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

M. Meijer[1,2], J.A.M.P. Houbraken[2], S. Dalhuijsen[1], R.A. Samson[2] and R.P. de Vries[1,2*]

[1]*Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands;* [2]*CBS-KNAW, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.*

**Correspondence*: Ronald P. de Vries, r.devries@cbs.knaw.nl

Abstract: Wild type *Aspergillus niger* isolates from different biotopes from all over the world were compared to each other and to the type strains of other black *Aspergillus* species with respect to growth and extracellular enzyme profiles. The origin of the *A. niger* isolate did not result in differences in growth profile with respect to monomeric or polymeric carbon sources. Differences were observed in the growth rate of the *A. niger* isolates, but these were observed on all carbon sources and not specific for a particular carbon source. In contrast, carbon source specific differences were observed between the different species. *Aspergillus brasiliensis* is the only species able to grow on D-galactose, and *A. aculeatus* had significantly better growth on Locus Bean gum than the other species. Only small differences were found in the extracellular enzyme profile of the *A. niger* isolates during growth on wheat bran, while large differences were observed in the profiles of the different black aspergilli. In addition, differences were observed in temperature profiles between the black *Aspergillus* species, but not between the *A. niger* isolates, demonstrating no isolate-specific adaptations to the environment.

These data indicate that the local environment does not result in stable adaptations of *A. niger* with respect to growth profile or enzyme production, but that the potential is maintained irrespective of the environmental parameters. It also demonstrates that growth, extracellular protein and temperature profiles can be used for species identification within the group of black aspergilli.

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 biotopes contain different plants (*e.g.* grasses *vs.* woods) and therefore different polysaccharides, different enzyme mixtures will be required for each biotope.

In light of this, one might expect that *Aspergillus* isolates from different biotopes 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 biotopes. 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 identidy 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.

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Table 1. Strains used in this study; T indicates type strain for that species.

Strain	Correct identification	Original identification	Origin (code)	β-tubulin Acc. No.	ITS Acc. No.
CBS 564.65 T	*A. acidus*		Japan (JAP)	FJ639280	FJ639329
CBS 106.47	*A. acidus*	*A. niger*	Switzerland (SWI)	FJ639281	FJ639330
CBS 124.49	*A. acidus*	*A. niger*	Central America (CA)	FJ639282	FJ639331
CBS 139.48	*A. acidus*	*A. niger*	Ukraine (UKR)	FJ639283	FJ639332
CBS 172.66 T	*A. aculeaus*		Tropics (TR)	FJ639271	FJ639320
CBS 101740 T	*A. brasiliensis*		Brasil (BRA)	FJ639272	FJ639321
CBS 246.65	*A. brasiliensis*	*A. niger*	Australia (AUS)	FJ639273	FJ639322
CBS 733.88	*A. brasiliensis*	*A. niger*	USA (USA)	FJ639274	FJ639323
CBS 116970	*A. brasiliensis*	*A. niger*	The Netherlands (NLD)	FJ639275	FJ639324
CBS 111.26 T	*A. carbonarius*		Unknown	FJ639276	FJ639325
CBS 115574 T	*A. costaricaensis*		Costa Rica (COS)	FJ639277	FJ639326
CBS 553.65	*A. costaricaensis*	*A. niger*	Costa Rica (COS)	FJ639278	FJ639327
CBS 707.79 T	*A. ellipicus*		Costa Rica (COS)	FJ639279	FJ639328
CBS 117.55 T	*A. heteromorphus*		Brasil (BRA)	FJ639284	FJ639333
CBS 101889 T	*A. homomorphus*		Israel (ISR)	FJ639285	FJ639334
CBS 114.51 T	*A. japonicus*		Unknown	FJ639286	FJ639335
CBS 101883 T	*A. niger*	*A. lacticoffeatus*	Indonesia (INA)	FJ639287	FJ639336
CBS 554.65 T	*A. niger*		USA (USA)	FJ639288	FJ639337
CBS 120.49	*A. niger*		USA (USA)	FJ639289	FJ639338
CBS 113.50	*A. niger*		Germany (GER)	FJ639290	FJ639339
CBS 139.54	*A. niger*		Namibia (NAM)	FJ639291	FJ639340
CBS 242.93	*A. niger*		The Netherlands (NLD)	FJ639292	FJ639341
CBS 101698	*A. niger*		Kenya (KEN)	FJ639293	FJ639342
CBS 101705	*A. niger*		Canada (CAN)	FJ639294	FJ639343
CBS 117785	*A. niger*		Morocco (MOR)	FJ639295	FJ639344
CBS 118725	*A. niger*		The Netherlands (NLD)	FJ639296	FJ639345
CBS 112.32	*A. niger*		Japan (JAP)	FJ639297	FJ639346
CBS 139.52	*A. niger*		Japan (JAP)	FJ639298	FJ639347
CBS 118.36	*A. niger*		USA (USA)	FJ639299	FJ639348
CBS 630.78	*A. niger*		South-Pacific Islands (SPI)	FJ639300	FJ639349
CBS 115989	*A. niger*		Nigeria (NIG) (DSM genome)	FJ639301	FJ639350
CBS 113.46	*A. niger*		USA (USA) (JGI genome)	FJ639302	FJ639351
CBS 112811 T	*A. piperis*		Denmark (DEN)	FJ639303	FJ639352
CBS 134.48 T	*A. tubingensis*		Unknown	FJ639305	FJ639354
CBS 126.52	*A. tubingensis*		Unknown	FJ639306	FJ639355
CBS 103.12	*A. tubingensis*	*A. niger*	Germany (GER)	FJ639307	FJ639356
CBS 116.36	*A. tubingensis*	*A. niger*	Russia (RUS)	FJ639308	FJ639357
CBS 122.49	*A. tubingensis*	*A. niger*	Japan (JAP)	FJ639309	FJ639358
CBS 130.52	*A. tubingensis*	*A. niger*	USA (USA)	FJ639310	FJ639359
CBS 121600	*A. tubingensis*	*A. niger*	Egypt (EGY)	FJ639311	FJ639360
CBS 626.66	*A. tubingensis*	*A. niger*	France (FRA)	FJ639312	FJ639361
CBS 522.85	*A. tubingensis*	*A. niger*	India (IND)	FJ639313	FJ639362
CBS 116417	*A. tubingensis*	*A. niger*	Iran (IRA)	FJ639314	FJ639363
CBS 425.65	*A. tubingensis*	*A. niger*	Japan (JAP)	FJ639315	FJ639364
CBS 161.79	*A. tubingensis*	*A. niger*	India (IND)	FJ639316	FJ639365
CBS 306.80	*A. tubingensis*	*A. niger*	Spain (SPA)	FJ639317	FJ639366
CBS 107.55	*A. tubingensis*	*A. niger*	Brasil (BRA)	FJ639318	FJ639367
CBS 113365 T	*A. vadensis*		Unknown	FJ639319	FJ639368

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 5×10^4 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 5×10^5 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 LS266 (GCATTCCCAAACAACTCGACTC) and V9G [TTACGTCCCTGCCCTTTGTA, (Gerrits van den Ende & de Hoog 1999)]. Amplification of part of the β-tubulin gene was performed using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b [ACCCTCAGTGTAGTGACCCTTGGC, (Glass & Donaldson 1995)]. 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 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 Na_2CO_3. 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-galactopyranoside, *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, xyloglucan 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 T	+	-	-	+	+	±
A. aculeatus	CBS 172.66 T	+++	-	-	+++	+++	++
A. brasiliensis	CBS 101740 T	+++	+	+	+++	+++	+
A. carbonarius	CBS 111.26 T	+++	-	-	+++	+++	++
A. costaricaensis	CBS 115574 T	+++	-	+	+++	+++	++
A. ellipicus	CBS 707.79 T	-	-	-	-	-	-
A. heteromorphus	CBS 117.55 T	-	-	-	-	-	-
A. homomorphus	CBS 101889 T	++	-	+	++	++	+
A. japonicus	CBS 114.51 T	+++	-	+	+++	+++	++
A. lacicoffeaus	CBS 101883 T	+++	-	+	+++	+++	++
A. piperis	CBS 112811 T	-	-	-	-	-	-
A. tubingensis	CBS 134.48 T	+++	-	+	+++	+++	++
A. vadensis	CBS 113365 T	++	-	-	++	++	±
A. niger	CBS 554.65 T	++++	-	±	++++	++++	+
	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 T	+	-	+	+	-	+
A. aculeatus	CBS 172.66 T	+++	+	+	++	+	++
A. brasiliensis	CBS 101740 T	+++	-	+	+++	±	++
A. carbonarius	CBS 111.26 T	+++	-	+	+	+	++
A. costaricaensis	CBS 115574 T	+++	-	+	++	+	++
A. ellipicus	CBS 707.79 T	-	-	-	-	-	-
A. heteromorphus	CBS 117.55 T	-	-	±	±	-	±
A. homomorphus	CBS 101889 T	++	+	+	+	++	++
A. japonicus	CBS 114.51 T	+++	+	+	+	+	++
A. lacicoffeaus	CBS 101883 T	+++	-	+	+++	+	++
A. piperis	CBS 112811 T	-	-	-	-	-	-
A. tubingensis	CBS 134.48 T	+++	-	+	+	+	++
A. vadensis	CBS 113365 T	++	-	+	+	-	+
A. niger	CBS 554.65 T	++++	-	+	++	+	+++
	CBS 120.49	+++	-	++	++	++	+++
	CBS 113.50	+++	-	++	++	++	+++

Table 3. (Continued).

Species	Strain	Starch	CEL	ABG	LBG	BWX	CP
A. niger	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	++	-	++	++	+	+++

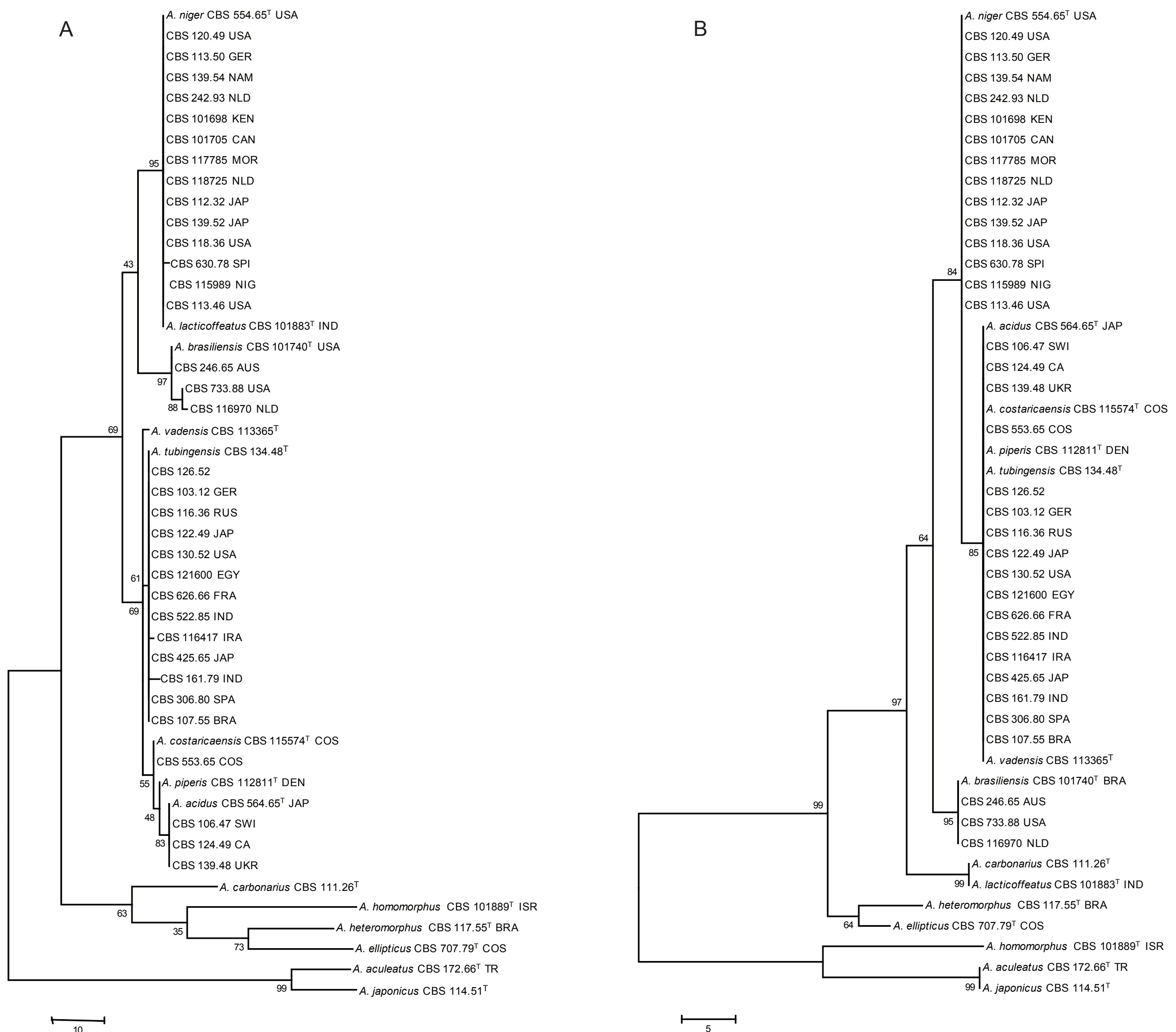

Fig. 1. Phylogeny of the strains used in this study. A. Maximum Parsimony tree based on the β-tubulin sequence. B. Maximum Parsimony tree based on the ITS sequence. The origin abbreviation refers to Table 1.

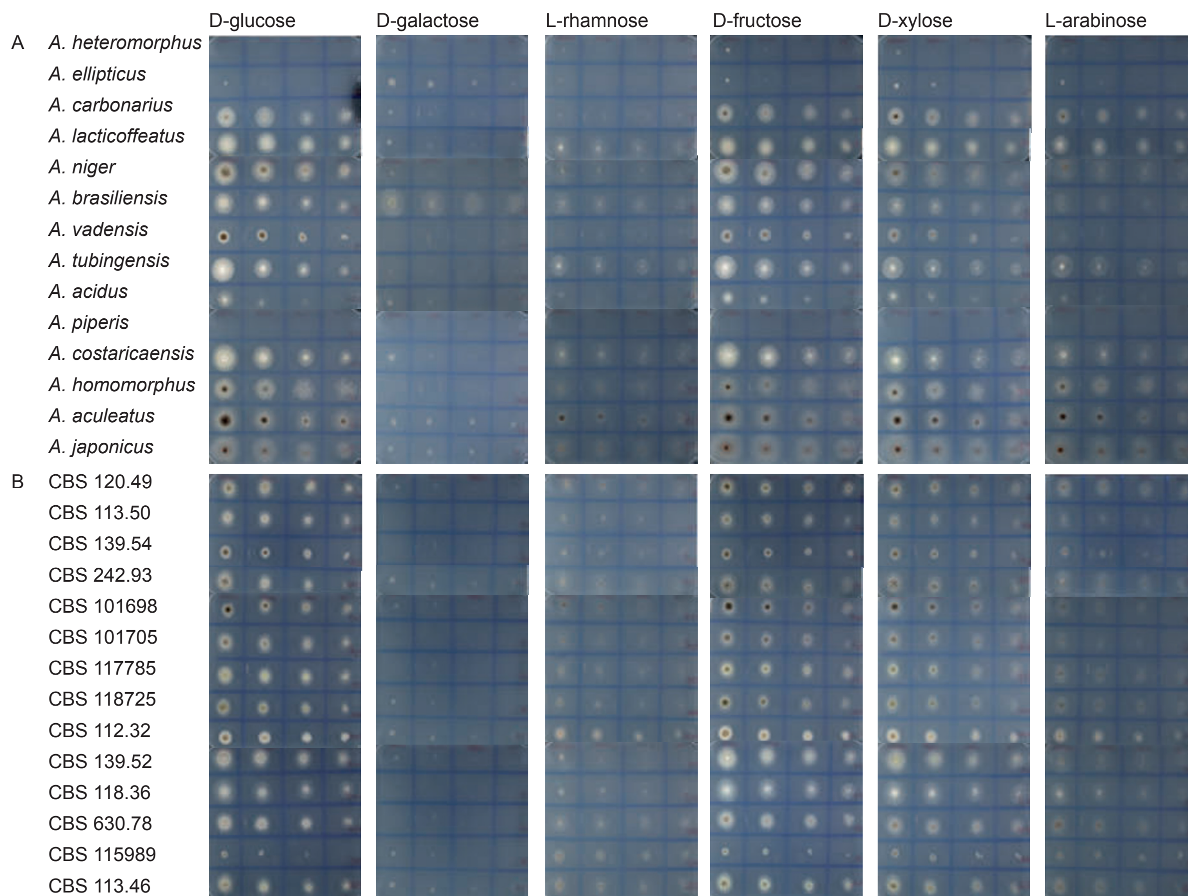

Fig. 2. Growth of ex-type strains of *Aspergillus* section *Nigri* (A) and *A. niger* isolates (B) on monomeric carbon sources. Strains were inoculated as serial dilutions (left to right) of 10000, 1000, 100 and 10 spores.

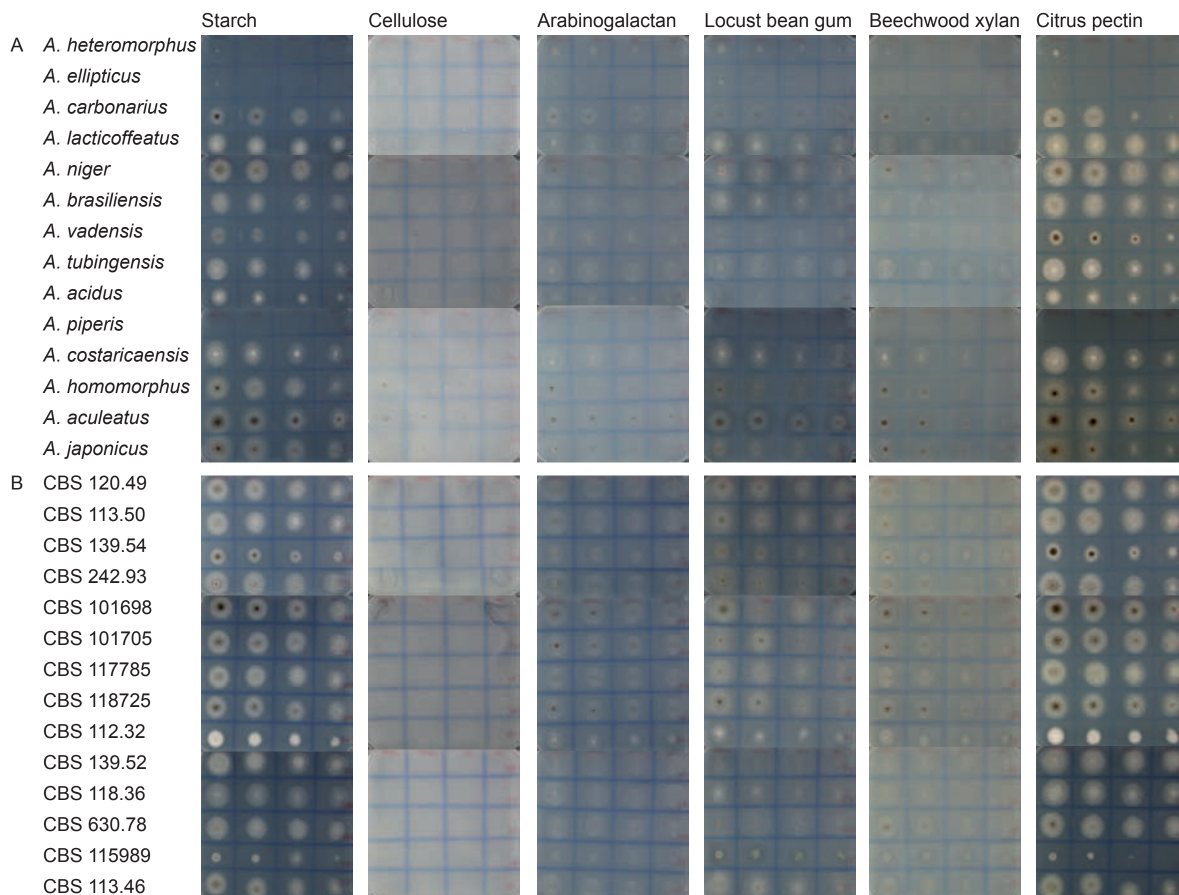

Fig. 3. Growth of ex-type strains of *Aspergillus* section *Nigri* (A) and *A. niger* isolates (B) on polymeric carbon sources. Strains were inoculated as serial dilutions (left to right) of 10000, 1000, 100 and 10 spores.

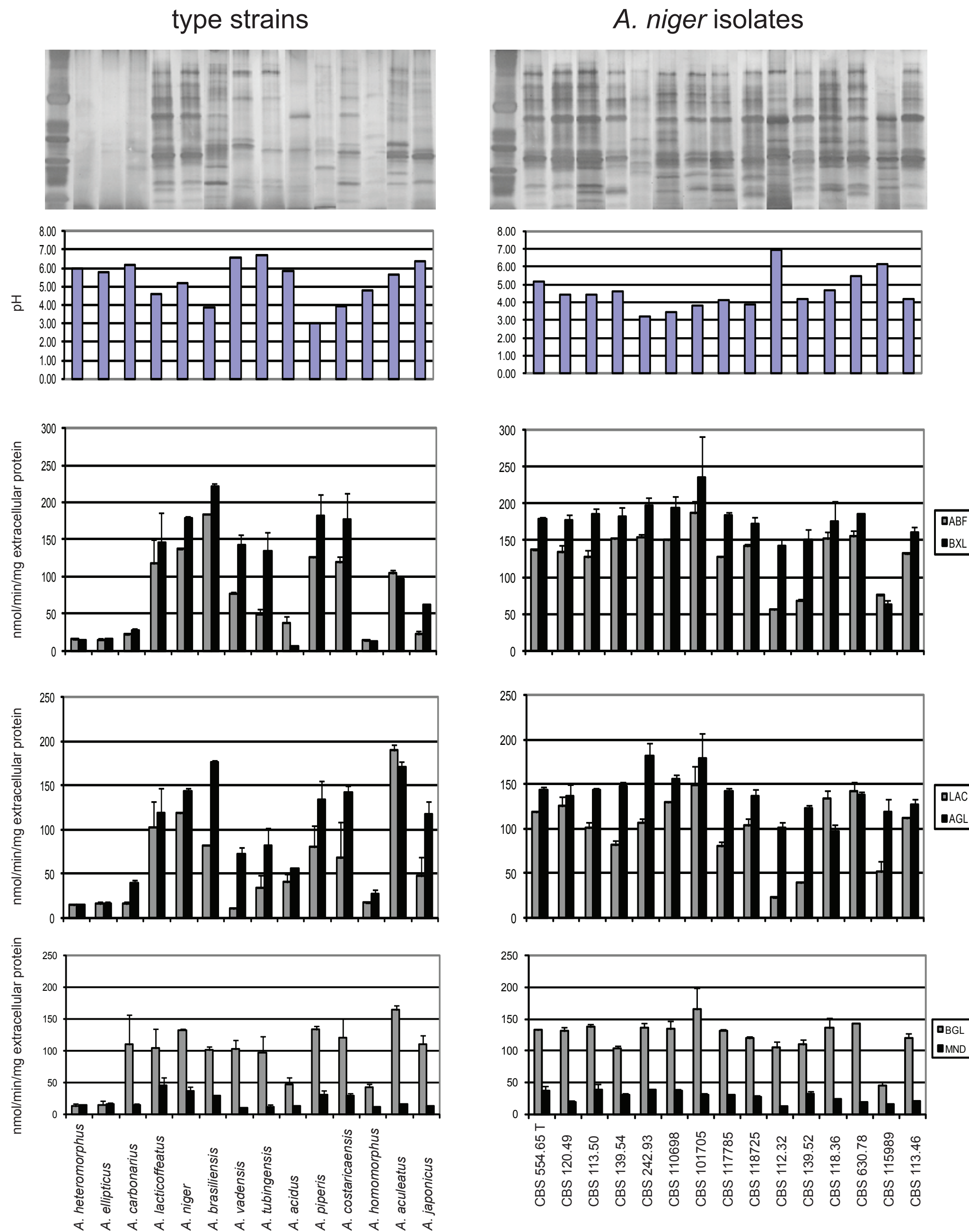

Fig. 4. SDS-PAGE profiles, pH, total secreted protein and hydrolytic activities of *A. niger* isolates and ex-type strains of *Aspergillus* section *Nigri* after growth on wheat bran. ABF = α-L-arabinofuranosidase, BXL = β-xylosidase, LAC = β-galactosidase, AGL = α-galactosidase, BGL = β-glucosidase, MND = β-mannosidase.

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

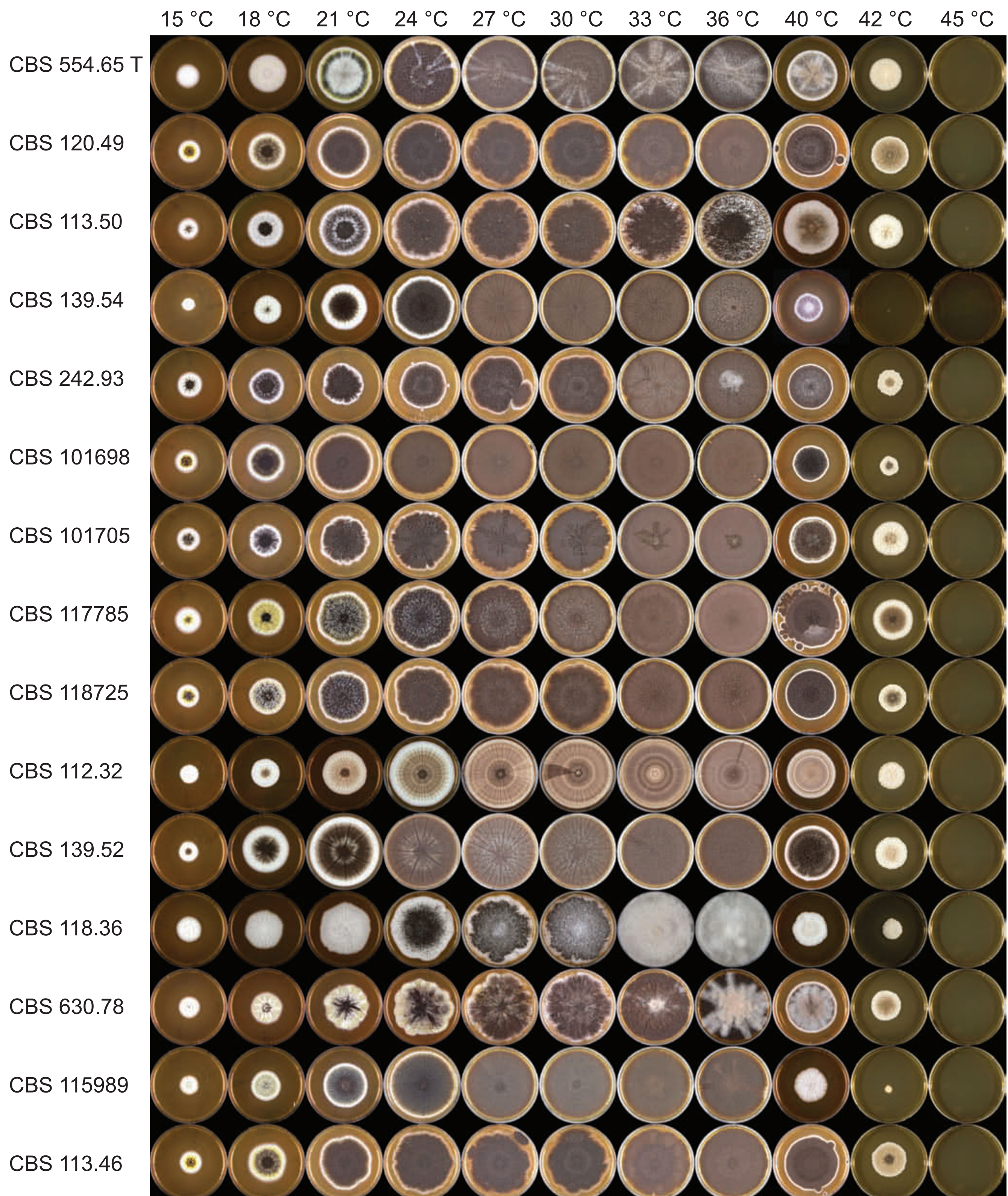

Fig. 5. Growth of the *A. niger* isolates at different temperatures. Pictures were taken after 10 d.

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

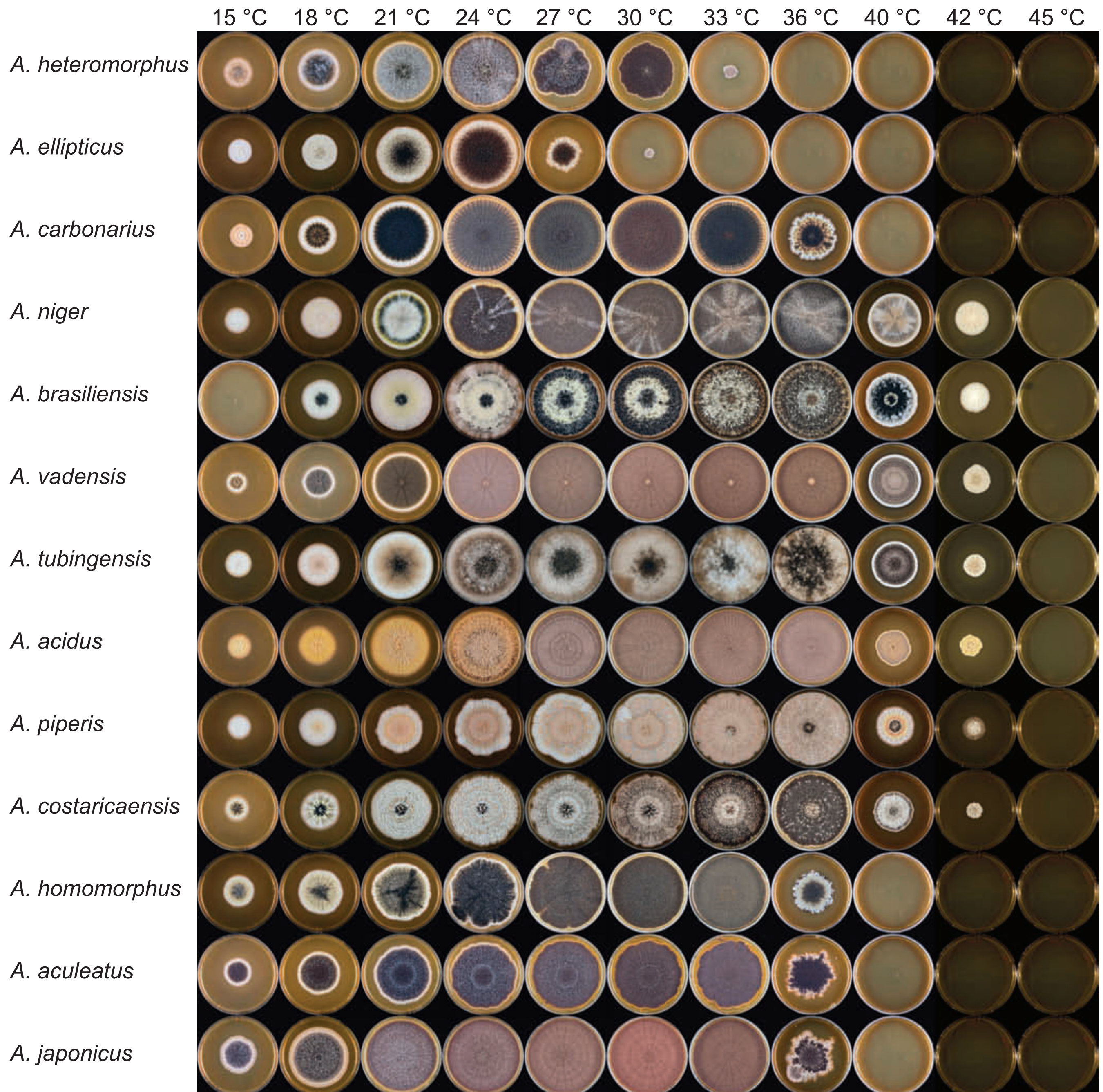

Fig. 6. Growth of ex-type strains of *Aspergillus* section *Nigri* at different temperatures. Pictures were taken after 10 d.

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

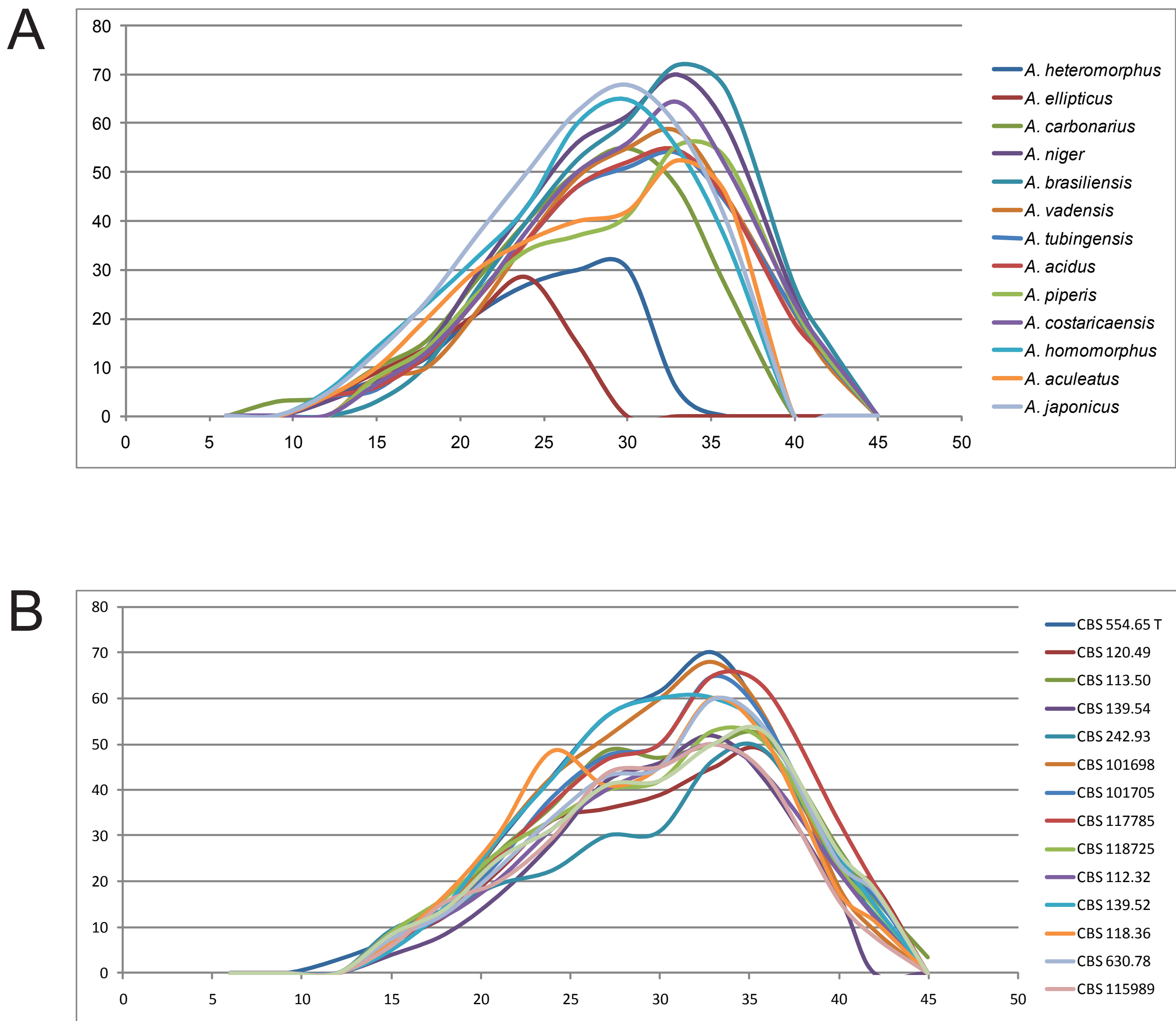

Fig. 7. Growth curves of the type strains (A) and the *A. niger* isolates (B). Growth curves were determined by the colony diameter (mm) after 4 d incubation.

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 *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 *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 *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 *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 *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 *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 *A. ellipticus* strain that was tested showed the same temperature profile. *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 *A. piperus*, especially since

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 *A. piperus* 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 *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 *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 *Aspergillus* (Yamane *et al.* 2002, Kang *et al.* 2004). Strain CBS 115989 overall had lower levels of activity than the other *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 BGL, 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 production strains or for understanding the factors (*e.g.* regulators, metabolic differences) that affect production of specific enzymes.

Similar to the growth experiments, much larger differences in hydrolase production were observed between the *Aspergillus* species than between the *A. niger* isolates. Production of all hydrolases was particularly low in *A. ellipticus*, *A. acidus*, *A. heteromorphus*, *A. homomorphus* and *A. carbonarius* (except for BGL). For *A. ellipticus* this can be explained by poor growth at this temperature, while in the case of *A. acidus* this is partly caused by a high extracellular protein production, but a low absolute enzyme activity. Except for *A. acidus*, all species with low activity cluster together in the phylogeny of the black aspergilli (Samson *et al.* 2007), suggesting that this phenomenon can be traced back to the combined origin of these species. The strong similarity between *A. lacticoffeatus* and the *A. niger* isolates is easily explained as recent studies showed that *A. lacticoffeatus* is in fact the same as *A. niger* (Varga *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 *A. niger*, *A. nidulans*, *A. flavus* and *A. fumigatus* (Rath 2001). However, these profiles are more complex and more sensitive to variation (de Vries *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 *A. niger* and *A. aculeatus*, while some have also been reported from *A. acidus*, *A. japonicus*, *A. tubingensis*, *A. carbonarius* and *A. brasiliensis* (Takada *et al.* 1999, Brumbauer *et al.* 2000, van Casteren *et al.* 2000, Decker *et al.* 2000, Ademark *et al.* 2001a, 2001b, de Vries & Visser 2001, Kiss *et al.* 2002, el-Gindy 2003, Liu *et al.* 2007, Pedersen *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. A. piperis*) could be interesting sources of hydrolytic enzymes, which may have different properties from those described previously.

In summary, this study demonstrates that *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 *Aspergillus* species, indicating that these parameters may be considered species characteristics.

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