Improved strategies to efficiently isolate thermophilic, thermotolerant, and heat-resistant fungi from compost and soil

Frederick Witfeld¹ · Dominik Begerow¹ · Marco Alexandre Guerreiro¹

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Abstract

Thermophilic, thermotolerant and heat-resistant fungi developed different physiological traits, enabling them to sustain or even flourish under elevated temperatures, which are life-hostile for most other eukaryotes. With the growing demand of heat-stable molecules in biotechnology and industry, the awareness of heat-adapted fungi as a promising source of respective enzymes and biomolecules is still increasing. The aim of this study was to test two different strategies for the efficient isolation and identification of distinctly heat-adapted fungi from easily accessible substrates and locations. Eight compost piles and ten soil sites were sampled in combination with different culture-dependent approaches to describe suitable strategies for the isolation and selection of thermophilous fungi. Additionally, an approach with a heat-shock treatment, but without elevated temperature incubation led to the isolation of heat-resistant mesophilic species. The cultures were identified based on morphology, DNA barcodes, and microsatellite fingerprinting. In total, 191 obtained isolates were assigned to 31 fungal species, from which half are truly thermophilic or thermotolerant, while the other half are heat-resistant fungi. A numerous amount of heat-adapted fungi was isolated from both compost and soil samples, indicating the suitability of the used approaches and that the richness and availability of those organisms in such environments are substantially high.

Keywords Heat-adapted fungi · Thermophily · Thermotolerance · Heat-resistance · Compost · Soil

Introduction

Fungi can grow in a wide range of conditions and inhabit a multitude of environments. Adaptation to specific environmental conditions can provide a considerable ecological advantage in contrast to less adapted organisms. Ecological niches characterized by extreme abiotic factors can be occupied to avoid competition situations or to gain advantages in habitats with both stable and fluctuating environmental conditions. Based on their growth temperature ranges, fungi are classified as psychrophilic, mesophilic, thermophilic, or thermotolerant. Psychrophily and mesophily, as the abilities to grow at low (under 20 °C) and moderate (20–40 °C) temperatures, respectively, are widespread features among fungi. In contrast, the ability to grow at higher temperatures as thermophilous, i.e., as

thermotolerant or as thermophile, is extremely rare. A consistent classification of heat-adaptations in the fungal kingdom is still missing. The most common definition by Cooney and Emerson (1964) classifies fungi with a minimum growth temperature above 20 °C and a maximum growth temperature above 50 °C as thermophilic, and those which can grow between temperatures lower than 20 °C and up to 50 °C as thermotolerant.

Among the 100,000 fungal species currently identified (Kirk et al. 2008), only 75 species are described to be thermophilic (Salar 2018). Additional temperature-adapted features include heat-resistance, which allows usually mesophilic fungi to survive extremely high temperatures (75–100 °C) for a limited amount of time (Samson et al. 2000). Heat-resistance is independent from thermotolerance and thermophily. Although some thermophilous fungi (e.g., *Aspergillus fumigatus*) are known to be heat-resistant, most heat-resistant fungi are actually mesophilic (e.g., *Basidioascus undulatus*) (Fergus and Amelung 1971; Ali et al. 2009; Nguyen et al. 2015).

Thermophilous and heat-resistant fungi possess a repertoire of physiological and molecular adaptations to high temperatures. These adaptations include thick sclerotia (Izzo et al. 2006; Samson and Dijksterhuis 2007), unique compositions

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✉ Marco Alexandre Guerreiro
Marco.Guerreiro@rub.de

¹ Faculty of Biology and Biotechnology, Ruhr-University Bochum, Universitätsstraße 150, 44801 Bochum, Germany

of lipid membranes (Ianutsevich et al. 2016), synthesis of protective compounds enhancing molecular and cellular stability (Jepsen and Jensen 2004), possibly a rapid transcription and translation machinery (Caspeta et al. 2016), and proteins and enzymes with increased heat stability (McPhillips et al. 2014; Robledo et al. 2016). Moreover, the broad repertoire of secondary metabolite production and heat-stable enzymes makes thermophilous fungi biotechnologically valuable (Busk and Lange 2013; Sharma et al. 2016; Liu et al. 2017). Enzymes derived from thermophilous fungi can be used in paper processing (e.g., xylanases), cosmetics (e.g., lipases), and pharmaceutical (e.g., amidase) (Sevo et al. 2002; Ghatora et al. 2006; Maalej-Achouri et al. 2012; Sen et al. 2016; Rigoldi et al. 2018). Many heat-resistant fungi cause spoilage of heat-processed food products (Scaramuzza and Berni 2014; dos Santos et al. 2018) and produce toxic secondary metabolites (Piecková and Jesenská 1997; Puel et al. 2005). Therefore, besides the valuable aspects for biotechnology, heat-adapted fungi are also relevant for economy and medicine. Cultivation of thermophilic and thermotolerant fungi enables experimental approaches, which enhance the understanding of their physiology, evolutionary adaptations, and possible applications. The cultivation of most thermophilous fungi requires media supplemented with easily accessible carbon sources and temperatures between 35 and 50 °C (Maheshwari et al. 2000; Salar 2018). However, studying these organisms is challenging because their isolation and handling is troublesome.

The main difficulties include fast overgrowth of certain species (Tansey 1971), overall low isolation success (Langarica-Fuentes et al. 2014a), potential pathogenicity (De Gannes et al. 2013; Sandona et al. 2019), and demanding culture maintenance at high temperatures. Since some common fast-growing thermophilous fungi outperform others, the isolation of rare species can be difficult (Kumar and Aneja 1999; Morgenstern et al. 2012). Isolation techniques developed to overcome those problems and to isolate thermophilous fungi from various substrates (see Salar 2018) make use of humidity chambers (Buxton and Mellanby 1934), dilution plating (Apinis 1963), and paired petri dish plating (Cooney and Emerson 1964). Additionally, recent studies performed enrichment cultivation by using selective temperature ranges and distinct carbon sources (Anastasi et al. 2005; Nazir et al. 2007; Langarica-Fuentes et al. 2014a). However, different combinations of these techniques and factors might be necessary to cover the physiological requirements of differently heat-adapted species from various substrates.

Thermophilous fungi often colonize habitats, characterized by temporary or frequent heating or associated with dead plant material. For instance, plant compost, herbivore dung, municipal waste, bird nests, wood chip piles (Korniłowicz-Kowalska and Kitowski 2013; Ahirwar et al. 2017; Noreen et al. 2019), as well as fermenting foods are known habitats for

thermophilous fungi (Zhang et al. 2016). Furthermore, the detection of thermophilous fungi in various types of soil has been often reported (Eggins and Malik 1969; Powell et al. 2012; Ahirwar et al. 2017). As a result of the unstable temperatures, the occurrence of mesophilic fungi, including heat-resistant species, is common in these habitats. Due to their unique adaptation to temperature, heat-resistant fungi have advantages in colonizing habitats with periodically extremely high temperature disturbances, e.g., flare pits, forest fire regions, or areas with slash-and-burn agriculture. Additionally, heat-resistant fungi have been repeatedly detected in heat-sterilized food products, e.g., fruit juices or canned foods (Tournas and Traxler 1994; Kotzekidou 1997), leading to spoilage of such products and economic losses. However, habitats exclusive for one group of these heat-adapted fungi are extremely rare. In compost temperatures fluctuate between ambient temperature and over 70 °C during the different phases of composting (Lechner et al. 2005; Fischer and Glaser 2012; Langarica-Fuentes et al. 2014b). Thus, compost harbors a highly dynamic and rich diversity of heat-adapted and mesophilic saprotrophic species (Langarica-Fuentes et al. 2014b; Galitskaya et al. 2017). Therefore, compost is a promising source of fungal species with biotechnological potential due to the broad repertoire of heat-stable enzymes.

The aim of this study was to develop an approach suitable for the isolation of a broad range of thermotolerant, thermophilic, and heat-resistant fungal species with promising biotechnological application. For this, we sampled common garden composts and soil. For the isolation of thermophilous fungi, the incubations were performed at two distinct temperatures on five different media. Additionally, we applied a heat-shock treatment to select for heat-resistant thermophilous and heat-resistant mesophilic species. Variations of the sampling, substrate-processing and cultivation were applied in order to increase isolation efficiency of heat-adapted fungi.

Material and methods

Study design

This study aimed for the efficient isolation and cultivation of differently heat-adapted fungi with different enzymatic repertoires and temperature ranges by using two strategies (Table 1, Fig. 1). The heat-adapted fungi were categorized as thermophilic (growing from 20 to > 50 °C) and thermotolerant (growing from < 20 to 50 °C) as defined by Cooney and Emerson (1964) and heat-resistant (resisting 75 °C for 30 min) according to Samson et al. (2000). We collected compost and soil samples from distinct locations and isolated fungi with variations of a flotation-based approach. The first strategy (strategies 1a and 1b), based on the combination of distinct temperature treatments (incubation at 45 °C or 55 °C) and standard

Table 1 Variation of parameters used for the sampling and cultivation strategies. Different carbon sources, incubation temperatures at 45 °C, 55 °C, and room temperature (RT), combined with a heat-shock (HS) treatment and usage of various dilutions for inoculation were tested for isolation of thermophilous and heat-resistant fungi from both compost and soil

Parameter	Strategy 1a	Strategy 1b	Strategy 2
Carbon source	Potato dextrose	Potato dextrose	Potato dextrose
	Starch	Starch	
	Cellulose	Cellulose	
	Dextrose	Xylan	
	Xylan		
Temperature	55 °C	55 °C	55 °C
	45 °C	45 °C	55 °C+HS RT+HS
Compost plots	3	3	2
Soil plots	-	-	10
Dilution	1:10	1:15	Undiluted
	1:25	1:30	1:10
Isolated species	13	12	19
Plates	120	96	215

(potato dextrose, dextrose) or enrichment media (starch, cellulose, xylan), was used for isolation of thermophilous fungi (i.e., thermophilic and thermotolerant) from compost material. Strategy 1 comprises two samplings, and strategy 1b represents an optimization of strategy 1a, with a reduced carbon source set. A second strategy (strategy 2) was used to isolate thermophilic fungi as well as heat-resistant mesophilic and heat-resistant thermophilic fungi from both compost and soil material. Therefore, an additional heat-shock treatment (30 min, 75 °C) was applied for a subset of samples, followed by an incubation at room temperature (RT) or 55 °C.

Sampling sites and collection

Eight compost and ten soil samples were collected between September 2018 and March 2019 from two different locations in Bochum, Germany (Online Resource 1). Six domestic compost piles of different age, containing various green waste (e.g., lawn cuttings, hay, vegetable waste, and fruit tree cuttings) were sampled at an allotment garden area (51° 28' 16.4" N 7° 13' 59.7" E). Located in the botanical garden of the Ruhr-University Bochum (51° 26' 33.6" N 7° 16' 04.3" E), two domestic compost piles consisted of botanical waste of both annual and persistent ornamental plants and the ten soil sites were associated to different plant communities (Online Resource 1).

Compost samples were collected from both the upper and the lower layer from a pile to obtain differently matured compost material. A volume of 300 to 400 ml was sieved (mesh

Fig. 1 Sampling and cultivation strategies for the isolation of thermophilic, heat-tolerant, and heat-resistant fungi. Two different strategies were conducted to isolate heat-adapted fungi from soil and compost piles. Soil substrate was taken from the A-horizon. Compost substrate was sampled from both the upper (surface) and lower (core) layers of the pile. Material was mixed 1:9 with peptone solution, roughly mixed and incubated for 30 min with 300 rpm at 45 °C. Alternatively, for the isolation of heat-resistant species, the samples were treated with a heat-shock at 75 °C for 30 min. Several dilutions (1:10, 1:15, 1:25, 1:30, and undiluted) and five different media (starch, xylan, cellulose, potato dextrose, dextrose) were tested. For strategy 2, only potato dextrose medium was used. The plates were inoculated in triplicates with 150 µl and incubated at 45 °C, 55 °C, or room temperature (RT)

size of 1 cm), roughly mixed and stored in plastic bags at RT for up to 24 h until processing. Prior to the sampling of soil material, the overlying surface layer (e.g., litter, grass) was removed. Using a steel cylinder (diameter of 5.5 cm and height of 4 cm) 200 to 300-ml bulk soil of the A-horizon were sampled. Soil in direct association with roots was avoided. Bigger solids and root residues were removed if present. The samples were stored at RT up to 24 h until processing.

Sample treatment and selective culture isolation

The first two sampling approaches applied similar strategies (strategies 1a and 1b), while the third sampling (strategy 2) differed considerably regarding sample types and treatments. For the first two samplings, a subsample of 4 g, while for the third sampling a subsample of 25 g was mixed 1:9 with sterile 0.1% proteose peptone solution (Fig. 1). Briefly, manual shaking was followed by an incubation in a water bath at 45 °C for 30 min to isolate either thermophilous fungi or at 75 °C for 30 min (at 300 rpm) to isolate heat-resistant fungi. Afterwards, the supernatant was combined with 0.1% proteose peptone solution to create differently diluted aliquots of the samples (Fig. 1). The aliquots were vortexed to avoid sedimentation of particles within the suspension and 150 µl each were evenly dispensed on petri dishes with solid growth media. To prevent bacterial growth and decelerate fungal growth speed, rose bengal (Ottow 1972) and chloramphenicol (Hunter et al. 1974) were added to the growth media.

The media contained various carbon-sources (Table 1) to promote growth of fungal species with different nutrition capabilities. Potato dextrose medium contained 39 g/l potato dextrose agar (Carl Roth) according to manufacturer instructions and pH adjusted to 6.2. Dextrose medium contained 10 g/l dextrose (Fisher Scientific), 5 g/l soy peptone, 1 g/l KH_2PO_4 , 0.5 g/l MgSO_4 , and 15 g/l agar and pH adjusted to 6.2 (see Salar 2018). Starch medium (YpSs) contained 4 g/l yeast extract, 15 g/l starch (Carl Roth), 1 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 20 g/l agar, and pH adjusted to 6.2 (see Salar 2018). Medium with cellulose as carbon sources consisted of 20 g/l cellulose (Sigma-Aldrich), 1 g/l K_3PO_4 , 0.5 g/l of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g/l L-Asparagin, 0.5 g/l KCl and yeast extract, 0.2 g/l MgSO_4 , 0.1 g/l CaCl_2 and 15 g/l agar, and pH adjusted to 6.2 (adapted from the formula provided by Hardy Diagnostics). Xylan medium contained 3 g/l yeast extract, 1.5 g/l peptone, 3.5 g/l NaCl, 1 g/l NaNO_3 and KH_2PO_4 each, 0.3 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g/l xylan (beechwood xylan, Sigma-Aldrich), and 20 g/l agar and pH adjusted to 6.2 (adapted from Kalim and Ali 2016). Triplicates of each medium were inoculated and incubated at different temperatures (strategies 1a and 1b: 45 °C, 55 °C; strategy 2: RT, 55 °C). To facilitate the growth of mainly thermophilic fungi, 55 °C was used as incubation temperature, while 45 °C was chosen to favor the growth of more thermotolerant species. The growth of mesophilic, but heat-resistant fungi was facilitated by incubation at RT, and the selection for heat-resistant and thermophilic species was achieved by incubating previously heat-shocked samples at 55 °C. The plates incubated at 45 °C or 55 °C were monitored daily for 7 days. Longer incubation of samples at increased temperatures was not feasible, due to desiccation of the agar or overgrowth of the plate, caused by single fungal colonies. Plates incubated at RT were monitored daily for 21 days, giving fungi, whose viability might be

decreased due to the heat-shock treatment, enough time to germinate and grow. Colonies with distinct morphology (e.g., growth, colorization, mycelia branching) were sub-cultured and purified from the environmental plates of each sampling site, carbon source, and incubation temperature. Taxonomic assignment was achieved by rDNA barcode sequencing or microsatellite fingerprinting (Online Resource 2).

Identification of thermophilous fungal isolates via ITS-rDNA barcoding and microsatellite fingerprinting

After purification, isolates were categorized based on their morphology into morphotypes. One representative of each morphotype, each substrate, sample, and treatment was chosen for further analysis. Genomic DNA of representatives of each morphotype was extracted by a phenol-chloroform-based approach (Mülhardt 2013). Therefore, a few milligrams of freshly grown mycelia were used. DNA extracts were used for PCR to amplify the fungal barcode ITS-rDNA region (Schoch et al. 2012; Stielow et al. 2015). The fungal specific primer combination ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used. Each PCR reaction had a volume of 12.5 µl and contained 6.25 µl GoTaq G2 Hot Start Colorless Master Mix (Promega), 5.25 µl ddH₂O, 0.25 µl of each primer (10 µM), and 0.5 µl of DNA extract. The PCR program was the following: initial denaturation at 95 °C (3 min), 32 cycles of denaturation at 94 °C (27 s), annealing at 57 °C (60 s), and elongation at 72 °C (90 s), followed by final elongation at 72 °C (7 min). If the ITS amplification was not successful or if this barcode was not suitable for identification on species level, other barcodes (LSU, β -tubulin or calmodulin) were additionally amplified. The primer combination LR0R (Cubeta et al. 1991) and LR6 (Vilgalys and Hester 1990) was used to amplify the adjacent LSU-region (Vu et al. 2019). The used PCR program was initial denaturation for 96 °C (2 min), 35 cycles of denaturation at 96 °C (20 s), annealing at 45 °C (40 s) and elongation at 72 °C (90 s), and a final elongation at 72 °C (10 min). For the *Aspergillus* species, the primers CF1 and CF4 (Peterson 2004) were used for amplification of the partial calmodulin barcode sequence. The PCR program contained the following steps: initial denaturation at 94 °C (5 min), 35 cycles of denaturation at 94 °C (45 s), annealing at 55 °C (45 s), and elongation at 72 °C (60 s), followed by final elongation at 72 °C (10 min) (Yin et al. 2017). For species identified as *Penicillium*, primers Bt2a and Bt2b (Glass and Donaldson 1995) were used to amplify the partial β -tubulin barcode sequence. The PCR started with the initial denaturation at 95 °C (5 min), followed by 5 cycles of denaturation (94 °C, 60 s), annealing (68 °C decreasing 1 °C/cycle, 90 s), and elongation (72 °C, 2 min); afterwards, the PCR proceeded with 25 cycles of denaturation at 94 °C (60 s), annealing at 64 °C (90 s), and elongation at 72 °C (2 min), and a final elongation at 72 °C for 10 min (Frisvad et al. 2004).

Amplicons were cleaned with Exonuclease I and Shrimp alkaline phosphatase (NEB) according to manufacturer conditions, but with 1:5 diluted enzyme concentration and sequenced by the sequencing facility of the Ruhr-University Bochum with a capillary sequencer (3130xl Genetic Analyzer, Applied Biosystems). The obtained sequences were checked against the GenBank database (<https://blast.ncbi.nlm.nih.gov>) with the BLASTn algorithm (Online Resource 2).

Besides sequencing the barcode region of representative isolates, extracted gDNA of the isolates was used for microsatellite-primed PCR (MSP-PCR). This enabled a differentiation of morphologically similar isolates by obtaining a genetic fingerprint for distinct species, without a barcode sequencing of each culture (Supplementary Table S2). Therefore, the (GTG)₅ (Lieckfeldt et al. 1993; De Vuyst et al. 2008) primer was used in a PCR reaction containing 6.25 µl GoTaq G2 Hot Start Colorless Master Mix (Promega), 5.5 µl ddH₂O, 0.25 µl (GTG)₅ (10 µM), and 0.5 µl DNA extract in a total volume of 12.5 µl. The PCR program started with an initial denaturation at 95 °C (2 min), followed by 35 cycles of denaturation at 95 °C (30 s), annealing at 55 °C (40 s), and elongation at 72 °C (90 s), and a final elongation at 72 °C (7 min). The MSP-PCR products were tested by agarose gel electrophoresis with standardized conditions.

Availability of data and material

Sequences of taxonomically assigned isolates were deposited at the European Nucleotide Archive (ENA) database under accession numbers LR881294–LR881364, LR881366–LR881403, LR881444–LR881452, and LR993201–LR993205, LR993215–LR993238 (Online Resource 2). Representative isolates of each species were deposited at the DSMZ culture collection (Online Resource 2).

Results

Isolation efficiency of different isolation and cultivation strategies

For the first sampling (strategy 1a), three compost piles were sampled (Table 1). The processed substrate was incubated for 1 week at two temperatures on five media, on a total of 120 agar plates (Fig. 1). After 3 days of incubation, a variety of fungi was visible on most plates. Purification of morphologically distinct colonies led to 30 single cultures, which were taxonomically assigned to 13 different species (Table 1) based on molecular barcoding. For the second sampling (strategy 1b), 96 agar plates were inoculated with compost substrate from three different piles. For strategy 1b, similar conditions were applied as in strategy 1a, but only four media were used

(Table 1). From this sampling, 46 morphologically different cultures were purified and assigned to 12 distinct species. In total, 16 different species were isolated by using the two complementary strategies (1a and 1b), with 9 species being isolated by both. Four and three species were only isolated with strategy 1a and strategy 1b, respectively. Strategy 2 (third sampling) included only one medium and an additional heat-shock treatment (Table 1, Fig. 1). From this, 115 cultures were purified from 216 agar plates. These cultures were taxonomically assigned to 19 different species (Table 1), of which 15 were exclusively isolated with this strategy. Using strategy 2, eight species were isolated from compost substrate, while 4 of those were only isolated in this strategy and by using the heat-shock treatment.

The molecular identification of the isolates based on barcode sequences resulted in a taxonomic assignment on species level for 179 isolates. The identification of 12 isolates was only possible on the genus level (*Fibulochlamys* sp., *Lichtheimia* sp., and *Triangularia* sp.) due to low similarity with reference material (Online resource 2), which might, therefore, represent novel-undescribed species. Besides that, intraspecific diversity has also been indicated by microsatellite fingerprinting. For 9 species or genera (e.g., *R. emersonii*, *T. aurantiacus*), the microsatellite fingerprints of several isolates displayed different patterns intraspecifically, which could indicate the isolation of multiple strains or lineages of the same species. However, isolates assigned to ten species or genera showed identical microsatellite fingerprints (Online resource 2).

Diversity of thermophilous and heat-resistant fungi in compost and soil

Within this study, we obtained 191 fungal isolates from compost (92 isolates) and soil (99 isolates) samples, belonging to 31 different species (Table 2). The main proportion of isolates was assigned to Ascomycota with 23 species belonging mainly to the orders Eurotiales and Sordariales. The Mucoromycetes was the second most common phylum, represented by seven species of the orders Mucorales and Mortierellales, followed by Basidiomycota with one species of the order Agaricales. *Aspergillus lacinosus*, *A. fumigatus*, *Chaetomium thermophilum*, *Rasamsonia emersonii*, *Thermoascus aurantiacus*, *Triangularia* sp., and *Trichocladium pyriforme* were isolated from both substrates. In contrast, 14 species were detected only in one sample, e.g., *Malbranchea cinnamomea*, *Thermomyces lanuginosus*, *Fibulochlamys* sp., *Rasamsonia byssochlamydoides*, and *R. composticola*.

Diversity in compost From compost, 20 species were isolated of which 13 were assigned to Ascomycota and 7 species to Mucoromycota. On average, six species were isolated from each compost pile. *A. fumigatus* (87.5%), *C. thermophilum*

Table 2 Fungal species isolated from various compost (C) and soil (S) substrates, using different sampling strategies (see Table 1). Isolates were obtained using different carbon sources (C1: potato dextrose, C2: xylan, C3: cellulose, C4: starch, C5: dextrose), in combination with incubation at 45 °C, 55 °C or room temperature (RT), and a heat-shock (HS) treatment. Taxonomic order and phylum are indicated, as well documented relevance of the species for biotechnology (BT), food industry (F), pathology (P), and pharmacy (PH)

Species	Strategy		Medium					Substrate	Treatment	Order	Potential relevance
	1a	1b	2	C1	C2	C3	C4				
<i>Devriesia thermodurans</i>				•	•			S	HS + RT	Capnodiales ^a	Unknown -
<i>Aspergillus fischeri</i>				•	•			S	HS + RT	Eurotiales ^a	F, P dos Santos et al. 2018; Balajee et al. 2005
<i>Aspergillus fumigatus</i>	•	•	•	•	•	•	•	C + S	45 °C, 55 °C, HS + RT	Eurotiales ^a	F, BT, P dos Santos et al. 2018; Latgé 1999; Hogaboam et al. 2000; Rodriguez et al. 2000
<i>Aspergillus laciniatus</i>				•	•			C + S	HS + RT	Eurotiales ^a	F dos Santos et al. 2018
<i>Aspergillus neoglaber</i>				•	•			S	HS + RT	Eurotiales ^a	F, P dos Santos et al. 2018; Jayasuriya et al. 2009; Berni et al. 2017
<i>Aspergillus nishimurae</i>				•	•			S	HS + RT	Eurotiales ^a	PH Hosoe et al. 2011
<i>Aspergillus spinulosporus</i>	•	•	•	•	•	•	•	C	45 °C	Eurotiales ^a	Unknown -
<i>Aspergillus thermomutatus</i>				•	•			S	HS + RT	Eurotiales ^a	F, P Berni et al. 2017; Balajee et al. 2005
<i>Paecilomyces niveus</i>				•	•			S	HS + RT	Eurotiales ^a	F Engel and Teuber 1991; da Rocha Ferreira et al. 2009
<i>Penicillium lapidosum</i>				•	•			S	HS + RT	Eurotiales ^a	F Tourmas 1994
<i>Penicillium turbatum</i>				•	•			S	HS + RT	Eurotiales ^a	Unknown -
<i>Rasamsonia byssochlamydoides</i>				•	•			S	HS + 55 °C	Eurotiales ^a	Unknown -
<i>Rasamsonia compositicola</i>				•	•			C	HS + 55 °C	Eurotiales ^a	Unknown -
<i>Rasamsonia emersonii</i>	•	•	•	•	•		•	C + S	55 °C, HS + 55 °C	Eurotiales ^a	BT Dwyer 2019; Schiano-di-Cola et al. 2020; Raheja et al. 2020
<i>Talaromyces columbinus</i>		•		•				C	45 °C	Eurotiales ^a	P Peterson and Jurjević 2013
<i>Thermoascus aurantiacus</i>	•	•	•	•				C + S	55 °C, HS + 55 °C	Eurotiales ^a	BT Schuerg et al. 2017; Oliveira et al. 2014
<i>Thermomyces lanuginosus</i>	•	•	•	•				C	55 °C	Eurotiales ^a	BT, P Khan et al. 2015; Sivagnanam et al. 2013
<i>Chaetomium thermophilum</i>	•	•	•	•	•		•	C + S	45 °C, 55 °C	Sordariales ^a	BT Tong et al. 2015; Hua et al. 2018
<i>Thermothelomyces heterothallicus</i>				•				C	55 °C	Sordariales ^a	BT, P Nourisson et al. 2017; Singh 2016; Le Naourès et al. 2011
<i>Triangularia</i> sp.				•	•			C + S	HS + RT	Sordariales ^a	Unknown -
<i>Trichocladium pyriforme</i>				•	•			C + S	HS + RT	Sordariales ^a	Unknown -
<i>Malbranchea cinnamomea</i>	•					•		C	55 °C	Onygenales ^a	BT Mahajan et al. 2016; Basotra et al. 2019
<i>Curvularia buchloes</i>				•	•			S	HS + RT	Pleosporales ^a	Unknown -
<i>Fibulochlamys</i> sp.				•	•			S	HS + RT	Agaricales ^b	Unknown -
<i>Mortierella wolfii</i>						•	•	C	45 °C	Mortierellales ^c	P Munday et al. 2006; Corbel and Eades 1991
<i>Lichtheimia ramosa</i>	•	•	•	•	•		•	C	45 °C	Mucorales ^c	BT, P Gonçalves et al. 2013; De Oliveira et al. 2016; Mouronte-Roibás et al. 2016
<i>Lichtheimia</i> sp.	•			•				C	45 °C	Mucorales ^c	Unknown -
<i>Rhizomucor miehei</i>	•	•		•	•			C	55 °C	Mucorales ^c	BT Rodrigues and Fernandez-Lafuente 2010; Gomes et al. 2011
<i>Rhizomucor pusillus</i>	•	•	•	•	•	•	•	C	45 °C, 55 °C	Mucorales ^c	BT, P Dien Bard et al. 2014; Hüttner et al. 2018; Kimura et al. 2009

Table 2 (continued)

Species	Strategy		Medium					Substrate	Treatment	Order	Potential relevance
	1a	1b	2	C1	C2	C3	C4	C5			
<i>Rhizopus microsporus</i>	•	•	•	•	•	•	•	•	45 °C	Mucorales ^c	BT, P
<i>Thermomucor indica-veudaticae</i>	•	•	•	•	•	•	•	•	55 °C	Mucorales ^c	BT

^a Ascomycota, ^b Basidiomycota, ^c Mucoromycota, •: Strategies and carbon sources leading to the isolation of a fungal species

(62.5%), and *T. aurantiacus*, *Rhizomucor pusillus*, *Rhizopus microsporus*, and *A. spinulosporus* (all 50%) showed the highest occurrence in compost piles. Ten species were isolated with an incubation temperature of 55 °C and can therefore be considered as thermophilic fungi, while 8 species were only isolated at 45 °C (strategy 1) and are rather thermotolerant. Two thermophilic, three mesophilic fungi, and *A. fumigatus* were isolated in combination with the heat-shock approach (strategy 2), resulting in the isolation of 6 heat-resistant species from compost.

Diversity in soil Isolates obtained from soil substrate, were assigned to 18 species, of which 17 belonged to Ascomycota and one species to Basidiomycota. On average, five species were isolated from each soil sample. The majority of species (14) were isolated with a heat-shock treatment in combination with incubation at room temperature (strategy 2) and can therefore be considered as mesophilic heat-resistant fungi. Although an incubation temperature of 55 °C was used as in previous samplings, only 4 species were isolated from soil. The highest occurrence in soil samples was observed for the thermophilic *R. emersonii* and *T. aurantiacus* (both 80%) and for the mesophilic heat-resistant *A. nishimurae* (60%).

Isolation efficiency of different carbon sources

The isolation success of species differed among different media. Less complex carbon sources (potato dextrose, dextrose medium) showed increased fungal growth. This resulted in quick overgrowth, hampering the purification of species. However, 29 species were isolated using potato dextrose medium, while seven and six species were isolated from either starch and xylan media and seven species from cellulose-containing medium. Although, ten species were obtained from multiple media, 22 species were isolated from only one carbon source (Table 2). *Aspergillus fumigatus* was the only species isolated from all media. Notably, the application of the non-standard media containing xylan, cellulose, and starch led to the isolation of biotechnologically relevant species, e.g., *Malbranchea cinnamomea*, *Thermothelomyces heterothallicus*, and *Rasamsonia emersonii*.

Effect of temperature for the isolation of heat-adapted fungi

Application of different incubation temperatures resulted in diverse fungal colonies with different growth rates. Plates incubated at 45 °C showed quick fungal overgrowth, which was a limiting factor for the isolation success. Incubations at 55 °C displayed no overgrowth, and plates were colonized by a reduced number of fungal colonies.

Different fungal species were obtained from each incubation temperature. *Chaetomium thermophilum*, *R. pusillus*, and

A. fumigatus are the only species obtained from both 45 °C and 55 °C. Incubation at 45 °C resulted in nine isolated species (Table 2), including most of the isolated Mucoromycota (e.g., *Lichtheimia* spp., *Rhizopus microsporus*, *Mortierella wolfii*, *Rhizomucor pusillus*). Thirteen species were isolated at 55 °C (Table 2), including the biotechnologically important species *Malbranchea cinnamomea*, *Thermothelomyces heterothallicus*, *Rasamsonia emersonii*, *Thermoascus aurantiacus*, and *Thermomyces lanuginosus*.

The combination of a heat-shock treatment at 75 °C with incubation at 55 °C resulted in the isolation of *Rasamsonia byssochlamydoides*, *R. composticola*, *R. emersonii*, and *Thermoascus aurantiacus*. From the combination of the heat-shock with incubation at RT, 14 fungal species were obtained (Table 2), which were not isolated from the previous approaches (e.g., various members of the genus *Aspergillus*).

Discussion

Isolation of thermophilous species from compost and soil

A total amount of 31 fungal species were isolated from compost (20 isolates) and soil (18 isolates) samples, from which 18 can be considered as thermophilous (i.e., thermotolerant, or thermophilic) and 13 as mesophilic heat-resistant species (Table 2). The number of isolated thermophilous fungal species from compost or soil was higher than in recent studies (Chadha et al. 2004; Lee et al. 2014; Langarica-Fuentes et al. 2014a, 2015). Distinct temperature treatments, the use of two different substrates and multiple sampling sites, as well as the variation of carbon sources (for compost samples) was shown to be an efficient combination of factors to isolate heat-adapted fungi. For instance, five species were only obtained from one carbon source within the first two complementary samplings of compost (strategies 1a and 1b) when multiple carbon sources were applied (Table 2). In contrast, sampling 3 (strategy 2) used only one medium (PD), but both compost and soil substrates were sampled to investigate the potential of soil as a substrate for isolating thermophilic fungi instead of the influence of used cultivation media. The additional heat-shock treatment in strategy 2 seemed to have influenced the outcome of the isolation of thermophiles. *R. byssochlamydoides* and *R. composticola* were only isolated from samples heat-shocked before incubation at increased temperatures, while *C. thermophilum* (9 isolates from 4 different locations) was solely isolated without the heat-shock. Moreover, in this study, six and five species were only isolated at 45 °C and 55 °C, respectively (Online Resource 2). This is in accordance with other studies, in which different temperatures (strategy 1 and strategy 2) and carbon sources (strategies 1a and 1b) led to the isolation of different fungal species (Tansey 1971; Anastasi et al. 2005; Langarica-Fuentes et al. 2014a; Birajdar et al. 2020).

Furthermore, we isolated thirteen species only from a single sampling site (7 species from soil and 6 species from compost), indicating the suitability of distinct sampling locations. Strategy 1 led to the isolation of five of those species, while eight were obtained with strategy 2 (Online Resource 2).

In our study, each variation of the sampling and isolation process seemed to affect the outcome of the applied strategy. Therefore, we consider the combination of strategies including different parameters to be suitable to isolate a high diversity of heat-adapted fungi. The usage of multiple media (strategies 1a and 1b) is assumed to help isolating fungi with most probably different nutritional requirements. However, the isolation success was influenced more by temperature variations (strategy 1: 45 °C, 55 °C; strategy 2: 55 °C, heat-shock, RT) and the sampling of different locations. The inclusion of an additional substrate in strategy 2 also slightly increased the isolation success of thermophilic fungi, since one species (*R. byssochlamydoides*) was only isolated from soil.

Thermophilous fungi have specific physiological and ecological requirements; however, different species in this group display different adaptations to temperature ranges and carbon sources (Powell et al. 2012; Morgenstern et al. 2012; Thanh et al. 2019). This diversity allows thermophilous fungi to colonize several niches and sub-habitats (Ahirwar et al. 2017; Salar 2018). Compost provides very heterogeneous sub-habitats that are dependent on the feedstock plant material (Neher et al. 2013) and the composting phase and process (Galitskaya et al. 2017). These factors shape fungal community composition and influence community succession (Langarica-Fuentes et al. 2014b; Meng et al. 2019; Jiang et al. 2020). Therefore, for the isolation of differently heat-adapted fungal species, it is necessary to consider and mimic this environmental niche diversity through variations in the sample collection, processing, and cultivation. For the isolation of thermotolerant and thermophilic species, the use of distinct temperature regimes (e.g., 45 °C, 55 °C) is advantageous. Cultivation between 40 and 45 °C rather promotes growth of thermotolerant fungi and prevents growth of mesophiles (Dix and Webster 1995; Houbraken et al. 2012). In order to promote the growth of thermophiles, incubation temperatures above 50 °C are recommended, since many thermotolerant species show minor or no growth at these temperatures (Maheshwari et al. 2000; Morgenstern et al. 2012). An incubation temperature of 50 °C could provide suitable temperature conditions for either very thermotolerant or less thermophilic fungi. However, incubation temperatures at 45 °C and 55 °C facilitate isolation of thermotolerant and thermophilic fungi, respectively. The thermophilic species *Chaetomium thermophilum*, *Malbranchea cinnamomea*, as well as *Thermoascus aurantiacus* grow at 55 °C (Morgenstern et al. 2012, Table 2), but might display different fitness on distinct carbon sources. These differences in the efficiency to breakdown different carbon sources, were

especially useful to prevent the overgrowth of *Aspergillus fumigatus*, which is a fast-growing fungus with a broad range of growth conditions (Jensen 1931; Kozakiewicz and Smith 1994; Tekaiia and Latgé 2005; Rhodes 2006). In this study, *Aspergillus fumigatus* was isolated from all used carbon sources (strategies 1a, 1b, and 2), but showed reduced growth on cellulose and xylan. Additionally, the usage of cellulose led to the isolation of a novel *Lichtheimia* species (Table 2, Online resource 2). This demonstrates that the combination of different temperatures with different carbon sources, further facilitates the selection of different species.

Previous studies attempted to isolate thermophilous fungi by performing increased sampling depth of several substrates and intensive cultivation approaches (Nazir et al. 2007; Rajavaram et al. 2010; Ahirwar et al. 2017). For instance, Ahirwar et al. (2017) isolated 19 thermophilous fungal species from 79 samples of eight different habitats. However, only seven different species were isolated from 12 compost samples. In our study, by combining different cultivation conditions (Table 1), we were able to obtain a higher yield from a smaller sampling size (eight compost samples). Seventeen thermophilic or thermotolerant and additionally three heat-resistant fungal species were isolated from compost. Sixteen of those thermophilous species were isolated with strategy 1 (1a and 1b) from six different composts, while eight species (4 thermophilic, 3 heat-resistant, 1 heat-resistant thermophilic) were isolated with strategy 2 from only two composts. From soil substrates, previous studies isolated 12 thermophilous fungal species from 46 soil samples from three geothermal sites in China (Pan et al. 2010), while 10 thermotolerant and six thermophilic species were isolated from 40 soil samples from four different sites in India (Salar and Aneja 2006). By applying a restrictive approach (strategy 2, Fig. 1), we were able to isolate five thermophilic species from 10 soil samples (Table 2). Despite the small sampling size and the restrictive cultivation approach, we were able to isolate thermophilic fungi with a high rate of success.

It is assumed that thermophilous fungi growing in suitable conditions, disperse by aerosols to reach new habitats (Le Goff et al. 2010). After sedimentation in a new environment (e.g., soil), they remain in a dormant stage until favorable conditions are present. This explains their occurrence in non-favorable habitats without elevated temperatures, which are mainly dominated by mesophilic fungi. The growth of thermophilous fungi in soil is assumed to be due to temporary sun-heating (Tansey and Jack 1976). However, molecular data is still missing to verify their activity in soil. The application of cultivation-independent approaches based on RNA might be used to provide insights into the ecology of thermophilous fungi.

Selective isolation of heat-resistant species

Most studies isolate heat-resistant species from soil substrates and heat-processed food products (Frąc et al. 2015). So far,

only few studies tested for heat-resistance of fungi isolated from compost (e.g., Fergus and Amelung 1971). To our knowledge, our study is the first in attempting to selectively isolate heat-resistant fungi from both soil and compost with a heat-shock approach. Previous studies attempting to isolate heat-resistant fungi from soil were able to obtain between six and 16 species (Okagbue 1989; Jesenská et al. 1992; Horie et al. 2003; Ali et al. 2009; Sezen and Demirel 2019). In this study, we isolated 18 heat-resistant fungal species, of which four species (*Rasamsonia byssochlamydoides*, *R. composticola*, *Curvularia buchloes*, and *Trichocladium pyriforme*) and two novel taxa (*Fibulochlamys* sp. and *Triangularia* sp.) are here reported for the first time as heat-resistant. Most of the heat-resistant species (11) were isolated exclusively from soil, while only *Rasamsonia composticola* was exclusively found in compost. Notably, *Rasamsonia composticola* was here found for the first time since its original isolation from compost (Su and Cai 2013). Furthermore, a third member of the undersampled *Fibulochlamys* genus (Mahajan et al. 2016) was isolated. The most frequently isolated heat-resistant species belong to the genera *Aspergillus* and *Thermoascus*, which is supported by previous studies. Members of the genus *Aspergillus* are among the most frequently isolated heat-resistant species from soil (Jesenská et al. 1993; Jesenská and Piecková 1995; Ali et al. 2009), while the genus *Thermoascus* comprises mainly soil fungi, which produce heat-resistant spores able to survive up to 90 °C (King et al. 1969; Hosoya et al. 2014; Scaramuzza and Berni 2014). Furthermore, we successfully isolated *Rasamsonia emersonii*, *Devriesia thermoturans*, *Paecilomyces niveus*, and *Penicillium* species, which were previously obtained from soil using a heat-treatment (Jesenská et al. 1992; Jesenská et al. 1993; Ali et al. 2009; Seifert et al. 2004). Usually, heat-resistance of fungi underlies the production of heat-resistant ascospores, which are described for many species isolated in this study, e.g., many *Aspergillus* species, *T. aurantiacus*, *R. emersonii*, *R. byssochlamydoides*, *R. composticola*, and *Paecilomyces niveus* (Houbraken et al. 2012; Su and Cai 2013; Berni et al. 2017; Biango-Daniels et al. 2019). Notably, several of the genera isolated in this study include species known to contaminate various heat-processed food products with their heat-resistant ascospores. For instance, species belonging to the genus *Thermoascus* are detected in processed tea and fruit juices (Hosoya et al. 2014). Members of *Byssochlamys* (anamorph: *Paecilomyces*), *Neosartorya* (anamorph: *Aspergillus*), and *Talaromyces* are among the commonly isolated molds causing spoilage of raw foods, juices, heat-processed cheese, canned fruits and pasteurized foods (Tournas 1994; Kotzekidou 1997; Pitt and Hocking 2009; Frąc et al. 2015; Tranquillini et al. 2017; dos Santos et al. 2018). Therefore, several studies investigated heat-resistance of ascospores under conditions, used in processing and

sterilization in food industries (Piecková and Samson 2000; Salomão et al. 2014). However, ascospores are not reported for all species isolated with the heat-shock approach in this study, but heat-resistant structures other than ascospores have already been described for several fungi (Samson and Dijksterhuis 2007). For instance, *Devriesia thermodurans* produces heat-resistant chlamydospores, requiring a heat activation prior germination (Seifert et al. 2004). A *Fibulochlamys* species is known to produce thick-walled conidia which might cause a certain heat-resistance (Madrid et al. 2010) and although for *T. pyriforme*, no information about ascospores is available, for several *Trichocladium* species, heat-resistant ascospores or chlamydospores have been reported (Wang et al. 2019). Our results suggest that both compost and soil harbor a promising diversity of heat-resistant fungi. Since differently adapted fungal species were detected in these habitats, we hypothesize that a higher diversity of heat-resistant fungi is present in habitats not considered so far. Therefore, further studies should analyze the ecology and diversity of heat-resistant fungi across habitats.

Strategies to increase isolation success

The adaptation to distinct temperature regimes by specific species is useable as an initial and powerful tool for selective approaches to isolate differently heat-adapted species. High temperatures (50–60 °C) facilitate the isolation success of truly thermophilic fungi, while moderately increased temperatures (40–45 °C) lead to the isolation of mainly thermotolerant species (Maheshwari et al. 2000). Additionally, in combination with a heat-shock treatment (strategy 2), the isolation success can be selective towards heat-resistant species. The overlapping growth temperatures of many thermophilic and thermotolerant species (Morgenstern et al. 2012) make additional levels of selection necessary to avoid overgrowth of species and increase the isolation success. The usage of multiple carbon sources, with varying complexity, in combination with different temperature conditions (strategy 1), diversifies the cultivation, which influences the isolation success based on the different enzymatic properties of the fungal species. Most heat-adapted species will grow on rich media with easily accessible carbon sources (e.g., potato dextrose). Aiming for the isolation of a high diversity of species, usage of such media might be sufficient, when enough technical replicates are prepared. However, the isolation strategy can be adapted to the aim of the respective study. Aiming for the isolation of rare or slow growing species, or specialists regarding the utilization of different carbon sources, a diversified approach with multiple media might be preferred (strategies 1a and 1b). Sampling of various habitats and substrates (strategy 2) can increase the number of isolated species, if suitable cultivation conditions are applied (Ahirwar et al. 2017). Furthermore, dilution plating and addition of growth inhibitors (strategies 1 and 2) into the cultivation medium is

useful to reduce overgrowth of single species. Categorization into morphotypes serves as an additional level of selection, and the application of microsatellite fingerprinting simplifies the identification and differentiation of isolates on a genetic level, allowing detection of intraspecific diversity. The combination of these screening approaches reduces workload, handling time, and associated costs. The presented effective and time-efficient approaches to isolate differently heat-adapted fungi benefit from the combination of parallelized enrichment cultivation using different incubation temperatures and carbon sources.

In this study, we provide with strategy 1 (1a and 1b) an example for an efficient and convenient approach to isolate both thermophilic and thermotolerant fungi, occupying different ecological niches, from the same substrate. With strategy 2, we were able to create a complementary approach, aiming for both the selective isolation of truly thermophilic fungi and additionally the isolation of heat-resistant thermophilic and mesophilic fungi from two substrates (Fig. 1). These two strategies allowed us to isolate 31 heat-adapted fungal species, including three novel undescribed fungal species. Thus, we exploited differences among and within at least three ecophysiological groups to efficiently isolate differently heat-adapted fungi (Table 2), by applying two distinct approaches. Such approaches can consist of multiple, parallelized selective parameters. This facilitates growth of a broader range of species and reduces overgrowth and loss of potentially slow growing isolates, which leads to the isolation of a higher diversity of heat-adapted fungi, including rare species. The high demand for thermostable enzymes and biomolecules in biotechnology is increasing in the last decades. Although enzyme engineering is expanding quickly (Chowdhury and Maranas 2019), a highly diverse repertoire of natural enzymes is still required. Since fungi are able to breakdown a broad range of recalcitrant compounds, thermophilic fungi are a promising source of enzymes. Currently, only around 75 thermophilic fungal species are known. Therefore, isolation of thermophiles and heat-resistant fungi provides a great opportunity to identify and characterize species with biotechnological potential. Cultivation-independent approaches are useful to characterize microbial community structure, composition, and diversity in the environment. However, for experimental approaches and downstream applications, the isolation of heat-adapted fungi from environmental samples is essential.

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Author contribution FW and DB designed the sampling campaign, FW conducted the isolation, cultivation, and molecular work with the help of students. FW and MG evaluated the results and performed the taxonomic assignment. FW wrote the manuscript. MG and DB critically reviewed the manuscript. MG edited the manuscript. All authors read and approved the final manuscript.

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Data availability and materials availability All data and materials generated during this study are included in this manuscript, on its supplementary information or publicly available on databases, or microbial culture collections.

Code availability Not applicable.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Not applicable

Consent for publication Not applicable

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