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Fungi open new possibilities for anaerobic fermentation of organic residues

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Abstract

Background: Large amounts of fibre-rich organic waste material from public green and private gardens have to be treated environmentally friendly; however, this fibre-rich biomass has low biogas yields. This study investigated the presence of fungi in full-scale biogas plants as well as in laboratory reactors and elucidated the importance of fungi for the biogas process.

Methods: The dominating members of the eukaryotic community were identified by analyzing 18S rRNA gene and internal transcribed spacer 1 (ITS1) region fragments of clone libraries. These identifications were accompanied by diverse microscopic techniques such as fluorescence microscopy and conventional scanning electron microscopy.

Results: Cells of presumably fungal origin were characterized by intensive fluorescence and were about 1 order of magnitude larger than prokaryotic cells. Molecular techniques enabled to identify fungi from the subphyla *Agaricomycotina*, *Mucoromycotina*, *Pezizomycotina*, *Pucciniomycotina* and *Saccharomycotina* and from the class *Neocallimastigomycetes*. Members of these groups can be important for microbial degradation of complex compounds, due to the ability to penetrate cell walls, and thus open the cells for the influx of bacteria, further enhancing degradation.

Conclusions: Optimal treatment of biowaste depends on the amount of lignocelluloses. Targeted application of fungi to the biogas process will open wider possibilities for anaerobic treatment of fibre-rich biomass and can result in better biomass utilization as a renewable energy resource. Due to higher temperature optima of fungal cellulolytic enzymes, the thermophilic process is suggested for anaerobic degradation of fibre-rich biomass.

Keywords: Biogas; Biowaste treatment; Fibre-rich biomass; Fungal ITS1 sequences; Lignocelluloses

Background

Energy crops and organic residues can serve as substrates for anaerobic digestion with further utilization of the obtained methane as a source of sustainable energy [1]. Amounts of municipal solid waste (MSW) as well as of organic waste are closely correlated to gross domestic product (GDP) and are therefore increasing sharply in many countries [2]. Household waste is collected regularly in most countries at public expense and subsequently incinerated or landfilled [3]. In Germany, the organic fraction of MSW amounts up to 3.8 Mt a^{-1} and source sorting is implemented at 72% of the municipalities with a further increasing share. Another large source of organic waste material is urban gardening waste from public green and

private gardens. This amount is estimated to about 4 Mt a^{-1} in Germany [4] and can also be utilized for energy generation. The advantage of biowaste over energy crops such as maize silage, sugar beet, etc. is the avoided competition between the substrate production for energy use and food production.

The anaerobic decomposition of organic compounds in the first steps of biogas production provides the basic substrates for the methanogens [5,6]. Aside from the known fermentative bacteria [7], microorganisms from the eukaryotic domain can also be involved in the fermentation processes. For instance, it is known that obligate anaerobic protozoa are part of the anaerobic ruminal microbiota [8-10]. The presence of episymbiotic methanogens in ruminal ciliated protozoa was already proven in the early 1980s [11]. Ten years later, Teunissen et al. [12] found that the most abundant fermentation products of

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fungi in the anaerobic process are acetate, formate and hydrogen. Furthermore, anaerobic fungi are known to form co-cultures with ruminal methanogenic archaea which utilize the fungal hydrogen production [13]. The role of ruminal fungi in the degradation of plant fibres has been studied extensively [9,10,14-17]. The fungi can attach to the most lignified plant tissues [18] and are in turn followed by the ingress of cellulolytic bacteria which then gain access to the interior of otherwise less fermentable plant material. Fungal penetration therefore results in faster and more complete decomposition of fodder that enters the rumen [13]. Such fungal enhancement of decomposition could also be used in the biogas process; however, until recently, there is only limited knowledge about the occurrence of fungi in a biogas plant.

Most of the latest studies were focusing on the methane-generating archaea (methanogens) [7,14] which are the key microorganisms in the biogas-forming process [1]. The long-term stability of the whole microbial community of a mesophilic biogas plant supplied with pig slurry, sanitized food waste, stale bread and other residues was reported by Bengelsdorf et al. [19], who provided the first evidence of the continuous presence of fungi in a biogas reactor. In the current paper, we further refer to the eukaryotic fungal community members identified by different culture-independent approaches using clone libraries, epifluorescence and conventional scanning electron microscopy (CSEM).

The presence of fungi in the biogas reactors was therefore put in the context of anaerobic treatment of not only fibre-rich materials such as gardening waste and municipal green waste but also agricultural residues such as straw. The current practice used in dealing with these substrates takes only scarcely the path of the anaerobic digestion, mostly because of limited degradability of fibrous substrates as well as technological problems such as floating layers in the most common wet digestion in completely stirred tank reactors. Then, biomass burning in incineration plants or composting are the most widespread treatments. Therefore, a brief analysis is provided for treatment approaches for different types of organic waste, analyzing their potential for regenerative energy generation under consideration of fungal fibrolytic potential.

Methods
Specifications of the biogas plants and sampling
Microbial communities were assessed in biogas plants located in southern Germany near Aulendorf (biogas plant 1, BP1) and Hermaringen (BP2) in Baden-Württemberg. BP1 utilizes predominantly sanitized food residues together with stale bread and occasionally also other substrates such as pig slurry, maize silage, potato peelings and grain husk. The biogas plant with an installed electric

output of 380 kWh consists of three mesophilic reactors with 350-, 450- and 1,200-m^3 volume at a mean hydraulic residence time of 100 days. The investigated biogas reactor (350 m^3) with a pH value of the slurry of 7.9 had an organic loading rate of 3.5 to 4.0 kg volatile solids (VS) m^{-3} day^{-1} with a total solid content of 4.5%. The total solids contained on average ($n = 14$) 34% total carbon (C) and 4% total nitrogen (N), giving a rather low C/N ratio of 8.5. The BP2 (installed electric output of 280 kWh) was being fed with maize silage, cattle manure, grass silage and crop residues with a slurry pH value of 8. The two mesophilic reactors have a total volume of 1,600 m^3 operating at a total hydraulic residence time of 120 days with a rather low organic loading rate of 0.98 kg VS m^{-3} day^{-1}.

The analyzed reactor of BP1 was sampled several times during the period from December 2008 to May 2011. The other biogas reactor (BP2) was sampled in August 2013. Prior the sampling, reactors were stirred for 10 min. From 10 L of the collected slurry, four sub-samples, a total of 20 mL, were mixed with 20 mL of 99.8% ethanol and stored at −20°C for genomic DNA extraction.

For verification of cell density by fluorescence microscopy, two extra samples were drawn from the reactor of BP1 in March and May 2010. The reactor content was fixed according to the protocol of [20] using either a fixative solution (4% paraformaldehyde, PFA) or 99.8% ethanol and incubated for 4 h on ice. Afterwards, the cells were spun down by centrifugation (5,000 × g, 3 min, 4°C) and washed three times with phosphate-buffered saline (PBS) buffer. Finally, all cell pellets were suspended in one volume 1× PBS buffer and one volume 99.8% ethanol and stored at −20°C.

Further investigations on fungal presence were done in anaerobic laboratory reactors of 10-L volume. In these experiments, polypropylene discs were exposed for variable time span and recovered, and the abundance of the present organism was assessed by different techniques. These experiments used similar substrates as described above as well as slaughter house waste material with an inoculum from the biogas plant described above. More details on the digestion experiments are given in [21].

Fluorescence microscopy
To determine the number of cells, diluted (100- to 200-fold) cell suspension and 100 mg of sterile glass beads (diameter 0.1 mm) were filled in a 2-mL microtube (Sarstedt, Nümbrecht, Germany) and homogenized using the RiboLyser (Hybaid Ltd., Middlesex, UK) at 4 m s^{-1} for 20 s. The homogeneous cell suspension (15 μL) was dropped onto each well of a Teflon-coated slide (eight wells, diameter 6 mm, Menzel GmbH & Co KG, Karlsruhe, Germany) and incubated for 15 min at 60°C. Cells per well were stained with 20 μL of a 10,000-fold diluted SYBR® Gold solution (Molecular Probes Inc.,

Eugene, OR, USA) per well for 10 min at room temperature, flushed with cold ddH$_2$O and immediately dried with compressed air. An analogous procedure was done with 4′,6′-diamidino-2-phenylindole (DAPI; 2.5 mg mL^{-1}) instead of SYBR® Gold. Fluorescence was detected using a Leica microscope (Leica, Solms, Germany). The microscope suitable for epifluorescence microscopy (resolution × 1,000) was equipped with a 100-W mercury high-pressure bulb (HBO 103 W/2) and adequate filter cubes. Digital images were taken with an AxioCam MRc 5 (Carl Zeiss, Jena, Germany) and the software AxioVision Rel. 4.8. Ten microscopic pictures (randomly chosen, magnification of × 1,000) were made per well. The following formula was used to calculate the total cell counts (TC$_c$) per millilitre of reactor content: TC$_c$ = A/A_{mi} × C_n × D_f. A is the total area of a well (28.27 mm^2), A_{mi} is the area of the microscopic image (0.015552 mm^2), C_n is the average cell number per microscopic image and D_f is the dilution factor per millilitre of reactor content.

Conventional scanning electron microscopy

The conventional scanning electron microscope (CSEM) DSM 942 (Carl Zeiss AG, Germany) was used for high-resolution visualization of the samples (×5,000). Polypropylene (PP) discs with a diameter of 9 mm were used as biofilm carriers. Samples for CSEM were either fixed in paraformaldehyde solution (4%, *w/v*) or ethanol. After fixation of biofilms attached to PP discs, samples were dehydrated for 1 day in 80% and 90% ethanol and 100% isopropyl alcohol, respectively. After dehydration, samples were critical point-dried (Polaron E 3000, Polaron Equipment Limited, Watford, England) and gold-coated. Structures were visualized by CSEM DSM 942 in high vacuum mode. The resolution under this mode reaches up to 4 nm at 30 kV. Signalling electrons were detected by a secondary electron detector and visualized on monitor. The optimal quality of micrographs was reached at a high voltage of 5 to 10 kV, a pressure of $2 × 10^{-7}$ hPa, a working distance of 7 to 12 mm and a spot size of 9.

DNA isolation and purification

Genomic DNA was isolated from the initial treatment of 2 mL of fixed reactor content according to [7]. Afterwards, the genomic DNA was extracted following the protocol of the 'High Pure PCR Template Preparation Kit' from Roche (Mannheim, Germany). For further purification of genomic DNA, the RNA was removed by RNase digestion (20 ng mL^{-1}) for 20 min at 37°C, followed by a standard phenol chloroform extraction and ethanol precipitation. Yields of the genomic DNA extracted were determined photometrically with the Ultraspec 3100 pro (GE Healthcare Bioscience AB, Uppsala, Sweden). The eukaryotic (fungal) members were characterized by creating clone libraries based on 18S rRNA gene and internal

transcribed spacer 1 (ITS1) fragments of eukaryotes. Colony polymerase chain reaction (PCR) was performed using a thermocycler (MWG-Biotech AG, Ebersberg, Germany). All employed primers were synthesized by biomers.net GmbH (Ulm, Germany). Eukaryotic 18S rRNA gene was amplified using the primer set Euk1a and Euk516r-GC [22] with the DF *Taq* DNA polymerase (Genaxxon bioscience GmbH). 18S rRNA gene fragments of approximately 500 bp were generated with the following protocol: initial denaturation at 95°C for 5 min; 8 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; 20 cycles of 92°C for 30 s, 52°C for 30 s and 72°C for 1 min; followed by final extension at 72°C for 10 min. The amplification of the fungal ITS1 region was performed using the primer set ITS1-F [23] and Neo-qPCR Rev [24]. ITS1 fragments of approximately 400 bp were amplified using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific Inc.) by the following protocol (modified from Fliegerová et al. [25]): initial denaturation at 98°C for 30 s; 10 cycles of 98°C for 10 s, 62.5°C for 45 s and 72°C for 15 s; 30 cycles of 98°C for 10 s, 67.5°C for 45 s and 72°C for 15 s; followed by final extension at 72°C for 10 min.

Cloning and sequencing

Amplified 18S rRNA gene and ITS1 fragments were cloned into either the pDrive Cloning Vector (QIAGEN GmbH, Hilden, Germany) or pJET1.2/blunt Cloning Vector also according to the instructions provided by the manufacturer. Cold competent *Escherichia coli* DH5α cells were used for heat shock transformation of plasmid DNA [26]. Colony PCR was carried out in order to recover the cloned DNA fragment from the recombinant plasmid, either of the 18S rRNA gene or the ITS1 fragments, respectively. Restriction fragment length polymorphism (RFLP) analysis was performed for 18S rRNA gene PCR fragments. Fragments of the expected size were combined with an appropriate restriction enzyme mix and the appropriate enzyme buffer. Electrophoresis to separate restriction fragments was done using 2% agarose gels in a TAE buffer system (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA; pH 8) at 80 V. The resulting RFLP banding patterns were compared visually. In case of identical RFLP banding patterns, one representative DNA fragment was chosen for DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). The resulting representative DNA sequences and the corresponding RFLP banding patterns, respectively, were defined as an operational taxonomic unit (OTU). In case of ITS1 PCR fragments, all amplified fragments were sequenced, and therefore, no OTU had to be defined.

The reference sequences used to analyze the 18S rRNA gene and ITS1 region sequences were retrieved from GenBank (National Center for Biotechnology Information database) and were analyzed using BLASTn [27].

All obtained nucleotide sequences were checked for chimeric artefacts with the Mallard software tool (version 1.02) [28]. All nucleotide sequences obtained in this study were deposited in the NCBI GenBank database. The fungal 18S rRNA gene sequences and ITS1 region sequences are available under the accession numbers JF421674 to JF421678, JF421681 and KF977129 to KF977143, respectively.

Results and discussion

Microscopical analysis

In total, 87 evaluated fluorescent micrographs gave on average 1.44 (± 0.3) $\times 10^{10}$ prokaryotic cells mL^{-1} reactor content of BP1. Autofluorescent methanogenic cells (coenzyme F_{420}) were counted on 75 micrographs providing an average abundance of 3.5 (± 0.78) $\times 10^{8}$ archaea mL^{-1} reactor content. This indicates that archaea accounts for 2.3% of the total prokaryotic cells (see [19] for more details). DAPI-stained fungal cells (Figure 1) were assessed on a total of 26 micrographs and were in the same order of magnitude (2.03 (± 1.05) $\times 10^{8}$ cells per mL) as the methanogens. Eukaryotic fungal cells were distinguished by an intensive fluorescence and had a mean circumference of 7.7 (± 1.8) µm and an expanse of 2.8 (± 1.0) μm^{2}, thus significantly larger than prokaryotic bacterial and archaeal cells. CSEM micrographs confirmed the size of prokaryotic organisms. Precise measurement of unstained fungal cells (about 10 µm in diameter) could be achieved by up to five times higher resolution compared to epifluorescence microscopy.

CSEM micrographs (Figure 2A,B) show fungal cells where almost all other cells were removed due to PFA fixation. The size difference between the eukaryotic fungal cells and the prokaryotic microorganisms is visible on images in Figure 2A,C. Fungi are 1 order of magnitude larger as can be seen from the large fungal bodies surrounded by prokaryotes. Micrographs in Figure 2C,D display fungal cells embedded in a prokaryotic biofilm layer, which was preserved by ethanol fixation. Such spatial arrangement with prokaryotes and fungi embedded in the common matrix of extracellular polysaccharides (EPS) guarantee fast transfer of metabolites such as acetate and hydrogen towards the methanogens.

The evidence of facultative anaerobic fungi in the studied biogas reactor of BP1 was based on their intensive fluorescence and the cell size and was demonstrated further by the presence of fungal 18S rRNA gene sequences. The genome of eukaryotic fungal cells is much larger than that of the prokaryotic ones. Thus, fungal cells contain higher amounts of DNA resulting in high fluorescence (Figure 1). Already in Figure 1, the several times larger circumference and expanse of presumably eukaryotic fungal cells are obvious compared to other prokaryotic cells stained by DAPI. The fungal cells found (Figure 2) with the diameter of 10 µm are comparable to other anaerobic fungi of the genus *Saccharomyces* [29] and others described in a review by Ho et al. [30].

Molecular and phylogenetic analysis

Clones were randomly chosen from several hundred colonies obtained after transformation. Six clones from BP1 contained eukaryotic 18S rRNA gene sequences, and further 15 ITS1 region sequences were determined in samples from the BP2 (Table 1). These fungal 18S rRNA gene sequences were classified into the subphyla *Agaricomycotina*, *Mucoromycotina*, *Pucciniomycotina* and *Saccharomycotina*. The fungal ITS1 region sequences were identified as belonging to the fungal subphyla *Agaricomycotina* and *Pezizomycotina* and to the class *Neocallimastigomycetes*. Sequences from uncultured fungal clones were assigned to the phylogenetically closest species within the respective subphylum using one of the top five BLAST hits. The exact determination of the uncultured *Pucciniomycotina* clone (Euk 12 pD), uncultured soil fungus clone (K73) and
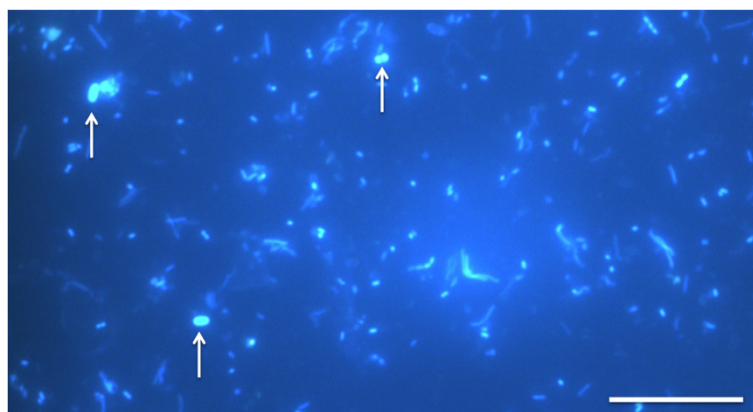

Figure 1 Epifluorescence micrograph showing cells in the biogas reactor content. Cells were stained with DAPI. Cells marked by arrows are presumably of fungal origin (scale bar 20 µm).
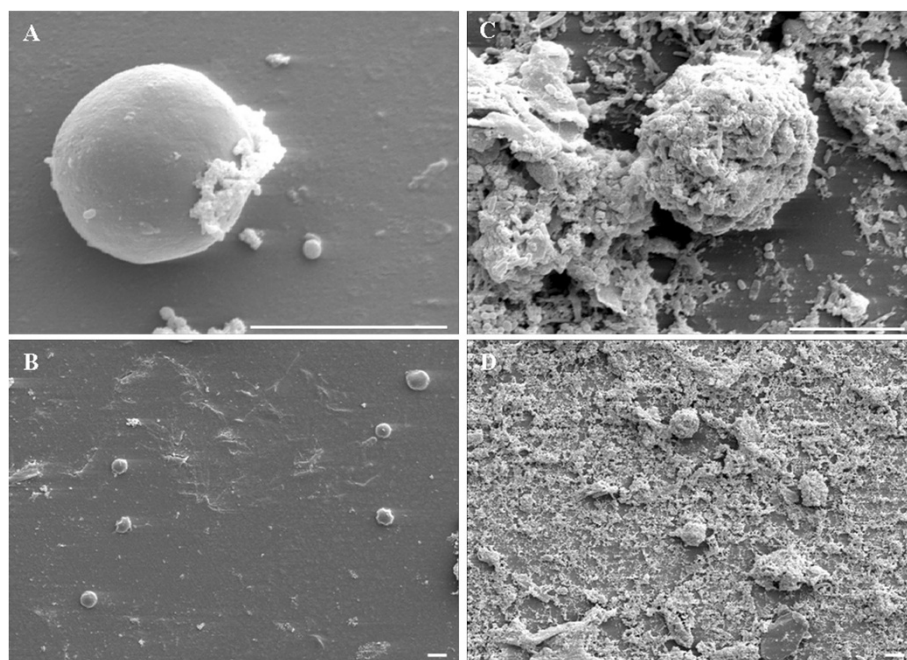
Figure 2 CSEM micrographs of fungal cells on biofilm carriers (polypropylene discs). (A, B) Sole fungal cells. **(C, D)** Fungal cells embedded in a prokaryotic biofilm layer (scale bar 10 μm).

uncultured *Neocallimastigales* clones (K76, K81) could not be assigned on the species level. However, at decreasing precision, 18S rRNA gene and 15 ITS1 region clone sequences gave the following identification: clone Euk 12 pD *Malassezia pachydermatis* isolate AFTOL-ID 856, identity 96%; clone K76 *Anaeromyces* sp. FFEX4, identity 85%; clone K81 no cultivated species matching; clone K73 *Pezizales* sp. G-P4, identity 90%.

The identified four subphyla (based on 18S rRNA gene sequences) of facultative anaerobic fungi, the *Agaricomycotina*, *Mucoromycotina*, *Pucciniomycotina* and *Saccharomycotina*, as well as the identified species from the genera *Mucor* and *Saccharomyces* (Table 1) were shown to be consistently present within the highly diverse reactor community of BP1 over the period of more than 1 year [19]. Addressing the ITS1 region sequences obtained from BP2 gave new evidence concerning the presence of the strongly anaerobic fungal class *Neocallimastigomycetes*. Both molecular approaches enlarged significantly our insight into the fungal diversity of biogas reactors. Our current knowledge about the role of fungi in biogas reactors is, however, still low. Fungi were not found in previous microscopic surveys [7,14,31,32], which only focused on the bacterial and archaeal communities. However, rumen liquor as well as faeces from ruminants and non-ruminants contains fungi belonging to *Anaeromyces*, *Orpinomyces*, *Caecomyces*, *Piromyces* and *Neocallimastix* [9,10]. Therefore, it can be assumed that most of biogas reactors supplied with liquid or solid manure are inhabited by facultative or even obligatory anaerobic fungi (Table 1).

Role of fungi in biogas reactors

The average abundance of 2.03 (±1.05) × 10^8 fungal cells per mL reactor content (BP1) is comparable to the relative presence of methanogenic archaea found in the same reactor [19]. A significantly larger size of fungal cells (Figures 1 and 2) suggests a high substrate requirement to maintain the metabolic activity of fungal cells. It must be highlighted that the same relative abundance of fungi as that of methanogens must have consequences for the anaerobic degradation process. Furthermore, the eukaryotic community dominated by fungi did not change, and the fungi remained present over the time period of 1 year [19]. Also, Ravella et al. [33] demonstrated that a laboratory biogas reactor is a suitable habitat for fungi, and the authors were able to isolate different viable fungal strains.

A review article [30] distinguished several fungi attached to fibrous plant material. After motile flagellated zoospores from the fluid become attached to the digestible materials, vegetative stages produce germ tubes that elongate very rapidly and build a network of rhizoids or hyphae in the plant tissues. Fungi from anaerobic environments (e.g. *Piromyces*, *Neocallimastix* [12], *Orpinomyces*, *Anaeromyces* [10]) are possessing fibrolytic activity and are able to enhance the digestibility of fibrous feeds as was documented for cattle [34], as well as wild-living

Table 1 Clone sequences of fungal 18S rRNA gene and ITS1 region fragments from BP1 and BP2

Phylum	Subphylum/class	Clone	Sequence related to	Identity (% cpa)	Accession number
Fungal 18S rRNA gene sequence					
Ascomycota	*Saccharomycotina*	Euk 06 pD	*Saccharomyces cerevisiae* strain YJM789	99	JQ277730
		Euk 1-4 pJ	*S. cerevisiae* strain YJM789	99	JQ277730
Basidiomycota	*Pucciniomycotina*	Euk 12 pD	Uncultured *Pucciniomycotina* clone D0735_42_M	99	EU647044
	Agaricomycotina	Euk 21 pD	*Sclerotium* sp. BSC-97	99	AF010303
Incertae sedis	*Mucoromycotina*	Euk 2-8 pJ	*Mucor circinelloides* f. *circinelloides* strain WA0000017591	99	HM641689
		Euk 57 pD	*Rhizomucor endophyticus* strain CBS 385.95	99	HM623313
Fungal ITS1 region sequence					
Ascomycota	*Pezizomycotina*	K01	*Hypocreales* sp. Vega851	96	EF694655
		K27	*Hypocrea* sp. KBS0814F	98	JQ437611
		K38	*Cladosporium* sp. AF13	99	JX173100
		K39	*Aspergillus fumigatus* strain ATCC 1022	99	HQ026746
		K58	*Pleosporales* sp. 5 TMS-2011	89	HQ631052
		K91	*Cladosporium* sp. F0910-49U4	99	HG008746
Basidiomycota	*Agaricomycotina*	K21	*Basidiomycota* sp. 54 OA-2013	95	JX507646
		K24	*Wallemia* sp. F53	97	FJ755832
		K31	*Mrakia* sp. CBS 8907	99	AY038836
		K79	*Guehomyces pullulans* isolate ANT03-093	99	JX171177
		K87	*Mrakia* sp. CBS 8907	99	AY038836
		K83	*Mrakia* sp. CBS 8907	99	AY038836
Neocallimastigomycota	*Neocallimastigomycetes* (class)	K76	Uncultured *Neocallimastigales* clone 238S18	98	KC431216
		K81	Uncultured *Neocallimastigales* clone 8SC4cg07	90	GU909951
Unclassified	Unclassified	K73	Uncultured soil fungus clone C152	97	JX489840

Corresponding sequences were retrieved from the NCBI database by the nucleotide BLAST algorithm. Accession numbers: fungal ITS1 region sequences, KF977129-KF977143; fungal 18S rRNA gene sequences, JF421674-JF421678, JF421681s.

herbivores like buffalo [35] and elephants [9]. Celluloses and hemicelluloses are forming plant cell walls, and their penetration and disintegration are the limiting steps in the anaerobic digestion of fibrous material, especially if they are embedded within the lignocellulose complex [36]. The beneficial role of fungi in the anaerobe biogas process is based on their ability to adhere on plant surfaces and to penetrate the cell walls. Through this, they open the cells for numerous members of the bacterial community and speed up the whole decomposition process.

The genus *Mucor*, which was found in the present study, has members present worldwide in soil and environmental samples [37]. They are characterized by high protease activity [38] that could improve the digestion of organic residues in a biogas plant. The also identified subphlyum *Pucciniomycotina* includes more than 8,000 described species, which are known as saprophytes and parasites of plants, animals and other fungi. Therefore, they are able to disintegrate organic materials. More specific estimates on their role in the biogas reactor (BP1) were not possible however. Although the applied detection methods in the current study could not provide any

information regarding fungal enzymatic activity, there is increasing evidence supporting the importance of facultative anaerobic fungi in producing enzymes for degradation of fibre-rich substrates [9,12,34]. Enzyme essays showed especially high cellulase, carboxymethylcellulase, xylanase and avicelase activities [9,10].

There are also positive effects of anaerobic fungi known mainly from animal breeding. Cultures of *Saccharomyces cerevisiae* and their extracts are in use as feed additive for cattle in ruminal fermentation for many years [17,39]. Lila et al. [40] showed that living cells of *S. cerevisiae* increased the numbers of total viable bacteria, especially cellulolytic ones in the cattle rumen. Newbold et al. [41] reported the same beneficial effect on the rumen of sheep. Thus, *S. cerevisiae* in biogas plants may have similar positive effects for the abundance of cellulolytic bacteria. Although the investigated biogas reactor of BP1 was not utilizing residues from cattle breeding but pig manure, the *Saccharomyces* were found as a sustaining member of the microbial community.

Still, further targeted investigations are needed on the presence and activity of fungi in order to relate them to
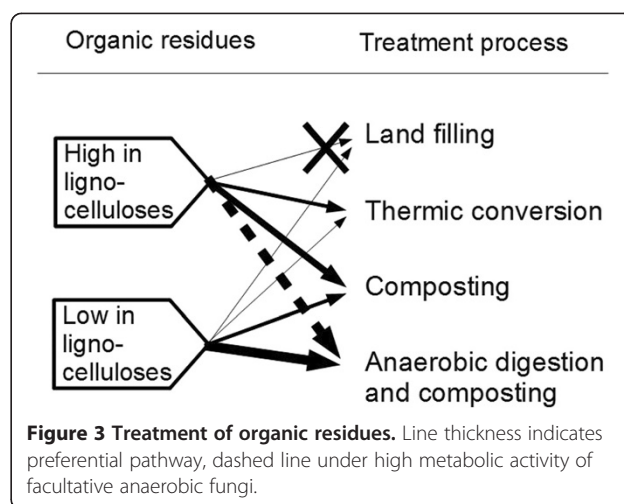
the used substrates. It is necessary to test how strongly the fungal presence depends on the regular and repeated inoculation by animal manure [34]. As facultative anaerobic fungi [37] are widely distributed in the environment, biogas reactors fed by plant material may offer preferable living conditions, even without ruminant manure (c.f. BP1). The metabolic activity of fungi in the anaerobic process can be assessed by volatile fatty acid (VFA) concentration. In fungal isolates incubated on filter paper [9] or wheat straw [10], the VFA concentration increased significantly. Teunissen et al. [12] reported the highest production of acetate and formate, together with hydrogen, by the investigated strains of *Neocallimastix* and *Piromyces*. Furthermore, anaerobic fungi are building syntrophic interactions with archaea where the latter utilizes the fungi-produced hydrogen for methanogenesis [10], thus keeping the hydrogen partial pressure low - a prerequisite for a stable biogas process. Markedly, the larger fungal cells had a similar share (based on cell numbers) within the reactor community as the methanogenic archaea, all closely embedded in the EPS matrix (see Figure 2).

In summary, the presence of fungi in biogas reactors (Table 1) increases and possibly also speeds up the decomposition of substrates rich in lignocelluloses as reported for cattle [34] and can broaden the utilization of various substrates for biogas production. Therefore, it is necessary to consider further possible substrate applications and implications for sustainable biomass use.

Implications for sustainable use of biogas substrates

Human population exploits large quantities of biogenic resources. Based on FAO statistics for 1995, estimates showed that about 20% (11.5 Pg C) of the terrestrial net primary production (NPP) is acquired by humans, from which food accounts for 4.1 Pg C [42]. It is evident that the exploitation of NPP increased significantly since 1995 along with the amount of organic waste material. Many municipalities around the world are facing increasing problems in dealing with domestic organic waste and urban greening waste [43].

Figure 3 lists the most widespread treatments for biowaste, while the amount of lignocellulosis is one important criterion for the treatment. Generally, organic material must not end in landfills, especially due to uncontrolled long-lasting methane emissions and other environmental impacts, which are most prominent in fast-developing countries [44] and megacities [45]. Applying thermic conversion like incineration and pyrolysis, air pollution problems must be solved and initial investments are very high [44,45]. Moreover, large quantities of organic waste have high water content, often over 80%, meaning that even additional energy might be needed when burning such material. One often neglected disadvantage of thermal treatment of organic material is that plant nutrients

Figure 3 Treatment of organic residues. Line thickness indicates preferential pathway, dashed line under high metabolic activity of facultative anaerobic fungi.

from the waste are going to get lost. Burning of organic waste with an average nitrogen concentration about 15 mg N g^{-1} waste [46] leads to nitrous gas emissions. Also, other valuable elements like phosphorus and potassium have to be replenished in agricultural soils as the ash and slag produced in incineration plants must be deposited in landfills.

Composting of organic material rich in lignocelluloses is the most applied treatment. It requires comparably low initial investment, and the operation of a composting plant is simpler than other technologies. However, composting itself is also an energy-demanding process. Depending on the technical operation, up to 100 kWh t^{-1} fresh material is needed for handling and aeration [4]. During the self-heating of the compost heap, high CO_2 emissions due to oxidation of easily degradable compounds are additionally emitted. Further major shortcomings of composting - at least for nutrient-rich agricultural waste - are nutrient losses, leading to reduced fertilizer value, and possibly point-source pollution [47] such as methane and leachate [48].

Dry digestion can be considered as an appropriate treatment for the organic fraction of MSW and fibre-rich material owing several advantages for these heterogeneous substrates of TS over 30% [44,46]. Compared to the mesophilic process, thermophilic dry fermentation proved to lead to higher methane yields and VS degradation when applied to cow dung [49] and to fibre-rich green yard waste (M Zak, personal communication). In fact, cellulase and xylanase activities in anaerobic fungal cultures had their optima at 50°C [12], which supports the importance of fungi in the anaerobic degradation of material rich in lignocelluloses.

Conclusions

For the aforementioned reasons, anaerobic digestion is the most appropriate treatment of organic waste in order to

achieve waste stabilization and energy generation [44,50,51]. Therefore, anaerobic treatment can produce a high amount of renewable energy, and subsequent composting will retain plant nutrients in the substrate for further use as organic fertilizer. However, appropriate biogas technology for fibre-rich substrates like straw, material from landscaping, urban greening, etc. is needed [46]. The thermophilic process might be appropriate [12,49] to generate higher methane yields. At this point, fungi play a key role in opening up the less accessible lignocellulosic biomass and in increasing the biogas gain from anaerobic digestion.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MK made an interpretation of the findings towards sustainable use of waste biomass and drafted the manuscript. SL made the microscopic analysis and interpretation of the micrographs. FRB collected the samples for molecular analyses, performed the related evaluation and, together with SL, contributed to the manuscript draft. All authors read and approved the final manuscript.

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