Growth and hydrodase profiles can be used as characteristics to distinguish Aspergillus niger and other black aspergilli

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INTRODUCTION

The genus Aspergillus consists of a large number of species, including several opportunistic pathogens (e.g. A. fumigatus, A. terreus), toxin producers (e.g. A. flavus, A. parasiticus) and industrial species (A. niger, A. aculeatus, A. oryzae). The genus is divided into several sections, such as the yellow and the black aspergilli. The black aspergilli (Aspergillus section Nigri) are cosmopolitan, and contain the most commonly used industrial species, A. niger.

Aspergillus niger has been collected from locations around the globe and is often among the most common species found in fungal communities, indicating that this species is able to propagate efficiently in a wide range of environments. Aspergillus niger and other black aspergilli grow predominantly on dead plant material, which consists mainly of cell walls. These cell walls contain polymeric components, such as cellulose, hemicellulose, pectin, lignin and proteins, of which the polysaccharides make up about 80 % of the biomass (de Vries & Visser 2001). Aspergillus cannot import polymeric compounds into the cell and therefore relies on enzymatic degradation to produce monomeric and small oligomeric carbon sources (de Vries & Visser 2001, de Vries 2003). Due to the large structural differences of the various plant polysaccharides, efficient degradation of these compounds relies on the production of a broad range of different enzymes. In addition, a tight regulatory system is required to ensure production of the right mixture of enzymes in the presence of a specific polysaccharide (de Vries & Visser 2001, de Vries 2003). Since different biotypes contain different plants (e.g. grasses vs. woods) and therefore different polysaccharides, different enzyme mixtures will be required for each biotype.

In light of this, one might expect that Aspergillus isolates from different biotypes have adapted to the available carbon source and produce different mixtures of enzymes to optimally utilise the available nutrients. Individual strains that have adapted to their environment might therefore grow less efficient in a different biotope. To study whether adaptation to the environment occurs we have compared 14 A. niger isolates from different global locations with respect to physiology, growth on different carbon sources, enzyme production and temperature profiles. In addition we also compared the ex-type strains of 14 species of black aspergilli to determine whether the differences between these species are larger than the differences between A. niger isolates from different biotypes. It was shown previously that A. niger can be distinguished from the other black aspergilli by the ability to grow in the presence of 20 % tannic acid, while the other species would only tolerate up to 5 % (Rippel 1939, van Diepeningen 2004). In this study we test a variety of non-toxic naturally occurring carbon sources to identify species-specific differences in carbon utilisation.

MATERIALS AND METHODS

Strains, media and growth conditions

All strains used in this study are listed in Table 1. Strains were grown on Malt Extract Agar (MEA) or Minimal Medium, pH 6.0 (MM) (de Vries et al. 2004) as indicated in the text. For growth on solid MM medium, 1.6 % (w/v) agar was added to the medium before autoclaving. For the generation of spore suspensions, strains were grown for 14 d on MEA plates at 25 °C except for A. piperis CBS 112811. This strain was cultivated at 37 °C, because it sporulated poorly at 25 °C. Temperature profiles were also obtained on MEA plates.

All strains and isolates were grown at 30 °C, for carbon source analysis. As a positive control, 1 % glucose was added to the MM media. Polysaccharides were added to a final concentration of 0.5 %, while monosaccharides were added to a final concentration of 25 mM.
| Strain | Correct identification | Original identification | Origin (code) | β-tubulin Acc. No. | ITS Acc. No. |
|--------|------------------------|-------------------------|---------------|-------------------|-------------|
| CBS 564.65 | A. acidus | Japan (JAP) | FJ639280 | FJ639329 |
| CBS 106.47 | A. acidus | Switzerland (SWI) | FJ639281 | FJ639330 |
| CBS 124.49 | A. acidus | Central America (CA) | FJ639282 | FJ639331 |
| CBS 139.48 | A. acidus | Ukraine (UKR) | FJ639283 | FJ639332 |
| CBS 172.66 | A. aculeatus | Tropics (TR) | FJ639271 | FJ639320 |
| CBS 101740 | A. brasiliensis | Brasil (BRA) | FJ639272 | FJ639321 |
| CBS 246.65 | A. brasiliensis | Australia (AUS) | FJ639273 | FJ639322 |
| CBS 533.88 | A. costaricaensis | Costa Rica (COS) | FJ639277 | FJ639326 |
| CBS 115574 | A. costaricaensis | Costa Rica (COS) | FJ639278 | FJ639327 |
| CBS 707.79 | A. ellipicus | Costa Rica (COS) | FJ639279 | FJ639328 |
| CBS 111.26 | A. carbonarius | Unknown | FJ639276 | FJ639325 |
| CBS 113.50 | A. carbonarius | USA (USA) | FJ639275 | FJ639324 |
| CBS 139.52 | A. carbonarius | Japan (JAP) | FJ639274 | FJ639323 |
| CBS 101883 | A. carbonarius | Indonesia (INA) | FJ639273 | FJ639322 |
| CBS 113.32 | A. carbonarius | Germany (GER) | FJ639272 | FJ639321 |
| CBS 101889 | A. carbonarius | Unknown | FJ639271 | FJ639320 |
| CBS 112.36 | A. carbonarius | Japan (JAP) | FJ639270 | FJ639319 |
| CBS 122.49 | A. carbonarius | USA (USA) | FJ639270 | FJ639319 |
| CBS 139.48 | A. carbonarius | South-Pacific Islands (SPI) | FJ639270 | FJ639319 |
| CBS 115989 | A. carbonarius | Nigeria (NIG) (DSM genome) | FJ639270 | FJ639319 |
| CBS 113.46 | A. carbonarius | USA (USA) (JGI genome) | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Denmark (DEN) | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Unknown | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Unknown | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Unknown | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Unknown | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Unknown | FJ639270 | FJ639319 |
| CBS 121600 | A. carbonarius | Unknown | FJ639270 | FJ639319 |
Plates were inoculated with 2 μL spore suspension of each strain. Cultivations for the crude polysaccharide assay were done with spore suspensions with a concentration of 5 × 10^6 spores/mL. For serial dilutions, spore suspensions of 5 × 10^6, 5 × 10^5, 5 × 10^4 and 5 × 10^3 spores/mL were used. For temperature profiles, a concentration of 5 × 10^6 spores/mL was used. Liquid cultures for enzyme analysis were performed in MM with 1 % wheat bran (WB) and were inoculated to a final concentration of 0.5 × 10^6 spores/mL and were incubated at 30 °C for 2 d. Liquid cultures for chromosomal DNA analysis were performed using malt peptone (MP) broth containing 10 % (v/v) malt extract and 0.1 % (w/v) bacto-peptone, and were incubated at 25 °C for 3–4 d. All standard chemicals and carbon sources were obtained from Sigma.

**Molecular Biology methods**

DNA was extracted from mycelial samples using the Masterpure yeast DNA purification kit according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using the primers LS268 (GACATCCCAAACAATCGACTC) and V9G [TTAGGTCCCTGCGCTTTGTA, (Gerrits van den Ende & de Hoog 1999)]. Amplification of part of the β-tubulin gene was performed using the primers Bt2a (GGTAACCAAATCGGTGCTTTC) and Bt2b [ACCCTCTAGTGTAGTGACCCTTGGC, (Glass & Donaldson 1999)]. Amplification of part of the β-tubulin gene was performed using the primers Bt2a (GGTAACCAAATCGGTGCTTTC) and Bt2b [ACCCTCTAGTGTAGTGACCCTTGGC, (Glass & Donaldson 1999)]. Both strands of the PCR fragments were sequenced with the ABI Prism® Big Dye™ Terminator v. 3.0 Ready Reaction Cycle sequencing Kit. Samples were analysed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the Genetic Analyzer and contigs were assembled using the forward sequencing Kit. Samples were analysed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package. Sequences were aligned in Molecular Evolutionary Genetics Analysis (MEGA) v. 4 using CLUSTALW. The Phylogenetic trees were established with Maximum Parsimony method in MEGA v. 4. To determine the support for each clade, a bootstrap analysis was performed with 500 replications.

**Enzyme assays and protein profiles**

Extracellular hydrolytic activities were assayed using 0.01 % substrate, 20–40 μL sample and 25 mM sodium acetate pH 5.0 in a total volume of 100 μL. The mixtures were incubated for 1 h at 30 °C after which the reaction was stopped by adding 100 μL 0.25 M Na2CO3. Absorbance was measured at 405 nm in a microtiter plate reader. The activity was calculated using a standard curve of p-nitrophenol. The substrates used for enzyme assays were all obtained from Sigma and were p-nitrophenol-α-arabinofuranoside, p-nitrophenol-β-xylopyranoside, p-nitrophenol-β-galactopyranoside, p-nitrophenol-α-galactopiyranoside, p-nitrophenol-β-glucopyranoside and p-nitrophenol-β-mannopyranoside to measure α-arabinofuranosidase, β-xylosidase, β-galactosidase, α-galactosidase, β-glucosidase and β-mannosidase, respectively. Culture filtrate samples were separated on 10 % SDS-PAGE gels and stained using silver-staining.

**RESULTS**

Identification of putative *A. niger* wild isolates

The CBS database was searched for *A. niger* isolates obtained from a wide variety of locations around the world, resulting in 34 isolates. In addition to these, the parent of the *A. niger* strain sequenced by DSM (Pel et al. 2007) and the strain sequenced by the Joint Genome Institute of the US Department of Energy (Baker 2006) were also included in the study. To confirm that these strains were true *A. niger* strains, the ITS and β-tubulin sequences of these strains were compared to those of the ex-type strains of the different black aspergilli (Fig. 1). This demonstrated that from the 34 isolates only 14 were *A. niger* strains. The other strains were members of *A. tubingensis* (13), *A. brasiliensis* (3), *A. acidus* (3) and *A. costaricaensis* (1). The 14 *A. niger* isolates as well as the sequenced strains were used for the rest of the study in comparison to the ex-type strains of the different black aspergilli, while the other isolates were eliminated from the study. The remaining *A. niger* isolates still represent a worldwide distribution.

Growth profiles of *A. niger* isolates and type strains from *Aspergillus* section *Nigri*

All *A. niger* isolates have similar growth profiles on monosaccharides (Table 2, Fig. 2). CBS 115989 grows significantly slower than the other isolates on all monomeric carbon sources. In contrast, carbon source specific differences were observed between the different black aspergilli (Table 2, Fig. 2). *Aspergillus brasiliensis* was the only species that was able to grow on D-galactose, and this species characteristic was confirmed for three other *A. brasiliensis* strains (data not shown). No or minimal growth was detected for *A. piperis*, *A. ellipticus* and *A. heteromorphus* on all carbon sources. Growth on L-rhamnose was only observed for *A. lacticoffeatus*, *A. niger*, *A. brasiliensis*, *A. tubingensis*, *A. costaricaensis* and *A. aculeatus* (Table 2, Fig. 2).

Growth on plant polysaccharides was also tested, as they are a major natural carbon source of aspergilli. The strain specific growth differences of the *A. niger* isolates observed on monomeric carbon sources were also observed on polysaccharides. All *A. niger* isolates grew best on starch and pectin, while slower growth was observed on xylan, arabinogalactan and Locust Bean gum (contains mainly galactomannan) (Table 3, Fig. 3). Very poor growth was observed on cellulose (Table 3, Fig. 3). In contrast, significant differences were observed when the *Aspergillus* ex-type strains were compared. Similar to the monomeric carbon sources, no growth was observed on any of the polysaccharides for *A. piperis* and *A. ellipticus*, but growth of *A. heteromorphus* on arabinogalactan and Locus Bean gum was better than on any of the monomeric carbon sources (Table 3, Fig. 3). Nearly all the other species preferred starch and pectin, as was observed for the *A. niger* isolates (Table 3, Fig. 3).

An exception was *A. aculeatus*, which grew equally well on Locust Bean gum, pectin and starch. *Aspergillus niger*, *A. carbonarius*, *A. tubingensis*, *A. costaricaensis*, *A. homomorphus*, *A. aculeatus* and *A. japonicus* grew better on xylan than the other species, while significant growth on cellulose was only observed for *A. aculeatus*, *A. japonicus* and *A. homomorphus* (Table 3, Fig. 3).

Protein and enzyme profiles of *A. niger* isolates and ex-type strains from *Aspergillus* section *Nigri*

Growth on polysaccharides is dependent on the production of extracellular enzymes that degrade these polymers to monomeric and small oligomeric compounds that can be taken up by the fungus. We therefore determined the extracellular protein profile and assayed the production of six polysaccharide hydrolases during growth on wheat bran: α-arabinofuranosidase (ABF, involved in xylan, xyloligucan and pectin degradation), β-xylosidase (BXL,
### Table 2. Growth of the *A. niger* strains on monosaccharides in comparison to the ex-type strains of the black aspergilli. Glc = D-glucose, Gal = D-galactose, Rha = L-rhamnose, Frc = D-fructose, Xyl = D-xylose, Ara = L-arabinose.

| Species       | Strain       | Glc | Gal | Rha | Frc | Xyl | Ara |
|---------------|--------------|-----|-----|-----|-----|-----|-----|
| *A. acidus*   | CBS 564.65   | +   | -   | -   | +   | +   | ±   |
|               | CBS 172.66   | +++ | -   | -   | +++ | +++ | ++  |
| *A. brasiliensis* | CBS 101740 | +++ | +   | +   | +++ | +++ | +   |
| *A. carbonarius* | CBS 111.26 | +++ | -   | -   | +++ | +++ | ++  |
| *A. costaricaensis* | CBS 115574 | +++ | -   | +   | +++ | +++ | ++  |
| *A. ellipicus* | CBS 707.79   | -   | -   | -   | -   | -   | -   |
| *A. heteromorphus* | CBS 117.55 | -   | -   | -   | -   | -   | -   |
| *A. homomorphus* | CBS 101889 | ++  | -   | +   | ++  | ++  | +   |
| *A. japonicus* | CBS 114.51   | +++ | -   | +   | +++ | +++ | ++  |
| *A. lacicoffeaus* | CBS 101883 | +++ | -   | +   | +++ | +++ | ++  |
| *A. piperis*  | CBS 112811   | -   | -   | -   | -   | -   | -   |
| *A. tubingensis* | CBS 134.48 | +++ | -   | +   | +++ | +++ | ++  |
| *A. vadensis* | CBS 113365   | +++ | -   | +   | +++ | +++ | ++  |
| *A. niger*    | CBS 554.65   | +++ | -   | +   | +++ | +++ | ++  |
| CBS 120.49    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 113.50    | ++           | -   | ±   | ++  | ++  | ±   |
| CBS 139.54    | ++           | -   | +   | ++  | ++  | ±   |
| CBS 262.65    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 242.93    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 101698    | +++          | -   | ±   | +++ | +++ | +   |
| CBS 101705    | +++          | -   | ±   | +++ | +++ | +   |
| CBS 117785    | +++          | -   | ±   | +++ | +++ | +   |
| CBS 118725    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 112.32    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 139.52    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 118.36    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 630.78    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 115989    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 113.46    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 564.65    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 120.49    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 113.50    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 139.54    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 262.65    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 242.93    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 101698    | +++          | -   | ±   | +++ | +++ | +   |
| CBS 101705    | +++          | -   | ±   | +++ | +++ | +   |
| CBS 117785    | +++          | -   | ±   | +++ | +++ | +   |
| CBS 118725    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 112.32    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 139.52    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 118.36    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 630.78    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 115989    | +++          | -   | +   | +++ | +++ | ++  |
| CBS 113.46    | +++          | -   | +   | +++ | +++ | ++  |

### Table 3. Growth of the *A. niger* strains on polysaccharides in comparison to the ex-type strains of the black aspergilli. CEL = cellulose, ABG = arabinogalactan, LBG = locust bean gum (galactomannan), BWX = beechwood xylan, CP = citrus pectin.

| Species       | Strain       | Starch | CEL | ABG | LBG | BWX | CP |
|---------------|--------------|--------|-----|-----|-----|-----|----|
| *A. acidus*   | CBS 564.65   | +      | -   | +   | +   | -   | +  |
|               | CBS 172.66   | +++    | +   | +   | +   | ++  | +  |
| *A. brasiliensis* | CBS 101740 | +++    | -   | +   | +++ | ±   | ++ |
| *A. carbonarius* | CBS 111.26 | +++    | -   | +   | ++  | +   | ++ |
| *A. costaricaensis* | CBS 115574 | +++    | -   | +   | ++  | +   | ++ |
| *A. ellipicus* | CBS 707.79   | -      | -   | ±   | ±   | -   | ±  |
| *A. heteromorphus* | CBS 117.55 | -      | -   | ±   | ±   | -   | ±  |
| *A. homomorphus* | CBS 101889 | ++    | +   | +   | ++  | ++  | +  |
| *A. japonicus* | CBS 114.51   | +++    | -   | +   | ++  | +   | ++ |
| *A. lacicoffeaus* | CBS 101883 | +++    | -   | +   | +++ | +   | ++ |
| *A. piperis*  | CBS 112811   | -      | -   | -   | -   | -   | -  |
| *A. tubingensis* | CBS 134.48 | +++    | -   | +   | +++ | +++ | ++ |
| *A. vadensis* | CBS 113365   | +++    | -   | +   | ++  | +   | ++ |
| *A. niger*    | CBS 554.65   | +++    | -   | +   | +++ | +++ | ++ |
| CBS 120.49    | +++          | -   | ++  | ++  | +   | +++ | ++ |
| CBS 113.50    | +++          | -   | ++  | ++  | +   | +++ | ++ |
Fig. 1. Phylogeny of the strains used in this study. A. Maximum Parsimony tree based on the β-tubulin sequence. B. Maximum Parsimony tree based on the ITS sequence. The origin abbreviation refers to Table 1.
|      | D-glucose | D-galactose | L-rhamnose | D-fructose | D-xylose | L-arabinose |
|------|-----------|-------------|------------|------------|----------|-------------|
| **A** | A. heteromorphus | A. ellipticus | A. carbonarius | A. lacticoffeatus | A. niger | A. brasilensis | A. vadensis | A. tubingensis | A. acidus | A. piperis | A. costaricaensis | A. homomorphus | A. aculeatus | A. japonicus |
| **B** | Starch | Cellulose | Arabinogalactan | Locust bean gum | Beechwood xylan | Citrus pectin |

Fig. 2. Growth of ex-type strains of *Aspergillus* section *Nigri* (A) and *A. niger* isolates (B) on monomeric carbon sources. Strains were inoculated as serial dilutions (left to right) of 10000, 1000, 100 and 10 spores.

Fig. 3. Growth of ex-type strains of *Aspergillus* section *Nigri* (A) and *A. niger* isolates (B) on polymeric carbon sources. Strains were inoculated as serial dilutions (left to right) of 10000, 1000, 100 and 10 spores.
involved in xylan degradation), β-galactosidase (LAC, involved in xylan, xyloglucan, pectin and galactomannan degradation), α-galactosidase (AGL, involved in galactomannan degradation), β-glucosidase (involved in cellulose and galactoglucomannan degradation) and β-mannosidase (involved in galactomannan degradation). The protein profiles were highly similar for the A. niger isolates and A. lacticoffeatus, while significant differences were detected between the other species (Fig. 4). The pH at the moment of sampling varied both between the species and within the A. niger group, although most A. niger isolates acidified the medium (Fig. 4).

The enzyme activity profiles of the A. niger isolates were also highly similar (Fig. 4). Some variation in activity levels were detected with CBS 112.32 and CBS 115989 often producing lower levels than the other A. niger isolates. Larger differences were observed between the different Aspergillus species (Fig. 4). Aspergillus carbonarius, A. ellipticus (poor growth), A. acidus, A. heteromorphus (poor growth) and A. homomorphus has significantly lower production of ABF, BXL, LAC, AGL, BGL and MND than the other species. The same applies for A. japonicus for ABF and BXL. The highest ABF and BXL activity was observed for A. brasiliensis, while the highest LAC and BGL activity was observed for A. aculeatus and the highest AGL activity for A. brasiliensis and A. aculeatus (Fig. 4). MND activity was low for all strains in comparison with the other enzyme activities.

**Temperature profiles of the A. niger isolates and ex-type strains from Aspergillus section Nigri**

The absence of growth of A. piperis and A. ellipticus on all carbon sources on solid media, but not in liquid media with wheat bran raised questions about the temperature tolerance of these species on solid media. To determine whether there were significant differences in the temperature profiles of the strains of this study, they were grown on MEA plates at temperatures ranging from
6 °C to 45 °C. All *A. niger* isolates had nearly identical temperature profiles, with 33–36 °C as optimal temperature (Fig. 6). More differences were observed between the different *Aspergillus* species (Fig. 5). *Aspergillus brasiliensis* grew very poorly at 15 °C. *Aspergillus ellipticus* only showed residual growth at 30 °C (Fig. 7), which was confirmed for a second *A. ellipticus* isolate (data not shown). *Aspergillus heteromorphus* showed only minimal growth at 33 °C, while the same was true at 36 °C for *A. japonicus*, *A. aculeatus*, *A. homomorphus* and *A. carbonarius*. The other species were still able to grow at 42 °C, but none of the species were able to grow at 45 °C.

**DISCUSSION**

*Aspergillus niger* is commonly found throughout the world and is therefore capable of growing in a large variety of biotopes with highly
different environmental conditions, such as nature of available carbon sources and other nutrients, temperature and humidity. In this study we evaluated whether the global origin of an *A. niger* isolate affects its carbon source profile as this would indicate that the isolates adapt to their local environment. Sequence-based identification of the 34 *A. niger* isolates selected from the CBS database, demonstrated that only 14 were true *A. niger* strains. The others mainly belonged to species that were previously shown to be closely related to *A. niger* (Samson *et al.* 2007) and this result demonstrates that the classification based on morphology is not sufficient for species identification. A previous study (van Diepeningen 2004) demonstrated that 40 % of black aspergilli isolates from soil belong to *A. niger* and another 40 % to *A. tubingensis*, providing a similar species dispersion as obtained in our study.

The 14 remaining *A. niger* isolates still represent a global distribution as they include 3 isolates from North-America, 4 isolates from North-western Europe, 4 isolates from Africa, 2 isolates from Asia and 1 isolate from the South-Pacific islands. As the climates and biotopes are very different in these areas it can be concluded that the strains were isolated from significantly different environments. Unfortunately, for most isolates the material they were collected from was not indicated, so it is impossible to describe the strains based on their natural carbon source at the moment of isolation.

Although some *A. niger* isolates grow faster than others, no carbon source specific differences were found between the strains, either on monomeric or polymeric carbon sources. This indicates that the ability to grow on the range of carbon sources tested in this study is maintained among all the isolates, even though they were isolated from environments that differ strongly in their carbon source composition. It can therefore be concluded that adaptation to the natural environment does not occur at the genetic level for *A. niger* and its ability to utilise various carbon sources. It could

Fig. 6. Growth of ex-type strains of *Aspergillus* section *Nigri* at different temperatures. Pictures were taken after 10 d.
be that metabolic adaptation occurs during growth in different environments, but this does not result in a permanent alteration of the ability of the strain to consume a wide range of carbon sources. A previous study (van Diepeningen 2004) suggested that the air-borne and UV-resistant characteristics of the spores result in world-wide well-mixed population of \textit{A. niger} isolates. Wind-based distribution would result in highly varied biotopes for the spores of a particular isolate. Specialisation to specific carbon sources would then be a disadvantage to an isolate. A recent study by Rokas et al. (2007) compared the two \textit{A. niger} strains that were used for genome sequencing, CBS 513.88 (a descendent from CBS 115989) and ATCC 1015 (CBS 113.48). They identified differences between the strains at the level of colony morphology. Another study (Pal et al. 2007) demonstrated that the two strains were heterokaryon incompatible, indicating that they do not have a (recent) clonal relation. Non clonal linkages often vary in gene expression and growth rates that in some cases can be attributed to the occurrence of dsRNA mycoviruses (van Diepeningen et al. 2006). In the current study the main difference between CBS 115989 and CBS 113.46 was the slower growth of CBS 115989, which confirms that these strains are not identical. However, they did not differ in their carbon source growth profile.

In contrast, significant differences were observed between the different black \textit{Aspergillus} species, demonstrating that the interspecies variation with respect to carbon source utilisation is larger than the variation within a species. The absence of growth on D-galactose for all the black aspergilli has been reported before (de Vries et al. 2005), but our study demonstrate that \textit{A. brasiliensis} is able to grow on this substrate. This suggests a significant difference between this species and the other black aspergilli. Whether the difference is at the level of sugar transport or metabolism is not clear at this point. Previous studies with an \textit{A. niger} high affinity hexose transporter demonstrated that this protein could transport D-glucose, D-fructose and D-mannose, but not D-galactose (vanKuyk et al. 2004), indicating that D-galactose transport may be different from the other hexoses.

The absence of growth on plates of \textit{A. ellipticus} can be explained by its temperature profile, as this strain is not able to grow above 27 °C and the experiment was performed at 30 °C. This appears to be a species characteristic, as a second \textit{A. ellipticus} strain that was tested showed the same temperature profile. \textit{Aspergillus ellipticus} did show slow growth at 30 °C in liquid shaken culture, indicating that the culture set-up affects its ability to cope with high temperatures. The culture conditions cannot explain the absence of growth on carbon source test plates for \textit{A. piperus}, especially since

\begin{figure}
\centering
\includegraphics[width=\textwidth]{growth_curves.png}
\caption{Growth curves of the type strains (A) and the \textit{A. niger} isolates (B). Growth curves were determined by the colony diameter (mm) after 4 d incubation.}
\end{figure}
the same strain grew very well in liquid culture at 30 °C and also was able to grow on malt extract agar plates at temperatures up to 42 °C. Possibly, minimal medium lacks a specific component (e.g. an amino acid) that cannot be synthesised sufficiently by \textit{A. piperius} itself, but that is present in both MEA and wheat bran.

These results suggest that growth profiles on defined media and at different temperatures can be used as a first step in the identification of different black \textit{Aspergillus} species, as they do not differentiate between strains of the same species isolated from different environments.

No strong differences were observed in hydrolase production between the \textit{A. niger} isolates during growth on wheat bran. Wheat bran was used as a substrate as it has been shown to induce the production of a large variety of hydrolases by \textit{Aspergillus} (Yamane \textit{et al.} 2002, Kang \textit{et al.} 2004). Strain CBS 115989 overall had lower levels of activity than the other \textit{A. niger} isolates, but this strain also grew significantly slower on all substrates than the other isolates. Based on the activity profile, CBS 101705 is the best producer of ABF, BXL, LAC and BOL, while CBS 242.93 is the best producer of AGL. These differences demonstrate that the variety among natural isolates with respect to enzyme production could be exploited for selection of novel metabolic differences that affect production of specific enzymes.

Similar to the growth experiments, much larger differences in hydrolase production were observed between the \textit{Aspergillus} species than between the \textit{A. niger} isolates. Production of all hydrolases was particularly low in \textit{A. ellipticus}, \textit{A. acidus}, \textit{A. heteromorphus}, \textit{A. homomorphus} and \textit{A. carbonarius} (except for BGL). For \textit{A. ellipticus} this can be explained by poor growth at this temperature, while in the case of \textit{A. acidus} this is partly caused by a high extracellular protein production, but a low absolute enzyme activity. Except for \textit{A. acidus}, all species with low activity cluster together in the phylogeny of the black aspergilli (Samson \textit{et al.} 2007), suggesting that this phenomenon can be traced back to the combined origin of these species. The strong similarity between \textit{A. lacticoffeatus} and the \textit{A. niger} isolates is easily explained as recent studies showed that \textit{A. lacticoffeatus} is in fact the same as \textit{A. niger} (Varga \textit{et al.} 2011). This suggests that species identification can already largely be determined using SDS-PAGE profiles after growth on wheat bran for the black aspergilli, which would be a relative easy tool that could also be applied in low-tech facilities. SDS-PAGE profiles of intracellular samples have been used previously for species identification when comparing isolates of \textit{A. niger}, \textit{A. nidulans}, \textit{A. flavus} and \textit{A. fumigatus} (Rath 2001). However, these profiles are more complex and more sensitive to variation (de Vries \textit{et al.}, unpubl. results).

Identification of the proteins that are secreted by these species would be interesting as this may shed some light on their physiology in the presence of crude carbon sources. Polysaccharide hydrolases have mainly been purified from \textit{A. niger} and \textit{A. aculeatus}, while some have also been reported from \textit{A. acidus}, \textit{A. japonicus}, \textit{A. tubingenis}, \textit{A. carbonarius} and \textit{A. brasiliensis} (Takada \textit{et al.} 1999, Brumbauer \textit{et al.} 2000, van Casteren \textit{et al.} 2000, Decker \textit{et al.} 2000, Ademark \textit{et al.} 2001a, 2001b; de Vries \& Visser 2001, Kiss \textit{et al.} 2002, el-Gindy 2003, Liu \textit{et al.} 2007, Pedersen \textit{et al.} 2007). No papers about polysaccharide hydrolases have been reported for any of the other species. The data of the current study indicates that some of these species (e.g. \textit{A. piperius}) could be interesting sources of hydrolytic enzymes, which may have different properties from those described previously.

In summary, this study demonstrates that \textit{A. niger} isolates have a similar potential for growth on monomeric and polymeric sugars as well as their polysaccharide hydrolase profiles, even when they have been isolated from significantly different biotopes. In contrast, strong differences were found in growth and hydrolase profiles among closely related \textit{Aspergillus} species, indicating that these parameters may be considered species characteristics.

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REFERENCES

Ademark P, Larsson M, Tjerneld F, Stalbrand H (2001a). Multiple a-galactosidases from \textit{Aspergillus niger}; purification, characterization, and substrate specificities. \textit{Enzyme & Microbial Technology} 29: 441–448.

Ademark P, Vries RP de, Hagglund P, Stalbrand H, Visser J (2001a). Cloning and characterization of \textit{Aspergillus niger} genes encoding an alpha-galactosidase and a beta-mannosidase involved in galactomannan degradation. \textit{European Journal of Biochemistry} 268: 2962–2990.

Baker SE (2006). \textit{Aspergillus niger} genomics: past, present and into the future. \textit{Medical Mycology} 44 Suppl 1: S17–21.

Brumbauer A, Johansson G, Reczcy K (2000). Study on heterogeneity of beta-galactosidase from \textit{Aspergillus} species by using counter-current distribution. \textit{Journal of Chromatography B Biomedical and Scientific Applications} 743: 247–254.

Casteren WHM van, Eimermann M, Broek LAM van den, Vincken J-P, Schols HA, Voragen AGJ (2000). Purification and characterisation of a beta-galactosidase from \textit{Aspergillus aculeatus} with activity towards (modified) exopolysaccharides from \textit{Lactococcus lactis} subsp. cremoris B39 and B891. \textit{Carbohydrate Research} 329: 75–85.

Decker CH, Visser J, Schreier P (2000). beta-galactosidases from five black \textit{Aspergillus} species: Study of their physico-chemical and biocatalytic properties. \textit{Journal of Agricultural and Food Chemistry} 48: 4929–4936.

Diepningen AD van, Debets AJ, Hoekstra RF (2006). Dynamics of dsRNA mycoviruses in black \textit{Aspergillus} populations. \textit{Fungal Genetics & Biology} 43: 446–452.

Diepningen AD van, Debets AJ, Varga J, Gaag M van der, Swart K, Hoekstra RF (2004). Efficient degradation of tannic acid by black \textit{Aspergillus} species. \textit{Mycology Research} 108: 919–925.

el-Gindy A (2003). Production, partial purification and some properties of beta-galactosidase from \textit{Aspergillus carbonarius}. \textit{Folia Microbiologica (Praha)} 48: 581–584.

Gentts van den Erde AHG, Hoog GS de (1999). Variability and molecular diagnostics of the neurotropic species \textit{Cladosporium herbarum} bantiana. \textit{Studies in Mycology} 43: 151–162.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. \textit{Applied and Environmental Microbiology} 61: 1233–1330.

Kang SW, Park YS, Lee JS, Hong SI, Kim SW (2004). Production of cellulases and hemicellulases by \textit{Aspergillus niger} KK2 from lignocellulosic biomass. \textit{Bioresource Technology} 91: 153–156.

Kiss T, Erdei A, Kiss L (2002). Investigation of the active site of the extracellular [beta]-xylanase from \textit{Aspergillus carbonarius}. \textit{Archives of Biochemistry and Biophysics} 399: 188–194.

Liu CQ, Chen QH, Cheng QJ, Wang JL, He GQ (2007). Effect of culturing conditions on alpha-galactosidase production by a novel \textit{Aspergillus foetidus} Zu-G1 strain in solid-state fermentation. \textit{Journal of Zhejiang University. Science. B} 8: 371–378.

Pal K, Diepningen AD van, Varga J, Hoekstra RF, Dyer PS, Debets AJ (2007). Sexual and vegetative compatibility genes in the aspergilli. \textit{Studies in Mycology} 59: 19–30.

Pedersen M, Lauritzen H, Frisvad J, Hofmann G, et al. (2007). Genome sequence of \textit{Aspergillus niger} strain CBS 513.88: a versatile cell factory. \textit{Nature Biotechnology} 25: 221–231.

Rath PM (2001). Phenotypic and genotypic characterization of reference strains of the genus \textit{Aspergillus}. \textit{Myccoses} 44: 65–72.
Rippel A (1939). Ueber die Verbreitung von Aspergillus niger ins besondere in Deutschland. Archiv für Mikrobiologie 11: 1–32.

Rokas A, Payne G, Fedorova ND, Baker SE, Machida M, et al. (2007). What can comparative genomics tell us about species concepts in the genus Aspergillus? Studies in Mycology 59: 11–17.

Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J (2007). Diagnostic tools to identify black aspergilli. Studies in Mycology 59: 129–145.

Takada G, Kawaguchi T, Kaga T, Sumitani J-I, Arai M (1999). Cloning and sequencing of b-mannosidase gene from Aspergillus aculeatus No.F-50. Bioscience Biotechnology and Biochemistry 63: 206–209.

van Kuyk PA, Diderich JA, MacCabe AP, Hererro O, Ruijter GJG, Visser J (2004). Aspergillus niger mstA encodes a high-affinity sugar/H+ symporter which is regulated in response to extracellular pH. Biochemical Journal 379: 375–383.

Varga J, Frisvad JC, Kocsubé S, Brankovics B, Tóth B, et al. (2011). New and revisited species in Aspergillus section Nigri. Studies in Mycology 69: 1–17.

Vries RP de (2003). Regulation of Aspergillus genes encoding plant cell wall polysaccharide degrading enzymes; relevance for industrial production. Applied Microbiology and Biotechnology 61: 10–20.

Vries RP de, Burgers K, Vandervoort PJ, van de, Frisvad JC, Samson RA, Visser J (2004). A new black Aspergillus species, A. vadensis, is a promising host for homologous and heterologous protein production. Applied and Environmental Microbiology 70: 3954–3959.

Vries RP de, Burgers K, Vandervoort PJ, van de, Frisvad JC, Samson RA, Visser J (2005). Aspergillus vadensis, a new species of the group of black aspergilli. Antonie Van Leeuwenhoek 87: 195–203.

Vries RP de, Visser J (2001). Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. Microbiology and Molecular Biology Reviews 65: 497–522.

Yamane Y-I, Fujita J, Shimizu R-I, Hiyoshi A, Fukuda H, Kizaki Y, Wakabayashi S (2002). Production of cellulose- and xylan-degrading enzymes by a koji mold, aspergillus oryzae, and their contribution to the maceration of rice endosperm cell wall. Journal of Bioscience and Bioengineering 93: 9–14.