Efficient plant biomass degradation by the thermophilic fungus *Myceliophthora heterothallica*

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Rapid and efficient enzymatic degradation of plant biomass into fermentable sugars is a major challenge for the sustainable production of biochemicals and biofuels. More thermostable enzymes (up to 70°C) will use shorter reaction times for complete saccharification of plant polysaccharides compared to hydrolytic enzymes of mesophilic fungi such as *Trichoderma* and *Aspergillus* species. The genus *Myceliophthora* contains four thermophilic fungi producing industrially-relevant thermostable enzymes. Within this genus, isolates belonging to *M. heterothallica* were recently separated from the well-described species *M. thermophila*. This paper evaluates the potential of *M. heterothallica* isolates to produce efficient enzymes mixtures for biomass degradation. Compared to the other thermophilic *Myceliophthora* species, isolates belonging to *M. heterothallica* and *M. thermophila* grew faster on pretreated spruce, wheat straw, and giant reed. According to their protein profiles and *in vitro* assays after growth on wheat straw, (hemi-)cellulolytic activities differed strongly between *M. thermophila* and *M. heterothallica* isolates. Compared to *M. thermophila*, *M. heterothallica* isolates were better in releasing sugars from mildly pretreated wheat straw (with 5% HCl) with a high content of xylan. The high levels of residual xylobiose revealed that enzyme mixtures of *Myceliophthora* species lack sufficient β-xylosidase activity. Sexual crossing of two *M. heterothallica* showed that progenies had a large genetic and physiological diversity. In the future, this will allow further improvement of the plant biomass-degrading enzyme mixtures of *M. heterothallica*. 
Introduction

Replacing petrochemical-based fuels and chemicals with truly sustainable alternatives requires biological conversion of agricultural waste plant material to fermentable sugars. The enzyme mixtures for the degradation of biomass-derived polysaccharides (e.g. cellulose, hemicellulose and pectin) are most commonly produced by fungal strains belonging to the genera Trichoderma and Aspergillus (1). However these mixtures are not sufficient for economically viable production of low-value products such as biofuels. A major hurdle of the enzyme mixtures from these mesophilic ascomycetes is that they are most effective at temperatures around 50°C (2-4).

Higher thermostability of enzymes allows saccharification of biomass polysaccharides at elevated temperatures. Consequently, reaction times will shorten drastically, mass transfer will increase, and substrate viscosity will reduce (5, 6). Another issue is the initial treatment by physical and/or chemical means (e.g. high temperature, acid or base treatment). This pretreatment should preferably be as mild as possible, since it involves undesirable chemicals and/or a high energy input in the process (7). Unfortunately current enzyme mixtures are incapable of releasing efficiently all available monomeric sugars from mildly pretreated biomass.

These issues can be solved by searching for other plant-biomass degrading fungi that produce enzymes with a higher efficiency and thermostability. The division of Ascomycetes contains several thermophilic fungi that produce hydrolytic enzymes with stability up to 70 °C (2, 4, 8, 9).

Several of these thermostable enzymes have been characterized (e.g. lipases, amylases, laccases, and phytases (10)). The value of thermophilic fungal enzymes has also been studied for plant-biomass degradation, in particular cellulases and xylanases (11-15). Myceliophthora is a genus consisting of mesophilic and thermophilic fungi. Their taxonomy has only recently been elucidated by phylogenetic analysis (Fig. 1) (16). The genus now includes ten species, of which three originally belonged to the genus Corynascus (e.g. C. thermophilus was renamed to M. fergusii). Furthermore, based on phylogeny and ability to cross sexually, the isolates originally belonging to M. thermophila have been divided in two species, M. thermophila and M. heterothallica. Four species, M. thermophila, M. heterothallica, M. hinnulea, and M. fergusii, have been described as thermophilic based on their optimal growth at 45°C and were suggested as producers of industrially interesting enzymes with a stability up to 70°C (2, 14). M. thermophila has been extensively described as a producer of thermostable enzymes such as...
amylase (17), keratinase (18), laccase (19-21), cellulase (22-24), and aldonolactonase (25), and its genome has been completely sequenced (6). *Myceliophthora* species can also hydrolyse and grow efficiently on plant substrates (14, 26).

In this study, isolates of *M. heterothallica* were analyzed for their ability to grow on, and degrade, mildly pretreated plant biomass. The physiology of this species is largely unknown even though its thermophilic character is very interesting for applications involving thermostable enzymes. In contrast to the better studied and closely related species *M. thermophila*, *M. heterothallica* has the ability to cross sexually, which is particularly useful for strain improvement. *M. heterothallica* isolates were compared to other thermophilic *Myceliophthora* isolates for their growth on five industrial biomasses with different contents of xylan. The enzyme mixtures of *M. heterothallica* isolates with the most promising growth profiles were compared to the ones of *M. thermophila* and mesophilic enzyme producers *Aspergillus niger* and *Trichoderma reesei*. Furthermore, sexual crossing of *M. heterothallica* isolates was assessed as a strategy to improve enzyme mixtures for plant biomass degradation.
Materials and Methods

Strains and growth conditions
All *Myceliophthora* isolates examined in this study are listed in Table 1. *Aspergillus niger* NW249 and *Trichoderma reesei* QM9414 were used for the comparison between *M. heterothallica* and mesophilic fungi. Growth profiling on solid media was performed on Minimal Media (27) containing 1.5% agar and 3% pretreated plant biomass. All strains were initially grown on Malt Extract Agar (MEA) (31). A small agar plug containing mycelium (1 mm diameter) was transferred from the edge of a vigorously growing 1-day-old colony to the center of the Petri dishes with the different media. The cultures were incubated in the dark at 40°C. The growth test was conducted twice for each strain. Growth profiling on 32 different carbon sources is explained in Supplemental material 1.

Growth experiments in liquid media were performed in 250 mL shakeflasks containing 50 mL Minimal Media and 1.5 g pretreated plant biomass. All strains were initially grown on MEA. Three agar plugs containing mycelium (5 mm diameter) were transferred from the edge of a vigorously growing 1-day-old colony to the shakeflasks with the different media. The cultures were incubated at 200 rpm and 45°C.

Materials
Three sources of plant biomass were used for growth and saccharification experiments: spruce (provided by Sekab E-Technology, Sweden), giant reed (*Arundo donax*; provided by Chemtex, Italy) and wheat straw (WS; provided by GreenSugar, Germany). Batches of spruce, giant reed and wheat straw were exposed to 20% hydrochloric acid. Another batch of spruce was pretreated with sulfur dioxide and 6-8 minutes steam at 195°C, and giant reed was pretreated by exposure to steam for 3 minutes at 200°C. Their different pretreatments, and their glucan and xylan content of the dry matter are given in Table 2. To remove the easily accessible sugars, the pretreated plant biomasses were dissolved in demineralized water, autoclaved and filtered (using filter papers, quality 4, Whatman) before using them.
Construction of phylogenetic tree

The construction of parsimonious consensus tree was performed according to van den Brink et al. (16).

Protein analysis

Protein concentrations were determined using Modified Lowry Protein Assay Reagent Kit adapted for 96-wells microtiter plates (no. 23240, Pierce). The extracellular proteins within the culture filtrates were separated on SDS-PAGE and stained with a coomassie stain using GelCode Blue Stain Reagent (no. 24590, Pierce).

Enzyme assays

Specific enzyme activities in the culture filtrates were measured using p-nitrophenol linked substrates (4-nitrophenyl α-D-galactopyranoside, 4-nitrophenyl β-D-galactopyranoside, 4-nitrophenyl β-D-xylopyranoside, 4-nitrophenyl α-L-arabinofuranoside, 4-nitrophenyl β-D-cellobioside, 4-nitrophenyl β-D-glucopyranoside of Sigma-Aldrich, Germany). The assays contained a total volume of 100 μL using 10-40 μL of the culture filtrates, 10 μL of 0.01 % p-nitrophenol linked substrates, and 25 mM sodium acetate pH 5.0. Samples were incubated in microtiter plates for 60 min at 45°C. Reactions were stopped by addition of 100 μL 0.25 M Na₂CO₃. Absorbance was measured at 405 nm in a microtiter platereader (FLUOstar OPTIMA, BMG LabTech). The activities were calculated using a standard curve ranging from 0 to 80 nmol p-nitrophenol per assay volume.

Cellulase, xyloluglucanase and xylanase activities were determined against carboxymethyl cellulose (Sigma-Aldrich, Germany), tamarind xyloglucan (Megazyme, Ireland), and beechwood xylan (Sigma-Aldrich, Germany). The assays contained a total volume of 200 μL using 10-50 μL of culture filtrates and 150 μL of 1% substrate in 50 mM sodium acetate pH 5.0. The samples were incubated in microtiter plates for 30-120 min at 50 and 70°C. Subsequently, 100 μL of supernatant was mixed with 150 μL 3,5-dinitrosalicylic acid (DNS) solution (28). After an incubation of 25 min at 95°C, absorbance was measured at 540 nm in a microtiter platereader (FLUOstar OPTIMA, BMG LabTech). The activities were calculated using a standard curve ranging from 0 to 2 g·L⁻¹ glucose.
Saccharification of pretreated plant biomasses

Three large agar plugs of mycelium (5 mm diameter) from the edge of a vigorously growing 1-day-old colony were used to inoculate a 250 mL shakeflask with 50 mL minimal medium (27) and 1.5 g pretreated plant biomass. Before harvesting the culture filtrate, culture flasks were incubated for 3 days at 200 rpm and 45°C. 4 mL of culture filtrate were mixed with 16 mL medium consisting of 50 mM NaAc buffer (pH 5.0) and 0.6 g pretreated plant biomass. The hydrolysates were sampled each day for four days, boiled for 10 min, and filtered for saccharide analysis.

The analysis of saccharides was performed using a Dionex ICS-3000 HPLC system equipped with a Dionex CarboPac PA-10 (2 mm ID x 250 mm) column in combination with a CarboPac PA guard column (1 mm ID x 25 mm) and a Dionex ED1 PAD-detector (Dionex Co., Sunnyvale). An isocratic step was performed at a flow rate of 0.25 mL·min⁻¹ with 16 mM NaOH for 15 min, followed by a gradient of 16-100 mM NaOH for 5 min. Then a gradient of sodium acetate was performed in 0.1 M NaOH: 0-200 mM in 20 min and 200-1000 mM in 5 min. Each elution was followed by a washing step of 5 min of 1000 mM sodium acetate in 0.1 M NaOH and a re-equilibration step of 15 min of 16 mM NaOH. Appropriate dilutions (25 µL) were injected on the column by means of an autosampler. Standards (arabinose, galactose, glucose, xylose, cellobiose and xylobiose) were included at concentrations of 0.5-4 µg·mL⁻¹ in order to quantify those saccharides.

Mating

A small agar plug containing mycelium (1 mm diameter) from the edge of a vigorously growing 1-day-old colony on MEA medium was transferred to the Petri dishes with MEA medium and were incubated in the dark at 37°C (16, 29, 30). After three weeks, ascomata were taken out of the agar and ascospores were separated and diluted in ACES buffer (31). The ascospores were cultured on Minimal Media containing 1.5% agar and 3% ‘5% HCl’ pretreated wheat straw.

The genotype of parents *M. heterothallica* CBS203.75 & CBS663.74 and their 14 progenies was determined using Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis, as described previously by Boekhout *et al.* (32).
Results

*M. heterothallica* and *M. thermophila* grow well on plant substrates with high xylan content

The potential of *M. heterothallica* to degrade plant biomass was evaluated by growth profiling on plant biomass. Five *M. heterothallica* isolates were compared to six *M. hinnulea*, two *M. fergussi*, and five *M. thermophila* isolates for their ability to grow on pretreated plant biomass (Fig. 2). The five tested substrates, all pretreated differently, contained a high amount of cellulose (>35% w/w), but varied strongly in their amount of xylan (Table 2). The pretreated spruce did not contain any xylan, while giant reed and wheat straw contain significant amounts of xylan (>8% w/w). In general *Myceliophthora* isolates grew better with an increasing content of xylan in the media. Compared to isolates of the other three species, *M. hinnulea* isolates grew slowly on the plant biomass (Fig. 2). Only *M. hinnulea* isolate CBS 542.82 showed densely grown colonies after an incubation of 11 days. The two isolates of *M. fergussii* showed dense colonies after 4 days on medium with giant reed and wheat straw. Still, the densest colonies after two days of incubation were observed for the ten isolates of the genetically-related species, *M. thermophila* and *M. heterothallica*. Using visual inspection, ATCC 42464 grew better amongst the *M. thermophila* isolates on xylan-rich substrates, while CBS 866.85 and CBS 669.85 grew better on cellulose-rich and xylan-poor spruce. *M. heterothallica* CBS 663.74, CBS 202.75, and CBS 375.69 grew better than the two other isolates of this species.

The isolates of *M. thermophila* and *M. heterothallica* were grown on media containing 35 different media with monomeric-, dimeric- and polymeric sugars, and crude substrates (Supplemental material 1A). Differences in growth of *M. thermophila* isolates were mostly visible on media with monomeric and dimeric sugars. Compared to the other isolates, *M. heterothallica* CBS 131.65, CBS 375.69, CBS 202.75 and CBS 663.74 grew better on most monomeric and dimeric sugars. However, *M. thermophila* ATCC 42464, CBS 866.85, CBS 173.70 and CBS 117.65 grew better on medium with lactose. The differences were less pronounced for growth on polymers and crude substrates. Except for medium with α-cellulose, all *M. thermophila* and *M. heterothallica* isolates grew well on polymeric and crude substrates.
M. thermophila and M. heterothallica produce different enzyme mixtures

Good growth of M. thermophila and M. heterothallica on plant biomass indicated that these fungi produce enzyme mixtures which efficiently degrade complex polysaccharides. To test the enzyme mixtures produced, the fastest growing M. heterothallica isolates on plant substrates, CBS 202.75 and CBS 663.74, were compared to M. thermophila ATCC 42464 and the mesophilic enzyme producers Aspergillus niger and Trichoderma reesei. The five strains were grown in liquid media containing 3% wheat straw (WS) as a substrate. The Myceliophthora strains produced most protein after 4 days growth at 45°C, while most protein of A. niger and T. reesei were produced after 5 days growth at 30°C. The enzyme mixtures were evaluated by measuring (hemi-)cellulolytic activities against cellulose, xyloglucan and xylan substrates at 50 and 70°C. Measuring at 50°C is most optimal for mesophilic enzymes, while 70°C is the optimum temperature for Myceliophthora strains (Supplemental material 1B shows activities at different temperatures). The activities between the five strains were all in the same range (Fig. 3). However, the activities at 70°C of A. niger and T. reesei were similar or lower compared to 50°C, while the activities of Myceliophthora strains were much higher at 70°C. The cellulase, xyloglucanase and xylanase activities did differ strongly between A. niger and T. reesei. T. reesei had higher cellulase and xyloglucanase activity, whereas A. niger had higher xylanase activity. The (hemi-)cellulolytic activities of M. heterothallica were between activities of A. niger and T. reesei. In general, the activities of M. heterothallica isolates were slightly lower compared to M. thermophila after growth on WS pretreated with 20% HCl.

To analyze the enzyme mixtures in more detail, M. heterothallica CBS 202.75 and CBS 663.74 were compared to M. thermophila by growing them on liquid media containing two different WS substrates. One substrate was pretreated with 20% HCl and contained 12% xylan, while the other was exposed to 5% HCl and contained 21% xylan. Between both WS substrates tested, no differences were found in the protein profiles of the isolates. However, between the three isolates there was a large difference in the protein patterns (Supplemental material 1C). M. thermophila ATCC 42464 grew the fastest and produced the broadest protein set. The two M. heterothallica isolates were very different in their protein profiles. M. heterothallica CBS 663.74 grew the slowest and produced its highest amount of proteins after three days growth (Supplemental material 1C). The enzyme activities of six main (hemi-)cellulolytic activities were also very
diverse during 4 days of culturing on both WS substrates. The two cellulolytic activities, cellobiohydrolase (CBH) and \( \beta \)-glucosidase (BGL), showed a similar pattern and activity levels for all three isolates on 20% HCl WS (Fig. 4A–B). On 5% HCl WS, CBH and BGL activities of CBS 202.75 were highest, while CBS 663.74 showed the lowest activities (Fig. 4G–H). The hemicellulolytic activities \( \beta \)-xylosidase (BXL), \( \alpha \)-arabinofuranosidase (ABF), \( \alpha \)-galactosidase (AGL), and \( \beta \)-galactosidase (LAC) showed diverse patterns for the isolates (Fig. 4C–F, I–L). All hemicellulolytic activities of *M. heterothallica* CBS 663.74 increased most after 3 days of culturing, which correlated with the extracellular protein profiles. BXL activities were highest for *M. heterothallica* CBS 202.75 on 20% HCl WS and low for all three isolates on 5% HCl WS. This does not correlate with the xylan content of WS. On both WS substrates, ABF and AGL activities were highest for *M. thermophila* ATCC 42464, while LAC activities were higher for the *M. heterothallica* isolates.

Based on the protein profiles and enzyme activities described above, saccharification of 3% WS solutions was performed using filtrates of the three-day old culture. The mono- and disaccharides in the solution were identified by HPLC during 4 days of incubation (Fig. 5). Most sugars were mainly released during the first day of incubation, although glucose, xylobiose, xylose and galactose were still increasing at the end of the incubation (Fig. 5B–E, H–J). In contrast, cellobiose increased strongly during the initial stages of saccharification followed by a gradual decrease. Especially the culture filtrate of CBS 663.74 released a high amount of cellobiose initially from the 5% HCl WS (Fig. 5G). This likely reflected the inability of the BGLs to cope with the high level of cellobiose release. Glucose levels of 20% HCl and 5% HCl WS were similar for the enzyme mixtures of CBS 202.75 and ATCC 42464. Glucose release by enzyme mixtures of CBS 663.74 was much better in the 5% HCl WS. The higher hemicellulose fraction in the 5% HCl WS resulted in a higher amount of released xylobiose, xylose, galactose, and arabinose. Xylobiose and xylose release was at least four times higher in the 5% HCl WS. Galactose and arabinose release was absent or negligible in the 20% HCl WS, while in the 5% HCl WS ATCC 42464 released 30 and 213 g·l\(^{-1}\), respectively. That the concentration of both sugars were the highest using ATCC 42464 enzymes, reflected the higher activities of ABF and AGL in the ATCC 42464 enzyme mixtures. Interestingly, this saccharification experiment
showed high concentrations of xylobiose in all *Myceliophthora* samples, which indicates a problem in xylobiose to xylose conversion.

**M. heterothallica** isolates produce genetically diverse progeny

Isolates with opposite mating type, CBS 663.74 and CBS 203.75, were used to evaluate the strategy of sexual crosses for improving enzyme mixtures produced by *M. heterothallica*. CBS 663.74, isolated from soil under a baobab tree in Senegal, was shown to be genetically different from CBS 203.75, isolated from soil in Indiana, USA (16). CBS 663.74 and CBS 203.75 produced ascomata in the agar media at their contact zone after three weeks. The dark brown ascospores were released from the ascomata and plated on media containing 5% HCl WS. Fourteen colonies with rapid growth were transferred to new plates with 5% HCl WS before further analysis.

The genetic diversity of the 14 selected progenies and their parents was investigated by Amplified Fragment Length Polymorphism (AFLP). The banding patterns of AFLP were clustered in 5 main groups (Fig. 6). The pattern of progeny FP 711.01 was very similar to CBS 663.74, while the patterns of FP 711.10 and FP 711.13 were very similar to CBS 203.75. Progenies FP 711.04, FP 711.05, FP 711.06, FP 711.07, FP 711.08 and FP 711.14 were similar to the pattern of CBS 663.74, but had also some bands fitting to the pattern of CBS 202.75. The two patterns of FP 711.09 and FP 711.11 showed a limited amount of bands, whereas the patterns of FP 711.02 and FP 711.03 showed a mix between bands of CBS 203.75 and CBS 663.74.

Compared to their parents, most progeny had a similar growth rate and enzyme activities after growth on wheat straw. However after 3 days growth on 5% HCl wheat straw, progenies FP 711.02 and FP 711.14 showed higher CBH activities than their parents (data not shown). FP 711.02 and FP 711.14 were therefore chosen for a saccharification experiment with their parents. Similar to the previous section, the filtrates of three-day cultures were used for the saccharification of a 3% wheat straw solution at 45°C. To have a more gradual increase of the sugar concentrations during the four days, less protein was loaded compared to the saccharification experiments of the previous section (Fig. 7). The glucose and galactose concentrations were lower after incubation with FP 711.02 and FP 711.14 filtrates than the parental strains. Xylobiose, xylose and arabinose concentrations of CBS 203.75 and FP 711.02
were similar to each other, and CBS 663.74 and FP 711.14 followed a comparable trend. This similarity in trend correlated with the clustering of the AFLP pattern of FP 711.14 and CBS 663.74. Nevertheless, CBS 663.74 released more mono- and disaccharides than CBS 203.75 and the two progenies.

**Discussion**

This study showed that *M. heterothallica* and *M. thermophila* rapidly grew on industrial media at temperatures above 40°C. Most of their strains formed dense colonies on media containing xylose, mannose, beechwood xylan, birchwood xylan and, in particular, industrial substrates with a high content of xylans (e.g. giant reed and wheat straw). Only *M. thermophila* isolates CBS 866.85 and CBS 669.85 grew faster on cellulose-rich and xylan-poor spruce, which could be expected as they were selected for their ability to produce cellulases (9). Fast growth of *M. thermophila* and *M. heterothallica* on plant biomass indicated that these fungi produce enzyme mixtures which efficiently degrade complex polysaccharides. *M. thermophila* has already been established as a producer of efficient plant-degrading enzyme mixtures (6, 26). However, the characteristics of enzyme mixtures produced by *M. heterothallica* isolates were unknown. The hydrolytic enzymes of *M. heterothallica* had a similar thermostability compared to *M. fergussi* and *M. thermophila* with optimal activities around 67-70°C (Supplemental material 1B) (14). The (hemi-)cellulolytic activities of *M. heterothallica* are comparable with *A. niger* and *T. reesei*, even though *T. reesei* is the most commonly used industrial fungus for cellulose production and *A. niger* is renowned for its xylanase production (33, 34). This study also showed that the enzymes of *M. heterothallica* were able to efficiently release fermentable sugars from plant biomass. Compared to *M. thermophila*, enzyme mixtures of *M. heterothallica* released more sugars from milder pretreated wheat straw with higher xylan content. The experiments also showed that *M. heterothallica* isolates were very different in their physiology, which supported their genetic diversity as previously shown using AFLP patterns (16).

A remarkable observation in the saccharification experiments was the high concentrations of xylobiose compared to the other released sugars. It seems that *Myceliophthora* isolates were limited in converting xylobiose to xylose; although the enzyme mixtures had activity against *p*-nitrophenyl-β-D-xylopyranoside and putative β-xylosidases have been identified (6, 35). The β-xylosidases of *Myceliophthora* species could be cell-bound, have high transglycolysation...
activity, or have strong product inhibition (36-38). Since activity has been measured in the culture filtrate and xylotriose was absent during the saccharification, the conversion of xylobiose to xylose was likely prevented by product inhibition of the β-xylosidases. This strong competitive type of inhibition by xylose has already been described for other fungal β-xylosidases (38-40). The introduction of a heterologous β-xylosidase with a lower inhibitor affinity can improve the release of fermentable xylose residues (41). This will be feasible as heterologous expression has already been achieved with the closely-related \textit{M. thermophila} (26).

The main advantage of \textit{M. heterothallica} isolates compared to \textit{M. thermophila} is their natural ability to cross sexually, which is rare amongst industrially-used fungi. Although a larger experiment is needed, the crossings in the pilot test showed the potential to create progeny with changed physiological characteristics. In conclusion, this study showed that \textit{M. heterothallica} has a high potential to become an enzyme producer for the efficient degradation of mildly pretreated plant biomass.
Acknowledgements

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References


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Table 1. Thermophilic *Myceliophthora* strains examined in this study.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Accession no.</th>
<th>Source</th>
<th>Mating type</th>
</tr>
</thead>
<tbody>
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<td>CBS 117.65</td>
<td>Dry pasture soil, UK</td>
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<td>CBS 137.70</td>
<td>Wheat straw compost, UK</td>
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<td>Unknown source; mutant of CBS 866.85</td>
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<td></td>
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<td></td>
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<td>MT–</td>
</tr>
<tr>
<td>CBS 202.75</td>
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<td>MT+</td>
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<tr>
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<td>Wood pulp, New Brunswick, Canada</td>
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<td>MT+</td>
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<td>Soil from cultivated garden, New Zealand</td>
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Table 2. Pretreated biomasses of spruce, giant cane and wheat straw and their glucan and xylan content.

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<th>Type plant biomass</th>
<th>Pretreatment</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Company</th>
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Downloaded from http://aem.asm.org/ on September 12, 2017 by guest
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<th>Material</th>
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<th>(% w/w)</th>
<th>(% w/w)</th>
<th>Company</th>
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<td>GreenSugar</td>
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<td>Giant reed</td>
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Figure 1. Parsimonious consensus tree of ITS1 region of *Myceliophthora* species (based on (16)). The numbers next to the branches indicate the percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates).

Figure 2. Growth of thermophilic isolates of *Myceliophthora* on five industrial substrates at 40°C. Spruce, wheat straw, and giant reed (*Arundo donax*) were pretreated with 20% HCl or with a steam treatment (indicated between brackets). The pictures of *M. thermophila* and *M. heterothallica* were taken after 2 days, *M. fergussi* after 4 days, and *M. hinnulea* after 11 days.

Figure 3. Cellulase, xyloglucanase and xylanase activities of *Aspergillus niger* (An), *Trichoderma reesei* (Tr), *M. thermophila* ATCC 42464 (Mt), and *M. heterothallica* CBS 202.75 and CBS 663.74 after growth on wheat straw pretreated with 20% HCl. The activities are measured by the amount of reduced sugar released and are given in nmol or μmol reduced sugar per min per mg total protein. The averages and standard deviations represent two independent cultivations and at least four technical replicates.

Figure 4. Enzyme activities of *M. thermophila* ATCC 42464 and *M. heterothallica* CBS 202.75 and CBS 663.74 during 4 days growth on wheat straw pretreated with 20% HCl (A–F) or 5% HCl (G–L). The enzymatic activities are measured against *p*-nitrophenol (PNP) linked substrates and given in nmol PNP per min per mg total protein. The averages and standard deviations represent two independent cultivations and six technical replicates.

Figure 5. Saccharification of wheat straw pretreated with 20% HCl (A–F) or 5% HCl (G–L) during 4 days at 45°C using an enzyme mixture of *M. thermophila* ATCC 42464 and *M. heterothallica* CBS 202.75 and CBS 663.74. The sugar concentrations are given in mg per L filtrate. The black lines are representing the sugar concentrations in wheat straw without an enzyme mixture. The averages and standard deviations represent two independent cultivations and six technical replicates.

Figure 6. Hierarchical clustering (UPGMA) of AFLP banding patterns of *Myceliophthora* progenies and their parents *M. heterothallica* CBS 203.75 and CBS 663.74. Similarity of the banding patterns is given in percentage.
Figure 7. Saccharification of mildly pretreated wheat straw (5% HCl) with an enzyme mixture of *M. heterothallica* CBS 203.75 and CBS 663.74 and their two progeny ‘FP 711.02’ and ‘FP 711.14’ during 4 days at 45 °C. The sugar concentrations are given in mg per L filtrate. The black lines are representing the sugar concentrations in wheat straw without an enzyme mixture. The averages and standard deviations represent two independent cultures.