A time course analysis of the extracellular proteome of Aspergillus nidulans growing on sorghum stover

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Abstract

Background: Fungi are important players in the turnover of plant biomass because they produce a broad range of degradative enzymes. Aspergillus nidulans, a well-studied saprophyte and close homologue to industrially important species such as A. niger and A. oryzae, was selected for this study.

Results: A. nidulans was grown on sorghum stover under solid-state culture conditions for 1, 2, 3, 5, 7 and 14 days. Based on analysis of chitin content, A. nidulans grew to be 4-5% of the total biomass in the culture after 2 days and then maintained a steady state of 4% of the total biomass for the next 12 days. A hyphal mat developed on the surface of the sorghum by day one and as seen by scanning electron microscopy the hyphae enmeshed the sorghum particles by day 5. After 14 days hyphae had penetrated the entire sorghum slurry. Analysis (1-D PAGE LC-MS/MS) of the secretome of A. nidulans, and analysis of the breakdown products from the sorghum stover showed a wide range of enzymes secreted. A total of 294 extracellular proteins were identified with hemicellulases, cellulases, polygalacturonases, chitinases, esterases and lipases predominating the secretome. Time course analysis revealed a total of 196, 166, 172 and 182 proteins on day 1, 3, 7 and 14 respectively. The fungus used 20% of the xylan and cellulose by day 7 and 30% by day 14. Cellobiose dehydrogenase, feruloyl esterases, and CAZy family 61 endoglucanases, all of which are thought to reduce the recalcitrance of biomass to hydrolysis, were found in high abundance.

Conclusions: Our results show that A. nidulans secretes a wide array of enzymes to degrade the major polysaccharides and lipids (but probably not lignin) by 1 day of growth on sorghum. The data suggests simultaneous breakdown of hemicellulose, cellulose and pectin. Despite secretion of most of the enzymes on day 1, changes in the relative abundances of enzymes over the time course indicates that the set of enzymes secreted is tailored to the specific substrates available. Our findings reveal that A. nidulans is capable of degrading the major polysaccharides in sorghum without any chemical pre-treatment.

Keywords: Cellulose, Biofuels, Lignocellulosic biocoversion, A. nidulans, Sorghum, Enzymatic hydrolysis, Proteome
Fungi achieve lignocellulosic conversion under mild conditions, albeit rather slowly. Fungi are known to secrete hydrolytic enzymes that are responsible for polysaccharide degradation, but also must produce ligninolytic systems and enzymes to decrease the crystallinity of cellulose [1,10-12]. Here we present a description of the enzymes secreted by *A. nidulans* to support its growth on powdered sorghum stover. *A. nidulans* is a model saprophytic fungus, with a sequenced and annotated genome [13-15]. It is also a well-known producer of plant cell wall degrading enzymes [16,17]. Several proteomic studies on extracellular proteins from aspergillus species growing on various carbon sources have been reported [11,12,18-20]. However, no studies have been reported on growth of *A. nidulans* on sorghum to elucidate the comprehensive strategy of *A. nidulans* for degradation of plant cell walls.

In this study we grew *A. nidulans* on sorghum stover under solid state culture conditions to simulate the natural environment of the fungus. We aimed to identify all secreted enzymes involved in degradation of the sorghum over a time course of 1, 2, 3, 5, 7 and 14 days and in 1% glucose grown cultures. Results from the study of growth, enzyme activities, quantification of breakdown products from the enzymes, and the nature of the remaining undigested sorghum should enhance our understanding of the plant cell wall degradation process and help us to devise ways to accelerate the process of lignocellulosic bioconversion using in vitro enzyme mixtures.

**Results**

To visualize the growth of *A. nidulans* in solid state sorghum cultures, *A. nidulans* samples grown on sorghum stover were sampled on 0, 3 and 5 days after inoculation and analyzed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM image in Figure 1A shows uninoculated sorghum particles as a control. Figure 1B indicates dense growth of *A. nidulans* on sorghum stover on day 5 by SEM.

![Figure 1](image)

**Figure 1 Growth of A. nidulans on sorghum stover.** A. Scanning electron micrograph (SEM) of sorghum particles without any fungal inoculation, B. Scanning electron micrograph of sorghum particles enmeshed with fungal mycelia on day 5 showing substantial growth of *A. nidulans*. C. Transmission electron micrograph of cross section of sorghum particles without any fungal inoculations. D. Cross section of sorghum particles showing presence of fungal particles inside sorghum cells.
Figure 1C is a control depicting only sorghum cell walls imaged by TEM. Figure 1D depicts fungal cells surrounding and within the sorghum cells on day 5 by TEM. By day 1 we saw a mat of fungus covering the surface of the sorghum slurry. The mat appeared to be getting thicker and penetrated throughout the sorghum slurry by day 14.

To quantitate the growth of *A. nidulans* in the solid-state cultures, the total chitin content of the cultures was measured. We selected this approach because fungal biomass is difficult to recover and separate from the sorghum particles as the fungal hyphae enmesh and bind tightly to the substrate [21]. Chitin is a long-chain polymer of N-acetyl glucosamine and a key constituent of the fungal cell walls. As no chitin-like materials occur in sorghum stover, determination of chitin content is a good measure of fungal growth. Results from the chitin estimation revealed that *A. nidulans* grew rapidly on day 1 reaching to 3.8% of the total dry biomass on the plate. After day 2 it reached a maximum (4.4% of the total dry biomass) but decreased on day 3 and then became relatively constant for rest of the days (Figure 2).

Enzyme activities in the extracellular filtrate (ECF) varied during the growth of *A. nidulans* on the sorghum (Figure 3). Xylanases showed high activity (0.98 U/ml) on day 1 and the activity increased slowly over the next 6 days. Polygalacturonase activity was lower than xylanase activity on days 1 and 2 (0.23 and 0.59 U/ml respectively) with the polygalacturonase activity becoming constant subsequently. Cellulase activity, as measured using carboxymethylcellulose as substrate, reached a fairly constant level of around 0.27 U/ml after day 1.
Mannanase activity was not detectable by colorimetric methods. Capillary zone electrophoresis (CZE) analysis, of fluorescently labeled substrates revealed a range of hemicellulase, cellulase, pectinase and mannanase activities in the A. nidulans cultures (Data not shown). We did not detect any of the above polysaccharide degrading enzyme activities in ECF collected from glucose control.

The next logical step after determining the enzymatic activities was to identify the individual extracellular enzymes involved in degradation of the sorghum. The A. nidulans secretome was analyzed using 1D PAGE LC-MS/MS. Spectrum counts were used to show the relative abundance of the proteins in extracellular filtrates.

Spectrum counts are the total number of tandem spectra assigned to each protein and are commonly used to determine relative protein abundances because previous studies have demonstrated a linear correlation between spectrum count and protein abundance in complex samples [22]. The results of the analysis of the secretome are presented in Tables 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 and additional file 1: Table S1. A total of 7 proteins were identified in the ECF of 1% glucose grown cultures. These proteins were a hypothetical protein (AN4575), thioredoxin reductase (AN8218), N-acetyl-6-hydroxytryptophan oxidase (AN0231), heat shock protein (AN5129), alkaline protease (AN5558), nucleoside

### Table 1: Identified hemicellulose-degrading proteins and spectrum counts on 1, 3, 7, and 14 days

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN8401           | GH3       | Beta-1,4-xylosidase | 82       | 60    | 98    | 107   | 132    | Y       |
| AN2217           | GH3       | Beta 1,4-Xylosidase | 83       | 39    | 48    | 58    | 79     | Y       |
| AN2359           | GH3       | Beta-xylosidase     | 87       | 53    | 115   | 57    | 0      | Y       |
| AN1818           | GH10      | Beta-1,4-endoxylanase | 34     | 101   | 142   | 576   | 720    | Y       |
| AN7401           | GH10      | Beta-1,4-endoxylanase | 38     | 0     | 4     | 11    | 31     | Y       |
| AN3613           | GH1i      | Beta-1,4-endoxylanase A precursor | 24 | 188   | 174   | 194   | 140    | Y       |
| AN7152           | GH27      | Alpha-1,4-galactosidase | 69   | 67    | 138   | 124   | 121    | Y       |
| AN8138           | GH36      | Alpha-1,4-galactosidase | 82   | 0     | 0     | 27    | 24     | Y       |
| AN7117           | GH39      | Xylosidase          | 50       | 0     | 9     | 13    | 12     | Y       |
| AN8007           | GH43      | Endoarabinase       | 34       | 6     | 29    | 20    | 19     | Y       |
| AN2533           | GH43      | Alpha N-arabinofuranosidase | 36   | 0     | 13    | 10    | 7      | Y       |
| AN7781           | GH43      | Arabinosidase, putative | 38   | 32    | 74    | 52    | 60     | Y       |
| AN2534           | GH43      | Endoarabinase       | 41       | 0     | 13    | 12    | 7      | Y       |
| AN10919          | GH43      | 1,4-endoxylanase D precursor | 42 | 2     | 39    | 50    | 48     | Y       |
| AN7313           | GH43      | Alpha L-arabinofuranosidase C | 52 | 0     | 5     | 0     | 0      | Y       |
| AN2727           | GH43      | Putative xylosidase | 55       | 0     | 0     | 0     | 24     | Y       |
| AN8477           | GH43      | Xylosidase/arabinofuranosidase | 60 | 37    | 69    | 64    | 97     | N       |
| AN5727           | GH53      | Beta-1,4-endogalactanase | 41   | 11    | 19    | 16    | 18     | Y       |
| AN1571           | GH54      | Alpha-arabinofuranosidase | 53   | 45    | 98    | 80    | 96     | Y       |
| AN2632           | GH62      | Arabinoxylan/arabinofuranohydrolase | 33 | 13    | 30    | 29    | 21     | Y       |
| AN7908           | GH62      | Arabinoxylan/arabinofuranohydrolase | 36 | 27    | 106   | 90    | 113    | Y       |
| AN9286           | GH67      | Alpha-glucuronidase | 94       | 14    | 17    | 69    | 104    | Y       |
| AN5061           | GH74      | Xyloglucanase       | 88       | 0     | 0     | 0     | 7      | Y       |
| AN2060           | GH93      | Exo-arabinanase     | 43       | 17    | 24    | 24    | 27     | Y       |
| AN6093           | CE1       | Acetyl xylan esterase | 34   | 9     | 6     | 4     | 4      | Y       |
| AN1320           | CE1       | Beta-1,4-endoxylanase B | 28   | 10    | 36    | 46    | 55     | Y       |
| AN6673           | CE1       | Alpha-fucosidase   | 92       | -     | -     | 30    | 31     | Y       |
| AN9380           | CE1       | Bifunctional xylanase/ deacetylase | 26 | 10    | 6     | 9     | 14     | Y       |

*aAccession numbers along with protein information and glycosyl hydrolase (GH) family information was obtained from Pedant [23].

*bHypothetical molecular weight of the proteins.

*Quantifying changes in protein abundance between samples from different time points was done using the spectral count method, yielding a semiquantitative analysis.

*dSignalP was used to predict secretion signals [23,24].

*eSignalP as reported at [25].

*fNot found by SignalP (N-terminal may be incorrectly annotated, a novel signal peptide may be present, or the protein is normally intracellular but was released by autolysis).
diphosphate kinase (AN8216) and catalase B precursor (AN9339). We identified a total of 196, 166, 172 and 182 proteins on days 1, 3, 7 and 14 respectively during the growth of *A. nidulans* on the sorghum stover. In the sorghum grown samples, eighty-nine proteins were present on all days. Eighty-proteins were exclusively secreted on day 1, whereas only 7, 5 and 19 proteins were unique to days 3, 7 and 14 respectively. Results of LC-MS/MS studies on *A. nidulans* are described below for enzymes directed against different polymers or processes. Where possible the CAZy family affiliation for each enzyme has been noted.

### Hemicellulose degradation

Hemicellulose is the second most abundant fraction (25-35%) of lignocellulosic biomass [20]. Hydrolysis of hemicelluloses into simple sugars requires multiple enzymes including beta-1,4-endoxylanase, beta-xylosidase, alpha-glucuronidase, alpha-L-arabinofuranosidase and acetyl xylan esterase [26,27]. Beta-endoxylanases cleaves the backbone of xylan and beta-xylosidase releases the xylose units from xylobiose and xylooligomers. Removal of xylan side chains is catalyzed by acetyl xylan esterases, ferulic acid esterases, alpha-L-arabinofuranosidas and alpha-D-glucuronidas [28,29]. Beta-1,4-endoxylanase and beta-1,4-glucosidase cleaves xyloglucan backbone and beta-1,4-endomannanase and beta-1,4-mannosidase acts on (galacto-) mannan backbone [28,30]. Twenty-eight different hemicelluloses degrading enzymes were identified in our study (Table 1). A total of twenty *A. nidulans* ORFs have been assigned to GH3 family with three of them being beta-xylosidases [31]. We found all three of these beta-xylosidases (AN8401, AN2359, AN2217). Beta-xylosidases (AN8401 and AN2217) were present in abundance on day 1 and showed a steady increase in spectrum count until day 14. Another beta-xylosidase (AN2359) was present on day 1, 3 and 7 but not on day 14. AN7275, a putative GH43 family xylosidase, only appeared on day 14. We found two of the potential three endoxylanases belonging to GH10 family (AN1804 and AN10482) and one out of the two belonging to GH11 family (AN1273). Beta-1,4-

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN9183           | GH1       | Beta−1,4-glucosidase| 66       | 11    | 14    | 22    | 14     | Y       |
| AN2227           | GH3       | Beta-1,4-glucosidase| 92       | 0     | 0     | 0     | 0      | N       |
| AN2828           | GH3       | Beta-1,4-glucosidase| 78       | 33    | 144   | 131   | 156    | Y       |
| AN4102           | GH3       | Beta glucosidase    | 92       | 78    | 222   | 204   | 215    | Y       |
| AN5976           | GH3       | Beta glucosidase    | 89       | 53    | 105   | 22    | 0      | Y       |
| AN7396           | GH3       | Beta glucosidase    | 84       | 0     | 116   | 107   | 59     | Y       |
| AN1804           | GH3       | Beta-1,4-glucosidase| 68       | 4     | 4     | 49    | 31     | Y       |
| AN10482          | GH3       | Beta-1,4-glucosidase| 94       | 0     | 9     | 21    | 10     | Y       |
| AN1285           | GH5       | Beta-1,4-endoglucanase| 36     | 21    | 49    | 38    | 42     | Y       |
| AN8068           | GH5       | Putative endoglucanase| 63   | 0     | 20    | 46    | 28     | Y       |
| AN9166           | GH5       | Cellulase family protein | 45 | 0     | 9     | 0     | 5      | Y       |
| AN1273           | GH6       | Cellobiohydrolase    | 41       | 12    | 37    | 23    | 39     | Y       |
| AN5282           | GH6       | Cellobiohydrolase    | 47       | 0     | 15    | 49    | 54     | Y       |
| AN0494           | GH7       | Cellobiohydrolase    | 56       | 15    | 33    | 58    | 80     | Y       |
| AN5176           | GH7       | Cellobiohydrolase    | 48       | 63    | 142   | 195   | 234    | Y       |
| AN3418           | GH7       | Beta-1,4-endoglucanase| 46     | 65    | 82    | 76    | 88     | Y       |
| AN2664           | GH43      | Beta-glucanase, putative | 55 | 0     | 0     | 0     | 7      | Y       |
| AN3046           | GH61      | Endoglucanase, putative | 32 | 44    | 0     | 0     | 0      | Y       |
| AN3860           | GH61      | Endoglucanase IV precursor | 26 | 5     | 0     | 14    | 17     | Y       |
| AN10419          | GH61      | Beta-1,4-endoglucanase| 29     | 0     | 10    | 10    | 16     | Y       |
| AN6428           | GH61      | Endoglucanase 4      | 24       | 2     | 0     | 5     | 7      | Y       |

*aAccession numbers along with protein information and glycosyl hydrolase (GH) family information was obtained from Pedant [23].

*bHypothetical molecular weight of the proteins.

*cQuantifying changes in protein abundance between samples from different time points was done using the spectral count method, yielding a semiquantitative analysis.

*dSignalP was used to predict secretion signals [23,24].

*eSignalP as reported at [25].

*fNot found by SignalP (N-terminal may be incorrectly annotated, a novel signal peptide may be present, or the protein is normally intracellular but was released by autolysis).
endoxylanase (AN1818) showed a dramatic increase in spectrum count from 101 on day 1 to 720 on day 14. AN3613 started with a high spectrum count on day 1 and then reached a plateau. AN7401 (which has a CBM) was present in lower abundance compared to the other xylanases. An alpha-1,4-galactosidase belonging to family GH27 and another belonging to GH36 (AN7152 and AN8138) were identified. AN7152 was found in high abundance.

Table 3 Identified pectin-degrading proteins and spectrum counts on 1, 3, 7, and 14 days

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN2463           | GH2       | Beta-galactosidase  | 115      | 0     | 0     | 50    | 96     | N       |
| AN2395           | GH2       | Beta-galactosidase/mannosidase | 69 | 25 | 70 | 83 | 81 | Y       |
| AN8761           | GH28      | Exopolygalacturonase | 48       | 49    | 38    | 18    | 0      | Y       |
| AN8891           | GH28      | Exopolygalacturonase | 49       | 30    | 20    | 0     | 0      | Y       |
| AN10274          | GH28      | Exo-polygalacturonase, putative | 46 | 0 | 4 | 0 | 0 | Y       |
| AN0980           | GH35      | Beta-galactosidase  | 109      | 2     | 14    | 8     | 25     | Y       |
| AN0756           | GH35      | Beta-galactosidase  | 109      | 0     | 5     | 2     | 8      | Y       |
| AN7151           | GH78      | Alpha-rhamnosidase  | 100      | 4     | 14    | 64    | 83     | N       |
| AN7828           | GH88      | Unsaturated rhamnogalacturonan hydrolase | 44 | 11 | 0 | 0 | 0 | Y       |
| AN9383           | GH105     | Unsaturated rhamnogalacturonan hydrolase | 43 | 92 | 54 | 60 | 39 | Y       |
| AN7041           | PL1       | Pectate lyase precursor | 35 | 7 | 41 | 28 | 41 | Y       |
| AN2331           | PL1       | Pectin lyase A precursor | 41 | 17 | 0 | 0 | 0 | Y       |
| AN2569           | PL1       | Pectin lyase A precursor | 39 | 32 | 29 | 47 | 31 | Y       |
| AN7646           | PL1       | Pectate lyase C      | 35       | 4     | 3     | 19    | 18     | Y       |
| AN6106           | PL3       | Pectate lyase C      | 26       | 6     | 22    | 20    | 23     | Y       |
| AN8453           | PL3       | Pectate lyase C      | 28       | 10    | 0     | 5     | 3      | Y       |
| AN7135           | PL4       | Rhamnogalacturonan lyase | 56 | 13 | 71 | 71 | 80 | Y       |
| AN4139           | PL4       | Rhamnogalacturonan lyase | 117 | 6 | 15 | 3 | 5 | Y       |
| AN3390           | CE8       | Pectin methylesterase | 35 | 0 | 19 | 11 | 16 | Y       |
| AN4860           | CE8       | Pectin methylesterase | 42 | 27 | 3 | 0 | 0 | Y       |
| AN2528           | CE12      | Rhamnogalacturonan acetyl esterase | 26 | 4 | 0 | 16 | 16 | Y       |
| AN2537           | PL1       | Exopolygalacturonate lyase | 44 | 4 | 12 | 6 | 5 | Y       |

*Accession numbers along with protein information and glycosyl hydrolase (GH) family information was obtained from Pedant [23].
*Hypothetical molecular weight of the proteins.
*Quantifying changes in protein abundance between samples from different time points was done using the spectral count method, yielding a semiquantitative analysis.
*SignalP was used to predict secretion signals [23,24].
*SignalP as reported at [25].
*Not found by SignalP (N-terminal may be incorrectly annotated, a novel signal peptide may be present, or the protein is normally intracellular but was released by autolysis).

Table 4 Identified starch degrading proteins and spectrum counts on 1, 3, 7, and 14 days

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN3388           | GH13      | Alpha amylase       | 50       | 33    | 0     | 49    | 41     | Y       |
| AN3402           | GH13      | Alpha amylase       | 69       | 11    | 0     | 0     | 0      | Y       |
| AN7402           | GH15      | Glucoamylase        | 71       | 7     | 43    | 24    | 15     | Y       |
| AN2017           | GH31      | Alpha-1,4-glucosidase | 110 | 5 | 12 | 5 | 6 | Y       |
| AN8953           | GH31      | Alpha-1,4-glucosidase B | 108 | 85 | 117 | 95 | 121 | Y       |
| AN0941           | GH31      | Alpha-1,4-glucosidase | 94       | 23    | 24    | 2     | 5      | Y       |

*Accession numbers along with protein information and glycosyl hydrolase (GH) family information was obtained from Pedant [23].
*Hypothetical molecular weight of the proteins.
*Quantifying changes in protein abundance between samples from different time points was done using the spectral count method, yielding a semiquantitative analysis.
*SignalP was used to predict secretion signals [23,24].
*SignalP as reported at [25].
abundance on all 4 days, whereas AN8138 was only secreted on day 7 and 14. Eight proteins (AN2533, AN7781, AN8007, AN2534, AN7275, AN8477, AN10919 and AN7313) belonging to family GH43 were identified. Out of these eight proteins, AN7275 and AN8477 do not have a signal peptide. AN7781 was secreted on all days. Its spectrum count increased more than twice from day 1 to day 3 and then it was constant on day 7 and 14. We identified an arabinosidase from family GH53 (AN1571), and two other arabinosidases (AN7908 and AN2632) belonging to family GH62. We also found one enzyme belonging to family GH53 (endogalactanase), one from GH67 (alpha-glucuronidase), and one from GH93 (exo-arabinase). Most of the hemicellulases were secreted on all days except for nine hemicellulases, which were absent on day 1 but secreted on all other days. One xyloglucanase (AN5061) appeared on day 14 only whereas galactosidase (AN8138) was found on only day 7 and 14.

### Cellulose degradation
The complete hydrolysis of cellulose requires random cleavage of internal bonds by endoglucanases, removal of cellobiose by cellobiohydrolases (exoglucanases), and

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN0933           | GH16      | Extracellular cell wall glucanase | 42       | 18    | 35    | 11    | 7      | Y       |
| AN0245           | GH16      | Beta-1,3(4)-endoglucanase, putative | 37       | 0     | 33    | 15    | 29     | Y       |
| AN6620           | GH16      | Beta-1,3(4)-endoglucanase, putative | 42       | 4     | 0     | 0     | 0      | Y       |
| AN6819           | GH16      | Endo-1,3 (4)-glucanase | 32       | 9     | 7     | 8     | 7      | Y       |
| AN7950           | GH17      | Cell wall beta-1,3-endoglucanase | 47       | 17    | 32    | 32    | 26     | Y       |
| AN4871           | GH18      | Protein similar to class V chitinase A | 44       | 5     | 224   | 277   | 317    | Nf      |
| AN8241           | GH18      | Class III Chi A chitinase | 97       | 0     | 5     | 2     | 0      | Y       |
| AN1502           | GH20      | Protein similar to N-acetylglucosaminidase | 68       | 11    | 101   | 124   | 176    | Y       |
| AN0779           | GH55      | Putative beta-1,3-exoglucanase | 84       | 0     | 19    | 19    | 15     | Y       |
| AN4825           | GH55      | Glucan 1,3-beta glucosidase precursor | 97       | 0     | 102   | 108   | 135    | Y       |
| AN9042           | GH71      | Putative alpha 1,3- glucanase | 69       | 0     | 51    | 55    | 60     | Y       |
| AN7657           | GH72      | 1,3-beta-glucanosyltransferase | 49       | 14    | 37    | 0     | 4      | Y       |
| AN0472           | GH81      | Putative beta-1,3-endoglucanase | 98       | 0     | 102   | 99    | 146    | Y       |
| AN9339           | Catalase B precursor | 79       | 58    | 111   | 109   | 108   | Y       |
| AN4390           | GPI-anchored cell wall organization protein Ecm33 | 41       | 4     | 7     | -     | -     | Y       |
| AN2385           | GPI-anchored beta-1,3(4)-endoglucanase, putative | 65       | 3     | -     | -     | -     | -       |

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN1772           | CE1       | Feruloyl esterase type B | 58       | 105   | 148   | 154   | 142    | Y       |
| AN5267           | CE1       | Feruloyl esterase | 28       | 21    | 12    | 56    | 65     | Y       |
| AN5311           | Putative tyrosinase | 42       | 14    | 10    | 19    | 19    | Y       |
| AN7230           | Cellobiose dehydrogenase | 83       | 0     | 17    | 39    | 77    | Y       |

Table 6 Identified proteins involved in various plant cell wall modifications and spectrum counts on 1, 3, 7, and 14 days

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN1772           | CE1       | Feruloyl esterase type B | 58       | 105   | 148   | 154   | 142    | Y       |
| AN5267           | CE1       | Feruloyl esterase | 28       | 21    | 12    | 56    | 65     | Y       |
| AN5311           | Putative tyrosinase | 42       | 14    | 10    | 19    | 19    | Y       |
| AN7230           | Cellobiose dehydrogenase | 83       | 0     | 17    | 39    | 77    | Y       |
release of glucose from cellobiose by beta-glucosidase [32-35]. Twenty-one different enzymes likely to be involved in cellulose degradation were identified in our cultures (Table 2). One beta-glucosidase (AN9183) belonging to family GH1 and seven beta-glucosidases belonging to family GH3 were detected. All of the family GH3 enzymes except AN2227 had recognizable signal peptide sequences. Beta-1,4-glucosidase (AN2828 and AN4102) showed high spectrum counts on all days, whereas AN7396 and AN5976 were absent on day 1 and day 14 respectively. Cellobiohydrolases are assigned to family GH6 and GH7. To date only family GH7 cellobiohydrolases have been characterized in A. nidulans [36] out of four predicted cellobiohydrolases belonging to family GH3 were detected. All of the family GH3 enzymes except AN2227 had recognizable signal peptide sequences. Beta-1,4-glucosidase (AN2828 and AN4102) showed high spectrum counts on all days, whereas AN7396 and AN5976 were absent on day 1 and day 14 respectively. Cellobiohydrolases are assigned to family GH6 and GH7. To date only family GH7 cellobiohydrolases have been characterized in A. nidulans [36] out of four predicted cellobiohydrolases belonging to family GH6 and GH7 from the A. nidulans genome sequence. We identified two cellobiohydrolases (AN1273 and AN5282) from family GH6 and two cellobiohydrolases (AN0494 and AN1576) belonging to family GH7. All showed a gradual increase in spectrum count from day 1 to day 14. Notably cellobiohydrolase (AN5176) showed high spectrum count of 234 on day 14 as compared to other cellobiohydrolases, whereas AN5282 was absent on day 1. We found one beta-1,4-endoglucanase (AN3418), from family GH7 and three beta-endoglucanases belonging to family GH5. Endoglucanases (AN1285 and AN8068) showed relatively low spectrum counts compared to the beta-endoglucanases from family GH7. A cellulase family protein (AN9166) was only present on day 3 and 14. We identified four beta-1,4-endoglucanases from family GH61 (AN10419, AN6428, AN3860 and AN3046) out of nine predicted ORFs for GH61 family proteins in the genome. Out of a total of twenty-one cellulases, seven enzymes were not secreted on day 1 whereas one putative endoglucanase AN2664 appeared only on day 14.

**Pectin degradation**

Due to the complex structure of pectin an array of enzymes are needed for its degradation [37,38]. We identified twenty-two enzymes involved in pectin degradation (Table 3). The GH2 family contains beta-galactosidase, beta-mannosidase and beta-glucuronidases. We found two GH2 family members (AN2935 and AN2463). Out of these two GH2 family members, AN2935 showed increased spectrum count across all days. The GH28 family consists of endo- and exo-polygalacturonases, rhamnogalacturonan hydrolases and xylogalacturonan hydrolases. Based on the A. nidulans genome nine ORFs have been assigned to this family. We identified three exopolygalacturonases (AN8761, AN8891, and AN10274) in the secretome. Out of three GH28 family exopolygalacturonases only AN8761 was identified.

### Table 7 Identified mannan degrading proteins and spectrum counts on 1, 3, 7, and 14 days

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN5361           | GH2       | Beta-1,4-mannosidase | 71       | 0     | 3     | 0     | 0      | Y       |
| AN9276           | GH5       | Beta-1,4-endomannanase | 42       | 0     | 5     | 8     | 15     | Y       |
| AN6427           | GH5       | Beta-1,4-endomannanase | 45       | 0     | 0     | 4     | 6      | Y       |
| AN2936           | GH38      | Alpha-mannosidase    | 124      | 0     | 4     | 38    | 72     | N       |
| AN0787           | GH47      | Similar to class I alpha-mannosidase 1B | 56 | 10 | 48 | 53 | 58 | Y       |
| AN2325           | GH92      | Alpha-1,2-mannosidase | 82       | 0     | 11    | 15    | 7      | Y       |
| AN1197           | GH92      | Alpha-1,2-mannosidase | 88       | 3     | 7     | 6     | Y       |

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**Table 8 Identified bacterial wall-degrading proteins and spectrum counts on 1, 3, 7, and 14 days**

| Accession number | GH family | Identified proteins | MW (kDa) | Day 1 | Day 3 | Day 7 | Day 14 | SignalP |
|------------------|-----------|---------------------|----------|-------|-------|-------|--------|---------|
| AN6470           | GH25      | Putative N,O-diacytly muramidase | 23       | 32    | 32    | 23    | 17     | Y       |
| AN8969           | GH25      | N,O-diacytly muramidase | 23       | 0     | 12    | 0     | 0      | Y       |

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*Accession numbers along with protein information and glycosyl hydrolase (GH) family information was obtained from Pedant [23,24].
*Hypothetical molecular weight of the proteins.
*Quantifying changes in protein abundance between samples from different time points was done using the spectral count method, yielding a semiquantitative analysis.
*SignalP was used to predict secretion signals [23,24].
secreted with high abundance on all days. So far GH78 family contains alpha-L-rhamnosidase exclusively. Seven A. nidulans ORFs have been assigned to this family. We found one alpha-rhamnoside from GH78 (AN7151) and it showed a gradual increase in spectrum count across all the days. We identified two pectate and two pectin lyases from PL1 family and two from PL3 family. Four A. nidulans ORFs have been assigned to rhamnogalacturonan lyases from family PL4. We identified two members of PL4. Two unsaturated rhamnogalacturonan hydrolases (AN7828 and AN9383), two pectin methylesterases (AN4860 and AN3390), and one rhamnogalacturonan acetylenesterase (AN2528) were also identified. With the exception of members from GH2, GH78, GH105 and PL4, the pectinases were expressed at higher levels only in the early growth stages.

Starch degradation

Ten A. nidulans ORFs have been assigned to family GH31. We identified only three alpha-1,4-glucosidases from family GH31, two alpha-amylases from family GH13 and one amylase from family GH15 (Table 4).

| Table 9 Identified proteases and spectrum counts on 1, 3, 7, and 14 days |
|---------------------------------------------------------------|
| **Accession number** | **GH family** | **Identified proteins** | **MW (kDa)** | **Day 1** | **Day 3** | **Day 7** | **Day 14** | **SignalP** |
|----------------------|---------------|-------------------------|--------------|-----------|-----------|-----------|-----------|-------------|
| AN7962               |               | Penicilloysin/deuterolysin metalloprotease | 37           | 170       | 232       | 302       | 318       | Y          |
| AN5558               |               | Alkaline protease       | 42           | 18        | 194       | 65        | 67        | Y          |
| AN2903               |               | Vacular aspartyl protease (protease A) | 43           | 0         | 41        | 51        | 58        | Y          |
| AN7159               |               | Hypothetical tripeptidyl-peptidase | 71           | 21        | 0         | 0         | 0         | Y          |
| AN10030              |               | Hypothetical serine protease | 50           | 0         | 12        | 18        | 31        | Y          |
| AN2366               |               | Hypothetical serine protease | 25           | 11        | 12        | 9         | 6         | Y          |
| AN8445               |               | Putative aminopeptidases | 54           | 40        | 72        | 74        | 69        | Y          |
| AN1426               |               | Serine carboxypeptidase | 62           | 10        | 52        | 51        | 31        | Y          |
| AN7231               |               | Serine carboxypeptidase | 57           | 8         | 16        | 9         | 15        | Y          |
| AN0224               |               | Membrane dipeptidase    | 46           | 7         | 9         | 23        | 32        | Y          |
| AN2572               |               | Dipetidyl dipeptidase   | 79           | 9         | 0         | 43        | 91        | Y          |

| Table 10 Identified esterases and lipases and spectrum counts on 1, 3, 7, and 14 days |
|---------------------------------------------------------------|
| **Accession number** | **GH family** | **Identified proteins** | **MW (kDa)** | **Day 1** | **Day 3** | **Day 7** | **Day 14** | **SignalP** |
|----------------------|---------------|-------------------------|--------------|-----------|-----------|-----------|-----------|-------------|
| AN5309               | CES           | Putative cutinase       | 22           | 17        | 15        | 39        | 48        | Y          |
| AN7541               | CES           | Cutinase, putative      | 26           | 23        | 0         | 0         | 0         | Y          |
| AN2834               | CE12          | Esterase, putative      | 27           | 6         | 0         | 0         | 0         | Y          |
| AN6422               | CE16          | Cellulose-binding GDSL Lipase/Acylhydrolase | 33           | 0         | 4         | 10        | 9         | Y          |
| AN5321               |               | Extracellular lipase putative | 62           | 75        | 103       | 71        | 50        | Y          |
| AN9287               | GDSL lipase/acylhydrolase | 47           | 23        | 52        | 49        | 50        | 50        | Y          |
| AN7691               | Phosphoesterase superfamily protein | 50           | 23        | 55        | 44        | 13        | 13        | Y          |
| AN1792               | GDSL lipase/acylhydrolase | 38           | 16        | 54        | 35        | 37        | 37        | Y          |
| AN8046               | Putative extracellular lipase | 31           | 11        | 48        | 44        | 47        | 47        | Y          |
| AN1433               | Putative triacylglycerol lipase | 60           | 24        | 16        | 6         | 0         | 0         | Y          |
| AN1799               | Putative lipase | 48           | 0         | 0         | 13        | 39        | 39        | Y          |
| AN7046               | Similar to triacylglycerol lipase | 25           | 3         | 0         | 0         | 0         | 0         | Y          |
| AN9361               | Lipase        | 63                      | 5         | -         | -         | -         | Y          |

**Accession numbers along with protein information and glycosyl hydrolase (GH) family information was obtained from Pedant [23].**

**Hypothetical molecular weight of the proteins.**

**Quantifying changes in protein abundance between samples from different time points was done using the spectral count method, yielding a semiquantitative analysis.**

**SignalP was used to predict secretion signals [23,24].**
Fungal cell wall remodeling enzymes
Sixteen enzymes, including chitinases and beta-1,3-endoglucanases involved in fungal cell wall modification/ degradation were identified (Table 5). Though chitinases and beta-1,3-endoglucanases were present on day 1, the spectrum count of most of them increased notably on day 3.

Enzymes involved in various plant cell wall modifications
Table 6 reports four enzymes, two feruloyl esterases, a cellobiose dehydrogenase, and a tyrosinase potentially involved in modification of lignin and other phenolics. The cellobiose dehydrogenase may also be involved in modification of cellulose.

Mannan and bacterial cell wall degrading enzymes
Table 7 and Table 8 show seven mannan degrading enzymes and two bacterial cell wall degrading enzymes respectively.

Proteases
Table 9 lists eleven fungal extracellular proteases, the production of which increased over time. They included one metalloprotease (AN7962) one alkaline protease, one aspartyl protease and two hypothetical serine proteases, two serine carboxypeptidases (AN1226, AN2237, AN7121), two putative aminopeptidase, an alkaline protease (AN5558), a putative dipeptidyl aminopeptidase (AN6438), and a membrane peptidase (AN7159). Table 10 show thirteen esterases and lipases secreted by A. nidulans. Out of these 12 esterases and lipases, seven were secreted with high abundance throughout the time course, whereas the rest of them were only present on one or two time points.

Miscellaneous proteins
Total 164 proteins were identified in this category including several hypothetical proteins (Additional file 1: Table S1).

The presence of high enzyme activities in the extracellular fluid of A. nidulans led us to quantify the solubilized breakdown products from the sorghum in the extracellular filtrate (ECF). Soluble polysaccharides and oligosaccharides present in ECF were estimated by gas chromatography after depolymerization by methanolysis and conversion of trimethylsilyl methyl glycosides. Between 0.01% and 0.23% of the total dry mass of the initial sorghum was soluble as each sugar type (Figure 4). The galacturonic acid and glucose content fell dramatically on day 1 and day 2 compared to the control suggesting that these already soluble sugars were readily converted into a form that could be taken up by the fungus. The amounts of arabinose and xylose containing soluble material increased compared to the control, reflecting digestion of the xylan by the enzymes and perhaps a lower ability to take up the solubilized products. The amount of mannose increased fairly consistently from day 1 to day 14. Rhamnose and galactose levels remained relatively constant. The amount of sugars at the different time points was probably a result of the dynamic balance between enzymatic breakdown of sorghum polysaccharides and uptake of sugars by fungus.

To find out how effective the fungus was at digesting the sorghum polysaccharides, we quantified the amount

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![Figure 4](Link_to_image)  
**Figure 4** Estimation of soluble sugars in the extracellular medium of A. nidulans grown on sorghum. This figure depicts breakdown products in the extracellular media after 1, 2, 3, 5, 7 and 14 days. The amounts of each sugar are shown as μg of sugars / mg of dry mass of sorghum. Soluble sugars in the un-inoculated control samples are designated as UC. Data represent mean ± SE.
of each sugar type remaining on the plate after various times of culture growth (Figure 5). Apart from a slight increase in glucose, which may have come from growth of the fungus or reflect experimental variability, the sorghum sugars decreased steadily. By day 7, 20% of the measurable glucose and xylose had been consumed and by day 14, 30% of these sugars had been utilized. The appearance of mannose and galactose, not present in the uninoculated sorghum, probably reflected the synthesis of fungal galactomannoproteins and mannans.

Discussion

A major goal of this study was to discover the enzymes *A. nidulans* secretes to digest sorghum stover while growing under conditions mimicking its natural habitat. These findings should help us to design a mixture of enzymes that can convert sorghum stover into readily fermentable sugars in vitro.

From the visual development of a thick fungal mat, the appearance of numerous fungal hyphae in electron microscopic images of the cultures (Figure 1), and the accumulation of chitin during the first two days of culture (Figure 2) we conclude that *A. nidulans* grows vigorously on a semi-solid sorghum slurry. However, although *A. nidulans* maintains a certain level of healthy biomass over a prolonged period, the actual fungal growth seems to reach a plateau after two days. From day 3 onwards we observed high levels of autolytic enzymes in the secretome indicating autolysis of the fungus. Class V chitinase, ChiB, AN4871, belonging to family GH18, N-acetylglucosaminidase, NagA, AN1502, belonging to family GH20, beta-1,3-endoglucanases AN4825 and AN0472, from family GH55 and GH81 respectively, the alkaline protease PrtA, AN5558, metallo-protease PepJ, AN7962, and dipeptidase AN2572 have all been shown to be associated with autolysis [39-42]. All were found to be highly induced from day 3 onwards (Table 5). On the other hand, Catalase B, an enzyme associated with growing and developing hyphae was found in high abundance throughout the time course [43]. Similarly enzymes associated with fungal cell wall synthesis and remodeling such as GPI-anchored cell wall organization protein Ecm33 (AN4390), and putative GPI anchored beta-1,3(4)-endoglucanase (AN2385) [44] were present in the secretome. Based on these findings, we propose that *A. nidulans* is maintaining growth throughout the time course but at the same time is recycling the older hyphae to support growth of hyphal tips and lateral branching. It is well established that lateral branching can be induced by fungal interaction with plants presumably leading to enhanced surface area and nutritional assimilation [45].

Powell et al. [2] studied the chemical composition of conventional grain sorghum, intermediate type sorghum and forage sorghum stover. They reported 31.3% cellulose content in grain type sorghum stover, 25.3% for intermediate sorghum stover, and 29.1% for forage type sorghum on a dry mass basis. The hemicellulose content was found to be 28.2% in grain sorghum stover, 21.7% in intermediate type sorghum stover, and 23.9% in forage type sorghum. The lignin content was between 7 to 7.3% for the three types of sorghum stover. In this study, the cellulose content was found to be 26.6% and the hemicellulose content was 22.3% of the total dry mass (Figure 5).

From the amount of xylanase and cellulase activities we found in the extracellular fluid collected from the culture on day 1 there should have been sufficient

![Figure 5](http://www.biotechnologyforbiofuels.com/content/5/1/52)

**Figure 5** Estimation of residual sugars of sorghum collected from *A. nidulans* grown on sorghum. The sugars were estimated using Saeman hydrolysis. The results show the total sugar remaining from the initial 3 g of sorghum after 1, 7 and 14 days of growth of fungus on sorghum. The bars designated as UC show the amount of each sugar in 3 g of un-inoculated sorghum. Data represent mean ± SE.
enzyme activity to break down all the xylan and cellulose in the sorghum in less than a day. However the concentration of soluble sugars (break-down products from the enzymatic hydrolysis), in the extracellular fluid was low (less than 1 mM), and less than 5% of the total xylan had been digested and seemingly none of the cellulose. This emphasizes the well-known recalcitrance of plant biomass to enzymatic degradation and suggests that the fungus is growing under carbon limited conditions. However, over time, the fungus did appear to be able to digest all classes of cell wall polysaccharides present and it did continue to maintain a certain level of healthy biomass.

We were interested in determining if the fungus attacked the most easily digestible polymers first and then moved on to using the more recalcitrant ones. It has been suggested that fungi first degrade the pectin in plant cell walls to make the hemicelluloses and cellulose more accessible [46]. Sugars characteristic of pectin, i.e. rhamnose and galacturonic acid, were not detected in total hydrolysates of sorghum stover, although both of these sugars were found in the solubilized sugars rinsed from the cultures. This indicates the presence of a small amount of pectin in sorghum. Polygalacturonase activity was induced rapidly in the culture but xylanase and cellulase activity increased at the same time. Looking at the proteomics results one can deduce that almost all of the many enzymes involved in hemicellulose and cellulose degradation are induced in parallel. However in pectin degradation there is a steady decrease over time in exopolygalacturonases, one of the pectin methyl esterases, and one of the pectin lyases. In contrast there was a steep increase in other pectate lyases and rhamnogalacturonan hydrolases.

The recalcitrance of the sorghum or any other plant biomass to digestion is thought to arise from physical inaccessibility of the polymers to the enzymes designed to digest them. Two major factors are the incrustation of the polysaccharides with lignin and the crystalinity of the cellulose. We identified three classes of proteins which might play a key role in overcoming the recalcitrance of the sorghum to digestion.

There is strong evidence that some of the ferulic acids esterified to xylans crosslink to make diferulates which link xylans together thus making them less accessible to enzyme digestion. The ferulates can also be incorporated into lignin thus attaching xylan directly to lignin [47]. Hydrolysis of the ferulate ester bonds is expected to decrease the recalcitrance of biomass to enzymatic hydrolysis [48-50]. Two feruloyl esterases were secreted by A. nidulans in high abundance throughout the time course.

Another enzyme in our cultures which might play a role in overcoming recalcitrance of the sorghum to digestion is cellobiose dehydrogenase (CDH). CDHs oxidize cellobiose, or other reducing sugars, and transfer the electrons to Fe^{3+}, quinones, radicals, or oxygen to make hydrogen peroxide [51]. The combination of the resulting Fe^{2+} and hydrogen peroxide can lead to the generation of hydroxyl radical which will attack polysaccharides and lignin. At least three putative sequences for CDHs are present in the A. nidulans genome. We only detected one form. Interestingly CDH was not present on day 1 and then increased gradually showing a spectrum count of 17 on day 3 up to 77 on day 14. No other studies have reported the secretion of cellobiose dehydrogenases by A. nidulans on solid state cultures. The presence of cellobiose dehydrogenase after day 3 may indicate its important role in the degradation of crosslinks between lignin, hemicelluloses and cellulose.

Whenever investigated, GH61 family endoglucanases lack measurable endoglucanase activity, but recently they have been reported to accelerate the hydrolysis of cellulose in cellulase preparations [52,53]. GH61 was initially described as an endoglucanase, but more recently has been shown to be members of the family are copper mono-oxygenases that catalyse cleavage of cellulose oxidatively, releasing cellobextrins [53,54]. Their active site contains a type II copper site, which, after being reduced to Cu^{+} by a reductant such as ascorbate or gallate, is thought to activate oxygen which can then oxidize glycosidic linkages on the surface of crystalline cellulose. This generates new chain ends rendering the substrate far more prone to attack by the classical endoglucanases and cellobiohydrolases [52,53]. Cellobiose dehydrogenase appears to be able to mediate the reduction of the copper site [54]. Recent studies have shown that cellobiose dehydrogenase may enhance cellulose degradation by coupling the oxidation of cellobiose to the reductive activation of copper-dependent polysaccharide monoxygenases [53,54]. Four GH61 enzymes were found in our study.

Studies similar to ours were conducted by Schneider et al. [11] who grew A. nidulans and the mesophilic bacteria Pectobacterium carotovorum on leaf litter for 20 days, both individually and in co-culture, and identified the proteins secreted into the medium by 1-D PAGE-LC-MS/MS. The aim of their study was to determine the relative contribution of the fungus and the bacterium to the decomposition. They reported a total of 90 proteins secreted by A. nidulans on leaf litter during the course of 20 days in comparison with the 294 proteins identified in our sorghum cultures. Seventy- two proteins were found to be common in both sorghum and leaf litter cultures, 59 proteins were exclusively found in the sorghum cultures and 13 were only identified in leaf litter cultures. The identities of the proteins in the two cultures are reported in Additional file 2: Table S2. More
than half of the cellulases and xylanases were common to both cultures with two xylanases and four endoglucanases unique to leaf litter cultures. Only one pectinase out of 22 was unique to leaf litter. Interestingly enzymes involved in degradation of crosslinks between lignin, cellulose and hemicelluloses such as cellobiose dehydrogenase and feruloyl esterases were only identified in sorghum cultures. Only half the proteases and less than one third of cell wall remodeling enzymes were common to both the cultures. In another recent study, Couturier et al. [55] identified total 66 proteins in the secretome of *A. nidulans* grown on maize bran. Out of 19 GHs identified in their study in *A. nidulans* secretome, seven hemicellulases belonging to family GH10, GH11, GH39, GH43, GH62 and GH93 and five beta-1,3-glucanases from the GH17, GH55 and GH81 families were identified. They could not detect any beta-1,4-endoglucanase or cellobiohydrolase or any enzyme activity on CMC [55]. The difference in growth and enzyme activities of *A. nidulans* on sorghum and on leaf litter may be attributed to different substrates compositions and growth conditions [12].

**Conclusions**

We have identified extracellular proteins secreted during the entire time course of cultivation of *A. nidulans* on sorghum stover. The aim was to learn the cocktail of enzymes we can devise to hydrolyze lignocellulosic biomass efficiently under mild conditions. In this study we identified a total of 294 proteins including cellulases, hemicellulases, pectinases, carbohydrate esterases, chitinases, and many proteins of unknown function. The enzymes such as feruloyl esterases, cellobiose dehydrogenase, and the family GH61 endoglucanases can be important in accelerating biomass conversion by reducing the recalcitrance of cellulose. Some of the hypothetical proteins we found may work as non-hydrolytic accessory proteins aiding hydrolytic enzymatic efficiency. Further work involving global/whole transcriptome studies of *A. nidulans* during its growth on sorghum may give insight into their function. Cloning and expression of the enzymes such as feruloyl esterases, cellobiose dehydrogenase and family 61 endoglucanases will let us test directly if they have beneficial effects.

**Materials and methods**

**Growth conditions**

*Aspergillus nidulans* Strain A78 was obtained from the fungal genetic stock center (FGSC) [56]. 3 g of ground sorghum (variety mix of AtX2752/RtX2783 and AtX2752/RtX430) was ground in a Thomas Wiley® Mini-Mill (Thomas Scientific, Swedesboro, NJ, USA) by passing through a 60-mesh screen. Fungal spores were counted using a hemacytometer (Spectrum Scientifics, Philadelphia, PA, USA) and adjusted to a million spores per milliliter in water. The ground sorghum was moistened with 6 ml water in a petri plate and autoclaved for an hour at 121°C. To these petri plates 15 ml of minimal media (pH 6.5; 95 ml of water, 5 ml 20X nitrate salts and 0.1 ml 1000X trace elements) and 1 ml of spore suspension containing 10^8 spores was added. Then the petri plates were incubated at 37°C and 70% of relative humidity for 1, 2, 3, 5, 7 and 14 days. Uninoculated sorghum stover incubated in same conditions as inoculated stover was used as control.

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Fungus grown on borosilicate 3 mm solid glass beads (Aldrich, Milwaukee, WI) in 1% glucose for 34 hours was used as a control for comparison of secretomes. *A. nidulans* cultured in 1% glucose for 34 hrs utilized only 1/4th of the initial glucose and thus should not be undergoing starvation.

**Sample preparations for microscopy**

For scanning electron microscopy (SEM), fungal samples approximately, 5 mm squares, were cut from the plates and placed in the first fixative solution (2.5% glutaraldehyde in 0.2 M sodium phosphate, pH 7.2) and incubated for 2 h. The glutaraldehyde solution was removed and replaced with 0.2 M sodium phosphate buffer, pH 7.2 and samples were washed 3X for 20 min each. Post fixation was done in 1% osmium tetroxide in dH2O for an hour. Dehydration was done with a series of ethanol solutions (50, 70, 90, 95,100,100,100%), for 15 minutes each. Samples were critical point dried and mounted on aluminum stubs using silver paint adhesive followed by coating with gold/palladium. Samples were observed using an FEI quanta 600 electron microscopy (FEI, Hillsboro, OR, USA).

For transmission electron microscopy (TEM), the fixation was done as for the SEM samples. Post fixation was done in 1% osmium tetroxide /1.5% potassium ferrocyanide in 0.1% sodium cacodylate buffer for 1 hour. Using a series of ethanol solutions (as described in SEM protocol), samples were dehydrated for 15 minutes each. The dehydrated samples were washed 2X with 100% propylene oxide/polyrexin/bed 812 overnight (Polysciences Inc., Warrington, PA, USA) followed by immersion in polyrexin/bed 812 and polymerized at 60°C. Samples were sectioned using a Leica EMCU 6 ultramicrotome (Midwest Lab Equipment, Iowa City, IA, USA). Sectioned samples were placed on nickel; carbon/formavar coated grids and stained with uranyl acetate and lead citrate and observed using a JEOL 2100 Transmission electron microscope (JEOL, Austin, TX, USA).
Estimation of fungal growth by chitin estimation
The entire plate contents of A. nidulans cultures after day 1, 2, 3, 5, and 14 was freeze-dried and then mixed thoroughly. One mg samples were transferred to vials with teflon lined caps and hydrolyzed in 3 ml of 6 N HCl, at 100°C for 6 h. The HCl was evaporated overnight in a speed-vac [57]. Once the extracts were dried, 10 μl of 1 M ammonium hydroxide was added to neutralize any residual HCl. Then 10 μl of 100 nanomoles methyl glucamine was added as an internal standard, followed by 100 μl of 20 mg/ml potassium boroxyhide in DMSO and incubated at 40°C for 90 min to reduce hexosamines to hexosaminitols. After 90 min the reaction was stopped by adding 10 μl glacial acetic acid, followed by addition of 20 μl methyl imidazole and 200 μl of acetic anhydride and incubated for 10 min at room temperature to acylate the hexosaminitols [58,59]. The reaction was stopped by adding 500 μl water and the alditol acetates were purified by adsorbing them to a C18 sep-pak and desorbing them with methylene chloride to remove the polyphenols. The dichloromethane was then evaporated to dryness. 25 μl of ethyl acetate was added to the dried sample, out of which, 1 μl was injected into the gas chromatograph (Agilent, Santa Clara, CA, USA). A standard curve was prepared using known amounts of glucosamine ranging from 10 to 250 nmol. The glucosamine content of 1 mg aliquots of dried A. nidulans grown in liquid media (49.50 μg) was used as a conversion factor to estimate milligrams of fungus per milligram of dry mass.

Estimation of enzyme activities by capillary zone electrophoresis (CZE) and dinitrosalicylic acid (DNS) assay
Extracellular extracts were collected by washing the whole petri plate contents with 5 ml of autoclaved water and filtering the extract using Whatman no 1 filter paper. Enzyme activities were detected semi-quantitatively by CZE using 8-aminopyrene-1,3,6-trisulfonate (APTS) labeled substrates (xylohexose, cellopentose, arabinohexaose, mannohexose) [60]. 1 μl aliquots of extracellular extract and 2 μl of respective APTS labeled substrates were added to 22 μl of 50 mM ammonium acetate buffer of optimum pH for the respective enzymes to make a total volume of 25 μl. Samples were incubated for 1 h at 37°C and the progress of enzymes was followed by CZE (Bio-Rad, Hercules, CA, USA).

The DNS assay, developed by Sumner and Graham [61] for determination of reducing sugar, was used for quantitative determination of enzyme activity. We used a DNS reagent composed of 0.75% di-nitrosalicylic acid, 0.5% phenol, 0.5% sodium metabisulfite, 1.4% sodium hydroxide, and 21% sodium potassium tartrate [62,63]. To 10 μl of aliquot of extracellular extract, 20 μl of 50 mM ammonium buffer of optimum pH and 20 μl of appropriate substrate (1%W/V) prepared in water, were added. The reaction was carried out in a 96 well PCR plate (Corning, NY, USA) and allowed to incubate for 30 minutes at 37°C. The reaction was terminated by addition of 40 μl of DNS mixture to each well and the plate was heated at 100°C for 5 min. We read the plate in a 96 well plate reader at 550 nanomoles (Tecan, San Jose, CA, USA). After subtracting substrate blanks from the reading enzyme activities were calculate from standard graphs.

Estimation of solubilized sugars in extracellular filtrate
To identify and quantitate the breakdown products of the polysaccharides, oligosaccharides present in extracellular filtrate were estimated by gas chromatographic (GC) analysis of trimethylsilyl methyl glycosides. Methanolysis and derivatization were performed using the protocol of Chaplin [64] modified by Komalavilas and Mort [65]. A 25 μl aliquot of sample and 100 nanomoles of inositol as an internal standard were dried in 1 dram glass vials in speed vac. 200 μl of methanol 1.5 M of HCl was added to each vial followed by addition of 100 μl of methyl acetate and kept in a heating block at 80°C for minimum 3 hours. The vials were cooled, followed by addition of few drops of t-butanol and then evaporated under a stream of nitrogen at room temperatures. Butanol co-evaporates with the HCl, helping to remove the HCl without degrading sugars. 25 μl of 1:1:5 mixture of hexamethyldisilazane: trimethylchlorosilane: pyridine (TMS) was added to all samples. TMS in all the samples was evaporated using nitrogen gas. Dried samples were re-dissolved in 300 μl of isoctane. 1 μl of sample was injected in the gas chromatograph (Agilent, Santa Clara, CA, USA). Standards were made by taking aliquot of 100 nanomoles of each sugar standards and 100 nanomoles of inositol. From the integrator we calculated relative peak areas and used these to calculate how much of each sugar in sample. Area of sugar peak in sample/area of inositol peak in the standards X 100 = number of nanomoles in the sample.

Liquid chromatography-tandem mass spectroscopy
The fungal cultures grown on sorghum for day 1, 3, 7 and 14 and fungal culture grown in 1% glucose were washed with 5 ml autoclaved water and the filtrates were collected. Concentration of total protein in these extracts was measured by Bradford assay. Small aliquots of the extracellular filtrates containing 50 μg of total protein were run on SDS-PAGE. Gel bands were excised, reduced with Tris (2-carboxyethyl) phosphine), alkylated with 2-Lodoacetamide, and digested for 16 hrs with 8 μg/ml trypsin using ammonium bicarbonate buffer. Peptides were extracted and analyzed by LC-MS/MS.
using an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). For this analysis, an Eksigent split less LC pump (Eksigent, Dublin, CA, USA) was used to separate peptide populations on analytical C18 nano columns (Bruker-Michrom Inc., Auburn, CA, USA), with the column effluent being sprayed directly into a new objective pico view ion source. Using a “Big Three” MS/MS method, the Orbitrap analyzer collected an ultra-accurate (R = 60,000) scan of intact peptides, whilst the LTQ ion trap simultaneously performed MS/MS fragmentation analysis of the each of three most abundant peptides eluting in that chromatographic fractions. The LC-MS/MS raw files were used for database searching via the software application Mascot (v2.2.2 from Matrix Science, Boston, MA, USA) against sequences from A. nidulans as well as against a sorghum database [23,66]. Searches were validated using Scaffold (Proteome Software Inc., Portland, OR, USA) and the Peptide Prophet algorithm [67]. Criteria for accepting protein identification, was only protein probability thresholds greater than 99% were accepted and at least two peptides needed to be identified, each with 95% certainty. Protein candidates containing similar peptides were grouped to fulfill the principles of parsimony. Search results were also checked for false discoveries using reversed decoy sequence databases, and no decoy sequences were detected, thus bringing our false discovery rate to zero. Changes in protein expression between samples from different time points were examined using the spectral count method [68]. The complete peptide reports from the Scaffold results for samples taken from days 1, 3, 7, and 14 are given in Additional file 3: Table S3, Additional file 4: Table S4, Additional file 5: Table S5, and Additional file 6: Table S6 respectively. SignalP was used to predict secretion signals in the identified proteins [69,70]. Additional information for the presence of a signal peptide was obtained by accessing the following URL [25].

Estimation of residual sorghum hydrolysates by saeman hydrolysis

Saeman hydrolysis was done on the total biomass on the plates after 1, 7, and 14 days of fungal growth to estimate total sugars [30]. Three hundred mg samples of the thoroughly mixed freeze dried biomass from each petri plate were suspended in 3 ml of 72% sulfuric acid. The samples were incubated at 300°C for 60 min with stirring every 5–10 min followed by dilution of sulfuric acid to 4% by adding 84 ml of water and autoclaved for an hour at 121°C. 2 ml aliquot of each sample was neutralized with calcium carbonate to pH 5–6. 25 µl of sample was processed for methanolysis, trimethylsilylation, and gas liquid chromatography (GLC) analysis as described above.

Additional Files

- **Additional file 1:** Table S1. Identified miscellaneous proteins and spectrum counts on 1, 3, 7, and 14 days.
- **Additional file 2:** Table S2. Comparison of identified proteins in A. nidulans cultures from different carbon sources.
- **Additional file 3:** Table S3. Protein report of A. nidulans grown on sorghum as carbon source on day 1.
- **Additional file 4:** Table S4. Protein report of A. nidulans grown on sorghum as carbon source on day 3.
- **Additional file 5:** Table S5. Protein report of A. nidulans grown on sorghum as carbon source on day 7.
- **Additional file 6:** Table S6. Protein report of A. nidulans grown on sorghum as carbon source on day 14.

Abbreviations

1D-PAGE: One dimensional polyacrylamide electrophoresis; LC-MS/MS: Liquid chromatography-tandem mass spectroscopy; CAZY families: Carbohydrate active enzyme families; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy; CZE: Capillary zone electrophoresis; ORF: Open reading frame; GH: Glycosyl hydrolase; AN: Accession number; CBM: Carbohydrate binding module; PL: Pectin lyase; ECF: Extracellular filtrate; CDH: Cellobiose dehydrogenase; CMC: Carboxymethyl cellulose; FGSC: Fungal genetic stock center; APTS: 8-aminopyrene-1,3,6-trisulfonate; DMS: Dinitrosalicylic acid; PCR: Polymer chain reaction; GC: Gas chromatography; TMS: Hexamethyldisilazane: trimethylchlorosilane: pyridine.

Competing interest

The authors declare that they have no competing interest.

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