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Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants

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Abstract

Bioaugmentation with anaerobic fungi (AF[1]) is promising for improved biogas generation from lignocelluloses-rich substrates. However, before implementing AF into biogas processes it is necessary to investigate their natural occurrence, community structure and transcriptional activity in agricultural biogas plants. Thus, AF were detected with three specific PCR based methods: (i) Copies of their 18S genes were found in 7 of 10 biogas plants. (ii) transcripts of a GH5 endoglucanase gene were present at low level in two digesters, indicating transcriptional cellulolytic activity of AF. (iii) Phylogeny of the AF-community was inferred with the 28S gene. A new *Piromyces* species was isolated from a PCR-positive digester.

Evidence for AF was only found in biogas plants operated with high proportions of animal feces. Thus, AF were most likely transferred into digesters with animal derived substrates. Additionally, high process temperatures in combination with long retention times seemed to impede AF survival and activity.

Keywords

anaerobic fungi, biogas, lignocellulose utilization, transcriptional activity, strain isolation

1 Introduction

The biogas industry has in several countries mainly focused on the utilization of easily-degradable energy crops such as maize, from which high amounts of methane are generated at high efficiency (Lebuhn *et al.*, 2014). The wisdom of converting food resources to energy (the "food versus fuel" conflict) is hotly debated (Tomei & Helliwell, 2016) but this conflict can be avoided by use of waste

[1] Abbreviations: AF= anaerobic fungi; LCB= lignocellulosic biomass; PB= pilot biogas plant; GH5=glycoside hydrolase family 5; ITS1= internal transcribed spacer region 1; D= digester; PD= post-digester; FR= final repository; HRT= hydraulic retention time; MPN= most probable number; LoB= limit of blank; LoD= limit of detection; LoQ= limit of quantification; LSU= large ribosomal subunit; rRNA=ribosomal ribonucleic acid; SSU= small ribosomal subunit;.

lignocellulosic biomass (LCB; e.g. wastes from agriculture, landscaping care or urban gardening) in biogas production. Worldwide, organic matter is the most storable renewable resource, and LCB is the most abundant reservoir of carbohydrates suitable for sustainable energy generation (Divya *et al.*, 2015). A technical report by the European Environment Agency from 2007 stated that LCB such as grasses will form the next generation of ecologically sustainable substrates for the production of biogas (Petersen *et al.*, 2007). However, to date LCB remains rather unused due to its recalcitrant nature and its low degradability in the existing standard biogas fermentations (Christy *et al.*, 2014; Procházka *et al.*, 2012).

The bottleneck in utilization of LCB is its complex structure, consisting of cellulose, hemicellulose and lignin, with the latter causing the greatest problems during hydrolysis. Lignin is enzymatically not degraded under anaerobic conditions and protects the more easily degradable carbohydrate polymers from rapid decomposition. Therefore pretreatment strategies enabling physical disruption of the plant cell wall would lead to increased accessibility of carbohydrates to microbial enzymatic attack and improved substrate digestibility (Sárvári Horváth *et al.*, 2016).

Anaerobic fungi (AF) are efficient degraders of LCB in the digestive tracts of their host animals and are regarded as a promising reservoir for bioaugmentation in biogas production processes (Gruninger *et al.*, 2014; Procházka *et al.*, 2012). The typical biogas fermentation process is carried out by consortia of primary and secondary fermenting bacteria which degrade cellulosic substrates mainly to volatile fatty acids, CO_2 + H_2, and methanogenic archaea which convert these products to methane (Weiland, 2010). A similar biocenosis comprising bacteria, methanogenic archaea, protozoa and AF exists in the herbivore gut (Kittelmann *et al.*, 2013), wherein AF act as primary colonizers and degraders, attaching within minutes to ingested forage and initiating both physical disintegration and catabolism of lignocellulose polymers (Gruninger *et al.*, 2014; Solomon *et al.*, 2016). The latter process is mediated by cellulases, hemicellulases and phenolic acid esterases and can be coordinated

in multienzyme complexes called cellulosomes (Fontes & Gilbert, 2010). Carbohydrate active enzymes and cellulosomes have to date been identified in most AF (Chen *et al.*, 1995; Chen *et al.*, 2014; Harhangi *et al.*, 2003; Hodrova *et al.*, 1998; Steenbakkers *et al.*, 2002). Genome analysis of *Orpinomyces* strain C1A revealed superior fiber degrading characteristics, 357 glycoside hydrolase genes, 24 polysaccharide lyases and 92 carbohydrate esterases were identified (Youssef *et al.*, 2013). AF are able to utilize a multitude of recalcitrant lignocellulosic substrates (e.g. wheat straw (Callaghan *et al.*, 2015; Dagar *et al.*, 2015), lucerne and grass stems (Bauchop, 1979), reed canary grass, alfalfa stems, switch grass and corn stover (Solomon *et al.*, 2016)) and degrade the comprised oligosaccharides. In the herbivore gut, some intermediates such as volatile fatty acids produced by AF and associated bacteria are ingested by the host, with 'waste' CO_2/H_2 being metabolized to methane by methanogenic archaea.

Attempts have been made to enhance biogas generation from plant biomass by addition of AF leading to higher biogas output (Procházka *et al.*, 2012) and quicker initial H_2 and CH_4 production combined with improved volatile fatty acid degradation (Nkemka *et al.*, 2015), principally demonstrating the potential of AF to improve fiber digestion. However, before bioaugmentation may be expanded to current full-scale biogas plants, it is first important to determine if AF are already present and particularly whether they are metabolically active in existing biogas reactors. Kazda et al. (2014) demonstrated the occurrence of AF DNA in two German biogas digesters. Here a more extensive and detailed study across ten separate agricultural biogas plants in Bavaria was performed, using a diverse range of methods to determine the presence (DNA) and the transcriptional cellulolytic activity (mRNA) of AF in these habitats.

Samples were examined with three PCR based detection methods recently published by Dollhofer *et al.* (2016). These tools comprise: (1) qPCR assay AF-SSU quantifying the gene of the small ribosomal subunit (SSU, 18S rRNA) of AF. The 18S rRNA gene is present in multiple copies per

anaerobic fungal cell. It is highly conserved within the phylum Neocallimastigomycota and allows the specific detection of the group of interest. Quantification of AF 18S rRNA gene copies determines the relative abundance of AF within examined samples. (2) PCR assay AF-LSU specifically targeting the gene of the phylogenetically informative (Callaghan *et al.*, 2015; Dagar *et al.*, 2011; Wang *et al.*, 2017) large ribosomal subunit (LSU, 28S rRNA) of AF. The 28S rRNA gene delivers good phylogenetic resolution of the known AF genera and even below, and is becoming the new gold standard for taxonomic identification of the AF (Callaghan *et al.*, 2015; Dagar *et al.*, 2011; Wang *et al.*, 2017). Compared to phylogenetic analysis of AF communities with the to date mostly used internal transcribed spacer region 1 (ITS1) (Liggenstoffer *et al.*, 2010) AF LSU sequences are less variable, produce unequivocal results, and are thus easier to analyze. (3) qPCR assay AF-Endo specifically targeting an AF glycoside hydrolase family 5 (GH5) endoglucanase (EC 3.2.4.1) gene transcript. Endoglucanases are hydrolyzing (1→4)-β-D-glucosidic bonds in cellulose, and transcription of this gene is known to be significantly upregulated in AF during lignocellulose degradation (Couger *et al.*, 2015; Solomon *et al.*, 2016). Overall these three approaches thus allow to determine not only the relative abundance of AF but also which species are present and how transcriptionally active they are. Further, a cultivation based assay was performed on two AF positive digesters to see if isolation of AF is possible.

Thus the main goals of this study were to determine if (1) AF are native part of the biogas producing community, (2) which AF are present in the tested biogas digesters and (3) if the detected AF were transcriptionally active in cellulose degradation.

2 Material and methods

2.1 Samples from agricultural biogas plants

Samples were taken from ten individual biogas plants across Bavaria. These plants were part of a monitoring study by the Institute for Agricultural Engineering and Animal Husbandry at the Bavarian State Research Center for Agriculture, Freising (Ebertseder *et al.*, 2012). An overview of the sampled biogas plants, their technical specifications and substrates used therein can be found in Table 1. These biogas plants were either operated with high amounts of animal derived substrates (PB 14, PB 22), mainly with renewable plant biomass (PB 15, PB 17) or with mixtures of animal and plant derived substrates (PB 10, PB 16, PB 18, PB 19, PB 22 and PB 25). The digesters of PB 22 and the primary digester of PB 19 were operated at thermophilic conditions (53 and 52 °C, respectively). The digesters of the other biogas plants were operated at mesophilic (38-42 °C) or high mesophilic (46 °C PB 10 and 44 °C PB 17) conditions. The hydraulic retention time (HRT) in the digesters ranged between 32 to 90 days, the only exception being PB 22 (12 days). Sludge samples were directly taken from the exhaust valve of nine digesters (D), two post-digesters (PD) and two final repositories (FR; Table 1). Samples were quickly transported to the laboratory in insulated sealed containers, and nucleic acids were immediately extracted.

2.2 Extraction of nucleic acids

2.2.1 Sample preparation and DNA extraction

Prior to nucleic acid extraction, samples were washed with sterile 0.85% KCl to remove water soluble inhibitory compounds. For DNA extraction, 40 µl of the washed sample were processed with a Fast-DNA Spin Kit for soil (MP Biomedicals) in a FastPrep-24 system (MP Biomedicals, 40 s bead beating at speed 6.0). DNA was eluted in 100 µl Millipore™ water. The extraction was performed following the protocol published by Lebuhn *et al.* (2003). A more detailed description of the methods and techniques used for nucleic acid extractions is provided in Dollhofer *et al.* (2016).

2.2.2 mRNA extraction

Extraction of mRNA was performed with the Dynabeads® mRNA DIRECT™ Purification Kit (Life Technologies) following the protocol published in Dollhofer *et al.* (2016). In brief: 80 µl of washed sample (see 2.2.1) were transferred to a Lysis Matrix E tube (MP Biomedicals) and lysed in 1,250 µl of Lysis/Binding buffer (Life Technologies) with bead beating for 60 s at speed 5.5 in a FastPrep-24 system (MP Biomedicals). After centrifugation for 5 min at 20,000 g, the supernatant was transferred into a 1.5 ml reaction tube and mixed with 250 µl Dynabeads in Lysis/Binding buffer. mRNA was bound to the magnetic beads by shaking (Thermomixer MHR 11 by HRC Biotech) the samples at 200 rpm for 7 min at room temperature. The samples were placed in the Dynal MPC®-S Magnetic Particle Concentrator (Dynal Biotech) for 2 min to accumulate the beads and discard the supernatant. The samples were washed with 1 ml of washing buffer A and washing buffer B. Each washing step was performed twice. mRNA was eluted at 74 °C at 200 rpm in a rocker (Thermomixer MHR 11 by HRC Biotech) for 2 min in 25 µl Tris/HCL and separated from the beads by the magnet. The elution step was repeated, resulting in a final volume of 50 µl mRNA extract.

2.3 cDNA synthesis

Following the suppliers protocol, 20 µl of mRNA extract were digested with 2 µl of 50 x Turbo DNA-free Buffer and 1µl of Turbo DNAse from the TURBO DNA-free ™ Kit (Ambion). The reaction was carried out at 37 °C for 45 min in a thermocycler (Tprofessional Thermocycler by Biometra or Flexcycler by Analytik Jena). DNAse was inactivated by adding 2.3 µl of TurboDNA-free Inactivation reagent. Samples were centrifuged at 11,000 g for 1.5 min, and the supernatant was transferred into a clean 1.5 ml DNA LoBind reaction tube (Eppendorf). Aliquots were taken for reverse transcription and qPCR control reactions.

Reverse transcription of mRNA was performed with the ThermoScript™ RT-PCR System (Life Technologies). 5 µl of DNA-free mRNA were added to 0.6 µl primer AF-Endo reverse (see section 2.4), 2 µl of 10 mM dNTP Mix and 4.4 µl of DEPC-water, leading to a total volume of 12 µl. After

RNA denaturation at 65 °C for 5 min in a thermocycler (Tprofessional Thermocycler by Biometra), the mixture was placed on ice, and 8 µl of reverse transcription mastermix comprising 4 µl 5x cDNA synthesis buffer, 1 µl 0.1 M DTT, 1 µl RNase Out™ (40 U/µl), 1 µl DEPC-water and 1 µl of ThermoScript™ RT (15 U/µl), was added. Reverse transcription was performed at 51 °C for 60 min and stopped by termination at 85 °C for 5 min. cDNA was stored at -20°C until further analysis.

2.4 PCR and qPCR assays

One PCR and two qPCR assays, including standards for quantification, had been designed, optimized and validated in a previous study (Dollhofer *et al.* 2016). Reagents from the Platinum® Taq DNA Polymerase system (Life Technologies) were used for all PCR and qPCR reactions. For quantification of the 18S rRNA gene copies, the primer pair AF-SSU (AF-SSU forward: 5'-CTAGGGATCGGACGACGTTT-3'; AF-SSU reverse: 5'-GGACCTYCCGATCAAGGATG-3') and probe AF-SSU (5'-FAM-ATTCGCGTAACTATTTAGCAGGTTAAGGT-BHQ1-3') were used. qPCR reactions with assay AF-SSU were performed in a reaction volume of 25 µl consisting of: 2.5 µl 10 x PCR buffer (no $MgCl_2$), 3 µl 50 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each), 1 µl 10 µM primer AF-SSU forward, 1 µl 10 µM primer AF-SSU reverse (final primer concentration of 400 nM), 1.5 µl 10 µM AF-SSU probe, 0.15 µl Platinum™ Taq DNA Polymerase (5 U/µl), 2.5 µl of DNA template and Millipore™ H_2O to reach the total volume of 25 µl. Amplification was performed in a two-step qPCR program: 3 min initial denaturation/activation at 94 °C, followed by 45 cycles consisting of 15 s denaturation at 94 °C and combined annealing/extension for 1 min at 64 °C.

Transcripts of a celluloytic endoglucanase (EC 3.2.1.4) of the GH5 were quantified in a qPCR assay with primer pair AF-Endo (AF-Endo forward: 5'- CGTATTCCAACYACTTGGWSYGG-3'; AF-Endo reverse: 5'-CCRKTRTTTAAGGCAAARTTRTAYGGA-3'). qPCR reactions with assay AF-Endo were performed in a reaction volume of 25 µl consisting of: 2.5 µl 10 x PCR buffer (no $MgCl_2$), 3 µl 50 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each), 0.5 µl 10 µM primer AF-Endo forward, 0.5 µl 10

µM primer AF-Endo reverse (final primer concentration of 200 nM), 1 µl EvaGreen Dye, 0.15 µl Platinum™ Taq DNA Polymerase (5 U/µl) and 2.5 µl of cDNA template. The reaction volume was adjusted to 25 µl by adding Millipore™ H_2O. qPCR was performed in a two-step program: 3 min initial denaturation/activation at 94 °C, followed by 45 cycles consisting of 15 s denaturation at 94 °C, combined annealing/extension for 1 min at 64 °C and denaturation at 82 °C for 10 s. Dissociation curve analysis was performed by one cycle comprising denaturation at 95 °C for 1 min, cool down to 55 °C for 30 s and reheating to 95 °C for 30 s. qPCR reactions were performed on an Mx3005P qPCR system (Agilent Technologies).

Escherichia coli (OneShot Top10, Invitrogen) clones carrying the corresponding target amplicon were used as whole cell quantification standards in each assay. Standards were initially quantified by most probable number (MPN) qPCR of 10 fold dilution series (undiluted to 10^{-8}) and parallel cell counting, allowing determination of the number of positive inserts per cell and thus the number of copies present in the standard cell suspension. The standard equation $Y = -3.230 \times LOG(X) + 38.37$ (Y = fluorescence in dR; X = initial quantity of copies) and a qPCR efficiency of 104% was obtained for assay AF-SSU and the standard equation $Y = -3.415 \times LOG(X) + 37.90$ (qPCR efficiency of 96.3%) for assay AF-Endo. For both qPCR assays, the lower analytical limits, comprising the Limit of Blank (LoB), the Limit of Detection (LoD) and the Limit of Quantification (LoQ), were assessed according to the method of (Francy *et al.*, 2015). For definitions and mathematical details see Dollhofer *et al.* (2016). For qPCR method AF-SSU, 11 copies of AF 18S rDNA per reaction was the LoD and 35 18S rDNA copies per reaction was the lowest accurately quantifiable copy number (LoQ). For qPCR assay AF-Endo 7.76 copies per reaction were calculated as LoD and 13.11 copies per reaction as LoQ. To rule out PCR inhibition, each sample was tested undiluted and in a 1:10 dilution.

PCR for phylogenetic classification of AF was performed using primer pair AF-LSU (AF-LSU forward: 5'-GCTCAAAYTTGAAATCTTMAAG-3'; AF-LSU reverse: 5'-

CTTGTTAAMYRAAAAGTGCATT-3'), targeting the large ribosomal subunit (LSU, 28S rRNA gene). Endpoint PCR with primer pair AF-LSU for cloning and sequencing was performed in a reaction volume of 50 µl. The reaction mix consisted of: 2.5 µl 10 x PCR buffer (no $MgCl_2$), 3 µl 50 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each), 1 µl 10 µM primer AF-LSU forward, 1 µl 10 µM primer AF-LSU reverse (final primer concentration of 200 nM), 0.15 µl Platinum™ Taq DNA Polymerase (5 U/µl) and 5 µl of DNA template. Millipore™ H_2O was added to reach the total volume of 50 µl. A three-step PCR program was performed: 3 min initial denaturation/activation at 94 °C, 35 cycles comprising 20 s denaturation at 94 °C, 45 s annealing at 61 °C and 45 s extension at 72 °C. PCR reactions were performed on a TProfessional Thermocycler (Biometra) or a Flexcycler (Analytik Jena).

2.5 Cloning and sequencing

Amplicons were generated with primer pair AF-LSU in a three step PCR using Platinum® Taq DNA Polymerase (Invitrogen) according to the conditions presented in section 2.4. PCR products were purified with the MinElute PCR Purification Kit (Qiagen) and used for cloning with the TOPO-TA cloning Kit (Invitrogen) with the pCR 4-TOPO vector and OneShot TOP10 chemically competent cells. Clones carrying the plasmid were identified by propagation on LB agar plates containing ampicillin. Clones were checked for the right insert size by colony PCR using primer pair M13. Positive clones were sequenced at Eurofins MWG operon. The received clone sequences were checked for accuracy and implemented in existing alignments of the 28S rRNA gene in MEGA 6.06 (Tamura *et al.*, 2013) or Geneious 6.06 (Kearse *et al.*, 2012). For phylogenetic analysis, alignments contained all sequences belonging to the Phylum Neocallimastigomycota available from NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and the Silva high-quality ribosomal RNA databases (https://www.arb-silva.de/). Clone sequences were analyzed for chimeric sequences with

Bellerophon (Huber et al., 2004), Uchime (Edgar *et al.*, 2011) and visually. Identified chimeras were excluded from further analysis.

2.6 Isolation of anaerobic fungi

Isolation of AF was performed at the University of Aberystwyth, Wales, UK, following the protocol of Callaghan *et al.* (2015), using a rumen fluid-based medium termed enrichment medium, containing wheat straw (0.5%), soluble xylan (0.2%) and cellobiose (0.2%). Two biogas digester sludge samples (from PB 18 and PB 21; Table 1) were selected for isolation of AF. The samples were cooled to 4 °C and packed in anaerobic bags (AnaeroGen™ W-Zip Compact Gas Generator System; Oxoid) for transport to Aberystwyth. In order to minimize the effect of inhibitors and detach the fungal cells from plant material, samples were diluted and stomached in an anaerobic salt solution as described by Callaghan *et al.* (2015).

All isolation and subculturing procedures were conducted under gas flow or gas atmosphere of 100% CO_2. Liquid enrichment medium (60 ml in 100 ml serum bottle), containing milled and sieved wheat straw (2 mm) as substrate, were inoculated with different amounts of diluted sample (3, 5 and 9 ml). Antibiotic mixture of penicillin G and streptomycin-sulfate (2 mg/ml of each in final medium) was used to inhibit bacterial growth. Enrichment cultures were incubated at 39 °C for 3-15 days until growth of AF was detected by gas generation, microscopy and visually through the formation of floating mats or balls formed from the enrichment substrate.

The mixed cultures were maintained by transferring to fresh enrichment medium, and pure cultures of AF were obtained through inoculation of agar-containing roll-tubes comprising only the soluble carbon sources xylan (0.3%) and cellobiose (0.3%) (Callaghan *et al.*, 2015; Joblin, 1981). Individual fungal colonies were picked and new enrichment cultures were inoculated. This allowed separating different colony types from each other. Roll-tubing was repeated several times to ensure that AF

cultures were pure. The isolates were identified morphologically under the microscope and through sequencing of their 28S rRNA gene (see 2.4, 2.5) from extracted DNA.

3 Results and Discussion

3.1 Quantification of anaerobic fungal gene copies and transcriptional activity

Samples were taken from different compartments of ten Bavarian biogas plants, from nine digesters, two post-digesters and two final repositories. The biogas plants, their characteristics and the fed substrates are described in Table 1. DNA and mRNA were extracted from the samples. First the concentration of anaerobic fungal 18S rDNA gene copies in the samples was quantified by qPCR with primer and probe combination AF-SSU (see 2.4 PCR and qPCR assays). AF 18S rDNA was detected in seven of the sampled biogas plants (Figure 1); data for biogas plant PB 25 was previously published (Dollhofer *et al.*, 2016) but is presented again here for comparison. The three other sampled biogas plants (PB 15, PB 17 and PB 19) showed no evidence for the presence of AF. For AF SSU rDNA, 4.38×10^3 to 1.65×10^9 copies/ml were detected in the six sampled main digesters, 5.76×10^8 copies/ml in the post-digester of PB 21 and 3.79×10^7 copies/ml in the final repository of PB 18 (Figure 1). AF 18S rDNA gene copies were thus detected in most examined agricultural biogas plants, but at levels lower than in cattle rumen fluid (1.69×10^{10} copies/ml; SD=3.88×10^9) and cattle slurries used as substrate in PB 14 (1.88×10^9 copies/ml; SD=3.3×10^8) and PB 22 (6×10^9; SD=1.16×10^9 copies/ml) (Dollhofer *et al.*, 2016). An exception was the digester of PB 22 in which 1.65×10^9 copies/ml sludge were found, which is close to the values measured in cattle slurry. AF generally occurred at levels 10 to 100-fold lower than in the rumen or cattle slurry.

However, the lower quantity of AF in the biogas plants does not exclude a function of AF in the biogas process, as AF are also not the dominant microbes by biomass in the rumen, but they are key players in fiber degradation in this ecosystem (Gruninger *et al.*, 2014).

To date, AF have most commonly been isolated from the digestive tracts of larger mammalian herbivores, but recently evidence for the occurrence of AF outside such habitats are accumulating. Their occurrence seems to be widespread ranging from the reptile gut (Liggenstoffer *et al.*, 2010), to pond sediments (Wubah & Kim, 1995), and landfill sites treating cellulosic wastes in the United Kingdom (Lockhart *et al.*, 2006). However, these findings were based on the detection of DNA or isolation of strains. This is no proof for the activity and growth of AF in these habitats. Several AF species produce aerotolerant resting spores enabling them to endure inhospitable aerobic conditions (Brookman *et al.*, 2000; Wubah *et al.*, 1991), allowing propagation of AF between host animals (Milne *et al.*, 1989). The persistence of aerotolerant AF resting stages could be a possible explanation for their detection in atypical habitats.

AF 18S rDNA was only detected in biogas plants fed with cattle manure (21.6% to 77.3% of total substrates) or slurry (1.7% to 44.9% of total substrates). The highest concentration of AF 18S rDNA was found in PB 22, which also received the highest input of cattle manure (77.3%). AF are known to be present and viable in animal feces for periods from weeks to months (Davies *et al.*, 1993; McGranaghan *et al.*, 1999), and the slurries fed in PB 14 and PB 22 were additionally tested positive for AF presence (see above).

In biogas plants operated with no (PB 15) or relatively low amounts of cattle manure (18% in PB 17), no AF were detected. Despite a moderate input of cattle slurry (36%), no AF were detected in biogas plant PB 19. As discussed later, the absence of AF was likely due to their long exposition (HRT= 90 days) to the high process temperature (52 °C) in PB 19 digester (Table 1). Taken together this suggests that AF detected in the digesters originated from the constant input of animal derived substrates.

Since detection of AF 18S rDNA does not prove that the detected AF are viable and active in the biogas production process, metabolic activity of AF was determined by quantification of transcripts of

a GH5 endoglucanase gene. Endoglucanases hydrolyze non-crystalline cellulose and have been shown to be part of the enzymatic lignocellulose degradation machinery of AF (Couger *et al.*, 2015; Solomon *et al.*, 2016). Detection of GH5 endoglucanase transcripts thus indicates the presence of viable AF, being active in cellulose degradation.

Although 18S rDNA was detected in 7 out of 10 biogas plants, evidence for transcriptional activity of GH5 endoglucanases was detected only in two plants (PB 21 and PB 22), where $4.46x10^{1}$ GH5 transcripts/ml and $1.8x10^{2}$ GH5 transcripts/ml were found. Transcript numbers were thus lower than in the rumen of cattle, where $1.88x10^{3}$ to $2.83x10^{5}$ AF GH5 endoglucanase transcripts/ml were detected using the same method (Dollhofer *et al.*, 2016).

Since the number of copies per reaction in the biogas sludge samples was lower than the calculated limit of reliable quantification (13.11 copies per reaction in the assay AF-Endo, see section 2.4), this did not allow accurate absolute quantification. However, specific peaks visible in qPCR melting curve analysis for the GH5 endoglucanase RT-amplicons confirmed the presence of these transcripts and thus the presence of transcriptionally active AF. As expected for biogas plants PB 21 and PB 22, where GH5 endoglucanase transcripts were detected, also the highest levels of AF 18S rDNA were found (Figure 1).

The temperature (53 °C) at which PB 22 was operated, is significantly higher than in mammalian digestive tracts (38-41°C). The detection of metabolically active AF in this digester was thus unexpected at a first glance.

However, two factors may explain the detected AF transcriptional activity: First, the constant input of cattle manure used in PB 22 (77.3%) was highest among the sampled biogas plants, and cattle manure is known to contain viable AF biomass (Davies *et al.*, 1993; McGranaghan *et al.*, 1999). Second, the HRT of 12 days in the digesters of PB 22 (Table 1) was extremely short. The AF were thus exposed to the adverse digester conditions only for a relatively short time period. In the other sampled

digesters which were operated in the high mesophilic or thermophilic range, PB 10 (47 °C) and PB 19 (52 °C), the HRT was longer (52 days and 90 days), and no evidence for transcriptionally active AF and in the latter no evidence for AF at all was found although they received considerable cattle slurry/manure input (Table 1). This suggests that longer exposure to thermophilic conditions was not endured by the AF.

Moreover, the results of previous studies in which AF cultures were inoculated into biogas digesters to improve biogas production rates showed that the AF were not able to persist in the biogas environment and died within the first 10 to 15 days after their implementation (Nkemka *et al.*, 2015; Procházka *et al.*, 2012). Taken together, the results suggest that at least the tested biogas fermenter environments with the given conditions do not favor AF growth and activity. The conditions appear to eventually kill the AF or render them inactive. For this reason, it seems that conventional bioaugmentation with addition of AF cultures to biogas plants is not promising. Alternative strategies should be developed considering specifically the needs of existing AF. Additionally, specific strains which can grow under the present biogas conditions could be identified and selected for bioaugmentation purposes.

3.2 Community composition of the anaerobic fungal populations in agricultural biogas plants

Analysis of the composition of biogas plant AF populations was performed by cloning and sequencing of a 441 bp amplicon of the 28S rRNA gene (amplified specifically with primer pair AF-LSU, see section 2.4). All samples of biogas plants showing amplification of AF 18S rDNA (see Figure 1 and section 3.1) were tested with the LSU primers. Amplicon sequences were subject to quality control (see section 2.5) to ensure that no chimeric sequences were present and deposited in NCBI GenBank: 31 sequences from PB 10 D (KX889576-KX889605), 23 from PB 14 D (KX889553-KX889575), 6 from PB 16 D (KX889547-KX889552), 45 from PB 21 D (KX889447-KX889490), 27 from PB 22 D (KX889521-KX889546), 30 from PB 25 D (KX164374-KX164403;

Dollhofer *et al.* (2016)), 30 from PB 21 PD (KX889491-KX889520) and 19 from PB 18 FR (KX889606-KX889625). Phylogenetic analysis of these sequences revealed the presence of six of the eight known genera of AF (*Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Cyllamyces*, *Piromyces*, *Anaeromyces*; Figure 2). The most recently described genera *Oontomyces* and *Buwchfawromyces* were not detected. In addition, four clades representing hitherto unclassified AF genera, named "novel clades" A to D were also detected. For better visualization, identical LSU clone sequences were removed from the phylogenetic tree (Figure 2). The community structure of AF populations in the studied samples as obtained by LSU sequence analysis is shown in Figure 3. Of the 230 clone sequences analyzed, the monocentric genera *Neocallimastix* and *Piromyces* were the most abundant representing 35.6% (present in 2/7 plants) and 27.3% (present in 5/7 plants), respectively. Third most abundant was novel clade A, represented by 46 clones (20% of total) from three different biogas plants. This clade was most closely related to the genus *Buwchfawromyces*. Fungi belonging to the genera *Orpinomyces*, *Anaeromyces*, *Cyllamyces*, *Caecomyces* and the novel clades B,C and D were present at lower abundance and were less widespread (in only 1 or 2 plants each).

A study by Liggenstoffer *et al.* (2010) on the AF community composition in feces of diverse zoo animals also found *Piromyces* and *Neocallimastix*, alongside with hitherto unclassified novel AF, to be the most widespread and abundant. Since both, the AF detected in the current study and those reported by Liggenstoffer *et al.* (2010) originated from manure/slurry and feces, respectively, the observed similarity of AF communities in the biogas plant samples was not unexpected.

The digester and post-digester of plant PB 21 were both examined. In the digester D2, all clones (n=45) were closely related to *N. cameroonii*. The post-digester PD (n=34) was also dominated (61.8%) by this species, accompanied by sequences belonging to novel clades B (14.7%) and D (23.5%). This difference may be due to the relatively low number of clones analyzed but it may also reflect the differential survival of different species DNA during the biogas fermentation stages.

Among AF sequences obtained from the digester of PB 22 (n=31), representatives of the *Neocallimastix* clade with the type species *N. frontalis* were dominant (51.6% of clone species) followed by sequences affiliated to the genus *Orpinomyces* (19.3%), *Piromyces* (16.2%), *Cyllamyces* (9.7%) and *Anaeromyces* (3.2%; n=1). Thus the sample from PB 22 showed the highest diversity of LSU sequences. The facility consisted of two small digesters with 115 m^3 volume each, which were continuously fed with very high amounts of cattle manure (77.3%). As all the mentioned genera are known from bovine feces, it is plausible, as mentioned in section 3.1, that the AF population in the feces remained almost unaffected, resulting in the diverse mix of AF sequences found.

Of the biogas plants showing no signal for transcriptionally active AF, PB 25 contained the highest level of anaerobic fungal 18S rDNA copies. Based on the LSU sequence data the vast majority (90%) of the sequences (total n=27) detected in PB 25 clustered in novel clade A, and the rest of the sequences clustered with the genera *Anaeromyces* and novel clade B (Dollhofer *et al.*, 2016). The widespread occurrence of hitherto unidentified clades of AF may have caused bias in qRT-PCR analysis of GH5 endoglucanase expression, since the AF-Endo primers were designed based on sequences from the cultured isolates from the genera *Neocallimastix*, *Orpinomyces* and *Piromyces*. It is possible that there are mismatches to the GH5 endoglucanase gene of the novel clades leading to poor or no amplification, for example in plant PB 25 where novel clade A was dominant and no GH5 transcripts were detected.

In the digester of PB 14 (total n=25) sequences of novel clade A accounted for nearly half of all clones 44% followed by sequences belonging to the bulbous groups with 32% and a minor portion of *Piromyces* sequences (not shown in Figure 2) with 24%.

Biogas plant PB 16, in which only a low amount of AF 18S rDNA was detected, gave rise to many chimeric sequences in the clone library, possibly due to the low amount of target DNA present. The chimeric sequences were split into their parental fractions and counted for the proportional clone

analysis (but not submitted to NCBI GenBank) as follows: 33% were belonging to "Novel clade D", 26.6% to "Novel Clade C", 20% to the genus *Cyllamyces*, 13% to the genus *Piromyces* and 6% to the genus *Caecomyces* (Figure 3).

3.3 Isolation of an anaerobic fungus from a biogas plant

Isolation of AF was attempted from two biogas plants (PB 21 and PB 18) which were both fed with a comparable substrate mix comprising high amounts of grass silage and moderate amounts of cattle manure/slurry (Table 1). 18S rDNA of AF was detected from both facilities, while GH5 endoglucanase transcripts were detected only in PB 21. No AF were isolated from the digester of PB 18, but an AF strain (CaDo16a) was isolated from sludge in parallel digester 1 (=D1) of plant PB 21. Strain CaDo16a showed monocentric growth forming a single sporangium on each thallus and filamentous branched rhizoids. Zoospores were abundant and monoflagellated. Isolate CaDo16a was assigned to the genus *Piromyces* consistent with its morphological characteristics according to the identification key of Ho and Barr (1995) and its LSU sequence (KY364902) which fell within the *Piromyces* clade (Figure 2). The most similar LSU sequences, were all from environmental samples (JX848540; from sheep rumen, Iran (unpublished)), KX164364 from cattle rumen fluid Dollhofer *et al.* (2016)) but not from pure cultures. An identical sequence was also detected in the clone library from a digester sludge sample from biogas plant PB 22 but it was not detected in the clone library from biogas plant PB 21 from which strain CaDo16a was isolated. Given its unique LSU sequence and its morphological characteristics it is likely that CaDo16a represents a new species of the genus *Piromyces*. It is the first Neocallimastigomycota strain that was isolated from a biogas digester. Comparison with the biogas clone sequences derived from parallel digester 2 of PB 21 showed no sequences related to the genus *Piromyces*, standing in contrast to the cultivation based results. It has to be considered that not the full diversity will be depicted by PCR-cloning and sequencing of a given sample (Hughes *et al.*, 2001) and that some AF might not be detected due to the limited depth of this

analysis. Thus, results from sequencing are often not comparable to the results from cultivation (Kautz *et al.*, 2013). This could be valid for work with the AF too, as it is known from sequencing studies that the majority of AF has not been cultured to date (Liggenstoffer *et al.*, 2010). Although isolate CaDo16a does not appear to be a dominant species in the digester, it might be more easily cultivable with the applied method than the *Neocallimastix* sp. for which evidence was found by cloning and sequencing (Figure 3). Further, the applied cultivation approach is based on methods that were successful for isolation of AF from rumen fluid and animal feces. More comprehensive results may be achieved if the isolation technique is specifically adjusted to the conditions of the examined habitats.

Although the given results indicate that most biogas digesters are not a suitable environment for optimum growth and activity of AF, the isolation of strain CaDo16a and the detection of AF GH5 endoglucanase transcripts support the hypothesis that AF can at least transitionally be an active part of biogas biocenoses and that they might be more widely distributed than currently thought.

4 Conclusions

This study shows that AF are present in agricultural biogas plants, can occasionally display low transcriptional cellulolytic activity therein, and can be isolated from digester sludge. Based on present knowledge, AF and their survival structures seem to be transported into the biogas plants with the daily load of animal derived substrates. Data suggests that they can survive only for a short period of time in conventional biogas processes with their fate strongly depending on the present process conditions. Modifying conventional biogas production with consideration of existing AF needs thus seems to be necessary to make efficient bioaugmentation with AF possible.

5 Acknowledgements

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6 Appendix A Supplementary Data

Supplementary data associated with this article can be found, in the online version, at XXX

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8 Figures Captions

Table 1: Technical specifications of sampled biogas plants

Figure 1: Number of anaerobic fungal SSU rDNA gene copies (black) and GH5 endoglucanase transcripts (grey) detected per ml fermenter sludge or digestate of AF positive biogas plants (D= digester; PD= post-digester; FR= final repository; none= not detected). Error bars indicating standard deviation.

Figure 2: Maximum likelihood tree based on an 453bp alignment of 78 AF 28S rDNA sequences. The sequences are representative of all described AF genera, along with clone sequences (in black) derived from the examined biogas plants and rumen fluid in this and the previous study (Dollhofer *et al.*, 2016). An aerobic chytrid *Polychytrium* sp. (HQ901712) was used to root the tree. Only bootstrap (1000 replicates) values over 70% are shown and scale bar shows substitutions per site. The different genera are colour coded: *Anaeromyces* (green), *Buwchfawromyces* (brown), *Caecomyces* and *Cyllamyces* (yellow), *Neocallimastix* (pink), *Oontomyces* (grey), *Orpinomyces* (blue) and *Piromyces* (red).

Figure 3: Composition of the LSU DNA sequences derived from the 7 AF positive biogas plant samples with phylogenetic resolution at the genus level. For biogas plant PB 21 an additional sample from the post-digester was analyzed. For abbreviations see Figure 1.

9 Tables and Figures

Table 1:

Biogas plant - ID	PB 10	PB 14	PB 16	PB 18	PB 21
Digester volume [m³]	800	800	900	1,200	2 x 1,200 parallel
Post-digester volume [m³]	850	800	2,280	absent	2,400
Final repository volume [m³]	2 x 2,700	I: 410 + II: 320	2,700	2,700	3,600
Temperature [°C]*	47 (D)	38-39 (D + PD)	42 (D)	42 (D), 40 (FR)	40 (D1, D2), 46 (FR)
HRT of first process step [d]*	51	53	32	65	61
OLR ($kg_{VS} \times m^{-3} \times d^{-1}$)*	4.5	2.3	7.7	3.1	3.3
NH_3-N ($mg \times L^{-1}$)*	338.7 (D)	67.49 (D)	80.50 (D)	149.71 (FR)	66.44 (D1), 91.15 (D2)
Plant-derived substrates	45% Maize silage	6.7% Maize silage	7.9% Maize silage	3.0% Maize silage	-
	7% Grass silage	8.5% Grass silage	59.1% Clover-grass silage	64.5% Grass silage	68.6% Grass silage
	5% Whole plant silage	1.8% Whole plant silage	-	-	-
	3% Corn-Cob-Mix	0.7% Corn-Cob-Mix	-	-	-
		0.2% Shredded grain	0.8% Shredded grain	-	2% Grain
Animal-derived substrates	37% Cattle manure	72% Cattle manure	32.2% Cattle manure	-	21.6% Cattle manure
	3% Cattle slurry	10.1% Cattle slurry	-	32.5% Cattle slurry	7.8% Cattle slurry
Sampled compartment	D	D	D	FR	D1, D2, PD

Biogas plant - ID	PB 22	PB 25	PB 15	PB 17	PB 19
Digester volume [m³]	2 x 115 parallel	700	800	1,200	1,200
Post-digester volume [m³]	absent	550	-	1,200	600
Final repository volume [m³]	1,460	1,600	1,200	3,000	I: 1,200, II:1,400
Temperature [°C]*	53 (D)	40 (D)	42 (D)	44 (D); 47 (PD)	52 (D)
HRT of first process step* [d]	12	52	63	69	90
OLR ($kg_{VS} \times m^{-3} \times d^{-1}$)*	10.1	4.3	5.1	5	1.8
NH_3-N ($mg \times L^{-1}$)*	120.43 (D1)	82.83 (D)	426.87 (D)	1,142.01 (D)	266.62 (D)
Plant-derived substrates	19% Maize silage	35.1% Maize silage	17.9% Maize silage	6% Maize silage	20% Maize silage
	1.3% Grass silage	8.8% Grass silage	77.3% Clover-grass silage	56% Clover-grass silage	44% Grass silage
	-	2.2% Whole plant silage	0.4% Whole plant silage	-	-
	-	-	-	1% Corn-Cob-Mix	-
	0.7% Shredded grain	2.4% Shredded grain	1.0% Shredded grain	2% Grain	-
	-	6.7% Sugar beets	1.8% Topinambur	-	-
Animal-derived substrates	77.3% Cattle manure	-	-	18% Cattle manure	-
	1.7% Cattle slurry	44.9% Cattle slurry	-	-	36% Cattle slurry
	-	-	1. 5% Poultry manure	17% Poultry manure	-
Sampled compartment	D1	D	D	D, PD, FR	D

HRT= hydraulic retention time; OLR= organic loading rate; D= digester; D1= digester 1; PD= post-digester, FR= final repository; *= yearly mean value

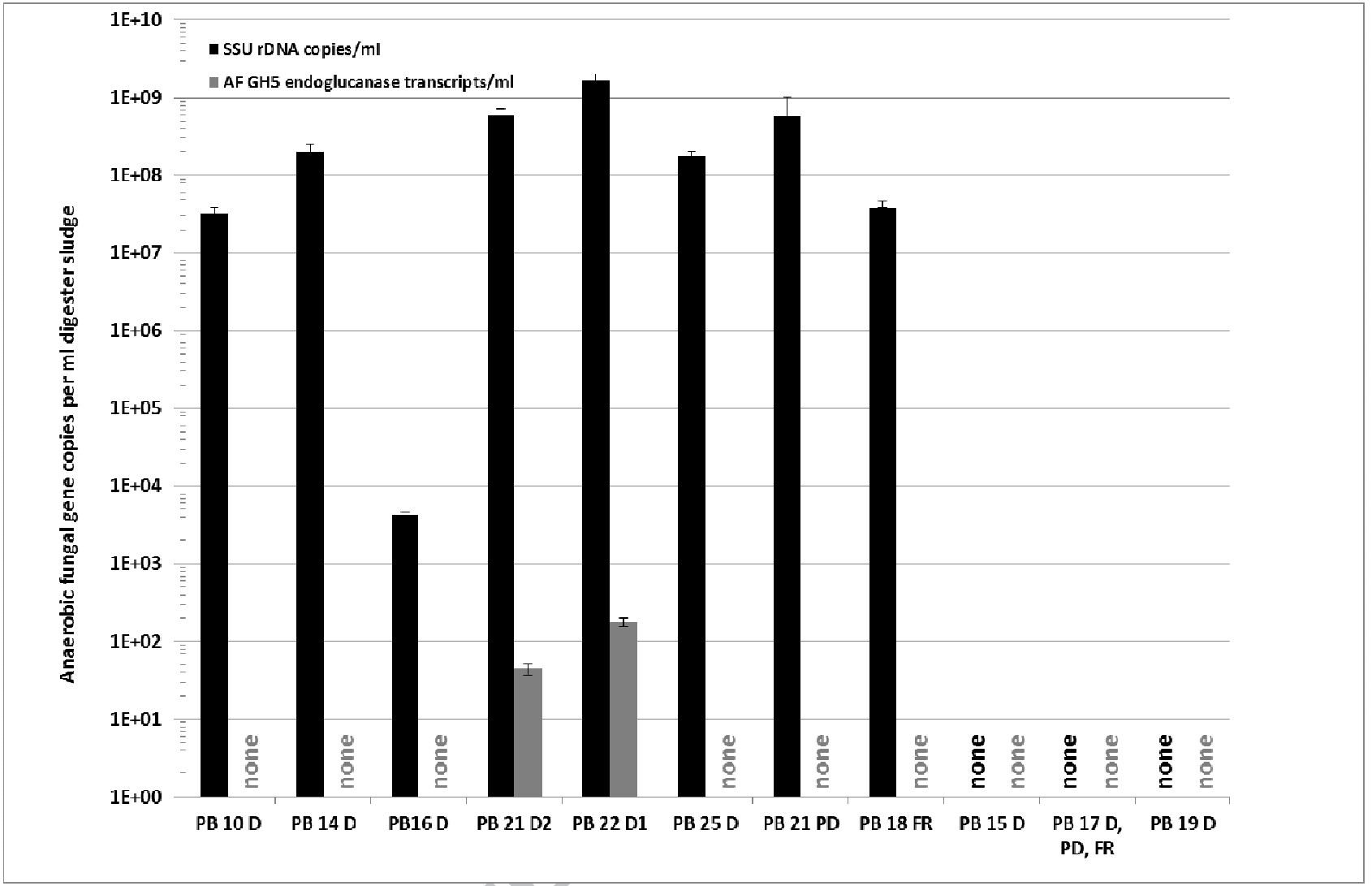

Figure 1:

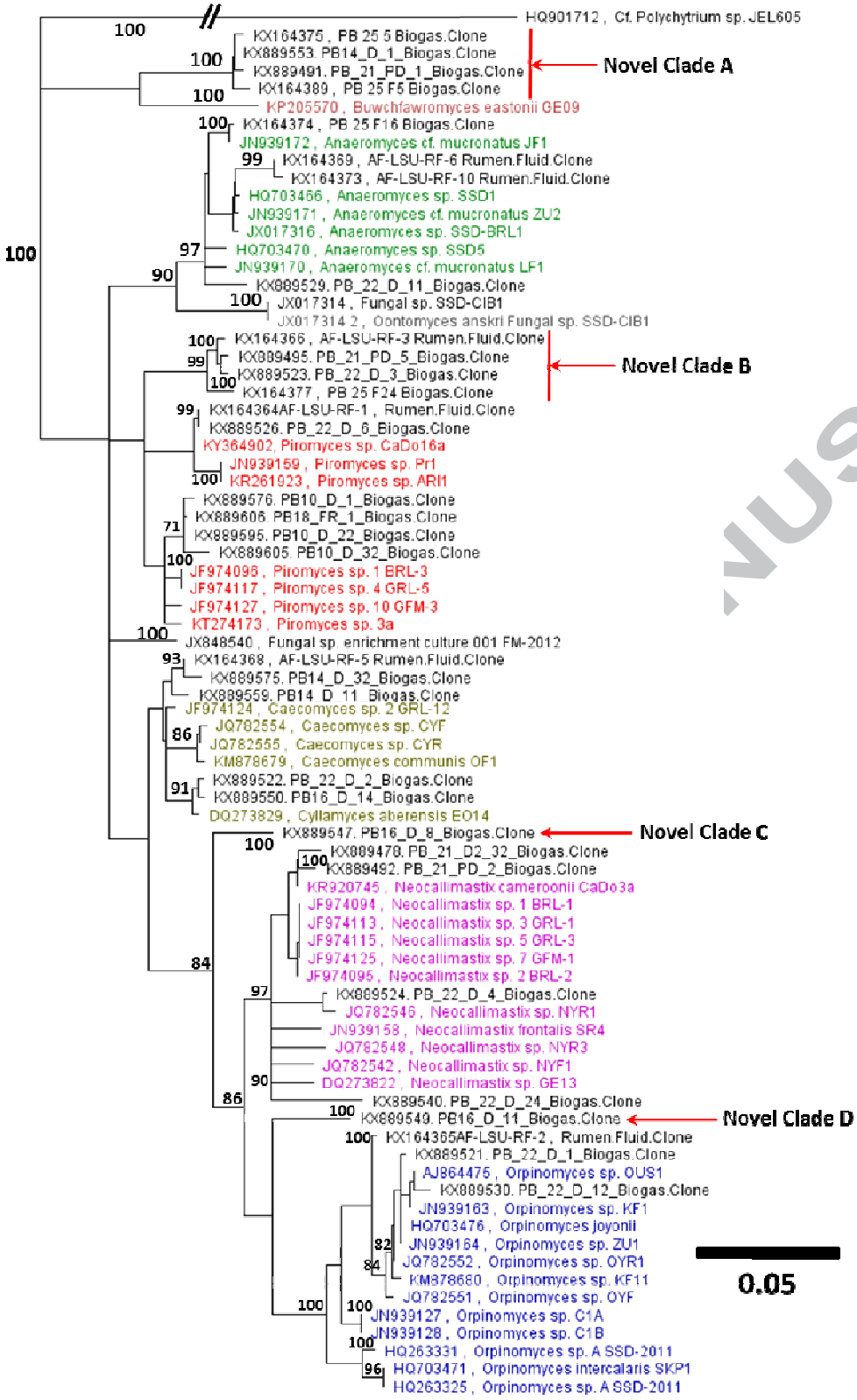

Figure 2:

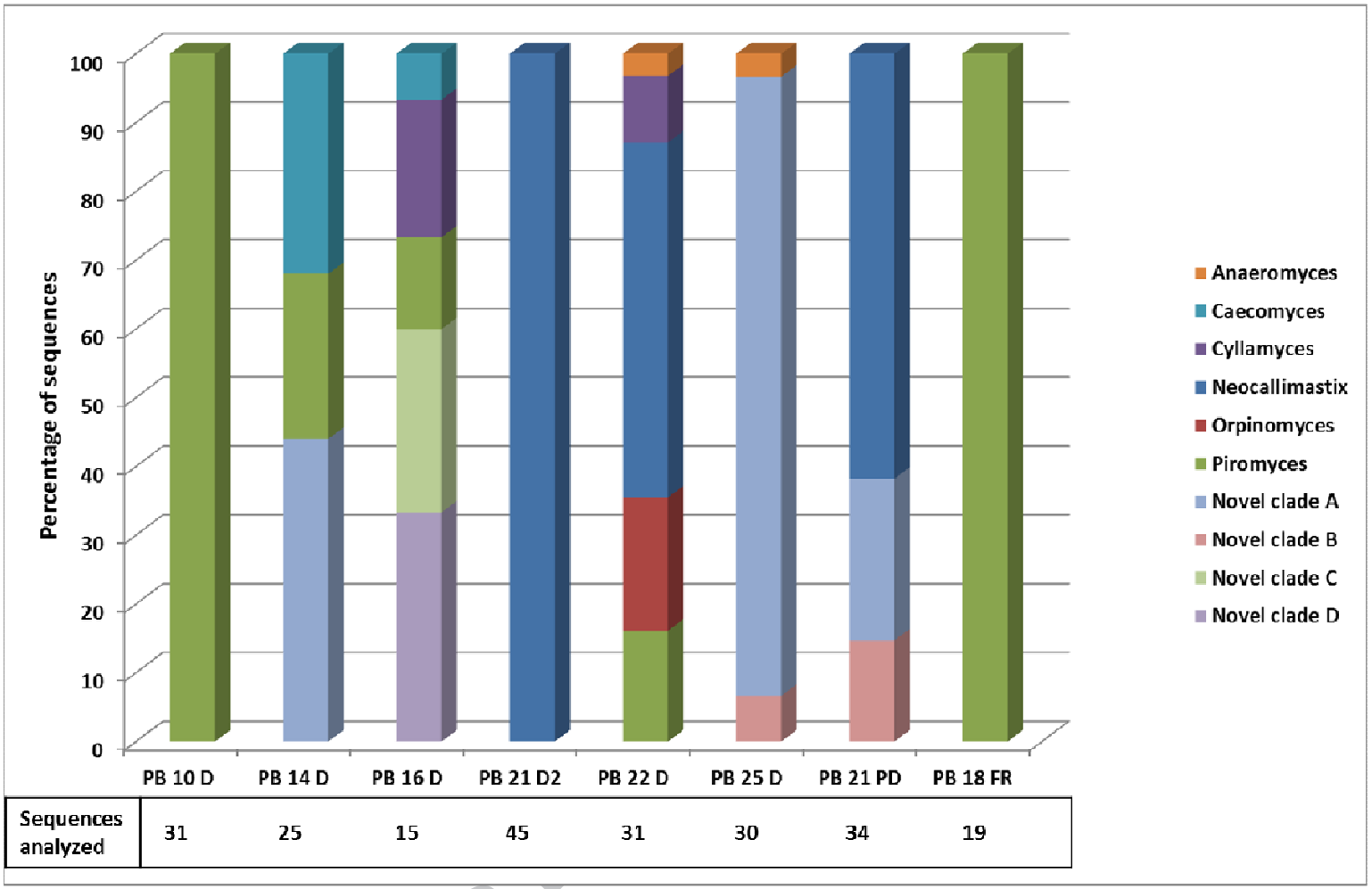

Figure 3:

Highlights

- **Specific study on occurrence of anaerobic fungi in agricultural biogas plants**
- **Anaerobic fungal 18S DNA only detected in biogas plants operated with cattle manure**
- **GH5 endoglucanase transcripts found only in 2 anaerobic fungi positive digesters**
- **Anaerobic fungi are probably transferred to digesters via animal manure**
- **Description of a novel *Piromyces* species isolated from a digester**