Microbiology (2016), **162**, 1147–1156 DOI 10.1099/mic.0.000306

Fungal communities in pressmud composting harbour beneficial and detrimental fungi for human welfare

Tássio Brito de Oliveira,[1] Viviane Cristina Padilha Lopes,[1] Fernando Nogueira Barbosa,[2] Milene Ferro,[3] Lucas Andrade Meirelles,[1,4] Lara Durães Sette,[1] Eleni Gomes[2] and Andre Rodrigues[1]

Correspondence
Andre Rodrigues
andrer@rc.unesp.br

[1]Department of Biochemistry and Microbiology, UNESP – Univ Estadual Paulista, Rio Claro, SP, Brazil

[2]Department of Biology, UNESP – Univ Estadual Paulista, Sao Jose do Rio Preto, SP, Brazil

[3]Center for the Study of Social Insects, UNESP – Univ Estadual Paulista, Rio Claro, SP, Brazil

[4]Division of Biology and Biological Engineering, Caltech – California Institute of Technology, Pasadena, CA, USA

Received 4 March 2016
Accepted 6 May 2016

Pressmud is a substrate derived from sugarcane juice filtrate, and around 26–40 kg of this residue are produced per ton of sugarcane. It is mainly used as fertilizer in crops, and its application in the field is often made without any prior treatment, but, in this research, it was studied for the risk this practice poses for human health. This research was stimulated by previous results indicating the presence of opportunistic pathogens in residues used in various composting systems and the extensive use of fresh pressmud in agriculture. Here, It was assessed the fungal diversity present in both fresh and composting pressmud using 454 pyrosequencing. In addition, heat-tolerant fungi were isolated and surveyed for their enzymatic repertoire of biomass-degrading enzymes (cellulase, xylanase, laccase and polygalacturonase). A wide range of opportunistic pathogens was found among the most abundant taxa in the fresh pressmud, such as *Lomentospora prolificans* (43.13 %), *Trichosporon* sp. (10.07 %), *Candida tropicalis* (7.91 %), and *Hormographiella aspergillata* (8.19 %). This indicates that fresh pressmud might be a putative source of human pathogenic fungi, presenting a potential threat to human health if applied as fertilizer without any treatment. With regard to the heat-tolerant fungi found in this substrate, all the 110 isolates screened were able to produce at least one of the tested enzymes. The pressmud composting process not only effectively reduces the load of pathogenic fungi, but also creates an interesting environment for fungi able to produce thermostable hydrolytic and oxidative enzymes with biotechnological applications.

INTRODUCTION

Brazil is the largest sugar producer worldwide, with an estimated production of 654.6 million tons of sugarcane in 2015 (CONAB, 2015). Considerable amounts of by-products (i.e. pressmud, bagasse and molasses) are generated during the production process. Pressmud (or filter cake) is derived from sugarcane juice filtrate; it is rich in phosphorus, nitrogen, and organic matter and has high moisture content (Prado *et al.*, 2013). Approximately 26–40 kg of this residue is produced after crushing one ton of sugarcane (Bhosale *et al.*, 2012).

Pressmud is largely used as fertilizer in crops and it is often applied without any treatment (as for example, composting) (Balakrishnan & Batra, 2011). Composting is one of the possible treatments where a biotransformation of solid organic matter into a matured and stabilized substrate, due to microbial action under aerobic conditions, takes place. Usually, three different phases can be recognized during the composting process: (1) mesophilic phase (up to 40 °C), which usually lasts for a couple of days; (2) thermophilic

GenBank (NCBI) sequence database under accession numbers KU855380-KU855387. The de-multiplexed ITS dataset obtained using pyrosequencing was deposited in MG-RAST under project number 254695 (accession numbers 4689389.3–4689394.3).

Two supplementary figures and three supplementary tables are available with the online Supplementary Material.

(over 40 °C), which can last from a few days to several days; and (3) mesophilic curing or maturation phase (up to 40 °C), which can last for several months (Mehta *et al.*, 2014).

The potential advantages of composting pressmud include: (i) the production of a marketable product, (ii) reduction of offensive odours caused by rotting, (iii) reduction of environmental pollution by solid waste, (iv) weight reduction of the residue as a result of drying and decomposition of organic matter and (v) an increase in the nutrient concentration in the pressmud due to the removal of water and cellulose (Bernhardt & Notcutt, 1993).

Although several environments have been studied using massive parallel sequencing, only a few composting systems have been investigated (De Gannes *et al.*, 2013; Langarica-Fuentes *et al.*, 2014a, 2015). These studies revealed a large diversity of fungi including several sequences that could not be assigned to known taxa and probably represent novel species. In addition, opportunistic human pathogens have been found in residues used in composting processes (De Gannes *et al.*, 2013), but their occurrence in fresh pressmud is still unknown. However, because composting often reaches high temperatures, it may harbour a high diversity of heat-tolerant fungi with relevance for biotechnological purposes (Oliveira *et al.*, 2015).

Here, the diversity and succession of fungi in a pressmud composting system were characterized using high-throughput 454 pyrosequencing of ITS libraries from the fresh pressmud and during the composting process (thermophilic and maturation phases). Furthermore, the occurrence of opportunistic pathogens in the fresh pressmud and throughout the composting process was evaluated. In addition, heat-tolerant fungi were isolated in the fresh pressmud and in the different stages of the composting process to access their enzymatic potential for biomass conversion.

METHODS

Experimental heaps. Approximately 1 ton of fresh pressmud was sampled to assemble three composting heaps (A, B and C, see Fig. S1 available in the online Supplementary Material). The substrate was collected from Usina Santa Lucia (Araras, SP, Brazil) during the sugarcane harvesting season in 2013. Initially, the whole raw material was mixed and moistened. Then, three piles were assembled in a well-ventilated and sheltered area using the windrow method, which consists of placing the residue in long narrow piles that are generally revolved to improve oxygen content. The piles were placed 1 m apart from one another and each was 0.8 m high, 1 m wide and 1.5 m long.

Sampling and physico-chemical analysis. After mixing and moistening the fresh pressmud, samples from 5 to 10 cm below the surface were taken at various points from the raw substrate, then combined into a composite sample and stored in polyethylene bags. The same procedure was done for each pile during the thermophilic and maturation phases, except for the thermophilic phase of pile A which lasted only three days and did not reach high temperatures.

The organic carbon content of the compost was estimated by the weight loss, using the combustion method at 430 °C for 24 h (Nelson & Sommers, 1982), and the total amount of carbon in the sample was determined by dividing the percentage of observed organic matter content in the sample by the correction factor 1.8, based on the assumption that humified organic matter contains around 54 % of carbon (Jiménez & García, 1992). Total nitrogen was measured by the Kjeldahl method following digestion in sulfuric acid with catalysts (Bremner & Mulvaney, 1982).

The temperature of the piles was monitored daily over the first month of composting and twice a week during the second month using a digital soil thermometer with a 50 cm rod-shaped sensor (Instrutherm, São Paulo, SP, Brazil).

Assessing fungal diversity by pyrosequencing. A total of six samples were collected as described (Fresh pressmud; thermophilic phase of piles B and C; and maturation phase of piles A, B and C) and maintained at −80 °C. DNA extraction was carried out using PowerSoil DNA Isolation Kit (MO-BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. A total of 0.3 g of substrate from each pile was used for DNA extraction. DNA concentration and quality were checked in NanoDrop (Thermo Scientific, Waltham, MA, USA) and stored at −20 °C.

Internal transcribed spacer (ITS) amplification, library preparation and sequencing were performed by Macrogen (http://www.macrogen.com, Seoul, Korea). Briefly, ITS region of fungi belonging to each sample was amplified using the ITS1F-ITS4 primer pair. These primers were connected with a barcode sequence to identify each library and an adaptor region. Amplicons derived from all samples were pooled together in an equimolar ratio and the sequencing was performed in a 454 pyrosequencing Genome Sequencer FLX Plus (Life Sciences, Branford, CT, USA).

Analysis of pyrosequencing data. Sequence processing, grouping in Operational Taxonomic Units (OTUs), taxonomic affiliation and diversity metrics were performed in MacQIIME v.1.8.0 (Caporaso *et al.*, 2010). The raw sequences were filtered for quality (Phred>25), length (>400 bp) and homopolymer (length >6 bp). Quality-checked sequences were then submitted to *de novo* chimera detection in UCHIME (Edgar *et al.*, 2011) using the USEARCH v. 5.2.236 (Edgar, 2010) pipeline in MacQIIME.

After processing, sequences with 97 % similarity were clustered in OTUs in USEARCH. A representative sequence of each OTU was selected using *pick_rep_set.py* script in MacQIIME. For taxonomic assignment, the RDP classifier (minimum confidence of 0.5) (Wang *et al.*, 2007) was used against UNITE+INSDC database (https://unite.ut.ee, Abarenkov *et al.*, 2010). To confirm the taxonomic assignment of the most abundant OTUs, sequences were compared with similar sequences available in NCBI-GenBank database (http://www.ncbi.nlm.nih.gov/) using MegaBLAST algorithm (Morgulis *et al.*, 2008). After reviewing the results of the OTU clustering, there were still OTUs unassigned to any fungal phyla; BLAST searches of representative sequences of these OTUs revealed that they were likely chimera sequences that passed through to the *de novo* detection; therefore, such OTUs were removed before the diversity analyses.

Fungal diversity in composting heaps was evaluated by alpha diversity indices (Shannon and Simpson), richness estimator (Chao1) and rarefaction curves. Beta-diversity between fresh pressmud and different composting phases was evaluated by the Bray–Curtis similarity index and principal coordinate analysis (PCoA) calculated in MacQIIME. To determine differences in fungal community structure between the samples from the different composting phases, an analysis of similarity (ANOSIM) was used based on the Bray–Curtis distance matrix. Then, differences in the initial and final proportion of pathogenic fungi were assessed by the Chi-square test ($P<0.05$) in R v. 3.0.1 (R Development Core Team 2013).

The de-multiplexed ITS dataset obtained using pyrosequencing was deposited in MG-RAST under project number 254695 (accession numbers 4689389.3–4689394.3).

Isolation and identification of thermophilic and thermotolerant fungi. To obtain heat-tolerant fungi with biomass-degrading potential, samples were taken from the fresh pressmud and in the thermophilic and maturation phases of composting. Samples of 10 g from each pile and at each composting stage (as described in the "sampling" section) were subjected to serial dilution. Then, 100 µl from the 10^{-3} to 10^{-5} dilutions were spread onto two standard culture media widely used for thermophilic fungi (Salar & Aneja, 2007) (in g l^{-1}): (1) Yeast Starch Agar (4 g yeast extract, 15 g soluble starch, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 20 g agar); (2) Yeast Glucose Agar (5 g yeast extract, 10 g glucose and 20 g agar) and onto a third medium (nutrient-balanced medium): (3) Malt Extract Agar 2 % (MA2 %) (20 g malt extract and 15 g agar). Both the yeast starch agar and yeast glucose agar were supplemented with 50 mg l^{-1} rose bengal to restrict the development of fast-growing fungi. All media were supplied with 30 mg l^{-1} streptomycin sulfate and penicillin G (Sigma-Aldrich, St Louis, MO, USA) to inhibit bacterial growth. Plates were incubated at 45 °C for five days and monitored daily.

All fungi recovered from the substrates were purified on MA2 % and subjected to morphological screening. Representative isolates of each morphospecies were identified using morphological (macro and microscopic characteristics) and molecular approaches.

For each morphospecies, genomic DNA from representative isolates was extracted by physical lysis with glass beads (425–600 µm diameter) following a combined protocol from Moller *et al.* (1992) and Gerardo *et al.* (2004) using fresh mycelia. After DNA extraction, the ITS region or β-tubulin gene were amplified with primers ITS4 and ITS5 (White *et al.*, 1990) for the ITS region or βT2a and βT2b (Glass & Donaldson, 1995) for β-tubulin. Amplification reactions consisted of 0.2 mM each of dNTP, 5× KCl buffer, 1.5 mM $MgCl_2$, 0.5 µM each of the primer and 1 U of *Taq* polymerase (Promega, Madison, WI, USA) in a final volume of 25 µl. Amplicon purification was performed using the Wizard SV Gel and PCR Clean-up System kit (Promega, Madison, WI, USA), and amplicons were quantified in the NanoDrop (Thermo Scientific, Waltham, MA, USA). The sequencing reaction was performed using the BigDye Terminator Cycle Sequencing kit v.3.1 (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol and applied to the ABI 3330 sequencer (Life Technologies, Carlsbad, CA, USA).

Forward and reverse sequences were compiled in contigs using BioEdit v7.1.3. The contigs were compared with homologous sequences deposited in NCBI-GenBank and the database of the Fungal Biodiversity Centre (CBS-KNAW). To confirm the species identification, the sequences were subjected to phylogenetic analysis in MEGA v.5.05 using additional sequences from reference strains (mostly from CBS-KNAW). The sequences were deposited in GenBank under accession numbers KU855380–KU855387. The isolate TBO264 was identified only by morphology.

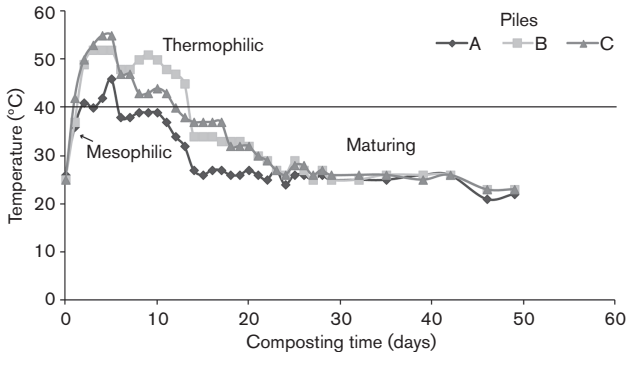

Fig. 1. Temperature monitoring during composting of pressmud in three heaps (A, B and C). Composting stages are denoted in the figure.

Enzymatic profiling of heat-tolerant fungi. To test the enzymatic potential of the heat-tolerant strains for biomass conversion, a screening of four different enzymes involved in the process (cellulase, xylanase, laccase and polygalacturonase) was undertaken. A total of 110 fungi isolated from the composting heaps were submitted for enzymatic screening. Isolates identified as *Aspergillus fumigatus* were excluded from the screening because this fungus is considered to be a potential opportunistic pathogen, although it is a good producer of some of the evaluated enzymes. The strains maintained on MA2 % slants at 25 °C were subcultured in MA2 % and incubated at 45 °C for five days prior to enzymatic screening.

The screening for fungal producers of hemicellulolytic enzymes (cellulase and xylanase) was performed using a method that cultivated fungi in microtubes (2 ml) containing 1.1 ml of specific liquid medium to induce enzyme production. The tubes were incubated for eight days at 45 °C under agitation (120 rpm). Then, the tubes were centrifuged at 3000 rpm for 10 min and the supernatant was recovered for enzyme extraction. Next, 50 µl of the extracts were added to sterilized plastic cylinders and arranged in plates containing medium specific for each enzyme. The plates were incubated at 45 °C in the dark for 24 h.

Culture media used to induce the production of cellulase and xylanase and the visualization of the results (based on halo formation) was performed according to the methods of Kasana *et al.* (2008) and Strauss *et al.* (2001), respectively. The screening for polygalacturonase and laccase-producing fungi was performed in individual plates using the methods of Mckay (1988) and D'Souza *et al.* (2006), respectively. The presence of a halo around the fungal colony indicated a positive result.

RESULTS

Physico-chemical analysis of pressmud composting

The composting heaps quickly reached the thermophilic phase. Pile C reached the thermophilic phase (temperature above 40 °C) on the second day of composting, while piles A and B reached the thermophilic phase on the third day (Fig. 1). Pile A remained in the thermophilic phase from the 3rd to the 5th day and reached 46 °C (Fig. 1). Pile B remained in the thermophilic phase from the 3rd to the 14th day of composting and reached a temperature peak of 52 °C. Pile C remained in the thermophilic phase from the 2nd to the 13th day and reached the highest temperature peak of 55 °C (Fig. 1). Because pile A reached a maximum temperature of 46 °C followed by the maturation stage, no sampling was performed during the thermophilic phase for this heap.

The C/N ratio of the initial compost heaps was 29.10 (Table 1). This ratio decreased during the maturation stage for the three piles, mostly due to the increased N content compared to the beginning of the process. Pile A showed the lowest change in the C/N ratio in comparison to the fresh pressmud (Table 1).

Table 1. Chemical characterization of pressmud composting of three piles during different phases

Sample	Nitrogen (%)	Organic matter (%)	Carbon (%)	C/N
Fresh pressmud*	1.41	73.81	41.10	29.10
T2B	1.84	77.65	43.14	23.46
T2C	1.77	74.33	41.29	23.29
T3A	1.70	79.87	44.37	26.12
T3B	1.94	69.39	38.55	19.86
T3C	1.98	65.70	36.15	18.27

*Fresh pressmud, mesophilic phase. T2, thermophilic phase; T3, maturation phase; A, B and C indicate the different composting piles.

Massive parallel sequencing of pressmud composting system

A total of 148 643 sequences were obtained from the six samples analysed. After processing, the final dataset consisted of 85 605 sequences, with an average of 14 267.33 ±1 712.38 sequences per library. All sequences were clustered in 175 OTUs (see Table S1).

Fungi of the phylum Ascomycota were predominant throughout the composting process (70.3 % of the total sequences), followed by Basidomycota (17.7 %). Representatives of the phylum Chytridiomycota (fungi that need water to reproduce), although in low amounts (lower than 0.01 %), were also found. The remaining sequences (12 %) were classified as unidentified fungi.

Regarding the taxa comprising the phylum Ascomycota, the most abundant order was Microascales (24.6 % of the sequences), followed by Eurotiales and Sordariales (16.8 % both, Fig. 2). Considering the phylum Basidiomycota, the most abundant order was Agaricales (6.9 %, Fig. 2). The most abundant OTUs (>1 % of the total sequences) found in the composting piles are summarized in Table 2 (the full list is available in Table S2).

A marked predominance of *Lomentospora prolificans* (Microascales, 43.13 % of the sequences) was observed in the fresh pressmud. In addition, *Thermomyces lanuginosus* (Eurotiales, 10.44 %), a thermophilic fungus often associated with composting systems, and *Trichosporon* sp2 (Trichosporonales, 10.06 %, Table 2) were found as the most abundant.

In the thermophlic phase, *L. prolificans* was found as the most abundant OTU in pile B (43.65 %); however, in pile C, which achieved the highest temperature (55 °C), there was a drastic reduction in the abundance of this fungus (14.91 %). In contrast, the abundance of *T. lanuginosus* during this phase was reduced in the pile B but increased in pile C (7.47 and 21.57 %, respectively). Furthermore, *Mycothermus thermophilum* (Leotiomycetidae), also considered a thermophilic fungus, and *Zopfiella* species (Sordariales) were the most abundant in pile C (11.35 and 13.17 %, respectively).

During the maturation phase of the compost, the abundance of *L. prolificans* was reduced in piles B and C (5.67 and 8.97 %, respectively), but the same did not occur in pile A (19.42 %), which did not reach high temperature (maximum of 46 °C) and the thermophilic phase lasted for only three days. Furthermore, *T. lanuginosus* became the most abundant in piles B and C during the maturation phase. However, in pile A there was a higher growth of Basidiomycetes fungi, such as *Coprinus cordisporus*, *Trichosporon* sp1 and *Coprinus* sp. (12.16, 10.14 and 5.72 %, respectively), when compared to the other piles.

It is worth mentioning that the presence of a wide range of fungi considered to be opportunistic pathogens, such as *L. prolificans* (43.13 %), *Trichosporon* sp. (10.07 %), *Candida tropicalis* (Saccharomycetales, 7.91 %) and *Hormographiella aspergillata* (Agaricales, 8.19 %), are among the most abundant taxa in fresh pressmud. However, all these fungi had a remarkably reduced load ($P<0.05$) after the thermophilic stage, especially in piles B and C, where temperatures were higher.

Along the pressmud composting process, there was a slight tendency to increase the number of species (see Table S3). Fresh pressmud showed 83 OTUs; 80 and 89 OTUs were observed during the thermophilic phase of piles B and C, respectively. Lastly, the highest number of OTUs was observed in the maturation phase in the three composting piles (mean of 116.7±6.1 OTUs, see Table S2).

The species richness estimator Chao1 showed that the number of observed taxa was close to the expected in almost all samples (see Table S2), corroborating the rarefaction analysis of OTUs at 97 % similarity (see Fig. S2); this indicates that sampling successfully assessed a comprehensive part of the fungal diversity present in the pressmud composting. Alpha diversity indices (Shannon and Simpson) followed a similar pattern as OTUs richness, showing a tendency to increase throughout the composting process (see Table S2). Furthermore, Bray–Curtis similarity index showed that different piles share an average of 58.5±9.3 % of OTUs, even considering the same stages of composting.

The ANOSIM test confirmed that the observed community composition was not significantly different among the

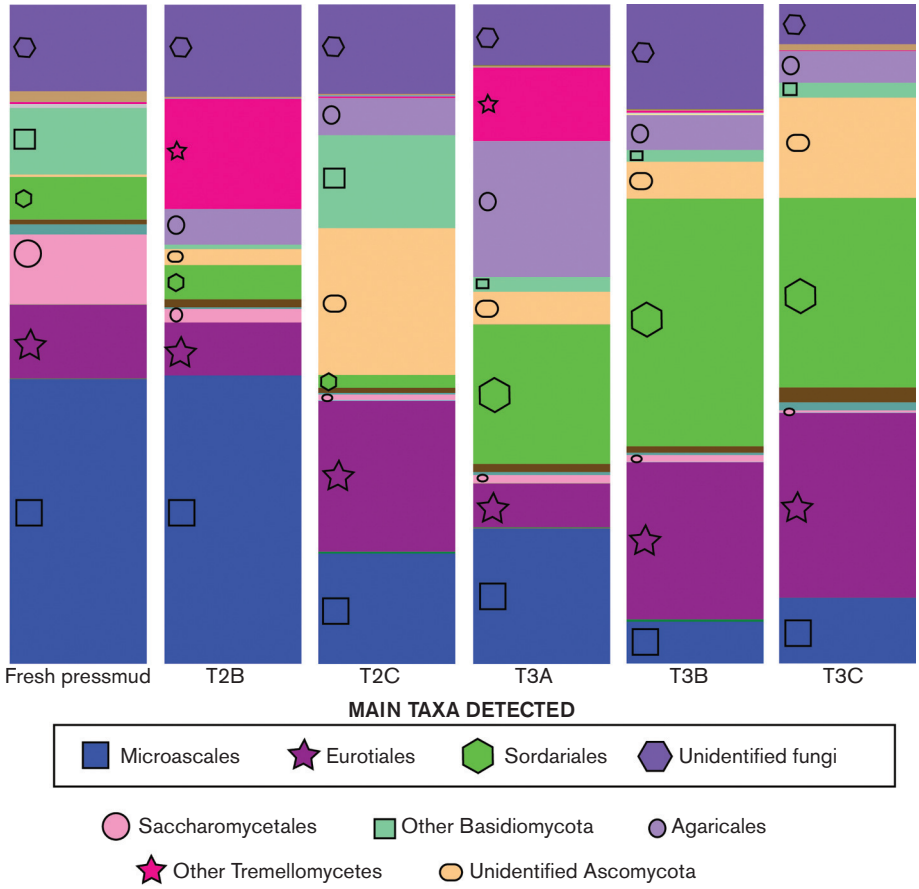

Fig. 2. Mycota (ranked by order) of the fresh pressmud and of the different composting stages. The different fungal orders are represented by different colors as well as by symbols. Fresh pressmud, mesophilic phase; T2, thermophilic phase; T3, maturation phase. A, B and C indicate the different piles.

different stages (fresh pressmud, thermophilic and maturation phases) (R=0.545, P>0.1). However, as the diversity increased in the piles, fungal diversity became more distinct, indicating succession over the composing process; the greatest differences were found mainly when comparing the thermophilic (T2) to the maturation phase (T3) (see Table S2). PCoA confirms that the fungal community structure at the end of the composting process (maturation phase) tends to be different from the initial and thermophilic phases (Fig. 3). However, PCoA also confirmed that distinguishable microbial communities were found when comparing different piles at the same stage, possibly because of the different temperatures reached by the piles in the thermophilic phase (e.g. piles B and C ended up with a similar fungal community in the maturation phase, different from pile A, which did not reach high temperatures; Fig. 3). The change in dominance of the opportunistic pathogen *L. prolificans* seems to be the main influencing factor in this variance. PCoA corroborates this fact, since very high percentages were found in the fresh pressmud sample and in the thermophilic phase for pile B, but lower values were found for the other samples, especially for the mesophilic phase in piles B and C (Figs 2 and 3).

Assessment of heat-tolerant fungi and their biomass-degrading enzymes

A total of 332 heat-tolerant fungi were isolated from the six data points (fresh pressmud, thermophilic stage of piles B and C and maturation stage of piles A, B and C, Table 3). The temperature of the fresh pressmud was 25 °C, and eight fungal species were obtained from this substrate. During the thermophilic phase, seven and six fungal species were recovered from piles B (52 °C) and C (55 °C), respectively. During the maturation phase, the temperature of pile A was 27 °C and the temperature of piles B and C was 32 °C. Three, five and eight fungal species were obtained from these piles, respectively (Table 3).

Among all the isolates obtained, five species belonged to the phylum Ascomycota (91.6 % of the isolates) and four to the subphylum Mucoromycotina (8.4 %). Fungi in the phylum Ascomycota comprised the orders Eurotiales (*Aspergillus*

Table 2. Relative abundance (%) of the most abundant taxa (OTUs) in different stages of pressmud composting*

Fungal taxa	Sample					
	Fresh pressmud**	T2B	T2C	T3A	T3B	T3C
Lomentospora prolificans	43.13	43.65	14.91	19.42	5.67	8.97
Thermomyces lanuginosus	10.44	7.47	21.57	5.59	22.26	26.54
Mycothermus thermophilum	0.35	2.22	20.99	3.87	4.0	11.35
Trichosporon sp1	0.01	16.58	0.26	11.09	0.3	0.12
Trichosporon sp2	10.06	0.52	14.10	2.05	1.17	1.06
Zygopleurage zygospora	3.61	1.3	0.13	5.88	11.46	1.33
Coprinopsis sp.	0.04	9.4	10.82	0.49	0.19	0.28
Unidentified fungi	4.23	0.72	0.65	3.67	10.42	1.92
Coprinus cordisporus	0.02	1.44	1.32	13.39	1.57	3.28
Zopfiella sp.	0.16	0.36	0.36	2.3	4.33	13.17
Coprinus sp.	–	3.5	3.82	6.25	1.49	1.15
Cercophora coronata	1.59	1.61	0.05	0.7	9.69	1.39
Candida tropicalis	7.91	1.8	0.65	0.3	0.64	0.29
Sordariales	0.04	0.63	0.53	2.4	2.11	6.64
Hormographiella aspergillata	8.19	1.3	1.08	0.03	0.02	0.04
Podospora communis	0.16	1.59	0.01	2.63	3.37	1.14
Cercophora sp.	0.12	0.82	0.42	0.35	5.17	0.43

*Considering the total number of sequences representing more than 1 % of the sequences in each library.
**Fresh pressmud, mesophilic phase; T2, thermophilic phase; T3, maturation phase; A, B and C indicate the different composting piles.

fumigatus and *T. lanuginosus*), Onygenales (*Myceliophthora fergusii* and *M. thermophila*) and Leotiomycetidae (*Mycothermus thermophilus*). All representatives of Mucoromycotina belonged to the order Mucorales (*Lichtheimia ramosa, Rhizomucor miehei, Rhizopus microsporus* and *Thermomucor indicae-seudaticae*).

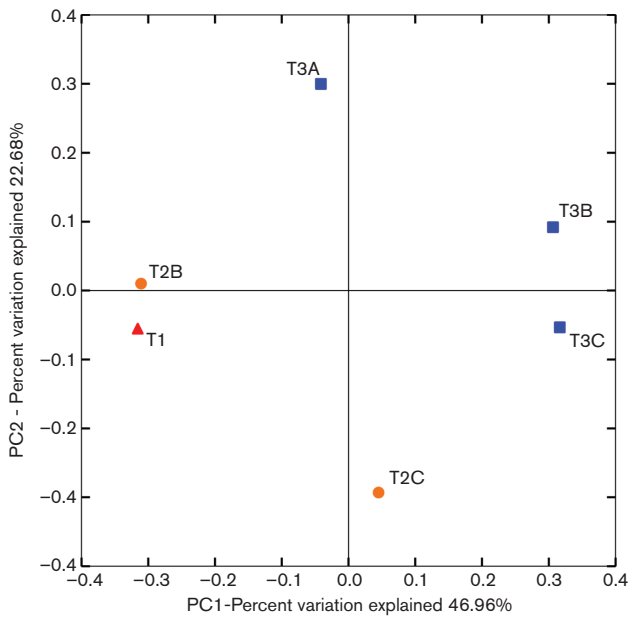

Fig. 3. Principal coordinate analysis (PCoA) of the fungal communities in a pressmud composting system. Fresh pressmud (red), thermophilic phase (orange), maturation phase (blue).

A. fumigatus was the most abundant species isolated in culture (66.3 % of the isolates), followed by *T. lanuginosus* (16 %). Both fungi were prevalent and consistently isolated in all samples. Other species were also observed in the different stages of composting, although they were not common to all stages of each pile (i.e. *M. fergusii, M. thermophila, R. pusillus* and *T. indicae-seudaticae*, Table 3). The thermotolerant species *L. ramosa* was found only in the fresh pressmud. *M. thermophilum* was present in the fresh pressmud but was not observed during the thermophilic phase. Later in the maturation stage, this species reappeared in piles B and C. *Rhizopus microsporus* appeared during the thermophilic phase and remained during the maturation phase.

With regard to the enzymatic potential of the fungi, all isolates were positive for at least one of the tested enzymes (Table 4). Strains belonging to the same species presented the same enzymatic profile. The majority of fungi was positive for xylanase (60.9 % of the total number of isolates), followed by cellulase and laccase (44.5 % for both) and polygalacturonase (30.9 %).

The production of laccase was observed only for *T. lanuginosus* isolates, which were negative for cellulase. The fungal genera *Lichtheimia, Rhizomucor, Rhizopus* and *Thermomucor* produced polygalacturonase and xylanase. Furthermore, *T. indicae-seudaticae* was the only one able to produce three different enzymes (Table 4). Fungi in the genus

Table 3. Abundance (number of isolates) of thermophilic and thermotolerant fungi during composting of pressmud

Fungal species	Mesophilic	Thermophilic (T2)			Maturing (T3)			Total	%
	Fresh pressmud	A*	B	C	A	B	C		
Aspergillus fumigatus	60		26	44	26	34	30	220	66.3
Lichtheimia ramosa	1							1	0.3
Myceliophthora fergusii	3		4	6			6	19	5.7
Myceliophthora thermophila	1		3	1	1		2	8	2.4
Rhizomucor pusillus	4		2	2			1	9	2.7
Rhizopus microsporus			3	1		5	2	11	3.3
Mycothermus thermophilum	1					2	1	4	1.2
Thermomucor indicae-seudaticae	1		2			2	2	7	2.1
Thermomyces lanuginosus	4		11	7	13	12	6	53	16
Total	75		51	61	40	55	50	332	100

*Because pile A did not reach >50 °C, it was not sampled during the thermophilic phase (see methods). A, B and C indicate the different composting piles.

Myceliophthora are most likely involved in the production of cellulolytic enzymes and were the only fungi that did not show the production of xylanase.

DISCUSSION

The massive ITS sequencing revealed the dynamics of the fungal communities present in fresh pressmud and their change over the composting process, raising new information about the species present in the system. An increase of fungal diversity from the fresh pressmud to the maturation phase was observed. In contrast, other studies with non-sugarcane material, which also used culture-independent techniques to assess fungal diversity, showed an opposite trend, with reduction of fungal diversity after the thermophilic phase (Tiquia, 2005; Bonito *et al.*, 2010; Hultman *et al.*, 2010). However, De Gannes *et al.* (2013) evaluated three different composting systems (rice straw, sugarcane bagasse and coffee hulls) and observed that the sugarcane bagasse composting follows the same pattern found in pressmud composting, indicating that the increasing of fungal diversity might be a characteristic of sugarcane residues and its composting process. Furthermore, Ascomycota is often the prevalent phylum in composting systems as observed in pressmud composting, generally comprising more than 60 % of the sequences (Langarica-Fuentes *et al.*, 2014a, 2015) or in some cases reaching up to 93 % (De Gannes *et al.*, 2013).

Ghazifard *et al.* (2001) reported that the increase in temperature caused a modification of the microbiota, reducing the diversity of mesophilic fungi and leading to an increase in the diversity of heat-tolerant fungi. In pressmud composting, the increase in temperature most likely led to an increase in the microbial load of some species (mainly heat-tolerant species) over the reduction of others (mesophilic species and pathogens), which did not withstand the temperature reached during the thermophilic phase, instead of alter species composition.

Fresh pressmud is often applied directly to sugarcane crops, without any prior treatment (Balakrishnan & Batra, 2011). However, it is noteworthy that, *L. prolificans* along with *C. tropicalis* accounted for more than 50 % of the sequences in the fresh pressmud, and both are classified as

Table 4. Enzymatic profile of thermophilic and thermotolerant fungi isolated from a pressmud composting system

Fungal species	Enzymes			
	Cellulase	Xylanase	Polygalacturonase	Laccase
Lichtheimia ramosa		+	+	
Myceliophthora fergusii	+			
Myceliophthora thermophila	+			
Rhizomucor pusillus		+	+	
Rhizopus microsporus		+	+	
Mycothermus thermophilum	+	+		
Thermomucor indicae-seudaticae	+	+	+	
Thermomyces lanuginosus		+		+

Biosafety Level 2 microorganisms by the American Biological Safety Association (ABSA).

In particular, *L. prolificans* is an emerging opportunistic pathogen with high levels of resistance to most antifungal drugs, and infections caused by this fungus are often fatal (Berenguer *et al.*, 1997; Song *et al.*, 2011). However, basic aspects of its biology, such as the natural reservoir, are still poorly known; clarifying these issues is necessary to prevent exposure of immuno-compromised individuals to this fungus (Thornton *et al.*, 2015). Previously, the prevalence of *L. prolificans* was also observed in the mesophilic phase of sugarcane bagasse composting (De Gannes *et al.*, 2013) suggesting that sugarcane, among other environments, might be a natural reservoir of this fungus.

Langarica-Fuentes *et al.* (2014b) evaluated the diversity of fungi in two commercial compounds in room temperature and after exposure to heating (50 °C). In one of the compounds at room temperature, the authors observed the presence of *L. prolificans,* although in low abundance (1.6 %). However, this fungus was not present among the most abundant taxa (>0.3 %) after heating both compounds up to 50 °C.

Supported by indirect results from De Gannes *et al.* (2013) and Langarica-Fuentes *et al.* (2014b), the notable reduction of *L. prolificans* at the end of the pressmud composting process highlights composting as a promissing treatment for pressmud residues before their direct application to agriculture. Specifically, the reduction in *L. prolificans* is possibly because of the natural community succession, since this fungus is not adapted to high temperatures. Therefore, the heat treatment derived from pressmud composting systems could potentially reduce the chances of handlers being exposed to high amounts of the emerging pathogen during application of this substrate in the field. This is also valid for *C. tropicalis* and the other pathogenic fungi present in the pressmud, which also decreased in abundance over the composting process.

Studies on the diversity of fungi in composting systems employing culture-dependent tools commonly report *Aspergillus fumigatus* as prevalent (Ghazifard *et al.*, 2001; Dehghani *et al.*, 2012). *A. fumigatus* was also found as the prevalent species isolated in piles of pressmud composting (66.3 % of the total isolates). However, here this fungus was not among the most abundant when using a culture-independent method, suggesting that it does not play an important role in the system but it has the advantage that it grows rapidly in the culture media (i.e. a copiotrophic fungus). On the other hand, *T. lanuginosus* was found to be among the most abundant when it was assessed by both culture-dependent and culture-independent methods. This fungus is thermophilic and often associated with various composting systems in which it is found among the most abundant taxa (Mchunu *et al.*, 2013; Langarica-Fuentes *et al.*, 2014a, b). During the isolation survey *L. prolificans* was not obtained, possibly because it was performed at high temperatures (45 °C), targeting isolation of thermophilic fungi.

Although fresh pressmud is rich in pathogenic fungi, the composting process contains a variety of fungi with biotechnological potential, for example, the production of enzymes for biomass conversion. *T. lanuginosus* has been reported to be the largest producer of cellulase-free xylanases, which has prompted the use of genome sequencing to assess the genetic potential of this species for industrial applications (Mchunu *et al.*, 2013). Zhou *et al.* (2014) exploited the genome of *Rhizomucor miehei* and reported the existence of a large number of genes encoding proteolytic, amylolytic and lipolytic enzymes including xylanase and β-glucanase, revealing the potential of this fungus for the degradation of organic matter.

Several studies have suggested that *M. thermophila* (Moretti *et al.*, 2012; Pereira *et al.*, 2015) and *M. heterothallica* (van den Brink *et al.*, 2013) are interesting bioconverters of lignocellulosic residues to simple sugars. Furthermore, the genomic study of *M. thermophila* and *Thielavia terrestris* suggested that they were capable of hydrolyzing all the major polysaccharides present in the plant biomass (Berka *et al.*, 2011). *M. thermophila* has the largest number of hemicellulolytic enzymes and accessory enzymes observed to date; it contains eight genes encoding endoglucanases, seven cellobiohydrolases, nine β-glucosidases, 25 lytic polysaccharide monooxygenases (LPMOs), and other enzymes of the group including xylanase, arabinases, mannanase, pectinases and esterases (Karnaouri *et al.*, 2014).

Therefore, we raise an alert for the use of fresh pressmud, because it is a putative source of human pathogenic fungi, and it presents a potential threat if applied as fertilizer without any treatment. However, composting with the thermophilic phase minimizes the amount of opportunistic pathogenic fungi in this substrate. At the same time, the pressmud composting process creates an interesting environment to study fungi that can produce thermostable hydrolytic and oxidative enzymes. Such fungi have the potential to be used as a starter culture consortium to accelerate the composting process of pressmud, other plant wastes or for industrial purposes.

ACKNOWLEDGEMENTS

The authors would like to thank FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for providing financial support to AR (Young Research Award # 2011/16765–0) and for a scholarship to TBO (# 2012/14594–7). We are also grateful to all members of our research group for providing helpful comments on this manuscript. This work was performed under permit #010554/2014–9 issued by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

REFERENCES

Abarenkov, K., Henrik Nilsson, R., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E. & other

authors (2010). The UNITE database for molecular identification of fungi-recent updates and future perspectives. *New Phytol* **186**, 281–285.

Balakrishnan, M. & Batra, V. S. (2011). Valorization of solid waste in sugar factories with possible applications in India: a review. *J Environ Manage* **92**, 2886–2891.

Berenguer, J., Rodríguez-Tudela, J. L., Richard, C., Alvarez, M., Sanz, M. A., Gaztelurrutia, L., Ayats, J. & Martinez-Suarez, J. V. (1997). Deep infections caused by *Scedosporium prolificans*. A report on 16 cases in Spain and a review of the literature. Scedosporium Prolificans Spanish Study Group. *Medicine* **76**, 256–265.

Berka, R. M., Grigoriev, I. V., Otillar, R., Salamov, A., Grimwood, J., Reid, I., Ishmael, N., John, T., Darmond, C. & other authors (2011). Comparative genomic analysis of the thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. *Nat Biotechnol* **29**, 922–929.

Bernhardt, H. W. & Notcutt, P. (1993). Composting of filter cake from a sugar factory. *South African Sugar Technologists' Association*, 185–187.

Bhosale, P. R., Chonde, S. G., Nakade, D. B. & Raut, P. D. (2012). Studies on physico-chemical characteristics os waxed and dewaxed pressmud and its effect on water holding capacity of soil. *Int J Biol Sci* **1**, 35–41.

Bonito, G., Isikhuemhen, O. S. & Vilgalys, R. (2010). Identification of fungi associated with municipal compost using DNA-based techniques. *Bioresour Technol* **101**, 1021–1027.

Bremner, J. M. & Mulvaney, C. S. (1982). Nitrogen total. In *Methods of Soil Analysis*, Part 2 pp. 371–378. Edited by A. L Page, R. H. Miller & D. R. Keeney. Madison: Am. Soc. Agron.

CONAB (2015). Companhia Nacional de Abastecimento. *Acompanhamento Da Safra Brasileira Cana-De-Açúcar*, pp. 1–28. Brasília: Primeiro Levantamento.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K. & other authors (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336.

D'Souzaa, D. T., Tiwari, R., Sah, A. K. & Raghukumar, C. (2006). Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. *Enzyme Microb Tech* **38**, 504–511.

de Cassia Pereira, J., Paganini Marques, N., Rodrigues, A., Brito de Oliveira, T., Boscolo, M., da Silva, R., Gomes, E. & Bocchini Martins, D. A. & Pereira, J. C. (2015). Thermophilic fungi as new sources for production of cellulases and xylanases with potential use in sugarcane bagasse saccharification. *J Appl Microbiol* **118**, 928–939.

De Gannes, V., Eudoxie, G. & Hickey, W. J. (2013). Insights into fungal communities in composts revealed by 454-pyrosequencing: implications for human health and safety. *Front Microbiol* **4**, 164.

Dehghani, R., Asadi, M. A., Charkhloo, E., Mostafaie, G., Saffari, M., Mousavi, G. A. & Pourbabaei, M. (2012). Identification of fungal communities in producing compost by windrow method. *J Environ Protect* **3**, 61–67.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–2200.

Gerardo, N. M., Currie, C. R., Price, S. L. & Mueller, U. G. (2004). Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ants symbiosis. *Proc R Soc B B* **271**, 1791–1798.

Ghazifard, A., Kasra-Kermanshahi, R. & Far, Z. E. (2001). Identification of thermophilic and mesophilic bacteria and fungi in Esfahan (Iran) municipal solid waste compost. *Waste Management & Research* **19**, 257–261.

Glass, N. L. & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* **61**, 1323–1330.

Hultman, J., Vasara, T., Partanen, P., Kurola, J., Kontro, M. H., Paulin, L., Auvinen, P. & Romantschuk, M. (2010). Determination of fungal succession during municipal solid waste composting using a cloning-based analysis. *J Appl Microbiol* **108**, 472–487.

Jiménez, E. I. & García, V. P. (1992). Relationships between organic carbon and total organic matter in municipal solid wastes and city refuse composts. *Bioresour Technol* **41**, 265–272.

Karnaouri, A., Topakas, E., Antonopoulou, I. & Christakopoulos, P. (2014). Genomic insights into the fungal lignocellulolytic system of *Myceliophthora thermophila*. *Front Microbiol* **5**.

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S. & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. *Curr Microbiol* **57**, 503–507.

Langarica-Fuentes, A., Zafar, U., Heyworth, A., Brown, T., Fox, G. & Robson, G. D. & Box, G. (2014a). Fungal succession in an in-vessel composting system characterized using 454 pyrosequencing. *FEMS Microbiol Ecol* **88**, 296–308.

Langarica-Fuentes, A., Handley, P. S., Houlden, A., Fox, G. & Robson, G. D. & Foz, G. (2014b). An investigation of the biodiversity of thermophilic and thermotolerant fungal species in composts using culture-based and molecular techniques. *Fungal Ecol* **11**, 132–144.

Langarica-Fuentes, A., Fox, G. & Robson, G. D. (2015). Metabarcoding analysis of home composts reveals distinctive fungal communities with a high number of unassigned sequences. *Microbiology* **161**, 1921–1932.

McBride, M. B., Prado, R. M., Caione, G. & Campos, C. N. S. (2013). Arsenic and lead uptake by vegetable crops grown on historically contaminated orchard soils. *Appl Environ Soil Sci* **2013**.

Mchunu, N. P., Permaul, K., Abdul Rahman, A. Y., Saito, J. A., Singh, S. & Alam, M. & Rahman, A. Y. A. (2013). Xylanase superproducer: genome sequence of a compost-loving thermophilic fungus, *Thermomyces lanuginosus* strain SSBP. *Genome Announc* **1**, e00388-13.

Mckay, A. M. (1988). A fungal assay method for the detection of fungal polygalacturonase secretion. *FEMS Microbiol Lett* **55**, 355–358.

Mehta, C. M., Palni, U., Franke-Whittle, I. H. & Sharma, A. K. (2014). Compost: its role, mechanism and impact on reducing soil-borne plant diseases. *Waste Manag* **34**, 607–622.

Moretti, M. M., Bocchini-Martins, D. A., Silva, R. D., Rodrigues, A., Sette, L. D. & Gomes, E. (2012). Selection of thermophilic and thermotolerant fungi for the production of cellulases and xylanases under solid-state fermentation. *Braz J Microbiol* **43**, 1062–1071.

Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T. L., Agarwala, R. & Schäffer, A. A. (2008). Database indexing for production MegaBLAST searches. *Bioinformatics* **15**, 1757–1764.

Möller, E. M., Bahnweg, G., Sandermann, H. & Geiger, H. H. (1992). A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res* **20**, 6115–6116.

Nelson, D. W. & Sommers, L. E. (1982). Total carbon, organic carbon and organic matter. In *Methods of Soil Analysis*. Edited by A. L Page. Madison: Part II American Society of Agronomers.

Oliveira, T. B., Gomes, E. & Rodrigues, A. (2015). Thermophilic fungi in the new age of fungal taxonomy. *Extremophiles* **19**, 31–37.

Salar, R. K. & Aneja, K. R. (2007). Thermophilic fungi: taxonomy and biogeography. *Journal of Agricultural Technology* **3**, 77–107.

Song, M. J., Lee, J. H. & Lee, N. Y. (2011). Fatal *Scedosporium prolificans* infection in a paediatric patient with acute lymphoblastic leukaemia. *Mycoses* **54**, 81–83.

Strauss, M. L., Jolly, N. P., Lambrechts, M. G. & van Rensburg, P. (2001). Screening for the production of extracellular hydrolytic enzymes by non-Saccharomyces wine yeasts. *J Appl Microbiol* **91**, 182–190.

Thornton, C. R., Ryder, L. S., Le Cocq, K. & Soanes, D. M. (2015). Identifying the emerging human pathogen *Scedosporium prolificans* by using a species-specific monoclonal antibody that binds to the melanin biosynthetic enzyme tetrahydroxynaphthalene reductase. *Environ Microbiol* **17**, 1023–1038.

Tiquia, S. M. (2005). Microbial community dynamics in manure composts based on 16s and 18s rdna t-rflp profiles. *Environ Tech* **27**, 1101–1114.

van den Brink, J., van Muiswinkel, G. C., Theelen, B., Hinz, S. W. & de Vries, R. P. (2013). Efficient plant biomass degradation by thermophilic fungus *Myceliophthora heterothallica*. *Appl Environ Microbiol* **79**, 1316–1324.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261–5267.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and Direct 599 Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, pp. 315–332. Edited by M. A Innis, D. H Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.

Zhou, P., Zhang, G., Chen, S., Jiang, Z., Tang, Y., Henrissat, B., Yan, Q., Yang, S., Chen, C. F. & other authors (2014). Genome sequence and transcriptome analyses of the thermophilic zygomycete fungus *Rhizomucor miehei*. *BMC Genomics* **15**, 294.

Edited by: V. Cid