced by the carbon source [13]. The secretome of the xylose-grown *A. niger* contained a variety of plant cell wall degrading enzymes, with xylanase and ferulic acid esterase being the most abundant. **A comparison of our dataset** of *A. niger* grown on WAX with the xylose-grown cultures [13] revealed a large overlap in the identified proteins with a few exceptions, such as α-galactosidase C (melibiase), which catalyze the hydrolysis α-1,6-linked galactose residues from oligomeric (e.g. melibiose and raffinose) and polymeric (e.g., xylan galactomannan) compounds [51]. This is expected since the backbone of arabinoxylan is a xylan composed of xylose units, but the mono- or double arabinosyl substitutions require specific enzymes for liberation. The enzyme β-glucuronidase (*A. niger*) was identified in our dataset, **while the yeast homologue and** the cell wall protein PhiA essential for phialide and conidium-spore development, was only found in cultures grown with xylose [13]. β-mannosidase (spot 65) and xyloglucanase (spot 64) were only identified
when grown on barley flour. Evidently, discrepancies are found when comparing several datasets, due to differences in the experimental designs, procedures and culture conditions (e.g. complex medium and substrate concentration).

4.4 Profiling the 2D-secretome of *Fusarium poae* grown on barley and WAX

*Fusarium poae* has been reported as one of the most frequent *Fusarium* species isolated from cereal grains in Finland, Japan, Norway and Sweden [52–54]. *F. poae* is a pathogenic filamentous fungus reported to produce several mycotoxins, including trichothecenes, such as deoxynivalenol, nivalenol and fusarenone-X [55–57] and to inhibit mitochondrial function and protein synthesis [58]. Little work has been performed on *F. poae*, despite its pathogenicity and hazard imposed to human health.

In the present study, secretome analysis of *F. poae* grown on either WAX or barley flour showed lower protein content compared to *A. niger* cultures, despite the same amount of spores used for inoculation. The difference may be due to different growth rates and secretory capacity of the two fungi. Notably, the protein content is influenced by several factors, including inoculum size and composition of fungal mycelium. Secretome of *F. poae* gave different profiles when grown on WAX and barley flour (Fig. 3). Thirty proteins were identified by mass spectrometry (Table 5).

On WAX, fungal endo-1,4-β-xylanase (GH11, spot 52) and chitinase (GH18, spot 48) were identified, while due to the lower protein content the secretome of *F. poae* grown with barley flour was dominated by plant proteins, and only three spots were found to contain fungal proteins: endo-1,4-β-xylanase A (GH10, spot 12), ubiquitin (spot 34) and a hypothetical protein (FG04936.1, spot 11) displaying homology to aminopeptidase Y. Noticeably, xylanases of family GH11 were detected in the
secretome of *F. poae* growing on WAX, while a GH10 member was detected when
grown on barley flour. It has been reported that xylanases of GH11 are more efficient
than the GH10 in hydrolysis of wheat bran and display two-fold higher affinity for
wheat bran and 6-fold turnover rate [59]. Xylanases of GH11 are known to have a
lower catalytic versatility than GH10 and preferentially cleave unsubstituted regions
of arabinoxylan, whereas GH10 xylanases have broader substrate specificity and
hydrolyse the AX main chain within decorated regions.

It is clear from the SDS-PAGE secretome profiles (Fig. 2) that different *Fusarium*
species exhibit distinctly different protein patterns. Thus the secretome pattern of *F.
graminearum* (lanes W5 and F5) differs from that of *F. poae* (lanes W8 and F8).
Further optimisation and analysis of the *F. poae* secretome therefore holds promise for
identification of proteins with specific roles in *F. poae* pathogenicity.

### 5. Conclusions

The present study provides an overview of the fungal community on barley grains,
their secreted proteins and xylanolytic activities. The fungi isolated from barley grains
have secretomes reflecting their enzymatic potential, which varies according to species
and growth substrate. Analysis of one well-characterized (*A. niger*) and one poorly
characterized (*F. poae*) fungus grown on barley flour and WAX enabled identification
of new proteins, including enzymes involved in cell wall degradation and carbohydrate
catabolism. This approach can provide valuable insight into secretory capacity and
pathogenicity of the studied organisms as well as the molecular interactions between
fungi and host plant.
Author information

*Corresponding author: Christine Finnie, Carlsberg Research Laboratory, J.C. Jacobsens Gade 4, DK-1799 Copenhagen, Denmark. Phone: +45 33275225; E-mail address: Christine.Finnie@carlsberg.com

Notes: All authors have given approval to the final version of the manuscript.

Acknowledgements

This work was funded by the Directorate for Food, Fisheries and Agri Business (DFFE) through the project “Exploiting barley first wave enzyme activities for better feed” (grant no. 3304-FVFP-08-M-07-02, 2008) and a PhD stipend from the Technical University of Denmark (DTU). MALDI-TOF/TOF MS was funded by the Enzyme and Protein Chemistry (EPC) group and the Danish Center for Advanced Food Studies (LMC). Maria Bach and Julie Sørensen are thanked for screening the fungal isolates.

References


A.D. Hocking, J.I. Pitt, Dichloran-glycerol medium for enumeration of


35
colloidal Coomassie G-250 staining for proteome analysis, Electrophoresis 25

digestion procedure for the micropreparation of internal protein fragments for

improved method of sample preparation on AnchorChipTM targets for MALDI
MS and MS/MS and its application in the liver proteome project, Proteomics 7

[31] O. Pechanova, T. Pechan, Maize-pathogen interactions: An ongoing combat

*Fusarium graminearum* and its interactions with cereal heads: studies in the

[33] P. Vandenkoornhuyse, A. Quaiser, M. Duhamel, A. Le Van, A. Dufresne, The
importance of the microbiome of the plant holobiont, New Phytol. 206 (2015)

[34] S.A. Watson, C.J. Mirocha, A.W. Hayes, Analysis for trichothecenes in samples
from Southeast Asia associated with “Yellow rain”, Fundam. Appl. Toxicol. 4

[35] A.M. Corder, R.J. Henry, Carbohydrate-degrading enzymes in germinating

Variability in xylanase and xylanase inhibition activities in different cereals in
the HEALTHGRAIN diversity screen and contribution of environment and
genotype to this variability in common wheat, J. Agric. Food Chem. 58 (2010)
9362–9371. doi:10.1021/jf100474m.

[37] R. Chávez, P. Bull, J. Eyzaguirre, The xylanolytic enzyme system from the


[39] G.H. Hansen, M. Lübeck, J.C. Frisvad, P.S. Lübeck, B. Andersen, Production of
cellulolytic enzymes from ascomycetes: Comparison of solid state and
doi:10.1016/j.procbio.2015.05.017.

[40] M.I. Ja’afaru, Screening of fungi isolated from environmental samples for

Paes Leme, F.M. Squina, M. Buckeridge, G.H. Goldman, J.V. de C. Oliveira,
Comparative secretome analysis of *Trichoderma reesei* and *Aspergillus niger*
doi:10.1371/journal.pone.0129275.

[42] D.J. Jiménez, M. Maruthamuthu, J.D. van Elsas, Metasecretome analysis of a
lignocellulolytic microbial consortium grown on wheat straw, xylan and xylose,

[43] J. Van Den Brink, R.P. De Vries, Fungal enzyme sets for plant polysaccharide


Delcour, G. Volckaert, Mutational analysis of endoxylanases XylA and XylB
from the phytopathogen Fusarium graminearum reveals comprehensive insights
into their inhibitor insensitivity, Appl. Environ. Microbiol. 73 (2007) 4602–

[47] C. Finnie, B. Svensson, Barley seed proteomics from spots to structures, J.

[48] M. Monod, S. Capoccia, B. Léchenne, C. Zaugg, M. Holdom, O. Jousson,


Dutkiewicz, Enzymatic activities of Aspergillus fumigatus strains isolated from

Slámová, K. Bezouška, V. Křen, R. Ettrich, The α-galactosidase type A gene


Figure captions

Fig. 1. Plate zymograms of culture supernatants (5 μL) from fungi grown on barley flour (f) and wheat arabinoxylan (w). Numbers refer to fungal isolates in Table 1.

Fig. 2. SDS-PAGE of culture supernatants from fungal isolates (Table 1) grown on barley flour (F) and wheat arabinoxylan (W) (20 μg protein). The gel was stained with Coomassie blue and numbered bands were excised for identification by MALDI-MS (Table 3).

Fig. 3. 2D-gel electrophoresis of the secretome (50 μg) of Aspergillus niger (A and B) and Fusarium poae (C and D) grown in medium containing wheat arabinoxylan (A and C) and barley flour (B and D) as sole carbon source. The numbered spots were selected for analysis by mass spectrometry. Spots a1-a105 (A. niger) and f1-f55 (F. poae) were excised for identification by MALDI-TOF/TOF MS (Table 5; Supplementary Table S1). Molecular mass markers and pI range are indicated.
Figure 2
Click here to download Figure: Figure2.pptx
Supplementary Table S1
Click here to download Supplementary material: Supplementary TableS1.docx
Conflict of Interest
Click here to download Conflict of Interest: coi_disclosure_Finnie.pdf
*Conflict of Interest
Click here to download Conflict of Interest: coi_disclosure_Sultan.pdf
*Conflict of Interest

Click here to download Conflict of Interest: coi_disclosure_Andersen.pdf
Click here to download Conflict of Interest: coi_disclosure_Svensson.pdf