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BIOENERGY AND BIOFUELS

Screening of ecologically diverse fungi for their potential to pretreat lignocellulosic bioenergy feedstock

Sukhwinder Singh · Hauke Harms · Dietmar Schlosser

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Abstract A widespread and hitherto by far underexploited potential among ecologically diverse fungi to pretreat wheat straw and digestate from maize silage in the future perspective of using such lignocellulosic feedstock for fermentative bioenergy production was inferred from a screening of nine freshwater ascomycetes, 76 isolates from constructed wetlands, nine peatland isolates and ten basidiomycetes. Wheat straw pretreatment was most efficient with three ascomycetes belonging to the genera *Acephala* (peatland isolate) and *Stachybotrys* (constructed wetland isolates) and two white-rot fungi (*Hypholoma fasciculare* and *Stropharia rugosoannulata*) as it increased the amounts of water-extractable total sugars by more than 50 % and sometimes up to 150 % above the untreated control. The ascomycetes delignified wheat straw at rates (lignin losses between about 31 and 40 % of the initial content) coming close to those observed with white-rot fungi (about 40 to 57 % lignin removal). Overall, fungal delignification was indicated as a major process facilitating the digestibility of wheat straw. Digestate was generally more resistant to fungal decomposition than wheat straw. Nevertheless, certain ascomycetes delignified this substrate to extents sometimes even exceeding delignification by basidiomycetes. Total sugar amounts of about 20 to 60 % above the control value were obtained with the most efficient fungi (one ascomycete of the genus *Phoma*, the unspecific wood-rot basidiomycete *Agrocybe aegerita* and one unidentified constructed wetland isolate). This was accompanied by lignin losses of about 47 to 56 % of the initial content. Overall, digestate delignification was implied to be less decisive for high yields of fermentable sugars than wheat straw delignification.

Keywords Bioenergy · Digestate · Fungi · Lignin degradation · Lignocellulosic feedstock · Pretreatment · Wheat straw

Introduction

Among all agricultural residues, wheat straw represents the largest lignocellulosic feedstock in Europe and the second largest in the world (Dashtban et al. 2009). Its high polysaccharide content suggests that hydrolysis could yield monomeric sugars for subsequent biofuel (biogas, bioethanol, biohydrogen) production (Dashtban et al. 2009; Kaparaju et al. 2009). However, the accessibility of cellulose for hydrolysing enzymes is generally hampered by its close association with lignin and hemicelluloses and by cellulose crystallinity. Pretreatment before hydrolysis to enhance biomass digestibility is therefore a prerequisite (Agbor et al. 2011; Dashtban et al. 2009). During biogas fermentation of plant biomass, readily available carbohydrates are degraded, whereas lignin persists and the NH_4-N content increases. This results in a lowered C/N ratio of the remaining digestate, which is frequently used as an organic fertiliser in agriculture (Chen et al. 2012; Klimiuk et al. 2010). However, the residual organic matter in digestate could still yield economically attractive

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S. Singh
Department of Bioenergy, Helmholtz Centre for Environmental Research−UFZ, Permoserstrasse 15, 04318 Leipzig, Germany

H. Harms
German Centre for Integrative Biodiversity Research iDiv, University of Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany

H. Harms · D. Schlosser (✉)
Department of Environmental Microbiology, Helmholtz Centre for Environmental Research−UFZ, Permoserstrasse 15, 04318 Leipzig, Germany
e-mail: dietmar.schlosser@ufz.de

amounts of biogas (Menardo et al. 2011), raising the question whether it could be qualified for biogas production under appropriate conditions.

Fungal pretreatment of lignocelluloses provides several advantages over abiotic pretreatments. These are mild reaction conditions, the specificity of enzymatic hydrolysis, the avoidance of inhibitory compounds and low energy requirements. Disadvantages include more time-consuming and not always sufficiently efficient enzymatic reactions (Agbor et al. 2011; Dashtban et al. 2009). White-rot basidiomycetes delignify plant materials with the help of various extracellular lignin-modifying peroxidases and an oxidase (the multicopper oxidase laccase), resulting in a polysaccharide-enriched residue (Dashtban et al. 2009; Liers et al. 2011). Wheat straw pretreatment using white-rot fungi can increase in vitro gas production, substrate saccharification and ethanol fermentation yields (Dias et al. 2010; Salvachúa et al. 2011; Tuyen et al. 2012). Unlike terrestrial white-rot basidiomycetes inhabiting wood, fungi of other habitats and ecophysiological groups have only scarcely been investigated with respect to lignocellulosic feedstock pretreatment. Certain topsoil-dwelling and litter-decomposing mushrooms and basidiomycetes colonising soil litter and compact wood also cause white rot (Liers et al. 2011). Brown-rot fungi primarily degrade cellulose and hemicelluloses while modifying lignin to some extent (Dashtban et al. 2009; Yelle et al. 2008). Other terrestrial basidiomycetes cause a rather unspecific rot which is difficult to assign to a certain lignocellulose decay type (Liers et al. 2011). Plant biomass-feeding freshwater ascomycetes and their anamorphs have been demonstrated to metabolise recalcitrant environmental pollutants, but information regarding their capabilities to act on lignin is scarce (Krauss et al. 2011). Wetlands, either constructed for bioremediation or naturally existing as, e.g. peatland, represent another source of fungi with promising degradation capabilities (Artz et al. 2007; Giraud et al. 2001).

In this study, we describe a two-stage screening of fungi from only rarely investigated environments and different ecophysiological groups. We investigated their potential for the (pre)treatment of wheat straw and digestate remaining from maize silage-based biogas production in the future perspective of (re)using these substrates in fermentation processes for bioenergy purposes. For this, we focused on delignification considered as one prerequisite for the efficient hydrolytic saccharification of lignocellulosic feedstock (Agbor et al. 2011; Dashtban et al. 2009; Kaparaju et al. 2009). Alterations in the lignin and sugar components of the water-soluble fractions of wheat straw and digestate, which were caused by the fungal colonization of these substrates, were concomitantly assessed. A total of nine already partly characterised ascomycete anamorphs from various freshwater environments (Junghanns et al. 2008), 76 filamentous fungal strains newly isolated from pilot-scale constructed wetlands designed for contaminated groundwater treatment (Fütterer et al. 2010; Seeger et al. 2011) and nine fungal isolates derived from a rewetted area of a peatland drained for cutting of peat in the past were employed. In addition, the litter-decomposing *Stropharia rugosoannulata* and the soil litter- and wood-inhabiting *Hypholoma fasciculare* were included as basidiomycetes causing a white-rot decay type of lignocellulose (Liers et al. 2011). *Coriolopsis polyzona*, *Pycnoporus sanguineus* and *Trametes versicolor* served as primarily wood-colonising white-rot references. The hard wood-inhabiting *Agrocybe aegerita* was included as an unspecific wood-rot basidiomycete (Liers et al. 2011). The brown-rot basidiomycete species *Gloeophyllum striatum*, *Gloeophyllum trabeum* (Fahr et al. 1999) and *Laetiporus sulphureus* were also included.

Materials and methods

Source, maintenance and identification of fungal strains

The strains compiled in Tables 1 and 2 were employed within the present study. Promising newly isolated strains were sent to the Belgian Coordinated Collections of Microorganisms/Mycothèque de L'Université catholique de Louvain (BCCM/MUCL, Louvain-la-Neuve, Belgium) for identification and deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The assigned DSMZ numbers are contained in Table 2.

A total of 76 fungal strains was newly isolated from different parts of the pilot-scale groundwater treatment plant "Compartment Transfer", closely located to the former refinery and industrial site of Leuna, Saxony-Anhalt, Germany (Seeger et al. 2011). Samples were taken from different types of horizontal-flow constructed wetlands basically consisting of steel basins filled with fine gravel (with a part of them planted with reed and/or additionally containing an open water body at the outflow) and from two vertical-flow soil filters (a roughing and a polishing filter, the latter being planted with willows on the top) (for details, see Fütterer et al. 2010; Seeger et al. 2011), within different sampling campaigns between 2007 and 2009. Samples of solid materials were taken in the respective middle of horizontal-flow constructed wetlands from the vadose (upper 10 cm) and the saturated zones (depths of 10 to 20, 40 and 80 cm; depending on the type of wetland and time point of sampling) and from vertical-flow soil filters at depths of 75 cm (roughing filter) and 30 to 35 cm (polishing filter). Water samples were taken from saturated zones of horizontal-flow constructed wetlands using a peristaltic pump (Seeger et al. 2011). Additional samples were taken from the respective outflow of horizontal-flow constructed wetlands.

Table 1 Fungal strains used within the present study

Fungal strains	Number of strains used	Supplier or site of isolation
Aquatic hypomycetes	2	
Clavariopsis aquatica De Wild. WD(A)-00-01	1	UFZ culture collection
Varicosporium sp. SpW08-1	1	UFZ culture collection
Other (partly) identified aquatic mitosporic isolates	7	
Alternaria sp. Tt-S01	1	UFZ culture collection
Coniothyrium sp. Kl-S05	1	UFZ culture collection
Cylindrocarpon didymum (Hartig) Wollenweber P10-10-3	1	UFZ culture collection
Myrioconium sp. UHH 1-6-18-4	1	UFZ culture collection
Myrioconium sp. UHH 1-13-18-4	1	UFZ culture collection
Phoma sp. SaSe-B6	1	UFZ culture collection
Phoma sp. UHH 5-1-03 (DSM 22425)	1	UFZ culture collection
Newly isolated and mostly unidentified fungal strains	85	
Isolates from constructed wetlands and vertical-flow soil filters	76	Compartment transfer pilot-scale groundwater treatment plant (Leuna, Saxony-Anhalt, Germany)
Isolates from peatland	9	Peatland "Hollweger Moor" (Westerstede, Lower Saxony, Germany)
Brown-rot basidiomycetes	4	
Gloeophyllum striatum (Swartz: Fries) Murrill DSM 10335	1	DSMZ
Gloeophyllum trabeum (Persoon: Fries) Murrill DSM 3087	1	DSMZ
G. trabeum (Persoon: Fries) Murrill WP 0992	1	UFZ culture collection
Laetiporus sulphureus (Bulliard: Fries) Murrill DSM 11211	1	DSMZ
Unspecific wood-rot basidiomycetes	1	
Agrocybe aegerita (Briganti) Singer DSM 22459	1	DSMZ
White-rot basidiomycetes	5	
Coriolopsis polyzona (Persoon) Ryvarden MUCL 38443	1	BCCM/MUCL
Hypholoma fasciculare (Hudson: Fries) Kummer UHH 1-4-03	1	UFZ culture collection
Pleurotus sanguineus (Linnaeus: Fries) Murrill MUCL 38531	1	BCCM/MUCL
Stropharia rugosoannulata Farlow ex Murrill DSM 11372	1	DSMZ
Trametes versicolor (Linneaus: Fries) Pilát DSM 11269	1	DSMZ

UFZ culture collection culture collection of the Department of Environmental Microbiology (Helmholtz Centre for Environmental Research—UFZ, Leipzig, Germany), *BCCM/MUCL* Belgian Coordinated Collections of Microorganisms/Mycothèque de L'Université catholique de Louvain (Louvain-la-Neuve, Belgium), *DSMZ* German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany)

Fungal strains were also isolated from peat samples taken from a soil profile of a re-wetted area of the peatland "Hollweger Moor" (about 0.91 km^2; close to the city of Westerstede, Lower Saxony, Germany) at depths of 32, 102 and 182 cm in 2009. This peatland was drained for cutting of peat from the beginning of the twentieth century until the 1960s and partly re-naturated after becoming a nature reserve in 1991.

Solid samples (10 g) were extracted using sodium pyrophosphate as previously described (Junghanns et al. 2008). After sedimentation of solids, aliquots (150 μl) from serial dilutions of the supernatants were poured onto malt agar plates containing 1 % (*w*/*v*) malt extract (0.1 % malt extract for peatland samples), 1.5 % agar, 0.25 g/l tetracycline and 0.4 g/l streptomycin (pH 5.6–5.8 for samples from constructed wetlands and soil filters, pH 3.5 for peatland samples). Additional malt extract plates with antibiotics were inoculated with aliquots (150 μl) of serial dilutions of water samples from constructed wetlands. Agar plates were incubated at 14 °C for several days in the dark. To obtain pure cultures, single colonies of filamentous fungi were transferred onto fresh agar plates. Those contained 1 % malt extract and 1.5 % agar (pH 5.6–5.8) and were routinely used to maintain fungal strains at 14 °C in the dark.

Table 2 Fungal strains selected for six-well plate cultivations on lignocellulosic substrates and their growth on agar plates

Fungal strain	Growth on agar plates[a]		Characteristics/comments
	Colony radius (cm)	Difference in colony radius (cm)[b]	
Cultivation on wheat straw			
B-2008-9	4.00±0.20	2.00±0.00	Constructed wetland isolate
S. rugosoannulata DSM 11372	4.00±0.00	1.50±0.00	White-rot basidiomycete
B-2008-12	3.83±0.29	1.67±0.41	Constructed wetland isolate
Phoma sp. UHH 5-1-03 (DSM 22425)	3.67±0.29	0.67±0.58	Mitosporic aquatic ascomycete
A-2008-11	3.50±0.29	1.92±0.14	Constructed wetland isolate
B-2008-7	3.50±0.58	1.17±0.58	Constructed wetland isolate
H. fasciculare UHH 1-4-03	3.50±0.00	1.50±0.00	White-rot basidiomycete
AP2-2009-10	3.33±0.41	1.17±0.41	Constructed wetland isolate
Myrioconium sp. UHH 1-6-18-4	3.33±0.29	1.50±0.41	Mitosporic aquatic ascomycete
AP2-2009-1	3.17±0.29	1.50±0.41	Constructed wetland isolate
Stachybotrys chlorohalonata Andersen & Trane A-2008-2 (DSM 27588)	3.00±0.41	0.92±0.14	Constructed wetland isolate
B-2008-2	3.00±0.41	1.17±0.58	Constructed wetland isolate
2007-23	2.67±0.00	0.42±0.29	Constructed wetland isolate
L. sulphureus DSM 11211	2.67±0.41	0.33±0.82	Brown-rot basidiomycete
Acephala sp. JU-A-2 (DSM 27592)	2.50±0.41	0.83±0.29	Peatland isolate
Stachybotrys cfr. *chlorohalonata* A-2008-4 (DSM 27589)	1.17±0.29	0.17±0.29	Constructed wetland isolate
Cultivation on digestates			
A-2008-11	4.00±0.00	1.17±0.29	Constructed wetland isolate
C. didymum P10-10-3	4.00±0.00	1.00±0.00	Mitosporic aquatic ascomycete
AP2-2009-11	3.33±0.29	0.50±0.41	Constructed wetland isolate
Alternaria sp. Tt-S01	3.33±0.29	0.83±0.14	Mitosporic aquatic ascomycete
Phoma sp. AP2-2009-3 (DSM 27590)	3.17±0.00	1.33±0.41	Constructed wetland isolate
A. aegerita DSM 22459	3.17±0.29	0.75±0.48	Unspecific wood-rot basidiomycete
Cladosporium herbarum (Persoon: Link) Fries AP2-2009-7 (DSM 27591)	3.08±0.00	1.58±0.14	Constructed wetland isolate
AP2-2009-8	3.08±0.32	0.58±0.14	Constructed wetland isolate
G. trabeum DSM 3087	3.00±0.00	2.08±0.14	Brown-rot basidiomycete
Phoma sp. UHH 5-1-03 (DSM 22425)	2.67±0.32	−0.83±0.29	Mitosporic aquatic ascomycete
Acephala sp. JU-A-2 (DSM 27592)	2.50±0.14	0.50±0.00	Peatland isolate
JU-M-3	2.50±0.32	1.17±0.29	Peatland isolate
Trichosporon porosum (Stautz) Middelhoven, Scorzetti & Fell JU-K-2 (DSM 27593)	2.17±0.29	1.33±0.41	Peatland isolate
H. fasciculare UHH 1-4-03	2.17±0.29	1.50±1.53	White-rot basidiomycete
B-2008-18	1.50±0.00	−1.50±0.00	Constructed wetland isolate
B-2008-6	1.50±0.00	−0.50±0.00	Constructed wetland isolate

[a] Agar plates contained wheat straw (in case of fungi employed in six-well plate cultivations on wheat straw) or digestate (for fungi used in six-well plate cultivations on digestate) as sole carbon and energy source. Data represent means±standard deviations (calculated according to Gaussian error propagation rules in case of colony radius differences) for triplicate plates after 2 weeks of cultivation

[b] Differences between fungal colony radii on wheat straw- or digestate-containing test plates and those of corresponding control plates

Agar plate-based screening for fungal growth on wheat straw and digestate

Agar plates used to assess fungal growth on wheat straw and digestate contained 1.5 % agar, 50 μM $CuSO_4$, 50 μM $MnSO_4$, 20 μM $FeSO_4$ and 10 g/l (dry mass-based) of either milled wheat straw (about 2 mm particle size) or digestate from biogas fermentation of maize silage (dried at 85 °C for 3 days before use) in tap water (final pH 6.5 to 7.0). Both wheat straw and digestate were provided by the German Biomass Research Centre (Deutsches Biomasseforschungszentrum), Leipzig, Germany. Plates

omitting wheat straw and digestate served as controls. Agar plate media were autoclaved (121 °C, 20 min) twice to ensure sterile conditions. Cu, Mn and Fe all participate in certain fungal lignocellulose degradation activities (Fahr et al. 1999; Liers et al. 2011) and were aseptically added as the respective sulphates from a freshly prepared and sterile-filtered stock solution after autoclaving. For each fungus, triplicate agar plates of each type were inoculated by placing one mycelium-containing agar plug (derived from the edge of fungal colonies on malt agar plates as described before) into the centre of each plate, respectively. Plates were incubated at ambient temperature (about 21 °C) in the dark. After 2 weeks of incubation, fungal colony diameters were recorded twice per plate (i.e. at right angles to each other) and used to calculate the corresponding colony radii.

Six-well culture plate-based cultivations on solid lignocellulosic substrates

Selected fungi listed in Table 2 were grown on solid wheat straw and/or digestate in six-well tissue culture plates (35 mm well diameter, flat bottom, sterile, with lid; Iwaki brand, Asahi Techno Glass Corporation, Tokyo, Japan). For this, 0.5 g of either dry wheat straw (about 2 mm particle size) or dry digestate was supplemented with 2 ml tap water and autoclaved (121 °C, 20 min) twice. Thereafter, 50 μM $CuSO_4$, 50 μM $MnSO_4$ and 20 μM $FeSO_4$ (final concentrations, as based on the volume of the liquid phase) were aseptically added from a freshly prepared and sterile-filtered stock solution. The resulting wet solid substrates (final pH of the aqueous phase 6.5 to 7.0) were then aseptically added to the wells of six-well culture plates mentioned before (corresponding to 0.5 g dry substrate per well of a six-well plate, respectively). For inoculation with fungi, two agar plugs per well (derived from the edges of fungal colonies grown on malt agar plates described above) were used. Six-well plates remaining un-inoculated served as controls. After capping following inoculation, plates were additionally sealed with parafilm and covered with aluminium foil to minimise evaporation and incubated at ambient temperature without agitation in the dark.

Triplicate wells per fungus and substrate were harvested after 18 and 28 days of incubation and stored frozen at −20 °C until analysis. After thawing, the contents of wells were completely transferred into Falcon tubes (50 ml; VWR International, Darmstadt, Germany). Two millilitres of McIlvaine buffer (pH 7.0) was added, and the Falcon tubes were shaken (horizontal shaker 3015; GFL, Burgwedel, Germany) at ambient temperature and maximum amplitude for 30 min, in order to extract water-soluble compounds from the (pre)treated solid substrates. After centrifugation at 7,000×*g* and 4 °C for 10 min (centrifuge 5810R, Eppendorf, Hamburg, Germany), the resulting aqueous supernatants were separated and stored at −20 °C until further analysis as described below. The whole extraction procedure was repeated two more times, and the resulting aqueous supernatants were also stored until further analysis as described. The remaining solids were dried on pre-weighted filter papers (Whatman no.1, Maidstone, UK) at 50 °C for 48 h. After dry mass determination, apparently biomass-free aliquots were subjected to Fourier transform mid-infrared (FT-mIR) spectroscopy for lignin analysis as described below.

Determination of total sugars

Amounts of total sugars in aqueous extracts of solid substrates were photometrically determined after acidic hydrolysis, based on the phenol-sulphuric acid method (Dubois et al. 1956). For absorbance readings at 492 nm, a Genios+ microplate reader (Tecan, Crailsheim, Germany) was used. A calibration curve was established from a mixture of equal amounts of D-glucose and D-fructose.

Absorbance measurements

Aqueous extracts derived from wheat straw and digestate (pre)treated with fungi were 5- and 10-fold diluted with distilled water, respectively. The absorbances of the resulting dilutions were recorded at 360 nm (within the absorbance range of lignin and also fulvic acids and humic-like substances; Kluczek-Turpeinen et al. 2005; Liers et al. 2011; Yang et al. 2010), using the Genios+ microplate reader mentioned before.

Size exclusion chromatography

Size exclusion chromatography (SEC) was employed to analyse fungal effects on water-soluble aromatic lignocellulose fragments and/or lignocellulose-derived humic-like substances of wheat straw and digestate. Equal amounts of aqueous extracts derived from three successive extractions of fungi-treated substrates were combined and subjected to SEC either without dilution (samples from wheat straw) or 10-fold diluted (digestate samples). SEC analysis was carried out as previously described (Junghanns et al. 2005), except that isocratic elution using 20 % (*v*/*v*) acetonitrile, 80 % double distilled water, 5 g/l $NaNO_3$ (5 g/L) and 2 g/l K_2HPO_4 (adjusted to pH 10 with 1 M NaOH) was applied at a constant flow rate of 1 ml/min. Chromatograms were recorded at a wavelength range from 250 to 550 nm (monitoring wavelength 250 nm). Polystyrene sulphonate sodium salt molecular mass standards (PSS GmbH, Mainz, Germany) were used for calibration.

Lignin determination using Fourier transform mid-infrared spectroscopy

Solid wheat straw and digestate samples (20 mg) were ground with the help of a mortar and pestle, mixed with 980 mg KBr (FT-IR grade; Sigma-Aldrich, Munich, Germany) and then milled in a vibratory mill (Pulverisette 23, Fritsch GmbH, Idar-Oberstein, Germany) at 3,000 oscillations/min for 10 min to ensure a sufficiently powdered state. KBr pellets (13 mm) were prepared from aliquots of the milled sample preparations (200 mg in total, corresponding to 4 mg sample and 196 mg KBr) in a standard device under vacuum, applying a pressure of 55.16 to 68.95 MPa with the help of a 15-t press for 2 min. After this, the pressure was slowly released while removing the sample for spectral analysis. Using a PerkinElmer System 2000 FT-IR spectrophotometer (PerkinElmer, Rodgau, Germany), 128 scans per sample were collected in transmission mode (wavenumber range of 5,000 to 500 cm^{-1}; spectral resolution of 8 cm^{-1}). Spectra of three repeated measurements were averaged. For data analysis, the Thermo Scientific GRAMS® spectroscopy software version 5.0 (Thermo Fisher Scientific, Dreieich, Germany) was employed. Second derivatives to enhance signals from FT-mIR spectra were obtained by processing the latter by means of a 17-point smoothing filter and a second-order polynomial (Fackler et al. 2007). A band indicating aromatic skeletal lignin based on stretching vibrations of C=C bonds (reported wavenumber range of 1,505 to 1,518 cm^{-1}; Fackler et al. 2007; Krasznai et al. 2012; Shafiei et al. 2010) was detected at 1,510 and 1,518 cm^{-1} for wheat straw and digestate, respectively (Fig. 1 a, c), and used to quantify the lignin contents of the lignocellulosic substrates. For this, second derivatives of spectra intended to minimize band overlap thus yielding sufficiently resolved lignin-specific negative peaks (Fig. 1 b, d), and areas of such peaks were calculated using the GRAMS® spectroscopy software mentioned before. Calibration curves were generated by adding varying amounts of hydrolytic lignin (Sigma-Aldrich; up to 50 mg for lignin determinations of wheat straw and up to 20 mg for those of digestate) to a background of 20 mg ground wheat straw and digestate, respectively, supplementation with KBr to yield a final mass of 1,000 mg (thus finally corresponding to 5 and 2 % hydrolytic lignin for wheat straw and digestate calibration curves, respectively) and further processing as already described. Linear regression used to establish calibration functions yielded coefficients of determination (R^2) of 0.981 and 0.954 for wheat straw and digestate, respectively.

Statistical analysis

The software OriginPro 8.6.0G Sr2 (OriginLab Corp., Northampton, MA, USA) was used to establish linear correlations for all pairwise combinations of analysed parameters of aqueous extracts of lignocellulosic substrates (total sugars, absorbances) and those of the lignocellulosic solids (lignin concentrations, dry masses) and to perform principal component analyses (PCA) as indicated in the text.

Nucleotide sequence data deposition

(Partial) nucleotide sequences acquired at BCCM/MUCL to aid the identification of promising newly isolated strains (Table 2) were deposited in the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena/data/view/HG530745-HG530756) under the following accession numbers: HG530745 (internal transcribed spacer (ITS)1, 5.8S rRNA gene and ITS2 of *Phoma* sp. AP2-2009-3), HG530752 (28S rRNA gene of *Phoma* sp. AP2-2009-3), HG530746 (ITS1, 5.8S rRNA gene and ITS2 of *Acephala* sp. JU-A-2), HG530747 (ITS1, 5.8S rRNA gene and ITS2 of *Cladosporium herbarum* AP2-2009-7), HG530748 (ITS1, 5.8S rRNA gene and ITS2 of *Trichosporon porosum* JU-K-2), HG530749 (28S rRNA gene of *T. porosum* JU-K-2), HG530750 (ITS1, 5.8S rRNA gene and ITS2 of *Stachybotrys chlorohalonata* A-2008-2), HG530753 (β-tubulin gene of *S. chlorohalonata* A-2008-2), HG530755 (translation elongation factor 1 alpha gene of *S. chlorohalonata* A-2008-2), HG530751 (ITS1, 5.8S rRNA gene and ITS2 of *S.* cfr. *chlorohalonata* A-2008-4), HG530754 (β-tubulin gene of *S.* cfr. *chlorohalonata* A-2008-4) and HG530756 (translation elongation factor 1 alpha gene of *S.* cfr. *chlorohalonata* A-2008-4).

Results

Fungal growth on wheat straw- and digestate-containing agar plates

Fungal growth on agar plates was recorded after 2 weeks. The majority of the 104 tested strains (Table 1) showed radial growth to varying extents also on control plates without wheat straw and digestate (except 16 strains not growing on control plates). Continuation of growth on control plates was most likely due to nutrients still contained in the mycelia-overgrown agar plugs used for inoculation and/or fungal storage compounds. The distribution of colony radii in terms of absolute values and differences to controls is compiled in Fig. S1 in the Supplementary Material. Overall, the tested strains responded to the lignocellulosic substrates in quite different ways. On wheat straw plates, 16 out of the 104 tested strains were not able to grow. Among the remaining 88 strains, seven strains exhibited colony radii not differing from those on control plates, and five strains were growing more slowly on wheat straw plates compared to controls (Fig. S1b). A

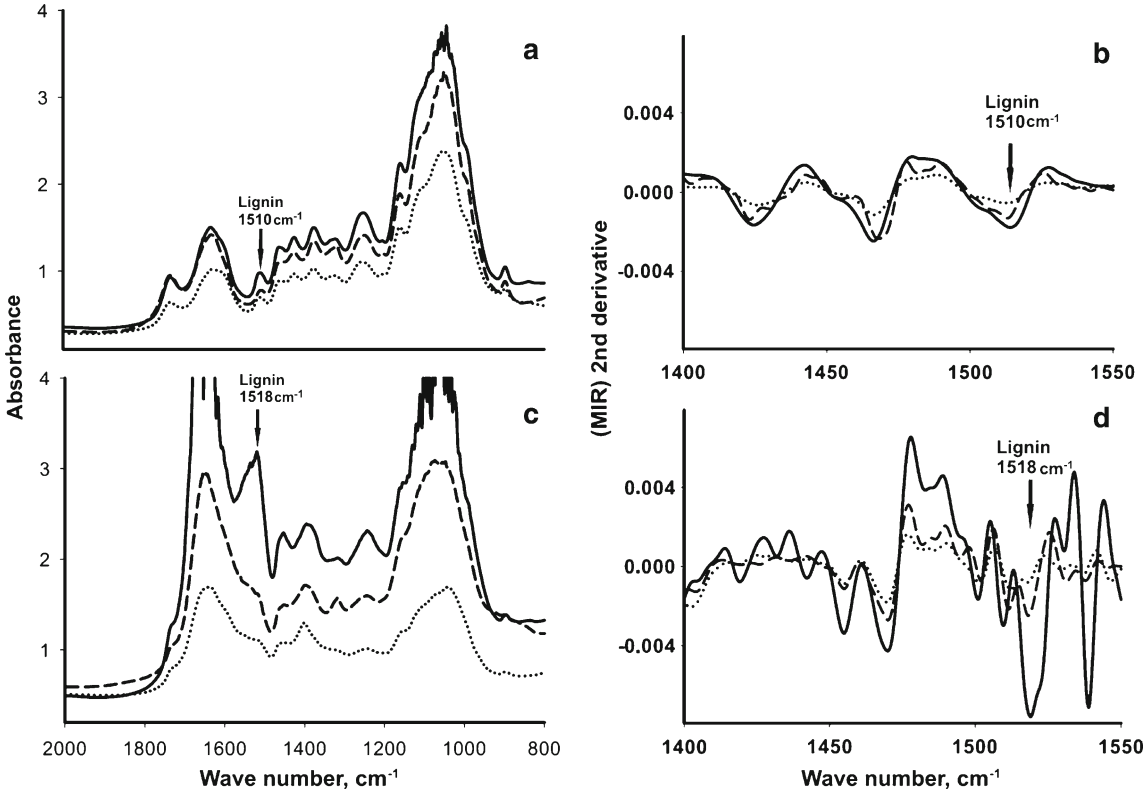

Fig. 1 Representative FT-mIR spectra (*a*, *c*) and corresponding second derivative spectra (*b*, *d*) of wheat straw (*a*, *b*) and digestate (*c*, *d*) treated with fungi for 28 days. Respective spectra of the untreated substrates (controls) are also shown. Lignin-indicative peaks used for determinations of lignin concentrations are indicated. Wheat straw treatments (*a*, *b*): *dashed line*, *S. chlorohalonata* A-2008-2; *dotted line*, *S. rugosoannulata* DSM 11372; *solid line*, control. Digestate treatments (*c*, *d*): *dashed line*, *G. trabeum*; *dotted line*, *Acephala* sp. JU-A-2; *solid line*, control

majority of 75 strains grew faster on wheat straw plates than on controls, indicating fungal utilisation of wheat straw components for growth. Using digestate, radial growth could not be observed for 20 of the tested strains. Three out of the remaining 84 strains grew equally on digestate and on control plates. Fungal utilisation of digestate components for growth was suggested by a total of 37 strains showing larger colony radii on digestate plates, as compared to controls. Inhibition of fungal growth by digestate was seen for 44 strains growing more slowly on digestate than on control plates (Fig. S1d). This exceeded by far the number of strains inhibited in the presence of wheat straw (Fig. S1b).

The numbers of those fungi among the tested strains, which were selected for the assessment of fungal treatment of wheat straw and digestate, respectively (Table 2), are indicated in Fig. S1 in the Supplementary Material. Criteria for the selection of strains were a representative coverage of (a) the growth behaviour in response to wheat straw and digestate on agar plates, thereby also targeting more slowly growing strains potentially causing less loss of fermentable carbon during (pre)treatment than faster growing strains; (b) different habitats serving as sources of strains, (c) strains representing different ecophysiological groups of fungi and (d) strains collectively used for both lignocellulosic substrates to enable direct comparison of their effects.

Identification of newly isolated strains

Due to striking abilities to increase the amount of water-extractable carbohydrates from wheat straw and digestate and/or to delignify these substrates (Figs. 1, 2, 3 and 5; Tables S1–S4 in the Supplementary Material), the newly isolated strains A-2008-2, A-2008-4, AP-2009-3, AP-2009-7, JU-A-2 and JU-K-2 (Table 2) were submitted to BCCM/MUCL for identification. ENA accession numbers of nucleotide sequences acquired for identification purposes at BCCM/MUCL are contained in the "Materials and methods" section. According to morphological and genetic characteristics, the isolates A-2008-2 and A-2008-4 belong to the *Stachybotrys chartarum* complex. Strain A-2008-2 was identified as *S. chlorohalonata* Andersen & Trane. Isolate A-2008-4 has a colony and conidia morphology slightly different from that of A-2008-2 but is genetically closely related to *S. chlorohalonata* (herein after referred to as *S.* cfr. *chlorohalonata* A-2008-4). Strain AP2-2009-3 is an anamorph and was identified as a species of *Phoma*,

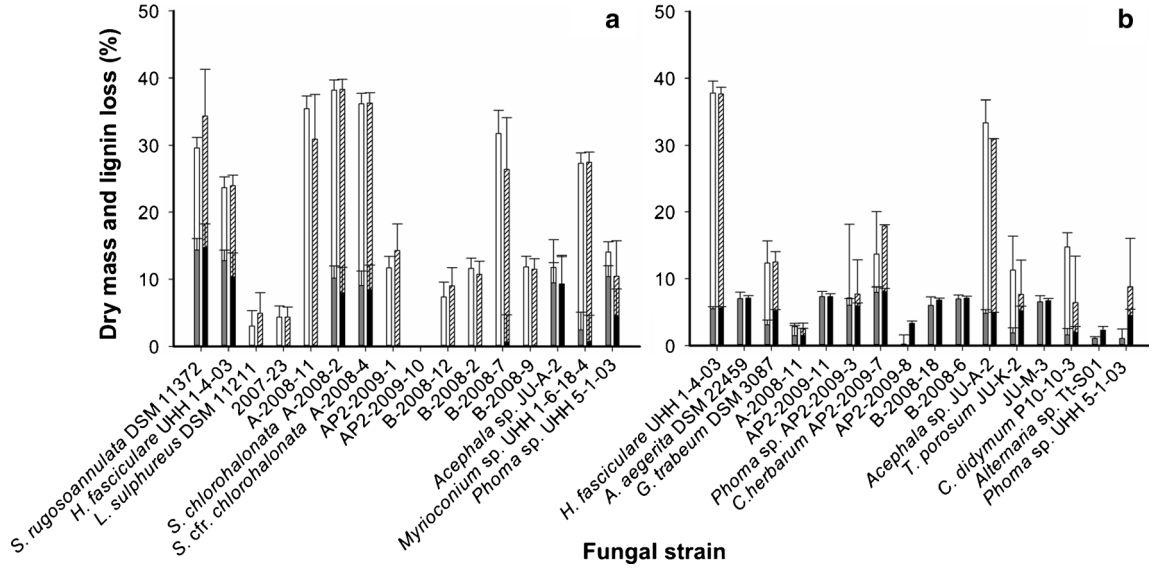

Fig. 2 Relative losses (% of initial substrate dry masses, as corrected by the respective control values) in substrate dry masses (*white* and *hatched bars*) and lignin contents (*grey* and *black bars*) of wheat straw (*a*) and digestate (*b*) treated with fungi for 18 (*white* and *grey bars*) and 28 days (*hatched* and *black bars*). All data represent means±standard deviations (calculated according to Gaussian error propagation rules) from triplicate cultivations. The underlying dry masses and lignin contents in terms of absolute quantities are compiled in Tables S1–S4 in the Supplementary Material

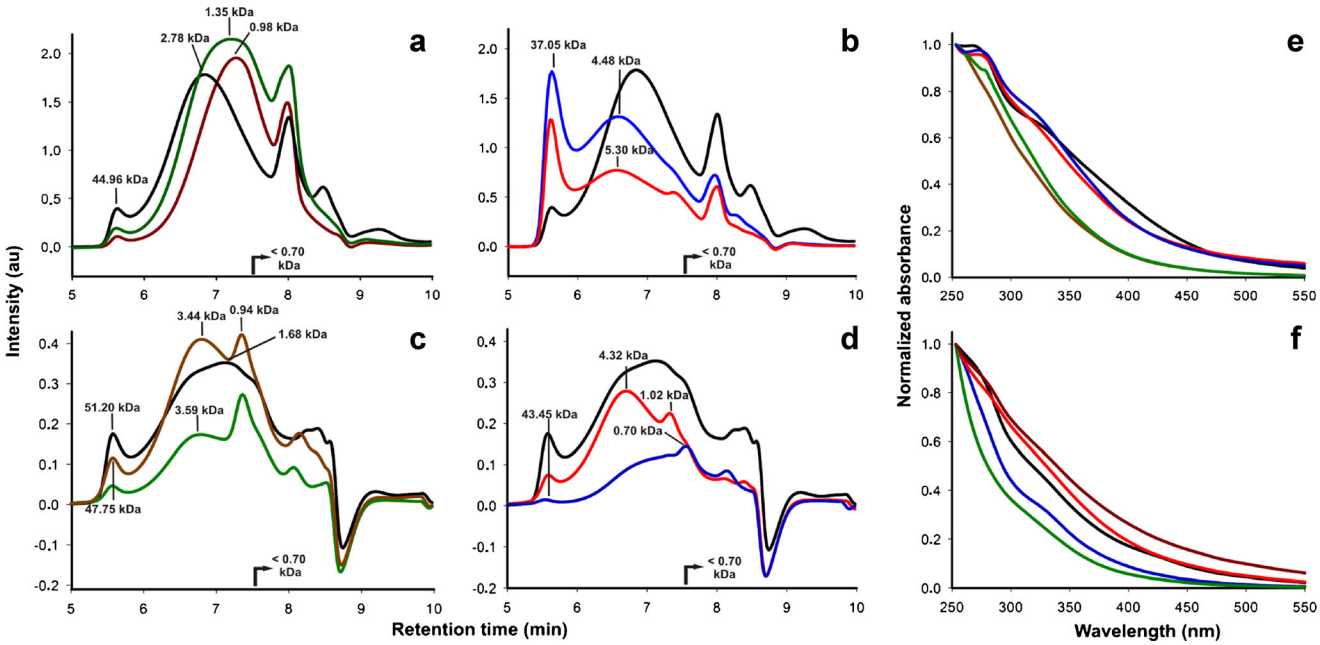

Fig. 3 Representative SEC chromatograms of samples from aqueous extracts of wheat straw (*a*, *b*) and digestate (*c*, *d*) treated with fungi for 28 days and normalised UV/Vis spectra corresponding to peaks of water-soluble aromatic lignocellulose fragments or structurally related compounds detected in wheat straw (*e*) and digestate samples (*f*). Molecular masses of peaks corresponding to water-soluble aromatic lignocellulose fragments or structurally related compounds as obtained from SEC calibration using molecular mass standards are indicated in SEC chromatograms (*a*–*d*), together with molecular mass ranges <0.7 kDa (i.e. below the molecular mass of the smallest molecular mass marker applied) falling below the calibration range. Wheat straw treatments (*a*, *b*, *e*): *green*, *H. fasciculare*; *brown*, *S. rugosoannulata*; *blue*, AP2-2009-1; *red*, AP2-2009-10; *black*, control (untreated wheat straw). Digestate treatments (*c*, *d*, *f*): *green*, *Phoma* sp. UHH 5-1-03; *brown*, *C. herbarum*; *blue*, *T. porosum*; *red*, *A. aegerita*; *black*, control (untreated digestate). Peaks in *a* and *b* correspond to UV/Vis spectra shown in *e* as follows: 2.78 kDa (*a*)=control (*e*); 1.35 kDa (*a*)=*H. fasciculare* (*e*); 0.98 kDa (*a*)=*S. rugosoannulata* (*e*); 5.30 kDa (*b*)=AP2-2009-10 (*e*); 4.48 kDa (*b*)=AP2-2009-1 (*e*). Peaks in *c* and *d* correspond to UV/Vis spectra shown in *f* as follows: 1.68 kDa (*c*)=control (*f*); 3.59 kDa (*c*)=*Phoma* sp. UHH 5-1-03 (*f*); 3.44 kDa (*c*)=*C. herbarum* (*f*); 4.32 kDa (*d*)=*A. aegerita* (*f*); 0.70 kDa (*d*)=*T. porosum* (*f*)

possessing *Leptosphaeria* Ces. & De Not. relationships. Isolate AP2-2009-7 represents a strain of *C. herbarum* (Persoon: Link) Fries. Strain JU-A-2 is a mycelium sterile providing only poor morphological information. Sequencing of ITS of its nuclear ribosomal DNA (nrDNA) points to a species of *Acephala* known to include species with sterile mycelia. Based on sequencing of the 28S rDNA D1/D2 domain and nrDNA ITS, isolate JU-K-2 was identified as *T. porosum* (Stautz) Middelhoven, Scorzetti & Fell representing a basidiomycetous yeast.

Effects of fungal treatment of wheat straw and digestate on lignin contents and dry masses

The effects of fungal treatments on relative lignin and substrate dry mass losses (i.e. relation to the initial substrate dry masses, to enable a direct assignment of the portion of lignin removal accounting for the total mass loss) are depicted in Fig. 2. Related lignin concentrations (% dry mass), total lignin contents and substrate dry masses in terms of absolute quantities and the corresponding losses are compiled in Tables S1–S4 in the Supplementary Material. Figure 1 exemplifies associated decreases in intensities of lignin-indicative peaks in FT-mIR spectra (Fig. 1 a, c) and their second derivatives (Fig. 1 b, d) for representative fungi. Initial dry mass-based lignin concentrations of untreated wheat straw and digestate of 25.77±3.11 (mean± standard deviation from a 9-fold determination) and 12.62± 0.17 % (mean±standard deviation from a 6-fold determination), respectively, coincide with previously published data (wheat straw 24 %, Salvachúa et al. 2011; maize-based digestate: about 10 to 11 % lignin, Chen et al. 2012; Klimiuk et al. 2010). For wheat straw, most efficient delignification was observed with the white-rot basidiomycetes *S. rugosoannulata* and *H. fasciculare*, with a lignin removal accounting for the total mass loss to about 14 to 15 and 10 to 13 %, respectively (Fig. 2 a). This corresponds to lignin losses of about 56 to 57 and 40 to 50 % of the initial lignin content for *S. rugosoannulata* and *H. fasciculare*, respectively (Tables S1 and S2 in the Supplementary Material). Considerable delignification rates coming close to those of the tested white-rot fungi were also obtained with the ascomycetes *Acephala* sp., *S. chlorohalonata* A-2008-2, *S.* cfr. *chlorohalonata* A-2008-4 and *Phoma* sp. UHH 5-1-03, where losses in lignin accounted for the observed total mass reductions to approximately 8 to 10 % (corresponding to losses of about 31 to 40 % of the initial lignin content; Fig. 2 a; Tables S1 and S2). Some minor delignification is also indicated for the ascomycete *Myrioconium* sp., whereas all other tested strains did not delignify wheat straw (Fig. 2 a). Delignification was always completed after 18 days of cultivation and did not further proceed later on. The extent to which delignification has contributed to the dry mass loss of wheat straw considerably varied among the tested strains. For *Acephala* sp. and (although less unambiguous) *Phoma* sp. UHH 5-1-03, the observed total substrate mass loss was predominantly due to delignification, indicating a high selectivity for lignin degradation (Fig. 2 a; Tables S1 and S2). About half of the observed total mass loss was due to delignification for *H. fasciculare* and *S. rugosoannulata*, and approximately 20 to 25 % of the total mass reduction was caused by lignin removal for *S. chlorohalonata* A-2008-2 and *S.* cfr. *chlorohalonata* A-2008-4 (Fig. 2 a; Tables S1 and S2).

Most efficient delignification of digestate was monitored for the ascomycetes *C. herbarum* and *Phoma* sp. AP2-2009-3; the basidiomycetes *A. aegerita* and *H. fasciculare*; the unidentified constructed wetland isolates AP2-2009-11, B-2008-6 and B-2008-18 and the peatland isolate JU-M-3. In these strains, the observed lignin losses accounted for the total substrate mass reductions to about 6 to 8 % (corresponding to losses of approximately 44 to 64 % of the initial lignin content; Fig. 2 b; Tables S3 and S4 in the Supplementary Material). *Acephala* sp. (one of the most efficient ascomycete delignifiers of wheat straw; Fig. 2 a; Tables S1 and S2 in the Supplementary Material), *Phoma* sp. UHH 5-1-03, the brown-rot basidiomycete *G. trabeum* and the basidiomycetous yeast *T. porosum* caused a rather moderate lignin removal from digestate, accounting for total substrate mass losses to up to about 5 % (up to about 41 % lignin loss as based on the initial lignin content; Fig. 2 b; Tables S3 and S4). In all other strains, the contributions of lignin removal to total mass losses did not exceed about 3 % (corresponding to losses of up to approximately 26 % of the initial lignin content; Fig. 2 b; Tables S3 and S4). In contrast to fungal cultivations on wheat straw (Fig. 2 a), delignification of digestate proceeded until the end of the experiment (28 days of cultivation) for a range of strains (*Phoma* sp. UHH 5-1-03, *T. porosum* JU-K-2, *Alternaria* sp. Tt-S01, *G. trabeum* DSM 3087, AP2-2009-8; Fig. 2 b). For digestate, an only limited sensitivity and apparent methodological errors associated with the applied gravimetrical dry mass determination did not always allow to detect total substrate dry mass losses that the detectable lignin removals suggest (Tables S3 and S4). Nevertheless, a striking difference between fungal growth on wheat straw and digestate is represented by overall dry mass losses of wheat straw (12.94±10.30 and 12.88±9.79 % of the initially applied 500 mg of substrate after 18 and 28 days of fungal treatment, respectively; median±median absolute deviation from all tested strains as calculated from data compiled in Tables S1 and S2; dry mass losses were set to zero where not detectable) much exceeding those observed for digestate (1.43±1.43 and 4.50±4.50 % after 18 and 28 days of fungal treatment, respectively; based on data shown in Tables S3 and S4). The selectivity for lignin degradation in digestate considerably varied among fungal strains as also observed for wheat straw, ranging from (hypothetical) total substrate mass losses (assumed to be) essentially due to lignin losses (in cases where the total dry mass losses could not be measured as observed with the top delignifiers *A. aegerita*, AP2-2009-11, B-2008-6,

B-2008-18 and JU-M-3; but also in *Phoma* sp. AP2-2009-3) via fair (e.g. observed with *C. herbarum*, *Phoma* sp. UHH 5-1-03 and *T. porosum*) to very little selectivity where lignin removal had contributed to the total dry mass loss to an only minor extent (e.g. *Acephala* sp. and *H. fasciculare*) (Fig. 2 b; Tables S3 and S4).

Alterations in water-extractable organic compounds caused by fungal treatment of wheat straw and digestate

Analysis of aqueous wheat straw extracts by SEC enabled the detection of water-soluble aromatic lignocellulose fragments, as indicated by UV/Vis spectral characteristics of the eluted compounds (Fig. 3 e). For efficiently delignifying fungi as exemplified for *H. fasciculare* and *S. rugosoannulata* in Fig. 3 a, depolymerisation of lignocellulose fragments is evident from shifts in the molecular masses of predominant peaks of water-soluble aromatics towards smaller fragments (around 1 and 1.3 kDa observed upon fungal treatment vs. about 2.8 kDa observed with the control) and from a decrease of peaks in the range of 45 kDa found to be the highest in the control. By contrast, other strains not removing lignin such as AP2-2009-1 and AP2-2009-10 (Fig. 2 a; Tables S1 and S2 in the Supplementary Material) caused shifts in the molecular masses of prominent peaks towards larger lignocellulose fragments (about 4.5 and 5.3 kDa observed upon fungal treatment vs. around 2.8 kDa observed with the control) and led to an increase of peaks at about 37 kDa thus becoming predominant in fungal samples but being much less pronounced in the control (Fig. 3 b).

Aqueous digestate extracts generally showing a dark brownish-grey colour (vs. a light brown colour of wheat straw extracts) unless bleached by certain fungal strains (see below) contained clearly more water-soluble material than wheat straw extracts and therefore had to be appropriately diluted before application to SEC (see "Materials and methods" section). Similar to wheat straw, the elution of aromatic lignocellulose fragments and/or possibly also lignocellulose-derived humic-like substances is indicated by corresponding UV/Vis spectra (Fig. 3 f). For a range of strains, concomitant polymerisations and depolymerisations became evident from shifts in the molecular mass of a predominant peak present in the control sample at about 1.7 kDa towards both smaller (around 1 kDa) as well as larger fragments (about 3.4, 3.6 and 4.3 kDa; Fig. 3 c, d) and from decreases of the prominent 51.2-kDa control peak and accompanying slight shifts towards less large fragments in the range of about 43 to 48 kDa (Fig. 3 c, d). With particular fungi such as *T. porosum*, solely depolymerisation was indicated by the appearance of small lignocellulose fragments at about 0.7 kDa (Fig. 3 d).

To varying extents, fungal treatment led to a decrease in absorbance at 360 nm of aqueous wheat straw and digestate extracts (Fig. 4). Like for SEC analysis, the generally more strongly coloured digestate samples required a higher dilution than wheat straw samples before measurement to prevent signal overflow. Fungal strain-dependent bleaching was also obvious from visual observation of aqueous wheat straw and digestate extracts.

The water extractability of carbohydrates from the lignocellulosic substrates (in terms of absolute amounts of total sugars in aqueous extracts, hence also inherently considering carbohydrate losses during biological treatment) was used as a measure of sugars potentially accessible to fermentation for bioenergy purposes, with the total sugars derived from the first extraction step considered to be the most easily available carbohydrate fraction. Numerous of the tested fungi increased the amounts of total sugars that were water-extractable from fungi-treated wheat straw using just one extraction step, as compared to the non-treated control (Fig. 5 a). Particularly efficient fungi well exceeded a value of 150 % of the control after 18 and 28 days (the white-rot basidiomycete *H. fasciculare* and the peatland isolate *Acephala* sp.) and after 28 days of cultivation (the white-rot basidiomycete *S. rugosoannulata* and the constructed wetland isolates *S. chlorohalonata* A-2008-2 and *S.* cfr. *chlorohalonata* A-2008-4), sometimes yielding about 200 (*Acephala* sp., *H. fasciculare*, *S. chlorohalonata* A-2008-2) to 250 % of the control value (*S. rugosoannulata*). With all of these fungi, higher amounts of water-extractable total sugars than in the control were also obtained from three successive extraction steps after 28 days of cultivation (Fig. 5 c).

For fungi-treated digestate, higher amounts of water-extractable carbohydrates than in controls were only recorded after 28 days of cultivation (Fig. 5 b, d). As compared to wheat straw, a clearly lower number of fungal strains led to a higher total sugar value than the corresponding control for digestate. The unspecific wood-rot basidiomycete *A. aegerita* and the constructed wetland isolates *Phoma* sp. AP2-2009-3 and B-2008-6 represent the only strains substantially increasing the amounts of water-extractable total sugars, where values between approximately 120 and 160 % of the control were observed after the first extraction step only after a treatment time of 28 days (Fig. 5 b). Overall, the relative amounts of water-extractable total sugars of fungi-treated digestate were clearly lower than those of fungi-treated wheat straw, which also applies to the sugar amounts observed with fungal strains commonly used for both substrates (strains A-2008-11, *Acephala* sp., *H. fasciculare* and *Phoma* sp. UHH 5-1-03) (Fig. 5).

Correlations between parameters of lignocellulosic solids, their aqueous extracts and fungal strains

Linear correlation estimates between parameters of the lignocellulosic solids and aqueous extracts derived thereof always yielded the strongest significant correlations (α=0.05) between amounts of total sugars from the first and from three successive substrate extractions, respectively, where Pearson's

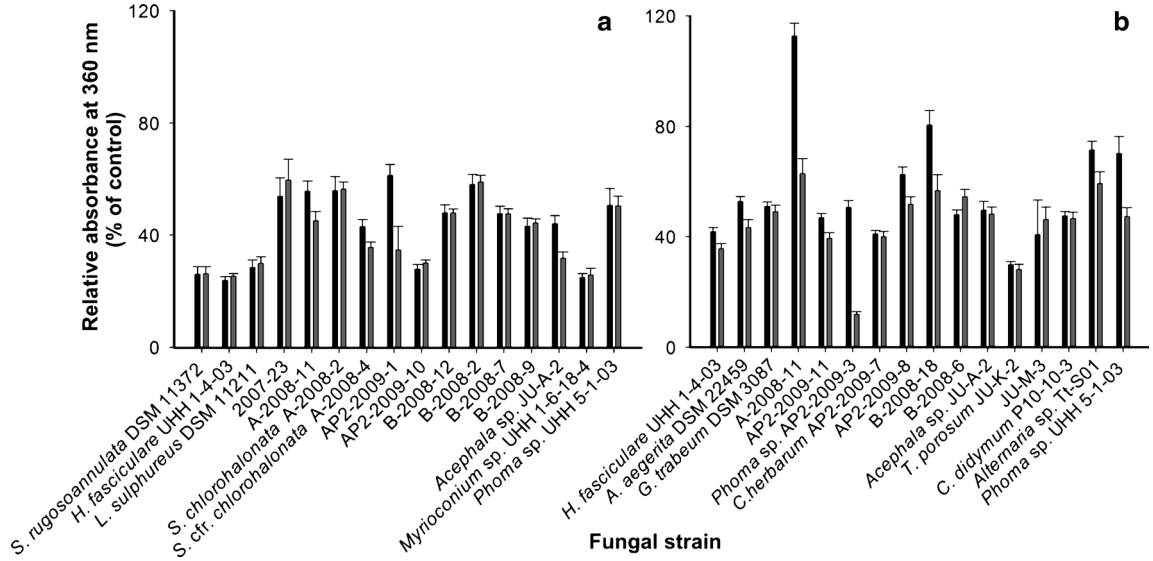

Fig. 4 Relative absorbances (% of control) at 360 nm of aqueous extracts of wheat straw (*a*) and digestate (*b*) treated with fungi for 18 (*black bars*) and 28 days (*grey bars*). All data refer to a sum of three successive extractions, respectively, and represent means±standard deviations from triplicate cultivations

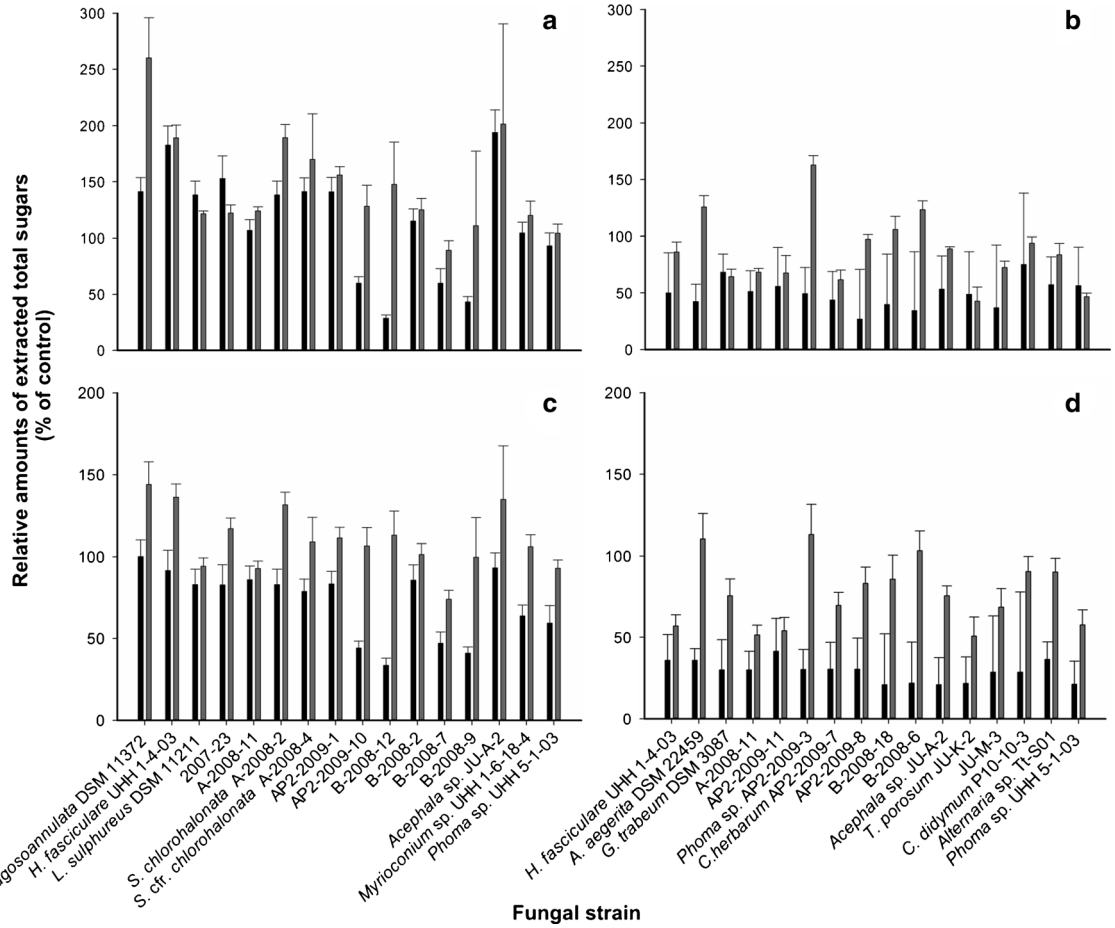

Fig. 5 Relative amounts (% of control) of total sugars in aqueous extracts of wheat straw (*a*, *c*) and digestate (*b*, *d*) treated with fungi for 18 (*black bars*) and 28 days (*grey bars*). Relative sugar amounts are shown for the first extraction (*a*, *b*) and as a sum of three successive extractions (*c*, *d*). All data represent means±standard deviations from triplicate cultivations. One hundred percent correspond to absolute amounts of total sugars of 694±61 (*a*, 18 days), 729±1 (*a*, 28 days), 905±123 (*b*, 18 days), 728±1 (*b*, 28 days), 2,369±213 (*c*, 18 days), 2,188±98 (*c*, 28 days), 2,866±153 (*d*, 18 days) and 2,148±100 μg (*d*, 28 days)

correlation coefficients (r)>0.9 (wheat straw) and >0.85 (digestate) were obtained (Figs. S2 and S3; Tables S5 and S6; all in the Supplementary Material). For wheat straw, fairly close and significant (α=0.05) inverse relationships between total sugar amounts and lignin concentrations (i.e. % dry mass) are evident from r values of about −0.685 and −0.785 (derived from exclusion of two strains showing data outside 95 % confidence ellipses; Fig. S2; Table S5). Such negative correlations are only cautiously and insignificantly indicated for digestate, if at all (Fig. S3; Table S6). Total sugars show only insignificant (α not below 0.15) and weak trends towards negative correlations with wheat straw dry masses (r not exceeding about −0.41; Table S5). The corresponding data for digestate reveal similarly weak and insignificant (albeit positive) tendencies (Fig. S3; Table S6). Absorbance values at 360 nm show a weak tendency (r= 0.524 at α=0.10) to reflect the lignin concentrations of wheat straw (Table S5). For digestate, absorbances rather tend to be weakly positively correlated with dry mass data (r=0.404 at α= 0.15; Table S6). No remarkable relationship was observed between lignin concentrations and dry masses of wheat straw samples after fungal treatment (Table S5), in line with a wide range of selectivity for lignin degradation covered by the tested strains as already mentioned. A modest negative correlation between lignin and dry mass data (r=−0.658 at α=0.05; Fig. S3; Table S6) was observed for digestate.

PCA was performed for the data sets used in Figs. S2 and S3, respectively. For wheat straw, fungi are separated along the PC1 axis (53 % of the variance) mainly according to total sugar amounts (the highest positive loadings) and lignin concentration (the highest negative loading) (PC2−22 %, highest positive loading: substrate dry mass, highest negative loading: absorbance at 360 nm; PC3−13 %, positive loadings: absorbance>dry mass>total sugar amounts; PC4−11 %, positive loadings: lignin concentration>total sugar amounts). Strain AP2-2009-10 was confirmed as a dry mass outlier (Tables S1, S2 and S5) in the PCA scores plot (data not shown). For digestate, PC1 (46 %) separates fungi by total sugars, dry mass (highest positive loadings) and lignin concentration (the highest negative loading) (PC2−29 %, positive loadings of absorbance and dry mass, negative loadings of all other parameters; PC3−19 %, positive loadings: absorbance> lignin concentration > total sugars). *Phoma* sp. AP2-2009-3 was confirmed as an outlier with respect to the absorbance at 360 nm (Table S6) in the PCA scores plot (data not shown). Neither for wheat straw nor for digestate PCA revealed a clustering of the tested fungal strains according to their ecophysiological groups or origins (data not shown).

Discussion

In this study, we have screened fungi from only scarcely investigated environments and different ecophysiological groups for their potential to pretreat lignocellulosic bioenergy feedstock such as wheat straw and digestate remaining from maize silage-based biogas production. The removal of lignin, considered as a major parameter hampering the enzymatic hydrolytic saccharification of lignocellulosic feedstock (Agbor et al. 2011; Dashtban et al. 2009; Kaparaju et al. 2009), from wheat straw by ascomycetes isolated from constructed wetlands (*S. chlorohalonata* A-2008-2, *S.* cfr. *chlorohalonata* A-2008-4; Table 2), peatland (*Acephala* sp.; Table 2) and river sediments (*Phoma* sp. UHH 5-1-03; Table 2; Junghanns et al. 2008) at rates that were only slightly lower than those observed with two white-rot references (the litter-decomposing *S. rugosoannulata* and the soil litter- and wood-inhabiting *H. fasciculare* (Fig. 2 a; Tables S1 and S2 in the Supplementary Material), indicates a considerable potential of hitherto only poorly investigated fungi for the pretreatment of this lignocellulosic substrate. Whereas lignin degradation is well documented for the white-rot basidiomycetes *H. fasciculare* and *S. rugosoannulata* (Buta et al. 1989; Liers et al. 2011; Šnajdr et al. 2010; Steffen et al. 2000), we are not aware of similar reports for the ascomycete genera *Acephala* and *Stachybotrys*. *Acephala* was implicated in soft-rot decay of mummified woods from the Arctic (Jurgens et al. 2009) and further involves certain plant root symbionts (Grünig and Sieber 2005). *Stachybotrys* species are known to produce potentially lignin-modifying laccases (Janssen et al. 2004). *Phoma* sp. UHH 5-1-03 and also the less efficiently delignifying *Myrioconium* sp. (Fig. 2 a) are known laccase producers (Junghanns et al. 2005, 2009), possibly indicating a role of this enzyme during delignification as already proposed for other ascomycetes (Liers et al. 2006). A particularly striking apparent increase in the wheat straw lignin content after fungal treatment observed with the constructed wetland isolate AP2-2009-1 (Tables S1 and S2) could be explained by the formation of structures potentially interfering with the applied lignin analysis, e.g. resulting from fungal lignin polymerisation and/or melanin synthesis (Liers et al. 2006, 2011) as also suggested by SEC analysis of aqueous wheat straw extracts (Fig. 3 b; see also below).

Similar to wheat straw, digestate delignification rates observed with various ascomycetes and unidentified fungal strains from constructed wetlands and peatland in the range of (and sometimes even exceeding) those observed with the most efficiently delignifying basidiomycetes *A. aegerita* and *H. fasciculare* (Fig. 2 b; Tables S3 and S4 in the Supplementary Material) indicate an as yet widely unexplored fungal potential for the pretreatment of this particular substrate. The unspecific wood-rot basidiomycete *A. aegerita* was previously shown to cause moderate changes in beech wood composition including slight delignification, and its extracellular aromatic peroxygenase is known to cleave non-phenolic lignin model compounds (Liers et al. 2011). It further degrades lignin when cultivated on combinations of

anaerobically digested poultry litter and wheat straw for mushroom production (Isikhuemhen et al. 2009), indicating a particular suitability for substrates rich in NH_4-N such as digestate from various sources. Ligninolytic capabilities including lignin peroxidase production have been reported for *C. herbarum* (Yadav et al. 2010). Potentially lignin-modifying laccase was demonstrated in the genus *Phoma* (Junghanns et al. 2009). Also, lignin cleavage has previously been proven for the brown-rot basidiomycete *G. trabeum* (Yelle et al. 2008). The basidiomycetous yeast *T. porosum* is known to assimilate phenolic compounds and hemicelluloses (Middelhoven et al. 2001) and hence perhaps could also act on phenolic lignin. A total substrate dry mass loss predominantly caused by delignification, which indicates a high selectivity for lignin degradation, was observed with a range of ascomycetes and isolates from constructed wetlands, peatland and river sediment (*Acephala* sp. and *Phoma* sp. UHH 5-1-03 during wheat straw and strains AP2-2009-11, B-2008-6, B-2008-18 and JU-M-3 during digestate treatment) but only one basidiomycete (*A. aegerita* during digestate treatment; Fig. 2 a, b). The highly selective lignin degraders reported so far are predominantly white-rot basidiomycetes (Dias et al. 2010; Salvachúa et al. 2011). By contrast, until now a high selectivity for lignin degradation could only rarely be inferred from studies dealing with ascomycetes (Bucher et al. 2004). A substrate specificity of the selectivity for lignin degradation is indicated by different extents of delignification contributing to total mass losses in dependence on the lignocellulosic substrate, as observed with *Acephala* sp. and *H. fasciculare* used in common for both substrates (Fig. 2 a, b).

The changes in the lignin contents of the solid substrates caused by fungal treatments (Fig. 2; Tables S1–S4 in the Supplementary Material) were reflected by alterations in the lignin-related components of the water-extractable fractions of the lignocellulosic substrates, as could be expected. Lignocellulose depolymerisation accompanied by the release of fragments with low molecular masses in the range of those observed with efficient delignifiers of wheat straw (Fig. 3 a) is well-known from white-rot basidiomycetes (Liers et al. 2011). Shifts in the molecular masses of prominent peaks towards larger lignocellulose/aromatic fragments and increasing peaks representing high molecular mass aromatic compounds, as monitored for strains AP2-2009-1 and AP2-2009-10 not showing lignin removal from wheat straw (Figs. 2 a and 3 b), indicate lignin and/or melanin polymerisation as previously implicated in ascomycetes (Liers et al. 2006, 2011). Depolymerisation of lignocellulose-related compounds, either as the only process that could be recorded or coexisting with polymerisation reactions, was observed upon fungal treatment of digestate (Fig. 3 c, d). Polymerisation and depolymerisation reactions simultaneously observed within the present study for the ascomycetes *Phoma* sp. UHH 5-1-03 and *C. herbarum* (Fig. 3 c), as well as for the basidiomycete *A. aegerita* (Fig. 3 d), partly resemble previously published data where an interim release of medium-sized (about 3 kDa) and a continuous increase in smaller lignocellulose fractions (around 0.8 kDa) was recorded during beech wood colonisation by laccase-producing ascomycetes and *A. aegerita* (Liers et al. 2006, 2011). The release of small fragments at around 0.7 to 1 kDa (Fig. 3 c, d) would also be in line with the reported partial depolymerisation of humic-like substances into smaller fulvic acid-like compounds by soil ascomycete anamorphs (Kluczek-Turpeinen et al. 2005). Like the results of the SEC analyses, the observed strain-specific decreases in absorbances at 360 nm of aqueous what straw and digestate extracts (Fig. 4) indicate oxidative biocatalytic alterations of lignocellulosic substrate structures as well-known from previous studies (Dorado et al. 1999). To directly link absorbances of aqueous extracts (Fig. 4) to depolymerisation or polymerisation reactions of lignocellulose-related compounds (Fig. 3) is complicated by potential interferences related to the individual amounts of water-extractable lignocellulose constituents and their respective absorbance characteristics. For instance, for strains showing depolymerisation abilities, increases (*H. fasciculare* and *S. rugosoannulata* in Fig. 3 a) as well as decreases in the respective amounts of water-soluble lignocellulose constituents compared to controls (*Phoma* sp. UHH 5-1-03 in Fig. 3 c, *T. porosum* in Fig. 3 d) are suggested from SEC analyses, whereas for all of these strains the absorbances at 360 nm are lower than those of the corresponding controls (Fig. 4). Similar complications arising from the application of different methods have previously been reported. In a study employing ^{14}C-labelled synthetic lignin to beech wood inoculated with *Xylaria polymorpha*, fungal $^{14}CO_2$ production (mineralization) was accompanied by an increase (compared to the control) in water-soluble lignocellulose fragments as analysed by SEC coupled to photometric detection, whereas the total amount of water-soluble lignin fragments as determined by analysing the ^{14}C-label decreased (or remained essentially unchanged, depending on the culture condition) upon fungal treatment (Liers et al. 2006).

Water-extractable carbohydrates from fungi-treated lignocellulosic substrates were used as a rapid and simple indicator for overall fermentable sugar yields, where the strains most efficiently increasing the amounts of water-extractable total sugars from wheat straw (*Acephala* sp., *H. fasciculare*, *S. rugosoannulata*, *S. chlorohalonata* A-2008-2 and *S.* cfr. *chlorohalonata* A-2008-4; Fig. 5 a) also belong to the strains most efficiently delignifying this substrate (Fig. 2 a). A fairly close negative correlation between total sugars released and lignin concentrations generally observed during fungal treatment of wheat straw (Fig. S2; Table S5; both in the Supplementary Material) confirms lignin to be an important parameter influencing the digestibility of this substrate, as already demonstrated in previous studies (Salvachúa et al. 2011; Tuyen et al. 2012). The importance of the lignin

concentration (% dry mass, thus reflecting the lignin to dry mass ratio) for available sugar yields hence suggested for wheat straw by our study corroborates previous results demonstrating a strong positive correlation between the lignin-to-cellulose loss ratio and the in vitro gas production from fungi-pretreated wheat straw (Tuyen et al. 2012). Since the group of fungi yielding the highest increases in total sugar amounts from wheat straw (Fig. 5 a) comprises strains with high (*Acephala* sp.), intermediate (*H. fasciculare*, *S. rugosoannulata*) and rather low selectivity for lignin degradation (*S. chlorohalonata* A-2008-2, *S.* cfr. *chlorohalonata* A-2008-4; Fig 2 a), this characteristic cannot be considered as the decisive parameter leading to high amounts of water-soluble total sugars for this substrate. This conclusion corroborates previous results regarding relationships between fermentable sugar yields and delignification patterns of white-rot fungi (Salvachúa et al. 2011). No general correlation between total sugars and lignin concentrations could be monitored during fungal treatment of digestate (Fig. S3; Table S6; both in the Supplementary Material), where only three (*A. aegerita*, *Phoma* sp. AP2-2009-3 and B-2008-6) out of the eight most efficiently delignifying strains (lignin removal above 44 % of the initial content; Tables S3 and S4 in the Supplementary Material) substantially increased the amounts of water-extractable sugars above the control value (Fig. 5 b). This observation suggests that for digestate delignification is less decisive for high yields of fermentable sugars than for wheat straw. Contrary to the strains, most efficiently increasing total sugar amounts from wheat straw, *A. aegerita*, *Phoma* sp. AP2-2009-3 and B-2008-6A all exhibited a rather high selectivity for lignin degradation on digestate (Fig. 2 b). This again may indicate different interrelations between fermentable sugar yields and delignification patterns for wheat straw and digestate, which remains to be clarified.

Within the present study, various observations consistently suggest that digestate is more recalcitrant to fungal decomposition than the seemingly more easily accessible wheat straw: (1) On agar plates, the number of strains showing growth inhibition in presence of digestate (Fig. S1d in the Supplementary Material) was much higher than that of strains inhibited in the presence of wheat straw (Fig. S1b in the Supplementary Material). Such growth-inhibiting effects of digestate are in line with a reported decrease of the specific microbial growth rate after soil amendment with digestate resulting from biogas fermentation of maize (Chen et al. 2012). (2) A higher proportion of the tested fungal strains showed enhanced growth (i.e. a larger colony radius than on the respective control plate) in the presence of wheat straw (75 strains in total, Fig. S1b) than in the presence of digestate (37 strains in total, Fig. S1d). Furthermore, on wheat straw, more fungal strains with a comparatively faster growth (corresponding to higher colony radius ranges as allocated in Fig. S1a in the Supplementary Material) than on digestate were observed (Fig. S1c in the Supplementary Material). These observations resemble previously published data where digestate derived from maize-based biogas fermentation was found to be more resistant to biodegradation by soil microorganisms than maize straw (Chen et al. 2012). (3) The overall total dry mass losses observed for wheat straw clearly exceeded those recorded for digestate (Fig. 2; Tables S1–S4 in the Supplementary Material). (4) For digestate, higher amounts of water-extractable total sugars than in controls were only detected after 28 days of cultivation (vs. after both 18 and 28 cultivation days for wheat straw). Also, a lower number of fungal strains increased the total sugar amounts above the control value for digestate, as compared to wheat straw. Next, for digestate, these increases did not reach the extents obtained with wheat straw. Finally, the relative amounts of water-extractable carbohydrates of fungi-treated digestate were generally lower than those of fungi-treated wheat straw (Fig. 5). (5) Delignification of wheat straw was always completed after 18 days of cultivation, whereas digestate delignification proceeded until the end of the experiment for about 30 % of the tested fungal strains (Fig. 2). The modest negative correlation between lignin and dry mass data observed for digestate (Fig. S3; Table S6; both in the Supplementary Material) suggests that fungal consumption of this substrate is generally not much influenced by its lignin content and additionally hampered by factors other than lignin, which still remain to be defined. The cellulose and hemicellulose contents of wheat straw (reported values of about 37 to 44 % and approximately 23 to 28 % for cellulose and hemicelluloses, respectively; Salvachúa et al. 2011; Tuyen et al. 2012) are higher than those of digestate from maize-based biogas production (about 15 to 25 % cellulose and approximately 12 to 20 % hemicelluloses; Chen et al. 2012; Klimiuk et al. 2010), and hence, wheat straw potentially represents a more rich carbohydrate source than digestate. Commonly, digestate remaining from biogas fermentation is enriched particularly in NH_4-N (Chen et al. 2012; Klimiuk et al. 2010). Fungi are known to be sensitive towards free ammonia, which can be formed at inhibitory concentrations if sufficient alkalinity is provided (DePasquale and Montville 1990; Veverka et al. 2007).

The fungal potential for the efficient (pre)treatment of wheat straw and digestate seems not to be restricted to just one particular ecophysiological group or habitat and goes well beyond the group of white-rot basidiomycetes so far predominantly investigated in this respect. The delignifying abilities of ascomycetes generally are thought to be limited and not being able to compete with those of potent lignin-degrading basidiomycetes (Liers et al. 2006, 2011; Šnajdr et al. 2010). Nevertheless, our results demonstrate that particular lignocellulosic substrates can be delignified by certain ascomycetes and fungi from only rarely explored environments to extents coming close to (wheat straw) or sometimes even exceeding (digestate) those observed with the most efficiently

delignifying basidiomycetes (Fig. 2; Tables S1–S4 in the Supplementary Material). Together with accompanying increases in amounts of water-extractable total sugars (Fig. 5), these results suggest an as yet by far underexploited fungal potential to pretreat lignocellulosic feedstock for fermentative biofuel production, which will be assessed in the future.

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