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Systematic Search for Cultivatable Fungi That Best Deconstruct Cell Walls of *Miscanthus* and Sugarcane in the Field▽†

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The goals of our project were to document the diversity and distributions of cultivable fungi associated with decaying *Miscanthus* and sugarcane plants in nature and to further assess biodegradation of host plant cell walls by these fungi in pure cultures. Late in 2008 and early in 2009 we collected decaying *Miscanthus* and *Saccharum* from 8 sites in Illinois and 11 sites in Louisiana, respectively. To recover fungi that truly decay plants and to recover slow-growing fungi, we washed the plant material repeatedly to remove spores and cultivated fungi from plant fragments small enough to harbor at most one mycelium. We randomly selected 950 fungal colonies out of 4,560 microwell colonies and used molecular identification to discover that the most frequently recovered fungal species resided in *Hypocreales* (*Sordariomycetes*), *Pleosporales* (*Dothideomycetes*), and *Chaetothryiales* (*Eurotiomycetes*) and that only a few weedy species were recovered. We were particularly interested in *Pleosporales* and *Chaetothyriales*, groups that have not been mined for plant decay fungi. To confirm that we had truly recovered fungi that deconstruct plant cell walls, we assayed the capacity of the fungi to consume whole, alkali-pretreated, ground *Miscanthus*. Solid substrate cultures of the nine most commonly encountered *Ascomycota* resulted in *Miscanthus* weight loss of 8 to 13% over 4 weeks. This is the first systematic, high-throughput, isolation and biodegradation assessment of fungi isolated from decaying bioenergy grasses.

The biological conversion process of lignocellulosic plant cell walls to make renewable transportation fuels relies on the activity of fungal enzymes that convert polysaccharides into sugars. Among the plants best suited for bioconversion to make transportation fuels are C4 energy crops, e.g., *Miscanthus* and *Saccharum* (52). However, most research on fungal deconstruction of plant cell walls has focused on wood, which has cell walls that are very different from grasses (6). In this study, we systematically searched for fungi found in decaying bioenergy grasses to find species whose enzymes would better convert biomass plant.

Prime candidates for bioenergy crops are the perennial grasses *Miscanthus* × *giganteus* and its close relative, *Saccharum officinarum* (sugarcane), which are found in temperate and tropical areas, respectively. Both species are C4 plants, which are more efficient than C3 plants at converting light, water, and nutrient into harvestable biomass (23, 26, 52). Sugarcane is widely used in Brazil, where sugarcane-derived fuel provides more than 40% of gasoline demand (18). *Miscanthus* × *giganteus* is an allotriploid (*M. sinensis* × *M. sacchariflorus*) (29) that has been extensively studied for biomass conversion in the European Union (30, 35) and, more recently, in the midwestern United States (22).

Most research on fungal decay of plants has focused on the fungi that decay wood of both angiosperm and conifers (5, 12, 13, 20, 25, 32, 37). Wood decay fungi either deconstruct the lignin to expose more polysaccharide (white rot) or deconstruct the polysaccharide with minor modification of the lignin (brown rot). These fungi, almost always basidiomycetes, are adapted to long-term decay of large lignocellulosic resources, i.e., trees and wood in service. However, grass cell walls are very different from the cell walls of conifers, other angiosperms, and even other monocots (6), especially in their lignins, which differ even between C3 and C4 grasses (21). In nature, the fungi that decay wood have not been reported to decay grasses and, therefore, are not likely to be optimal for deconstruction of grass cell walls. The fungal enzymes used to convert polysaccharides to sugars are mostly obtained from mutants of *Trichoderma reesei*, an industrial strain cultivated from relatively pure cellulose of cotton cloth. Again, the cellulolytic enzymes obtained from this fungus may not be optimal for bioconversion of different types of bioenergy plants.

To find enzymes best suited to bioconversion of promising bioenergy plants, we sought to bring into cultivation the fungi that bioconvert *Miscanthus* and sugarcane cell walls in agricultural fields. We adopted the dilution-to-extinction culture methods developed by the pharmaceutical industry (3, 42). These methods allow for high throughput and aim in bioprospecting to recover both fast- and slow-growing fungi that actually grow in decaying plants rather than those that are simply present as spores.

Fungal ecologists have made strong efforts to study fungi associated with the phyllosphere and rhizosphere of living plants (2, 8, 31, 46, 60) or fungi that cause disease in energy crops (1, 28, 29, 34, 45, 57), but surprisingly few studies have focused on fungi that decay plants (17, 39, 40, 47, 55), and

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no study has used high-throughput, dilution-to-extinction methods to cultivate fungi from bioenergy crops.

To test the hypothesis that the fungi recovered from decaying bioenergy plants actually are responsible for the decay, their ability to decay the substrate must be assayed. Although this step has not been taken with any systematic, high-throughput culturing study, it has been applied to fungi cultivated from oak by using oak as the substrate for decay (55) and to a collection of nine fungi by using *Miscanthus* as the substrate (40).

Ours is the first comprehensive study to both exhaustively cultivate fungi from biofuel crops (*Miscanthus* and sugarcane) and then challenge the ability of the fungi to bioconvert the biofuel plant. In fact, prior to our study only one fungal species actually isolated from *Miscanthus* had been evaluated for bioconversion of that plant (40).

Here we sampled the fungi that decay temperate and tropical energy grasses by using high-throughput cultivation of fungi, starting from pieces of plants washed free of spores and small enough to harbor at most 1 CFU (4, 9). From 950 cultures, we used rDNA sequence comparisons to GenBank sequences to identify 106 operational taxonomic units (OTUs). Rarefaction analyses of samples from 17 fields and two batches of stored or processed grass showed that our sampling and isolation techniques likely recovered all of the common fungi and provided an adequate approach for the rare fungi. Our solid substrate culture experiments with the nine most commonly cultivated fungi showed that all these fungi effectively bioconverted *Miscanthus* biomass. We hope that our study will provide a basis for further study of energy crop-associated fungi and their enzymes that deconstruct plant cell walls.

MATERIALS AND METHODS

Sample collection. Dead leaves and stems of *Miscanthus* in contact with soil or at the bottom of plants were collected on 26 September 2008 from 7 farming sites with standing *Miscanthus* at the University of Illinois at Urbana-Champaign with these geographical coordinates: 40°5′25″N, 88°12′54″W; 40°2′27″N, 88°13′27″W; 40°2′29″N, 88°13′28″W; 40°2′29″N, 88°13′30″W; 40°2′31″N, 88°13′28″W; 40°2′34″N, 88°13′31″W; 40°2′34″N, 88°14′17″W. The annual temperature and precipitation for Urbana, IL, are 50.6°F (10.3°C; September average, 19.4°C) and 1,350 mm (38). For the fungi that decay *Saccharum* (sugarcane) in the field, leaves and stems of sugarcane in contact with the soil were collected on 22 January 2009 from 10 plantation sites with no standing sugarcane near Baton Rouge, LA, with these geographical coordinates: 30°16′19″N, 91°5′43″W; 30°1′18″N, 90°47′00″W; 30°1′16″N, 90°47′00″W; 30°4′4″N, 90°41′48″W; 30°4′1″N, 90°41′42″W; 30°0′11″N, 90°44′34″W; 29°43′52″N, 90°35′51″W; 29°43′53″N, 90°35′54″W; 29°44′14″N, 90°36′28″W; 29°45′19″N, 90 42′09″W. The annual temperature and precipitation for Baton Rouge, LA, are 69°F (20.5°C; January average, 10.5°C) and 1,690 mm (38a). To sample fungi that decay stored *Miscanthus* or processed sugarcane, baled *Miscanthus* samples were collected on 26 September 2008 from a site at the University of Illinois (40°5′39″N, 88°14′3″W) and sugarcane bagasse samples were collected on 22 January 2009 from Raceland Raw Sugar Corporation, Raceland, LA (29°44′2″N, 90°35′26″W).

At each field or plantation site, 16 samples were taken with intersite distances ranging from 0.5 m to 11.3 m by sampling at the corners of nested squares with sides of 0.5, 1, 2, 4, and 8 m. *Miscanthus* bales and sugarcane bagasse were sampled where plant materials appeared decayed. The collected samples, in paper bags, were transported to the lab.

Sample processing, high-throughput culture, and isolation. The 16 samples from each of the 19 sites were air dried at room temperature for 2 days and then cut into 1-cm lengths. To isolate and cultivate fungi, the remaining cut material from the 16 samples at each collection site was combined to make one composite sample for each of the 19 field, plantation, and bulk samples.

We followed the particle filtration process described by Bills et al. (4) to obtain plant fragments with at most one culturable fungal CFU. For each composite sample, enough material to fill a 10-cm petri dish was mixed with 200 ml sterile water, and the mixture was blended (Waring blender; Waring Laboratory and Sciences, Torrington, CT) for 1 min. The particle slurry was then strained through a stack of three 51-mm-diameter polypropylene mesh screens (microsieve set, product number 378451000; Mini-Seive, Pequannock, NJ) with pore sizes of 1 mm, 210 μm, and 105 μm (Spectra Mesh woven filters; Spectrum Labs, Rancho Dominguez, CA). To remove the fungal spores that happened to be present on plant surfaces, the residues were washed in 2 liters of sterile water flowing through the sieve assembly under gravity assisted by vacuum. Particles collected on the 105-μm sieve were suspended in 30 ml of 0.2% aqueous carboxymethyl cellulose.

We tested a range of dilutions for each sample (i.e., undiluted and dilutions of 10-, 50-, 100-, and 200-fold) to determine the dilution appropriate to deliver at most 1 CFU to each well of a 48-microwell plate (Falcon plates, product no. 351178; Becton Dickinson and Company, Franklin Lakes, NJ). For each dilution of each sample, 5 μl was inoculated into one well containing 990 μl of YM broth (2 g yeast extract, 10 g malt extract, 1 liter deionized water) with antibiotics (final concentrations, 50 mg/liter each of streptomycin sulfate and oxytetracycline) as described by Bills et al. (4). The 48-microwell plates were sealed with lids and incubated at 25°C in constant light for 1 month.

To select filamentous fungal colonies likely to have arisen from a single CFU, mycelia were selected from plates where at least one-third (16 of 48) of the wells were not colonized. If 10 or fewer wells had mycelia, all were selected. If more than 10 wells had mycelia, 10 were randomly selected. Mycelia were transferred to YM agar (YM broth with 1.5% agar) plates with antibiotics (50 mg/liter each of streptomycin sulfate and oxytetracycline). The petri dishes were sealed with parafilm and incubated at 25°C in constant light for 2 weeks.

DNA extraction, PCR, rDNA sequencing, and BLAST searches. Extraction of DNA from colonies growing on agar involved sterile toothpick transfers of hyphae from YM agar plates into individual wells in a 96-microwell PCR plate, each containing 10 μl of extraction buffer (REDExtract-N-Amp plant PCR kit; Sigma Aldrich, St. Louis, MO). To mix transferred hyphae and extraction buffer, the PCR plates were centrifuged at 2,000 × *g* for 1 min in a benchtop centrifuge machine (Eppendorf centrifuge 5804; Brinkmann Instrument Inc., Westbury, NY). To extract DNA for use as PCR template, the 96-well plates were then heated in a thermocycler (PTC-100; MJ Research Inc., Watertown, MA) first at 65°C for 10 min and then at 95°C for another 10 min. Twenty microliters of dilution buffer (REDExtract-N-Amp plant PCR kit; Sigma Aldrich, St. Louis, MO) was added to each well, and the plates were sealed with 3M plastic tape, centrifuged at 2,000 × *g*, kept at room temperature for 2 to 3 h, and finally stored in a refrigerator at 4°C.

Two primer pairs, ITS1F/ITS4 (16, 59) and CTB6/LR3 (CTB6, GCATATCA ATAAGCGGAGG [unpublished data] and LR3 [27]) were used to amplify the internal transcribed spacer (ITS1, 5.8s, and ITS2) and portion of the large subunit (LSU) of nuclear rDNA (28s rDNA), respectively. For each reaction mixture, 2.5 μl of diluted template DNA was transferred into each well in a 96-well PCR plate, followed by 22.5 μl of the master mixture containing 2.5 μl 10× PCR buffer, 2.5 μl 10× deoxynucleoside triphosphates (dNTPs), 5 μl 50 μM primer pairs (1:1; ITS1F/ITS4 or CTB6/LR3), 0.25 μl of *Taq* polymerase, and 16.75 μl of deionized water. The plates were centrifuged at 2,000 × *g* for 1 min and then placed in a thermocycler that was programmed for 94°C for 1 min; 34 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min; 72°C for 8 min; 10°C hold.

The quality of PCR amplification was assessed by agarose gel electrophoresis of the PCR product in 1% agarose in Tris-acetate-EDTA (TAE) buffer for 2 h at 180 mA. The gel was then stained in 0.5 μg/ml ethidium bromide for 20 min, destained in the same buffer for 20 min, rinsed with water, and photographed with a charge-coupled-device camera using a UV imager (Eagle Eye; Stratagene, Agilent Technologies, La Jolla, CA).

PCR amplified fungal rDNA was purified from unused primers and unincorporated dNTPs by mixing 3.5 ml of PCR product with 1.5 ml of diluted Exosap-IT (1 μl deionized water and 0.5 ml Exosap-IT (USB Corporation, Cleveland, OH) in new PCR plates followed by centrifugation at 2,000 × *g* for 1 min, incubation at 37°C for 45 min, incubation at 80°C for 15 min, and storage at 8°C.

Both strands of the cleaned PCR products were sequenced using BigDye v3.1 (Applied Biosystems) and an Applied Biosystems 96 capillary 3730xl DNA analyzer. The resultant sequences were edited and corrected using the ABI Prism sequence navigator v1.0.1 (Perkin-Elmer, Waltham, MA), Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI), and CodonCode Aligner v3.0.3 (CodonCode Corporation, Dedham, MA).

We used the program CD-HIT (cluster database at high identity with tolerance; www.bioInformatics.org) to find the nonredundant set of sequences with similarity of 98%. To provisionally identify the DNA sequences as fungal OTUs

TABLE 1. Isolated fungal cultures from *Miscanthus* samples[a]

Sample no.	Isolate ID	Total no. of each OTU	OTU	GenBank accession no.	Closest BLAST match (GenBank accession no., species name)	% identity
1	MS5p50-7	98	*Hypocrea* aff. *koningii*	HQ630959	AJ301990, *Hypocrea koningii*	98.0
2	MS3p_50-23	40	*Hypocrea* aff. *lixii*	HQ630960	EF392757, *Hypocrea lixii*	100.0
3	MSbale50-22	28	*Arthrinium* aff. *sacchari*	HQ630961	AF393679, *Arthrinium sacchari*	99.2
4	MSbale50-9	26	*Trichoderma* aff. *spirale*	HQ630962	EU280068, *Trichoderma spirale*	99.8
5	MS5p50-9	15	*Phoma* aff. *herbarum*	HQ630963	AB369456, *Phoma herbarum*	100.0
6	MS7p50-21	12	*Fusarium* aff. *aethiopicum*	HQ630964	FJ240306, *Fusarium aethiopicum*	100.0
7	MS2-4	12	*Fusarium* aff. *proliferatum*	HQ630965	EF577235, *Fusarium proliferatum*	99.8
8	MS7p50-29	10	*Gibberella* aff. *moniliformis*	HQ630966	EU364864, *Gibberella moniliformis*	100.0
9	MS3p_50-12	9	*Arthrinium* aff. *phaeospermum*	HQ630967	AJ279447, *Arthrinium phaeospermum*	99.2
10	MS3p_50-38	9	*Cordyceps* aff. *bassiana*	HQ630968	AJ560684, *Cordyceps bassiana*	100.0
11	MSbale50-8	9	*Trichoderma* aff. *atroviride*	HQ630969	EU280107, *Trichoderma atroviride*	100.0
12	MS3p_50-33	8	*Alternaria* aff. *tenuissima*	HQ630970	AY154709, *Alternaria tenuissima*	100.0
13	MS6p50-33	6	*Cladosporium* aff. *cladosporioides*	HQ630971	AY251074, *Cladosporium cladosporioides*	99.8
14	MS7p50-17	6	*Epicoccum* aff. *nigrum*	HQ630972	AJ279448, *Epicoccum nigrum*	99.8
15	MS3p_50-35	4	*Cephalosporium* aff. *gramineum*	HQ630973	AY428791, *Cephalosporium gramineum*	97.9
16	MS3p_50-45	4	*Minimidochium* sp. 1	HQ630974	FN394724, *Minimidochium* sp. 3871	92.0
17	MS5p50-1	3	*Hypocrea* aff. *koningii*	HQ630975	AJ301990, *Hypocrea koningii*	98.8
18	MS6p50-29	3	*Fusarium* aff. *equiseti*	HQ630976	FJ481025, *Fusarium equiseti*	100.0
19	MS7p50-6	3	*Gibberella* aff. *avenacea*	HQ630977	AY147283, *Gibberella avenacea*	99.8
20	MSbale50-42	3	*Chloridium* sp. 1	HQ630978	GQ331985, *Chloridium* sp. GHJ-3	99.0
21	MS5p50-12	2	*Cephalosporium* aff. *gramineum*	HQ630979	AY428791, *Cephalosporium gramineum*	99.5
22	MS5p50-23	2	*Ceratobasidium* sp. 1	HQ630980	AF472298, *Ceratobasidium* sp. JTO115	97.0
23	MS5p50-32	2	*Microdochium* aff. *bolleyi*	HQ630981	AJ279454, *Microdochium bolleyi*	99.8
24	MS5p50-34	2	*Nigrospora* aff. *oryzae*	HQ630982	EU272503, *Nigrospora oryzae*	98.8
25	MS5p50-47	2	*Phaeosphaeriopsis* sp. 1	HQ630983	DQ885894, *Phaeosphaeriopsis musae*	92.9
26	MSbale50-11	2	*Sporothrix* aff. *lignivora*	HQ630984	EF127887, *Sporothrix lignivora*	99.8
27	MS1-48	2	*Arthrinium* aff. *phaeospermum*	HQ630985	AJ279447, *Arthrinium phaeospermum*	97.2
28	MS3p_50-2	1	*Trichoderma* aff. *saturnisporum*	HQ630986	Z48726, *Trichoderma saturnisporum*	100.0
29	MS3p_50-29	1	*Cephalosporium* sp.1	HQ630987	AY428791, *Cephalosporium gramineum*	93.6
30	MS3p_50-44	1	*Chalara* sp.1	HQ630988	EF029209, *Chalara dualis*	95.3
31	MS3p_50-5	1	*Mucor* aff. *hiemalis*	HQ630989	EU326196, *Mucor hiemalis*	100.0
32	MS4p_50-2	1	*Exophiala* aff. *salmonis*	HQ630990	AY213652, *Exophiala salmonis*	97.9
33	MS4p_50-34	1	*Hypocrea* aff. *lixii*	HQ630991	EU280078, *Hypocrea lixii*	98.3
34	MS5p50-27	1	*Phaeosphaeria* sp.1	HQ630992	AJ496632, *Phaeosphaeria pontiformis*	94.4
35	MS6p50-31	1	*Paraphaeosphaeria* aff. *michotii*	HQ630993	AF250817, *Paraphaeosphaeria michotii*	99.1
36	MSbale50-40	1	*Chaetosphaeria* aff. *chloroconia*	HQ630994	AF178542, *Chaetosphaeria chloroconia*	97.5
37	MS2-1	1	*Microdochium* sp. 1	HQ630995	AJ279454, *Microdochium bolleyi*	96.9
38	MS2-18	1	*Alternaria* aff. *longissima*	HQ630996	EU030349, *Alternaria longissima*	100.0
39	MS2-40	1	*Fusarium* aff. *sporotrichioides*	HQ630997	AF414972, *Fusarium sporotrichioides*	99.1
40	MS2-9	1	*Trichoderma* aff. *brevicompactum*	HQ630998	EU280087, *Trichoderma brevicompactum*	99.8

[a] Ribosomal nucleotide sequences (ITS1f) were matched against the closest BLAST-matched species. BLAST matches above 90% sequence similarity are shown. For ≥97% sequence similarity, the OTUs are reported as genus and species name with "aff." in between as a qualifier to note that they have affinity to the species matched. Matches between 97% and 93% were given the generic name of the match plus a number. Some OTUs were also given a genus name followed by a number when their nucleotide sequence matched ≥97% with the closest BLAST match that had only genera names without full species identification (for example, *Chloridium* sp. 1).

(≥97% sequence similarities), the nonredundant sequences were retained and compared, using the Basic Local Alignment Search Tool (BLAST), to the sequences of known fungi archived at GenBank, maintained by the National Center for Biotechnology Information. We have used the term *affinis* (aff.) to indicate that OTUs are similar but not necessarily identical to the described species.

Selection of biomass pretreatment and fungal biodegradation via solid substrate cultures. Ground (1-mm sieve size) *Miscanthus* was pretreated using three methods. Untreated *Miscanthus* was used as a control. The methods assessed were the following: (i) hot water, with autoclaving at 121°C for 1 h the ground *Miscanthus* in water, at a solid:liquid ratio of 1:10; (ii) dilute acid, with heating by microwave to 180°C for 2 min the ground *Miscanthus* in 1% (wt/vol) sulfuric acid, at a solid:liquid ratio of 1:10; (iii) mild alkali, with constant stirring at 25°C for 24 h the ground *Miscanthus* in 0.5% (wt/vol) sodium hydroxide, at a solid:liquid ratio of 1:10; (iv) no pretreatment (control).

Following pretreatment, the biomass residues were rinsed 3 times, each with 2 liters of deionized water, and the biomass was recovered by centrifugation at 8,631 × *g* (7,500 rpm) for 10 min. The residues were rinsed a final time with 2 liters of deionized water, and the pH was adjusted to 5 ± 0.2 by adding acid or alkali. Following a final centrifugation, all extra liquid was squeezed from the wet residues, which were then air dried for 2 days followed by 48 h of lyophilization.

We assessed fungal biodegradation of *Miscanthus* via a modified solid substrate fungal culture protocol (44, 48–50) to carry out high-throughput fungal culture in 14-ml polypropylene tubes (Falcon 352059; Becton, Dickinson and Company, Franklin Lakes, NJ) stoppered with a sterile plastic foam plug (catalog no. 14-127-40B; Fisher Scientific, Pittsburg, PA). Each tube contained 0.6 g of dry, pretreated *Miscanthus* material and three 5-mm glass beads. The tube, plug, and contents were weighed and then autoclaved. The tubes were then inoculated with 2 ml of standardized fungal inoculum in Vogel's broth with no added carbon source (58). To incorporate the average dry weights of fungal inocula into respective initial dry biomass weights, 2 ml of fungal inoculum per species was also collected in preweighed 5-ml polypropylene tubes, which were lyophilized and weighed. The plugged tubes were vortexed so that the glass beads would mix and uniformly spread the fungal inoculum and *Miscanthus* along the length of the tube, leaving a hollow space in the middle to promote gas exchange during growth. The tubes were incubated horizontally at 25 ± 2°C at high, constant

TABLE 2. Isolated fungal cultures from sugarcane samples[a]

Sample no.	Isolate ID	Total no. of each OTU	OTU	GenBank accession no.	Closest BLAST match (GenBank accession no., species)	% identity
1	sc13d50p14-6	99	*Phoma* aff. *glomerata*	HQ630999	EU273521, *Phoma glomerata*	100
2	sc14d1p16-14	50	*Phoma* aff. *herbarum*	HQ631000	AB369456, *Phoma herbarum*	99.8
3	sc12d100p8-2	24	*Trichoderma* aff. *atroviride*	HQ631001	EU280107, *Trichoderma atroviride*	100
4	sc15d10p10-10	23	Pleosporales sp. 1	HQ631002	DQ018090, *Dictyosporium heptasporum*	90.4
5	sc8d50p14-1	23	*Cladosporium* aff. *cladosporioides*	HQ631003	AJ300334, *Cladosporium cladosporioides*	99.8
6	sc12d1p13-6	18	*Hypocrea* aff. *lixii*	HQ631004	EU280078, *Hypocrea lixii*	99.8
7	sc8d100p16-14	12	*Fusarium* aff. *equiseti*	HQ631005	EU595566, *Fusarium equiseti*	100
8	sc15d200p6-2	9	*Pleosporales* sp. 2	HQ631006	DQ018094, *Dictyosporium subramanianii*	88.8
9	BGd100p3-1	8	*Penicillium* aff. *minioluteum*	HQ631007	AF380354, *Penicillium minioluteum*	100
10	sc10d50p8-8	7	*Dothideomycete* sp.	HQ631008	FJ752617, fungal sp. F1-1	98
11	sc9d100p9-2	6	*Bipolaris* sp. 1	HQ631009	GQ280376, *Bipolaris* sp. Vic-3	99
12	sc9d50p12-1	6	*Candida* aff. *fukuyamaensis*	HQ631010	AM158923, *Candida fukuyamaensis*	99
13	sc13d100p7-6	5	*Lecythophora* aff. *decumbens*	HQ631011	FN428890, *Lecythophora* aff. *decumbens*	99
14	sc8d50p14-4	5	*Dokmaia* sp. 1	HQ631012	GU973777, *Dokmaia* sp. ASR-227	98
15	sc8d50p14-8	4	*Aureobasidium* aff. *pullulans*	HQ631013	EF197817, *Aureobasidium pullulans*	100
16	sc13d1p11-4	4	*Cryptococcus* aff. *flavescens*	HQ631014	AB085803, *Cryptococcus flavescens*	98.1
17	sc8d10p9-6	4	*Pleosporales* sp. 3	HQ631015	EF060849, *Pleosporales* sp. LM561	94
18	sc10d1p11-1	3	*Fusarium* aff. *proliferatum*	HQ631016	FN868470, *Fusarium proliferatum*	99
19	sc17d100p18-12	3	*Occultifur* aff. *externus*	HQ631017	FN428928, *Occultifur* aff. *externus*	98
20	sc8d100p16-13	3	*Phaeosphaeria* sp. 2	HQ631018	DQ092527, *Phaeosphaeria* sp. HKC12	99
21	sc8d200p6-4	3	*Microbotryomycetes* sp. 1	HQ631019	DQ870625, *Rhodotorula* sp. CECT 11976	85
22	sc9d50p12-11	3	*Candida* sp. 1	HQ631020	FJ873586, *Candida* sp. GJ15M15	94
23	BGd1p19-4	2	*Aspergillus* aff. *fumigatus*	HQ631021	FJ227896, *Aspergillus fumigatus*	100
24	sc10d100p9-2	2	*Cyphellophora* sp. 1	HQ631022	EU035416, *Cyphellophora laciniata*	96.6
25	sc10d10p11-8	2	*Epicoccum* aff. *nigrum*	HQ631023	EU272495, *Epicoccum nigrum*	99.8
26	sc11d100p8-2	2	*Cryptococcus* sp. 1	HQ631024	FJ153175, *Cryptococcus* sp. SJ8L05	98
27	sc11d10p11-11	2	*Phoma* aff. *leveillei*	HQ631025	FJ571477, *Phoma leveillei*	98.1
28	sc11d10p11-3	2	*Leptoxyphium* aff. *madagascariens*	HQ631026	GQ303277, *Leptoxyphium madagascariens*	98
29	sc12d100p8-7	2	*Exophiala* aff. *spinifera*	HQ631027	AY484985, *Exophiala spinifera*	99.7
30	sc13d100p7-2	2	*Periconia* aff. *macrospinosa*	HQ631028	FJ536208, *Periconia macrospinosa*	97.3
31	sc13d100p7-5	2	*Candida* aff. *akabanensis*	HQ63129	EU100744, *Candida akabanensis*	99.4
32	sc13d10p12-7	2	*Pichia* aff. *membranifaciens*	HQ631030	DQ104722, *Pichia membranifaciens*	100
33	sc13d200p1-1	2	*Tremella* aff. *globispora*	HQ631031	FN428949, *Tremella* aff. *globispora* IMUFRJ	99
34	sc15d50p10-8	2	*Cryptococcus* sp. 2	HQ631032	GQ181171, *Cryptococcus* sp. QMW-2009a	99
35	sc16d50p9-9	2	*Dothideomycete* sp. 1	HQ631033	*Dothideomycete* sp. 1	
36	sc17d100p18-16	2	*Dothideomycete* sp. 2	HQ631034	*Dothideomycete* sp. 2	
37	sc17d200p8-1	2	*Tremella* aff. *globispora*	HQ631035	FN428922, *Tremella* aff. *globispora* IMUFRJ	99
38	Sc13-4-5	2	*Fusarium* aff. *sporotrichioides*	HQ631036	AF414972, *Fusarium sporotrichioides*	99.2
39	BGd100p3-2	1	*Scytalidium* sp. 1	HQ631037	HM214453, *Scytalidium lignicola*	95
40	BGd10p15-14	1	*Zopfiella* sp. 1	HQ631038	AY999128, *Zopfiella karachiensis*	95.9
41	BGd10p15-15	1	*Cercophora* sp. 1	HQ631039	AY999135, *Cercophora caudata*	93.7
42	BGd1p19-12	1	*Penicillium* aff. *daleae*	HQ631040	DQ132832, *Penicillium daleae*	99.2
43	BGd1p19-17	1	*Paecilomyces* sp. 1	HQ631041	EF550986, *Paecilomyces* sp. MTCC6328	98
44	BGd1p19-3	1	*Penicillium* aff. *pinophilum*	HQ631042	AB369480, *Penicillium pinophilum*	99.8
45	sc11d100p8-1	1	*Ascomycota* sp.	HQ631043	*Ascomycota* sp.	
46	sc11d100p8-8	1	*Hypocreales* sp. 1	HQ631044	AJ301999, *Myrothecium verrucaria*	90
47	sc11d10p11-8	1	*Capnodium* sp. 1	HQ631045	AY805548, *Capnodium* sp. olrim506	97
48	sc11d50p13-2	1	*Bullera* aff. *sinensis*	HQ631046	AF444468, *Bullera sinensis*	100
49	sc12d100p8-5	1	*Sordariomycete* sp. 1	HQ631047	*Sordariomycete* sp. 1	
50	sc12d10p12-12	1	*Phoma* sp. 2	HQ631048	AF218789, *Phoma* sp. 2	98
51	sc12d10p12-6	1	*Exophiala* aff. *salmonis*	HQ631049	AY213652, *Exophiala salmonis*	96.7
52	sc12d1p13-3	1	*Bipolaris* aff. *zeicola*	HQ631050	GQ167208, *Bipolaris zeicola*	98
53	sc12d200p4-3	1	*Pleosporales* sp. 4	HQ631051	GU230751A, *Scochyta manawaorae*	94
54	sc12d50p8-4	1	*Pleosporales* sp. 5	HQ631052	GU361946, *Dictyosporium heptasporum*	91
55	sc12d50p8-5	1	*Stilbella* sp. 1	HQ631053	DQ993633, *Stibella* sp. RM5-6	99
56	sc13d10p12-2	1	*Penicillium* sp. 1	HQ631054	DQ123635, *Penicillium* sp. NRRL 35186	98
57	sc13d1p11-8	1	*Hypocreales* sp. 2	HQ631055	HQ115699, *Hypocreales* sp. NG_p26	100
58	sc13d50p14-5	1	*Tremellaceae* sp. 1	HQ631056	EU673082, *Tremella encephala*	87.8
59	Sc14-14-2	1	*Fusarium* aff. *sacchari*	HQ631057	EF453121, *Fusarium sacchari*	99.8
60	Sc15-15-4	1	*Myrothecium* sp. 1	HQ631058	AJ301998, *Myrothecium* sp. BBA69174	99

Continued on following page

TABLE 2—*Continued*

Sample no.	Isolate ID	Total no. of each OTU	OTU	GenBank accession no.	Closest BLAST match (GenBank accession no., species)	% identity
61	sc15d100p10-3	1	*Pleosporales* sp. 5	HQ631059	*AY864822 Phoma herbarum*	90.4
62	sc15d100p10-8	1	*Dothideomycete* sp. 3	HQ631060	*Dothideomycete* sp. 3	
63	sc16d1p11-2	1	*Curvularia* sp. 1	HQ631061	GQ184733, *Curvularia* sp. HSAUP074064	100
64	sc17d100p18-10	1	*Myrmecridium* aff. *schulzeri*	HQ631062	EU041777, *Myrmecridium schulzeri*	99.8
65	sc17d100p18-11	1	*Exophiala* aff. *salmonis*	HQ631063	AY213652, *Exophiala salmonis*	99.7
66	sc17d100p18-15	1	*Acremonium* sp. 1	HQ631064	EF042104, *Acremonium* sp. CBS 109930	99
67	sc17d100p18-4	1	*Paraphaeosphaeria* sp. 1	HQ631065	GU973660, *Paraphaeosphaeria* sp. ASR-77	99
68	sc8d100p16-11	1	*Candida* aff. *metapsilosis*	HQ631066	EU564207, *Candida metapsilosis*	98.7
69	sc8d10p9-5	1	*Myrothecium* sp. 2	HQ631067	*Myrothecium* sp.	
70	sc8d50p14-5	1	*Dokmaia* sp. 2	HQ631068	GU973777, *Dokmaia* sp. ASR-227	99
71	sc9d10p14-10	1	*Ustilago* sp.	HQ631069	*Ustilago* sp.	
72	sc9d1p7-1	1	*Nigrospora* sp. 1	HQ631070	EU272498, *Nigrospora oryzae*	95
73	sc9d50p12-4	1	*Pichia* aff. *anomala*	HQ631071	AB469881, *Pichia anomala*	100

[a] Ribosomal nucleotide sequences (ITS1f) were matched against the closest BLAST-matched species. BLAST matches above 90% sequence similarity are reported here. For ≥97% sequence similarity, the OTUs are reported with the genus and species name, with aff. in between as a qualifier to note that they have affinity to the species matched. Matches between 97 and 93% were given a genus name for the match plus a number. Some OTUs were also given a genus name followed by numbers where their nucleotide sequences matched ≥97% with the closest BLAST match that had only genus names without full species identification (for example, *Bipolaris* sp. 1).

relative humidity (85% ± 5%) for as many as 28 days of growth. For each fungus, 12 replicate tubes were inoculated to provide for three sample tubes each on days 0, 7, 14, and 28. Each of 12 control tubes were inoculated with 2 ml Vogel's medium and no fungus.

Neurospora crassa (D140) was used to test *Miscanthus* pretreatment methods for further biodegradation studies of fungi cultivated from energy grasses. To prepare the inoculum, fungi were grown at 30°C and 220 rpm for 1 week in 125-ml Erlenmeyer flasks containing 50 ml YM broth with antibiotics (50 mg/liter

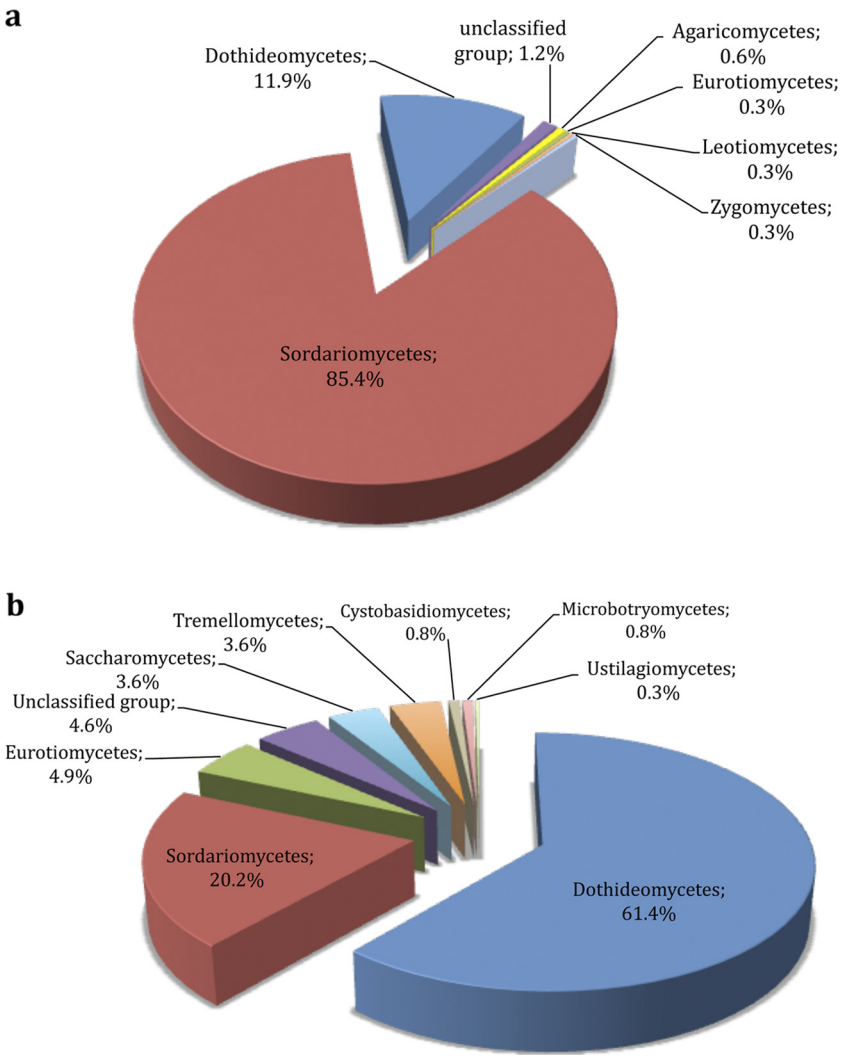

FIG. 1. Diversity of fugal OTUs isolated from *Miscanthus* and sugarcane samples.

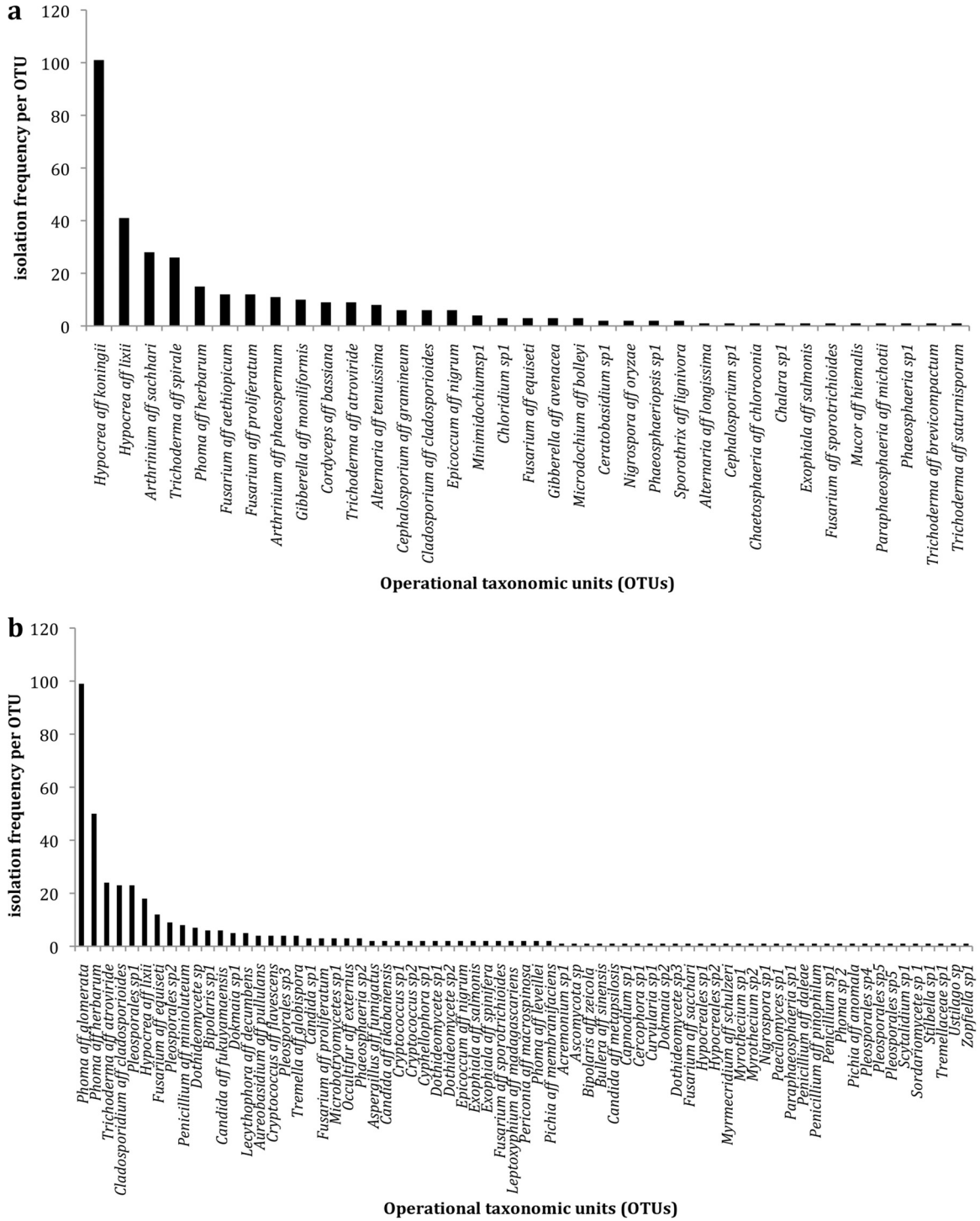

FIG. 2. Abundance curves for fungal OTUs isolated from *Miscanthus* (a) and sugarcane (b) fields.

streptomycin sulfate and oxytetracycline). The resulting mycelia were fragmented in sterilized laboratory Waring blenders using three, 10-s blendings, each followed by a 5-s pause. The hyphal fragment slurries were then poured back into the same 125-ml flasks and incubated for 24 h to produce many small mycelial colonies. The young mycelia were washed three times in sterile 0.85% saline (wt/vol; NaCl in water) and recovered each time by centrifugation (at 5,000 × *g* for 15 min at 4°C). The final hyphal pellet was resuspended in 40 ml of Vogel's medium with antibiotics (50 mg/liter streptomycin sulfate and oxytetracycline), mixed, and used to inoculate culture tubes as described above.

Analyses. (i) Statistical analyses on adequacy of sampling and fungal diversity. Fungal species abundance curves, rarefaction curves for each sampling site, and species dissimilarity indices across sampling sites were computed with EstimateS Mac 8.2 (10) using 500 data sets, for which the species order had been randomized by resampling without replacement. We made estimates of species

TABLE 3. Abundance of fungal species at each of the *Miscanthus* plantation sites (MS1 to MS7) and bale storage (MS bale) site

OTU	Total for all sites (n =335)	No. of indicated OTU at site (total no. of isolates at site)							
		MS1 (44)	MS2 (33)	MS3 (41)	MS4 (44)	MS5 (42)	MS6 (46)	MS7 (43)	MS bale (42)
Hypocrea aff. *koningii*	101	4	11	13	13	21	17	17	5
Hypocrea aff. *lixii*	41	1	2	2	24	1	6		5
Arthrinium aff. *sacchari*	28	22						5	1
Trichoderma aff. *spirale*	26				1		1		24
Phoma aff. *herbarum*	15	1	1		1	2	9	1	
Fusarium aff. *aethiopicum*	12	3	7	1				1	
Fusarium aff. *proliferatum*	12		2	1	1	1		7	
Arthrinium aff. *phaeospermum*	11	7		4					
Gibberella aff. *moniliformis*	10	5						5	
Cordyceps aff. *bassiana*	9			9					
Trichoderma aff. *atroviride*	9				1	4		3	1
Alternaria aff. *tenuissima*	8		2	1		2	2	1	
Cephalosporium aff. *gramineum*	6			3		2	1		
Cladosporium aff. *cladosporioides*	6			1		2	3		
Epicoccum aff. *nigrum*	6			1	1	1	2	1	
Minimidochium sp. 1	4		2	1			1		
Chlorodium sp. 1	3								3
Fusarium aff. *equiseti*	3		2				1		
Gibberella aff. *avenacea*	3	1						2	
Microdochium aff. *bolleyi*	3		2			1			
Ceratobasidium sp. 1	2					2			
Nigrospora aff. *oryzae*	2			1		1			
Phaeosphaeriopsis sp. 1	2					1	1		
Sporothrix aff. *lignivora*	2								2
Alternaria aff. *longissima*	1		1						
Cephalosporium sp. 1	1			1					
Chaetosphaeria aff. *chloroconia*	1								1
Chalara sp. 1	1				1				
Exophiala aff. *salmonis*	1						1		
Fusarium aff. *sporotrichioides*	1		1						
Mucor aff. *hiemalis*	1			1					
Paraphaeosphaeria aff. *michotii*	1						1		
Phaeosphaeria sp. 1	1			1					
Trichoderma aff. *brevicompactum*	1					1			
Trichoderma aff. *saturnisporum*	1				1				

number based on the species actually sampled, e.g., Mao Tau (10), and estimates of total species richness (nonparametric Jackknife 1 estimator [24, 51]). To investigate the relationship between the presence of species and spatial distance, we compared distance and community (or assemblage) dissimilarities (dissimilarity index = 1 − Jaccard similarity index [7]) matrices using the statistical program R 2.11.1 (43) and assessed significance by Mantel's test (36). To detect relationships, if any, between the spatially distributed *Miscanthus* and sugarcane plants and the respective fungal species compositions, we used the statistical R program for nonparametric multidimensional scaling (NMDS) analyses to graphically ordinate samples in two dimensions (54).

(ii) Percentage biomass weight loss as a measure of fungal biodegradation of *Miscanthus*. At each sampling, culture tubes were frozen overnight at −80°C and lyophilized to dryness over 48 h. Biomass weight loss was determined as the difference in initial and final dry weights as a percentage of the initial dry weight. The initial dry weight included the dry weight of culture tubes with ground *Miscanthus*, foam cap, and glass beads plus the average dry weight (n = 3) of each fungal inoculum. The culture residues were stored at −80°C for future analyses of sugar, proteins, and cell wall components.

RESULTS

Identification of fungal OTUs in *Miscanthus* and sugarcane samples. Using BLAST matches, we were able to identify OTUs for 724 of the 950 cultures; rDNA sequences for the remaining 226 samples were poor and not used. There were 335 sequence reads from *Miscanthus* that represented 35 fungal OTUs and 389 from sugarcane that represented 71 OTUs (Tables 1 and 2). Nine OTUs were found on both substrates. The results of this search for each cultivated fungus, based on GenBank accession numbers HQ630959 to HQ631071, are presented in Tables 1 and 2.

Ascomycetous fungi dominated (94%) the total fungal diversity of all the isolates from *Miscanthus* and sugarcane samples. *Basidiomycota* were the next most common at 3% of the total diversity, and a single *Mucoromycotina* species (*Mucor haemalis*) was isolated from a *Miscanthus* sample. Unclassified sequence comprised 3% of the fungal diversity. Most *Ascomycota* cultivated from *Miscanthus* (Fig. 1a) belonged to two classes: *Sordariomycetes* (85.4%) and *Dothideomycetes* (11.9%). Representatives of fungi belonging to other classes, i.e., *Agaricomycetes*, *Eurotiomycetes*, *Leotiomycetes*, and *Zygomycetes*, were 1.5% of the OTUs, and 1.2% could not be classified. With *Ascomycota* cultivated from sugarcane, the same two classes dominated, but *Dothideomycetes* were the most common (61.4%), with *Sordariomycetes* second (20.2%), followed by *Eurotiomycetes* (4.9%), *Saccharomycetes* (3.6%), and *Tremellomycetes* (3.6%) (Fig. 1b). Representatives of fungi belonging to other classes, i.e., *Cystobasidiomycetes*, *Microbotryomycetes*, and *Ustilagiomycetes*, accounted for 1.9% of OTUs, and 4.6% could not be classified.

TABLE 4. Abundance of fungal species at each of the sugarcane plantation sites (SC1 to SC10) and the bagasse site

OTU	Total for all sites (n = 398)	No. of indicated OTU at site (total no. of isolates at site)										
		SC1 (41)	SC2 (43)	SC3 (37)	SC4 (44)	SC5 (43)	SC6 (39)	SC7 (6)	SC8 (39)	SC9 (42)	SC10 (43)	Bagasse (12)
Phoma aff. *glomerata*	99	4	8	17	15	9	5	1	3	22	15	
Phoma aff. *herbarum*	50	2	5	5	5	2	2	2	10	7	10	
Trichoderma aff. *atroviride*	24					10	10		3	1		
Cladosporidium aff. *cladosporioides*	23	9	3	1	2	1	1	1	2	1	2	
Pleosporales sp. 1	23	3	2		1	3			11	1	2	
Hypocrea aff. *lixii*	18				2	6	4	1	1	2	1	1
Fusarium aff. *equiseti*	12	7			3					1		1
Pleosporales sp. 2	9	2				1			3	3		
Penicillium aff. *minioluteum*	8			6								2
Dothideomycete sp.	7		3	3					1			
Bipolaris sp. 1	6		6									
Candida aff. *fukuyamaensis*	6		1		4		1					
Dokmaia sp. 1	5	2	1		1						1	
Lecythophora aff. *decumbens*	5						5					
Aureobasidium aff. *pullulans*	4	1	1		1				1			
Cryptococcus aff. *flavescens*	4		2				1				1	
Pleosporales sp. 3	4	2			1	1						
Tremella aff. *globispora*	4		1				2				1	
Candida sp. 1	3		3									
Fusarium aff. *proliferatum*	3		1	1		1						
Microbotryomycetes sp. 1	3	2	1									
Occultifur aff. *externus*	3			1							2	
Phaeosphaeria sp. 2	3	2			1							
Aspergillus aff. *fumigatus*	2											2
Candida aff. *akabanensis*	2					1	1					
Cryptococcus sp. 1	2				2							
Cryptococcus sp. 2	2	1							1			
Cyphellophora sp. 1	2		1	1								
Dothideomycete sp. 1	2	1								1		
Dothideomycete sp. 2	2										2	
Epicoccum aff. *nigrum*	2			1						1		
Exophiala aff. *salmonis*	2					1					1	
Exophiala aff. *spinifera*	2					1					1	
Fusarium aff. *sporotrichioides*	2			1			1					
Periconia aff. *macrospinosa*	2		1				1					
Phoma aff. *leveillei*	2				1						1	
Pichia aff. *membranifaciens*	2						2					
Acremonium sp. 1	1										1	
Ascomycota sp.	1				1							
Bipolaris aff. *zeicola*	1					1						
Bullera aff. *sinensis*	1				1							
Candida aff. *metapsilosis*	1	1										
Capnodium sp. 1	1				1							
Cercophora sp. 1	1											1
Curvularia sp. 1	1									1		
Dokmaia sp. 2	1	1										
Dothideomycete sp. 3	1								1			
Fusarium aff. *sacchari*	1							1				
Hypocreales sp. 1	1				1							
Hypocreales sp. 2	1						1					
Myrmecridium aff. *schulzeri*	1										1	
Myrothecium sp. 1	1								1			
Myrothecium sp. 2	1	1										
Nigrospora sp. 1	1		1									
Paecilomyces sp. 1	1											1
Paraphaeosphaeria sp. 1	1										1	
Penicillium aff. *daleae*	1											1
Penicillium aff. *pinophilum*	1											1
Penicillium sp. 1	1						1					
Phoma sp. 2	1					1						
Pichia aff. *anomala*	1		1									
Pleosporales sp. 4	1					1						
Pleosporales sp. 5	1					1						
Pleosporales sp. 5	1								1			
Scytalidium sp. 1	1											1
Sordariomycete sp. 1	1					1						
Stilbella sp. 1	1					1						
Tremellaceae sp. 1	1						1					
Ustilago sp.	1		1									
Zopfiella sp. 1	1											1

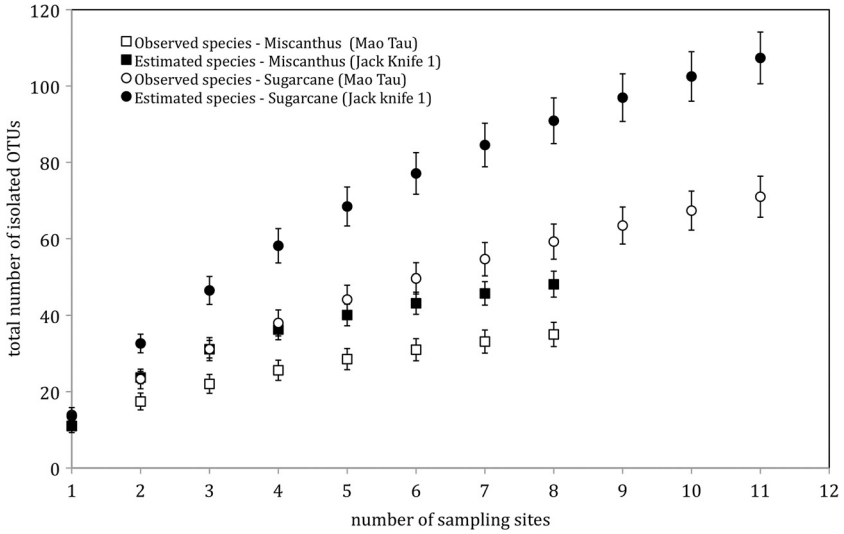

FIG. 3. Species richness curves for fungal OTUs isolated from *Miscanthus* and sugarcane fields.

Species abundance, sampling adequacy, and spatial diversity. The species abundance curves for fungal OTUs showed a few relatively abundant fungi and a long tail with many rarely isolated OTUs. The most common OTUs from *Miscanthus* belonged to the genera *Trichoderma* (teleomorph *Hypocrea*), *Fusarium*, *Cordyceps*, *Arthrinium*, and *Phoma* (Fig. 2a; Table 1). Similarly, the common OTUs isolated from sugarcane were from the genera *Phoma*, *Trichoderma* (teleomorph *Hypocrea*), *Cladosporium*, *Fusarium*, and *Penicillium* (Fig. 2b; Table 2).

The most commonly isolated fungal OTUs, i.e., those isolated between 10 and 100 times, were the OTUs most likely to be shared among *Miscanthus* or sugarcane fields (Table 3 and 4). These included *Hypocrea* aff. *koningii*, *Hypocrea* aff. *lixii*, *Phoma* aff. *herbarum*, and *Fusarium* aff. *proliferatum* from *Miscanthus* and *Phoma* aff. *glomerata*, *Phoma* aff. *herbarum*, *Pleosporales* sp. 1, *Cladosporium* aff. *cladosporioides*, and *Hypocrea* aff. *lixii* from sugarcane. The one site that stood out as different was sugarcane bagasse, because it contained only one of the commonly isolated species, *Hypocrea* aff. *lixii*, and 70% of its OTUs were unique to the site.

To determine the depth of our sampling, we estimated the increase in total fungal OTUs for each plant as additional sites were sampled (Fig. 3 [species richness curves]). The rate of new OTU discovery diminished as sampling sites were increased (Fig. 3) and also with additional isolates per sample site (Fig. 4 [rarefaction curves]). A greater fraction of rarefaction curves approached plateaus for *Micanthus* sample sites (MS1, MS2, MS5, and Mbale), while only three rarefaction curves corresponding to sugarcane sample sites, SC1, SC3, and SC8, reached plateaus. Again, the one site that stood apart was sugarcane bagasse, for which the rarefaction curve showed no indication of reaching a plateau (Fig. 4b).

Community dissimilarity among pairs of sites ranged from 50% to 84% with a mean of 70% for *Miscanthus* and from 50% to 92% with a mean of 77% for sugarcane (Table 5 and 6). There was no strong relationship between OTU dissimilarity and geographic distance for OTUs isolated from *Miscanthus* (Mantel $r = 0.326$, $P = 0.083$) or sugarcane (Mantel $r = 0.124$, $P = 0.586$). The NMDS test (Fig. 5) showed a clear difference in fungal communities between *Miscanthus* and sugarcane sampling sites (Mantel $r = 0.669$, $P = 0.001$).

***Miscanthus* biodegradation via high-throughput fungal cultures. (i) Effect of biomass pretreatment.** Three methods of biomass pretreatment, hot water at 121°C, mild alkali (0.5% [wt/vol] sodium hydroxide), and dilute acid (1% [wt/vol] sulfuric acid), were compared in preliminary studies of *Miscanthus* biodegradation using *Neurospora crassa* D140. Percentage biomass weight loss was the highest (data not shown) after the alkali and acid treatments, and there was no significant difference between the two pretreatments (weight loss, $P = 0.1653$). Alkali pretreatment being the easier to perform, we used alkali pretreated *Miscanthus* for all solid-substrate cultures.

(ii) Percentage biomass weight loss by the fungi. The 9 most commonly isolated fungi from *Miscanthus* samples showed substantial biomass loss when cultured on moist *Misccanthus* for 4 weeks (Fig. 6). Three OTUs (*Arthrinium* aff. *phaeospermum*, *Trichoderma* aff. *atroviride*, and *Phoma* aff. *herbarum*) removed more than 13% of *Miscanthus* biomass over 28 days, and the remaining six OTUs were able to remove at least 10% of the biomass over the same period (Fig. 6).

DISCUSSION

Systematic approach to estimate fungal biodiversity from environmental samples. The particle filtration and dilution-to-extinction culture method that we employed was successful in cultivating fungi that are not simply abundant spore producers or fast-growing weedy species. For example, only one *Penicillium* species, *Penicillium* aff. *minioluteum*, was among the 10 most commonly isolated fungi from sugarcane, and the most abundant *Cladosporium* species, *Cladosporium* aff. *cladosporioides*, was the 13th and 5th most common *Miscanthus* and sugarcane associate, respectively (Tables 1 and 2). The only *Aspergillus* species recovered (Table 2), *Aspergillus fumigatus*, probably is truly responsible for bioconversion in hot sugarcane bagasse pile (Table 2) due to its thermotolerance (56).

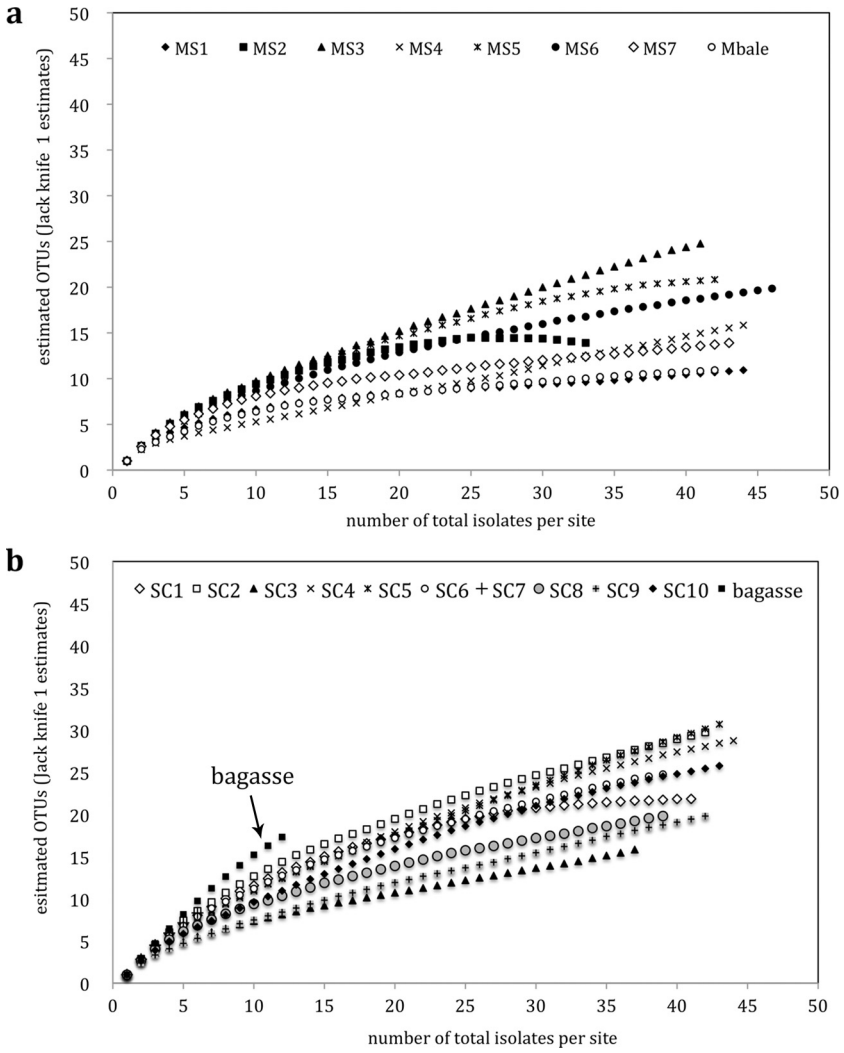

FIG. 4. Rarefaction curves for estimated fungal OTUs in different *Miscanthus* and sugarcane sites.

The observation that fungal species abundance curves associated with each plant (Fig. 3) and rarefaction curves for most sites (Fig. 4) approached or reached plateaus, taken together with the ranked abundance curves (Fig. 1 and 2) and the distribution of OTUs per site (Tables 3 and 4), indicate that our sampling was sufficient to find the common species but not all of the rare ones. The analyses also indicate that additional sampling would bring diminishing returns, particularly when adding additional isolates at specific sites. Other applications of the high-throughput cultivation approach have also found

TABLE 5. Community dissimilarity indices for fungi isolated from *Miscanthus* (MS1 to MS7) sites

Site	Dissimilarity index[a] with comparison site						
	MS1	MS2	MS3	MS4	MS5	MS6	MS7
MS1							
MS2	0.73						
MS3	0.79	0.7					
MS4	0.79	0.75	0.8				
MS5	0.84	0.68	0.62	0.65			
MS6	0.83	0.67	0.67	0.71	0.58		
MS7	0.5	0.69	0.75	0.64	0.67	0.79	

[a] Dissimilarity index = 1 − Jaccard index.

TABLE 6. Community dissimilarity indices for fungi isolated from sugarcane fields (sites SC1 to SC10)

Site	Dissimilarity index[a] with comparison site									
	SC1	SC2	SC3	SC4	SC5	SC6	SC7	SC8	SC9	SC10
SC1										
SC2	0.75									
SC3	0.87	0.74								
SC4	0.64	0.77	0.88							
SC5	0.79	0.84	0.83	0.80						
SC6	0.90	0.75	0.82	0.83	0.79					
SC7	0.83	0.86	0.75	0.79	0.79	0.77				
SC8	0.68	0.77	0.79	0.76	0.71	0.79	0.71			
SC9	0.67	0.85	0.78	0.70	0.70	0.78	0.69	0.61		
SC10	0.82	0.75	0.82	0.74	0.74	0.77	0.77	0.79	0.78	

[a] Dissimilarity index = 1 − Jaccard index.

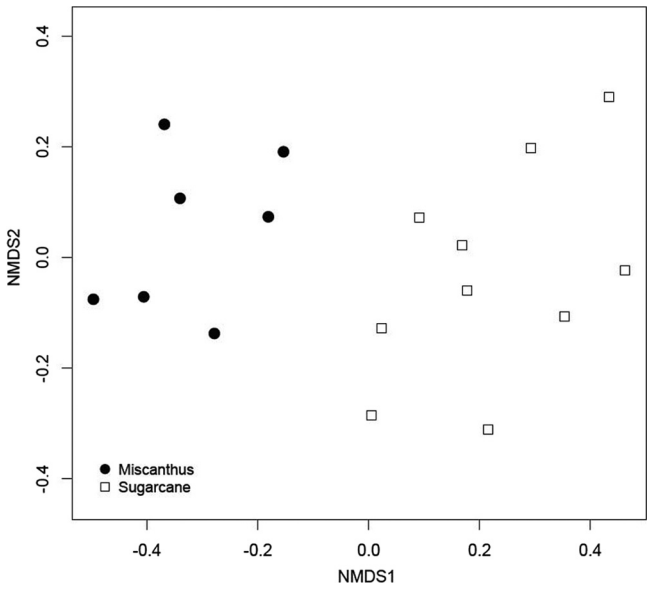

FIG. 5. Two-dimensional NDMS ordinate plots of fungal diversity from *Miscanthus* and sugarcane samples.

similar trends for species abundance and species rarefaction curves (3, 41, 42).

Spatial diversity of fungal OTUs. The community dissimilarity indices (Tables 5 and 6) ranged from 50 to 90% and showed that fungi found at the several sites are very different and that there is no strong relationship between geographic distance and species diversity, at least over distances between 48 m and tens of kilometers. The species abundance curves (Fig. 2a and b) showed that, although a few species were found repeatedly, by far the majority of species were rarely cultivated. When species composition was compared among sites (Tables 3 and 4), it was apparent that the most commonly isolated fungi were found in all or a majority of sites and the rarely found fungi were often unique to a single site. Therefore, it is the rarely detected fungi that contribute to the high dissimilarity indices. In terms of the adequacy of sampling, it seems unlikely that additional sites or samples would significantly increase the number of commonly found fungi and that it would likely increase the number of rare fungi, albeit at a lower rate than was seen from the initial samples.

The sugarcane bagasse site was unique. Seven out of 10 species were unique to the site, and only one OTU, *Hypocrea* aff. *lixii*, was shared by more than half the other sites. Clearly, additional sampling of bagasse is likely to uncover more fungi that decay sugarcane. Fungi isolated from sugarcane bagasse have been studied for their ability to detoxify phenanthrene, and other studies have involved fungal cultures on bagasse for cellulase enzyme production (11). However, we found no report regarding the ability of fungi cultivated from bagasse to deconstruct the host plant cell walls.

The fungi recovered from *Miscanthus* and sugarcane were largely different. Two classes of *Ascomycota* dominated the fungi recovered from both plants, *Sordariomycetes* and *Dothideomycetes*, and together these classes accounted for more than 97% of the diversity on *Miscanthus* and more than 81% of the diversity on sugarcane (Fig. 1a and b). The relative importance of these classes changes with the plant; *Sordariomyetes* was the most common on *Miscanthus*, and *Dothideomycetes* was the most common on sugarcane. Comparison of fungal diversity at sites for the two plants (Fig. 5) showed no overlap in the NMDS ordinate. This result could be due to a number of factors, including the plant species, geographic distance, or the very different environments of Illinois in September versus Louisiana in January. If one considers only those fungi that are found in at least 1/3 of the field or plantation sites (Tables 3 and 4), four OTUs were shared by *Miscanthus* and sugarcane: *Hypocrea* aff. *lixii* and *Trichoderma* aff. *atroviride* in the *Sordariomycetes* and *Phoma* aff. *herbarum* and *Cladosporium* aff. *cladosporioides* in the *Dothideomycetes*.

Compared with other studies (Table 7), our use of a high-

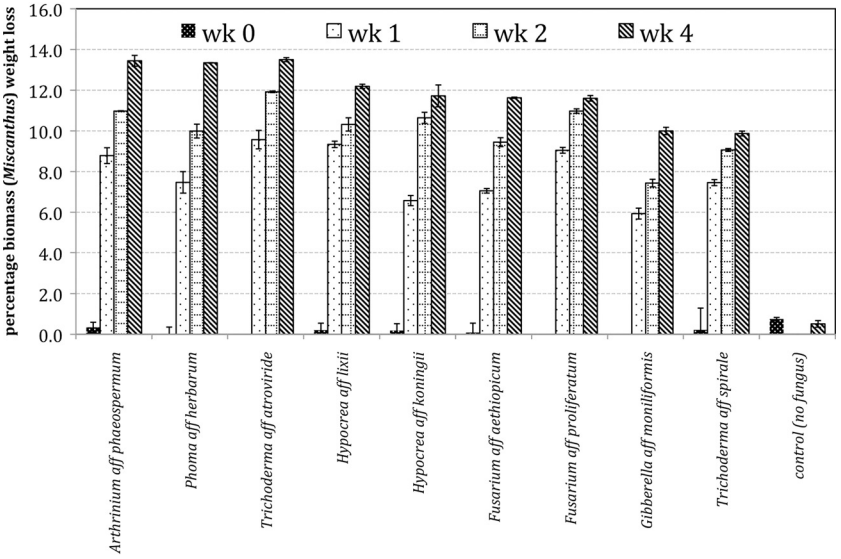

FIG. 6. Percent biomass weight loss during fungal biodegradation of alkali-pretreated *Miscanthus* material. Error bars are standard errors ($n = 3$).

TABLE 7. Comparison of fungal taxa associated with *Miscanthus* and sugarcane

Source or sample	Isolation and identification technique	Isolated fungal OTU	No. of species	Biodegradation study?	Reference
Miscanthus × *giganteus*	Particle filtration, dilutions to extinction, microwell and plate cultures, molecular identification via rDNA (ITS 1f→ITS4, CTB6→LR3) sequencing	*Hypocrea* aff. *Trichoderma*	6	Yes	This study
		Arthrinium	2		
		Phoma	1		
		Gibberella aff. *Fusarium*	6		
		Cordyceps	1		
		Alternaria	2		
		Cladosporium	1		
		Epicoccum	1		
		Cephalosporium	2		
		Minimidochium	1		
		Chloridium	1		
		Ceratobasidium	1		
		Microdochium	2		
		Nigrospora	1		
		Phaeosphaeriopsis	1		
		Sporothrix	1		
		Chalara	1		
		Mucor	1		
		Exophiala	1		
		Phaeosphaeria	1		
		Paraphaeosphaeria	1		
		Chaetosphaeria	1		
Miscanthus × *giganteus* (+ pig manure)	Composting and morphology	*Pythium*	1	No	Klamer et al. (33)
		Absidia	4		
		Mortierella	2		
		Mucor	4		
		Rhizopus	1		
		Acremonium	3		
		Aspergillus	2		
		Chaetomium	2		
		Chrysosporium	1		
		Corynascus	1		
		Nectria	1		
		Paecilomyces	2		
		Penicillium	7		
		Pseudallescheria	1		
		Scopulariopsis	1		
		Sepedonium	1		
		Sporothrix	1		
		Trichoderma	1		
		Trichothecium	1		
		Trichurus	1		
		Verticillium	1		
		Basidiomycete sp.	1		
		Rhizomucor	1		
		Myceliopthora	1		
		Scytalidium	1		
		Thermomyces	1		
M. sinensis	Surface disinfection	*Nigrospora*	1	Yes	Osono et al. (40)
M. sinensis × *M. floridulus*	rDNA (ITS) sequencing	*Cladosporium*	2	No	Chiang et al. (8)
		Fusarium	1		
		Basidiomycete sp.	1		
Sugarcane	Particle filtration, dilutions to extinction, microwell and plate cultures, molecular identification via rDNA (ITS 1f→ITS4, CTB6→LR3) sequencing	*Phoma*	4	Yes	This study
		Hypocrea aff. *Trichoderma*	2		
		Pleosporales sp	6		
		Cladosporium	1		
		Gibberella aff. *Fusarium*	4		
		Penicillium	4		
		Dothideomycete sp.	4		
		Bipolaris	2		
		Candida	4		
		Lecythophora	1		
		Dokmaia	2		
		Aureobasidium	1		

Continued on following page

TABLE 7—*Continued*

Source or sample	Isolation and identification technique	Isolated fungal OTU	No. of species	Biodegradation study?	Reference
		Cryptococcus	3		
		Occultifur	1		
		Phaeosphaeria	1		
		Microbotryomycetes sp.	1		
		Aspergillus	1		
		Cyphellophora	1		
		Epicoccum	1		
		Leptoxyphium	1		
		Exophiala	2		
		Periconia	1		
		Pichia	2		
		Tremella	1		
		Scytalidium	1		
		Zopfiella	1		
		Cercophora	1		
		Paecilomyces	1		
		Ascomycota sp.	1		
		Hypocreales sp.	2		
		Capnodium	1		
		Bullera	1		
		Sordariomycete sp.	1		
		Tremellaceae sp.	1		
		Myrothecium	2		
		Curvularia	1		
		Myrmecridium	1		
		Acremonium	1		
		Paraphaeosphaeria	1		
		Ustilago	1		
		Nigrospora	1		
Sugarcane	Dilution and plating	*Bullera*	1	No	De Azeredo et al. (14)
		Cryptococcus	7		
		Cystofilobasidium	1		
		Fellomyces	1		
		Filobasidiella	1		
		Leucosporidium	1		
		Rhodosporidium	1		
		Rhodotorula	6		
		Sporobolomyces	1		
		Sporidiobolus	1		
		Tremella	3		
		Trichosporon	4		
		Candida	7		
		Clavispora	1		
		Debaryomyces	1		
		Pichia	1		
		Saccharomyces	1		
		Torulaspora	1		
		Zygoascus	1		
Sugarcane bagasse compost	Dilution and plating	*Aspergillus*	4	No	Sandhu and Sidhu (47)
		Penicillium	1		
		Trichoderma	1		
		Rhizopus	1		
		Mucor	1		
		Agaric	1		

throughput culture isolation technique allowed us to isolate many more fungal taxa associated with *Miscanthus* or sugarcane. Chiang et al. (8) used PCR to identify *Miscanthus* endophytes, and they found two *Cladosporium* species and a *Fusarium* species, which raises the possibility that some of the decay fungi found by us could also be endophytes. Sandhu and Sidhu (47) reported 6 genera associated with sugarcane bagasse compost, three of which, *Penicillium*, *Aspergillus*, and *Trichoderma*, were also isolated from our bagasse samples. The abundance levels of some yeast genera, i.e., *Cryptococcus*, *Candida*, and *Tremella*, reported by others (14) were confirmed by us. A particularly interesting study is that of Klamer et al. (33), who investigated fungi responsible for decay of *Miscanthus* mixed with pig waste, because the mixture achieved high temperatures and resulted in the isolation of some thermophilic species. No *Dothideomycetes* were recovered in this study, but

the cultivation method for that study was not designed to recover fungi other than those that grow fast or that are present only as spores. Osono (40) reported on the decay of *Miscanthus* by several basidiomycota and one ascomycota: *Nigrospora sphaerica*, which was the only species tested that was actually cultivated from surface-sterilized *Miscanthus* leaves. We also found a species genus *Nigrospora* (*N. aff oryzae*). It was the 24th most common fungus on decaying *Miscanthus*, suggesting that *Nigrospora* is either less common in North America than Asia or that this endophyte does not persist well in the saprophytic communities that we sampled.

The most comprehensive studies that have been made of saprobic fungi found on grasses are those of Gessner and Goos on *Spartina* (17) and Wirsel et al. on *Phragmites* (60). The most common saprobes seen on *Spartina* were Dothideomycetes and those on *Phragmites* were both *Dothideomycetes* and *Hypocreales*, including several *Trichoderma* species. The pioneering fungal cultivation studies that introduced particle filtration and dilution to extinction (3, 42) were focused on tropical forests, and the most abundant species found in these studies were classified in *Hypocreales*, *Xylariales*, and *Dothideomycetes*. More recently, Paulus et al. (41) used high-throughput methods with small particles and washing to recover hundreds of morphologically distinct fungi from six tropical Australian trees. Again, the fungi were *Hypocreales*, *Xylariales*, and *Dothideomycetes*, along with *Chaetothyriales*, *Leotiales*, and *Eurotiales*. Fungal diversity was high, resulting in species abundance curves with long tails of singletons, and overlap of fungi recovered from the different tree species was low.

Ability of isolated fungal OTUs to biodegrade lignocellulosic biomass. The final step in bioprospecting is to test the ability of fungi isolated from decaying plants to actually decay the plant. Steffen et al. (55) tested the ability of fungi isolated from oak litter to bioconvert oak biomass, and Song et al. (53) tested the ability of fungi obtained from forest litter to reduce the biomass of pine needles and Formosan sweetgum leaves found in forest litter. Only the study of Osono (40) tested the hypothesis that a fungus, *Nigrospora sphaerica*, isolated from *Miscanthus* could actually bioconvert *Miscanthus* biomass.

To test our hypotheses that the fungi cultivated from field-collected *Miscanthus* or sugarcane are responsible for bioconversion of these grasses in nature, we used the nine fungi most frequently cultivated from *Miscanthus* and found that four of nine species caused biomass loss of 12% or higher in 4 weeks. The most weight loss, >13%, was achieved by three OTUs, *Arthrinium* aff. *phaeospermum* and *Trichoderma* aff. *atroviride* in the *Sordariomycetes* and *Phoma* aff. *herbarum* in the *Dothideomycetes.* These results indicate that we have isolated fungi that are responsible for deconstruction of grass cell walls in nature. We noted that *N. crassa* converted 16% of *Miscanthus* over the same period, showing that this model fungus is well suited to bioconversion, although the use of a laboratory-adapted strain, a mineral nutrition medium developed for *Neurospora* (58), and inoculation by conidia rather than hyphal fragments may have biased the outcome.

Our results may be compared to several studies of plant biomass conversion using fungi collected from nature. An early study (15) examined retting of hemp, where biomass weight loss over 20 days was reported to be 15.6% for a *Fusarium* sp. and 13.1% for a *Phoma* sp. Osono (40) assessed fungal biodegradation of *Miscanthus sinensis* over 12 weeks by nine litter-decomposing fungi. That author reported that *Trametes versicolor* showed the highest biomass weight loss (43%), whereas percentages for *Ascomycota* ranged from 7% to 20%. A bioconversion study, similar to ours in approach (55), reported that three basidiomycotous fungi, *Marasmius quercophilus*, *Pholiota lenta*, and *Mycena inclinata*, reduced biomass of oak leaves over 4 weeks by 19, 14, and 10%, respectively. In another recent study, 0.5 to 6.92% plant leaf biomass reduction over 5 weeks was reported for species of *Trichoderma*, *Aspergillus*, *Penicillium*, *Chaetomium*, *Mucor*, and *Cladosporium* (53). The percent biomass reduction that we found, from 10% to 13%, is similar to that seen for *Ascomycota*, but slightly lower than what has been reported for *Basidiomycota*. We are currently conducting comparative *Miscanthus* biodegradation and enzyme studies over longer periods using fungi isolated from the *Miscanthus* and sugarcane fields.

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REFERENCES

1. **Ahonsi, M. O., et al.** 2010. First report of *Pithomyces chartarum* causing a leaf blight of *Miscanthus* × *giganteus* in Kentucky. Plant Dis. **94:**480–481.
2. **Arnold, A. E., Z. Maynard, G. Gilbert, P. D. Coley, and T. A. Kursar.** 2000. Are tropical fungal endophytes hyperdiverse? Ecol. Lett. **3:**267–274.
3. **Bills, G. F., and J. D. Polishook.** 1994. Abundance and diversity of microfungi in leaf-litter of a lowland rain-forest in Costa Rica. Mycologia **86:**187–198.
4. **Bills, G. F., M. Christensen, M. Powell, and G. Thorn.** 2004. Saprobic soil fungi, p. 271–302. *In* G. Mueller, G. F. Bills, and M. S. Foster (ed.), Biodiversity of fungi, inventory and monitoring methods. Elsevier Academic Press, Oxford, England.
5. **Blanchette, R. A.** 1995. Degradation of the lignocellulose complex in wood. Can. J. Bot. **73:**S999–S1010.
6. **Carpita, N. C.** 1996. Structure and biogenesis of the cell walls of grasses. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47:**445–476.
7. **Chao, A., R. L. Chazdon, R. K. Colwell, and T.-J. Shen.** 2005. A new statistical approach for assessing compositional similarity based on incidence and abundance data. Ecol. Lett. **8:**148–159.
8. **Chiang, Y. C., C. H. Chou, P. R. Lee, and T. Y. Chiang.** 2001. Detection of leaf-associated fungi based on PCR and nucleotide sequence of the ribosomal internal transcribed spacer (ITS) in *Miscanthus*. Bot. Bull. Acad. Sin. **42:**39–44.
9. **Collado, J., G. Platas, B. Paulus, and G. F. Bills.** 2007. High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. FEMS Microbiol. Ecol. **60:**521–533.
10. **Colwell, R. K.** 2005. EstimateS: statistical estimation of species richness and shared species from samples, version 8.2. http://viceroy.eeb.uconn.edu/estimates.
11. **Cortes-Espinosa, D., et al.** 2006. Selection and identification of fungi isolated from sugarcane bagasse and their application for phenanthrene removal from soil. J. Environ. Sci. Health **41:**475–486.
12. **Cullen, D., and P. J. Kersten.** 1996. Enzymology and molecular biology of lignin degradation, p. 295–312. *In* K. Esser (ed.), The Mycota. III. A comprehensive treatise on fungi as experimental systems for basic and applied research: biochemistry and molecular biology. Springer Verlag, Berlin, Germany.
13. **Cullen, D.** 1997. Recent advances on the molecular genetics of ligninolytic fungi. J. Biotechnol. **53:**273–289.
14. **De Azeredo, L. A. I., E. A. T. Gomes, L. C. Mendonca-Hagler, and A. N. Hagler.** 1998. Yeast communities associated with sugarcane in Campos, Rio de Janeiro, Brazil. Int. Microbiol. **1:**205–208.

15. **Fuller, W. H., and A. G. Norman.** 1945. Biochemical changes involved in the decomposition of hemp bark by pure cultures of fungi. J. Bacteriol. **50:**667–671.
16. **Gardes, M., and T. D. Bruns.** 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. **2:**113–118.
17. **Gessner, R. V., and R. D. Goos.** 1973. Fungi from *Spartina alterniflora* in Rhode Island. Mycologia **65:**1296–1301.
18. **Goldemberg, J.** 2008. The Brazilian biofuels industry. Biotechnol. Biofuels **1:**6.
19. **Gutierrez-Correa, M., and R. P. Tengerdy.** 1997. Production of cellulase on sugarcane bagasse by fungal mixed culture solid substrate fermentation. Biotechnol. Lett. **19:**665–667.
20. **Hammel, K. E., A. N. Kapich, K. A. Jensen, Jr., and Z. C. Ryan.** 2002. Reactive oxygen species as agents of wood decay by fungi. Enzyme Microb. Technol. **30:**445–453.
21. **Hatfield, R. D., J. R. Wilson, and D. R. Mertens.** 1999. Composition of cell walls isolated from cell types of grain sorghum stems. J. Sci. Food Agric. **79:**891–899.
22. **Heaton, E. A., T. B. Voigt, and S. P. Long.** 2004. A quantitative review comparing the yields of two candidate C-4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass Bioenergy **27:**21–30.
23. **Heaton, E., F. G. Dohleman, and S. P. Long.** 2008. Meeting US biofuel goals with less land: the potential of *Miscanthus*. Global Change Biol. **14:**2000–2014.
24. **Heltshe, J., and N. E. Forrester.** 1983. Estimating species richness using the jackknife procedure. Biometrics **39:**1–11.
25. **Higuchi, Takayoshi.** 2004. Microbial degradation of lignin: role of lignin peroxidase, manganese peroxidase, and laccase. Proc. Jpn. Acad. B Phys. Biol. Sci. **80:**204–214.
26. **Hodkinson, T. R., M. W. Chase, M. D. Lledo, N. Salamin, and S. A. Renvoize.** 2002. Phylogenetics of *Miscanthus*, *Saccharum* and related genera (Saccharinae, Andropogoneae, Poaceae) based on DNA sequences from ITS nuclear ribosomal DNA and plastid trnL intron and trnL-F intergenic spacers. J. Plant Res. **115:**381–392.
27. **Hopple, J. S., and R. Vilgalys.** 1994. Phylogenetic relationship among Coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. Mycologia **86:**96–107.
28. **Hoy, J. W., and M. P. Grisham.** 1988. Spread and increase of sugarcane smut in Louisiana. Phytopathology **78:**1371–1376.
29. **Johnson, R. M., M. P. Grisham, and E. P. Richard.** 2007. Relationship between sugarcane smut rust severity and soil properties in Louisiana. Phytopathology **97:**748–755.
30. **Jones, M. B., and M. Walsh.** 2001. Miscanthus for energy and fibre. James & James Ltd., London, England.
31. **Jumpponen, A., and K. L. Jones.** 2010. Seasonally dynamic fungal communities in *Quercus macrocarpa* phyllosphere differ among urban and rural environments. New Phytol. **186:**496–513.
32. **Kersten, P., and D. Cullen.** 2007. Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Fungal Genet. Biol. **44:**77–87.
33. **Klamer, M., A. M. Lind, and W. Gams.** 2001. Fungal succession during composting of *Miscanthus* straw and pig slurry. Acta Hortic. **549:**37–46.
34. **Koike, H., D. Fontenot, K. Damann, and R. Schlub.** 1981. Smut of sugarcane in Louisiana. Plant Dis. **65:**1018.
35. **Lewandowski, I., J. C. Clifton-Brown, J. M. O. Scurlock, and W. Huisman.** 2000. *Miscanthus*: European experience with a novel energy crop. Biomass Bioenergy **19:**209–227.
36. **Mantel, N.** 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. **27:**209–220.
37. **Martinez, A. T., et al.** 2005. Biodegradation of lignocellulosics: microbial chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. **8:**195–204.
38. **National Climatic Data Center, National Oceanic and Atmospheric Administration Center.** 2010. Climatological data, vol. 113, no. 13. Annual summary, Illinois, 2008. http://www1.ncdc.noaa.gov/pub/orders/FA5708B1-F9C5-4FA7-83B1-696E0953038F.pdf.
38a. **National Climatic Data Center, National Oceanic and Atmospheric Administration.** 2010. Climatological data, vol. 114, no. 13. Annual summary, Louisiana, 2009. http://www1.ncdc.noaa.gov/pub/orders/EAD5A798-4550-414C-A682-1698B1D302CB.pdf.
39. **Osono, T., and H. Takeda.** 2002. Comparison of litter decomposing ability among diverse fungi in a cool temperate deciduous forest in Japan. Mycologia **94:**421–427.
40. **Osono, T.** 2010. Decomposition of grass leaves by ligninolytic litter-decomposing fungi. Grassland Sci. **56:**31–36.
41. **Paulus, B., J. Kanowski, P. A. Gadek, and K. D. Hyde.** 2006. Diversity and distribution of saprobic microfungi in leaf litter of an Australian tropical rainforest. Mycol. Res. **110:**1441–1454.
42. **Polishook, J. D., G. F. Bills, and D. J. Lodge.** 1996. Microfungi from decaying leaves of two rain forest trees in Puerto Rico. J. Ind. Microbiol. **17:**284–294.
43. **R Development Core Team.** 2010. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
44. **Rasmussen, M. L., P. Shrestha, S. K. Khanal, A. L. Pometto, and J. Van Leeuwen.** 2010. Sequential saccharification of corn fiber and ethanol production by the brown rot fungus *Gloeophyllum trabeum*. Bioresour. Technol. **101:**3526–3533.
45. **Remlein-Starosta, D.** 2007. Diseases of bioenergy crops. Prog. Plant Protect. **47:**351–357.
46. **Rodriguez, R., J. White, A. E. Arnold, and R. Redman.** 2009. Fungal endophytes: diversity and ecological roles. New Phytol. **182:**314–330.
47. **Sandhu, D. K., and M. S. Sidhu.** 1980. The fungal succession on decomposing sugarcane bagasse. Trans. Br. Mycol. Soc. **75:**281–286.
48. **Shrestha, P., M. L. Rasmussen, S. K. Khanal, A. L. Pometto, and J. Van Leeuwen.** 2008. Saccharification of corn fiber by *Phanerochaete chrysosporium* in solid-substrate fermentation and subsequent fermentation of hydrolyzate into ethanol. J. Agric. Food Chem. **56:**3918–3924.
49. **Shrestha, P., S. K. Khanal, A. L. Pometto, and J. Van Leeuwen.** 2009. Enzyme production by wood-rot and soft-rot fungi cultivated on corn fiber followed by hydrolyzate fermentation to ethanol. J. Agric. Food Chem. **57:**4156–4161.
50. **Shrestha, P., S. K. Khanal, A. L. Pometto, and J. Van Leeuwen.** 2010. Ethanol production via in-situ fungal saccharification and fermentation of mild alkali and steam pretreated corn fiber. Bioresour. Technol. **101:**8698–8705.
51. **Smith, E. P., and G. Van Belle.** 1984. Nonparametric estimation of species richness. Biometrics **40:**119–129.
52. **Somerville, C., H. Youngs, C. Taylor, S. C. Davis, and S. E. Long.** 2010. Feedstocks for lignocellulosic biofuels. Science **329:**790–792.
53. **Song, F., X. Tian, X. Fan, and X. He.** 2010. Decomposing ability of filamentous fungi on litter is involved in a subtropical mixed forest. Mycologia **102:**20–26.
54. **Sprules, W. G.** 1980. Nonmetric multidimensional scaling analyses of temporal variation in the structure of limnetic zooplankton communities. Hydrobiologia **69:**139–146.
55. **Steffen, K. T., T. Cajthaml, J. Snajdr, and P. Baldrian.** 2007. Differential degradation of oak (*Quercus petraea*) leaf litter by litter-decomposing basidiomycetes. Res. Microbiol. **158:**447–455.
56. **Toumela, M., M. Vikman, A. Hatakka, and M. Itavaara.** 2000. Biodegradation of lignin in a compost environment: a review. Bioresour. Technol. **72:**169–183.
57. **Vanky, K.** 2000. The smut fungi of *Saccharum* and related grasses. Austr. Plant Pathol. **29:**155–163.
58. **Vogel, H. J.** 1956. A convenient growth medium for *Neurospora* (medium N). Microb. Genet. Bull. **13:**42–43.
59. **White, T., T. Bruns, S. Lee, and J. Taylor.** 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of rRNA genes, p. 315–322. *In* M. Innis, D. Gelfand, J. Sninsky, and T. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Orlando, FL.
60. **Wirsel, S. G. R., W. Leibinger, M. Ernst, and K. Mendgen.** 2001. Genetic diversity of fungi closely associated with common reed. New Phytol. **149:**589–598.