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Anaerobic fungi – detection methods, fate in agricultural biogas plants and potential to improve gas production

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Dedication

This work is dedicated to my parents Brigitte and Manfred, who always supported me and always believed in the success of my work.

“Look wide, beyond your immediate surroundings and limits, and you see things in their right proportion. Look above the level of things around you and see a higher aim and possibility to your work.”

Lord Robert Stephenson Smyth Baden-Powell

Abstract

Biogas production from lignocellulosic residues (LCR) can help to reduce the use of fossil fuels in the future. However, biogas in Germany is currently mainly produced in agricultural biogas plants from energy crops such as maize and from slurry and manure. A key reason is, compared to energy crops, the lower digestibility of the LCR, in which a recalcitrant lignin layer protects the more easily digestible carbohydrates cellulose and hemicellulose from attack by hydrolytic bacteria. For biogas plant operators, the use of LCR is currently rather disadvantageous, as in addition to poor gas yields, long fibers can cause mechanical process disturbances.

Anaerobic fungi (AF, phylum *Neocallimastigomycota*) might improve the conversion of LCR to biogas and thus enable their profitable energetic use. In their natural habitat, the digestive tract of herbivorous vertebrates, anaerobic fungi contribute to the nutrition of the hosts. Therein, they colonize the ingested plant fibers, disintegrate them by rhizoidal growth and enzymatically split the lignocellulose structures.

In the present work, three PCR-based detection systems were developed and validated to detect anaerobic fungi, their composition on genus level and activity in biogas processes. Two assays are based on the specific analysis of ribosomal genes: (i) AF-SSU for quantification of 18S rDNA gene copies of anaerobic fungi using quantitative real-time PCR; (ii) AF-LSU for sequencing the 28S rDNA of anaerobic fungi and their phylogenetic placement; (iii) AF-Endo for the specific detection of the transcriptional activity of a GH5 endoglucanase from anaerobic fungi.

Anaerobic fungi were detected in seven out of ten agricultural biogas plants analyzed. Phylogenetic analysis of LSU sequences revealed the presence of seven anaerobic fungal genera, comprising the genera *Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Cyllamyces*, *Piromyces*, *Anaeromyces*, *Feramyces* and the putative novel genus *Khoyollomyces*. Anaerobic fungi were only detected in facilities operated with a high share of cattle slurry or cattle manure. In two of the seven positive tested biogas plants, low transcriptional activity for an anaerobic fungal GH5 endoglucanase was measured. Moreover, addition of anaerobic sludge led to fade out of transcriptional activity of anaerobic fungi, in the hydrolytic pre-treatment experiment of this work. Taken together, these results indicated that the anaerobic fungi were transferred into the biogas plants with the animal derived substrate and were inactivated in the fermenter after a short time.

Since the conditions in the biogas process apparently damaged the anaerobic fungi, a direct implementation of anaerobic fungi into biogas reactors seemed inappropriate. However, in order to achieve an improvement in biogas production from LCR, a hydrolytic pre-treatment with two different *Neocallimastix frontalis* strains was tested. In both pre-treatments, the initial biogas production was accelerated, as compared to control approaches with inactivated anaerobic fungi, dry matter degradation was improved, and the production of volatile fatty acids was increased. Thus, hydrolytic pre-treatment adapted to the needs of anaerobic fungi may represent a potential way to a more efficient energetic use of LCR.

Zusammenfassung

Die Gewinnung von Biogas aus lignocellulosereichen Reststoffen (LCR) kann künftig dazu beitragen, den Einsatz fossiler Energieträger zu reduzieren. Aktuell wird Biogas in Deutschland allerdings hauptsächlich in landwirtschaftlichen Biogasanlagen aus eigens dafür angebauten Energiepflanzen wie Mais sowie aus Gülle und Mist produziert. Ein wesentlicher Grund ist die im Vergleich zu Energiepflanzen schlechtere Vergärbarkeit der LCR, bei denen eine schwer zersetzbare Ligninschicht die leichter verwertbaren Kohlenhydrate Cellulose und Hemicellulose vor einem Angriff der hydrolytischen Bakterien schützt. Für Biogasanlagenbetreiber ist der Einsatz von LCR aktuell eher nachteilig, da neben schwachen Gasausbeuten auch mechanische Prozessstörungen durch das langfaserige Material auftreten können.

Anaerobe Pilze (Phylum *Neocallimastigomycota*) könnten dazu beitragen, die Umsetzung von LCR zu Biogas zu verbessern und so deren rentable energetische Nutzung zu ermöglichen. In ihrem natürlichen Lebensraum, dem Verdauungstrakt pflanzenfressender Wirbeltiere, tragen anaerobe Pilze zur Ernährung der Wirte bei. Sie besiedeln dort die aufgenommenen Pflanzenfasern, durchwachsen sie und spalten die Lignocellulose-Strukturen enzymatisch auf.
In der vorliegenden Arbeit wurden drei PCR-basierte Nachweissysteme entwickelt und validiert, um anaerobe Pilze, deren Zusammensetzung auf Gattungs-Ebene und deren Aktivität in Biogasprozessen zu erfassen. Zwei Assays basieren auf der spezifischen Analyse ribosomaler Gene: (i) AF-SSU zur Quantifizierung der 18S rDNA Genkopien von anaeroben Pilzen mittels quantitativer Real-time PCR; (ii) AF-LSU zur Sequenzierung der 28S rDNA anaerober Pilze und ihrer phylogenetischen Einordung; (iii) AF-Endo zum spezifischen Nachweis der transkriptionellen Aktivität einer GH5 Endoglukanase anaerober Pilze.
Anaerobe Pilze wurden in sieben von zehn untersuchten landwirtschaftlichen Biogasanlagen nachgewiesen. Die phylogenetische Auswertung der vorhandenen LSU-Gensequenzen zeigte, dass Vertreter aus sieben Gattungen anaerober Pilze, einschließlich der Gattungen *Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Cyllamyces*, *Piromyces*, *Anaeromyces*, *Feramyces* und der mutmaßlichen neuen Gattung *Khoyollomyces*, vorkamen. Anaerobe Pilze wurden nur in Anlagen mit hohem Einsatz von Rindergülle oder Rindermist gefunden. Nur in zwei der sieben positiv getesteten Anlagen konnte auch eine geringe Anzahl an GH5 Endoglukanase Transkripten anaerober Pilze gemessen werden, die auf eine cellulolytische Aktivität dieser hindeuten könnte. Auch in dem in dieser Arbeit durchgeführten hydrolytischen Vorbehandlungsexperiment war nach der Zugabe von Biogas-Gärgemisch keine transkriptionelle Aktivität der anaeroben Pilze mehr nachzuweisen. Diese Ergebnisse wiesen darauf hin, dass anaerobe Pilze mit tierischem Substrat in die Biogasanlagen gelangten, dort aber im Fermenter nach kurzer Zeit inaktiviert wurden.
Da die Bedingungen im Biogasprozess die anaeroben Pilze offenbar schädigten, erschien ein direkter Zusatz von anaeroben Pilzen in den Biogasreaktor wenig zielführend. Um dennoch eine Verbesserung der Biogasproduktion aus LCR zu erreichen, wurde eine hydrolytische Vorbehandlung mit zwei unterschiedlichen *Neocallimastix frontalis* Stämmen getestet. In beiden Vorbehandlungen war die initiale Biogasproduktion im Vergleich zu Kontrollansätzen mit inaktivierten anaeroben Pilzen beschleunigt, der Trockensubstanzabbau verbessert und die Produktion von flüchtigen Fettsäuren erhöht. Demnach könnte eine auf die Bedürfnisse der

anaeroben Pilze angepasste hydrolytische Vorbehandlung einen Weg zu einer effizienteren energetischen Nutzung von LCR darstellen.

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%	percent
°C	degree Celsius
$\Delta G_0'$	Gibbs free energy
µ	micro
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
AD	anaerobic digestion
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BS	bootstrap value
CAZyme	carbohydrate active enzyme
CBM	carbohydrate binding domain
CCM	corn-cob-mix
cDNA	complementary DNA
CGS	clover-grass silage
CHP	combined heat and power station
CM	cattle manure
CS	cattle slurry
CSTRs	continuous stirred tank reactors
d	day
D	digester
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ENAs	environmental nucleic acid sequences
e.g.	exempli gratia
et al.	et alteri
FR	final repository
g	gram
g	times gravity
GH	glycoside hydrolases
GH5	glycoside hydrolases family 5
GS	grass silage
h	hour
HGT	horizontal gene transfer
HRT	hydraulic retention time
ILT	Institute for Agricultural Engineering and Animal Husbandry at the Bavarian State Research Center for Agriculture
ITS 1	internal transcribed spacer region 1
L	liter
LCB	lignocellulosic biomass

LCR	lignocellulosic residues; lignocellulosereiche Reststoffe
LoB	Limit of Blank
LoD	Limit of Detection
LoQ	Limit of Quantification
LPMO	lytic polysaccharide monooxygenases
LSU	large ribosomal subunit
m	milli
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MPN	most probable number
mRNA	messenger RNA
MS	maize silage
MSW	municipal solid waste
NCDD	non-catalytic docking domains
nM	nanomolar
NTC	no template control
N/S	not specified
OLR	organic loading rate
OTUs	operational taxonomic units
PB	pilot biogas plant
PCR	polymerase chain reaction
PD	post-digester
pH	pondus Hydrogenii
Ph.D.	philosophiae doctor
qPCR	real-time quantitative PCR
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rxn	reaction
ScaA	scaffoldin protein
sp.	species
spp.	species pluralis
SSU	small ribosomal subunit
TCA	transcriptional cellulolytic activity for a GH5 endoglucanase gene
TFU	thallus forming units
Tm	melting temperature
TUM	Technical University Munich
TWh_{el}	terawatt-hour electric
U	units
VFAs	volatile fatty acids
v/v	volume per volume
w/v	weight per volume
WPS	whole plant silage

Chapter 1
Introduction

1.1 Biogas production

Mankind faces increasingly severe environmental impacts caused by the exploitation of fossil fuels. Fossil energy production is coupled to the emission of pollutants and carbon dioxide known to be a crucial cause of global warming (Strzalka *et al.* 2017). In order to alternate this development and since fossil energy sources are limited, alternative regenerative strategies will have to be strengthened to circumvent energy shortage and mitigate the most harmful environmental impacts. Governmental policies are thus at least starting to emphasize and support the transition to renewable energy. Exemplarily, the European Union has set goals that 20 % of the total energy share should be produced from renewable sources by 2020, rising to 27 % of the share in 2030 (European Parliament and Council 2009).

Biogas has already been discovered as a source of energy in ancient times (Lebuhn *et al.* 2014) and has advanced to date to one of the major techniques for renewable energy production in some countries. Especially in Germany, where 8700 biogas plants were operated in the year 2016, with 8200 of them built at agricultural sites, producing 29.5 TWh_{el} electricity (Daniel-Gromke *et al.* 2018). Biogas production is seen as one of the most promising alternatives to fossil energy, as biogas is derived from renewable resources and can be versatilely used for electricity, heat and fuel production (Westerholm *et al.* 2016). In addition, the digestate remaining as a by-product of biogas generation is a nutrient-rich resource, a highly welcome eco-friendly fertilizer for agriculture (Ehmann *et al.* 2018).

The transition to renewable energies is coupled to the phase-out of nuclear and fossil fuel-fired (e.g. coal, oil and gas) power plants, thus withdrawing reliable resources which guarantee baseload power (Boing *et al.* 2018). Wind and solar energy production is dependent on the weather and thus cannot constantly generate electricity. Further, in contrast to biogas production, the resources and the products of such energy forms can hardly be stored. Biogas is thus highly relevant to provide guaranteed electric capacity, as it allows permanent production and utilization, mitigating the electricity shortage associated with seasonal fluctuations (Lebuhn *et al.* 2014).

In principle, all biomass composed of digestible carbohydrates, proteins, or lipids can be used as input for biogas production (Hagos *et al.* 2017). The complex substrates are disintegrated in the anaerobic biogas process by a chain of microbial reactions (see section 1.1.1) to smaller organic compounds and finally to methane (50-75 %), carbon dioxide (25-50 %), water, small amounts of hydrogen, hydrogen sulfide, ammonia and other trace gases (Strzalka *et al.* 2017). The majority of European biogas plants is agricultural based (63 % in Europe, Strzalka *et al.* (2017)), only a minority of biogas plants is operated with bio-waste and/or sewage sludge. Thus, mainly energy crops, agricultural residues, and animal manure are used as substrates.

However, the extensive growth of energy crops in monocultures is seen as ecologically harmful, and the use of potential food or feed resources for energy production is criticized. The alternative use of agricultural residues, comprising e.g. animal manure and straw as by-product of crop production, is ecologically more favorable as it preserves food resources, helps to recycle nutrients and reduces methane (CH_4) and nitrous oxide (N_2O) emissions caused by conventional manure disposal and fertilization strategies (Meyer *et al.* 2018).

Biogas is mainly produced by wet fermentation in continuous stirred tank reactors (CSTRs) accounting for 90 % of modern biogas plants in Germany. Besides, solid-material fermentation in tubular plug-flow reactors and dry-fermentation in discontinuous systems are performed (Weiland 2010). Conventional biogas plants usually consist of several tanks, each with an individual purpose. The following tanks can be implemented: (i) a tank for mixing liquid and solid substrates (mixing tank), (ii) a (mostly gas-tight) hydrolysis tank separating fiber digestion from methane generation, (iii) a main digester (or more) with a gas-tight roof, (iv) a secondary digester (or more) with a gas-tight roof treating the digestate of the main fermenter, (v) a storage tank (or more) for the digestate from the main and secondary digesters (Weiland 2010). The tanks can be combined individually and should be arranged in a way that optimal digestion of the chosen substrates can be obtained. Most commonly, a mixing tank, a main fermenter, a secondary fermenter, and a storage tank are connected in series (Figure 1). During anaerobic digestion in this set-up, most microbial degradation processes take place simultaneously in each fermenter. In some biogas plants, microbial digestion of fibers is separated from methane production by including a hydrolysis tank prior to the fermenter. This can help to improve the digestion of recalcitrant substrates, as the conditions for the two microbial processes can be adjusted and optimized individually (Bachmann and Erep 2013). The produced biogas is then mostly combusted for electricity and heat production in a combined heat and power station (CHP). In addition, the produced biogas can be upgraded to convert it to fuel for transportation (e.g. liquefied natural gas, compressed natural gas) or to biomethane, reaching specifications similar to natural gas, allowing injection in the natural gas grid (Angelidaki *et al.* 2018).

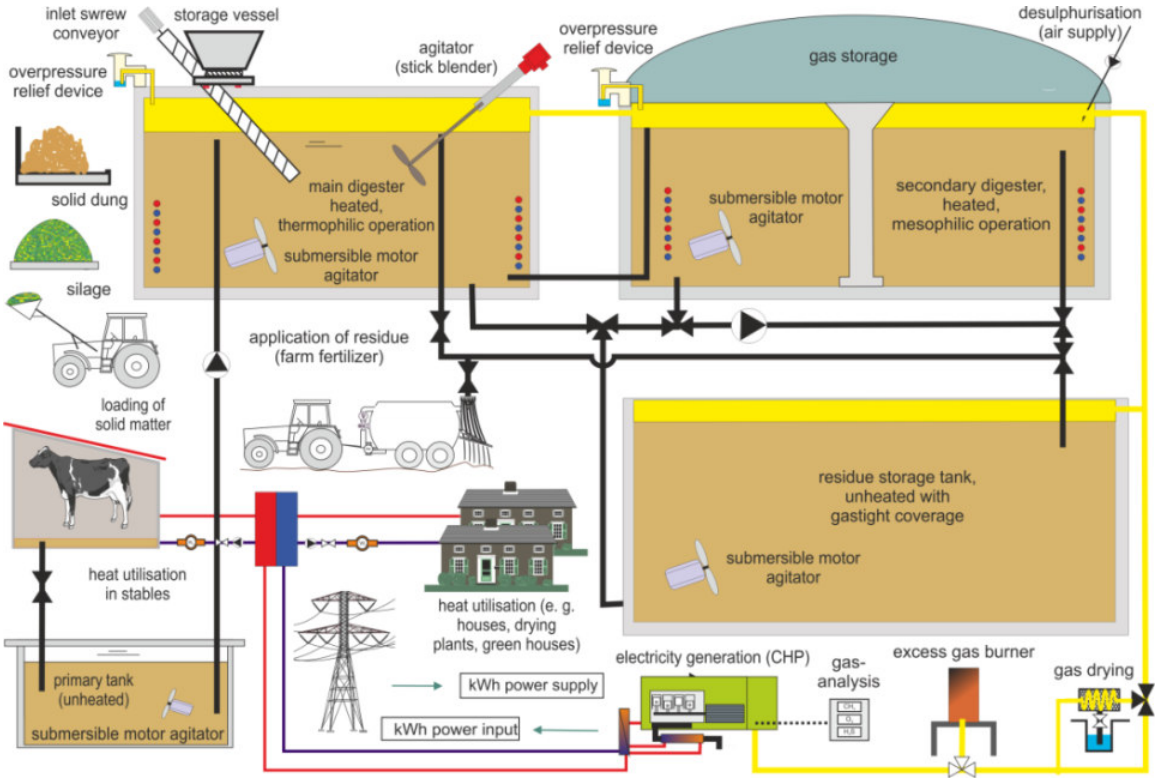


Figure 1: Typical two-stage agricultural biogas plant

Biogas reactors are typically operated between 38 – 55 °C, mostly in the higher mesophilic range. At thermophilic conditions, the enzymatic reactions of the microbial degradation process typically are running more quickly, thus shorter hydraulic retention times can be applied. However, the process is less stable at higher temperatures. Fluctuations in temperature, pH value or substrate composition can lead to process disturbance (Bachmann and Erep 2013). In addition, the risk of ammonia intoxication is rising at higher temperatures, as they foster the transition of ammonium (NH_4^+) to ammonia (NH_3), causing microbial inhibition (Lebuhn *et al.* 2014). Thus, to date, most biogas reactors are operated in a range from 38 – 44 °C, and some biogas plants contain an upstream thermophilic reactor.

1.1.1 Microorganisms in biogas processes

The degradation cascade in the biogas production process is mainly catalyzed by the two microbial key groups, bacteria and methanogenic archaea. Fungi may play a role in biogas processes, but only a few reports present evidence for and discuss their presence and activity (Bengelsdorf *et al.* 2013; Kazda *et al.* 2014; Dollhofer *et al.* 2017).

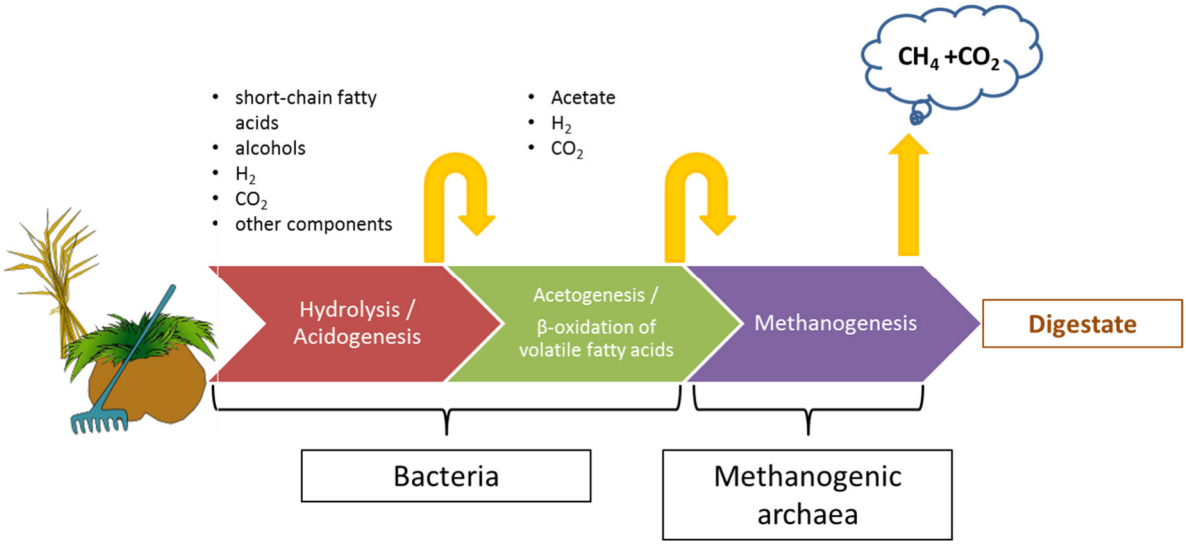


Figure 2: Schematic drawing of the biogas production process, illustrating which products result from each step and which is the performing microbial key group

The process steps hydrolysis / acidogenesis, acetogenesis and methanogenesis, are performed by specialized guilds from the two microbial key groups (Figure 2). The functional microbial groups in this chain are highly dependent on each other, meaning that the first guild produces the substrates for the following guild, which has a high impact on the former group by utilizing their metabolic products and avoiding feedback inhibition (Schink and Stams 2013). Thus, only if all necessary functional members are present and metabolically active, biogas can efficiently be produced.

During hydrolysis, the complex compounds in the substrates, carbohydrates, proteins, and lipids, are degraded to smaller monomers, fatty acids of different length, glycerol, amino acids and sugars. These are further metabolized to short-chain fatty acids, alcohols, H_2, CO_2, and other components during acidogenesis. The degradation is mainly catalyzed by extracellular and cell wall-bound enzymes of hydrolytic bacteria, fermentative bacteria and potentially fungi (Westerholm *et al.* 2016). The enzymatic arsenal present in these steps includes cellulases, hemicellulases, xylanases, pectinases, amylases, lipases and proteases. A special trait of cellulolytic bacteria of the genera *Clostridium*, *Acetivibrio*, *Bacteroides* and *Ruminococcus* are cellulosomes, multi-enzyme complexes containing a multitude of synergistically active cellulolytic and hemicellulolytic enzymes. Such cellulosomes are coupled to the bacterial cell wall and possess cellulose binding domains directing the

enzymatic attack to the targeted plant biomass (Azman *et al.* 2015). Similar complexes have been discovered in anaerobic fungi (Haitjema *et al.* 2017) of the phylum *Neocallimastigomycota* which have shown to be present and at least transiently transcriptionally active in biogas plants in the presented thesis (Chapter 6). If substrates are used which are recalcitrant to microbial degradation, their hydrolysis will be the rate-limiting step of the biogas production process (Zheng *et al.* 2014). Particularly hard to digest are feedstocks composed of lignocellulose-rich plant biomass. This is due to the high lignin content as this compound is almost not convertible by anaerobic digestion and functions like a shield, protecting the internal cellulose and hemicellulose from microbial degradation (Rodriguez *et al.* 2017). Thus, efficient hydrolysis of lignocellulosic biomass is one of the major challenges for future economic biogas production (see section 1.1.2).

In the next step, the acetogenesis, the intermediate compounds produced during hydrolysis/acidogenesis are further oxidized to acetate, H_2, and CO_2 by acetogenic bacteria, mostly performing β-oxidation of volatile fatty acids (Sousa *et al.* 2009). A different metabolic route of acetate metabolism is the Wood-Ljungdahl pathway, in which either two molecules of CO_2 are reduced via the formation of acetyl-CoA to acetate (reductive acetogenesis or homoacetogenesis, Drake *et al.* (2008)), or acetate is oxidized to H_2, CO_2 or formate in syntrophic acetate oxidation (Westerholm *et al.* 2016). Some of those chemical reactions display a positive Gibbs free energy (ΔG_0') at standard conditions, meaning that the reactions would cost the performing microorganism more energy than they deliver. In order to accomplish such endergonic reactions, syntrophic partnerships e.g. between acetate-oxidizing bacteria and methanogens have evolved. In such syntrophic interactions, two metabolically different microorganisms act as a team, in which the propagation of each partner depends on the presence and activity of the other (Schink and Stams 2013). These relationships are based on the transfer of reduction equivalents between the two partners. In biogas production processes, for example, hydrogen is transferred to the methanogenic partner which generates methane, thereby reducing the H_2 partial pressure and enabling the acetate-oxidizing partner to perform energetically unfavorable reactions (Müller *et al.* 2018).

At the end of the biogas production cascade, methanogenic archaea produce methane by methanogenesis. Mainly three methanogenic groups are known to be active during biogas production: (i) hydrogenotrophic methanogens utilizing H_2 and CO_2 to form CH_4 or (ii) acetoclastic methanogens splitting acetate to form CH_4 and CO_2 (Enzmann *et al.* 2018), and (iii) methylotrophic methanogens. Some groups can use additional alternative substrates: some hydrogenotrophs are able to utilize formate or H_2, while some methylotrophs are able to utilize hydrogen, CO, methylamines or methanol instead of or in addition to acetate to form CH_4 (Schnürer 2018). Typical methanogens found in biogas fermenters are hydrogenotrophic *Methanobacteriaceae* and *Methanomicrobiales*, versatile *Methanosarcinaceae*, acetoclastic *Methanosaetaceae* and obligate hydrogenotrophic / methylotrophic *Methanomassiliicoccaceae* (Lebuhn *et al.* 2014). All of the different methanogenic groups can occur in biogas production processes, depending on the given process conditions. Under stress conditions, e.g. high organic loading rates, high acetate or ammonia levels, unfavorable temperatures,

hydrogenotrophic methanogens, finally *Methanobacteria*, will outcompete the more delicate and slowly growing acetoclastic methanogens (Lebuhn *et al.* 2014).

1.1.2 Challenges for biogas production

Lignocellulosic biomass, the structural substance of plants, comprised for example in agricultural, forestry and biogenic residues, is a substrate of choice for future biogas production (see section 1.1) for environmental, bioeconomical and political reasons (Kumar *et al.* 2008). Despite the high abundance, good availability and the low costs of such resources, not many biogas plant owners use them to date. The main reasons are process disturbances caused by the fibrous structure and the recalcitrant chemical nature of lignocellulose (Figure 3). Its main compounds are interconnected with lignin (10-35 %) as outer layer coating the interwoven cellulose (35-50 %) and hemicellulose (15–35 %) (Kabir *et al.* 2015).

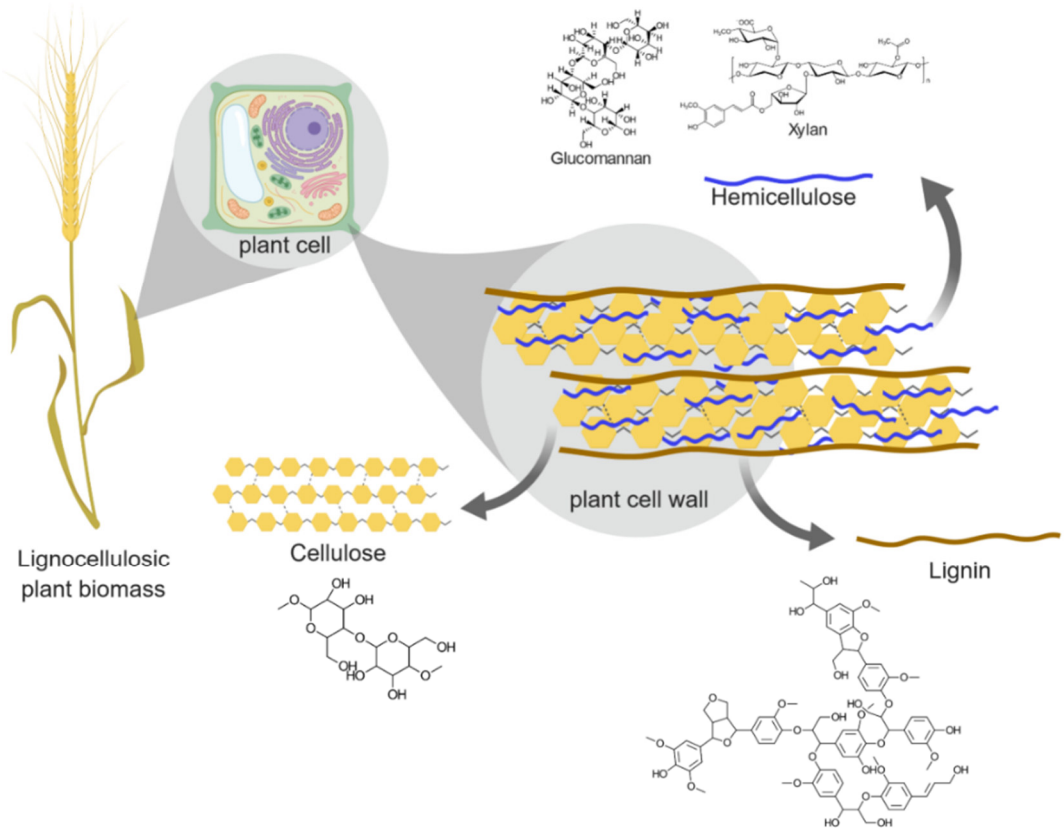


Figure 3: Composition and structure of lignocellulose. Created with BioRender.com

Bacteria and *Archaea* in the anaerobic biogas process are not able to crack the lignin layer and thus can only degrade the accessible parts of the inner lying cellulose and hemicellulose. This insufficient hydrolysis leads to lack of substrates for the downstream functional microbial groups, incomplete fermentation of the feedstock and thus to lower gas yields. In addition, the fibrous material is hard to transfer and handle in the biogas plant, as it poses problems for pumps and feeding devices, can damage the stirring units or form floating

layers. A major challenge for future biogas production is thus to adapt biogas plants and production processes to the challenging lignocellulosic biomass.

Different pre-treatment strategies are developed and tested to date to facilitate the use of lignocellulosic biomass for biogas production. Their main goals are to: (i) increase the accessible surface area for microbial attack, (ii) disrupt or remove the lignin coat and (iii) enhance the solubilization of cellulose and hemicellulose (Patinvoh *et al.* 2017). Techniques comprise mechanical (e.g. milling), chemical (alkaline or acid treatment), thermal (e.g. steam explosion) and microbiological (e.g. application of enzyme cocktails or aerobic lignocellulolytic fungi) approaches (Sibiya *et al.* 2018). The physical methods currently available and the production of recombinant enzyme cocktails impose high costs due to high energy consumption (Sharma *et al.* 2017) and the application of chemicals can harm the environment. Thus, the direct application of competent microorganisms is thought to be a cheaper alternative to achieve the main pre-treatment goals.

1.2 Anaerobic fungi and their potential for biogas production

As stated in section 1.1.2, a major challenge for future biogas production is to overcome the limited degradation of lignocellulosic biomass. Learning from nature, utilizing decomposing microorganisms for microbial pre-treatment is a promising strategy. Anaerobic fungi mostly inhabit the digestive tract of mammalian herbivores and are specialized to degrade fiber-rich feedstock for their hosts (Gruninger *et al.* 2014). They are needed, as the animals themselves do not express lignocellulolytic enzymes and are thus dependent on the combined activity of anaerobic fungi, bacteria, archaea and protozoa. Anaerobic fungi are key-players in fiber degradation (Edwards *et al.* 2008). They follow a life cycle during which they colonize ingested fibers, penetrate their surface by rhizoidal growth and degrade plant carbohydrates by a multitude of carbohydrate active enzymes (CAZYmes).

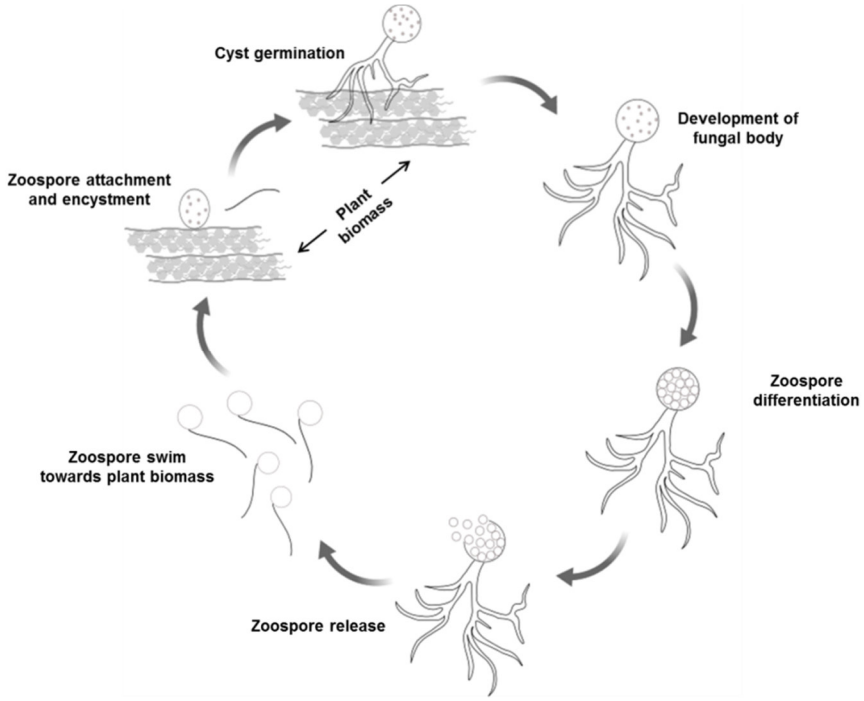


Figure 4: Schematic life cycle of a monocentric anaerobic fungus with small grey dots representing nuclei and small unfilled circles representing zoospores

A general overview on their taxonomy, physiology and potential for biogas production is given in the review article "Anaerobic fungi and their potential for biogas production", which was composed at the beginning of this Ph.D. thesis and published by Dollhofer *et al.* (2015) in the book series "Biogas Science and Technology, Advances in Biochemical Engineering/Biotechnology" (published version below).

1.2.1 [1]Publication 1: Anaerobic fungi and their potential for biogas production

Abstract: Plant biomass is the largest reservoir of environmentally friendly renewable energy on earth. However, the complex and recalcitrant structure of these lignocellulose-rich substrates is a severe limitation for biogas production. Microbial pro-ventricular anaerobic digestion of ruminants can serve as a model for improvement of converting lignocellulosic biomass into energy. Anaerobic fungi are key players in the digestive system of various animals, they produce a plethora of plant carbohydrate hydrolyzing enzymes. Combined with the invasive growth of their rhizoid system their contribution to cell wall polysaccharide decomposition may greatly exceed that of bacteria. The cellulolytic arsenal of anaerobic fungi consists of both secreted enzymes, as well as extracellular multi-enzyme complexes called cellulosomes. These complexes are extremely active, can degrade both amorphous and crystalline cellulose and are probably the main reason of cellulolytic efficiency of anaerobic fungi. The synergistic use of mechanical and enzymatic degradation makes anaerobic fungi promising candidates to improve biogas production from recalcitrant biomass. This chapter

[1] Was published in a similar version by Dollhofer, V., Podmirseg, S.M., Callaghan, T.M., Griffith, G.W., Fliegerová, K. 2015. Anaerobic Fungi and Their Potential for Biogas Production. in: Biogas Science and Technology, (Eds.) G.M. Guebitz, A. Bauer, G. Bochmann, A. Gronauer, S. Weiss, Springer International Publishing. Cham, pp. 41-61.

shall give an overview about their biology and their potential for implementation in the biogas process.

1.2.1.1 Anaerobic fungi: An overview

Anaerobic fungi belonging to the phylum *Neocallimastigomycota*, are the most basal lineage of the kingdom *Fungi*. These fungi are principally known from the digestive tracts of larger mammalian herbivores, where they play an important role as primary colonisers of ingested forage (Akin and Borneman 1990; Liggenstoffer *et al.* 2010). Recent studies indicate their appearance in herbivorous reptiles like the green iguana (Liggenstoffer *et al.* 2010) and termites (Lee *et al.* 2015) also. Anaerobic fungi are characterised by several distinctive traits which stem from their obligately anaerobic physiology; mitochondria, cytochromes and other biochemical features of the oxidative phosphorylation pathway are absent. Energy generation occurs in hydrogenosomes where ATP is formed by malate decarboxylation to form acetate, CO_2, and H_2 (Yarlett and Hackstein 2005; van der Giezen 2009). The *Neocallimastigales* are thought to be the only fungi that do not require molecular oxygen for any of their physiological processes, and for which the presence of oxygen is toxic. This trait raises the question how anaerobic fungi defend themselves against the toxic effects of oxygen, for instance when colonizing freshly ingested forage or during dispersal between host animals. Respective insights are presented in the following section “life cycle”. Additionally, their genomes are peculiar having the highest AT-content hitherto found (often exceeding 90 % in non-coding regions) and with a substantial expansion of important hydrolytic and cellulolytic gene families (Youssef *et al.* 2013).

Anaerobic fungi are the only fungi which possess cellulosomes. These extraordinary features are presented in more detail in section 1.2.1.2.1. The position of anaerobic fungi as a basal fungal lineage is reflected in the genome characteristics, which are also present in other early-branching fungal lineages and/or non-fungal opisthokonts, but are absent in the later diverging Dikarya (*Ascomycetes* and *Basidiomycetes*) genomes (Liu *et al.* 2006). Such phylogenetic determinants and unique taxonomy of anaerobic fungi are discussed in the following section 1.1.

1.2.1.1.1 Classical and pragmatic Taxonomy of anaerobic fungi

The atypical morphology and physiology of anaerobic fungi has caused some taxonomic uncertainty. After misleading classification as *Protozoa* (Liebetanz 1910), *Phycomycetes* (Orpin 1977a) and *Chytridiomycetes* (Barr 1980; Barr 1988) the anaerobic fungi were finally placed into the distinct phylum *Neocallimastigomycota* (Hibbett *et al.* 2007). The phylum contains only one order (*Neocallimastigales*) and one family (*Neocallimastigaceae*) within which seven genera are currently described: The monocentric rhizoidal genera *Neocallimastix*, *Piromyces*, *Oontomyces* and *Buwchfawromyces*, the polycentric rhizoidal genera *Anaeromyces* and *Orpinomyces,* and the two bulbous genera, monocentric *Caecomyces* and polycentric *Cyllamyces*, respectively (Gruninger *et al.* 2014; Callaghan *et al.* 2015).

The genera are defined based on thallus morphology, the formation of rhizoidal filaments or bulbous holdfasts within the substrate and their zoospore morphology. A distinction is made between monoflagellate and polyflagellate zoospores. The latter possessing 7 to 20 posterior flagella inserted in two rows. Formation of polyflagellate zoospores is a trait unique to *Orpinomyces* and *Neocallimastix* spp., not known from any other Opisthokonta, and these two genera form a distinct clade within the *Neocallimastigomycota* (Koetschan *et al.* 2014).

Differentiation by the shape of sporangia may additionally be possible but can be misleading as it is varying depending on culture conditions. Currently about 20 species have been described (Fliegerová *et al.* 2012b). Uncertainties created by difficulties in inter-lab comparisons and the loss of many viable type cultures, can only now be resolved by the use of DNA barcoding and the concerted effort to exchange cultures (Griffith *et al.* 2010).

Culture-independent analysis of environmental nucleic acid sequences provided evidence for much greater fungal diversity than previously suspected in the digestive tract of wild and domestic herbivores. Based on data from these more recent studies, it appears that twelve or more hitherto un-named genera may exist (Fliegerová *et al.* 2010; Liggenstoffer *et al.* 2010; Koetschan *et al.* 2014). Several of these novel clades are now recognized from sequences of cultured fungi (Koetschan *et al.* 2014) while other clades still consist of environmental nucleic acid sequences (ENAS) only.

1.2.1.1.2 Life Cycle

The life cycle of anaerobic fungi alternates between a motile zoospore stage and a non-motile vegetative stage. The latter consists of a thallus associated to plant material and fruiting bodies known as sporangia (Figure 5) (Gruninger *et al.* 2014). Flagellate zoospores (see Figure 5g) released from mature sporangia actively swim towards freshly ingested plant tissues using chemotactic response to soluble sugars and/or phenolic acids (Wubah and Kim 1996). After attachment to the feed particles, flagella are shed and a cyst is formed. The cyst then germinates to form the thallus. In all monocentric species (*Piromyces*, *Neocallimastix* and *Buwchfawromyces*), the nucleus remains in the enlarging cyst which forms the sporangium. In the polycentric species *Anaeromyces* and *Orpinomyces*, the nuclei migrate through the rhizoidal system to form multiple sporangia on a single thallus. The terms exogenous and endogenous germination (nuclei migrate into the thallus or not), that are widely used in describing chytrid development, are less clearly applicable to the bulbous anaerobic fungi which do not form rhizoids but do form multiple sporangia (i.e. *Cyllamyces*) (Ozkose *et al.* 2001).

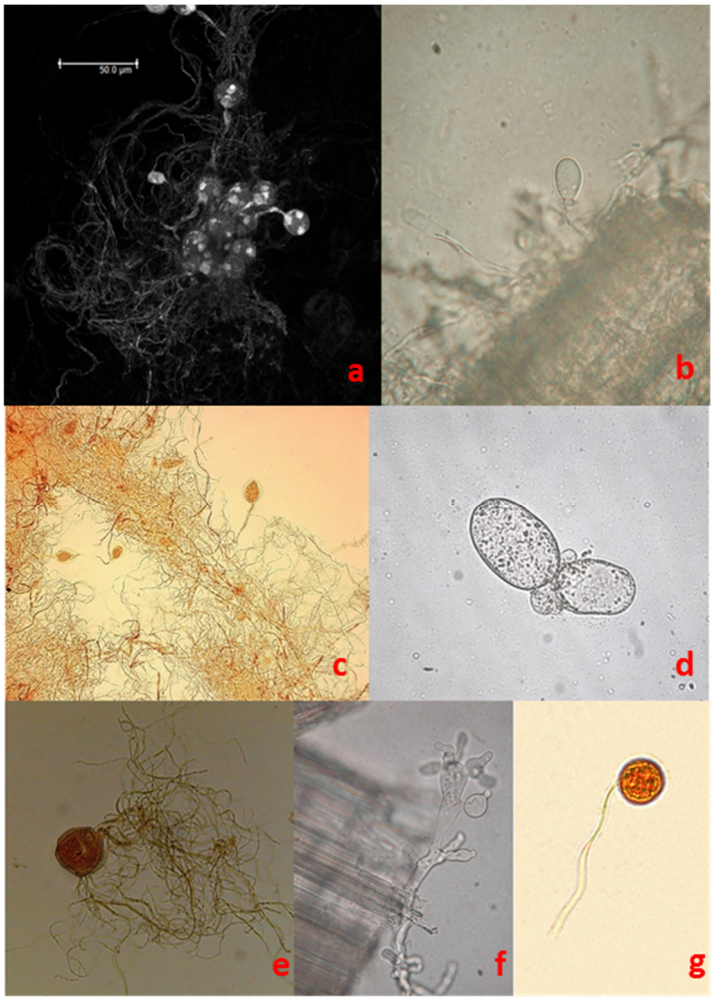


Figure 5: Different culture morphologies of anaerobic fungi: a) *Neocallimastix* sp. sporangia and rhizomycelium (CLSM: superimposed z-stacks (26.7 µm total depth) showing culture auto fluorescence (excitation at 561 nm and emission from 570 nm to 620 nm); b) *Piromyces* sp. light microscopy of native preparation; c) Rhizoid of *Anaeromyces mucronatus* with apical sporangia. Light microscopy of lugol-stained preparation (x 200); d) Bulbous species *Caecomyces communis*. Light microscopy of native preparation (x 400); e) *Neocallimastix frontalis* sporangium and rhizoid. Light microscopy (x 400); f) *Orpinomyces* sp. with sporangia and rhizoid. Light microscopy of native preparation; g) Light microscopy of a biflagellated zoospore of *Piromyces* sp. (x 1000)

The rhizoidal system penetrates the plant tissue by a combination of enzymatic activity and hydrostatic pressure using appressorium-like penetration structures (Ho *et al.* 1988; Ho and Bauchop 1991). In the non-rhizoidal bulbous species (*Caecomyces, Cyllamyces*), the expanding holdfast formed within the substrate causes a splitting of the plant fibers (Orpin 1977b; Lowe *et al.* 1987b; Orpin and Joblin 1997). Sporangium maturation and release of asexual zoospores can occur as quickly as eight hours after encystment (France *et al.* 1990; Theodorou *et al.* 1993).The complete life cycle, is conducted within 24-32 h (Lowe *et al.* 1987b). Propagules of the anaerobic fungi are known to survive up to and probably over a year in feces (McGranaghan *et al.* 1999) and have also been found to be transferred to neonatal hosts through saliva (Davies *et al.* 1993). Putative aero-tolerant survival structures have been observed only rarely (Wubah *et al.* 1991; Brookman *et al.* 2000b; Callaghan *et al.* 2015) and many questions as to the formation of these structures and their occurrence in the various genera of anaerobic fungi remain to be answered.

1.2.1.1.3 Anaerobic fungi and their interactions with methanogens and bacteria

Close association of anaerobic fungi with methanogens is well known (Orpin and Joblin 1997; Cheng *et al.* 2009), with inter-species hydrogen transfer leading to both methane production and also more efficient re-generation of oxidized nucleotides (NAD^+, $NADP^+$). Syntrophic co-cultivation markedly increases fungal growth rate, with increased rates of cellulolysis and xylanolysis, consequently enhancing dry matter reduction (Nakashimada *et al.* 2000). However, the anaerobic fungus – methanogen interaction is more complex than simple cross-feeding. Hydrogen transfer also influences fungal catabolic pathways and specific enzyme profiles, shifting fungal product formation away from more oxidized end products (lactate, ethanol) towards production of more reduced products (acetate, formate). Acetate, and in the rumen especially formate, are the preferred growth substrates for methanogens (Nakashimada *et al.* 2000; Cheng *et al.* 2009). This interaction is so pivotal, that some species of anaerobic fungi cannot be isolated as axenic cultures, but only in combination with the permanent archaeal symbiont (Leis *et al.* 2014).

Syntrophic interactions between acetogenic bacteria and methanogens are well known to occur in the biogas biocenosis (Stams 1994). Since anaerobic fungi show improved growth in the presence of methanogens, the idea of augmenting biogas reactors with this microbial group seems a logical step.

Interactions of anaerobic fungi with bacteria can be of antagonistic and symbiotic nature as shown by Bernalier and coworkers (Bernalier *et al.* 1992), who tested the degradation efficiency in different culture combinations of three anaerobic fungal and two cellulolytic bacterial strains. In general both groups are competing for the same ecological niche, but the breaking up of plant tissue through fungal rhizoids may also enhance the overall efficiency of cellulolytic bacteria (Bernalier *et al.* 1992). This improved degradation was also confirmed when testing the contribution of different microbial groups (fungi, bacteria, protozoa) on orchard grass decomposition (Lee *et al.* 2000). Presence of protozoa was, however attributed with lower degradation efficiency and inhibition of both, bacteria and fungi.

Most of these studies are based on *in vitro* co-cultures, that may not completely reflect conditions of whole rumen or biogas reactor consortia and still more research is needed in this field.

1.2.1.2 Anaerobic fungi and their potential for biogas production

Under oxygen-free conditions organic matter is decomposed by a complex of microorganisms which are so far divided into three functional groups: hydrolyzing and fermenting bacteria, obligate hydrogen-producing acetogenic bacteria, and methanogenic archaea. Only little is known on the role and the potential of anaerobic fungi for biogas production. Great potential lies in biogas production from lignocellulosic waste but, slow and inefficient degradation processes, the formation of toxic intermediates and the necessity for long incubation times are only a few examples of the problems encountered (Čater *et al.* 2014; Kabir *et al.* 2015). A promising strategy is the use of microorganisms, which are able to successfully perform such complicated degradation processes in their natural environment (Lynd *et al.* 2002; Wen *et al.*

2015). Herbivores as biogas reactors evolved the need for fungal symbionts for this purpose and over millions of years natural selection has created a highly specialized and niche specific community of anaerobic fungi.

The following paragraphs will give an overview about useful features of anaerobic fungi and will present the actual knowledge about anaerobic fungi and biogas production.

1.2.1.2.1 Lignocelluloytic enzymes of anaerobic fungi and their potential use

Lignin-embedded cellulose and hemicellulose (Cullen and Kersten 1992) represent a physical barrier against microbial and enzymatic attack. Known as the primary digesters of plant biomass in the rumen anaerobic fungi (Lee *et al.* 2000) have the ability to open up the plant tissue through rhizoidal growth and produce a cocktail of enzymes to degrade and separate the different compounds of lignocellulosic biomass, while lignin itself remains anaerobically indigestible. Some of these enzymes are secreted freely but most of them are bound to a multi-enzyme complex the so called cellulosome. Genome sequencing of *Orpinomyces* strain C1A revealed a broader enzyme range compared to aerobic fungi with a repertoire of 357 glycosyl hydrolases, 92 carbohydrate esterases and 24 pectate lyases (Youssef *et al.* 2013). Horizontal gene transfer from bacteria is suggested as one of the main reasons why anaerobic fungi have evolved such robust and impressive cellulolytic and hemicellulolytic capability.

A group of enzymes often termed cellulases synergistically hydrolyze β-1, 4 glucosidic bonds in cellulose through three discrete enzymatic activities involving three different types of enzymes. Endoglucanases (EC 3.2.1.4) cut within amorphous regions of cellulose strands, releasing oligosaccharides and creating new free chain ends for the enzymatic attack by exoglucanases (EC 3.2.1.176; EC 3.2.1.91). Since the latter liberate cellobiose disaccharides from either reducing (EC 3.2.1.176) or non-reducing (EC 3.2.1.91) ends, they are also termed cellobiohydrolases. In a cellulosomal complex extracted from a *Neocallimastix frontalis* culture, enzymes from glycosyl hydrolase family 5 (GH5) operated by the endo- and enzymes from GH6 and GH48 by the exo-mechanism (Wang *et al.* 2014). The residual cellobiose is then hydrolyzed to glucose by β-glucosidases (EC 3.2.1.21) (Lynd *et al.* 2002; Juturu and Wu 2014). Auxiliary enzymes like the recently discovered lytic polysaccharide monooxygenases (LPMO) (family AA9) have been reported to enhance or complete the utilization of cellulose in many fungal species (Morgenstern *et al.* 2014). In contrast to the hydrolyzing enzymes they cleave glucosidic bonds with a copper dependent oxidation mechanism and are able to attack crystalline regions of cellulose (Hemsworth *et al.* 2014). But it seems that basal fungal groups including the anaerobic fungi lack those enzymes (Morgenstern *et al.* 2014)

All three major cellulase types have been reported for the *Neocallimastigomycota* (Borneman *et al.* (1989); Borneman *et al.* (1990); Harhangi *et al.* (2002); Ljungdahl (2008); Youssef *et al.* (2013) and many more) confirming the potential of anaerobic fungi as a reservoir for highly efficient cellulases. The fact that glucose is the main product of anaerobic fungal cellulose degradation is an advantage for biotechnological applications. Cellobiose is not accumulated and therefore cannot act as end-product inhibitor for cellulose hydrolysis, as known for

Trichoderma reesi or many bacterial species. Thus costly addition of β-glucosidase becomes unnecessary (Dashtban *et al.* 2009).

Due to the heterogenous structure of hemicelluloses, several enzymes are needed for their catabolism. Until now anaerobic fungi have been reported to provide all enzymes needed to degrade the major hemicelluloses constituents of the plant cell wall, namely β-glucans, mannans and xylans. And in some cases xylanase activity was even higher than cellulase activity (Aylward *et al.* 1999). In contrast to aerobic higher fungi (Dikarya), anaerobic fungi lack the enzymatic machinery to catabolize lignin. The enzymatic reaction to cleave the aromatic ring requires oxygen and can therefore not take place in an anaerobic environment (Berg and Mc Claugherty 2014). But it was shown that a *Neocallimastix sp*. could mediate the loss of up to 34 % of plant biomass associated lignin, however this loss probably due to physical alteration or chemical modification of the lignin rather than enzymatic catabolism (McSweeney *et al.* 1994). Additional feruolyl (EC 3.1.1.73) esterases are produced which cleave the bond between hemicelluloses and lignin and by separating these two compounds, making cellulose and hemicellulose more easily accessible for further degradation (Nagpal *et al.* 2009).

1.2.1.2.2 Anaerobic fungal cellulosomes

As mentioned above, most of the cellulolytic and hemicellulolytic enzymes are part of a multi-enzyme complex known as the cellulosome. Cellulosomes were first identified in the bacterial family *Clostridiaceae* (Felix and Ljungdahl 1993) and the anaerobic fungi are the only eukaryotic representatives showing this feature. The fungal cellulosome is structurally and phylogenetically similar to that found in bacteria and is thought to have arisen through a horizontal gene transfer event (Garcia-Vallve *et al.* 2000). Up to now cellulosomes have been described for species of *Piromyces* (Steenbakkers *et al.* 2002; Nagy *et al.* 2007), *Orpinomyces* (Ljungdahl 2008), and *Neocallimastix* (Zhou *et al.* 1994; Aylward *et al.* 1999). Anaerobic fungi invade plant tissues with their rhizoid and it is assumed that in addition to the secretion of soluble enzymes, they form cellulosomes anchored to the cell walls of rhizoid tips (Nagpal *et al.* 2009). Unfortunately, the molecular structure of the anaerobic fungal cellulosome is still unclear and miscellaneous theories exist (see Haitjema *et al.* (2014) for a schematic overview). In anaerobic bacteria a non-catalytic protein, the ‘scaffolding protein’, is anchored to the cell wall and contains several repeating domains, the cohesins. This structure forms the backbone to which the enzymatic subunits assemble by non-catalytic domains, the dockerins. Additionally the scaffolding connects to the substrate, in this case the (hemi) cellulose molecules, via a cellulose-binding domain (Shoham *et al.* 1999).

Compared to the enzymes of anaerobic bacteria, which contain only one species-specific dockerin domain, the fungal enzymes contain one to three copies of dockerin domains which show an interspecies specificity. It is believed that the amount of dockerin regulates the affinity of the enzymes towards the scaffolding molecule (Fontes and Gilbert 2010). Recently it was reported that the anaerobic fungal cellulosome contains a scaffolding backbone as well, raising the suggestion that the catalytic components also interact with it via dockerin domains (Wang *et al.* 2014). Other studies have shown that some types of docking domains attach to

several individual proteins, concluding that there might be various different scaffolding proteins in anaerobic fungal cellulosomes (Steenbakkers *et al.* 2001). Additionally, it could be shown that a double-dockerin domain and a β-glucosidase enzymatic subunit from glycosyl hydrolase family 3 (GH3), both belonging to one fungal species, could bind to each other (Nagy *et al.* 2007; Haitjema *et al.* 2014). This leads to the third theory that dockerins mediate the binding of different secreted enzymes to each other, forming the cellulosome without scaffolding as structural molecule. Despite the detailed structure remaining unsolved, cellulosomes permit anaerobic fungi to use their cellulolytic enzymes in a synergistic and more efficient way, unequalled by individually secreted enzymes (Haitjema *et al.* 2014). It also provides protection against proteases from the surrounding environment in the form of a serine protease inhibitor named celpin (Steenbakkers *et al.* 2008).

1.2.1.2.3 Substrates utilized by anaerobic fungi

In addition to municipal solid waste (MSW) and animal waste, lignocellulose-rich materials potentially useful for biogas production are by-products of various industrial processes, including agriculture, forestry, pulp-, paper- and food production (Dashtban *et al.* 2009; Sánchez 2009). However, the recalcitrance and variability of these materials leads to low gas yields in biogas fermentations, thus making their exploitation uneconomical. Since anaerobic fungi are efficient physical and enzymatic degraders of lignocellulose-rich substrates (see Table 1), they have the potential to make the biogas production from these lignocellulose-rich materials more efficient and profitable.

Table 1: Examples for lignocellulosic residues degraded by anaerobic fungi

Lignocellulosic residue	Lignin content % (Sánchez 2009)	Organism	Reference
Wheat straw	16-21	*Neocallimastix frontalis*	(Li and Calza 1991)
Coastal Bermuda grass	6.4	*Piromyces* MC-1, *Orpinomyces* PC-1-3, *Neocallimastix* MC-2	(Borneman *et al.* 1990)
Sugar cane bagasse	19-24	*Piromyces* strain E2	(Teunissen *et al.* 1993)
Hard wood	18-25	*Neocallimastix sp.*	(Joblin and Naylor 1989)
Rice straw	18	*Piromyces* M014, *Orpinomyces* GSRI-001, *Neocallimastix* T010	(Lee *et al.* 2015)

1.2.1.2.4 Production of recombinant enzymes

One strategy to overcome the bottelneck of enzymatic hydrolysis of lignocellulose in the biogas production process is the development and use of recombinant potent polysaccharide-degrading enzymes. Such a strategy could involve the transfer of the cellulolytic genes of efficient degraders (e.g. anaerobic fungi) into other well-established enzyme production hosts or biofuel producers (e.g. yeast) or alternatively the modification of the genetic capability of the anaerobic fungi themselves. Improving the efficiency of known enzymes and the creation of optimized enzyme mixtures, along with the identification of new and more active enzymes has been the focus of some studies (Biswas *et al.* 2014). Efforts to produce recombinant

fibrolytic enzymes from anaerobic fungi have focused on expressing a range of carbohydrate-active enzymes into a number of aerobic fungal expression hosts. But catalytic activity of anaerobic fungal xylanases, cellulases, β-glucosidases, or cellobiohydrolases in the tested aerobic strains (*Saccharomyces cerevisiae*, *Hypocrea jecorina, Pichia pastoris* and *Pichia methanolica*) was low or else the recombinant proteins were not catalytically active (Li *et al.* 2007; Tsai and Huang 2008; van Wyk *et al.* 2010; O'Malley *et al.* 2012). Genetic modification of *Saccharomyces cerevisiae* integrating a xylose isomerase from anaerobic fungi allowing the yeast to metabolize monosaccharide xylose was more successful. Conversion of xylose into xylulose using the isomerases of *Piromyces* and/or *Orpinomyces* species (Kuyper *et al.* 2005; van Maris *et al.* 2007; Madhavan *et al.* 2009) represents at this time the most promising technique for improving the industrial production of ethanol (Bellissimi *et al.* 2009) and several patents have been filed so far (Teunissen and De Bont 2011). In addition to the incorporation of single enzymes, the creation of artificial cellulosomes and xylanosomes, to profit from the synergy between certain enzymes is on the rise. For example Doi and colleagues built a cellulosome from *Clostridium thermocellum* enzymes which show synergistic activity against cellulose (Sánchez 2009). Mingardon *et al*. designed mini-cellulosomes combining free fungal endoglucanase of glycosyl hydrolase family 6 from *Neocallimastix patriciarum* with bacterial cellulosomal endoglucanase of glycosyl hydrolase family 9 from *Clostridium cellulolyticum*, achieving superior cellulose activity, compared to complexes assembled only with bacterial enzymes (Mingardon *et al.* 2007). But even if recombinant anaerobic fungal enzymes could be produced and implemented in biotechnological processes, the physical degradation abilities of anaerobic fungi would still remain unused.

1.2.1.2.5 Anaerobic fungi in the biogas production process

A commonly encountered issue during anaerobic digestion is limited degradability of plant biomass, 40 – 60 % of organic carbon remain unused (Procházka *et al.* 2012). This problem is due to the physical structure and the recalcitrant chemical nature of these polymers. In detail, lignin remains indigestive under anaerobic conditions and shields cellulose and hemicellulose from enzymatic degradation. Thus, technologies that can improve anaerobic degradation of lignocellulosic biomass are needed. Partial disruption of plant tissues, can be achieved by mechanical (Nah *et al.* 2000), thermal (Bougrier *et al.* 2007; Climent *et al.* 2007), chemical (Tanaka and Kamiyama 2002), oxidative (Goel *et al.* 2003) or ultrasonic (Neis *et al.* 2000; Appels *et al.* 2008) pre-treatment.

However, in the rumen the natural biogas system these techniques, besides mechanical pre-treatment by mastication, are not available. There bacteria, archaea, protozoa and anaerobic fungi account for the key players in plant tissue degradation. Some important parameters of anaerobic digestion in biogas fermenters resemble conditions of the fermentation processes found in the rumen, namely a strong negative redox potential, a nearly neutral pH and a temperature between 37 °C ± 2 °C. Microbial pre-treatment or the implementations of rumen microorganisms into the biogas process seem to be possible strategies to deal with recalcitrant substrates.

Improvement of anaerobic biomass hydrolysis through the addition of specific microorganisms has been experimentally tested in several studies for bacteria. Miah and co-workers (Miah *et al.* 2004) described a 210 % increase in biogas production during thermophilic digestion (65 °C) of sewage sludge caused by the protease activity of a *Geobacillus* sp. strain. Similarly, Bagi and colleagues (Bagi *et al.* 2007) applied mesophilic *Enterobacter cloacae* and thermophilic *Caldicellulosiruptor saccharolyticus* strains during anaerobic digestion of waste water sludge, pig manure and dried plant biomass of artichoke, and achieved a remarkable increase of biogas production (160 %). This increase was explained by the enhanced H_2 level as both tested strains are excellent hydrogen producing bacteria, and *Caldicellulosiruptor saccharolyticus* has moreover cellulolytic activity. Also, introduction of an aerobic pre-treatment step for plant residues through e.g. white and brown rot fungi or the potent cellulose degrading *Trichoderma viride* has shown promising results on improving the subsequent anaerobic digestibility in biogas reactors (Ghosh and Bhattacharyya 1999; Wagner *et al.* 2013).

In contrast, the direct introduction of anaerobic fungi into these bioreactors would eliminate the requirement of an aerobic pre-digestion. With respect to the presented intention, of course only mesophilic conditions are eligible. In recent years, several studies have dealt with the application of anaerobic fungi to improve anaerobic digestion of cellulosic material (Lee *et al.* 2015). In more detail, the digestive tract of animals fed with very specific, fibre-rich diets have been chosen for the isolation of potent anaerobic fungal strains, that could be best suited for a technical implementation (Leis *et al.* 2014). The possibility of *Anaeromyces* and *Piromyces* strains to integrate into biogas-producing anaerobic sludge bacterial communities, to improve degradation of substrate polysaccharides and consequently to influence methane production has already been tested in laboratory conditions. Promising results were obtained during the bioaugmentation of swine manure fed biogas reactors with different strains of anaerobic fungi. Amendment with fungal biomass led to 4-22 % higher gas yields and up to 2.5 % higher methane concentration (Fliegerová *et al.* 2012a; Procházka *et al.* 2012). A recent study showed that bioaugmentation with anaerobic fungi didn't increase the overall methane yield, but that it speeds up initial gas production and thus may help to reduce retention time (Nkemka *et al.* 2015). In most cases, however, it was not possible to preserve fungal activity and the fungal beneficial effect on hydrolysis seems to decline after about ten days of incubation. The factors permitting fungal growth in habitats other than the digestive tract of their hosts still require thorough research and it is unclear if full-scale application of these microorganisms will become feasible.

1.2.1.3 Anaerobic fungi: Methodological state of the art

1.2.1.3.1 Detection techniques for anaerobic fungi

The monitoring of anaerobic fungi sampled from the digestive tract or feces of herbivores requires accurate and reliable detection techniques, and the same methods are also applicable to axenic cultures and industrial fermentations (Lockhart *et al.* 2006). Here we summarize the range of approaches that have been used so far, or which may be of relevance to detect and quantify the activity of anaerobic fungi.

Microscopy is still the most straightforward method for a general determination of growth status and initial phylogenetic classification of fungal biomass. However it requires a certain level of skill and experience to assign identity, and mistakes can be made even with the help of identification keys as found in e.g. Ho and Barr (1995) and Orpin (1994). Classification into rhizoidal or bulbous genera is relatively easy, for a more exact attribution of anaerobic fungi to the monocentric or polycentric group, the DNA binding fluorescent dyes DAPI (4',6-diamidino-2-phenylindole) or stains of the Hoechst-group (bisbenzimides) must be employed. A microscopic approach reaches its limit when differentiation between e.g. *Piromyces* and *Neocallimastix*, or *Orpinomyces* and *Anaeromyces* is needed and often no zoospore release can be witnessed to check for monoflagellate or polyflagellate zoospores. Another drawback, especially in microscopy of environmental samples that contain plant debris, is the clear differentiation of fungal- and plant biomass. During fluorescence microscopy, autofluorescene of plant material over a wide wavelength range clearly impedes distinct identification of fungal structures. Staining with calcofluor white (Darken 1961) or the more recently proposed stains Solophenyl Flavine 7GFE 500 and Pontamine Fast Scarlet 4B (Hoch *et al.* 2005) will help to highlight chitinous structures of the fungal biomass, such as cell walls, septa and bud scars, but the affinity of these dyes for cellulose and other sugar polymers can be problematic. Specific staining protocols can be performed to circumvent this issue. One possibility is the staining with lactofuchsin as described in Leis *et al.* (2014), an approach originally used to bring out plant root fungi, e.g. arbuscular mycorrhizas.

Measurement of fungal abundance with culture-dependent techniques i.e. thallus forming units (TFU) is generally performed through the most probable number (MPN) method (Theodorou *et al.* 1990; Davies *et al.* 1993) and by using the roll-tube method as described by Joblin (1981). However, this is a work that can be tedious and also requires certain expertise. The roll-tube approach is further well suited to obtain pure fungal cultures during the isolation procedure.

An indirect way to determine fungal biomass / growth is through their gas production that can be monitored by the use of a pressure transducer and then correlated to the amount of biomass (Theodorou *et al.* 1995).

Anaerobic fungi produce a wide range of potent enzymes, e.g. cellulase, endoglucanase, xylanase or amylase amongst others, that help to degrade plant material (Li *et al.* 1997; Novotná *et al.* 2010; Fliegerová *et al.* 2012a). Thus, enzyme activity can be used as indirect parameter for fungal activity. For instance Fliegerová and co-workers could, based on these parameters, demonstrate the improved hydrolytic activity of biogas reactors after fungal amendment, but also detected the relatively fast decrease of this enzyme activity over time (Fliegerová *et al.* 2012a).

Another very promising approach that has yet to be tested for anaerobic fungi is the raising of enzyme specific antibodies. Li and coworkers (Li *et al.* 1997) were able to produce specific antibodies for the catalytic domain of xylanases found in *Orpinomyces* and *Neocallimastix*. By fluorescence-labelling of these antibodies that could maybe also be raised for other fungi specific structures, an elegant detection technique could be established.

Culture independent, molecular techniques and DNA-based approaches have revolutionized microbial ecology over the last two decades and helped to confirm the monophyly of the *Neocallimastigomycota*. The most commonly used target genes, that allow not only for detection and community analysis of anaerobic fungi but also quantification through qPCR are the small ribosomal subunit (18S rRNA gene) and the internal transcribed spacer (ITS) region (Denman and McSweeney 2006; Fliegerová *et al.* 2006; Lockhart *et al.* 2006; Edwards *et al.* 2008; Cheng *et al.* 2009; Lwin *et al.* 2011; Marano *et al.* 2012; Koetschan *et al.* 2014). However, both gene regions also bear certain drawbacks that should be considered and are discussed in Gruninger *et al.* (2014). To summarize these drawbacks, the sequence of the 18S rRNA gene is too conserved within the *Neocallimastigomycota* phylum to allow for a clear differentiation of closely related taxa (Dagar *et al.* 2011), and the ITS region, despite its prevalent utilization in fungal phylogeny (Schoch *et al.* 2012), does not allow for direct sequencing of PCR products and exhibits high variability for this microbial group that might impair sequence alignments. The 28S rRNA gene however seems to be best suited for detection and phylogenetic assignment of anaerobic fungi and should be considered as the best target gene thus far utilized. A recent study even suggests to combine all three DNA regions (18S, 28S and ITS) for a more accurate representation of fungal diversity in environmental samples (Tan and Cao 2014), indicating that each chosen DNA region (18S, 28S and ITS) leads to a different result. Quantification of anaerobic fungi through qPCR gives a good insight into fungal abundance but is difficult to correlate with culture dependent enumeration results (TFU) or the actual biomass due to varying ratios of the DNA / biomass content within the *Neocallimastigomycota* members and depending on specific growth phase of each culture.

1.2.1.3.2 Cultivation techniques and cryopreservation

This chapter has highlighted the potential of this unusual group of fungi to address a range of problems associated with the degradation of lignocellulose-rich waste materials. The fact that these fungi are obligate anaerobes is an important component of their biotechnological potential, since scale-up issues are less problematic with anaerobic fermentation. However, the associated difficulty in the culturing and maintenance of obligate anaerobic fungi does impede the exchange of materials between scientists and could cause problems in future biotechnological deployment of these fungi. First there is a need for an international culture collection, with moves underway to exchange cryogenically stored cultures between interested parties. This will avoid the loss of cultures that has beset past research - we note with sadness that most of the type cultures that define the ca. 20 species are no longer extant. However, the growth in the routine use of DNA barcoding will facilitate the process of reliable identification of these fungi both in pure culture and from environmental samples.

Storage in liquid nitrogen appears to provide the only means for long term storage of anaerobic fungal cultures and it is strongly advised to store such cryovials in several locations. Storage at -80 °C is possible but there is progressive loss of viability of cultures over periods of more than a few months. Given the fragility of pure cultures, there is a need to elucidate the mechanism whereby these fungi form aerotolerant structures. It is clear, that all the anaerobic

fungi must be able to do this in order to disperse between hosts and furthermore it is clear, that they are very efficient in dispersal. The ability to generate such aerotolerant structures from axenic cultures would be extremely useful for long-term preservation of cultures and important in the context of this chapter for the inoculation of industrial fermentations with desired cultures or culture mixtures. Fliegerová *et al.* (2012a) has already demonstrated that biogas fermentation can be enhanced by addition of anaerobic fungi, as have Puniya *et al.* in their use of 'direct fed' microbials for the enhancement of the rumen fermentation (Sehgal *et al.* 2008). However, they used actively growing cultures, a process difficult to scale up. The ability to add aerotolerant structures to such fermentations would be most advantageous.

1.2.1.4 Conclusions

One of the major research goals in biogas science is to find an efficient tool to circumnavigate the bottleneck possessed by hydrolysis of lignocellulose-rich residues. Besides several physical, mechanical, chemical or microbial pretreatment techniques, the use of anaerobic lignocellulolytic fungi should be beneficial and even more cost-efficient. The rumen of herbivores can be seen as a natural resource for potent biomass degraders. Especially anaerobic fungi, known to act as primary digesters, could be good candidates.

They produce a superior set of hemi / cellulolytic enzymes which they excrete separately or combined in cellulosomes. Additionally, they are able to attack the plant material mechanically by their rhizoidal growth and open up the tissue for further digestion by bacteria. These two features are of capital interest to the biogas industry.

Until now several attempts have been made to produce recombinant anaerobic fungal enzymes for biotechnological application and even artificial cellulosomes have been built. Production in yeast has been the most profitable way, but still more research has to be done to provide recombinant enzymes in an industrial scale. Experiments to use anaerobic fungi directly in the biogas production process showed positive effects on gas production, but enzymatic activity and fungal growth decreased quickly under these conditions. Maybe anaerobic fungi cannot be implemented into conventional biogas reactors, but an individual anaerobic fungal pre-hydrolysis stage might be a possible solution facing this problem.

To summarize, anaerobic fungi have the potential to make biogas production much more efficient and the utilization of lignocellulose-rich substrates more viable. But for use in the industrial scale a greater understanding of the underlying ecology of these fungi and their cohorts is needed.

1.2.2 Current state of scientific knowledge on anaerobic fungi

Since the review article (presented above) has been published, progress in research on anaerobic fungi has been made. An overview of novel insights is given here in brief: In the year 2015 only eight anaerobic fungal genera had been described, to date the number has risen to 11 comprising: *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Neocallimastix*, *Orpinomyces*, *Piromyces*, *Buwchfawromyces*, *Feramyces*, *Oontomyces*, *Pecoramyces and Liebetanzomyces* (Joshi *et al.* 2018). They all were derived from feces or rumen fluid of mammalian herbivores, but molecular studies suggest that the diversity of anaerobic fungi (Paul *et al.* 2018), the

ecological niches and habitats colonized by them might be much broader. Suggestions of novel habitats range from the gut system of more exotic animals such as the green iguana (Liggenstoffer *et al.* 2010), wild western lowland gorillas (Schulz *et al.* 2018) over sediments from the marine coast (Picard 2017) and a freshwater lake (Wurzbacher *et al.* 2016) to the deep biosphere (Drake and Ivarsson 2018). The physiological status of the anaerobic fungi detected by molecular means was not determined. Thus, it is not known if anaerobic fungi are really members of the active microbial community outside mammalian herbivore gut systems or if the molecular evidence detected was coming from anaerobic fungal DNA introduced from more common sources. A hypothetical role for anaerobic fungi in non-gut habitats was described by Ivarsson *et al.* (2016) who considers them as potential hydrogen producers in subseafloor habitats cross-feeding the chemoautotrophic prokaryotic community.

Syntrophic interactions of anaerobic fungi and rumen methanogens are well characterized as summarized by Dollhofer *et al.* (2015). Mainly associations with *Methanobrevibacter* sp. and *Methanobacterium* sp. have been reported (Cheng *et al.* 2018). A novel methanogenic archaeon belonging to the so-called "Rumen Cluster" has been identified from a *Piromyces* sp. co-culture (Jin *et al.* 2014). The methanogenic partners can generate methane from H_2, CO_2, and formate, removing potential metabolic inhibitors from the growth medium. The anaerobic fungi in co-cultures were shown to increase their hydrogenosomal metabolism. It is thus believed that the accompanying methanogens facilitated the transport of carbohydrates into these organelles (Li *et al.* 2017). The increased hydrogenosomal activity further led to higher energy yields for the anaerobic fungi (Li *et al.* 2017). In addition, fungal diversity was higher in co-culture with methanogenic archaea (Cheng *et al.* 2018). The mentioned beneficial effects reported for co-cultures of anaerobic fungi and methanogens are of biotechnological relevance, as they could be the key to fully unlock the lignocellulolytic potential of anaerobic fungi. A novel strategy in this field of science is to optimize natural consortia or develop synthetic consortia to achieve most efficient lignocellulose degradation. Thus a computational strategy has been developed to identify potential microbial partner organisms for anaerobic fungi (Wilken *et al.* 2018).

Further progress in understanding anaerobic fungal genetics, ecology and physiology has been and will be made by modern sequencing approaches, deciphering anaerobic fungal genomes, transcriptomes, proteomes, and metabolomes (Edwards *et al.* 2017). Youssef *et al.* (2013) were the first to apply Illumina sequencing to analyze the genome of *Orpinomyces* C1A (now classified as *Pecoramyces ruminatium*). This publication already emphasized the plethora of CAZymes expressed by anaerobic fungi and gave further insights into their metabolic pathways for energy production in hydrogenosomes. Four more anaerobic fungal genomes have hitherto been sequenced from the genera *Piromyces*, *Anaeromyces* and *Neocallimastix* (Haitjema *et al.* 2017). The genome sequences and the transcriptomes determined by direct RNA sequencing allowed further understanding of the construction of anaerobic fungal cellulosomes (Haitjema *et al.* 2017) and CAZyme expression on different substrates (Solomon *et al.* 2016; Henske *et al.* 2017). Such information will help to develop approaches for the future industrial use of anaerobic fungi (Seppälä *et al.* 2017). As most of the

mentioned studies were focused on anaerobic fungal cellulosomes and CAZymes, they will be reviewed more detailed in the section on lignocellulolytic enzymes of anaerobic fungi (1.2.2.2).

In the following sections, the current state of scientific knowledge on the most relevant topics regarding anaerobic fungi for this Ph.D. thesis is presented in more detail.

1.2.2.1 Molecular detection techniques for anaerobic fungi

As not all anaerobic fungi might be cultivable with state-of-the-art methods, culture-independent detection and classification approaches are necessary to identify the full anaerobic fungal diversity from environmental samples. For phylogeny and community analysis, most commonly genes from the ribosomal operon have been used. They are highly abundant in repeated copies per genome, highly expressed and show different genetic variation (Edwards *et al.* 2017). The ITS 1 has mostly been targeted in environmental studies (Liggenstoffer *et al.* 2010; Kittelmann *et al.* 2012), as this region is the conventionally assessed marker for the kingdom *Fungi* (Schoch *et al.* 2012) and allows to record the entire known fungal diversity. This region is non-coding, leading to a high degree of genetic variation (Eckart *et al.* 2010). The high variability allows resolving to species and sometimes strain level (Eckart *et al.* 2010). However, the broad diversity of ITS 1 sequences entails disadvantages as well. Phylogenetic placement of anaerobic fungi based on this marker region is hampered by difficult alignment building due to size polymorphism (Edwards *et al.* 2008) and highly divergent regions (Edwards *et al.* 2017), limiting the number of informative sites to only a few for distantly related organisms. To overcome these issues, secondary structure based models have been developed that should facilitate alignment construction (Koetschan *et al.* 2014). Further, the ITS 1 region has been shown to differ up to 13 % within a single *Buwchfawromyces* strain (Callaghan *et al.* 2015) (see Chapter 4). Such intra-genomic variation brings by even more uncertainty for the phylogenetic placement of anaerobic fungal species and strains (see Chapter 4). Due to the draw-backs of ITS 1 based phylogeny, the large ribosomal subunit (LSU, 28S rRNA gene) got into focus as a phylogenetic marker for anaerobic fungi (Fliegerová *et al.* 2006; Dagar *et al.* 2011; Callaghan *et al.* 2015). This gene region shows less variability and less intra-genomic variation, and thus allows constructing alignments more easily (Edwards *et al.* 2017) (see Chapter 4). In a study by Wang *et al.* (2017); phylogeny based on ITS 1 and LSU sequences were compared for anaerobic fungal isolates derived from Yak. The phylogenetic trees were additionally linked to morphological characteristics of the known anaerobic fungal genera. LSU phylogeny supported the hitherto defined genera and even delivered a clearer picture of the relationships between them (Wang *et al.* 2017). Both genetic markers present advantages and disadvantages. Thus, using both in a combined approach actually seems to be the most suitable strategy to describe novel anaerobic fungi and assign the position of environmental detected sequences (Hanafy *et al.* 2017; Hanafy *et al.* 2018; Joshi *et al.* 2018).

Information on the anaerobic fungal community composition in environmental samples is necessary to understand their ecological niche and distribution. To further gain insights in their importance and activity under certain environmental and experimental conditions, tools

for molecular quantification are needed. However, molecular quantification methods for anaerobic fungi are still scarce. For real-time quantitative polymerase chain reaction (qPCR) three primer sets had been published, all of them target specific regions of the ribosomal operon (Li and Heath 1992; Denman and McSweeney 2006; Edwards *et al.* 2008; Kittelmann *et al.* 2012). They are still applied to monitor e.g. anaerobic fungi during bioaugmentation (Aydin *et al.* 2017) and animal feeding studies (Tomkins *et al.* 2015). Novel qPCR based approaches allowing the quantification of anaerobic fungi and their transcriptional activity were developed during this Ph.D. thesis and are presented in Chapter 4. In addition, Nagler *et al.* (2018) published a qPCR assay targeting the 28S rRNA gene, representing the most recent method for quantification of anaerobic fungi.

1.2.2.2 Lignocellulolytic enzymes of anaerobic fungi

The general enzymatic machinery of anaerobic fungi was already partly deciphered by enzymatic and molecular assays. Thus most of the general information on anaerobic fungal enzymes was already presented by Dollhofer *et al.* (2015). In brief, anaerobic fungi possess a large collection of CAZymes, which they either excrete freely or which are synergistically bound in cellulosomes. They can liberate cellulose and hemicellulose from lignin and can degrade the carbohydrates to glucose by the action of β-glucosidases. Since knowledge on the composition and regulation of the anaerobic fungal lignocellulolytic machinery is crucial to facilitate their application in biogas production and other biotechnological processes, the most recent major insights in this topic are presented below.

Novel Omics-techniques revealed that a much higher enzymatic potential than expected is still hidden in anaerobic fungi (Youssef *et al.* 2013; Solomon *et al.* 2016; Haitjema *et al.* 2017; Henske *et al.* 2017; Seppälä *et al.* 2017; Gruninger *et al.* 2018). A comparison of 27 fungal genomes concluded that anaerobic fungi provide a superior amount of cellulases, hemicellulases, and enzymes modifying pectin and lignin (Seppälä *et al.* 2017). Thus, they possess even greater potential for pre-treatment of lignocellulosic biomass than the commonly applied aerobic *Trichoderma* and *Aspergillus* species. In a study by Solomon *et al.* (2016), the transcription and expression of CAZymes by *Anaeromyces robustus*, *Neocallimastix californiae* and *Piromyces finnis* grown on fibrous and non-fibrous substrates was analyzed. In total, 2 % of the anaerobic fungal transcriptomes accounted for CAZymes transcripts with the majority of the CAZymes carrying non-catalytic docking domains (NCDD) which allow their arrangement in cellulosomes. An overview of CAZyme transcripts in all hitherto sequenced anaerobic fungal isolates is given in Table 2.

Table 2: CAZymes transcribed in sequenced anaerobic fungal isolates

		Number of transcripts in to date sequenced anaerobic fungi								
Enzyme type	Glycosyl hydrolase family	*Anaeromyces mucronatus*	*A. robustus*	*Caecomyces churrovis*	*Neocallimastix californiae*	*N. frontalis*	*Orpinomyces joyonii*	*Pecoramyces ruminantium*	*Piromyces finnis*	*P. rhizinflata*
Cellulases	GH1	5	11	20	16	11	11	N/S	9	6
	GH3	13	16	16	33	18	15	19	12	10
	GH5	24	22	19	48	40	31	50	27	49
	GH6	12	5	27	22	35	31	24	8	27
	GH8	1	1	2	4	2	4	3	1	2
	GH9	14	15	29	25	13	11	29	12	14
	GH16	6	9	3	15	12	8	N/S	5	8
	GH31	8	6	1	7	7	9	N/S	1	5
	GH45	14	13	26	24	22	16	22	11	20
	GH48	6	7	25	24	22	19	31	14	16
	GH74	1	N/S	N/S	N/S	5	2	7	N/S	2
	GH124	2	N/S	N/S	N/S	4	2	4	N/S	2
Hemicellulases	GH2	2	N/S	N/S	N/S	2	1	2	N/S	1
	GH10	10	15	15	67	35	23	37	16	25
	GH11	24	30	60	59	41	23	28	31	49
	GH11-12	N/S	30	63	67	N/S	N/S	N/S	35	N/S
	GH26	3	N/S	N/S	N/S	10	1	11	N/S	7
	GH30	2	2	2	2	2	1	N/S	1	2
	GH39	2	4	3	9	4	6	N/S	1	3
	GH43	24	N/S	N/S	N/S	37	30	35	N/S	18
	GH95	1	N/S	N/S	N/S	1	1	1	N/S	1
	GH115	1	N/S	N/S	N/S	5	2	5	N/S	3
Carbohydrate esterase	N/S	94	28	47	43	131	85	N/S	22	130
Pectin lyase	N/S	10	5	45	35	19	16	N/S	9	30
Polysaccharide deacetlyase	N/S	N/S	58	42	93	N/S	N/S	N/S	48	N/S
Rhamnogalacturonate lyase	N/S	3	3	4	4	4	3	N/S	2	7
Pectinesterase	GH88	N/S	0	0	2	N/S	N/S	N/S	1	N/S
	other	N/S	5	4	12	N/S	N/S	N/S	6	N/S

Data was derived from the following publications: *Anaeromyces robustus*, *Neocallimastix frontalis, Orpinomyces joyonii* and *Piromyces rhizinflata* Gruninger et al. (2018); *Anaeromyces robustus, Caecomyces churrovis*, *Neocallimastix californiae* and *Piromyces finnis* Henske et al. (2017); *Pecoramyces ruminantium* (formerly known as *Orpinomyces* sp. C1A) Youssef et al. (2013). N/S abbreviates “not specified” here.

The only sequenced bulbous growing isolate is represented by *Caecomyces churrovis*. A comparison of the transcriptomes of the two growth types showed, that the transcriptome of *Caecomyces churrovis* was only 37% similar to those of the rhizoidal strains *Anaeromyces robustus*, *Neocallimastix californiae* and *Piromyces finnis* (Henske *et al.* 2017). The bulbous species showed better growth on the soluble substrate and relied more on free enzymes (only 15% of transcripts contained NCDD allowing organization in cellulosomes), expressing a higher amount of hemicellulases (GH43, GH11/12), carbohydrate esterases and different endoglucanases (GH9). This suggests that the different enzymatic setup is needed to facilitate efficient plant cell wall degradation without rhizoidal growth (Henske *et al.* 2017).

Further, the structure of anaerobic fungal cellulosomes (Figure 6) was deciphered (Haitjema *et al.* 2017), constituting the basis for future biotechnological utilization of such multienzyme complexes. Anaerobic fungi have obtained cellulosomal CAZymes from bacteria by horizontal gene transfer (HGT) (Murphy *et al.* 2019). Bioinformatical comparison of dockerin domain proteins showed that anaerobic fungal cellulosomes are constituted by unique fungal enzymes, bacterial enzymes derived by HGT and enzymes representing a fusion of fungal and bacterial enzymes (Haitjema *et al.* 2017). Anaerobic fungal scaffoldin protein (ScaA) and dockerin domains show no genetic similarity to their bacterial counterpart. In contrast to species specificity known from bacterial scaffoldin backbones, the ScaA might be universal in the phylum of *Neocallimastigomycota*. Thus, anaerobic fungal cellulosomes could be composed of CAZymes from different anaerobic fungal species, which might be an advantage in competitive environments (Haitjema *et al.* 2017).

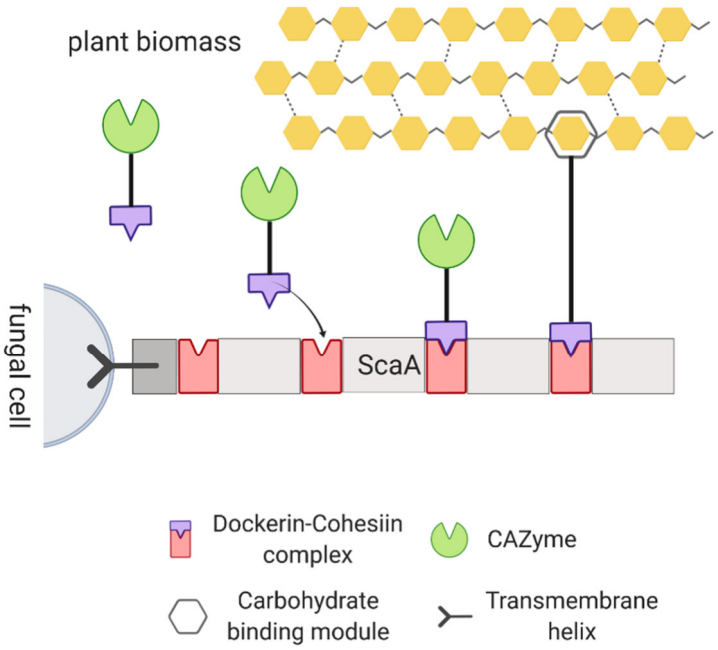


Figure 6: Simplified model of an anaerobic fungal cellulosome, showing the principal components of the multienzyme complex. Created with BioRender.com

In addition, CAZyme expression was shown to be catabolite repressed in anaerobic fungi (Henske *et al.* 2018), the expression of CAZymes is induced by complex substrates (e.g. lignocellulose) and repressed by simple sugars (e.g. glucose). Non-coding antisense transcripts accounting for 11 % of the *Piromyces* transcriptome are known to fulfill regulatory functions in higher fungi (Solomon *et al.* 2016). In anaerobic fungi, the transcription of non-coding antisense RNA might regulate the expression of CAZymes controlled by catabolite repression (Solomon *et al.* 2018).

1.2.2.3 Biogas production with anaerobic fungi

In contrast to studies on the enzymatic arsenal of anaerobic fungi, applied studies on biogas production with anaerobic fungi are scarce, and not much progress has been achieved since the publication of the review article presented above. Several recent studies tested the growth of anaerobic fungi on diverse plant-derived substrates with the perspective to improve their use in bioenergy production. Anaerobic fungi showed growth on Whatman N°1 filter paper, carboxymethyl cellulose (Atanasova-Pancevska and Kungulovski 2018), as well as on a diverse range of lignocellulosic substrates comprising biomass from C3 plants, C4 plants and even woody poplar biomass (Hooker *et al.* 2018). Less growth and activity was observed on hemicellulosic substrates leading to the conclusion that metabolic cycling of xylose is slow, and that hexoses are the preferred substrates (Hooker *et al.* 2018). Pre-treatment of plant biomass with anaerobic fungi seems thus to be a reasonable strategy which can be applied e.g. in biogas and bioethanol production processes. Regarding the latter, hydrolysis of alkaline pre-treated corn stover by *Orpinomyces* strain C1A (now renamed *Pecoramyces ruminantium*) prior to ethanol production by a genetically engineered *Escherichia coli* strain (K011) delivered proof that anaerobic fungi can be utilized to perform sugar extraction for bioethanol production. The hydrolysis yield was lower than with commercially produced enzyme cocktails, but the low costs for fungal application counterbalanced the lower effectiveness (Ranganathan *et al.* 2017).

Further, it was shown that differences in substrate degradation exist between monocentric and polycentric growth types (Dagar *et al.* 2018) and even between individual strains of one genus (Atanasova-Pancevska and Kungulovski 2018). It might thus be more efficient to use mixed cultures of anaerobic fungal isolates to exploit their full degradation potential. This approach was recently applied by Yildirim *et al.* (2017) using a mix of four fungal isolates (*Anaeromyces* sp., *Neocallimastix frontalis*, *Orpinomyces* sp. and *Piromyces* sp.) and Ferraro *et al.* (2018) who used a mix of a *Neocallimastix* and an *Orpinomyces* species combined with fermentative bacteria. Both studies report accelerated and higher methane production in the variants treated with anaerobic fungal inocula further emphasizing the potential of such fungi to improve biogas production from lignocellulosic residues. But both studies present weakness in the experimental design, as the fungi were applied together with media containing more easily metabolizable sugars along with antibiotics, and no adequate controls were provided. Thus, the increase in biogas and methane production might partly originate from the transferred media components and fungal biomass which may have been degraded by bacteria and archaea in the biogas reactors. Taken together, further research is needed to

define the optimal anaerobic fungal inoculum and develop methods for biogas production with anaerobic fungi.

Chapter 2
Hypotheses and organization of this Ph.D. thesis

2.1 Research topics and hypotheses

Anaerobic fungi with their skills to degrade lignocellulosic biomass mechanically and enzymatically might be used to improve biogas production from recalcitrant substrates. The major goals of this Ph.D. thesis were thus to identify methods for the detection of anaerobic fungi, to study their fate in agricultural biogas plants and to improve biogas production by their application. Two main research topics were addressed by the research questions of this Ph.D. thesis:

1. **Molecular detection of anaerobic fungi in biogas production processes**

   Research question No. 1: Which PCR based methods are suitable to detect anaerobic fungi in biogas production processes?

   Research question No. 2: Are anaerobic fungi part of the biogas producing microbial community in agricultural biogas plants?

2. **Hydrolytic pre-treatment of lignocellulosic biomass for biogas production with anaerobic fungi**

   Research question No. 3: Can biogas production from lignocellulosic feedstock be enhanced by hydrolytic pre-treatment with anaerobic fungi?

The presented three research questions were addressed testing the following research hypotheses by a step-wise experimental approach (see Chapters 4-6 and Figure 7):

Hypothesis #1: Anaerobic fungi can be detected in and quantified from animal derived and biogas sludge samples by a specific 18S rRNA gene targeting qPCR assay (see Chapter 4).

Hypothesis #2: Active transcription of a cellulolytic gene of anaerobic fungi can be assessed for animal-derived and biogas sludge samples by specific quantification of glycosyl hydrolase family 5 endoglucanase transcripts in messenger RNA extracts (see Chapter 4).

Hypothesis #3: The composition of the anaerobic fungal community within animal derived and biogas sludge samples can be assessed by cloning and sequencing the 28S rRNA gene (see Chapter 4).

Hypothesis #4: Anaerobic fungi can be present and transcriptionally active in agricultural biogas plants (see Chapter 5)

Hypothesis #5: Pre-treatment with anaerobic fungi, as exemplified with different *Neocallimastix frontalis* strains, can improve biogas production from hay (see Chapter 6)

Research topic	Hypotheses	Chapter	Journal article
Molecular detection of anaerobic fungi in biogas production processes	**Hypothesis #1:** Anaerobic fungi can be detected in and quantified from animal derived and biogas sludge samples by a specific 18S rRNA targeting qPCR assay	**4**	**Dollhofer et al., 2016:** *Journal of Microbiological Methods*, 127, 28-40.
	Hypothesis #2: Transcriptional cellulolytic activity (TCA) of anaerobic fungi can be assessed for animal derived and biogas sludge samples by specific quantification of glycosyl hydrolase family 5 endoglucanase transcripts in messenger RNA extracts		
	Hypothesis #3: The composition of the anaerobic fungal community within animal derived and biogas sludge samples can be assessed by cloning and sequencing the 28S rRNA gene		
	Hypothesis #4: Anaerobic fungi can be present and transcriptionally active in agricultural biogas plants	**5**	**Dollhofer et al., 2017:** *Bioresource Technology*, 235, 131-139.
Hydrolytic pre-treatment of lignocellulosic biomass for biogas production with anaerobic fungi	**Hypothesis #5:** Pre-treatment with anaerobic fungi, as exemplified with different *N. frontalis* strains, can improve biogas production from hay	**6**	**Dollhofer et al., 2018:** *Bioresource Technology*, 264, 219-227.

Figure 7: Overview of the two research topics and five hypotheses addressed in this Ph.D. thesis and links to the corresponding chapters and journal articles

2.2 Organization of this Ph.D. thesis

This Ph.D. thesis is organized according to the research topics and hypotheses listed in section 2.1 (chapters 4-6; see Figure 7). Chapter 3 gives a brief overview of used material and methods. In chapters 4 to 6, the five hypotheses are tested and the results are presented alongside the related background, experimental approaches, results, and discussions.

Chapter 4 describes the development of novel PCR based methods to determine the presence, transcriptional activity and the community structure of anaerobic fungi in animal-derived and biogas sludge samples. In chapter 5, the validated PCR based methods were applied to screen ten agricultural biogas plants for the presence and transcriptional activity of anaerobic fungi.

Chapter 6 describes the application of two *Neocallimastix frontalis* isolates to pre-treat hay prior to biogas production and describes the effect of the treatment.

In chapter 7, the major results of this thesis are discussed in context with the current scientific knowledge and literature.

Chapter 3
Material and methods

This Chapter provides a short summarized outline of the Materials and Methods applied to address the two main research topics: 1. Molecular detection of anaerobic fungi in biogas production processes; 2. Hydrolytic pre-treatment of lignocellulosic biomass for biogas production with anaerobic fungi. Detailed information and description of the experimental approaches used are given in the respective Chapters 4 - 6.

3.1 Development of molecular detection tools for anaerobic fungi in biogas processes

For the analysis of the presence of anaerobic fungi, their transcriptional activity and their community profile in a biogas sludge sample, specific detection tools had to be developed and tested. After a literature survey, three genes were identified as possible markers for these tasks: The 18S rRNA gene for detection and quantification of anaerobic fungal ribosomal DNA (assay AF-SSU), a glycosyl hydrolase family 5 endoglucanase gene to detect and quantify anaerobic fungal transcriptional activity (assay AF-Endo) and the 28S rRNA gene to characterize the anaerobic fungal community by phylogenetic means (AF-LSU). The procedure for creating and validating the polymerase chain reaction (PCR) based detection tools is described in the following sections.

3.1.1 Sequence collection, bioinformatics processing, and primer design

In order to develop suitable primer systems, first, all publicly accessible sequences of the respective target genes and of anaerobic fungi were registered in alignments. Ribosomal sequences were obtained from the online databases NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and SILVA - high-quality ribosomal RNA Database (http://www.arb-silva.de/). The database CAZy - Carbohydrate-Active enZYmes Database (http://www.cazy.org/) was queried to identify a suitable marker gene for anaerobic fungal transcriptional activity. It contains gene sequences of all enzymes capable of assembly (glycosyltransferases) and breakdown (glycoside hydrolases, polysaccharide lyases, carbohydrate esterases) of carbohydrates (Lombard *et al.* 2014). All carbohydrate active enzymes (CAZymes) in the CAZy database are sorted according to their affiliation to enzyme groups (e.g. glycosyl hydrolase families) and their occurrence in organisms.

The collected sequences were sorted according to their similarity in the program MEGA 6.0 (Tamura *et al.* 2013). Questionable sequences were discarded. Using BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), similar outgroup sequences of non-target organisms were also included in the alignment. Based on the processed alignments, suitable primer binding sites specific for the anaerobic fungal genes were identified. Primers for the specific detection by PCR should bind in relatively conserved regions of the chosen gene. They should be designed in a way that only the target organisms are detected specifically and that they

cover the targeted group completely. The chosen binding sites should not be present in non-target organisms or particularly the 3'- ends of the primer sites should be so strongly alienated that binding of the primers and thus a successful PCR with outgroup DNA is excluded. In order to identify suitable sequences within the alignment, the program Primrose (Ashelford *et al.* 2002) was used. Therein all sequences affiliating with anaerobic fungi were defined as a target. The program calculates possible primer sequences and delivers an output giving their location on the target gene and their melting temperature. The suggestions were examined for the best primer pair, taking into account a suitable melting temperature (Tm). Primer sets were selected which had a Tm around 60 °C and did not differ by more than two degrees Celsius between the forward and reverse primers. The primers were tested for the formation of secondary structures using the OligoAnalyzer (Integrated DNA Technologies, https://eu.idtdna.com/calc/analyzer). A strong formation of such structures can prevent the annealing of the primers during PCR and lead to poor functionality of the system. All primer sequences were tested for their specificity by *in-silico* BLAST analysis and validated for PCR and quantitative PCR.

3.1.2 Development and validation of qPCR assays: AF-SSU and AF-Endo

The whole procedure for development and validation of the qPCR assays developed in this Ph.D. thesis is given detailed in the methods paper presented in Chapter 4, thus only a brief description of the general approach is given here.

Real-time quantitative PCR (qPCR) allows real-time monitoring of the DNA increase during PCR and quantification of the amount of DNA in the sample. The addition of fluorescence dyes (e.g. EvaGreen®, Biotium) or a labeled oligonucleotide probe allows monitoring the increase of double-stranded DNA. For absolute quantification of the amount of DNA in a given sample, a standard is required. Such standards were generated in this thesis for each quantitative assay by cloning the target gene fragment in a plasmid (Figure 8). An overnight culture of a positive clone carrying the target gene in liquid LB_{AMP} medium was split, and two 10-fold dilution series were prepared. One was used to quantify the amount of cells / ml by plating on LB_{AMP} agar plates, the other one was washed and measured by qPCR after cell lysis. The number of gene copies in each dilution step was determined by the Most Probable Number (MPN) method and the number of gene copies per *Escherichia coli* cell was calculated. The standard was included in each qPCR run, and the concentration of gene copies in the template was calculated from the known standard concentration.

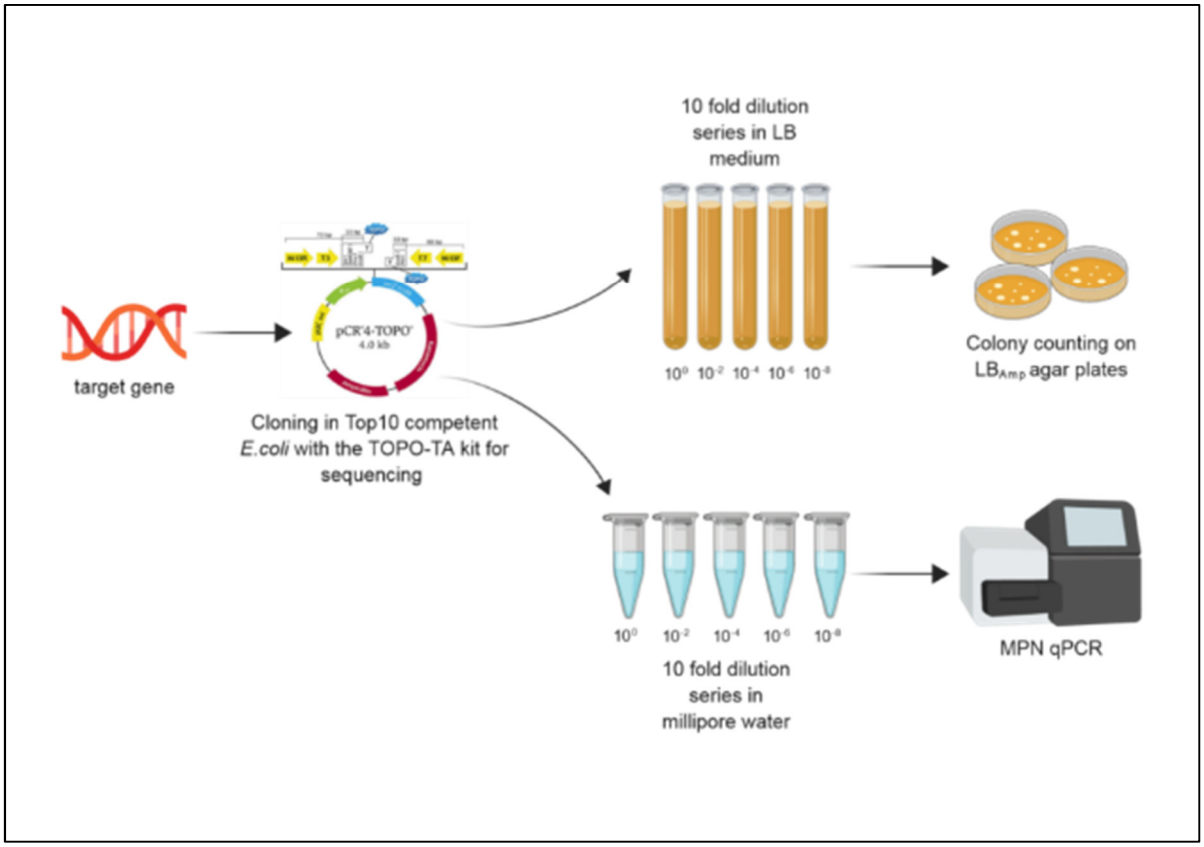


Figure 8: Production of a standard for absolute quantification. Created with BioRender.com

The two qPCR assays AF-SSU and AF-Endo were tested for specificity with samples derived from pure anaerobic cultures, rumen fluid, and biogas digester sludge. Further, for each qPCR assay detection limits and reproducibility were defined as described in Chapter 4.

3.2 Detection of anaerobic fungi in agricultural biogas plants

In order to clarify whether anaerobic fungi are present in agricultural biogas plants, samples of ten biogas plants were analyzed (Figure 9). The biogas plants were sampled and technically monitored within the projects "Nutzung von Grünland zur Biogaserzeugung – Teilprojekt: Betriebs-Monitoring Biogasanlagen" (Kissel *et al.* 2015) and "Monitoring von Biogasanlagen – Diversifizierung der Einsatzstoffe und Verfahrenstechnik" (Streicher *et al.* 2016) performed at the Institute of Agricultural Engineering and Animal Husbandry, Bavarian State Research Center for Agriculture (LfL) in Freising, Germany. A table summarizing the technical specifications of the tested biogas plants can be found in Chapter 5.

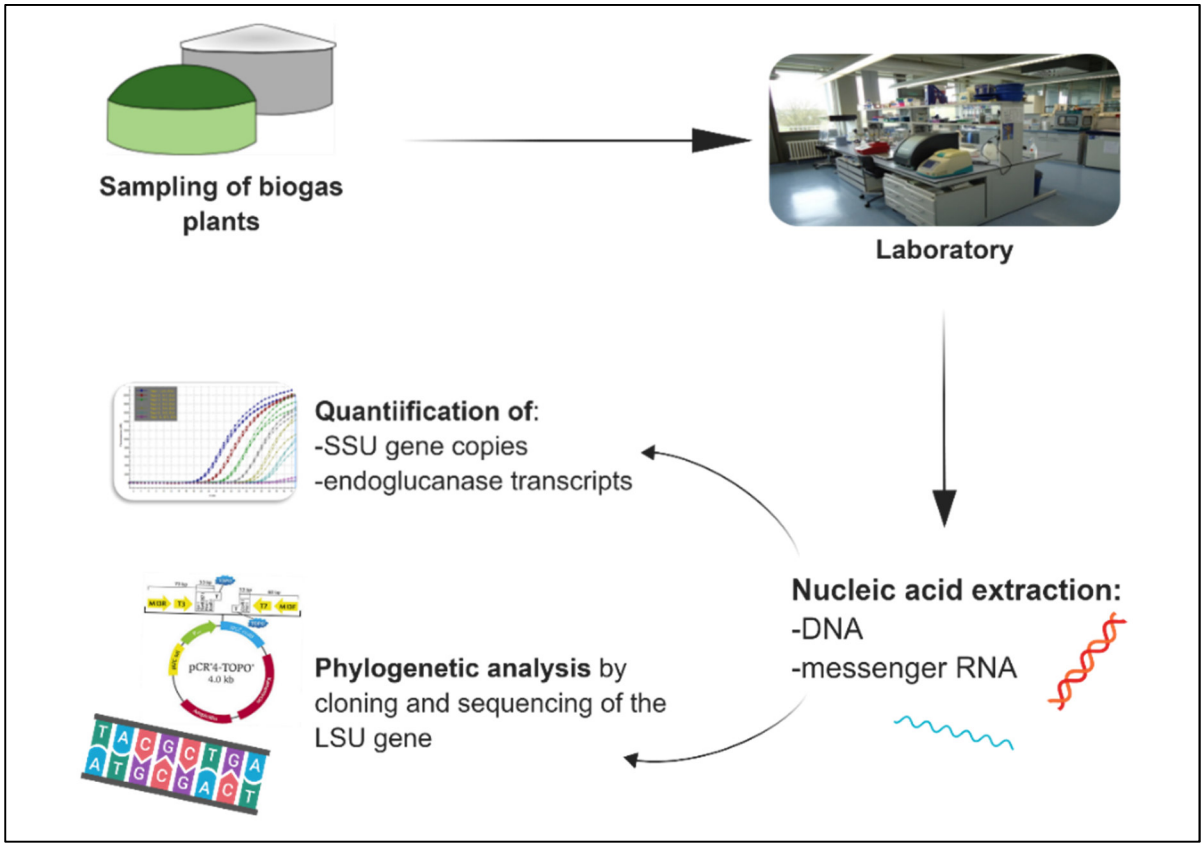


Figure 9: Different steps in sample processing to assess the presence of anaerobic fungi, their transcriptional activity and their community profile in agricultural biogas plants. Created with BioRender.com

Samples were mainly taken from digesters. Occasionally, post-digesters and final repositories were sampled in addition. The samples were transported to the laboratory in insulated, sealed bottles, and nucleic acids were extracted. For quantification of functional genes, mRNA was extracted with the Dynabeads® mRNA DIRECT™ Purification kit (Life Technologies). Each sample was tested for the presence and transcriptional activity of anaerobic fungi in qPCR with the assays AF-SSU and AF-Endo, respectively. To determine the composition of the present anaerobic fungal community, PCR, cloning and sequencing with assay AF-LSU was performed. Sequencing results were quality checked (e.g. sequencing errors and chimeric sequences identified, and faulty sequences discarded), aligned and phylogenetically analyzed as described in Chapter 4.

3.3 Cultivation of anaerobic fungi for specificity testing and hydrolytic pre-treatment

For the cultivation of anaerobic fungi, mainly full media are used. Their composition attempts to mimic conditions in the natural habitat, the digestive tract of herbivorous vertebrates (examples of nutrient media can be found in the following publications: (Joblin 1981; Lowe *et al.* 1985; Orpin and Greenwood 1986; Teunissen *et al.* 1991). The main ingredients of the

culture media are rumen fluid, salt solutions, protein and carbon sources. The latter are usually crude fiber-rich plant materials such as wheat straw, but also soluble plant sugars like xylan and cellobiose can be included. Growing anaerobic fungi on media without rumen fluid was successful only for some species (Lowe *et al.* 1985; Teunissen *et al.* 1991).

Within this Ph.D. project a rumen fluid-free medium comprising plant sugars and a high amount of hemin could be developed by Dr. Samart Dorn-In at the Chair of Animal Hygiene, TUM. In this medium, at least strains of the genera *Orpinomyces* sp. and *Neocallimastix* sp. were able to grow. The respective *Neocallimastix frontalis* isolates were applied in the experiments presented in Chapter 6. For specificity testing, a wider range of anaerobic fungal isolates was needed. A broader isolation and cultivation method (Callaghan *et al.* 2015) was studied therefore in the Mycology Research Group of Dr. Gareth Griffith, Aberystwyth University, Wales, UK. For isolation of anaerobic fungi, the following four processing steps were performed: Dilution of the sample, enrichment, transfer on different media and purification by passaging through agar-containing roll-tubes. A scheme of the isolation procedure from fecal and biogas plant samples is shown in Figure 10.

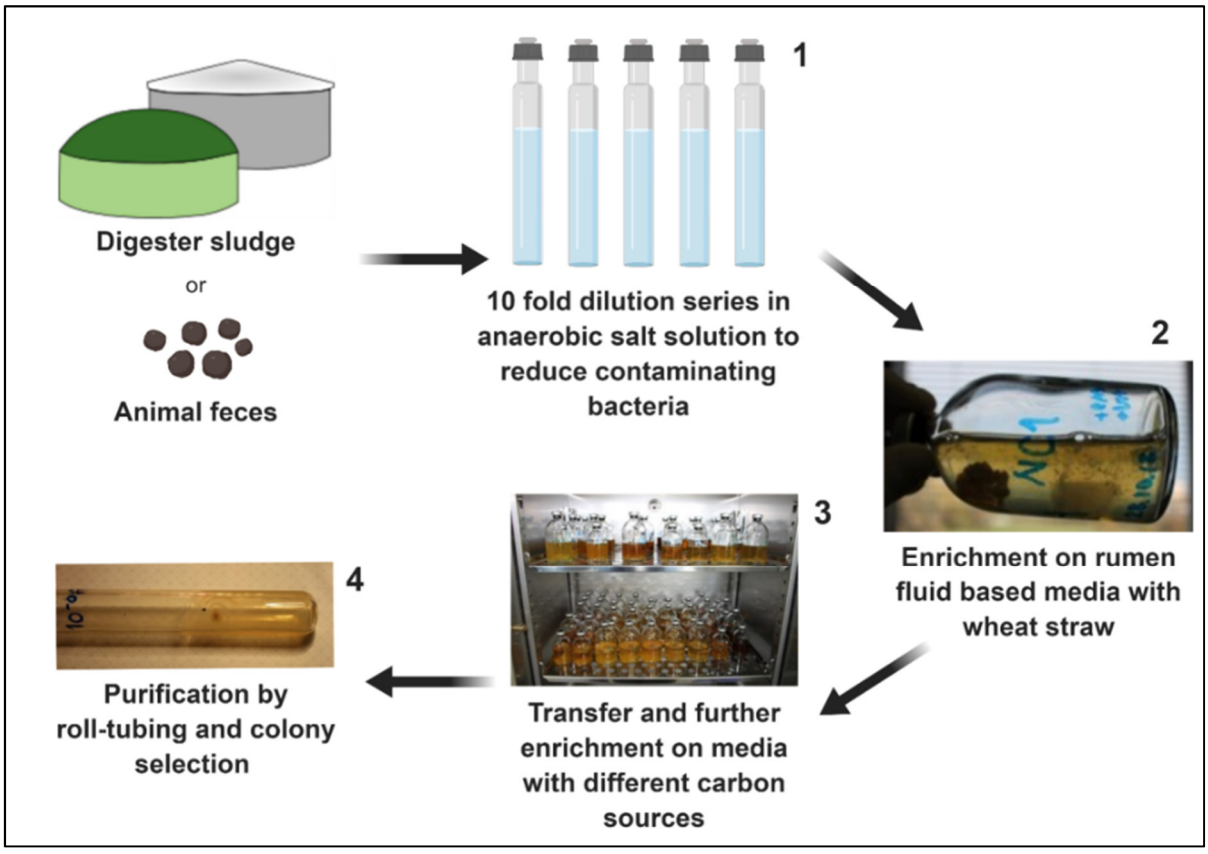


Figure 10: Isolation of anaerobic fungi from animal feces and digester sludge. Created with BioRender.com

First, 10 g of the respective sample were dispersed with 90 ml of anaerobic salt solution (see Callaghan *et al.* 2015) by vigorous shaking. A 10-fold dilution series was produced in salt solution containing the antibiotics streptomycin sulfate and penicillin G, both at a concentration of 120 $mg*ml^{-1}$ (step 1, Figure 10). This step is necessary to reduce contaminating bacteria. For the enrichment of anaerobic fungi, 5 ml of the different dilution steps were transferred to enrichment rumen fluid based media with wheat straw (5 $g*L^{-1}$) following the method by Callaghan *et al.* (2015) (step 2, Figure 10). Enrichment cultures were incubated at 39 °C for a period of 3-10 days in the dark. The cultures were checked daily for the typical formation of substrate mats by anaerobic fungi (see step 2, Figure 10). The growth of anaerobic fungi was verified by microscopy and documented by photography. If anaerobic fungi could be enriched, these were transferred in parallel to purification media containing wheat straw, cellobiose (3 $g*L^{-1}$), xylan (3 $g*L^{-1}$) or a mix of the mentioned carbon sources (step 3, Figure 10). Purification of isolates was performed by 3-fold passaging of enrichment cultures through agar-containing roll-tubes (step 4, Figure 10; Haitjema *et al.* (2014)). During each passage, colonies showing different morphology were separated and transferred to liquid media from which the next roll-tube was inoculated. Purity and identity of the obtained isolates were analyzed by microscopy, PCR, cloning, and sequencing. All culturing steps were carried out in gas-tight serum bottles under a 100 % CO_2 atmosphere. All media and solutions used were prepared anaerobically and sterilized by autoclaving (at 121 °C for 30 min). If open work was necessary, it was performed under continuous CO_2 gas flow. Application of the method described above led to a redundant strain collection and enabled working with reference strains from other laboratories.

3.4 Batch experiments for hydrolytic pre-treatment of lignocellulosic biomass for biogas production with anaerobic fungi

To analyze whether a hydrolysis step with anaerobic fungi has a positive effect on the biogas process, experiments in batch fermenters were performed. A detailed description of the experimental design is given in Chapter 6, thus only a brief overview of the experimental design is presented here.

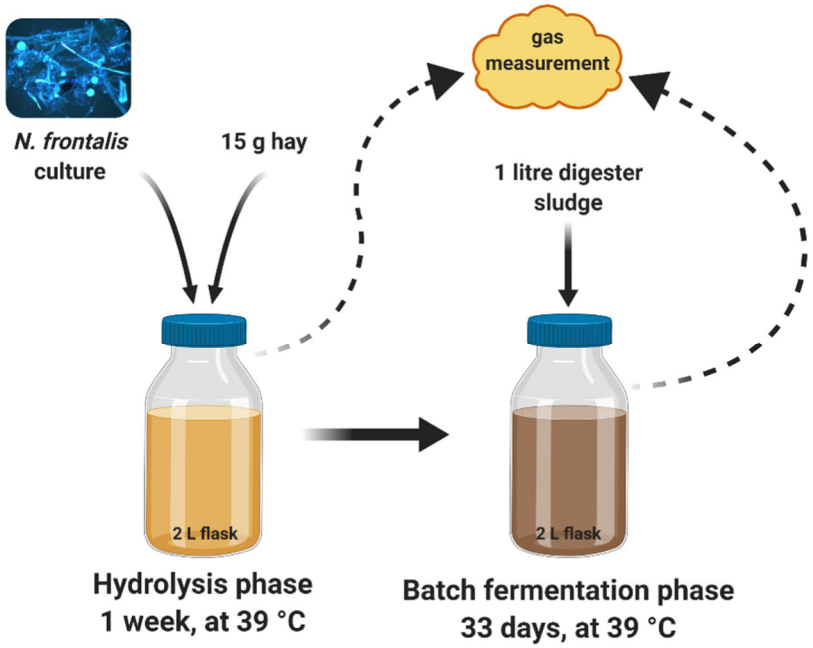


Figure 11: Experimental set-up for hydrolytic pre-treatment of hay with anaerobic fungi. Created with BioRender.com

The trials covered a period of 40 days and were divided into two process phases. One week of hydrolysis phase was followed by a biogas production / methanogenic phase in the batch fermentation mode (Figure 11). For the hydrolysis phase, only the hay used as substrate and the respective inoculum, either one of the individual *Neocallimastix frontalis* cultures or their heat-inactivated pendants, were mixed and incubated. Each variant was prepared in four repetitions. The fourth repetition was not transferred to the biogas production / methanogenic phase, as it was needed for chemical, microbiological and molecular genetic analyses. At the beginning of the biogas production / methanogenic phase, sludge from a biogas digester, operated by the Institute for Agricultural Engineering and Animal Husbandry at the Bavarian State Research Center for Agriculture (ILT), was added to induce biogas production. The sludge had been starved for a week prior to the experiment to avoid biogas production from residual comprised substrate. At the end of the experiment, wet chemical, microbiological and molecular genetic analyses were performed and the collected gas data was examined.

3.5 Adaptation of anaerobic fungi to a digestate based medium

Anaerobic fungi seem only to survive shortly in anaerobic reactor content (Procházka *et al.* 2012) hampering their direct implementation in the biogas production process. In order to examine growth and adaptability of anaerobic fungi under biogas conditions an anaerobic sludge based medium was developed. Digester effluent of a mono-maize fed biogas reactor operated by the ILT in Freising, Germany was used as base of the "digestate-medium". This anaerobic sludge was selected because it did not contain animal manure or slurry and thus could not have harbored anaerobic fungi. Solids were removed from the sludge by sieving and it was stored at -20 °C until medium was produced. For the "digestate medium", a "digestate-stock solution" was prepared by mixing the sieved anaerobic sludge with deoxygenized water (80 ml anaerobic sludge to 320 ml water). Sodium carbonate buffer, resazurin and L-cysteine were used at the same concentrations as reported in Callaghan *et al.* (2015). The "digestate stock solution" was flushed with CO_2 for about 3 h and the pH was adjusted to 6.8 with 5 M NaOH. Serum bottles (100 ml) were filled with 50 ml "digestate stock solution" sealed with butyl rubber stoppers, crimped and sterilized by autoclaving (15 min, 121 °C).

To allow adaptation of anaerobic fungi to the digestate, a "digestate medium" was created by mixing enrichment medium containing cellobiose (0.2 % w/v) and soluble xylan (0.3 % w/v) as carbon sources with different amounts of "digestate stock solution" (10 % to 80 % v/v which is equal to a range from 2 % to 16 % original digestate). Adaptation experiments were performed with four pure anaerobic fungal isolates obtained following the method described in Section 3.2 comprising *Neocallimastix cameroonii* (strain CaDo 3b; Griffith *et al.* (2015)), *Piromyces* sp. (KiDo 3a), *Cyllamyces* sp. (KiDo 2m) and a potentially novel genus strain KiDo 1h (Kinker 2015). Anaerobic fungi were transferred to the 2 % "digestate-medium" at the experiment start and subcultured on the next higher concentration only if good growth was confirmed by microscopy. The fate of the anaerobic fungal isolates in each "digestate-medium" concentration was monitored by light microscopy.

Chapter 4

Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi[2]

Anaerobic fungi decompose plant material with their rhizoid and multiple cellulolytic enzymes. They disintegrate the complex structure of lignocellulosic substrates, making them more accessible and suitable for further microbial degradation. There is also much interest in their use as biocatalysts for biotechnological applications. Here, three novel PCR-based methods for detecting anaerobic fungi and their transcriptional activity in *in vitro* cultures and environmental samples were developed. Two qPCR-based methods targeting anaerobic fungi were developed: AF-SSU was designed to quantify the 18S rRNA genes of anaerobic fungi. AF-Endo, measuring transcripts of an endoglucanase gene from the glycoside hydrolase family 5 (GH5), was developed to quantify their transcriptional cellulolytic activity. The third PCR based approach was designed for phylogenetical analysis. It targets the 28S rRNA gene (LSU) of anaerobic fungi revealing their phylogenetic affiliation. The *in silico*-designed primer / probe combinations were successfully tested for the specific amplification of anaerobic fungi from animal and biogas plant derived samples. In combination, these three methods represent useful tools for the analysis of anaerobic fungal transcriptional cellulolytic activity, their abundance and their phylogenetic placement.

[2] Was published in a similar version by Dollhofer, V., Callaghan, T.M., Dorn-In, S., Bauer, J., Lebuhn, M. 2016. Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. Journal of Microbiological Methods, 127, 28-40.

4.1 Introduction

Anaerobic fungi represent a basal phylum of the Kingdom *Fungi*, the *Neocallimastigomycota*. To date, five monocentric genera, *Neocallimastix*, *Piromyces*, *Caecomyces, Buwchfawromyces,* and *Oontomyces*, and three polycentric genera, *Anaeromyces*, *Cyllamyces*, and *Orpinomyces*, have been described (Griffith *et al.* 2010; Gruninger *et al.* 2014; Haitjema *et al.* 2014; Callaghan *et al.* 2015; Dagar *et al.* 2015). Their flagellated zoospores use chemotaxis to swim towards plant material and attach to the plant surface (Orpin and Bountiff 1978; Lowe *et al.* 1987b). Depending on the genus, they develop a filamentous rhizoid (*Neocallimastix*, *Piromyces*, *Anaeromyces*, *Orpinomyces, Buwchfawromyces* and *Oontomyces*) or bulbous holdfasts (*Caecomyces* and *Cyllamyces)*, both growing into the plant matter and rupturing the plant structure. During growth, the anaerobic fungi excrete a plethora of enzymes that enables them to digest different plant sugars and also to liberate cellulose and hemicellulose from their lignin coats (Borneman *et al.* 1990; Teunissen and Op den Camp 1993). The lignocellulolytic enzymes are secreted individually or are found combined in multi-enzyme complexes called cellulosomes (Fontes and Gilbert 2010; Haitjema *et al.* 2014). Cellulosomes were first described for cellulolytic bacteria from the family *Clostridiaceae*. Anaerobic fungi are the only eukaryotes hitherto known equipped with this unique feature. The combination of enzymes in cellulosomes mediates their synergistic attack and thereby enhances cellulolytic efficiency (Gruninger *et al.* 2014). With their ability to break down recalcitrant substrates mechanically and enzymatically, anaerobic fungi are ideal candidates for the anaerobic microbial pretreatment of lignocellulose-rich wastes (Nagpal *et al.* 2011; Procházka *et al.* 2012; Kazda *et al.* 2014) useful for several biotechnological approaches.

However, anaerobic fungi are notoriously difficult to cultivate and to preserve; and the lack of a centralized culture collection has hampered research (Griffith *et al.* 2010; Gruninger *et al.* 2014). Moreover, for the identification of promising strains of anaerobic fungi, up-to-date molecular biology tools applicable for the screening of environmental samples are a necessity. To our knowledge only three unique qPCR based quantification methods for anaerobic fungi have been reported, probably due to the handling issues mentioned above and the still relatively small number of sequences deposited in online databases. The first one is targeting a 120 bp region, at the 3’ end of the 18S rRNA gene (small ribosomal subunit, SSU) and the 5’end of the ITS 1 (Denman and McSweeney 2006). The second one is targeting a 110 bp region of the 5.8S rRNA gene and was developed and validated as being more specific than the previous method (Edwards *et al.* 2008). The third one is a qPCR based method using a 433 bp rRNA region, starting in the 18S rRNA gene, spanning the ITS 1 and parts of the 5.8S rRNA gene. It was first used by Li and Heath (1992) and was adapted by Kittelmann *et al.* (2012) for the qPCR based quantification of AF. Since a longer amplicon than that of Edwards *et al.* (2008) with higher internal sequence variability presents advantages, e.g. to design group-specific qPCR hydrolysis probes, possibly in a multiplexing approach, we designed primers and a probe (AF-SSU) specific for an 475 bp anaerobic fungal 18S rRNA segment on the basis of currently available sequences and used the AF-SSU primer / probe combination for quantification of the multicopy anaerobic fungal 18S rRNA genes. The primer sites for the mentioned qPCR assays, referring to an *Orpinomyces sp.* reference

sequence (AJ864475) are shown in Figure 25 in section 7.1. It has been attempted to relate qPCR DNA quantity to anaerobic fungal biomass using *in vitro* prepared standards (Denman and McSweeney 2006; Edwards *et al.* 2008). However, this approach is limited by several issues, such as differences in *in vitro* and *in vivo* growth and inter-generic variations in growth morphology, DNA content and even potentially rRNA copy numbers in different developmental stages. Therefore, we limited the determination of the abundance of anaerobic fungi to the quantification of 18S rRNA genes.

Knowledge of the quantity of anaerobic fungi present in environmental samples is interesting, but this is not the sole element. What is also important for identifying a strain that is suitable for biotechnological purposes (e.g. pretreatment) is the cellulolytic activity of a particular anaerobic fungal isolate. To date, activity testing of anaerobic fungi is typically based on fermentation (Paul *et al.* 2010) or enzymatic screening techniques (Aylward *et al.* 1999). However, a RT-qPCR-based activity measurement approach could be more time saving and would be independent of difficult cultivation techniques. Endoglucanases cleave glucosidic bonds in cellulose at amorphous non-crystalline sites, liberating oligosaccharides (Lynd *et al.* 2002). The expression of endoglucanases belonging to glycoside hydrolase family 5 (GH5) were upregulated during the degradation of plant material in an *Orpinomyces* species (Youssef *et al.* 2013). The gene sequences of cellulolytic enzymes of anaerobic fungi were thus screened in online databases in this study, and the gene sequences of endoglucanases belonging to glycoside hydrolases family 5 (GH5) were selected as a target of the novel primer pair AF-Endo. If anaerobic fungi are actively degrading cellulosic substrates, endoglucanases should be produced and the associated mRNA upregulated. For the quantitative assessment of GH5 specific transcriptional cellulolytic activity (TCA) of anaerobic fungi in samples, experiments were thus conducted at the mRNA level.

In addition to quantitative approaches, a tool for the phylogenetic characterization of anaerobic fungal populations was needed to be developed. The ITS 1 region has been proposed as a standard marker for fungal taxonomy (Schoch *et al.* 2012), and initial research has used this locus for identification of anaerobic fungal isolates (Li and Heath 1992; Brookman *et al.* 2000b). More recently it has been applied to study the environmental abundance of the anaerobic fungi in pyrosequencing studies (Liggenstoffer *et al.* 2010) and clone libraries (Kittelmann *et al.* 2012; Koetschan *et al.* 2014). However, the ITS 1 region confesses limitations attributable to high intra-genomic sequence variability. In a *Buwchfawromyces eastonii* strain, e.g. up to 12.9 % divergence among ITS 1 clones was observed (Callaghan *et al.* 2015). ITS 1 also shows significant size polymorphism among the AF. This has been exploited in ARISA based community finger printing studies (Edwards *et al.* 2008). The variability present makes phylogenetic assignments uncertain, and in next generation sequencing studies, false-positive clustering of anaerobic fungi (Eckart *et al.* 2010; Gruninger *et al.* 2014) was observed. Using the 18S rRNA gene as a phylogenetic marker is limited for anaerobic fungi, too, as this region is too conserved and does not allow differentiation between individual species (Eckart *et al.* 2010). The 28S rRNA (large ribosomal subunit, LSU) gene, however, was recently reported as a suitable marker for the differentiation of *Orpinomyces* spp. (Dagar *et al.* 2011). Tests with isolates from all known

anaerobic fungal genera showed better differentiation than ITS 1 (Kumar 2014). Accordingly, we developed a PCR based method, AF-LSU, which targets specifically the large ribosomal subunit of anaerobic fungi.

4.2 Material and methods

4.2.1 Isolates and samples

Isolates used for validation of PCR-based approaches were provided by the Academy of Sciences (Prague, Czech Republic), the University of Aberystwyth (Wales, UK) and by the Rowett Institute for Nutrition and Health, University of Aberdeen (Scotland, UK). The isolate names, their phylogenetical affiliation, and the source institutions are shown in Table 3. Cultivation of the isolates followed the method described by Callaghan *et al.* (2015).

Table 3: Isolates and DNA extracts used for PCR probe validation

Isolate	Affiliation	Sample type	Provided by
Re1	*Neocallimastix frontalis*	DNA	University of Aberdeen
P	*Piromyces communis* (Orpin type strain)	DNA	University of Aberdeen
KF 8	*Anaeromyces mucronatus*	Culture	Academy of Sciences, Czech Republic
OF 1	*Caecomyces communis*	Culture	Academy of Sciences, Czech Republic
Tmc 002.28xy	*Anaeromyces*	Culture	University of Aberystwyth
KiDo 3a	*Piromyces* sp.	Culture	This work
KiDo 1m	Putative novel genus	Culture	This work
KiDo 2m	*Cyllamyces* sp.	Culture	This work

Cattle rumen fluid, used for specificity testing of primer pairs AF-Endo and AF-LSU, was derived from fistulated cows maintained by the Chair of Animal Nutrition at Technische Universität München (TUM). DNA samples for specificity testing of primer pair AF-SSU were supplied by the Chair of Animal Hygiene, TUM. These samples comprised two types of pig forage (FM1, FM2), two samples of perennial ryegrass (G1 and G2), and a maize sample (G3).

Biogas plant 21 (PB 21, numbered to mask the associated operators identity) was operated at a temperature between 45 °C and 46 °C and fed with a mixture of 68.6 % grass silage, 2 % grain, 21.6 % cattle manure, and 7.8 % cattle slurry. Samples of fermenter 1, the post-digester, maize and grass silage were analyzed by PCR, cloning and sequencing to prove specificity of assay AF-SSU. For the showcase analysis (4.3.2), the fermenter of biogas plant 25 (PB 25) was sampled. It was maintained at a temperature of 40 °C and fed with a mixture of 2.4 % shredded grain, 6.7 % sugar beets, 8.8 % grass silage, 22 % whole plant silage, 35.1 % maize silage and 44.9 % cattle slurry.

All samples were collected in 1 L polyethylene bottles and transported at an ambient temperature to the laboratory where nucleic acids were extracted immediately. Subsamples were collected and stored at −20 °C. In addition, samples of cattle slurry used as substrates of biogas plants PB 14 and PB 22 were collected. The biogas plants were part of a monitoring study by the ILT (Ebertseder *et al.* 2012).

4.2.2 Nucleic acid extraction

Following the method of Lebuhn *et al*. (2003), 500 µl of the samples were transferred to a 2 ml reaction tube using a 1000 µl pipette with a tip cut at its end to facilitate flow-through of viscous samples and solids. The filling line was marked. Each sample was mixed with 1 ml of sterile 0.85 % KCl by shaking. After centrifugation at 20,000 *g* for 5 min, the supernatant was discarded, the washing step was repeated and the original volume was reconstituted with 0.85 % KCl. Soluble putative inhibitors, particularly humic compounds, were discarded with the supernatant and solids retained in the reaction tube.

If nucleic acids were extracted from fungal cultures, the whole cultures were transferred into 50 ml centrifugation tubes. Anaerobic fungal cells were pelleted by centrifugation at 5,000 *g* for 10 min. The anaerobic fungal cell pellet was washed twice with 5 ml of sterile 0.85 % KCl and processed for nucleic acid extractions.

4.2.2.1 DNA extraction

Washed samples (40 µl) were processed with a Fast-DNA Spin Kit for soil (MP Biomedicals) in a FastPrep-24 system (MP Biomedicals; bead beating for 40 s, at speed 6.0 m/s). The extraction was performed according to a previously published protocol (Lebuhn *et al.* 2003), yielding 100 µl of DNA-containing eluate. In a previous study, approximately 90 % of spiked DNA was recovered by performing DNA extraction with this method (Lebuhn *et al.* 2016). At this high recovery rate, absolute quantification of target organisms or DNA copies in a given sample is possible.

4.2.2.2 mRNA extraction and cDNA synthesis

mRNA was extracted using a Dynabeads® mRNA DIRECT™ Purification Kit (Life Technologies), following the supplier's instructions and some of the specifications of a published protocol for mRNA extraction from *Cryptosporidium parvum* (Garces-Sanchez *et al.* 2009). Combination of the two mentioned protocols led to the method presented in the following paragraph. The kit allows direct extraction of eukaryotic mRNA by its poly-(A)-tail via magnetic beads carrying an oligo (dT) extension. Dynabeads (250 µl suspension) were prepared following the supplier's protocol. The washed sample (80 µl) was transferred to a Lysis Matrix E tube (MP Biomedicals), and 1,250 µl of lysis/binding buffer was added. The tube was inverted 10 times and placed in a FastPrep-24 system (MP Biomedicals; bead beating for 60 s at a speed of 5.5 m/s) to rupture cells and solid material. Solids were spun down by centrifugation for 5 min at 20,000 *g*, and the supernatant was transferred to a 1.5 ml reaction tube containing 250 µl beads in lysis/binding buffer. The tube was placed in a laboratory shaker (MHR 11, HLC Biotech) and mixed for 7 min at room temperature at 200 rpm to allow mRNA binding to the magnetic beads. The sample was placed in the Dynal

MPC®-S Magnetic Particle Concentrator (Dynal Biotech) for 2 min to collect the beads and discard the supernatant. The mRNA bound to the beads was washed with 1 ml of washing buffer A and of washing buffer B, with each washing step performed twice. mRNA was eluted at 74 °C and 200 rpm in a laboratory shaker for 2 min in 25 µl Tris/HCL (10 mM, pH 7.5) and separated from the beads using the Dynal MPC®-S Magnetic Particle Concentrator. The elution step was repeated, and finally 50 µl of mRNA solution were obtained.

For the digestion of possibly co-extracted DNA, the extract (20 µl) was digested with the TURBO DNA-free ™ kit (Ambion) according to the suggestions of the supplier. 2 µl of 50 × Turbo DNA-free buffer and 1 µl of Turbo DNAse were added to the mRNA extract. Tubes were mixed by finger tapping and centrifuged. The reaction was performed at 37 °C for 45 min in a thermocycler (TProfessional Thermocycler by Biometra or Flexcycler by Analytik Jena) and stopped by addition of 2.3 µl Turbo DNA-free inactivation reagent. The samples were centrifuged at 11,000 *g* for 1.5 min, and the supernatants were transferred to 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). cDNA was generated from DNAse-treated mRNA (5 µl) in a reaction mixture comprising 0.6 µl of 10 µM AF-Endo reverse primer (Table 4), 2 µl of 10 mM dNTPs, and 4.4 µl of DEPC treated water (total volume, 12 µl). RNA was denatured at 65 °C for 5 min in a thermocycler (TProfessional Thermocycler by Biometra). Subsequently, the mixture was placed on ice, and 8 µl of reverse transcription master mix, containing 4 µl of 5 × cDNA synthesis buffer, 1 µl of 0.1 M DTT, 1 µl of RNase Out™ (40 U/µl), 1 µl of DEPC-water, and 1 µl of ThermoScript™ RT (15 U/µl) was added. Reverse transcription was performed for 60 min at 51 °C and terminated at 85 °C for 5 min. Synthesized cDNA was stored at −20 °C until further analysis.

4.2.3 qPCR and PCR based tools

4.2.3.1 Primer and probe development

Primer pairs and a probe were designed on the basis of alignments of target genes and outgroup sequences obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the Silva high-quality ribosomal RNA databases (http://www.arb-silva.de/) using MEGA 6.06 software (Tamura *et al.* 2013). Possible primer sites were identified with Primrose 2.17 (Ashelford *et al.* 2002). Three primer pairs and a hydrolysis probe (Table 4) were designed for the specific amplification of AF. Primers were chosen within a melting temperature (T_m) range from 55 °C to 65 °C, according to guide values (Taylor *et al.* 2010). Primer pairs were allowed to differ in T_m by ≤2 °C. Because mismatches at the 3′-end of primers prevent amplification at stringent conditions, mismatches in the last bases of the 3′-ends were strictly avoided.

For quantification, a primer pair and a corresponding 5' hydrolysis probe (AF-SSU) targeting conserved and specific signature positions of the anaerobic fungal 18S rRNA gene was developed (Table 4, Supplementary Figure 25).

The assay AF-Endo (Table 4) was designed for the specific amplification and quantification of anaerobic fungal GH5 cellulolytic endoglucanase (EC 3.2.1.4) genes and transcripts. Respective anaerobic fungal sequences were retrieved from the Carbohydrate-Active EnZymes database (http://www.cazy.org/) (Lombard *et al.* 2014). TCA was assessed by the quantification of cDNA transcribed from endoglucanase mRNA.

A primer pair (AF-LSU) for the phylogenetic classification of anaerobic fungi based on gene sequences of the large ribosomal subunit (LSU, 28S rDNA) was established (Table 4).

All oligonucleotides were checked for possible interfering self-complementary or hairpin formation using OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). *In silico* specificity of the designed oligonucleotides was verified using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the database Nucleotide collection (nr/nt). For the primer pair AF-Endo, *in silico* PCR was performed against genomes of bacteria known to express GH5 endoglucanases. *Fibrobacter succinogenes* subsp. succinogenes S85 and 56 *Clostridium* species including *Clostridium cellulolyticum* H10 and *Clostridium thermocellum* were tested for amplification with the software developed by Bikandi *et al.* (2004).

Alignments, building of phylogenetic trees, visualization of primer positions and counting of phylogenetic informative sites were performed with MEGA 6.06 (Tamura *et al.* 2013) and Geneious version 6.0.6 (Kearse *et al.* 2012).

Table 4: Primer pairs and the probe developed for the detection of AF. Position* refers to the GenBank reference sequence from the *Orpinomyces* sp. isolate OUS1 (AJ864475) Nicholson et al. (2005). Position† refers to *Neocallimastix patriciarum* cellulase (celA) mRNA (U38843). All rRNA primer positions are shown in Supplementary data Figure 25.

Oligonucleotide	Sequence 5′ --> 3′ (Position)	Tm (°C)	Amplicon size
AF-SSU forward	CTA GGG ATC GGA CGA CGT TT (75*)	59	475 bp
AF-SSU reverse	GGA CCT YCC GAT CAA GGA TG (532*)	59	
AF-SSU probe	6-FAM ATT CGC GTA ACT ATT TAG CAG GTT AAG GT-BHQ1 (369*)	62	-
AF-Endo forward	CGT ATT CCA ACY ACT TGG WSY GG (142†)	60	526 bp
AF-Endo reverse	CCR KTR TTT AAG GCA AAR TTR TAY GGA (642†)	60	
AF-LSU forward	GCT CAA AYT TGA AATCTT MAA G (1530*)	53	441 bp
AF-LSU reverse	CTT GTT AAM YRA AAA GTG CAT T (1950*)	51	

4.2.3.2 Quantification standards

For quantification with 5' hydrolysis assay AF-SSU and the EvaGreen based assay with primers AF-Endo, standards for the quantification of (c)DNA copies were generated. Specific PCR amplicons (see section 4.2.4) were cloned into competent *Escherichia coli* cells for each primer pair using the TOPO-TA® cloning kit with the PCR® 4-TOPO TA vector and OneShot® TOP10 chemically competent cells (Invitrogen). For each clone a 10 fold dilution series was quantified by parallel cell counting and most probable number (MPN) qPCR (Lebuhn *et al.* 2003; Munk *et al.* 2010), following the qPCR conditions described in sections 4.2.3.4 and 4.2.3.5. This approach allowed determining the number of positive inserts per cell, and thus, their concentration in the standard cell suspension volumes.

4.2.3.3 Analytical limits of qPCR assays

For each qPCR assay, the lower analytical limits were assessed by determination of the Limit of Blank (LoB), the Limit of Detection (LoD) and the Limit of Quantification (LoQ) according to the definitions by Francy *et al*. (2015). The LoB was defined as the lowest concentration reportable with 95 % confidence above the measured concentration of blanks. In our experiments, blanks comprised the qPCR reaction mix with Millipore^{TM} H_2O instead of a (c)DNA template (=no template controls). In case blanks did deliver a signal, the LoB was calculated as follows from the standard curve: $\log_{10}$ LoB= (($\overline{Cq}_{Blanks}$)-b)/-m ($\overline{Cq}_{Blanks}$= mean measured Cq Blanks; m= slope of regression line; b= y-axis intercept of regression line). After Francy *et al.* (2015), the "LoD is the lowest concentration that can be detected with 95 % confidence that it is a true detection and can be distinguished from the LoB." In addition, replicates of the dilution chosen as LoD had to deliver Cq values with a standard deviation below 1, and more than 95 % of the replicates had to give a detectable signal. The LoD was calculated as follows: $\log_{10}$ (LoD) = ($\overline{Cq}_{LoD}$)–b) / -m ($\overline{Cq}_{LoD}$= mean measured Cq values dilution chosen as LoD; m= slope of regression line; b= y-axis intercept of regression line). The LoD assigns a value for the lowest detectable copy number. However, for absolute quantification, defining the LoQ as the lowest concentration of copies that can accurately be

quantified is even more important. The Cq value of the LoQ (Cq_{LoQ}) was calculated from the $\overline{Cq}_{LoD}$ and the standard deviation of Cq_{LoD} ($\sigma[Cq_{LoD}]$): $Cq_{LoQ}=\overline{Cq}_{LoD}-2 \times (\sigma[Cq_{LoD}])$. The $LoQ_{conc.}$, could thus be calculated using the standard curve: $\log_{10}(LoQ_{conc.})= (Cq_{LoQ})– b) / -m$.

4.2.3.4 PCR reaction mixtures

For all PCR reactions, reagents from the Platinum® *Taq* DNA Polymerase system (Life Technologies) were used. For qPCR assays, the reaction volume was 25 µl. The basic reaction mixture contained 2.5 µl of 10 × PCR Buffer (no $MgCl_2$), 3 µl 50 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each), 10 µM forward primer, 10 µM reverse primer and 0.15 µl Platinum™ Taq DNA Polymerase (5 u/µl). In each reaction 2.5 µl of (c)DNA template were used, and Millipore™ H_2O was added to reach a total volume of 25 µl. For the assay AF-SSU, targeting the anaerobic fungal 18S rRNA gene, 1 µl of 10 µM primers were used to achieve a final concentration of 400 nM. For fluorescence detection, 1.5 µl of 10 µM AF-SSU hydrolysis probe were included. For the assay AF-Endo targeting an anaerobic fungal GH5 endoglucanase gene, 0.5 µl of 10 µM primers were applied to achieve a final concentration of 200 nM in the reaction mixture. 1 µl of 50 × EvaGreen Dye was used for fluorescent detection. The optimum concentration of primers (between 200 nM and 600 nM) derived from reactions leading to the lowest Cq and no or low production of unspecific products such as primer multimers, as visualized in melting analysis dissociation curves.

For cloning and sequencing an endpoint PCR with primer pair AF-LSU targeting the 28S rRNA gene of anaerobic fungi was performed. The reaction volume was 50 µl, and the mix comprised: 2.5 µl of 10 × PCR Buffer (no $MgCl_2$), 3 µl 50 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer and 0.15 µl Platinum™ Taq DNA Polymerase (5 u/µl). The reaction volume of 50 µl was adjusted by adding Millipore™ H_2O.

4.2.3.5 qPCR and PCR temperature programmes

For primer pair AF-SSU, a two-step qPCR program was performed, consisting of an initial denaturation / activation of 3 min at 94 °C, followed by 45 cycles comprising denaturation at 94 °C for 15 s, and combined annealing / extension at 64 °C for 1 min. For the specific primers AF-Endo, a two-step qPCR program was performed that consisted of an initial denaturation / activation for 3 min at 94 °C, followed by 45 cycles comprising denaturation at 94 °C for 15 s, combined annealing / extension at 64 °C for 1 min, 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.

For endpoint PCR with primer pair AF-LSU, a three-step PCR program was performed consisting of initial denaturation / activation for 3 min at 94 °C, followed by 35 cycles comprising denaturation at 94 °C for 20 s, annealing at 61 °C for 45 s, and extension at 72 °C for 45 s, followed by a final elongation at 72 °C for 10 min. qPCR reactions were performed on an Mx3005P qPCR System (Agilent Technologies) and PCR reactions on a TProfessional Thermocycler (Biometra) or a Flexcycler (AnalytikJena).For each primer pair, the optimum annealing temperature was defined by temperature gradient PCR (around the calculated Tm)

and visual comparison of band strength on gel electrophoresis of *Neocallimastix frontalis* and *Piromyces* sp. amplicons.

4.2.4 *In vitro* primer specificity

In addition to *in silico* primer specificity (see section 4.2.3.1), it was checked if false positive results could be obtained with the designed primer pairs and primer / probe combination. Nonspecific amplification of plant-derived nucleic acids was excluded for primer pair AF-SSU by testing against DNA from two different plant-based forages, two different cuts of perennial ryegrass and maize. Samples from pilot biogas plant 21 (PB 21) for qPCR assay AF-SSU and cattle rumen fluid for primer pairs AF-Endo and AF-LSU (see 4.2.1) were tested for positive amplification of anaerobic fungal genes. Positive amplicons were cloned, sequenced, and checked for correctness. Given that functional gene sequence database entries for anaerobic fungi are rare, primer pair AF-Endo was tested for the coverage of different anaerobic fungal reference specimens. The tested cDNA and DNA extracts included samples from the genera *Anaeromyces*, *Piromyces*, *Caecomyces*, *Cyllamyces*, and *Neocallimastix* as well as a putative novel genus (Isolate KiDo 1m).

4.2.5 Cloning and sequencing

Amplicons for cloning and sequencing were obtained by three-step PCR using Platinum® Taq DNA Polymerase (Invitrogen). Purified PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) with the pCR® 4-TOPO TA vector and OneShot® TOP10 chemically competent cells. *E. coli* clones carrying the plasmid were identified by propagation on lysogeny broth agar plates containing ampicillin. Clones were checked for the expected insert size by colony PCR using primer pair M13. Sequencing of all positive clones was performed by Eurofins MWG Operon. The received clone sequences were implemented in the existing alignments and analyzed in MEGA 6.06 or Geneious 6.06. Chimeric sequences were identified with Bellerophon (Huber *et al.* 2004) and excluded.

4.3 Results and discussion

4.3.1 PCR-based tools for quantification of anaerobic fungal 18S rRNA gene copies and transcriptional cellulolytic activity

4.3.1.1 qPCR assay AF-SSU for quantification of anaerobic fungal 18S rRNA gene copies

In PCR stringency evaluation with assay AF-SSU, amplicons sharing the correct size of 475 bp were specifically produced in an annealing temperature range between 62.5 °C and 68 °C. The brightest band intensity was generated at 64 °C. The annealing temperature was thus fixed at this temperature.

To assess the *in vitro* specificity of assay AF-SSU, DNA derived from different forages and from *Neocallimastix frontalis* was checked for amplification. An image of the gel is shown in supplementary Figure A2 (please see the online version of the article Dollhofer *et al.* (2016)). A clear band with the correct size of approximately 475 bp was visible for the *Neocallimastix frontalis* DNA. No amplification was observed for the negative control (H_2O) and for DNAs of the different forages, including two types of pig forage (FM1, FM2), two samples of perennial ryegrass (G1, G2) and a maize sample (G3).

Cloning and sequencing of DNA extracts from samples of different compartments of an agricultural biogas plant confirmed specific amplification of anaerobic fungal 18S rRNA genes with assay AF-SSU. From a total of 72 clones, 66 carried inserts of the expected size and were sequenced. Fifty-two high-quality sequences were obtained, aligned, and analyzed for their phylogenetic position. These sequences were deposited in NCBI GenBank (Accession numbers KX164294 to KX164345). Results of BLAST analysis, with the five top hits for each clone sequence, are listed in supplementary Table A1 (please see the online version of Dollhofer *et al.* (2016)). Most sequences showed 99 % sequence identity with an E-value of 0.00 to the top hit, entry AB665902, described as partial 16S rRNA gene of an uncultured rumen bacterium from sheep rumen (Fuma *et al.* 2012). Since (i) anaerobic fungal 18S rDNA is present in the sheep rumen, (ii) both primers used by Fuma *et al.*(2012), S-*-Univ-530-a-S-16 and S-*-Univ-1392-a-A-15, matched perfectly anaerobic fungal 18S rRNA genes (supplementary Table A2; please see the online version of Dollhofer *et al.* (2016)) and (iii) identical sequences were assigned to the *Neocallimastigomycota* (supplementary Table A1; please see the online version of Dollhofer *et al.* (2016)). It is obvious that amplification of anaerobic fungal 18S rDNA was the reason for incorrect annotation of sequence AB665902. As all other BLAST results were correctly assigned to anaerobic fungi, assay AF-SSU was determined to be specific for the amplification of anaerobic fungal 18S rRNA genes.

For the quantification of anaerobic fungal 18S rDNA in environmental samples, a standard for AF-SSU qPCR was established (see section 4.2.3.2). The standard was defined by measuring a dilution series of lysed recombinant *Escherichia coli* cells carrying the target sequence as an insert. The MPN qPCR results are shown in Figure 12a and the associated standard curve in Figure 12b. With an equation of Y = -3.230*LOG(X) + 38.37 (Y = fluorescence in dR; X = initial quantity of copies) and a qPCR efficiency of 104 %, the standard was acceptable for quantification (Taylor *et al.* 2010). In qPCR with method AF-SSU, 11 copies of anaerobic

fungal 18S rDNA per reaction was the lowest detectable copy number, resembling the Limit of Detection. The lowest accurately quantifiable copy number (LoQ) was 35 18S rDNA copies per reaction. Inter-assay reproducibility was calculated as 95.3 % for a standard aliquot containing 9.3 x 10^4 copies / reaction (rxn) over 7 individual qPCR runs

It is tempting to translate SSU copy numbers into cell biomass, similarly as this has been done for ITS 1 by Lwin *et al.* (2011) using the primers described by Denman and McSweeney (2006). However, this relation is influenced by many physiological factors (see Introduction), and results obtained from *in-vitro* assays may not adequately be valid for complex ecosystems such as biogas reactors, due to divergent growth conditions, potential sampling bias and differences in nucleic acid recovery rates. For these reasons we only used the qPCR determined SSU copy numbers to describe and compare the abundance of anaerobic fungi in samples, and not to translate gene copy numbers into cell biomass.

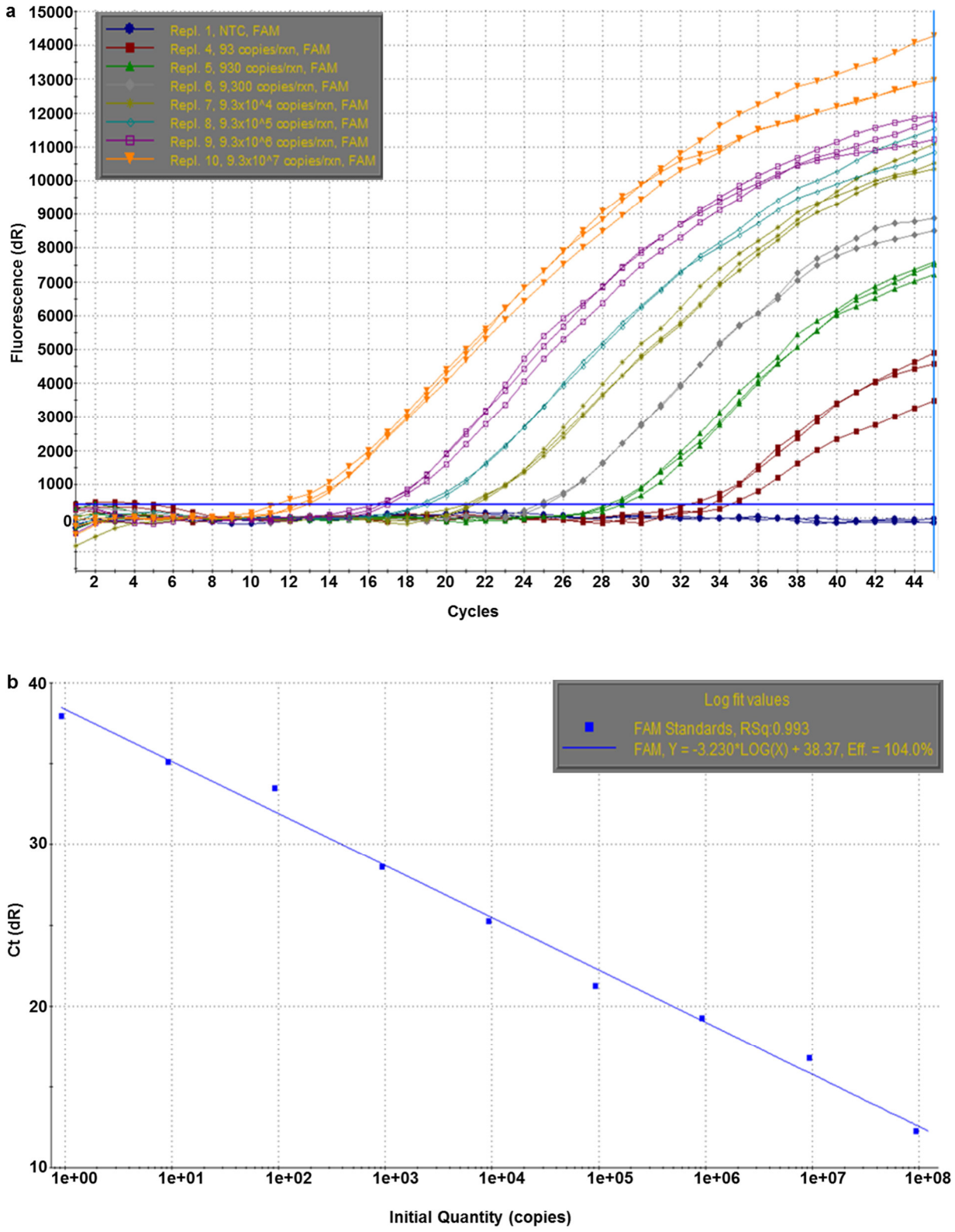


Figure 12: Real-Time qPCR amplification of a 10 fold dilution series of recombinant *Escherichia coli* cells carrying the anaerobic fungal SSU gene (a) with primer pair AF-SSU with associated standard curve (b)

In order to determine, to explain and to compare the abundance of anaerobic fungi in biogas reactors, we quantified 18S rDNA copies in DNA extracts from cattle rumen fluid (as positive control) and two cattle slurry samples used as substrates in biogas plants PB 14 and PB 22.

We measured 1.69×10^{10} (standard deviation, SD= 3.88×10^{9}) anaerobic fungal 18S rDNA copies per ml rumen fluid. In the slurry samples from PB 14 and PB 22, we detected 1.88×10^{9} (SD= 3.3×10^{8}) and 6×10^{9} (SD= 1.16×10^{9}) anaerobic fungal 18S rDNA copies x ml^{-1}. Anaerobic fungi are inhabitants of the digestive tract of herbivores and can account for 10^{6} cells per ml rumen fluid (Kumar *et al.* 2015), in addition anaerobic fungi are known to be present in animal feces (Davies *et al.* 1993). Thus, the findings of 18S rRNA gene copies in rumen fluid and cattle slurry are reasonable. Anaerobic fungal population size in the gastric, post-gastric organs and in feces was reported to be lower than in the rumen and the omasum (Davies *et al.* 1993), explaining why lower copy numbers were determined in cattle slurry than in the cattle rumen fluid sample.

4.3.1.2 qPCR assay AF-Endo for the quantification of anaerobic fungal transcriptional cellulolytic activity

Primer pair AF-Endo was designed to quantify the concentration of mRNA of glycoside hydrolases family 5 (GH5) endoglucanases (EC number 3.2.1.4) in order to estimate the transcriptional cellulolytic activity (TCA) of anaerobic fungi from environmental samples and to compare the TCA of individual strains. The functional genes and enzymes involved in lignocellulosic degradation, including the chosen GH5, have been monitored in transcriptomic studies recently published by Couger *et al.* (2015) and Solomon *et al.* (2016). Both studies show that genes encoding GH5 enzymes are transcribed on significant levels during lignocellulose degradation. Endoglucanases belonging to GH5 together with GH9 and GH45 endoglucanases represent 15 % of total endoglucanases at all tested growth conditions (Couger *et al.* 2015). Quantification using the RT-qPCR approach with primers AF-Endo can thus inform us if anaerobic fungi are actively transcribing genes for cellulose break down.

Only very few sequences of functional genes of anaerobic fungi are deposited in online databases. The AF-Endo primers were thus checked for positive amplification with cDNA from seven anaerobic fungal isolates. Figure 13 shows the amplification plots obtained with an *Neocallimastix frontalis* (Re1) DNA (as quantification standard) and cDNA from anaerobic fungal isolates assigned to the genera *Anaeromyces* (KF8, Tmc 002.28xy), *Piromyces* (KiDo 3a), *Caecomyces* (OF1), and *Cyllamyces* (KiDo 2m) as well as a putative novel bulbous genus (KiDo 1m) all resulting in a positive specific signal in qPCR with the primer pair AF-Endo. Since no isolate or DNA sample from the genera *Orpinomyces, Buwchfawromyces* or *Oontomyces* was available, their detection with the assay AF-Endo could not be proven. Bioinformatics analysis of sequences deposited in NCBI GenBank and the genome of *Orpinomyces* sp. C1A (Youssef *et al.* 2013) suggested that *Orpinomyces* sp. GH5 endoglucanase genes should be detected. However, GH5 sequences of *Buwchfawromyces* and *Oontomyces* strains were not available in online databases. Such information is needed to evaluate the applicability of the AF-Endo assay for all anaerobic fungi and possibly to improve the primers.

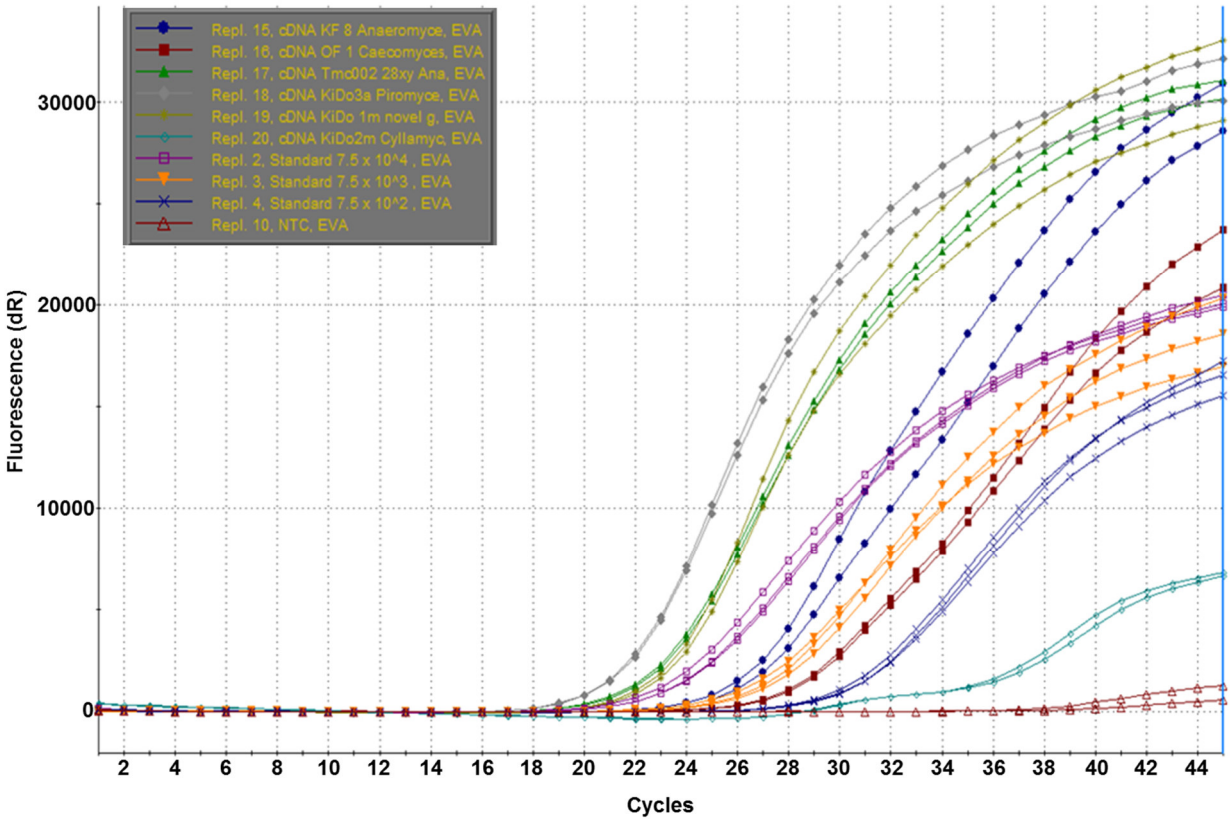


Figure 13: qPCR amplification plots with primer pair AF-Endo and cDNA of isolates of the anaerobic fungal genera *Anaeromyces* (KF8, Tmc002. 28xy), *Caecomyces* (OF1), *Piromyces* (KiDo 3a), *Cyllamyces* (KiDo 2m) a novel genus (KiDo 1m) and a *Neocallimastix frontalis* DNA derived quantification standard.

Considering that many anaerobic fungal genes for cellulolytic enzymes are obviously the result of horizontal gene transfer (Garcia-Vallve *et al.* 2000), differentiation of anaerobic fungal genes from bacterial genes encoding cellulolytic enzymes was an issue. *In silico* BLAST analysis of the AF-Endo primer pair showed that no bacterial GH5 sequences in NCBI GenBank possessed matching bases on relevant primer binding sites. For further confirmation of specificity of primer pair AF-Endo we evaluated in *in silico* PCR against the genomes of cellulolytically active bacteria like *Fibrobacter succinogenes* and 56 different *Clostridium* species, including *Clostridium cellulolyticum* and *Clostridium thermocellum*. Horizontal gene transfer for a GH5 endoglucanase has been shown for *Fibrobacter succinogenes* (Garcia-Vallve *et al.* 2000), implying that its genes might be closely related to the anaerobic fungal counterparts. No products were amplified in *in silico* PCR with primer pair AF-Endo for any of the tested genomes. This confirmed specificity of the primer pair AF-Endo for anaerobic fungal GH5 endoglucanase genes and transcripts. Another parameter supporting the specificity for anaerobic fungi is the chosen mRNA extraction method (see section 4.2.2.2). Binding of mRNA to the Dynabeads is restricted to oligo (dT) and poly-(A)-tail interactions. As polyadenylation is typical for eukaryotic mRNA, this should exclude bacterial mRNAs from extraction and thus from amplification in PCR. The *in-vitro* specificity of qPCR assay AF-Endo was tested by cloning and sequencing of DNA from cattle rumen

fluid. The resulting 15 high-quality clone sequences listed in Table 5 were assigned to anaerobic fungi and deposited in NCBI GenBank (Accession numbers KX164346 to KX164363). The most closely related bacterial sequence, with 62 % sequence identity in the BLAST results, was a representative of the family *Clostridiaceae*. With this low sequence identity, differentiation from anaerobic fungal genes was no problem.

Table 5: BLAST results of the clone sequences obtained with primer pair AF-Endo from cattle rumen fluid DNA

Name of clone	Accession nearest related	Description	Max score	Total score	Query coverage	E value	Max. identity
RF 1	AF053363	*Neocallimastix patriciarum* cellulase CelD (celD) mRNA, partial cds	580	1722	99 %	9.00E-162	87 %
RF 2	AJ277483	*Piromyces equi* mRNA for endoglucanase 5A (cel5A)	600	2394	100 %	9.00E-168	88 %
RF 3	AJ277483	*Piromyces equi* mRNA for endoglucanase 5A (cel5A)	609	2421	100 %	2.00E-170	88 %
RF 7	AJ277483	*Piromyces equi* mRNA for endoglucanase 5A (cel5A)	609	2421	100 %	2.00E-170	88 %
RF 9	AJ277483	*Piromyces equi* mRNA for endoglucanase 5A (cel5A)	600	2394	100 %	9.00E-168	88 %
RF 10	JF906704	*Neocallimastix patriciarum* isolate W5C-P putative cellulase mRNA, complete cds	634	634	99 %	5.00E-178	90 %
RF 11	AF053363	*Neocallimastix patriciarum* cellulase CelD (celD) mRNA, partial cds	587	1740	98 %	6.00E-164	88 %
RF 12	Z31364	*N. patriciarum* mRNA for endoglucanase B	493	493	96 %	1.00E-135	84 %
RF 13	AJ277483	*Piromyces equi* mRNA for endoglucanase 5A (cel5A)	600	2400	100 %	9.00E-168	88 %
RF 14	JF906704	*Neocallimastix patriciarum* isolate W5C-P putative cellulase mRNA, complete cds	639	639	99 %	1.00E-179	90 %
RF 16	AF053363	*Neocallimastix patriciarum* cellulase CelD (celD) mRNA, partial cds	576	1711	99 %	1.00E-160	87 %
RF 17	U57818	*Orpinomyces* sp. PC-2 cellulase (celB) gene, complete cds	504	504	99 %	6.00E-139	84 %
RF 19	JF906704	*Neocallimastix patriciarum* isolate W5C-P putative cellulase mRNA, complete cds	639	639	99 %	1.00E-179	90 %
RF 20	JF906704	*Neocallimastix patriciarum* isolate W5C-P putative cellulase mRNA, complete cds	506	506	98 %	2.00E-139	84 %
RF 24	JF906704	*Neocallimastix patriciarum* isolate W5C-P putative cellulase mRNA, complete cds	630	630	99 %	6.00E-177	89 %

Since only anaerobic fungal mRNA sequences were obtained from microbial biotopes with high diversity such as the rumen, this further supports that the qPCR assay AF-Endo is specific for the detection of the anaerobic fungal endoglucanase gene GH5. However, as mentioned above, the number of currently available anaerobic fungal GH5 sequences is very limited. Modification of the primers may be necessary if sequences with divergent primer binding sites are described.

A qPCR standard was defined by MPN qPCR with a dilution series of lysed recombinant *E. coli* cells carrying the target sequence as an insert. The amplification plots, the associated standard curve, and the dissociation curve are shown in Figure 14. The standard curve was defined by the equation $Y = -3.415 \times LOG(X) + 37.90$ (qPCR efficiency of 96.3 %). The Limit of Blank, the Limit of Detection and the Limit of Quantification were determined for the assay AF-Endo as described in section 4.2.3.3. For the qPCR assay with primer pair AF-Endo 0.91 endoglucanase gene copies per reaction were calculated as LoB, 7.76 copies per reaction as LoD and 13.11 copies per reaction as LoQ. Inter-assay reproducibility was calculated as 96.1 % for a standard aliquot containing 7500 copies/rxn over 7 individual qPCR runs. The annealing temperature was set to 64 °C according to the results of temperature gradient analysis. Melting curve analysis showed a specific peak at 86 °C, with a minimal unspecific primer peak between 74 °C and 80 °C. The fluorescence signal of this peak was not quantified owing to introduction of a denaturation step at 82 °C prior to fluorescence measurement in each cycle.

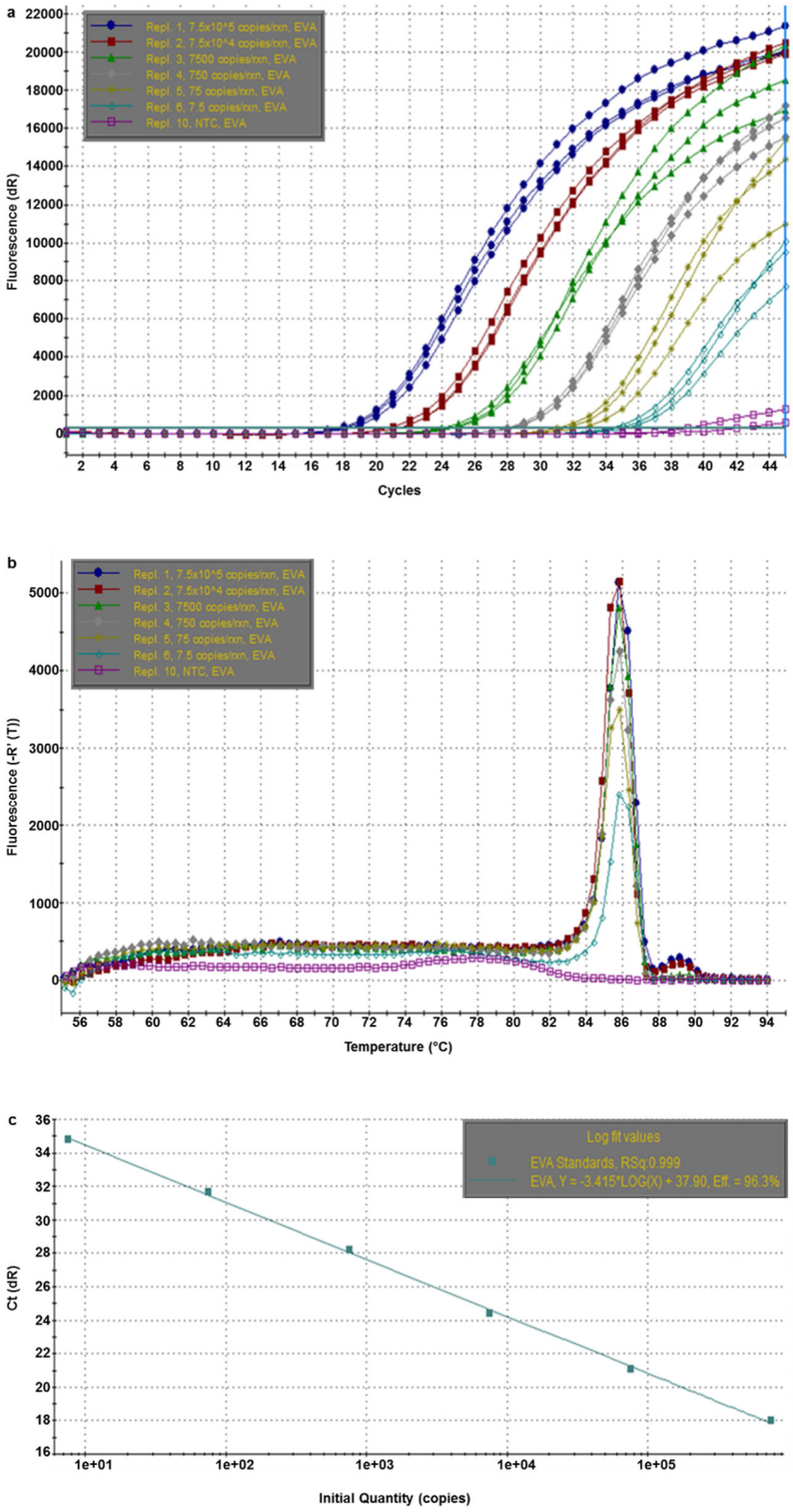


Figure 14: Real-Time qPCR amplification of a dilution series of recombinant *Escherichia coli* cells carrying an anaerobic fungal GH5 endoglucanase gene (a) with primer pair AF-Endo with associated dissociation curve (b) and standard curve (c)

Assuming 100 % mRNA extraction efficiency 6.65×10^2 to 1.28×10^6 anaerobic fungal GH5 endoglucanase transcript copies per ml of culture medium were determined (Figure 15) for the

tested actively growing anaerobic fungal isolates, mentioned above (Figure 13). For two of the tested anaerobic fungal isolates, Tmc 002 28xy and KiDo 1h, endoglucanase transcripts were measured from biological replicates after incubation at 39 °C for 72 h and showed satisfactory accordance (supplementary Figure A3; please see the online version of Dollhofer *et al.* (2016)). These values may systematically be underestimated since the extraction efficiency of the used method could not be assessed yet, but efficiencies between 5-70 % have been reported (Lebuhn *et al.* 2016). As the qPCR approach AF-Endo is used to compare the TCA levels of anaerobic fungi between samples with similar sample constitution, relative differences between results for individual samples remain invariable. Thus, defining the extraction efficiency is not necessary in this case. For absolute values, however, the extraction efficiency constant should be considered.

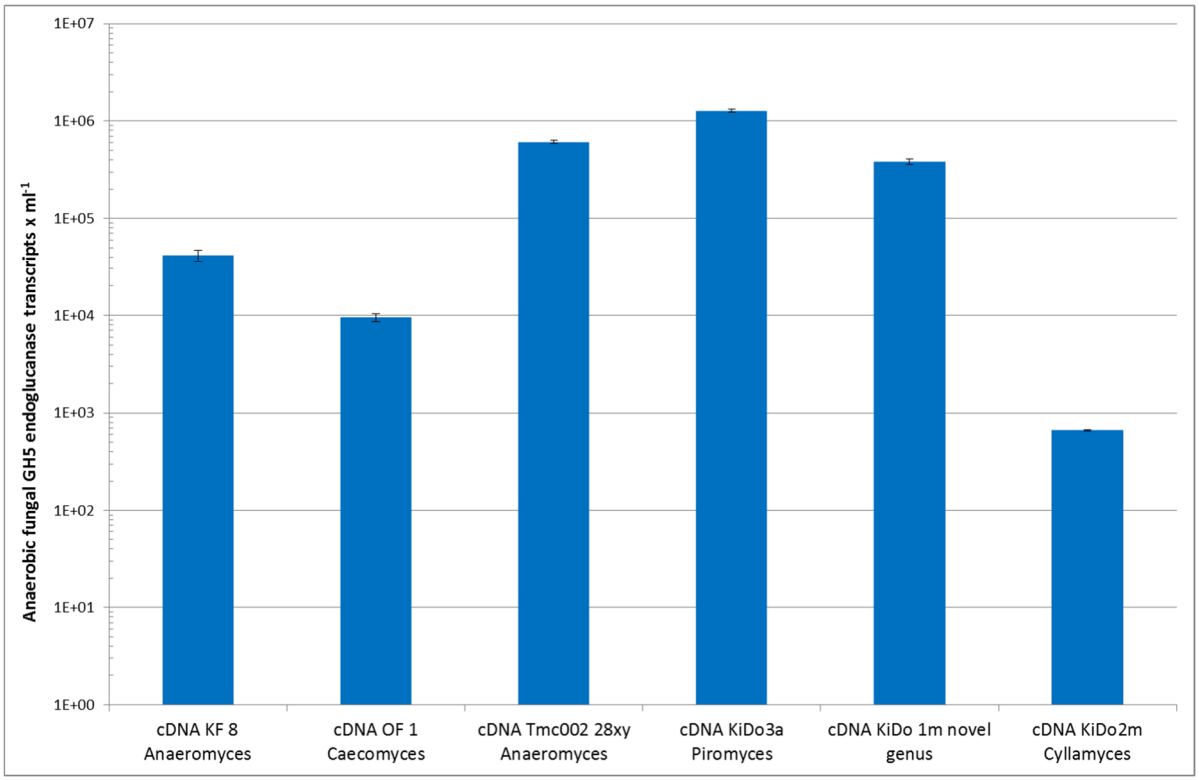


Figure 15: Quantification of endoglucanase transcripts with primer pair AF-Endo in actively growing cultures of the genera *Anaeromyces* (KF 8, Tmc 002 28xy), *Caecomyces* (OF 1), *Piromyces* (KiDo 3a), *Cyllamyces* (KiDo 2m) and a putative novel genus (KiDo 1m). Error bars denote averaging deviations

4.3.1.3 Primer pair AF-LSU for the assessment of anaerobic fungal phylogeny

Temperature gradient PCR with primer pair AF-LSU produced amplification signals from a *Piromyces communis* (isolate P, see Table 3) DNA in a temperature range between 50 °C and 62.1 °C. Since optimum amplification was found at an annealing temperature of 61 °C, this was used for subsequent reactions. The specificity of primer pair AF-LSU for the anaerobic fungal 28S rRNA gene was evaluated by cloning and sequencing of DNA from cattle rumen fluid and from biogas plant sludges. A total 40 clone sequences were analyzed, and all were

assigned to anaerobic fungi by phylogenetic analysis. A maximum likelihood tree of anaerobic fungal 28S rRNA gene sequences deposited in online databases (n=89) including the 10 clone sequences from cattle rumen fluid DNA, (Accession numbers KX164364-KX164373, described as "Rumen Fluid clone") and 30 from the biogas reactor of pilot biogas plant 25 (Accession numbers KX164374-KX164403, described as "Biogas.Clone") are shown in Figure 16. In order to improve visualization, clones with identical sequences are not shown, only representative sequences are displayed. Supplementary Table A3 (please see the online version of Dollhofer *et al.* (2016)) shows details of all reference sequences used in Figure 16 along with the rumen fluid and biogas clones included in the tree.

Most of the clone sequences from cattle rumen fluid clustered closely to the genera *Caecomyces*, *Piromyces* and *Anaeromyces* (Figure 16). Sequences grouping with clone AF-LSU-RF-3 appeared most closely related to those of the genus *Piromyces.* However, they formed a distinct clade (BS=91) and may represent a novel species. Clone sequence AF-LSU-RF 1, grouped distinctly with sequence JF848540 from an unclassified anaerobic fungus from a sheep and appeared basal in the tree. This clade is also a candidate for a new species. Clone AF-LSU-RF-2 clustered within the *Orpinomyces* sp. clade and clone AF-LSU-RF-5 between *Caecomyces* and *Cyllamyces*. Results for the clone sequences derived from the biogas reactor PB 25 are discussed in section 4.3.2.

Overall, phylogenetic analysis of the AF-LSU amplicons showed good resolution of all tested anaerobic fungal genera. The alignment with 125 LSU sequences of cultured anaerobic fungi and the clone sequences mentioned above spanned a length of 447 bp containing 97 parsimony informative sites (see supplementary alignment LSUtree.fas in the online version of Dollhofer *et al.* (2016)). The LSU alignment consisted of 322 conserved and 123 variable sites. The AF-LSU primers showed specific amplification of anaerobic fungal 28S rRNA genes. This is an advantage compared with the phylogenetic approach presented by Dagar *et al.* (2011) which is based on primers developed by O'Donnell *et al.* (1992) covering the complete Fungi kingdom. Moreover, alignment construction with AF-LSU amplicons is much easier than with ITS 1 sequences and insertion of gaps is much less frequent (See 4.3.1.4).

4.3.1.4 Comparison of LSU and ITS 1 trees

As mentioned in the introduction, phylogenetic analysis of anaerobic fungi has mostly been done by sequencing of the ITS 1 region, which is also a conventionally used phylogenetic marker for the whole kingdom Fungi. In order to evaluate the suitability of the AF-LSU amplicons phylogeny assessment, we compared the alignments and phylogenetic tree obtained with AF-LSU (Figure 16 and supplementary alignment LSUtreeAlignment.fas in the online version of Dollhofer *et al.* (2016)) with a corresponding ITS 1 based alignment and tree (supplementary alignment ITS 1treeAlignment.fas and supplementary Figure A4 in the online version of Dollhofer *et al.* (2016)). For better comparison, sequences from isolates that appear in the ITS 1 and in the AF-LSU tree are labeled in Figure 16. The alignment of 369 ITS 1 sequences of cultured anaerobic fungi spanned 510 bp containing 329 parsimony informative sites. Due to the high sequence variability in the alignment only 80 conserved sites were observed. The alignment appeared very 'gappy' due to the high degree of heterogeneity

between the sequences and the presence of large insertions / deletions in some sequences and most of the parismony informative sites were dominated by gaps greatly reducing their informative character. The AF-LSU alignment had much less gaps and, as shown above, was much more conserved with sequences showing a higher similarity to eachother. However, even with the higher degree of similarity, individual genera and species could be resolved using the AF-LSU primer assay: e.g. in AF-LSU tree, the genus *Orpinomyces* was separated into several clades. *Orpinomyces* sp. isolates C1B and C1A (Accession numbers JN939128 and JN939127) formed a distinct clade (Support. BS=96 %) and this clade was also significant in the ITS 1 tree (BS=73 %). All other *Orpinomyces* isolates group similarly in the LSU and the ITS 1 tree. LSU based species specific clustering in the genus *Orpinomyces* has also previously been reported by Dagar *et al*. (2011). The two significant *Neocallimastix* sp. clades (BS=86 %) visible in the LSU tree are not clearly distinguished in the ITS 1 tree. In general, the ITS 1 tree showed a complicated picture, e.g for the genus *Neocallimastix,* which formed several distinct clades. This could be partly due to misidentified sequences which have been deposited in NCBI GenBank (Gruninger *et al.* 2014), but also the high degree of size (≥ 20 bp based on ITS 1 ARISA data) and sequence polymorphisms within a single strain (Edwards *et al.* 2008; Callaghan 2014) may contribute. For example comparison of ITS 1 clones from a single *N. cameroonii* isolate (CaDo3a) showed a minimal sequence identity of 93.6 %, clones from a single *Cyllamyces* sp. (KiDo 2m) had minimum of 92.3 % identity and clones from a single *Buwchfawromyces* eastonii isolate (GE09) had a minimum identity of 88.5 %. Sequence analysis of the anaerobic fungal LSU gene however, showed no or little variation between clone sequences (e.g. ≥ 98.9 % identity in isolate KiDo2M).

The bulbous anaerobic fungi also formed several divergent clades in the ITS 1 tree, whereas the LSU tree shows them forming a distinct group with *Cyllamyces* and *Caecomyces* forming separate branches. For isolates in the divergent ITS 1 clades no corresponding LSU sequences were available. Were we have corresponding ITS 1 and LSU sequences for the same isolate, these form a distinct bulbous fungal clade with the *Cyllamyces* and *Caecomyces* genera separate. On the LSU tree *Cyllamyces sp.* isolate KiDo 2M is close to the type *C. aberensis* isolate and on the ITS 1 tree it appears to be supported within the *Cyllamyces* genus. However, on the ITS 1 tree there are several (potentially miss identified) *Caecomyces sp.* isolates which fall in between. The 14 KiDo 2M clones which are shown on the tree illustrate the difficulty in interpreting ITS 1 trees. Since these sequences are all from one single isolate they still form two distinct branches with significant bootstrap support. Reasons for the highly divergent *Caecomyces* and *Cyllamyces* clades in the ITS 1 tree are unknown, but this may partly be explained by reasons mentioned previously. Additionally Edwards *et al*. (2008) found two *Caecomyces sp.* isolates (GE42 and Isol1) that produced ITS 1 based ARISA fragments which were quite dissimilar in terms of their size.

Sequences assigned to the genus *Piromyces* formed a discrete clade both, in the LSU and mostly in the ITS 1 tree. The isolates which appear distinct based on the LSU tree also resolve on the ITS 1 tree namely isolate Prl which is part of a separate clade on both trees. The genus *Anaeromyces* formed a discrete clade in the LSU and mostly in the ITS 1 tree. However, the *Anaeromyces sp.* isolate BRL-3 (Accession JX017318) clustered within the *Anaeromyces*

clade on the LSU tree but was found in a separate cluster with other *Anaeromyces* sp. sequences in the ITS 1 tree (Accession JQ326215). This cluster is split from the main *Anaeromyces* clade by the *Oontomyces* sp. genus. Sequences from this cluster show a high degree of polymorphism between base pairs ca. 246-291 in the ITS 1 alignment (see supplementary alignment ITS 1tree.fas in the online version of Dollhofer *et al.* (2016)). Due to the heterogeneity of the ITS 1 region and the lack of information on this sequence, we cannot say if the observed variability is unique to this isolate or if it was only the particular clone that was used containing these insertions.

The observed high level of inter- and intra-genomic ITS 1 heterogeneity among ITS 1 sequences of the anaerobic fungi is not a new finding (Hausner *et al.* 2000; Nicholson *et al.* 2005; Eckart *et al.* 2010; Callaghan *et al.* 2015). The high level of inter-genomic (between genera) variation makes determination of phylogenetic relationships complicated, and this coupled with the high level of intra-genomic (within a single genome) variation makes phylogenetic analysis and determination of the identity and affiliation of a single strain difficult and time consuming. Since clones can display different ITS 1 sequences comparisons between researchers is hampered. In a phylogenetic tree, it is difficult to determine which diversity is due to inter-genomic heterogeneity and which is due to intra-genomic heterogeneity. The result of these two types of variation is the ambigious anaerobic fungal ITS 1 alignment (Supplementary alignment ITS 1tree.fas in the online version of Dollhofer *et al.* (2016)), with many insertions and deletions leading to an unreliable phylogenetic tree (see supplementary phylogenetic tree in Figure A4 in the online version of Dollhofer *et al.* (2016)). In comparison to phylogenetic analysis with the ITS 1 region, no misleading assignment owing to excessive intra-genomic variation was observed in LSU gene phylogeny.

The AF-LSU specific primer pair is thus more suitable for community analysis from environmental samples and to classify pure cultures of anaerobic fungi. Improvement of the anaerobic fungal sequence collection and sequencing of well described reference strains will further increase the phylogenetic significance and resolution of the AF-LSU assay, and consolidate the tree topology.

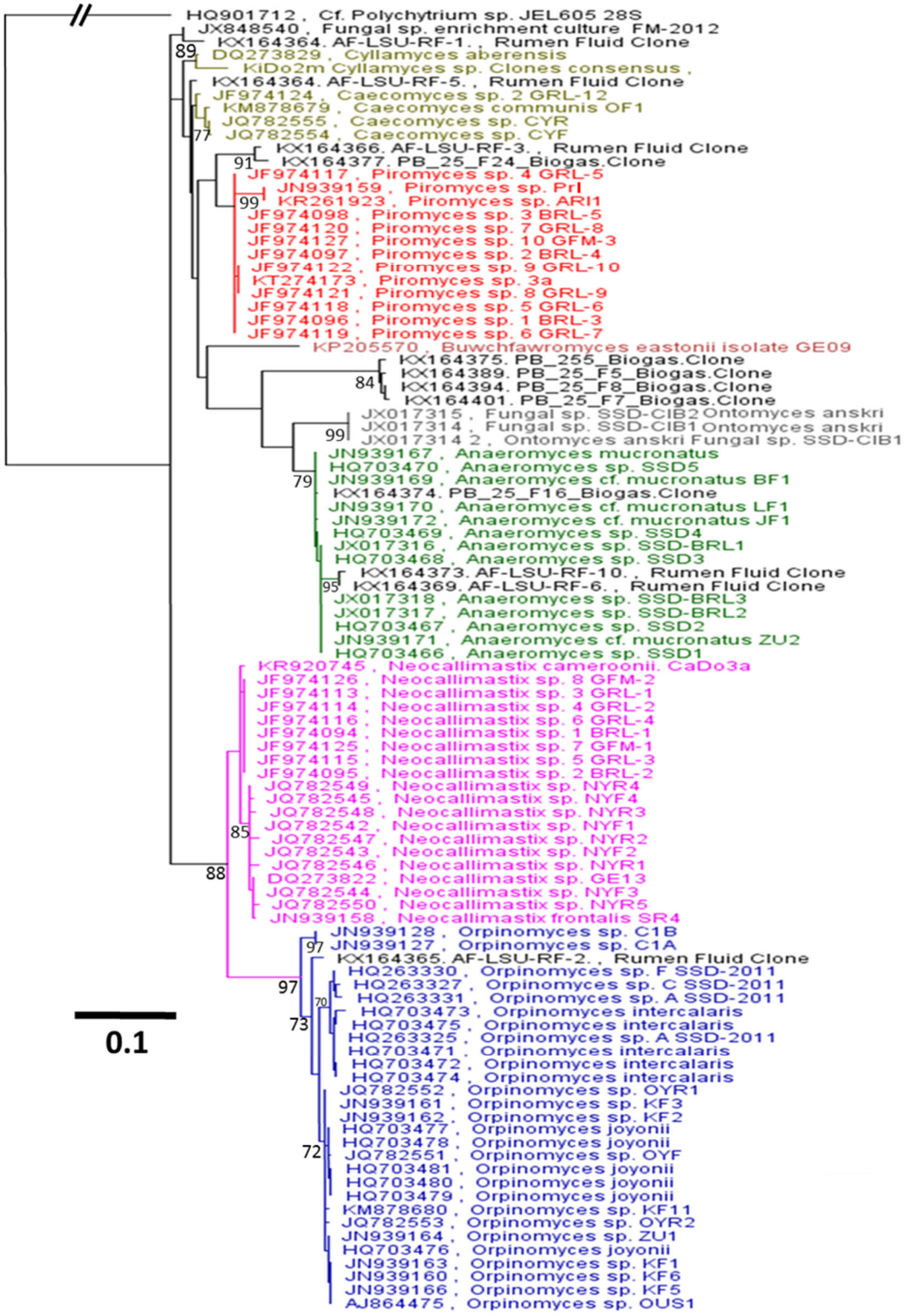


Figure 16: Maximum likelihood tree based on alignment (447 bp) of the 28S rRNA gene of all anaerobic fungal sequences available on NCBI GenBank along with clones derived from cattle rumen fluid and from the biogas reactor of PB 25 (marked black) sequenced in this study. An aerobic chytrid *Polychytrium sp.* (HQ901712) was used to root the tree and bootstrap values over 70 % are shown (1000 replicates). Scale bar shows substitutions per site. The different genera are colour coded; *Piromyces*, *Oontomyces*, *Anaeromyces*, the bulbous fungi *Caecomyces* and *Cyllamyces*, *Buwchfawromyces*, *Neocallimastix* and *Orpinomyces*.

4.3.2 Showcase analysis of agricultural biogas plant 25 with all three tools

In order to give an example of the performance of our three molecular tools, we analyzed the fermenter sludge of an agricultural biogas plant with all three assays.

The chosen biogas plant number 25 (PB25) consisted of one fermenter (700 m^3), one secondary fermenter (550 m^3) and a final repository (1600 m^3) and was operated at 40 °C. The sampled fermenter was fed with different plant biomass (see 4.2.1) and cattle slurry representing 44 % of the total substrate. We found 1.78 x 10^8 18S rRNA gene copies per ml fermenter sludge with qPCR assay AF-SSU. This is a lower concentration of anaerobic fungal 18S rRNA gene copies than was detected in rumen fluid (1.69 × 10^{10} copies/ml) or cattle slurry (1.88 x 10^9 and 6 × 10^9 copies/ml). The detection of ribosomal genes of anaerobic fungi outside of their natural habitat, the digestive tract of herbivores, is not novel. Several researchers have detected anaerobic fungal genes in non-gut environments such as landfill sites (Lockhart *et al.* 2006; McDonald *et al.* 2012). Kazda *et al.* (2014) also identified anaerobic fungi in two biogas plants in Germany using a ITS 1 based clone library method.

This evidence for anaerobic fungi in non-gut environments has thus far been DNA based and does not indicate if the *Neocallimastigomycota* present were active or viable. This matters as well for our finding of 18S rDNA in PB 25, as assay AF-Endo did not give a signal for TCA of anaerobic fungi (not shown). However, in a screening of several different Bavarian agricultural biogas plants, we detected TCA signals of anaerobic fungi in some samples (this data will be presented in a future publication). Biogas reactors are densely colonized by cellulolytic bacteria, and it has been shown that bacterial GH5 endoglucanases are significant in these environments (Wei *et al.* 2015). The negative result with primer pair AF-Endo in this reactor additionally confirms the specificity of our method, since no bacterial GH5s were amplified in this sample. Due to the fact that a high amount of animal derived substrate was used to operate the fermenter, anaerobic fungal DNA or cells were probably transferred into the biogas plant via the fed cattle slurry. Even if the process temperature of 40 °C lies near to the optimum growth temperature of anaerobic fungi, they apparently did not stay transcriptionally active.

Using assay AF-LSU, we analyzed the anaerobic fungal community in the PB 25 fermenter sludge. From the total 32 clones produced, 30 resulted in high quality sequences suitable for sequence analysis. The majority of the sequences (90 %) formed an individual clade representing a putative novel genus which was supported by high bootstrap values (BS=84) (Figure 16). Two clone sequences (PB_25_F24 and F27) clustered together with three clones derived from rumen fluid near to the genus *Piromyces,* as mentioned above this clade may representing a novel species or genus (BS=91). Clone sequence PB_25_F16 clustered within the *Anaeromyces* sp. clade near to the species *Anaeromyces mucronatus*. All LSU sequences obtained from PB 25 were marked black and described as “Biogas.Clone” in the presented phylogenetic tree (Figure 16).

4.4 Conclusion

Our objective was to create easy to-use and specific PCR-based detection methods for anaerobic fungi, enabling the investigation of environmental samples and monitoring of anaerobic fungal populations and their transcriptional activity. We developed three PCR-based detection methods suitable for; the determination of the anaerobic fungal 18S rRNA gene abundance (AF-SSU), the cellulolytic transcriptional activity (AF-Endo) and for community structure analysis and phylogenetic placement (AF-LSU). The AF-SSU primers are based on the currently available 18S rRNA gene sequence data from anaerobic fungi and proved to be specific for the phylum *Neocallimastigomycota*. It allows the determination of anaerobic fungal 18S rRNA gene copy numbers within a sample. The method can be used to compare anaerobic fungal loads among biogas reactors but not to quantify exact quanties of biomass. Further work is needed to explore the relationship between biomass and 18S rRNA gene copy numbers.

With primer pair AF-Endo targeting an anaerobic fungal endoglucanase (GH5) gene, we developed the first quantification method for anaerobic fungal transcriptional activity of a cellulolytic gene. This allows to determine whether anaerobic fungi are actively growing and participating in cellulose degradation in a given sample or habitat. Most if not all of the hitherto known anaerobic fungal genera can be detected with primer pair AF-Endo, and it provides sufficient differentiation from bacterial cellulolytic genes. With more anaerobic fungal genome information and sequences from reference strains, the primer pair AF-Endo may have to be adjusted.

The AF-LSU method targeting the 28S rRNA gene of anaerobic fungi allowed the specific detection and plylogenetic placement of AF. In a comparison of different methods of phylogenetic analysis, including the highly variable ITS 1 region and the too conserved anaerobic fungal 18S rRNA gene, the 28S rRNA gene appears to constitute a consistent and a more reliable phylogenetic barcode, which is able (at least occasionally) to differentiate between anaerobic fungi at the species level, as shown for the genera *Orpinomyces (Orpinomyces intercalaris* and *Orpinomyces joyonii)* and *Neocallimastix (Neocallimastix cameroonii and Neocallimastix frontalis).* Additionally, further work is needed to fully characterize ITS 1 intra and inter genomic heterogeneity among the anaerobic fungi so that previous studies can be better understood and integrated with this new barcode. With the growing pool of anaerobic fungal sequences and the creation of anaerobic fungal reference strains, 28S rRNA-based phylogeny will be useful to barcode and phylogenetically identify anaerobic fungal species. In summary, the three PCR approaches described in this study represent useful tools for the specific detection, quantification, phylogenetic placement and indication of transcriptional activity of AF.

Chapter 5

Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants[3]

Bioaugmentation with anaerobic fungi is promising for improved biogas generation from lignocelluloses-rich substrates. However, before implementing anaerobic fungi into biogas processes it is necessary to investigate their natural occurrence, community structure and transcriptional activity in agricultural biogas plants. Thus, anaerobic fungi 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 anaerobic fungi. (iii) Phylogeny of the anaerobic fungal community was inferred with the 28S gene. A new *Piromyces* species was isolated from a PCR-positive digester. Evidence for anaerobic fungi was only found in biogas plants operated with high proportions of animal feces. Thus, anaerobic fungi were most likely transferred into digesters with animal derived substrates. Additionally, high process temperatures in combination with long retention times seemed to impede anaerobic fungal survival and activity.

[3] Was published in a similar version by Dollhofer, V., Callaghan, T.M., Griffith, G.W., Lebuhn, M., Bauer, J. 2017. Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants. Bioresource Technology, 235, 131-139.

5.1 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 and Helliwell 2016) but this conflict can be avoided by use of waste 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 (Procházka *et al.* 2012; Christy *et al.* 2014). 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 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 (Procházka *et al.* 2012; Gruninger *et al.* 2014). 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 anaerobic fungi exists in the herbivore gut (Kittelmann *et al.* 2013), wherein anaerobic fungi 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 and Gilbert 2010). Carbohydrate active enzymes and cellulosomes have to date been identified in most anaerobic fungi (Chen *et al.* 1995; Hodrova *et al.* 1998; Steenbakkers *et al.* 2002; Harhangi *et al.* 2003). 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). Anaerobic fungi 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 1979b), 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 anaerobic fungi 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 anaerobic

fungi 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 anaerobic fungi to improve fiber digestion. However, before bioaugmentation may be expanded to current full-scale biogas plants, it is first important to determine if anaerobic fungi are already present and particularly whether they are metabolically active in existing biogas reactors. Kazda *et al.* (2014) demonstrated the occurrence of anaerobic fungal 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 anaerobic fungi 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 anaerobic fungal 18S rRNA gene copies determines the relative abundance of anaerobic fungi within examined samples. (2) PCR assay AF-LSU specifically targeting the gene of the phylogenetically informative (Dagar *et al.* 2011; Callaghan *et al.* 2015; Wang *et al.* 2017) large ribosomal subunit (LSU, 28S rRNA) of AF. The 28S rRNA gene delivers good phylogenetic resolution of the known anaerobic fungal genera and even below, and is becoming the new gold standard for taxonomic identification of the anaerobic fungi (Dagar *et al.* 2011; Callaghan *et al.* 2015; Wang *et al.* 2017). Compared to phylogenetic analysis of anaerobic fungal communities with the to date mostly used ITS 1 (Liggenstoffer *et al.* 2010) anaerobic fungal LSU sequences are less variable, produce unequivocal results, and are thus easier to analyze. (3) qPCR assay AF-Endo specifically targeting an anaerobic fungal 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 anaerobic fungi 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 anaerobic fungi but also which species are present and how transcriptionally active they are. Further, a cultivation based assay was performed on two anaerobic fungal positive digesters to see if isolation of anaerobic fungi is possible. Thus the main goals of this study were to determine if (1) anaerobic fungi are native part of the biogas producing community, (2) which anaerobic fungi are present in the tested biogas digesters and (3) if the detected anaerobic fungi were transcriptionally active in cellulose degradation.

5.2 Material and methods

5.2.1 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 6). Samples were quickly transported to the laboratory in insulated sealed containers, and nucleic acids were immediately extracted.

Table 6: Technical specifications of sampled biogas plants

Biogas plant - ID	**PB 10**	**PB 14**	**PB 16**	**PB 18**	**PB 21**
Digester volume [m^3]	800	800	900	1,200	2 x 1,200 parallel
Post-digester volume [m^3]	850	800	2,280	absent	2400
Final repository volume [m^3]	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 % MS	6.7 % MS	7.9 % MS	3.0 % MS	-
	7 % GS	8.5 % GS	59.1 % CGS	64.5 % GS	68.6 % GS
	5 % WPS	1.8 % WPS	-	-	-
	3 % CCM	0.7 % CCM	-	-	-
		0.2 % grain	0.8 % grain	-	2 % grain
Animal-derived substrates	37 % CM	72 % CM	32.2 % CM	-	21.6 % CM
	3 % CS	10.1 % CS	-	32.5 % CS	7.8 % CS
Sampled compartment	D	D	D	FR	D1, D2, PD
Biogas plant - ID	**PB 22**	**PB 25**	**PB 15**	**PB 17**	**PB 19**
Digester volume [m^3]	2 x 115 parallel	700	800	1,200	1,200
Post-digester volume [m^3]	absent	550	-	1,200	600
Final repository volume [m^3]	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 % MS	35.1 % MS	17.9 % MS	6.0 % MS	20 % MS
	1.3 % GS	8.8 % GS	77.3 % CGS	56 % CGS	44 % GS
	-	2.2 % WPS	0.4 % WPS	-	-
	-	-	-	1 % CCM	-
	0.7 % grain	2.4 % grain	1.0 % grain	2 % grain	-
	-	6.7 % sugar beets	1.8 % topinambur	-	-
Animal-derived substrates	77.3 % CM	-	-	18 % CM	-
	1.7 % CS	44.9 % CS	-	-	36 % CS
	-	-	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; MS = maize silage; GS = grass silage; CGS = clover-grass silage; WPS = whole plant silage; CCM = corn-cob-mix; CM = cattle manure; CS = cattle slurry. * =yearly mean value

5.2.2 Extraction of nucleic acids

5.2.2.1 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 ml 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 ml MilliporeTM 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).

5.2.2.2 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 ml of washed sample (see 5.2.2.1) were transferred to a Lysis Matrix E tube (MP Biomedicals) and lysed in 1250 ml 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 ml 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 ml Tris/HCL and separated from the beads by the magnet. The elution step was repeated, resulting in a final volume of 50 ml mRNA extract.

5.2.3 cDNA synthesis

Following the suppliers protocol, 20 ml of mRNA extract were digested with 2 ml of 50x Turbo DNA-free Buffer and 1 ml 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 ml 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 ml of DNA-free mRNA were added to 0.6 ml primer AF-Endo reverse (see Section 5.2.4), 2 ml of 10 mM dNTP Mix and 4.4 ml of DEPC-water, leading to a total volume of 12 ml. After RNA denaturation at 65 °C for 5 min in a thermocycler (Tprofessional Thermocycler by Biometra), the mixture was placed on ice, and 8 ml of reverse transcription mastermix comprising 4 ml 5x cDNA synthesis buffer, 1 ml 0.1 M DTT, 1 ml RNase Out™ (40 U/ml), 1 ml DEPC-water and 1 ml of ThermoScript™ RT (15 U/ml), 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.

5.2.4 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'-CTAGGGATCGGAC GACGTTT-3'; AF-SSU reverse: 5'-GGACCTYCCGATCAAGGATG-3') and probe AF-SSU (5'-FAM-ATTCGCGTAACTAT-TTAGCAGGTTAAGGT-BHQ1-3') were used. qPCR reactions with assay AF-SSU were performed in a reaction volume of 25 ml consisting of: 2.5 ml 10 x PCR buffer (no $MgCl_2$), 3 ml 50 mM $MgCl_2$, 0.5 ml dNTPs (10 mM each), 1 ml 10mM primer AF-SSU forward, 1 ml 10mM primer AF-SSU reverse (final primer concentration of 400 nM), 1.5 ml 10 mM AF-SSU probe, 0.15 ml Platinum™ Taq DNA Polymerase (5 U/ml), 2.5 ml of DNA template and Millipore™ H_2O to reach the total volume of 25 ml. 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 cellulolytic endoglucanase (EC 3.2.1.4) of the GH5 were quantified in a qPCR assay with primer pair AF-Endo (AF-Endo forward: 5'- CGTATTCC-AACYACTTGGWSYGG-3'; AF-Endo reverse: 5'-CCRKTRTTTAAGGCAAARTTRTAY-GGA-3'). qPCR reactions with assay AF-Endo were performed in a reaction volume of 25 ml consisting of: 2.5 ml 10 x PCR buffer (no $MgCl_2$), 3 ml 50 mM $MgCl_2$, 0.5 ml dNTPs (10 mM each), 0.5 ml 10 mM primer AF-Endo forward, 0.5 ml 10 mM primer AF-Endo reverse (final primer concentration of 200 nM), 1 ml EvaGreen Dye, 0.15 ml Platinum™ Taq DNA Polymerase (5 U/ml) and 2.5 ml of cDNA template. The reaction volume was adjusted to 25 ml 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 x 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 x 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 anaerobic fungal 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 anaerobic fungi 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 ml. The reaction mix consisted of: 2.5 ml 10 x PCR buffer (no $MgCl_2$), 3 ml 50 mM $MgCl_2$, 0.5 ml dNTPs (10 mM each), 1 ml 10 mM primer AF-LSU forward, 1 ml 10mM primer AF-LSU reverse (final primer concentration of 200 nM), 0.15 ml Platinum™ Taq DNA Polymerase (5 U/ml) and 5 ml of DNA template. Millipore™ H_2O was added to reach the total volume of 50 ml. 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).

5.2.5 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.

5.2.6 Isolation of anaerobic fungi

Isolation of anaerobic fungi 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 (AnaeroGenTM 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 anaerobic fungi 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 anaerobic fungi were obtained through inoculation of agar-containing roll-tubes comprising only the soluble carbon sources xylan (0.3 %) and cellobiose (0.3 %) (Joblin 1981; Callaghan *et al.* 2015). Individual fungal colonies were picked and new enrichment cultures were inoculated. This allowed separating different colony types from each other. Rolltubing was repeated several times to ensure that anaerobic fungal cultures were pure. The isolates were identified morphologically under the microscope and through sequencing of their 28S rRNA gene (see 5.2.4 and 5.2.5) from extracted DNA.

5.3 Results and discussion

5.3.1 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 5.2.4). Anaerobic fungal 18S rDNA was detected in seven of the sampled biogas plants (Figure 17); 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 anaerobic fungal 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 17). Anaerobic fungal 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. Anaerobic fungi generally occurred at levels 10 to 100-fold lower than in the rumen or cattle slurry. However, the lower quantity of anaerobic fungi in the biogas plants does not exclude a function of anaerobic fungi in the biogas process, as anaerobic fungi 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, anaerobic fungi have most commonly been isolated from the digestive tracts of larger mammalian herbivores, but recently evidence for the occurrence of anaerobic fungi outside such habitats are accumulating. Their occurrence seems to be widespread ranging from the reptile gut (Liggenstoffer *et al.* 2010), to pond sediments (Wubah and 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 anaerobic fungi in these habitats. Several anaerobic fungal species produce aerotolerant resting spores enabling them to endure inhospitable aerobic conditions (Wubah *et al.* 1991; Brookman *et al.* 2000b), allowing propagation of anaerobic fungi between host animals (Milne *et al.* 1989). The persistence of aerotolerant anaerobic fungal resting stages could be a possible explanation for their detection in atypical habitats. Anaerobic fungal 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 anaerobic fungal 18S rDNA was found in PB 22, which also received the highest input of cattle manure (77.3 %). Anaerobic fungi 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 anaerobic fungal presence (see above). In biogas plants operated with no (PB 15) or relatively low amounts of cattle manure (18 % in PB 17), no anaerobic fungi were detected. Despite a moderate input of cattle slurry (36 %), no anaerobic fungi were detected in biogas plant PB 19. As discussed later, the absence of anaerobic fungi was likely due to their long exposition (HRT = 90 days) to the high process temperature (52 °C) in PB 19 digester (Table 6). Taken together this suggests that anaerobic fungi detected in the digesters originated from the constant input of animal derived substrates. Since detection of anaerobic fungal 18S rDNA does not prove that the detected anaerobic fungi are viable and active in the biogas production process, metabolic activity of anaerobic fungi was determined by quantification of transcripts of a GH5 endoglucanase gene. Endoglucanases hydrolyze noncrystalline cellulose and have been shown to be part of the enzymatic lignocellulose degradation machinery of anaerobic fungi (Couger *et al.* 2015; Solomon *et al.* 2016). Detection of GH5 endoglucanase transcripts thus indicates the presence of viable anaerobic fungi, being active in cellulose degradation.

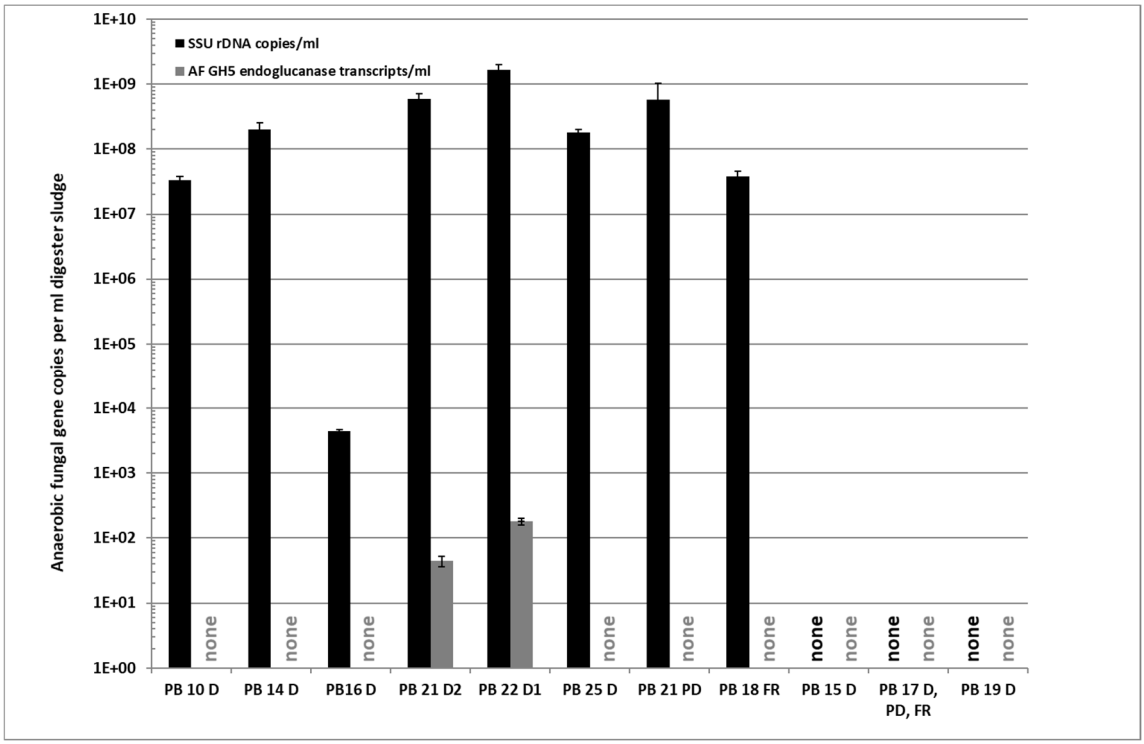


Figure 17: Number of anaerobic fungal SSU rDNA gene copies (black) and GH5 endoglucanase transcripts (grey) detected per ml fermenter sludge or digestate of anaerobic fungi positive biogas plants (D = digester; PD = post-digester; FR = final repository; none = not detected).

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 4.46 x 10^1 GH5 transcripts/ml and 1.8 x 10^2 GH5 transcripts/ml were found. Transcript numbers were thus lower than in the rumen of cattle, where 1.88 x 10^3 to 2.83 x 10^5 anaerobic fungal 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 5.2.4), 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 anaerobic fungi. As expected for biogas plants PB 21 and PB 22, where GH5 endoglucanase transcripts were detected, also the highest levels of anaerobic fungal 18S rDNA were found (Figure 17). 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 anaerobic fungi in this digester was thus unexpected at a first glance. However, two factors may explain the detected anaerobic fungal 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 anaerobic fungal biomass (Davies *et al.* 1993; McGranaghan *et al.* 1999). Second, the HRT of 12 days in the digesters of PB 22 (Table 6) was extremely short. The anaerobic fungi 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 anaerobic

fungi and in the latter no evidence for anaerobic fungi at all was found although they received considerable cattle slurry/manure input (Table 6). This suggests that longer exposure to thermophilic conditions was not endured by the AF. Moreover, the results of previous studies in which anaerobic fungal cultures were inoculated into biogas digesters to improve biogas production rates showed that the anaerobic fungi were not able to persist in the biogas environment and died within the first 10 to 15 days after their implementation (Procházka *et al.* 2012; Nkemka *et al.* 2015). Taken together, the results suggest that at least the tested biogas fermenter environments with the given conditions do not favor anaerobic fungal growth and activity. The conditions appear to eventually kill the anaerobic fungi or render them inactive. For this reason, it seems that conventional bioaugmentation with addition of anaerobic fungal cultures to biogas plants is not promising. Alternative strategies should be developed considering specifically the needs of existing anaerobic fungi. Additionally, specific strains which can grow under the present biogas conditions could be identified and selected for bioaugmentation purposes.

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

Analysis of the composition of biogas plant anaerobic fungal 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 5.2.4). All samples of biogas plants showing amplification of anaerobic fungal 18S rDNA (see Figure 17 and Section 5.3.1) were tested with the LSU primers. Amplicon sequences were subject to quality control (see Section 5.2.5) 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 anaerobic fungi (*Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Cyllamyces*, *Piromyces*, *Anaeromyces*; Figure 18). The most recently described genera *Oontomyces* and *Buwchfawromyces* were not detected. In addition, four clades representing hitherto unclassified anaerobic fungal genera, named ‘‘novel clades” A to D were also detected. For better visualization, identical LSU clone sequences were removed from the phylogenetic tree (Figure 18). The community structure of anaerobic fungal populations in the studied samples as obtained by LSU sequence analysis is shown in Figure 19. 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 community composition of anaerobic fungi in feces of diverse zoo animals

also found *Piromyces* and *Neocallimastix*, alongside with hitherto unclassified novel anaerobic fungi, to be the most widespread and abundant. Since both, the anaerobic fungi detected in the current study and those reported by Liggenstoffer *et al.* (2010) originated from manure/slurry and feces, respectively, the observed similarity of anaerobic fungal 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 *Neocallimastix 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 the anaerobic fungal sequences obtained from the digester of PB 22 (n = 31), representatives of the *Neocallimastix* clade with the type species *Neocallimastix 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 m3 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 5.3.1, that the anaerobic fungal population in the feces remained almost unaffected, resulting in the diverse mix of anaerobic fungal sequences found. Of the biogas plants showing no signal for transcriptionally active anaerobic fungi, 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 anaerobic fungi 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.

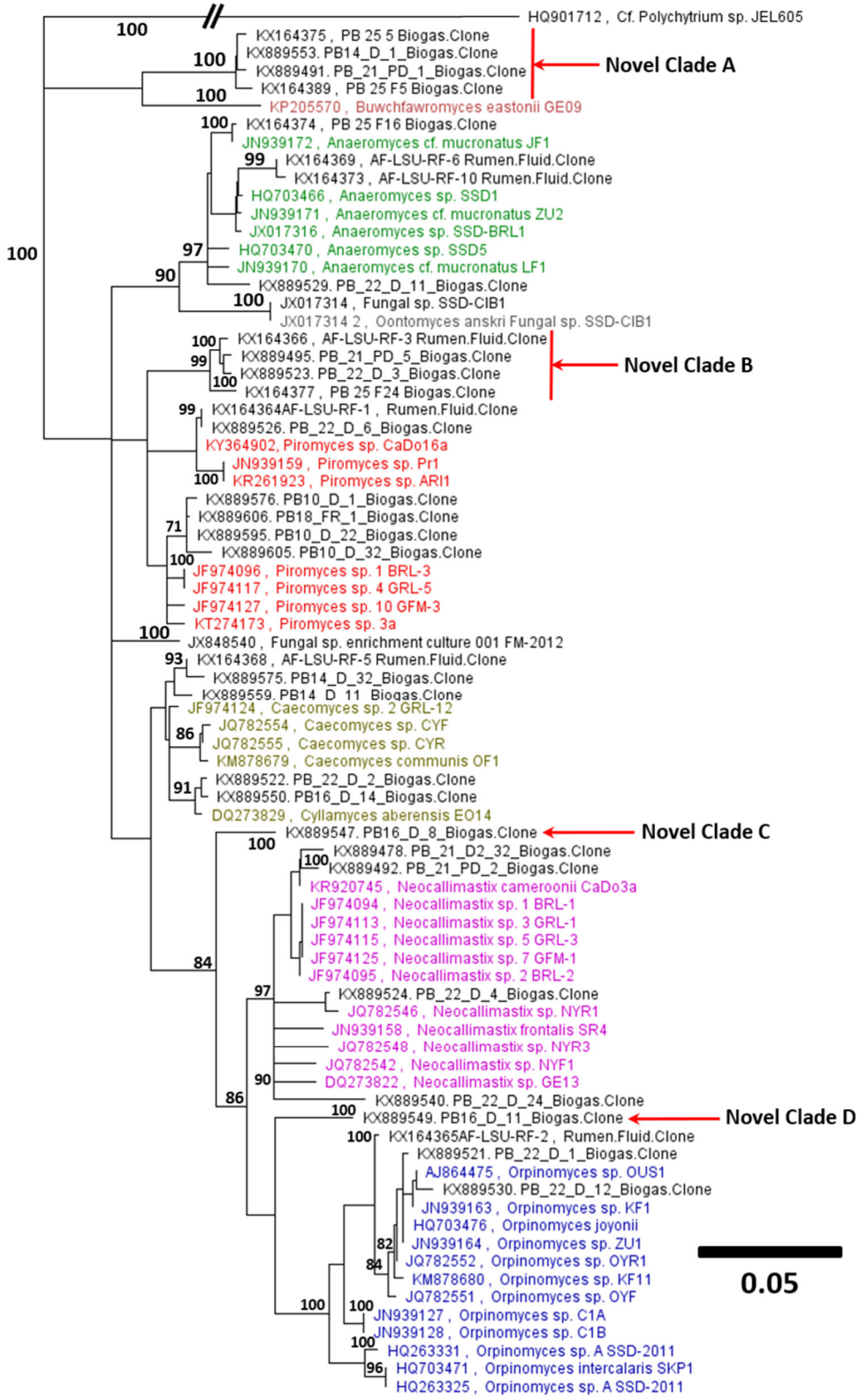


Figure 18: Maximum likelihood tree based on an 453 bp alignment of 78 28S rDNA sequences of anaerobic fungi. The sequences are representative of all described anaerobic fungal genera, along with clone sequences (in black) derived from the examined biogas plants and rumen fluid in this and the previous 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).

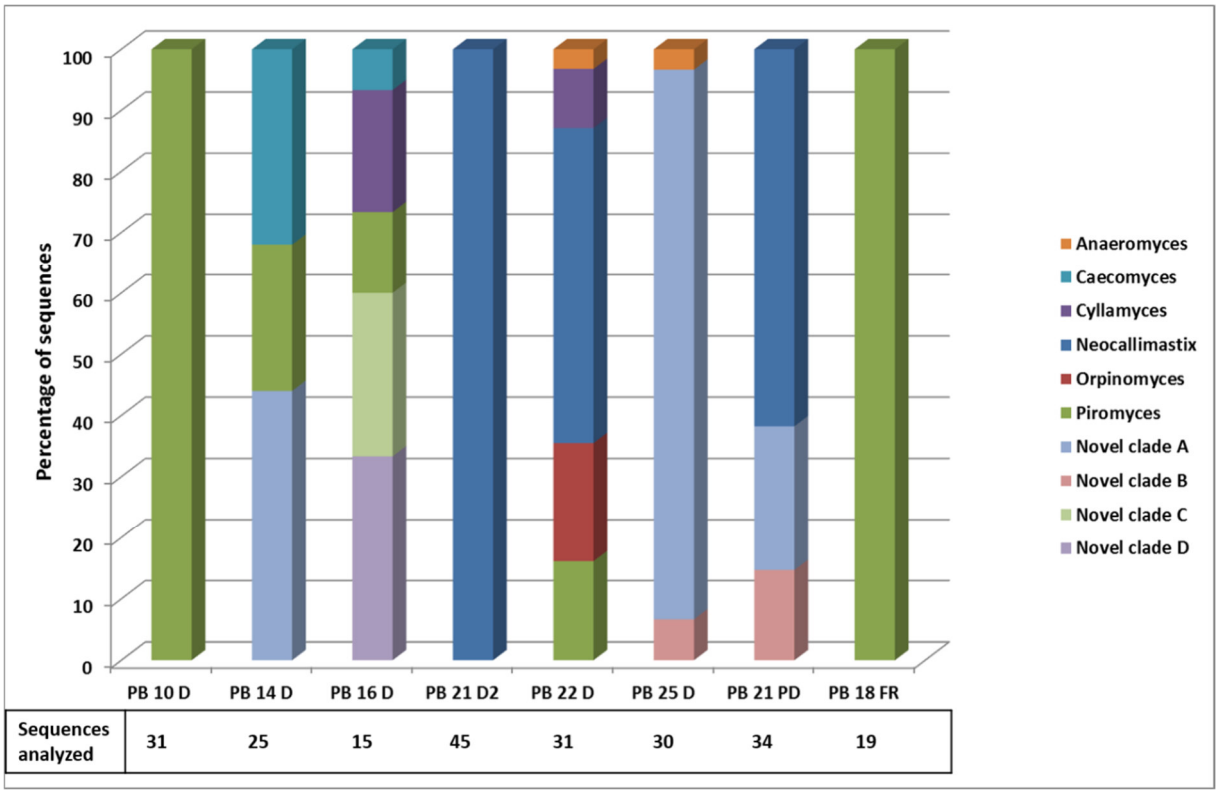


Figure 19: Composition of the LSU DNA sequences derived from the 7 biogas plant samples tested positive for anaerobic fungi with phylogenetic resolution at the genus level. For biogas plant PB 21 an additional sample from the post-digester was analyzed. For abbreviations see Figure 17.

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 18) with 24 %. Biogas plant PB 16, in which only a low amount of anaerobic fungal 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 19).

5.3.3 Isolation of an anaerobic fungus from a biogas plant

Isolation of anaerobic fungi 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 6). 18S rDNA of anaerobic fungi was detected from both facilities, while GH5 endoglucanase transcripts were detected only in PB 21. No anaerobic fungi were isolated from the digester of PB 18, but an anaerobic fungal 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 16). 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 anaerobic fungi 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 anaerobic fungi too, as it is known from sequencing studies that the majority of anaerobic fungi 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 19). Further, the applied cultivation approach is based on methods that were successful for isolation of anaerobic fungi 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 anaerobic fungi, the isolation of strain CaDo16a and the detection of anaerobic fungal GH5 endoglucanase transcripts support the hypothesis that anaerobic fungi can at least transitionally be an active part of biogas biocenoses and that they might be more widely distributed than currently thought.

5.4 Conclusion

This study shows that anaerobic fungi 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, anaerobic fungi 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 anaerobic fungal needs thus seems to be necessary to make efficient bioaugmentation with anaerobic fungi possible.

Chapter 6

Accelerated biogas production from lignocellulosic biomass after pre-treatment with *Neocallimastix frontalis*[4]

Two *Neocallimastix frontalis* strains, isolated from rumen fluid of a cow and of a chamois, were assessed for their ability to degrade lignocellulosic biomass. Two independent batch experiments were performed. Each experiment was split into two phases: hydrolysis phase and batch fermentation phase. The hydrolysis process during the *Neocallimastix frontalis* incubation led to an initial increase of biogas production, an accelerated degradation of dry matter and an increased concentration of volatile fatty acids. As monitored by quantitative PCR, the applied *Neocallimastix frontalis* strains were present and transcriptionally active during the hydrolysis phase but were fading during the batch fermentation phase. Thus, a separate hydrolytic pretreatment phase with anaerobic fungi, such as *Neocallimastix frontalis*, represents a feasible strategy to improve biogas production from lignocellulosic substrates.

[4] Was published in a similar version by Dollhofer, V., Dandikas, V., Dorn-In, S., Bauer, C., Lebuhn, M., Bauer, J. 2018. Accelerated biogas production from lignocellulosic biomass after pre-treatment with *Neocallimastix frontalis*. Bioresource Technology, 264, 219-227.

6.1 Introduction:

Transformation of lignocellulose-rich material into biogas or platform chemicals is an attractive strategy to face growing energy demands and mitigate greenhouse gas emissions from the exploitation of fossil energy resources. Lignocellulosic residues or waste, such as by-products from the agricultural (e.g. crop residues, green waste), the forestry (e.g. mill waste) or the municipal area (e.g. landscaping care material, food waste) are highly abundant (Gerbrandt *et al.* 2016; Wei 2016; Williams *et al.* 2017). Particularly interesting for biogas production are agricultural residues like maize stover, wheat straw and spare forage grass, waste from food crop production or animal husbandry. They are easily accessible, cheap in comparison to the cultivation of energy crops, do not require additional land to grow on and do not trigger "food or fuel" conflicts.

However, the complex recalcitrant chemical structure of such lignocellulosic waste is resistant to hydrolysis in conventional biogas production. The fibrous biomass is composed of interwoven cellulose and hemicellulose, coated by anaerobically almost undegradable lignin (Rodriguez *et al.* 2017). Bacteria and archaea in the biogas biocenosis are not efficient in disintegrating the lignin coat, leaving a considerable portion of the more easily convertible sugars untouched. Unsatisfactory biogas yields, process failure caused by undigested fibres and financial losses leave lignocellulose-rich substrates unattractive for biogas plant operators. Pre-treatment approaches to disintegrate the recalcitrant structures and enhance the accessibility for cellulolytic microbes allow a more efficient biogas production from lignocelluloses-rich waste (Patinvoh *et al.* 2017). However, currently available chemical or mechanical approaches are rather cost-intensive (Patinvoh *et al.* 2017) and mostly not cost-efficient in agricultural biogas production. Microbial pre-treatment utilizing the fibre degrading potentials of fungi may be a much cheaper alternative. Considerable research has been devoted to aerobic lignocellulose degrading fungi such as white-rot, brown-rot and soft-rot fungi. While soft-rot and brown-rot fungi were reported to have only a small effect on the lignin content of substrates, white-rot fungi are known to attack phenolic structures enzymatically and convert lignin components to CO_2 (Rouches *et al.* 2016). However, drawbacks of pre-treatment with aerobic fungi are loss of carbohydrates by respiration and biomass build-up and the requirement of long pretreatment periods (Isroi *et al.* 2011).

Anaerobic fungi from the phylum *Neocallimastigomycota* are natural inhabitants of the digestive tract of herbivorous animals (Bauchop 1979a; Liggenstoffer *et al.* 2010), which decompose a big share of the ingested forage. The *Neocallimastigales* attach to the plant material and crack the fibres mechanically by growth and expansion of their rhizoids or bulbous holdfasts (Ho *et al.* 1988; Akin and Borneman 1990). Thereby the protective lignin layers are disintegrated, making cellulose and hemicellulose accessible for further enzymatic digestion and other microbial attacks.

In addition to their mechanical effectiveness, anaerobic fungi possess cellulosomes attached to their cell wall (Dashtban *et al.* 2009). These multi-enzyme complexes contain a multitude of lignocellulolytic enzymes for the degradation of plant carbohydrates and the cleavage of ester bonds interlinking lignin and hemicelluloses. The assimilated carbohydrates are

metabolized mainly to lactate, acetate, formate and H_2 via mixed acid fermentation (Youssef *et al.* 2013). Additionally, ethanol, succinate and CO_2 can be produced. The metabolized compounds are generally used to generate energy in form of ATP to promote fungal growth and are representing potential substrates for methanogens living closely associated with anaerobic fungi (Leis *et al.* 2014). Similar syntrophic interactions are known from the biogas process where CO_2 and H_2 or acetate produced by bacteria are converted to CH_4 and H_2O by methanogenic archaea.

Pre-treatment of lignocellulosic biomass with anaerobic fungi could thus achieve the following advantages: (i) carbohydrates are better accessible for bacterial attack after disintegration of the lignin layer, (ii) metabolites from anaerobic fungal mixed acid fermentation can directly be used for bacterial fermentation and methanogenesis, (iii) energy is captured in biomass and mostly in the CH_4 produced at anaerobic conditions and not lost by respiration during the aerobic pretreatment process, (iv) pretreatment periods may be shorter than pretreatment with white-rot fungi, as anaerobic fungi can attack and degrade fibres within hours (Edwards *et al.* 2008).

The objective of this study was the application of two *Neocallimastix frontalis* strains to the hydrolysis phase in order to improve hydrolysis of lignocellulosic biomass. The applied isolates were obtained from animals living on a high fibre diet, namely a cow (*Bos taurus taurus*) and a chamois (*Rupicarpa rupicarpa*). The effects on biogas production of *Neocallimastix frontalis* from both animal species were assessed in two step batch experiments, comprised by a hydrolytic / acidogenic stage, followed by a methane production stage.

6.2 Material and methods

6.2.1 Samples

6.2.1.1 Rumen fluid samples

Two samples of rumen content were used for isolation of AF. One sample from a domestic cow maintained by the Chair of Animal Nutrition, Technical University Munich (TUM) and one sample from a chamois (*Rupicarpa rupicarpa*) hunted in the Bavarian Alps (Krün, Bavaria , Germany, 47°30'17.2"N 11°16'44.6"E)

To collect the rumen fluid sample from the cow, the rumen fistula was rinsed with distilled H_2O. A sterile 15 ml polypropylene tube (Greiner Bio-One) was dipped into the rumen and filled entirely with rumen fluid containing plant fibres. After that, the tube was promptly sealed in order to avoid oxic conditions in the sample. The samples were transported to the laboratory in a box filled with 39 °C warm water to keep the condition similar to rumen temperature. The isolation of anaerobic fungi from rumen fluid was performed within 1 h after sample collection. Additionally, rumen fluid from a cow was also used as media component for culturing the anaerobic fungi (see section 6.2.2).

A chamois was hunted in winter. Its whole digestive tract (oesophagus to rectum) was removed and was ligated at its cranial and its caudal end. The digestive tract was packed in a

plastic bag and was transported to the laboratory. The rumen was opened with a sterile scalpel. Rumen fluid and rumen contents were collected in a sterile 15 ml polypropylene tube (Greiner Bio-One). The cultivation of anaerobic fungi from ruminal contents of chamois was performed within 1 day (see section 6.2.2).

6.2.1.2 Inoculum for the batch experiments

Digester effluent of a biogas digester operated by the Institute for Agricultural Engineering and Animal Husbandry at the Bavarian State Research Center for Agriculture in Freising, Germany was used to inoculate batch fermentations. The digester was operated at a temperature of 38 °C ± 1 °C and was fed with 80 % cattle slurry and 20 % total mixed ration. Total mixed ration comprised 39.5 % grass silage, 44.4 % maize silage, 3.7 % hay, 1.2 % straw, 3.7 % molasses, 2.4 % Bovigold ® SojaPlus (comprising protein from shredded soya; BayWa) and 4.9 % concentrated feed (consisting of 23.4 % shredded barley, 23.4 % shredded maize, 46.9 % grain maize, 1.1 % calcium carbonate, 0.3 % cattle salt and 4.8 % VitalMiral Hofmix RKWSüd). The digester effluent was stored at batch test temperature without feeding to reduce endogenous gas potential. The degassed digester effluent was used as inoculum for the batch experiments (see section 6.2.3).

6.2.2 Cultivation and identification of anaerobic fungi

Medium A (

Table 7), modified from Teunissen *et al.* (1991) was used for isolation of AF. Cattle rumen fluid (see section 6.2.1) was clarified by centrifugation at 5000 *g* for 15 min. Salt solution I consisted of: 3 g KH_2PO_4, 3 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4$, 0.6 g $CaCl_2$ per l. Salt solution II comprised 3 g K_2HPO_4 per l. Hemin solution was prepared by dissolving 0.1 g hemin in 10 ml of absolute ethanol and filled up to 1 L with 0.05 M NaOH.

The medium preparation was adjusted to 1 L with distilled water and autoclaved for 20 min at 115 °C (Teunissen *et al.* 1991). After cooling to ca. 50 °C, vitamin solution and antibiotics solutions 1 and 2 were added. The vitamin solution contained 0.1 g thiamin-HCl, 0.2 g riboflavin, 0.6 g calcium D-pantothenate, 1 g nicotinic acid, 1 g nicotinamide, 0.05 g folic acid, 0.2 g cyanocobalamin, 0.2 g biotin, 0.1 g pyridoxine-HCl and 0.01 g p-aminobenzoic acid. The volume was adjusted to 1 L by adding sterile distilled H_2O. Antibiotics were used to suppress the growth of contaminanting microorganisms. Antibiotics solution 1 contained 2.4 g penicillin G (1,650 U / mg) and 0.4 g streptomycinsulfate (748 U / mg) in 100 ml sterile distilled H_2O. Antibiotics solution 2 was 5.0 g chloramphenicol dissolved in 100 ml 100 % ethanol.

Table 7: Media composition for growth of anaerobic fungi. Medium A for isolation and medium B for batch inocula production

Ingredients	Medium A	Medium B
Salt solution I (ml)	150.0	150.0
Salt solution II (ml)	150.0	150.0
Clarified rumen fluid (ml)	200.0	-
Bactocasitone (g)	10.0	5.0
Yeast extract (g)	2.5	1.4
$NaHCO_3$ (g)	6.0	6.0
L-Cystein-HCL (g)	1.0	1.0
Fructose (g)	2.0	-
Xylose (g)	2.0	-
D-(+)-Cellobiose (g)	2.0	-
Resazurin (0,1 % w/v; ml)	1.0	1.0
Hemin (ml)	10.0	10.0
Hay (autoclaved, cut to10 mm; g)	1.0	1.0
H_2O	adjusted to 1 l	adjusted to 1 l
Sterilized by autoclaving 20 min 115 °C		
Vitamin solution (ml)	1.0	-
Antibiotics solution 1 (ml)	10.0	-
Antibiotics solution 2 (ml)	5.0	-

For isolation of anaerobic fungi, pre-warmed medium A (39 °C) was inoculated with rumen content in a ratio of 9:1. Immediately after inoculation, the tubes were degassed with CO_2 prior to incubation. After 3-5 days of incubation at 39 °C, growth of anaerobic fungi was examined by microscopy.

In this study, anaerobic fungi were not cultivated on solid / agar containing medium, thus anaerobic fungi were not purified by picking of single colonies. Instead, the growing anaerobic fungi in medium A were further subcultured in medium B for at least 3 times in order to achieve adapted isolates. Medium B was used for culturing anaerobic fungi, which were applied as inoculum to the presented batch experiments. It contained similar components as medium A, except rumen fluid, sugars, vitamin solution and antibiotics solutions (see

Table 7). Hay used as substrate for the anaerobic fungi in medium B was cut into 10 mm long pieces and was autoclaved for 20 min at 115 °C. Autoclaved hay was weighed into sterile Hungate tubes or sterile serum bottles prior to filling them with medium (1 g / 100 ml medium). Tubes or bottles containing hay and Medium B were degassed with 100 % CO_2 to achieve an adequate anaerobic atmosphere and were pre-warmed for 3 to 4 h at 39 °C. Media showing evidence of microbial contamination or oxygenation were discarded.

After inoculation the media were degassed again with 100 % CO_2 and were incubated at 39 °C. Growth of anaerobic fungi in medium B was visually observed after 5 – 7 days of incubation by the formation of floating mats or balls of hay in the medium. Growth of

anaerobic fungi was confirmed by microscopy and the isolates were classified according to their morphological characteristics (Ho and Barr 1995).

As the purity of anaerobic fungal isolates could not be assured by microscopy, their ITS 1 was sequenced after Single Strand Conformation Polymorphism (SSCP) and polyacrylamide gel analysis (see section 6.2.5.3).

6.2.3 Batch experiments

Two independent batch experiments (ExpA and ExpB) were performed to assess if hydrolysis with *Neocallimastix frontalis* cultures could enhance fibre degradation and thus increase overall biogas and methane yield. The batch experiments were following the German technical guideline VDI 4630 (https://www.vdi.de/uploads/tx_vdirili/pdf/9703240.pdf). Each batch digester had a total volume of 2 l, was sealed with a rubber septum with a hole in its middle and connected to a MilliGascounter (Ritter Apparatebau GmbH, Bochum, Germany). The biogas volume was measured by flow through the MilliGascounter. Each tilting of the measurement chamber corresponds to approximately 1 ml of gas. The exact volume of each tilting of the MilliGascounters was determined by a mass flow controller (EL-Flow®, Bronkhorst High-Tech B.V., Netherlands). Gas composition was analysed by infrared measurement for CH_4 and CO_2 and by electrochemical measurement for O_2 with an Awiflex device (Awite Bioenergie GmbH, Langenbach, Germany).

In total there were 4 variants for each batch assay: hay inoculated with *Neocallimastix frontalis* isolates from a cow (*Neocallimastix frontalis* isolate 1) and from a chamois (*Neocallimastix frontalis* isolate 2), and hay inoculated with the inactivated corresponding *Neocallimastix frontalis* isolates. The *Neocallimastix frontalis* cultures were inactivated by autoclaving for 15 min at 121 °C. The experiment period was 40 days in total, separated in 7 days of a hydrolysis phase (pre-treatment) and 33 days of biogas production (batch fermentation phase). During the hydrolysis phase, 15 g autoclaved hay (ExpA 94,39 dry matter (DM) 92,71 organic dry matter (oDM); ExpB 94,42 DM; 92,65 oDM) as substrate was inoculated with 400 ml culture (in medium B) of *Neocallimastix frontalis* isolate 1 & 2 (see section 6.3.1) and incubated at 39 °C. For the biogas production and batch test, 1 L degassed digestate (see section 6.2.1) was added to each batch flask, resulting in a working volume of 1.4 l. Each variant was performed in 4 replicates. At the end of hydrolysis phase, three replicates of each variant were continued to the batch fermentation phase for CH_4 production whereas the fourth replicate was used for chemical, microbiological and molecular biological analyses. At the end of the batch fermentation phase, the three replicates of each variant were also subjected to molecular biological and microbiological analyses.

6.2.4 Process chemical analysis

Content and composition of monocarboxylic acids (C2 to C7) formed during anaerobic digestion provide information on the process state. Total acids were measured titrimetrically (DIN 38409-H7) as volatile fatty acids (VFAs) according to DIN 38414 S19. The spectrum of VFA patterns and their concentrations were measured in an Agilent 6890N Network Gas Chromatograph (Agilent Technologies, USA).

6.2.5 Molecular biological analysis

6.2.5.1 Extraction of nucleic acids

Samples of each variant were taken at the beginning of the batch experiment, after the hydrolysis and at the end of the methanogenic phase. DNA and mRNA were extracted from each sample following the protocols published by Dollhofer *et al.* (2016). In brief: Prior to nucleic acid extractions, each sample was washed twice with 1 ml 0.85 % sterile KCl. DNA was extracted from 40 µl washed sample with the FastDNA™ Spin Kit for Soil (MP Biomedicals), with a DNA recovery rate of 90 % (Lebuhn *et al.* 2016). Polyadenylated mRNA was extracted from 80 µl washed sample with the Dynabeads® mRNA DIRECT™ Kit (Life Technologies). The residual DNA in the extracts was digested with the TURBO DNA-*free*™ Kit (Life Technologies).

6.2.5.2 Quantitative PCR assays

Two quantitative PCR (qPCR) procedures, previously published by Dollhofer et al. (2016), were applied in this study. The assay AF-SSU was used to quantify the 18S rDNA (copies / ml sludge) and the assay AF-Endo was used to measure the transcriptional activity of anaerobic fungi (Table 8). The qPCR reactions with both assays were performed in a reaction volume of 25 µl on an Mx3005P qPCR system (Agilent Technologies).

For assay AF-SSU (Table 8) qPCR was performed with a hydrolysis probe and a cell suspension standard (standard curve equation of $Y = -3.230 \times LOG(X) + 38.37$; Y = fluorescence in dR; X = initial quantity of copies; qPCR efficiency of 104 %) allowing the specific quantification of anaerobic fungal 18S rDNA copies per ml sample. The limit of detection (LoD) was 11 copies and the limit of quantification (LoQ) was 35 copies per reaction for assay AF-SSU (Dollhofer *et al.* 2016).

Assay AF-Endo is targeting the gene of a glycoside family 5 (GH5) endoglucanase coding for an enzyme cutting cellulose at amorphous sites during degradation of plant fibres. The method is performed on mRNA level and thus measuring the transcripts of the anaerobic fungal GH5 endoglucanase gene. Total mRNA was specifically reverse transcribed to endoglucanase cDNA with ThermoScript™ Reverse Transcriptase. The cDNAs were further amplified by qPCR using EvaGreen dye for fluorescent detection and a cell suspension standard carrying the gene of interest for transcript quantification. AF-Endo standard curve was defined by the equation $Y = -3.415 \times LOG(X) + 37.90$ (qPCR efficiency of 96.3 %). The limit of detection (LoD) was 8 copies and the limit of quantification (LoQ) was 13 anaerobic fungal GH5 endoglucanase copies per reaction (Dollhofer *et al.* 2016).

Table 8: Oligonucleotides for classification and quantification of anaerobic fungi

Assay	Primer / probe	Sequence 5'→3'	Amplicon length	Reference
AF-SSU	AF-SSU forward	CTAGGGATCGGACGACGTTT	475 bp	Dollhofer *et al.* 2016
	AF-SSU reverse	GGACCTYCCGATCAAGGATG		
	AF-SSU probe	FAM-ATTCGCGTAACTATTTAGCAGGTTAAGGT-BHQ1		
AF-Endo	AF-Endo forward	CGTATTCCAACYACTTGGWSYGG	526 bp	
	AF-Endo reverse	CCRKTRTTTAAGGCAAARTTRTAYGGA		
ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA	350 bp	Gardes and Bruns 1993
	5.8S-R1	GAGATCCATTGTCAACAGTT		this study

6.2.5.3 SSCP, PCR and sequencing

Anaerobic fungal pure cultures isolated from rumen fluid samples of a cow and a chamois were identified by SSCP performed as described by Dorn-In *et al.* (2013) and sequencing of the ITS 1. DNA was extracted from 0.5 ml of anaerobic fungi on medium B (see section 6.2.2). The ITS 1 region was amplified with the primer pair ITS 1 & 5.8S-R1 (Table 8). PCR amplification of the ITS 1 region was performed in a Thermocycler (Biometra, Germany) using primer pair ITS1F and 5.8S-R (Table 8) in a reaction volume of 25 µl consisting of 2.5 µl 10 x PCR buffer (15 mM $MgCl_2$), 0.5 µl dNTP mix (10 mM each), 0.25 µl of each primer solution (50 µM), 0.13 µl HotStarTaq DNA Polymerase (5 U / µl) and 1 µl of DNA template. The reaction volume of 25 µl was reached by adding 20.37 µl nuclease-free H_2O. Amplification was performed in a three-step PCR program: 15 min initial activation at 95 °C, followed by 35 cycles consisting of 45 s denaturation at 94 °C, annealing for 1 min at 52 °C and extension for 1 min at 72 °C, and final elongation for 10 min at 72 °C. PCR products were purified using the GenElute PCR Clean Up Kit (Sigma Aldrich) and analyzed by SSCP and polyacrylamide gel electrophoresis. All single DNA bands visible on the polyacrylamide gel were purified and submitted for Sanger sequencing at Sequiserve (Vaterstetten, Germany). The obtained, quality checked sequences were deposited in NCBI GenBank with the accession numbers (MH127654 to MH127660).

6.2.6 Statistical analysis

The effects observed for hay treated with active and inactivated *Neocallimastix frontalis* isolates 1 & 2 during hydrolysis and initial batch fermentation phase were analyzed statistically as follows: The distribution of the gas production (GP) values in the batch experiments was generated by Monte Carlo simulation from a gamma distribution with the shape parameter $a = (E(x))^2 / Var(x)$ and the scale parameter $s = E(x) / Var(x)$; E = mean of the experimental gas production from the fungal treated variant or the control. Var $(x)^\star$ was

simulated for each step by random sampling from a χ^2-distribution with N-1 degrees of freedom according to the equation: $Var(x)^\star = (s(x))^2 \times (N-1) / \chi^2 (N-1)$ with $s(x)$ = the observed standard deviation of the gas production and N= the number of repetitions in each treatment group. The distribution of the ratio between the $GP_{treatment}$ and $GP_{control}$ was calculated by $GP_{treatment}^\star / GP_{control}^\star$ for R=100.000 times.

In order to test if gas production rates over time (t) were equal, two random effect models were compared. Thereby the coefficients (B_1–B_3) were calculated as a function of time. Model 1 is a random intercept model, where a separate intercept (B_{0I}) is calculated for each subject (I). Additionally, the coefficients (B_{1G}–B_{3G}) were separately calculated for each treatment group (G) in model 2.

Model 1: $Y_I = B_{0I} + B_1 * t + B_2 * t^2 + B_3 * t^3$

Model 2: $Y_{IG} = B_{0I} + B_{1G} * t + B_{2G} * t^2 + B_{3G} * t^3$

The two models were compared by likelihood statistics. All statistical analyses were performed in R (R Core Team 2015).

6.3 Results and discussion:

6.3.1 Isolation and identification of anaerobic fungi from rumen fluid samples

The morphological characteristics of anaerobic fungal isolates from rumen fluid of a cow and of a chamois were analyzed by microscopy according to the key published by Ho and Barr (1995). Both isolates were morphologically classified as *Neocallimastix* species. ITS 1 sequence analysis confirmed this result (accession numbers of *Neocallimastix frontalis* isolates MH127654 to MH127660). The DNA sequences obtained were aligned with all anaerobic fungal sequences provided in NCBI GenBank, The sequences obtained for the isolate from cow rumen content were identified as *Neocallimastix frontalis* with 97-100 % identity to the sequences provided in GenBank (NCBI, Accession numbers KF312489, KF312485, and JN939158). The isolate from a chamois was also identified as *Neocallimastix frontalis*, with an identity between 96-100 % to the sequence with KF312488, KF312485 and HQ585902. The identity of both isolates was 90 – 99 %. According to sequencing results, *Neocallimastix frontalis* isolated from both animals showed partially high similarity of the ITS 1 region.

The *Neocallimastix* isolates from both animals (isolate 1 from a cow, isolate 2 from a chamois) were further subcultured in medium B, and later used as inoculum for the hydrolysis phase in the batch experiments.

6.3.2 Biogas production from hay after hydrolysis with *Neocallimastix frontalis* isolates 1 & 2

6.3.2.1 Effect on biogas production

In this study, treatment with *Neocallimastix frontalis* cultures did not always increase the total biogas and methane yield after 40 experimental days (Figure 20). In the first experiment (ExpA), the total biogas yield was higher in the variants treated with *Neocallimastix frontalis* cultures (Figure 20 a). Since this effect was not observed in the second experiment (ExpB) it was not repeatable. However, an initial increase of biogas production was observed during the hydrolysis and the first days of the batch fermentation phase (Figure 20 a, c, d). This effect was apparent with each viable *Neocallimastix frontalis* isolate in the presented experiments, except for variant b in ExpA (Figure 20 b), where *Neocallimastix frontalis* isolate 2 was found inactive after surviving only for a short period of time, as confirmed by microscopical observation and no growth after re-isolation.

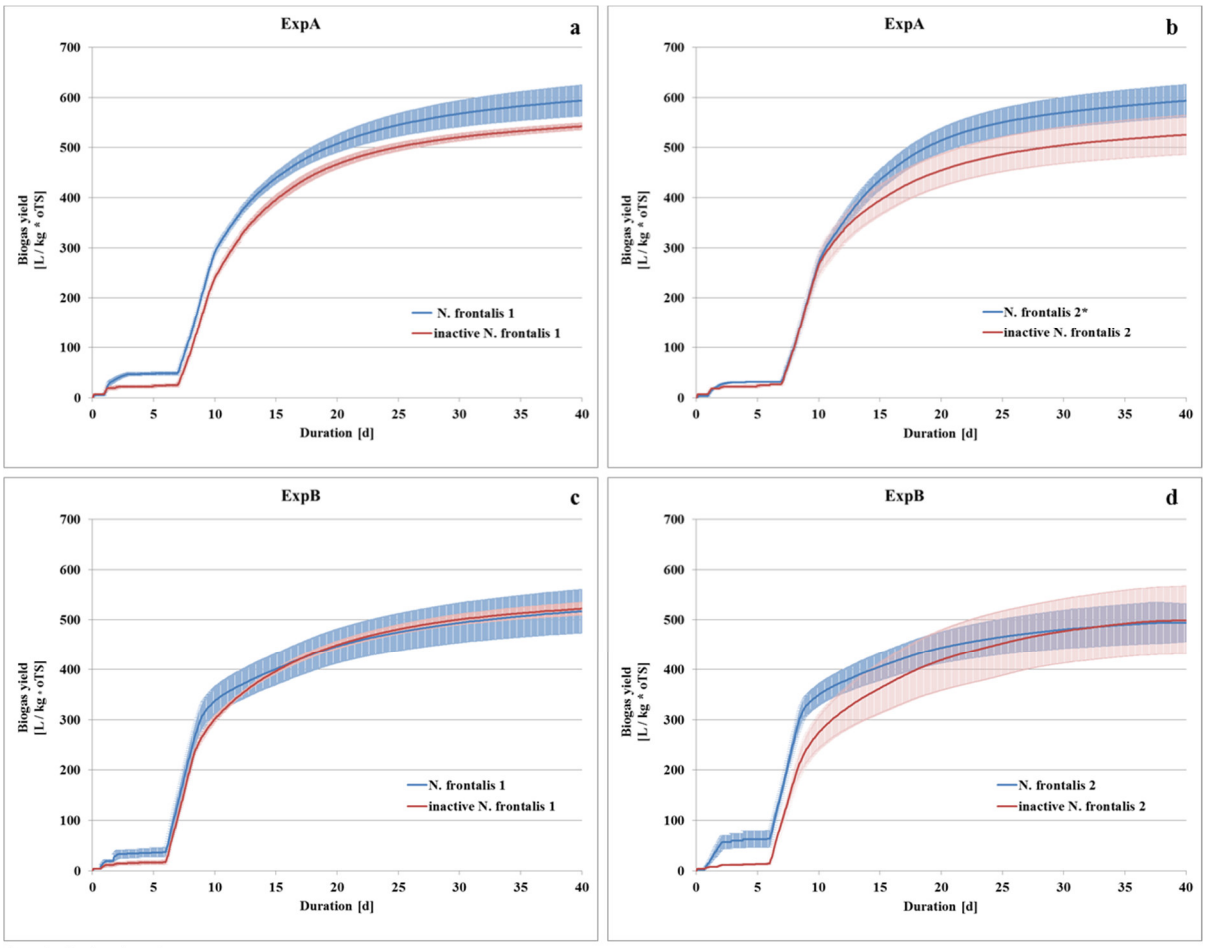


Figure 20: Biogas yield obtained from hay inoculated with *Neocallimastix frontalis* isolates 1 and 2 and the corresponding inactivated isolates in ExpA (a, b) and ExpB (c, d). Each curve is the average value of three replicates with standard deviation

The observed effects were statistically supported by the results of Monte Carlo simulation (section 6.2.6) presented in Table 9 and in Figure 21. The differences between the gas production curves (Figure 20) were statistically examined by comparison of gas production rates over time (see section 6.2.6). The comparison of "Model 1" und "Model 2" showed a significant difference of the likelihood with a p-value < 0.001, indicating that the gas production rate over time was significantly different between the variants treated with *Neocallimastix frontalis* and inactived *Neocallimastix frontalis* isolates.

Table 9: Confidence intervals calculated by Monte Carlo simulation for the different variants with *Neocallimastix frontalis* and inactivated *Neocallimastix frontalis* isolates

Experiment	*Neocallimastix frontalis* / inactivated *Neocallimastix frontalis*	Ratio	0.05	0.95
After one week of hydrolysis				
A	isolate 1 / inactivated isolate 1	1.9	1.36	3.03
B	isolate 1 / inactivated isolate 1	2.3	0.96	5.51
	isolate 2 / inactivated isolate 2	4.7	2.08	8.39
First days of batch digestion				
A	isolate 1 / inactivated isolate 1	1.1	1.04	1.22
B	isolate 1 / inactivated isolate 1	1	0.92	1.24
	isolate 2 / inactivated isolate 2	1.1	0.84	1.62

Bioaugmentation of biogas processes with anaerobic fungi has been tested in batch experiments, namely semicontinuous flow-through experiments (Procházka *et al.* 2012) and an two-stage system (Nkemka *et al.* 2015). Procházka *et al.* (2012) inoculated anaerobic sludge (90 % pig slurry and 10 % wastewater treatment sludge) from a biogas plant with anaerobic fungi from the genera *Anaeroymces*, *Piromyces* and *Orpinomyces* and analysed their influence on biogas production from maize silage and microcrystalline cellulose. Batch assays, fed-batch trials and semicontinuous flow-through experiments showed some improvement of the biogas production by addition of AF. However, a positive effect was seen only initially, indicating that the implemented fungi were not able to stay active under the given fermentation conditions. Nkemka *et al.* (2015) performed bioaugmentation experiments in a two-stage fermentation set-up, connecting a leach bed reactor to an upflow anaerobic sludge blanket (UASB) reactor operated with apple juice wastewater sludge. Leach bed reactors containing water and substrate (maize silage (*Zea mays*) or cattail (*Typha latifolia*)) were inoculated with a *Piromyces rhiziniflata* YM600 culture (10 % v/v). Compared to control reactions, bioaugmentation with *Piromyces rhiziniflata* improved the initial H_2 and CH_4 production and the volatile fatty acids (VFAs) production rate, substantiating the potential of anaerobic fungi for biogas production. Similarly to the results of the present study, Nkemka *et al.* (2015) reported that during bioaugmentation with *Piromyces rhiziniflata* the initial gas production was increased but total biogas and methane yield was not improved.

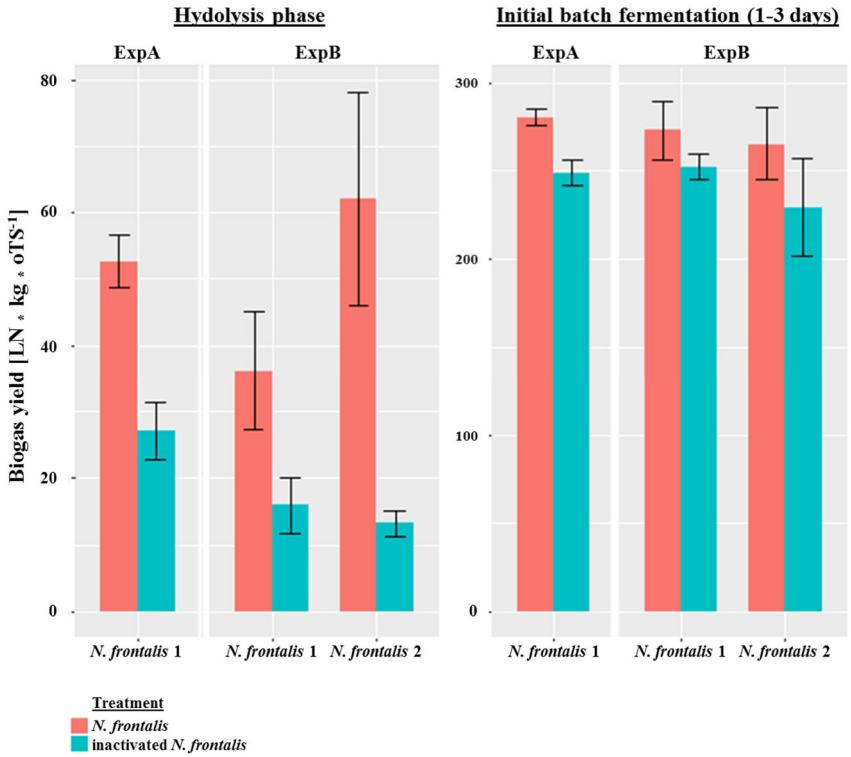
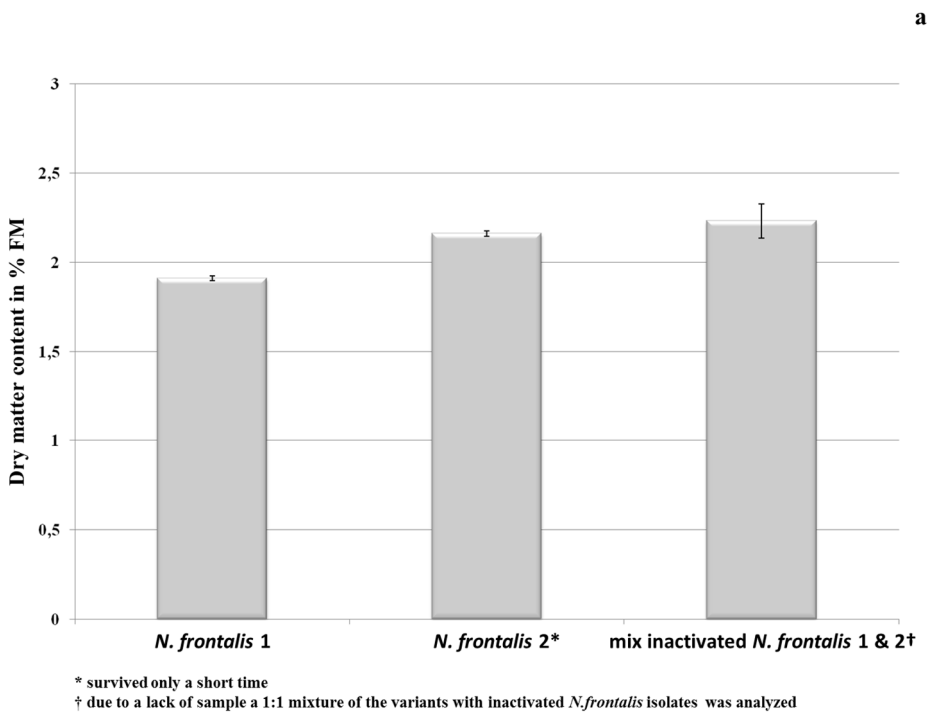


Figure 21: Biogas yield achieved from hay in experiments A and B with *Neocallimastix frontalis* isolates 1 & 2 and the corresponding inactivated isolates during the hydrolysis and the initial batch fermentation phase

6.3.2.2 Effects of anaerobic fungi on dry matter content and volatile fatty acid production

Variants treated with *Neocallimastix frontalis* and inactivated *Neocallimastix frontalis* cultures showed differences in their chemical composition at the end of the hydrolysis phase. Variants treated with *Neocallimastix frontalis* isolates showed a lower amount of dry matter (Figure 22) and a higher concentration of volatile fatty acids (Figure 23) than variants treated with inactivated *Neocallimastix frontalis* cultures, suggesting that the application of both *Neocallimastix frontalis* isolates enhanced fibre degradation, resulting in an increased conversion of plant organic matter to VFAs and other products.



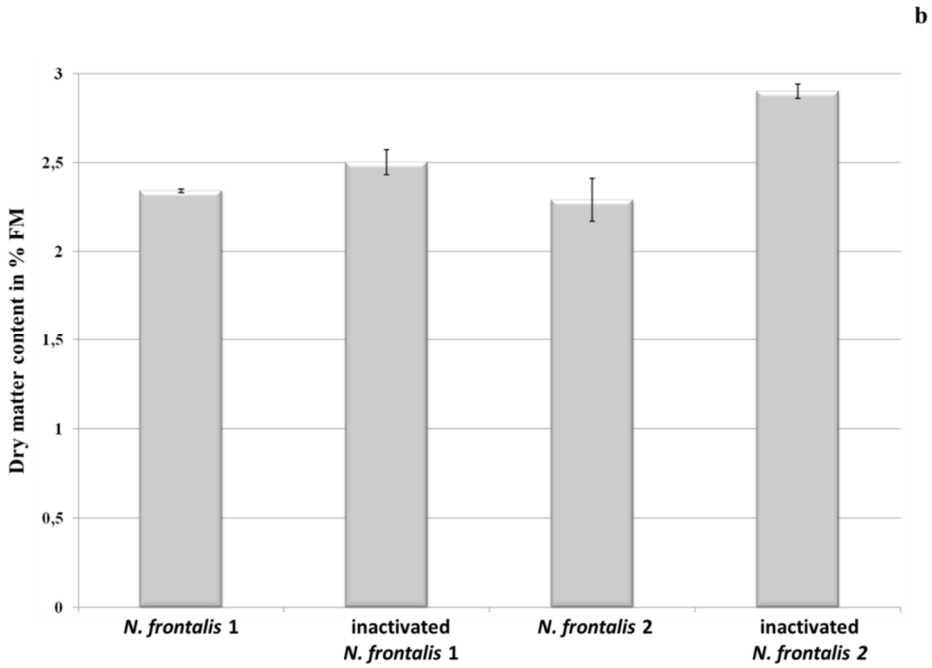


Figure 22: Dry matter contents (in % fresh matter) measured after one week of hydrolysis with *Neocallimastix frontalis* isolates 1 & 2 and the corresponding inactivated cultures during ExpA (a) and ExpB (b)

During degradation of plant matter, anaerobic fungi metabolize the comprised carbohydrates to H_2, CO_2, formate, acetate, lactate and ethanol by mixed acid fermentation (Theodorou *et al.* 1996; Gruninger *et al.* 2014). Acid production during the hydrolysis phase in variants

inoculated with *Neocallimastix frontalis* was higher than in variants inoculated with inactivated *Neocallimastix frontalis* in both experiments (Figure 23). Variants treated with *Neocallimastix frontalis* isolate 1 provided the highest production of VFAs during the hydrolysis phase (105.2 mmol * kg FM^{-1} in ExpA and 125.5 mmol * kg FM^{-1} in ExpB). The main acid compounds were acetic acid (70.9 (A) and 75.0 (B) mmol * kg FM^{-1}) followed by butyric acid (19.1 (A) and 36.9 (B) mmol * kg FM^{-1}). A low amount of propionic, isovaleric, isobutyric, valeric and caproic acid was also detected (acid concentrations see Figure 23). In the variants treated with *Neocallimastix frontalis* isolate 2 the total VFA production was 111.3 mmol * kg FM^{-1} in ExpB. In total, VFA production in variants inoculated with *Neocallimastix frontalis* isolate 2 was lower than in variants inoculated with *Neocallimastix frontalis* isolate 1. *Neocallimastix frontalis* isolate 2 from chamois survived only for a short period of time after its application to the hydrolysis phase in ExpA (see section 6.3.2.1). The total VFAs measured in this variant were with 83.2 mmol * kg FM^{-1} in ExpA still higher than in the inactivated variant. It can be assumed that in contrast to the variants with inactivated fungi, the acids were produced before the culture was applied to the batch reactors and that the acids were not disintegrated by autoclaving. The acid profile of variants inoculated with *Neocallimastix frontalis* isolate 2 after the hydrolysis phase was similar to variants inoculated with *Neocallimastix frontalis* isolate 1. Main acid product was acetic acid followed by butyric acid, propionic acid, isovaleric acid, isobutyric acid as well as low amounts of valeric acid (acid concentrations see Figure 23). Caproic acid was produced in higher amounts in variants with *Neocallimastix frontalis* isolate 2 with 12.4 mmol * kg FM-1 during ExpA and 8.9 mmol * kg FM-1 during ExpB.

In the acid profiles published by Nkemka *et al.* (2015) for bioaugmentation with the anaerobic fungus *Piromyces rhiziniflata*, the major proportions from digestion of maize silage were acetate, butyric acid and propionic acid whereas the digestion of cattail generated mainly butyric acid and propionic acid. The conclusion that acid production is dependent on amount and composition of acids in the substrates is in agreement with the results of the current study. Acid production from identical substrate differed between the two *Neocallimastix frontalis* isolates in the presented study. Different acid profiles produced by individual anaerobic fungal isolates and strains were also mentioned by Mountfort and Orpin (1994) and Teunissen *et al.* (1991).

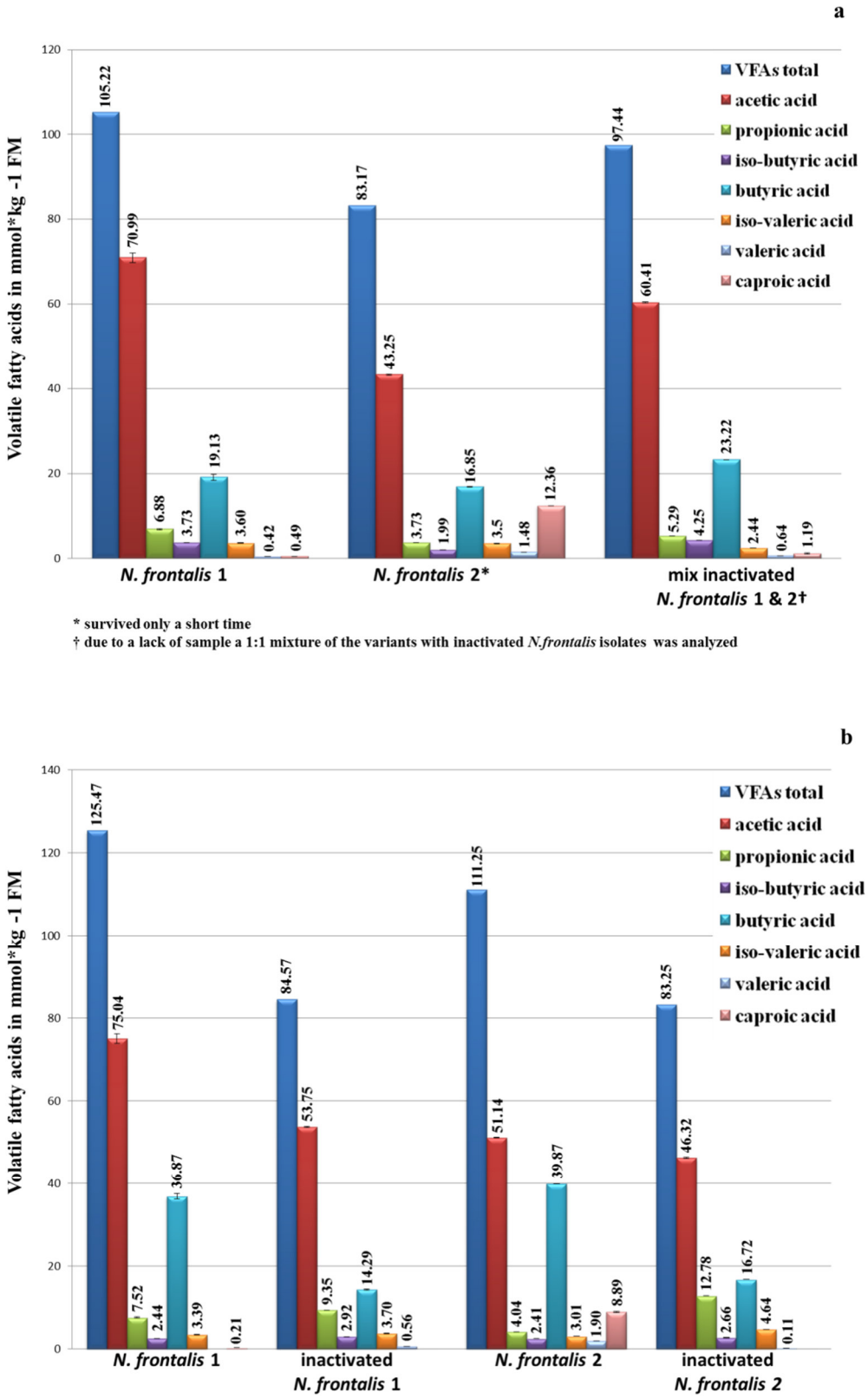


Figure 23: Concentrations of volatile fatty acids measured after one week of hydrolysis with *Neocallimastix frontalis* isolates 1 & 2/inactivated cultures during ExpA (a) and ExpB (b)

Noteworthy is the relatively high amount of caproic acid produced in variants treated with *Neocallimastix frontalis* isolate 2. Until now, caproic (C6) acid has not been described as

fermentation product of AF. Instead saturated fatty acids (SFA) including caprylic (C8) and capric (C10) acid have been shown to be inhibitory to cellulose digestion by *Neocallimastix frontalis* C5-1 (Ha *et al.* 2001). Medium chain fatty acids showed a stronger inhibitory effect than long chain fatty acids, thus the accumulation of caproic acid in variants treated with *Neocallimastix frontalis* isolate 2 is a potential indication that this strain might have struggled with the habitat conditions. This hypothesis is supported by the lower production of VFAs in total compared to hydrolysis with *Neocallimastix frontalis* isolate 1 and the short survival of *Neocallimastix frontalis* isolate 2 in ExpA. In addition medium chain fatty acids particularly caproic acid are used as antimicrobial feedstock supplement (Nair *et al.* 2005) in animal husbandry and are in demand as intermediate for production of fragrances and pharmaceuticals (Voulis 2012). Thus, besides the application of anaerobic fungi to enhance biogas production, anaerobic fungal cultures producing valuable fermentation products and intermediates could be of interest for the chemical and pharmaceutical industry. Future experiments should thus not only focus on the use of anaerobic fungi in the bioenergy production process, they should also consider anaerobic fungi as means to generate valuable chemicals.

6.3.2.3 Quantification of anaerobic fungal SSU gene copies and GH5 endoglucanase transcripts by qPCR

Neocallimastix frontalis concentrations were monitored during both experiments by quantification of their SSU gene and specific quantification of their GH5 endoglucanase transcripts. Samples for molecular biological analysis were taken from the *Neocallimastix frontalis* inocula, after the hydrolysis phase (day 7) and at the end of the batch fermentation phase (day 40). The concentration of anaerobic fungal SSU gene copies / ml digestate was decreasing over time in both experiments A and B. Exemplary, the result from ExpB are shown in Figure 24. The copies of SSU rDNA of *Neocallimastix frontalis* isolate 1 & 2 were 2×10^7 and 2.3×10^5 copies / ml in the inocula, 1.2×10^6 and 7.6×10^4 copies / ml at the end of the hydrolysis phase and 4.5×10^4 and 6×10^3 copies / ml at the end of the batch fermentation phase.

The transcription of the anaerobic fungal GH5 endoglucanase gene decreased throughout the experiment. In ExpA *Neocallimastix frontalis* isolates 1 & 2 used for inoculation contained 9.2×10^3 and 5.5×10^3 transcripts / ml, respectively. In this experiment, no GH5 endoglucanase transcripts of the applied anaerobic fungi were detected in a later phase of the experiment. In ExpB, 8.7×10^3 and 1.9×10^3 anaerobic fungal GH5 endoglucanase transcripts / ml culture were measured in the cultures of *Neocallimastix frontalis* isolate 1 & 2, respectively. At the end of the hydrolysis phase, the levels of anaerobic fungal GH5 endoglucanase transcripts were below the LoQ, but a specific signal above the LoD was detected in both variants containing *Neocallimastix frontalis* cultures. At the end of the experiments, no anaerobic fungal GH5 endoglucanase transcripts were detected. The decline in the number of GH5 endoglucanase transcripts and anaerobic fungal SSU rDNA copies throughout the experiments indicated that the applied anaerobic fungi were viable during the hydrolysis phase (except *Neocallimastix frontalis* isolate 2 in ExpA) but did not survive the batch fermentation phase. This assumption is supported by the higher VFA profiles in the

variants treated with *Neocallimastix frontalis* cultures after hydrolysis. A similar result was reported by Procházka *et al.* (2012). After their implementation into biogas reactors, anaerobic fungi survived only ten days in the biogas environment.

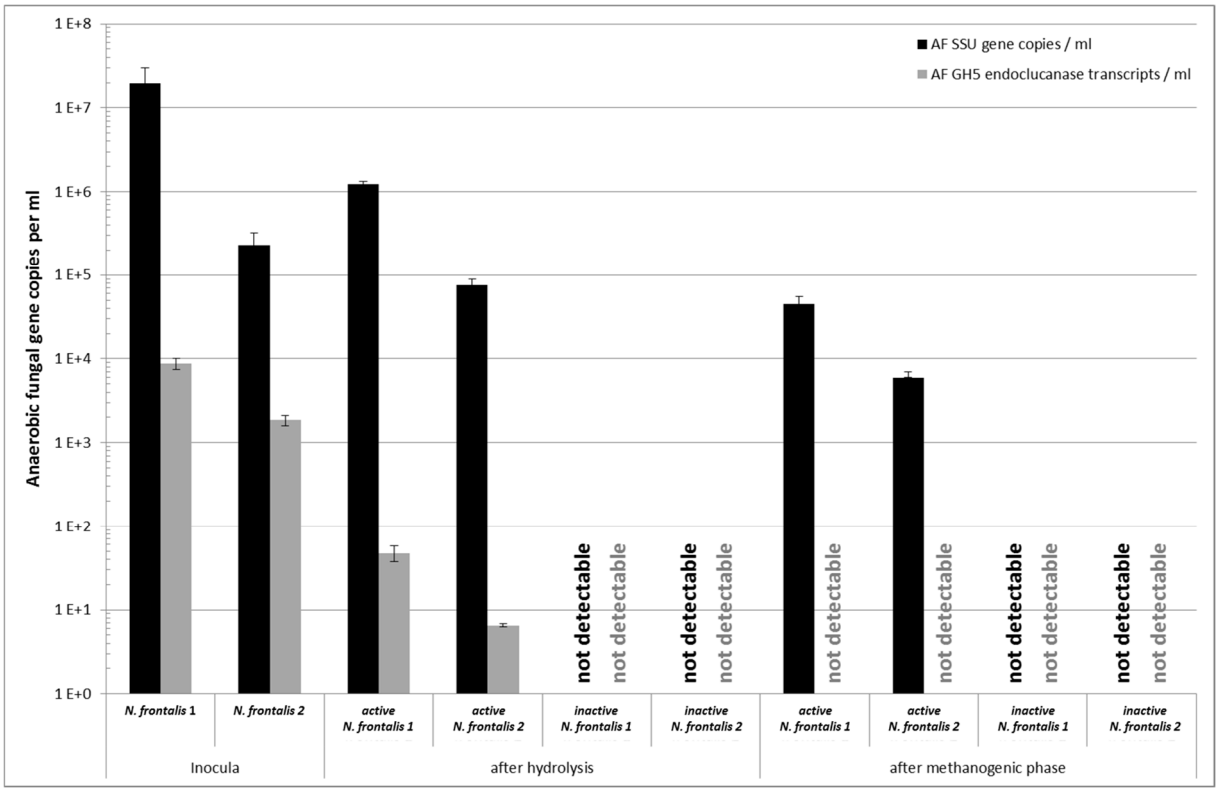


Figure 24: Number of anaerobic fungal SSU rDNA gene copies detected per ml culture in the inocula, the end of the hydrolysis phase and at the end of the batch fermentation phase quantified by qPCR during ExpB. Error bars indicating standard deviation

A screening of ten Bavarian biogas plants further showed that even if anaerobic fungal SSU rDNA was detected in seven biogas plants, transcripts of anaerobic fungal GH5 endoglucanase could only be detected in two biogas plants (Dollhofer *et al.* 2017). It was suggested that anaerobic fungi can only transitionally be active in biogas sludge and biogas reactors. Thus the approach of a hydrolytic pretreatment adjusted to the needs of anaerobic fungi, separated from the methanogenic phase, should be a strategy considered for future biotechnological utilization of fibre rich wastes.

6.4 Conclusion

Hydrolytic pretreatment of hay with two individual *Neocallimastix frontalis* isolates led to an increase in initial biogas production, higher VFA concentrations and accelerated degradation of dry matter. Activity and viability of *Neocallimastix frontalis* isolate 1 and isolate 2 decreased during the batch fermentation phase, suggesting that direct implementation into biogas reactors might not be effective. The results of this study highlight the potential of anaerobic fungi to pre-process lignocellulosic substrates for energy and platform chemical production and demonstrate the need for further research on anaerobic fungi in biotechnological applications.

Chapter 7 Discussion

This Ph.D. thesis is composed of two main research topics (see also Chapter 2)

(i) molecular detection of anaerobic fungi in biogas production processes and
(ii) hydrolytic pre-treatment of lignocellulosic biomass for biogas production with anaerobic fungi.

Each research topic was addressed by investigating the respective research questions and testing the related hypotheses in a series of experiments presented in Chapters 4 to 6.

The first research topic of this thesis was to develop and evaluate suitable PCR-based tools to detect anaerobic fungi in biogas production processes and animal derived samples (Chapter 4). Three individual detection strategies were needed to determine the anaerobic fungal quantity, transcriptional activity and anaerobic fungal community composition in a sample. These strategies were addressed by testing research hypotheses #1 to #3. The hypothesis that anaerobic fungi can be detected in and quantified from animal derived and biogas sludge samples by a specific 18S rRNA gene targeted qPCR assay (#1) could be confirmed. The designed primer and fluorogenic hydrolysis probe combination of the developed qPCR assay AF-SSU was proven to specifically detect and quantify anaerobic fungi. The second hypothesis was that active transcription of a cellulolytic gene of anaerobic fungi can be assessed for animal-derived and biogas sludge samples by specific quantification of GH5 endoglucanase transcripts. The primer set AF-Endo was designed and checked for specificity for anaerobic fungi by BLAST analysis and by in-silico PCR against cellulolytically active bacteria as non-target group. A qPCR assay AF-Endo was developed, and GH5 endoglucanase transcripts could be quantified from mRNA extracts from strains of six anaerobic fungal genera with this approach. Hypothesis #2 was thus verified, but further development of the method might be needed as qPCR assay AF-Endo could not be tested for all currently known anaerobic fungi, due to the lack of available cultures. The third tool was developed to describe the anaerobic fungal community composition in a sample. As molecular marker, the 28S rRNA gene was chosen, and assay AF-LSU was designed and developed. Phylogenetic analysis of LSU amplicons derived from a rumen and a biogas sludge sample showed sufficient resolution of the known anaerobic fungal genera, validating hypothesis #3. Research question No. 1 and the respective hypotheses are discussed in Section 7.1.

The three developed PCR-based tools allowed screening of ten agricultural biogas plants for the presence and transcriptional activity of anaerobic fungi (Chapter 5), in order to test hypothesis #4 of research question No. 2. The results revealed that anaerobic fungi were present in seven of the ten tested biogas plants. Signals for transcriptionally active anaerobic fungi were detected at low level in sludges of two agricultural biogas plants. Hypothesis #4 “Anaerobic fungi can be present and transcriptionally active in agricultural biogas plants.” was thus verified. This second research question is discussed in Section 7.2.

The second research topic of this thesis was to apply anaerobic fungi to pre-treat hay for biogas production (Chapter 6). In a two-stage batch fermentation experiment, hay was first hydrolyzed by two individual *Neocallimastix frontalis* strains before methane production was induced by the addition of sludge from a biogas reactor. Pre-treatment with *Neocallimastix frontalis* led to initially increased biogas production, accelerated degradation of dry matter and increased concentrations of volatile fatty acids. Hypothesis #5 "Pre-treatment with anaerobic fungi, as exemplified with different *Neocallimastix frontalis* strains, can improve biogas production from hay" was thus confirmed. The respective research is discussed in Section 7.3.

7.1 Tools for molecular detection of anaerobic fungi in biogas processes

Cultivation of certain microorganisms can be tedious and time-consuming, and the techniques are far from being perfect: after 150 years of cultivation of bacteria, only about 12,000 species could be isolated. Compared to the estimated existence of 10^7 to 10^9 bacterial species, this still remains a minor fraction (Overmann *et al.* 2017). This is true for the phylum of anaerobic fungi as well: in the first 40 years after their detection and placement in the fungal kingdom, only six genera have been described (Edwards *et al.* 2017). Till November 2019, 11 genera have been discovered but this is still not reflecting the wealth of 25 genera comprising 119 species suggested by environmental molecular biological studies (Joshi *et al.* 2018). This is mostly due to the general difficulty of cultivating these fungi (Callaghan *et al.* 2015) which is caused by the lack of knowledge of their physiology (this is even more problematic for hitherto uncultivable strains), growth optima and growth limitations. In order to screen a novel potential habitat for anaerobic fungi such as the biogas production process and to identify strains which can thrive in this environment, as attempted in research topic 1 of this Ph.D. thesis (Section 2.1), molecular biology tools are the more promising approach to investigate their biology and ecology.

Three molecular tools were thus developed and tested in this thesis to detect the presence, transcriptional activity and community profile of anaerobic fungi in rumen and biogas sludge samples (Chapter 4). As a first step, the available literature and sequence data were screened for suitable genetic markers. An overview of the genetic markers and primer sets for quantification and phylogenetic classification applied until 2012 is given in Table 10 and Table 12, respectively.

Table 10: Overview of primer sets for quantification of anaerobic fungi applied until 2012. Information on specificity for anaerobic fungi was judged from the referenced articles.

Target gene	Sample type	Nucleic acid type	Reference	Specific for anaerobic fungi
18S rRNA gene – ITS 1	Pure culture; rumen fluid	DNA	Denman and McSweeney (2006) Lwin *et al.* (2011)	Yes
	Rumen fluid	DNA	Khejornsart and Wanapat (2010)	Yes
	Rumen fluid	DNA	Khejornsart *et al.* (2011)	Yes
5.8S rRNA	Pure culture; rumen digesta	DNA	Edwards *et al.* (2008)	Yes
ITS 1	Rumen fluid and digesta	DNA	Kittelmann *et al.* (2012)	Yes

Prior to the work performed in this Ph.D. thesis, three different PCR based methods to specifically quantify anaerobic fungi in pure culture and rumen samples existed (see Subsection 1.2.2.1). All reported approaches are targeting different conserved parts of the ribosomal operon (Denman and McSweeney 2006; Edwards *et al.* 2008; Kittelmann *et al.* 2012), which are highly abundant in anaerobic fungal genomes (ca. 200 per genome; Edwards *et al.* (2017)). This is an advantage especially for analysis of environmental samples which might contain only a low number of anaerobic fungi but potentially still a sufficient amount of ribosomal sequences for PCR based quantification. Thus, in the presented Ph.D. thesis, the 18S rRNA gene (SSU gene) was chosen as marker for estimation of the amount of anaerobic fungi. As compared to the 28S rRNA gene (LSU gene) and even more the ITS regions, the SSU gene is more conserved and does not allow deep phylogenetic resolution (Edwards *et al.* 2017). This low degree of variation is an advantage for designing a quantitative PCR assay, as it simplifies alignment building and allows specific genetic demarcation of the phylum *Neocallimastigomycota*. Assay AF-SSU was thus developed specifically targeting a 475 bp region of the anaerobic fungal 18S rRNA gene (see Figure 25) to assess hypothesis #1 "Anaerobic fungi can be detected in and quantified from animal derived and biogas sludge samples by a specific 18S rRNA gene targeting qPCR assay".

Figure 25: Screenshot from Geneious version 6.0.6. of a 4868 bp rRNA (showing; 18S, ITS 1, 5.8S, ITS 2 and 28S) reference sequence (AJ864475) from an *Orpinomyces* sp. isolate. The positions of primers are annotated on the sequence. Primers shown were developed in the presented Ph.D. thesis or reported in key publications describing PCR based studies on anaerobic fungal phylogeny (Li and Heath 1992; Brookman *et al.* 2000a; Callaghan *et al.* 2015) or anaerobic fungal quantification by qPCR (Denman and McSweeney 2006; Edwards *et al.* 2008; Lwin *et al.* 2011; Kittelmann *et al.* 2012; Procházka *et al.* 2012). This picture was published as Supplementary Figure A1 by Dollhofer *et al.* (2016).

Assay AF-SSU was tested for specificity and applied for quantification of anaerobic fungi from rumen fluid and biogas sludge in the original study presented in Chapter 4. As this study was performed already in 2016, and some new taxa possibly with mismatches in relevant primer/probe hybridization regions have been described since, a bioinformatics re-evaluation was performed for this discussion. The alignment containing anaerobic fungal sequences and sequences of genetically close non-target organisms was updated by BLAST search and all novel sequences available from the NCBI GenBank nucleotide collection were integrated. Both primer sequences (AF-SSU forward see Figure 26; AF-SSU reverse see Figure 27) and the probe sequence (Figure 28) were marked in MEGA 7.0 (Kumar *et al.* 2016), showing their specific binding to the target group of anaerobic fungi. The integration of a 5'-hydrolysis probe further improved the specificity of assay AF-SSU, as in contrast to assays based on dyes binding to double stranded DNA (e.g. SYBR®-Green, EvaGreen®-Dye), both primers and the probe have to bind to the target template for a positive fluorescence signal, further avoiding false positive results (Hulley *et al.* 2019). This also differentiates assay AF-SSU from the quantitative PCR assays applied by Denman and McSweeney (2006) and (Kittelmann *et al.* 2012) which both involve SYBR®-Green chemistry.

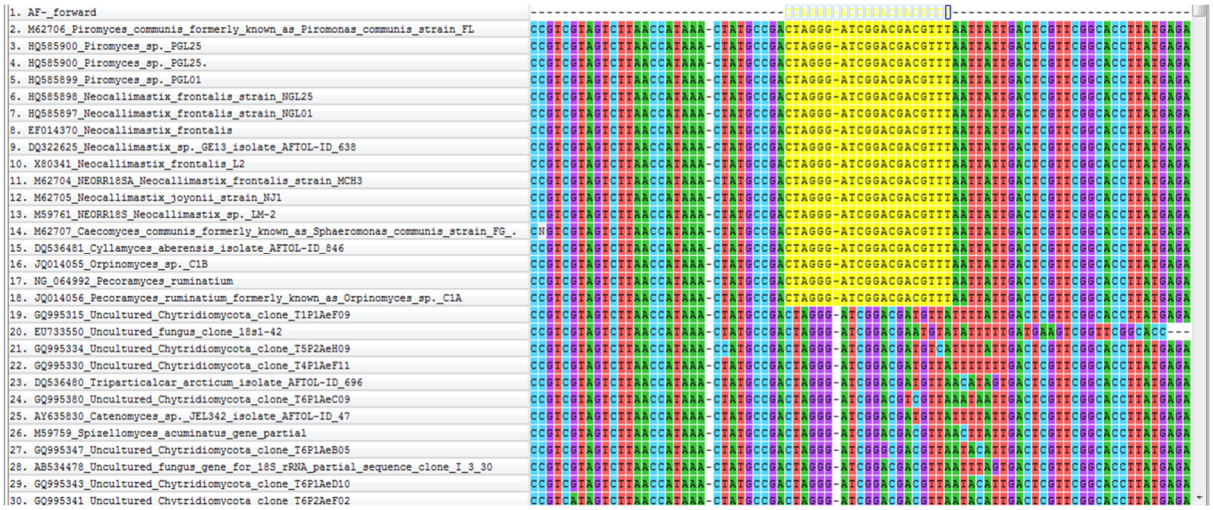


Figure 26: Screenshot from MEGA 7.0 showing the part of the updated 18S rRNA gene alignment with the forward primer binding site specifically found in the target group of anaerobic fungi (marked yellow)

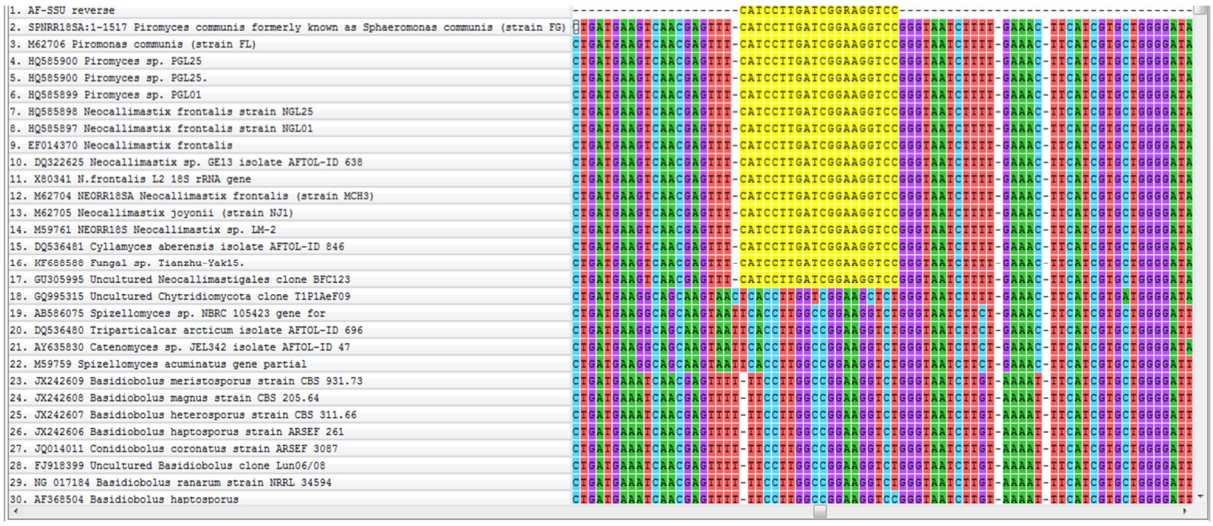


Figure 27: Screenshot from MEGA 7.0 showing the part of the updated 18S rRNA gene alignment with the reverse primer binding site specifically found in the target group of anaerobic fungi (marked yellow)

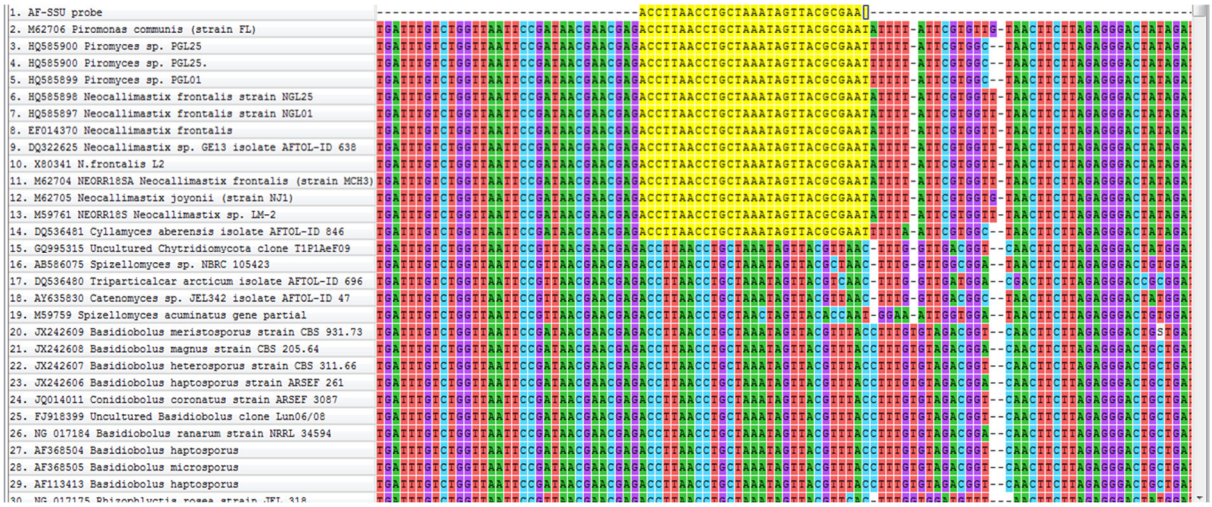


Figure 28: Screenshot from MEGA 7.0 showing the part of the updated 18S rRNA gene alignment with the probe binding site specifically found in the target group of anaerobic fungi (marked yellow)

However, as the SSU gene is not commonly applied for classification of anaerobic fungi or environmental barcoding studies, not many SSU gene sequences were added to the NCBI GenBank nucleotide database in the last three years. Only sequences of the genera *Caecomyces* (sequence only available for forward primer binding site), *Cyllamyces*, *Neocallimastix*, *Orpinomyces* (sequence only available for forward primer binding site), *Pecoramyces* (sequence only available for forward primer binding site) and *Piromyces* were available. Thus, specific detection for all known anaerobic fungal genera cannot be predicted by bioinformatics analysis to date. In order to demonstrate that assay AF-SSU allows detecting at least most of the actually described anaerobic fungal genera, endpoint PCR and agarose gel electrophoresis were performed with a collection of DNA extracts (Table 11) comprising strains of the species *Anaeromyces contortus*, *Anaeromyces mucronatus*, *Caecomyces* spp., *Cyllamyces* sp., *Feramyces austinii*, *Liebetanzomyces* sp., *Neocallimastix cameronii*, *Orpinomyces joyonii, Piromyces* sp. and *Pecoramyces ruminantium* (Figure 29). For the genera *Buwchfawromyces* and *Oontomyces*, no isolate or DNA extract was available.

Table 11: Isolates and DNA extracts used for re-validation of PCR assays

Isolate	Affiliation	Sample type	Provided by
G3C	*Anaeromyces contortus*	DNA	Dr. Mostafa Elshahed, Oklahoma State University
Brit-4	*Caecomyces* sp.	DNA	Dr. Mostafa Elshahed, Oklahoma State University
TSB-2	*Cyllamyces* sp.	DNA	Dr. Mostafa Elshahed, Oklahoma State University
F2a	*Feramyces austinii*	DNA	Dr. Mostafa Elshahed, Oklahoma State University
Cel 1a	*Liebetanzomyces* sp.	DNA	Dr. Mostafa Elshahed, Oklahoma State University
D3B	*Orpinomyces joyonii*	DNA	Dr. Mostafa Elshahed, Oklahoma State University
OS2	*Pecoramyces ruminantium*	DNA	Dr. Mostafa Elshahed, Oklahoma State University
Jen.1	*Piromyces* sp.	DNA	Dr. Mostafa Elshahed, Oklahoma State University
CaDo 3a	*Neocallimastix cameronii*	DNA	LfL collection
CaDo 13 a	*Caecomyces sp.*	DNA	LfL collection
YoDo3	*Anaeromyces mucronatus*	DNA	LfL collection

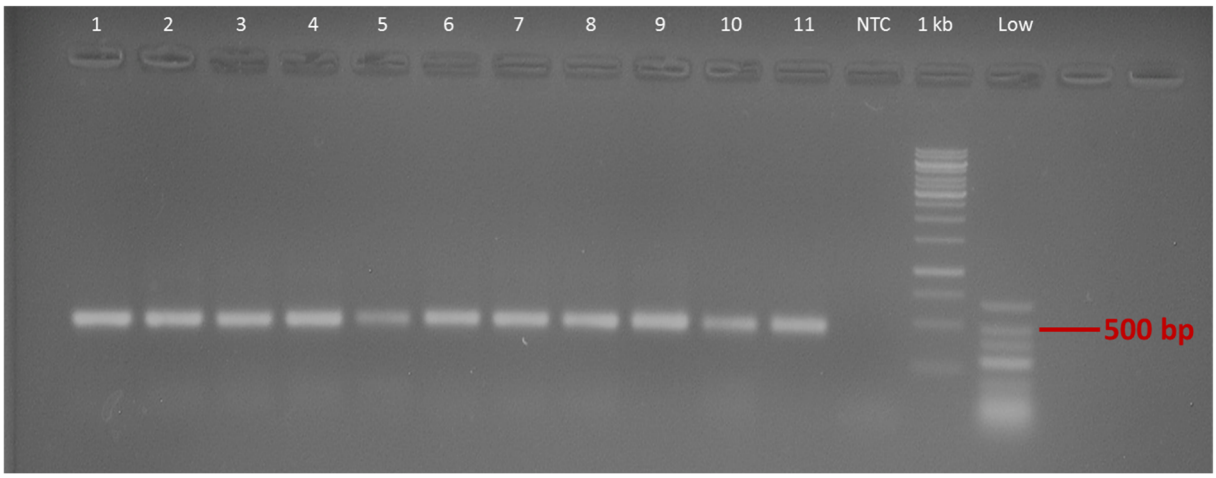


Figure 29: Amplicons (475 bp) derived by PCR with the primer pair AF-SSU on a 1.2 % agarosegel dyed with 2 µl of SERVAGreen, ran at 110 V for 35 min. Lanes: 1= *Anaeromyces contortus*, 2= *Caecomyces* sp., 3= *Cyllamyces* sp., 4= *Feramyces austinii*, 5= *Liebetanzomyces* sp., 6= *Orpinomyces joyonii*, 7= *Pecoramyces ruminantium*, 8= *Piromyces* sp., 9= *Neocallimastix cameronii*, 10= *Caecomyces* sp., 11= *Anaeromyces mucronatus*; NTC= No template control

As shown in Figure 29 specific amplification of the 475 bp long 18S rRNA gene sequence of all tested anaerobic fungal strains was achieved applying assay AF-SSU. Thus, this assay allowed specific detection of nine of the 11 known anaerobic fungal genera from pure culture DNA extracts. For the genera *Buwchfawromyces* and *Oontomyces*, no isolate or DNA extract was available, and it remains unclear if those will be amplified with assay AF-SSU. Further quantification from rumen fluid and biogas sludge samples delivered reasonable results (see Chapter 4), allowing acceptance of research hypothesis #1: "Anaerobic fungi can be detected in and quantified from animal derived and biogas sludge samples by a specific 18S rRNA gene targeting qPCR assay". Assay AF-SSU was further applied to quantify anaerobic fungi

by measuring their 18S rRNA gene copies in ten agricultural biogas plants (see Chapter 5) and to monitor the fate of the two *Neocallimastix frontalis* strains applied in the hydrolytic pre-treatment experiment performed in Chapter 6. In both studies, the assay performed well, and the results validated research hypothesis #1.

However, other researchers did not apply assay AF-SSU to monitor the abundance of anaerobic fungi during bioaugmentation experiments (Aydin *et al.* 2017; Yildirim *et al.* 2017) but continued using an older primer set published by Denman and McSweeney (2006). Due to mismatches of the forward primer with some *Neocallimastigomycota* and perfect matches with some outgroup sequences, this assay can produce biased results and cause limited coverage of anaerobic fungi Edwards *et al.* (2008).

In addition to the quantification tools discussed so far, Nagler *et al.* (2018) recently applied primer set GGNL1F and GGNL4R targeting a 573 bp fragment of the 28S rRNA gene for quantification of anaerobic fungi by qPCR. This approach seems valuable as researchers currently switch towards application of the LSU gene as a phylogenetic marker for anaerobic fungi (Edwards *et al.* 2017). Targeting the anaerobic fungal LSU gene for specific quantification and phylogenetic placement could simplify the detection of anaerobic fungi in various environments such as biogas processes and should be further evaluated.

Switching towards the LSU gene as phylogenetic marker is also depicted by the development of assay AF-LSU for phylogenetic classification of anaerobic fungi during this Ph.D. thesis (Chapter 4), testing hypothesis #3 “The composition of the anaerobic fungal community within animal derived and biogas sludge samples can be assessed by cloning and sequencing the 28S rRNA gene” (Section 2.1). As stated in the introduction (1.2.2.1), the ITS 1 region was most commonly used to analyze anaerobic fungal communities in environmental samples prior to the marker gene switch (see also below). In order to provide an overview, all primer sets applied for the phylogenetic placement of anaerobic fungi until the start of this Ph.D. thesis are listed in Table 12, and the positions of the most commonly used ones are visualized in Figure 25.

Table 12: Overview of primer sets for phylogenetic analysis of anaerobic fungi applied until 2012. Information on specificity for anaerobic fungi was judged from the referenced articles.

Target gene	Sample type	Nucleic acid type	Reference	Specific for anaerobic fungi
18S rRNA gene / SSU	Pure culture	RNA	Dore and Stahl (1991)	No
		DNA	Bowman *et al.* (1992)	No
		DNA	Fliegerová *et al.* (2006)	No
ITS 1	Pure culture	DNA	Li and Heath (1992)	No
		DNA	Brookman *et al.* (2000a)	No
		DNA	Hausner *et al.* (2000)	No
		DNA	Fliegerová *et al.* (2002)	No
		DNA	Tuckwell *et al.* (2005)	Yes
		DNA	Edwards *et al.* (2008)	Yes
	Pure culture; rumen fluid	DNA	Denman *et al.* (2008)	Yes
	Rumen digesta	DNA	Cheng *et al.* (2009)	Yes
	Animal feces	DNA	Liggenstoffer *et al.* (2010)	Yes
	Pig and cow manure	DNA	Fliegerová *et al.* (2010)	Partly specific
	Animal feces	DNA	Nicholson *et al.* (2010)	Yes
	Rumen fluid	DNA	Khejornsart and Wanapat (2010)	Yes
	Rumen fluid	DNA	Khejornsart *et al.* (2011)	Yes
	Rumen fluid; digesta	DNA	Kittelmann *et al.* (2012)	Yes
ITS 1 - 5.8S - ITS 2	Pure culture	DNA	Fliegerová *et al.* (2002); (2004; 2006)	No
		DNA	Tuckwell *et al.* (2005)	Yes
		DNA	Griffith *et al.* (2009)	Yes
		DNA	Nicholson *et al.* (2010)	Yes
28S rRNA gene / LSU	Pure culture	DNA	Hausner *et al.* (2000)	No
		DNA	Fliegerová *et al.* (2006)	No
		DNA	Dagar *et al.* (2011)	No
Intergenic spacer region	Pure culture	DNA	Hausner *et al.* (2000)	No

As already discussed in Chapter 4 and in the introduction (1.2.2.1), both relevant markers, ITS 1 and LSU, display advantages and disadvantages. The ITS 1 region is highly variable as it is non-coding (Eckart *et al.* 2010). This high variability can allow differentiating of closely related fungal taxa (if a good reference database is given) but it less suited for classifying evolutionary more distant taxa (Heeger *et al.* 2018). Due to the high sequence variability, alignment building is problematic (see also Chapter 4), impeding reliable phylogenetic classification (Heeger *et al.* 2019). Another drawback of the ITS 1 as genetic marker particularly for anaerobic fungi is that many strains show a high degree of intra-genomic variation between their ITS 1 sequences: E.g. *Buwchfawromyces* strain GE09 differs in its ITS 1 sequences by up to 13 % (Callaghan *et al.* 2015). Such variations were also detected for several other strains (see Chapter 4). This issue generally complicates classification of anaerobic fungi by their ITS 1 sequences. Such issues were not observed with assay AF-LSU or when LSU based phylogenetic analysis was performed. The LSU gene is more conserved, allows easier alignment building and delivers enough phylogenetic relevant information to

classify anaerobic fungi at genus and in some cases even at species level (Wang *et al.* 2017). Re-evaluation (performed as explained for assay AF-SSU above) of assay AF-LSU showed that the forward primer specifically binds to the target group of (known) anaerobic fungi and thus allows specific detection of all hitherto known anaerobic fungal genera (Figure 30).

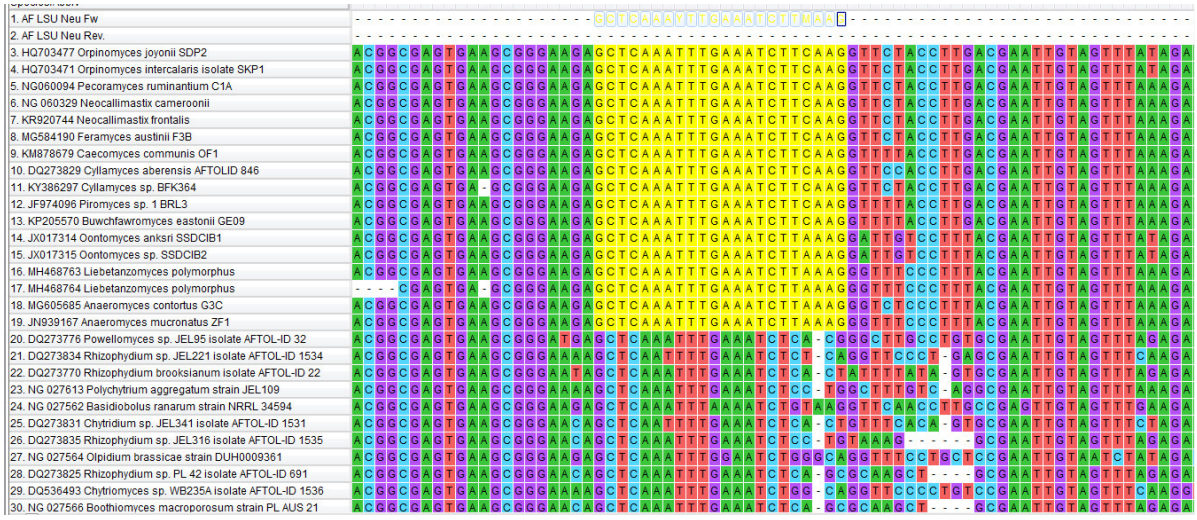


Figure 30: Screenshot from MEGA 7.0 showing the part of the updated 28S rRNA gene alignment with the forward primer binding site specifically found in the target group of anaerobic fungi (marked yellow)

For the reverse primer, two mismatches with the 28S rRNA gene sequence of the genus *Buwchfawromyces* were identified (Figure 31). However, as the mismatches are not located at the 3'-strand end of the reverse primer (base 15 and 16 from the 3'-strand end) annealing of the primer, attachment of the polymerase and amplification of the 28S rRNA gene of the genus *Buwchfawromyces* should still be possible.

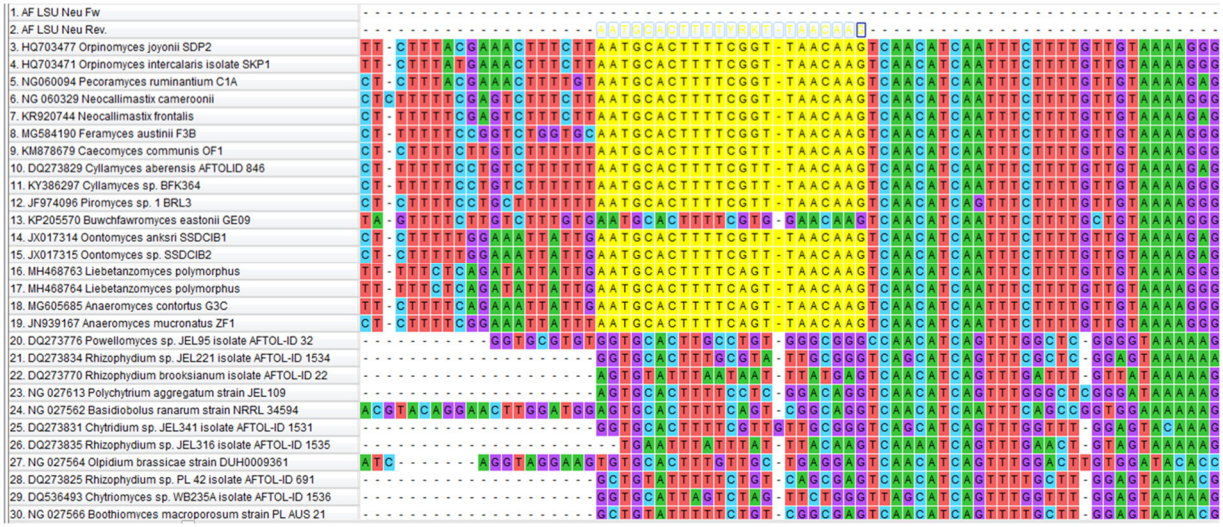


Figure 31: Screenshot from MEGA 7.0 showing the part of the updated 28S rRNA gene alignment with the reverse primer binding site specifically found in the target group of anaerobic fungi (marked yellow), except of the genus *Buwchfawromyces* sequence number 13

In order to further check the specificity of assay AF-SSU, amplification was tested with the DNA collection described above (Table 11).

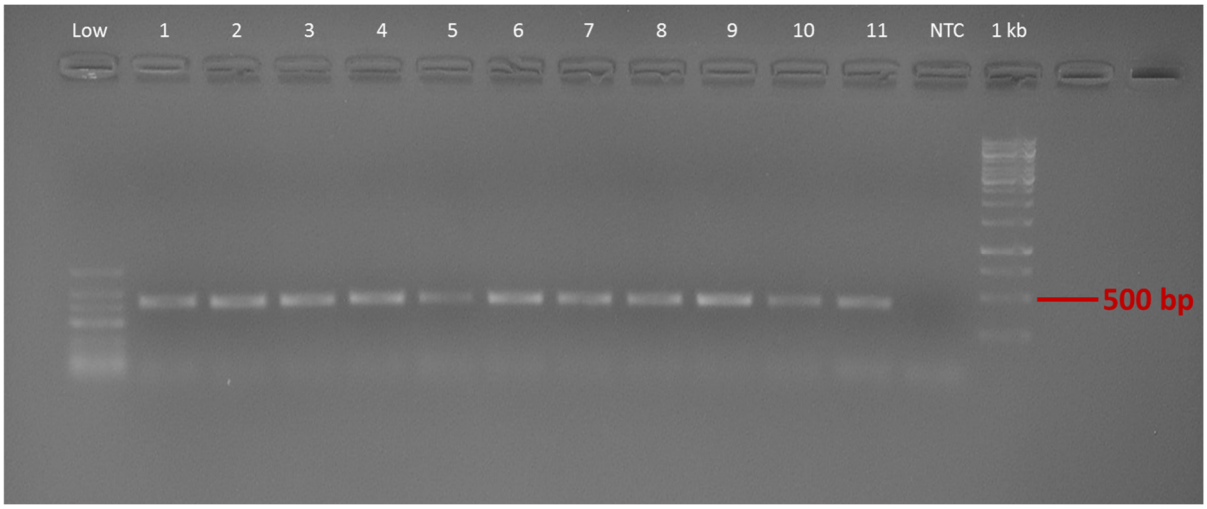


Figure 32: Amplicons (440 bp) derived by PCR with the primer pair AF-LSU on a 1.2 % agarosegel dyed with 2 µl of SERVAGreen, ran at 110 V for 35 min. Lanes: 1= *Anaeromyces contortus*, 2= *Caecomyces* sp., 3= *Cyllamyces* sp., 4= *Feramyces austinii*, 5= *Liebetanzomyces* sp., 6= *Orpinomyces joyonii*, 7= *Pecoramyces ruminantium*, 8= *Piromyces* sp., 9= *Neocallimastix cameronii*, 10= *Caecomyces* sp., 11= *Anaeromyces mucronatus*; NTC= No template control

As shown in Figure 32 all tested eleven anaerobic fungal strains covering 9 of the currently 11 known genera were amplified in PCR with assay AF-LSU. From the genera *Buwchfawromyces* and *Oontomyces*, which were not available for testing, the genus *Oontomyces* should be covered as shown by bioinformatics analyses (Figure 30 and Figure 31). For future studies it should be tested if the mismatches of the reverse primer cause exclusion of the genus *Buwchfawromyces*. Taken together, assay AF-LSU allowed specific detection of anaerobic fungi (as far as currently known) from a rumen and a biogas sludge sample and showed sufficient resolution of the known detected anaerobic fungal genera (see Chapter 4), validating hypothesis #3 “The composition of the anaerobic fungal community within animal derived and biogas sludge samples can be assessed by cloning and sequencing the 28S rRNA gene” (Section 2.1). In addition, re-evaluation of specificity and coverage of the phylum of *Neocallimastigomycota* qualify assay AF-LSU as a specific tool for phylogenetic analysis of anaerobic fungi.

Still, phylogenetic analysis of anaerobic fungi in environmental samples can be improved. One major issue is the lack of LSU reference sequences (Edwards *et al.* 2019) hampering classification in anaerobic fungal community analysis. Another issue is the difference between the informative sites of ITS 1 and LSU. Thus, both markers, ITS 1 and LSU, are used currently in parallel in most recent studies (Hanafy *et al.* 2018; Joshi *et al.* 2018; Li *et al.* 2019) allowing improved classification of anaerobic fungi and comparison with deposited environmental ITS 1 sequences. However, the parallel analysis can generate contradictory results, as the ITS 1 and the LSU based phylogenies can differ (Mura *et al.* 2019). This dilemma can be overcome by concatenation, by coupled amplification and analysis of both genetic markers, as recently performed by Heeger *et al.* (2019) for the conserved 5.8 S rRNA gene and the highly variable ITS 2 region in aquatic fungi. The additional information inferred from the 5.8S rRNA gene allowed classifying on higher taxonomic levels when ITS 2

taxonomy alone failed because no closely related gene sequences were available in the database. This facilitated the identification of a greater number of operational taxonomic units (OTUs) in an understudied basal fungal lineage (Heeger *et al.* 2019), comparable to the situation with the anaerobic fungi. As sequencing techniques are further improved and longer sequencing reads become possible, analysis of the complete ribosomal RNA operon comprising all hitherto utilized genetic markers (SSU, LSU, 5.8 S rRNA gene, ITS 1 and ITS 2), as already performed for aquatic fungi (Heeger *et al.* 2018), might be a more reliable strategy to assess the phylogeny of anaerobic fungi and should be evaluated in future studies.

The assays AF-SSU and AF-LSU discussed above give information on the presence and the community composition of anaerobic fungi in a sample. If DNA is analyzed, results do not give information if the present anaerobic fungi are also metabolically active. However, such information is necessary to assess activity e.g. in experiments in which the fiber degrading potential of anaerobic fungi shall be harnessed to improve biogas production from lignocellulosic residues, and to clarify if anaerobic fungi are an active part in conventional biogas production processes. Such information can be derived by an mRNA based approach illustrating gene transcription (Steiner *et al.* 2019). Addressing research hypothesis #2 (Section 2.1), assay AF-Endo was developed in this thesis to determine if anaerobic fungi in a sample were transcribing the gene of a glycosyl hydrolase family 5 (GH5) endoglucanase, known to be necessary for cellulose degradation (Chapter 4). In contrast to DNA which has been proven to endure days or even hundreds of years in dead tissues (Allentoft *et al.* 2012), mRNA in intact cells has a much shorter half-life time ranging from several minutes up to several hours (Clouet-d'Orval *et al.* 2018; Steiner *et al.* 2019). After cell lysis or damage, mRNA is exposed to extracellular ribonucleases and uncontrolled degradation (Deutscher 2015). This can further decrease its half-life time. It was therefore assumed that GH5 endoglucanase transcripts are only detected in samples containing viable and metabolically active anaerobic fungi.

As already discussed in Chapter 4, the chosen GH5 endoglucanase gene was shown to be transcribed at significant levels during lignocellulose degradation (Couger *et al.* 2015; Solomon *et al.* 2016). GH5 transcripts were further detected in a broad range of anaerobic fungal genera, comprising *Anaeromyces* spp., *Caecomyces* sp., *Feramyces* sp., *Neocallimastix* spp., *Orpinomyces* sp., *Pecoramyces* sp. and *Piromyces* spp. (see Table 1 or Murphy *et al.* 2019). The detection of anaerobic fungal GH5 endoglucanase transcripts shows that they were actively transcribing a cellulolytic gene. However, such findings cannot be directly correlated with protein abundance or the respective enzymatic activity (Greenbaum *et al.* 2003) and thus do not give information on the real-life cellulolytic performance of a particular anaerobic fungal isolate.

In order to survive in the highly competitive rumen gut environment, anaerobic fungi had bolstered their genetic repertoire and metabolic functions apparently at least partially by HGT (Murphy *et al.* 2019). The majority of anaerobic fungal CAZymes seems to be of non-fungal origin and was most probably acquired by HGT, including GH5 cellulases of which more than 50 % were likely obtained from *Clostridia* and unclassified *Bacteria* (Murphy *et al.* 2019). Thus, a crucial point in the development of assay AF-Endo was to rule out amplification of

bacterial GH5 endoglucanase genes. As described already in Chapter 4, this was done methodologically by choosing an mRNA extraction method excluding bacterial mRNA by restriction to polyadenylated mRNA, alleviated by a Poly-(A)-tail to Oligo(dT) binding step. Further specificity of assay AF-Endo was tested by *in silico* PCR against a database containing the genomes of HGT relevant cellulolytically active bacteria like *Fibrobacter succinogenes* and 56 different *Clostridium* species. As no positive amplification was detected *in silico* and no bacterial gene sequences were obtained by cloning and sequencing of amplicons from cattle rumen fluid (Chapter 4), AF-Endo preliminarily qualified to detect anaerobic fungal GH5 endoglucanase genes specifically.

An issue remains the low number of functional gene sequences deposited in online databases. Sequences of the GH5 endoglucanase gene of anaerobic fungi are not available for all described genera. As described in Chapter 4, testing of DNA of strains of the genera *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Neocallimastix* and *Piromyces* resulted in specific amplification. In addition, bioinformatics analysis suggests that according to the available sequences, the genera *Orpinomyces* and *Pecoramyces* may be covered by assay AF-Endo. As no sequences were available for the other anaerobic fungal genera, the DNA collection described in Table 11 was used to examine representatives of nine of the known genera. As stated already above, no sequence or DNA extract was available to test assay AF-Endo for amplification of a representative of the genera *Buwchfawromyces* and *Oontomyces*.

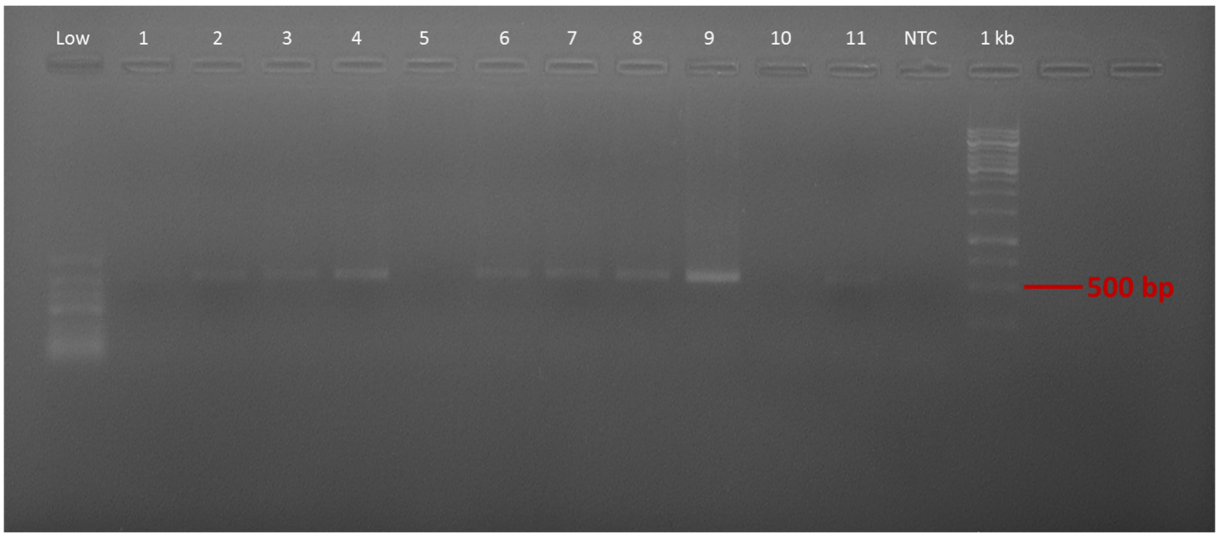


Figure 33: Amplicons (526 bp) derived by PCR with the primer pair AF-Endo on a 1.2 % agarosegel dyed with 2 µl of SERVAGreen, ran at 110 V for 35 min. Lanes: 1= *Anaeromyces contortus*, 2= *Caecomyces* sp., 3= *Cyllamyces* sp., 4= *Feramyces austinii*, 5= *Liebetanzomyces* sp., 6= *Orpinomyces joyonii*, 7= *Pecoramyces ruminantium*, 8= *Piromyces* sp., 9= *Neocallimastix cameronii*, 10= *Caecomyces* sp., 11= *Anaeromyces mucronatus*; NTC= No template control

From the tested genera not all were amplified with assay AF-Endo. The *Liebetanzomyces* strain, as well as one of the tested *Caecomyces* strains was not amplified. Further the two tested *Anaeromyces* species showed only weak bands on the agarosegel, which could be caused by poor primer binding leading to lower PCR efficiency for such strains.

As assay AF-Endo was tested positive for amplification of most anaerobic fungal genera (Chapter 4 and Figure 33) and for amplification of GH5 genes from cattle rumen fluid (Chapter 4) and biogas sludge samples (Chapter 5), research hypothesis #2 “Active transcription of a cellulolytic gene of anaerobic fungi can be assessed for animal-derived and biogas sludge samples by specific quantification of glycosyl hydrolase family 5 endoglucanase transcripts in mRNA extracts” was accepted. However, upon publication of new data and isolation of novel strains, assay AF-Endo should be re-evaluated and might possibly be modified.

Re-validation of the three tools developed in this Ph.D. thesis showed that they are still up to date and can detect most anaerobic fungal genera. Only for the genera *Buwchfawromyces* and *Oontomyces*, no testing was possible as no isolate or DNA extract was available. It remains thus unclear if all assays would detect members of these genera.

Using three individual assays each targeting an individual gene is time consuming. It would be an improvement if the analyses could be combined to simplify the detection of anaerobic fungi in biogas processes and environmental samples. However, applying one single tool for all three purposes will most probably not be possible, and as functional gene sequences allowing for activity screening are not available for all known anaerobic fungal genera, it is impossible to estimate their suitability for phylogenetic analyses yet. Still, quantification and phylogenetic placement will be combined by developing a qPCR method from assay AF-LSU in future studies.

7.2 Detection of anaerobic fungi in agricultural biogas plants

Industrial production of biogas is an engineered version of the natural anaerobic digestion process. It is running in soil, sediments, ruminants and other anaerobic habitats (Godon *et al.* 2013). In ruminants, this process has been evolutionarily optimized over millions of years leading to a superior fiber degradation strategy, from which bioengineers could learn. Both processes, anaerobic digestion in the rumen and biogas production, rely on the combined action of diverse microbial consortia to degrade plant biomass. In the rumen, bacteria, methanogenic archaea, anaerobic fungi, phages and protozoa live in close association (Huws *et al.* 2018). The role of phages and protozoa is still not fully understood. Lytic phages are known to infect rumen bacteria (Gilbert *et al.* 2017) and thus might shape the bacterial community in the rumen. The protozoa community is dominated by ciliates, of which some genera (e.g. *Epidinium* spp.) are known to be linked to fiber degradation (Huws *et al.* 2018). This was also proven by *in-vivo* defaunation experiments, where the elimination of protozoa caused a decrease in organic matter degradation (Newbold *et al.* 2015). However, the major part of fiber degradation is performed by bacteria (e.g. *Ruminococcus* spp, *Fibrobacter* spp.) and anaerobic fungi. Both groups express a multitude of cellulolytic and hemicellulolytic enzymes. The enzymes can be excreted freely or are synergistically operating due to cellulosomal organization (see Subsubsection 1.2.2.2). Rumen fungi work auxiliary to the bacterial action. They are known to be primary colonizers of ingested forage and to break up the plant material mechanically by rhizoidal growth (Edwards *et al.* 2008), increasing the accessible surface for bacterial attack. Further, they possess additional amylolytic and proteolytic enzymatic activities (Huws *et al.* 2018). The combined action of bacteria and fungi delivers energy, mainly in form of VFAs and microbial protein, to the host (Weimer *et al.* 2009). Just like in the biogas production process, the degradation cascade ends with methanogenic archaea producing methane which is released to the environment (Huws *et al.* 2018).

Both processes show similarities: they are running commonly at mesophilic temperatures, at pH values close to the neutral point, and they are utilizing metabolic pathways leading over acetate as the central metabolite at very low redox potentials. This limits oxidative degradation processes to carbonate respiration via C-disproportion and leads to almost equimolar CO_2 and CH_4 production (Table 13). Further, animal manure and slurry are often used as substrates for agricultural biogas production potentially transferring microorganisms from animal digestive tracts into the biogas plant. One could thus assume that some rumen microorganisms should thrive in both environments, and that anaerobic fungi as cellulolytic key players might also contribute to the biogas production process in industrial biogas plants.

Table 13: Comparison of the process conditions in the rumen and agricultural biogas plants. Rumen data was obtained from Weimer *et al.* (2009) biogas plant data from Lebuhn *et al.* (2014) and Weiland (2010).

	Rumen	Agricultural biogas plants
Substrates	Forage, water	Plant biomass, animal manure and slurry
Pre-treatment	Mastication	chemical, physical or microbial pre-treatments can be applied
Total solids	12 – 18 %	<10 – 14 %
Redox potential	-0.4 V	≤ -0,25 V
Temperature	38 – 40 °C	Mostly 38 – 44 °C
pH value	5.3 – 6.7	6.6 – 8.5
Mixing	Muscular movements	Mechanical stirring
Key microbial guilds	Mixed consortium of bacteria, archaea, anaerobic fungi and protozoa	Mixed consortium of bacteria and archaea.
Buffer system	Bicarbonate	Bicarbonate

This assumption led to research question No. 2 “Are anaerobic fungi part of the biogas producing microbial community in agricultural biogas plants?” and the accompanying hypothesis #4: Anaerobic fungi can be present and transcriptionally active in agricultural biogas plants (see Section 2.1). Ten agricultural biogas plants were thus screened with the PCR based tools developed in this thesis (Chapter 4, discussed in Section 7.1.) for the presence and transcriptional activity of anaerobic fungi.

Most of the biogas microbiome research has been focused on the two key groups, bacteria and methanogenic archaea (Stolze *et al.* 2015; e.g. Campanaro *et al.* 2016; Grohmann *et al.* 2018). Since eukaryotes were omitted in these studies, it remained unclear if they were part of the examined biogas production processes. The occurrence of fungi was first indicated in a biogas plant treating food waste by Bengelsdorf *et al.* (2013), who found DNA of the genera *Mucor* and *Saccharomyces* aside of two unclassified fungal clades. The study led to more targeted research on the fungal fraction in the already earlier sampled biogas plant treating food waste and an agricultural biogas plant operated with maize silage, cattle manure, grass silage and crop residues (Kazda *et al.* 2014). Fungi from the subphyla *Agaricomycotina*, *Mucoromycotina*, *Pucciniomycotina*, *Saccharomycotina*. *Pezizomycotina* and the class *Neocallimastigomycetes* were detected by means of their 18S rDNA and ITS 1 DNA sequences. While the other fungal clades were present in both biogas plants, the anaerobic fungi (*Neocallimastigomycota*) were only present in the biogas plant fed with cattle manure. In a recent study, Langer *et al.* (2019) analyzed the bacterial, archaeal and fungal communities in nine full-scale biogas plants with an RNA-based amplicon sequencing approach targeting 16S rRNA and 28S rRNA fragments. Fungi were identified in all nine reactors and belonged mainly to the phyla Ascomycota (*Pezizomycotina* and *Saccharomycotina*), Basidiomycota (*Agaricomycotina*, *Pucciniomycotina* and *Ustilaginomycotina*) and the subphylum *Mucoromycotina*, similarly as found by Kazda *et al.* (2014). The composition of the fungal communities was comparable between five of the sampled reactors and dominated by members of the family *Cladosporiaceae*. In two of the

other fermenters, the main proportion of the fungal community clustered with hitherto uncharacterized soil fungi. Anaerobic fungal sequences were not detected by Langer *et al.* (2019) in contrast to earlier work and the present study (Chapter 5), in which the presence of anaerobic fungi was shown by measuring 18S rDNA gene copy numbers in seven of the ten sampled biogas plants. The feedstock of these biogas plants containing anaerobic fungal DNA consisted of cattle manure (21.6 % to 77.3 % of total substrate) and/or slurry (1.7 % to 44.9 % of total substrate). The three biogas plants showing no evidence for anaerobic fungi contained no or lower amounts of cattle manure or presented adverse conditions (e.g. 90 days hydraulic retention time at 52 °C) for their survival. The presence of anaerobic fungi seemed thus to be linked to the input of cattle manure and/or slurry, coinciding with the findings of Kazda *et al.* (2014). However, as mentioned above, this observation is not supported by the recent study of Langer *et al.* (2019) in which no evidence for anaerobic fungi was found even if the biogas plants were operated with a share of cattle manure (18 to 99 % of the total feedstock) and cattle dung (2 to 21 % of the total feedstock).

In a follow-up study, the PCR based tools developed in this thesis (Chapter 4) were applied to screen two one-phase and two two-phase biogas plants for anaerobic fungi (Young *et al.* 2018). Alongside of anaerobic fungi, aerobic fungi were detected and classified by isolation and sequencing. The aim of operating biphasic biogas plants is to separate hydrolysis / acidogenesis and acetogenesis from methanogenesis, and to provide optimum growth conditions (e.g. pH value) for the different functional microbial guilds. As hydrolysis tanks were not analyzed for the occurrence of fungi before, this study gives additional information on their fate in and contribution to biogas production processes. Seventeen aerobic fungal isolates from the phyla *Zygomycota*, *Ascomycota* and *Basidiomycota* were obtained. Aerobic fungi were detected in all four biogas plants. They occurred mainly in the substrates, the mixing-tanks, the hydrolysis tanks and in fibers separated from the digestate. Their occurrence and diversity seemed to be linked to the type of fibrous feedstock used. Anaerobic fungi were detected only in the digester and digestate storage tank of one mesophilic one-phase biogas plants. This biogas plant was the only one in the study of Young *et al.* (2018) operated with cattle manure, emphasizing that cattle manure is a potential input source for anaerobic fungi.

In order to examine which anaerobic fungi were present in the ten agricultural biogas plants analyzed in Chapter 5, cloning and sequencing of a 441 bp long amplicon of the anaerobic fungal 28S rDNA genes was performed with primer pair AF-LSU (Chapter 4). Analysis of the derived LSU sequences resulted in a phylogenetic tree differentiating well between the individual anaerobic fungal genera (see Chapter 5). As the article (Chapter 5) was already published in 2017, many novel anaerobic fungal genera were discovered until publication of this thesis. Thus, the originally published dataset was updated with all available LSU sequences of the novel described genera and sequences of isolates which are candidates for further seven novel *Neocallimastigomycota* genera but not formally published and described yet (Hanafy *et al.* 2019). The addition of the novel genera and “candidate genera” allowed better classification of some of the novel clades detected in Chapter 5. Novel clade A comprising the sequences KX164375 Biogas.Clone PB_25_F5, KX889553 Biogas.Clone

PB_14_D_1, KX889491 Biogas.Clone PB_21_PD_1, KX164389 PB_25_5 and two sequences detected by Young *et al.* (2018) in the digestate storage tank of a mesophilic biogas plant (see Figure 18 Chapter 5 and Figure 34) clustered with the *Neocallimastigomycota* strains ZS, ZC 41 and ZC 42 isolated from Grevy Zebra (*Equus grevyi*) feces. The latter represent the putative novel genus *Khoyollomyces* (Hanafy *et al.* 2019). Representatives of this clade were the most abundant group in the biogas plant examined by Young *et al.* (2018) and the third most abundant and present in three of the ten tested biogas plants in the study performed during this thesis (Chapter 5). Further novel clade D (see Figure 18 Chapter 5) represented by sequence KX889549 Biogas.Clone PB_16_D_11 is most closely related to the recently discovered genus *Feramyces*, actually comprising only a single species, *Feramyces austinii* (Hanafy *et al.* 2018). The described *Feramyces austinii* strains were isolated from feces and rumen liquid of a wild Barbary sheep and a fallow deer. The strains showed the ability to utilize a broad range of carbohydrates, including substrates usually unfavorable for anaerobic fungal growth, and grew more quickly on plant biomass compared to the six other strains of the tested anaerobic fungal genera (Hanafy *et al.* 2018). The mentioned abilities of *Feramyces austinii* might also account for the closely related novel clade D and favor the occurrence of such anaerobic fungi in biogas plants. However, anaerobic fungal LSU sequences belonging to novel clade D were present in low abundance and only in one of the sampled biogas plants, and are thus potentially playing a minor role in the biogas production process. In addition, the placement of the sequences KX164369 Rumen.Fluid.Clone AF-LSU-RF-6 and KX164373 Rumen.Fluid.Clone AF-LSU-RF-10 made in Chapter 4 needs to be corrected, as these two sequences cluster more closely to the recently described genus *Liebetanzomyces* (Joshi *et al.* 2018) isolates from goat rumen than to species of the genus *Anaeromyces*. For the two remaining detected novel genera B and C (Figure 18, Chapter 5), still no closely related anaerobic fungal LSU gene sequences were available.

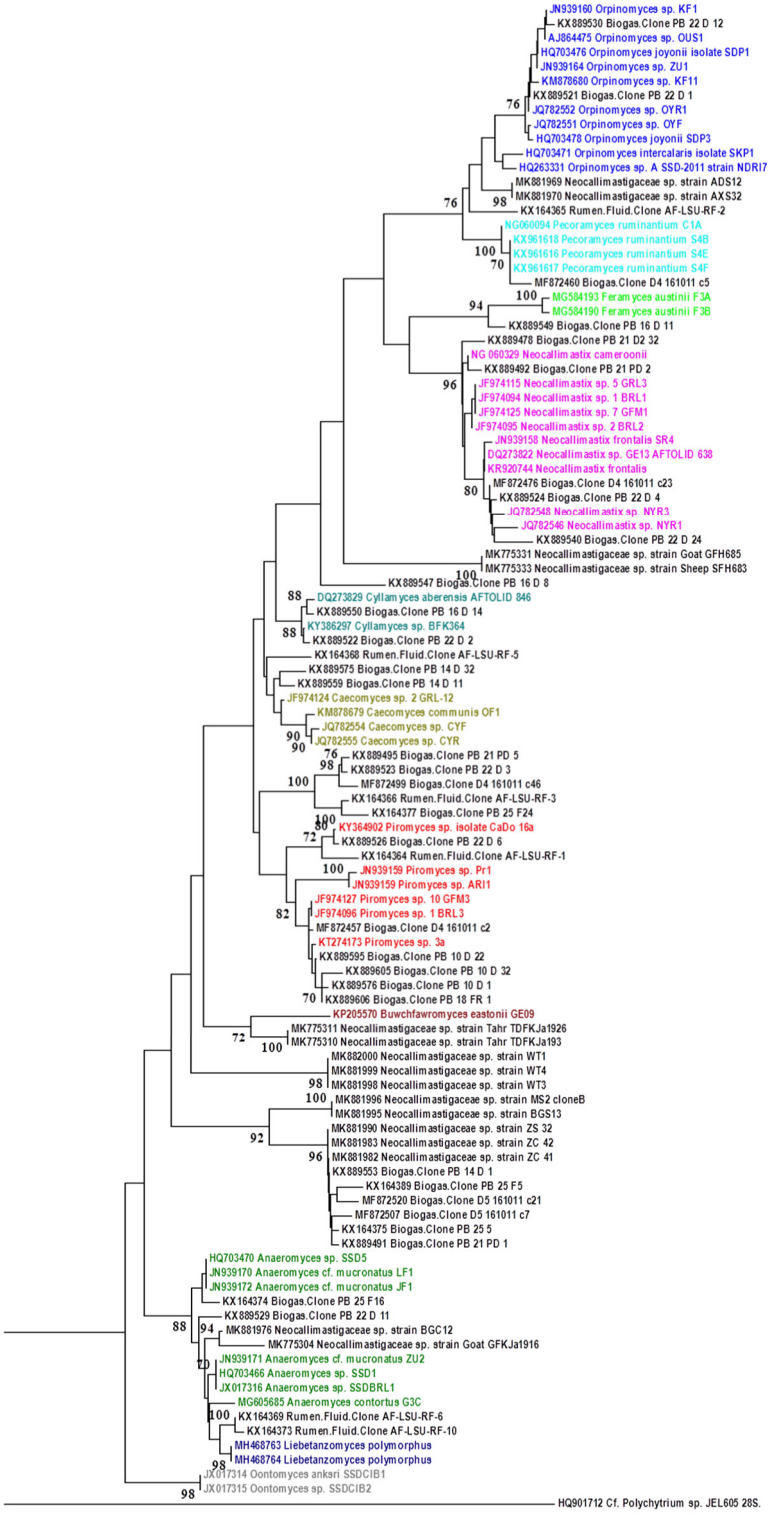


Figure 34: Neighbour joining tree based on a 409 bp alignment of 104 anaerobic fungal 28S rDNA sequences. Included are sequences of representatives of all described anaerobic fungal genera, along with clone sequences derived from the biogas plants examined in Chapter 5 of this thesis named as "Biogas.Clone PB", the biogas plants examined by (Young *et al.* 2018) named as Biogas.Clone D4 or Biogas.Clone D5 and clone sequences derived from rumen fluid examined in Chapter 4 of this thesis, named as Rumen.Fluid.Clone. An aerobic chytrid *Polychytrium* sp. (HQ901712) was used to root the tree. Only bootstrap values over 70 % are shown, the scale bar shows substitutions per site. The different genera are color coded as follows: *Anaeromyces* (green), *Buwchfawromyces* (brown), *Caecomyces* (light green), *Cyllamyces* (aquamarin), *Feramyces* (bright green), *Liebetanzomyces* (marineblue), *Neocallimastix* (pink), *Oontomyces* (grey), *Orpinomyces* (blue), *Pecoramyces* (turquoise), *Piromyces* (red).

Phylogenetic analysis of anaerobic fungal gene sequences found in biogas plants showed that all anaerobic fungal genera except for the genera *Oontomyces* and *Buwchfawromyces* were present. It cannot be excluded, that the absence of sequences of the genus *Buwchfawromyces* might have been caused by the mismatches occurring with the AF-LSU reverse primer. The genera *Neocallimastix*, *Piromyces* and the "candidate genus" *Khoyollomyces* were the most abundant and wide spread. As already discussed in Chapter 5, *Neocallimastix* and *Piromyces* sequences were detected in a broad range of animal derived samples in a study by (Liggenstoffer *et al.* 2010). Particularly high numbers were detected in samples from the family *Bovidae*, leading to the conclusion that cattle manure and slurry used in the sampled biogas plants should contain a high load of strains of the above mentioned anaerobic fungal genera. For the "candidate genus" *Khoyollomyces,* all hitherto isolated strains were derived from animals of the family *Equidae*. As none of the sampled biogas plants was operated with manure/slurry or feces of *Equidae*, the origin of the biogas plant sequences falling in this clade remains unclear. Supposedly, the spectrum of the "candidate genus" *Khoyollomyces* will get wider if more sequences and isolates are obtained from a broader range of animals, as emphasized by the study of Hanafy *et al.* (2019).

In addition to the presence of anaerobic fungi, their transcriptional activity was measured by quantification of GH 5 endoglucanase transcripts. If mRNA was extracted and the respective transcripts were detected, it was concluded that viable anaerobic fungi were present in the tested biogas sludge sample (Section 7.1). Low signals for GH5 transcriptional activity were only detected in the digesters of two of the seven biogas plants (PB 21 D2, PB 22 D1 see Chapter 5) that tested positive for the presence of anaerobic fungi. The digesters of these two biogas plants showed also the highest concentration of 18S rRNA gene copies $*$ ml^{-1} digester sludge and were both operated with medium to high amounts of cattle manure (21.6 % and 77.3 % of the total feedstock). The temperature was different in the digesters of the two biogas plants, digester D2 of biogas plant 21 was operated at 40 °C and the digester of biogas plant 22 at 53 °C. Thus, the latter exceeded the mean rumen temperature by 14 °C. As far as known, this temperature is too hot for the growth of anaerobic fungi (Lowe *et al.* 1987a). Still, the digester of biogas plant 22 showed the highest transcriptional activity of anaerobic fungi. This can be explained by the short hydraulic retention time of only 12 days and the high amount of daily fed cattle manure constantly supplying the digester with fresh anaerobic fungi. These fungi are most probably dispersed in the fermenter, and their nucleic acids, including mRNA, was still measured at the sampling point. The other sampled thermophilic biogas digester which was also fed with a high share of cattle slurry but had a longer hydraulic retention time (Chapter 5) showed no signal for metabolically active anaerobic fungi, indicating that these organisms, including their mRNA, are digested during the longer residence times. In addition to the findings in this thesis, transcriptional activity of anaerobic fungi was detected by Young *et al.* (2018) in the digester of a mesophilic biogas plant (32.5 °C) operated with 30 % cattle manure. Again, this supported the idea that anaerobic fungi can be present and metabolically active for a certain time in biogas plants operated with a considerable share of cattle manure.

To further understand the occurrence and fate of anaerobic fungi in biogas plants, isolation was performed for two digester sludge samples which had tested positive for the presence of anaerobic fungi: digester 2 of biogas plant 21 showed also transcriptional activity of anaerobic fungi and isolate CaDo 16a, a potentially novel *Piromyces* species, was obtained from the biogas plant that had tested positive for metabolically active anaerobic fungi. To date only a *Piromyces* strain was isolated from a pond sediment as a non-animal habitat Wubah and Kim (1995). Anaerobic fungal DNA has been detected in a much wider range of non-animal habitats (see Subsubsection1.2.2) but it was not examined if the fungi were metabolically active therein. An explanation for their potentially widespread occurrence contrasting with the low number of isolates derived from non-animal habitats may be that resistant structures of the anaerobic fungi, so called resting stages, are the source of molecular detection (Gruninger *et al.* 2014). Anaerobic fungi produce such stages to raise their chance for survival when they are exposed to adverse environmental conditions (Wubah *et al.* 1991; Brookman *et al.* 2000b; Callaghan *et al.* 2015).

Taken together, the above listed results have proven research hypothesis #4 “Anaerobic fungi can be present and transcriptionally active in agricultural biogas plants”, but with substantial restrictions: Anaerobic fungi were only present in biogas plants operated with a medium to high share of cattle manure in the feedstock. Signals for metabolically active anaerobic fungi were scarce and low in intensity. This leads to the conclusion that anaerobic fungi are transferred to biogas reactors via the fed cattle manure. The low signals of metabolic activity probably result from the constant input of active fungi with the fed cattle manure. Anaerobic fungi seem thus to play only a minor role in conventional biogas plants, as the data suggest that they are only able to stay transitionally active at the conditions in biogas reactors.

It remains unclear which physical, chemical or operational parameters impede the activity of anaerobic fungi during anaerobic digestion. While differences in bacterial and archaeal communities have been linked for example to process temperature and ammonium concentration, the abundance of aerobic fungi was only found to depend on the used substrate mixture, and the communities were comparable under a variety of conditions (Langer *et al.* 2019). Without a better understanding of the presumably inhibiting factors, it seemed to be a more promising strategy to apply anaerobic fungi as fiber pre-treatment step decoupled from anaerobic digestion, as performed in this thesis and discussed in section 7.3, than introducing them directly into a biogas fermenter.

7.3 Pre-treatment of hay with *Neocallimastix frontalis* leads to enhanced biogas production

Anaerobic fungi as excellent LCB degraders (see section 1.2) open new possibilities to improve biogas production of fibrous residues. Following this general idea, hay was pre-treated with two individual *Neocallimastix frontalis* strains in a two-stage batch fermentation experiment during this Ph.D. thesis (Chapter 6). The application of the fungal isolates led, compared to heat-inactivated controls, to increased dry matter reduction, coupled to increased VFA production and an initial increase in biogas generation. Thus, research question No. 3 "Can biogas production from lignocellulosic feedstock be enhanced by hydrolytic pre-treatment with anaerobic fungi?" could be positively answered at least for the two tested *Neocallimastix frontalis* strains by proof of hypothesis #5 "Pre-treatment with anaerobic fungi, as exemplified with different *Neocallimastix frontalis* strains, can improve biogas production from hay".

As summarized already in the introduction (Subsection 1.2.2.3) only a few attempts were made to improve anaerobic digestion by implementing anaerobic fungal pure isolates or mixed cultures. An overview of all published work regarding this topic is given in the timeline in Figure 35. These approaches were mainly devised to integrate anaerobic fungi or mixed anaerobic fungal cultures directly into methanation processes.

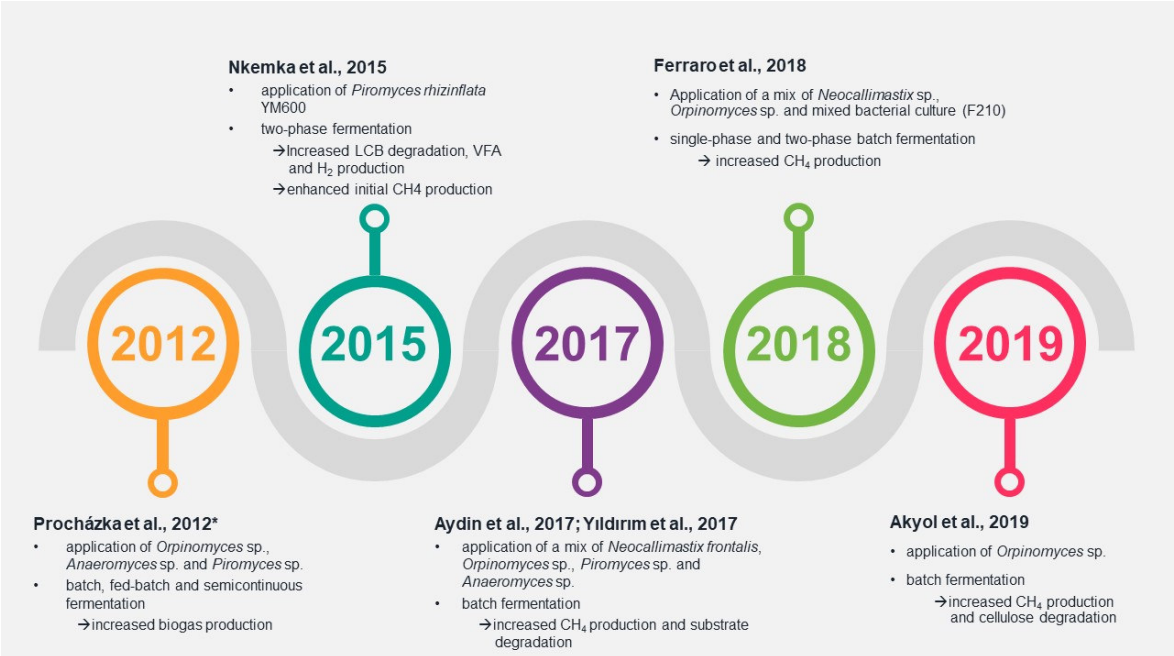


Figure 35: Timeline of research performed on biogas production with anaerobic fungi. * =adequate control assay included

The results of these studies seem encouraging at a first glance, as in each study the application of anaerobic fungi caused beneficial effects (e.g. increased methane yield, biogas production

or degradation of substrates; see Figure 35) on biogas production. Taking a closer look, some limitations or drawbacks become obvious explaining why anaerobic fungi are still not commonly used for biogas production and why the pre-treatment experiment was performed during this Ph.D. thesis:

Anaerobic fungi are commonly grown on complex culture media (see Section 3.3). Such media usually contain clarified rumen fluid, salt solutions, a diverse range of carbohydrates, nitrogen sources (e.g. yeast extract, casitone, tryptone), redox-buffer (e.g. $NaCO_3$ and L-Cysteine), resazurin, a volatile fatty acid solution, a vitamin solution and antibiotics (e.g. ampicillin, streptomycin, penicillin and/or chloramphenicol). These media were developed to provide optimum conditions for anaerobic fungal growth but not to be used in industrial processes. Limitations in the availability of rumen fluid and variability in the quality of each batch of rumen fluid and other complex media components hamper standardized and large scale media production (Podolsky *et al.* 2019).
Further, the ingredients are not only nutrients for anaerobic fungi, they also serve as feed for other microorganism e.g. bacteria and/or methanogenic archaea performing biogas production. Additional nutrients transferred to the anaerobic digestion assay together with the anaerobic fungal inocula can be converted to biogas and thus may be responsible for increased production rates and yields. Such a bias cannot be excluded for the studies listed above (Figure 35), except for the study by Procházka *et al.* (2012) in which adequate inactivated controls were used to define biogas production from the spent culture media and fungal biomass. In this thesis (Chapter 6) a less complex rumen fluid free medium (see Section 3.3) was thus developed. The *Neocallimastix frontalis* strains were adapted to this medium prior to their use for hydrolysis of hay. In addition, heat-inactivated (autoclaved for 15 min at 121 °C) controls were added for each tested culture. By this approach, the issues mentioned above were mitigated. Improvements of the minimal medium still seem to be necessary, as it was not suitable for routine subcultivation and long-term maintenance of anaerobic fungal isolates. This became obvious by the loss of *Neocallimastix frontalis* isolate 2 during the first experiment (Exp A) (see Chapter 6) which was most probably caused by transfer of the strain to the minimal medium and accumulation of inhibiting mid-chain fatty acids during the hydrolysis stage (see Chapter 6). Furthermore, the use of defined minimal culture media might limit the range of cultivable anaerobic fungi and thus limit the diversity of available strains for subsequent industrial applications (Podolsky *et al.* 2019).

Another issue is the short survival time of anaerobic fungi under biogas production conditions. Two of the studies mentioned above (Procházka *et al.* (2012); Yildirim *et al.* (2017); Figure 35) and this thesis (Chapter 6) had applied molecular detection techniques to monitor the survival of anaerobic fungi and delivered all comparable results: Signals for the presence of anaerobic fungi were found to decrease when the fungi were admixed to the anaerobic sludge and were not detectable after 7 to 10 days (Procházka *et al.* 2012; Yildirim *et al.* 2017). This is in agreement with the findings presented in Chapter 5 and the observations of Young *et al.* (2018) that anaerobic fungi do not seem to be generally active in conventional biogas production processes, although they are constantly introduced by cattle manure in the feedstock. This suggests that the conditions in the biogas process inhibit the viability of anaerobic fungi. In addition, results of experiments performed during this Ph.D.

thesis on the ability of four anaerobic fungal isolates to adapt to a digestate based medium further discouraged the approach to bioaugment biogas reactors directly with anaerobic fungi: Four anaerobic fungal isolates were individually transferred on “digestate-medium” (Section 3.5) starting with a concentration of 2 % digestate mixed into enrichment medium containing cellobiose and soluble xylan as carbon sources. After successful growth, the fungi were transferred to the next higher “digestate-medium” concentration in portions ranging from 2 to 20 % digestate. Growth was monitored by light microscopy and documented by pictures. Pictures of the adaptation trial with *Neocallimastix cameroonii* (CaDo 3b) are shown in Figure 36. For comparison healthy cultures of CaDo 3b grown on rumen fluid based medium are shown (Figure 36 a and b). Cultivation with digestate in concentrations of 2 % (see Figure 36 c) seemed already to affect growth in CaDo 3b. Sporangia were darker colored and the usually clearly visualizable (Figure 36 b) zoospores inside of the sporangia looked fuzzy. While during cultivation on 10 % digestate still fungal bodies could be observed (Figure 36 d), on 14 % digestate (Figure 36 e) only single ruptured and non-viable sporangia could be found occasionally. On the highest tested digestate concentration with 16 % (Figure 36 f) no growth of CaDo 3b could be observed at all.

The isolates KiDo 1h and *Cyllamyces* sp. KiDo 2m survived in a medium containing up to 14 % and 16 % digestate, while the *Piromyces* sp. (KiDo 3a) and the *Neocallimastix cameroonii* (CaDo 3b) died already at concentrations of 6 % and 10 % respectively. None of the tested fungal cultures was able to grow on the pure “digestate stock solution” containing 20 % digestate and no additional nutrients or adjuvants (Section 3.5). All tested isolates showed inhibited growth accompanied by changes in morphology when they were cultivated on “digestate-medium”

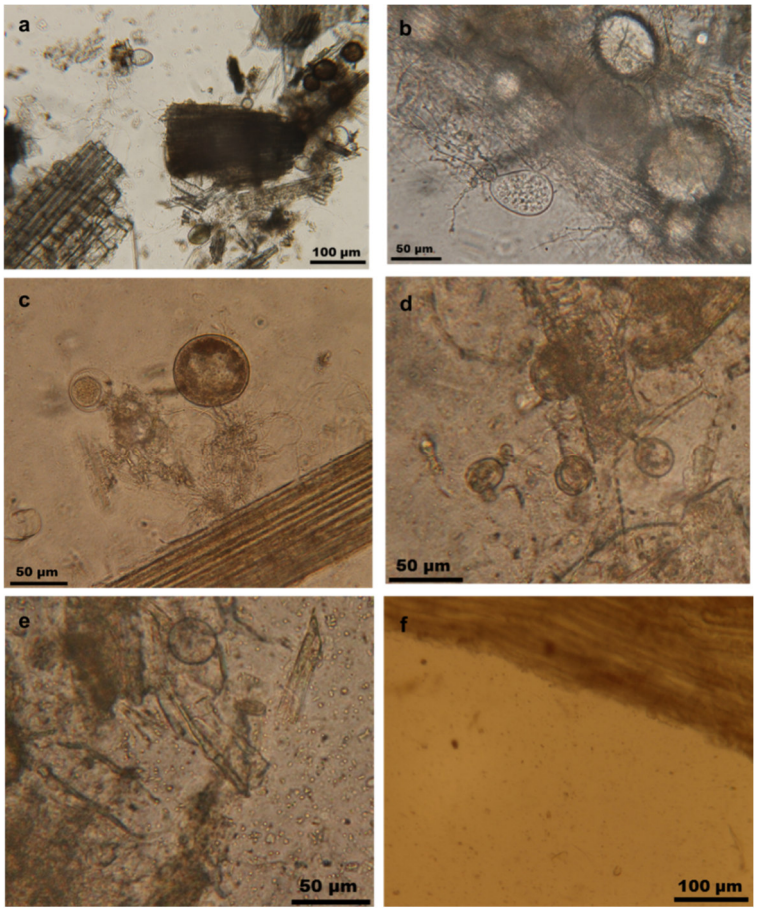


Figure 36: Exemplary picture series of *Neocallimastix cameroonii* strain CaDo 3b growing on rumen fluid based medium (a and b) and "digestate-medium" (c-e). a= Healthy culture, light microscopy 20x; b= Healthy sporangium, light microscopy 40x; c= CaDo 3b growing with 2 % digestate; d= CaDo 3b growing with 10 % digestate; e= CaDo 3b cultured with 14 % digestate; f= CaDo 3b cultured with 16 % digestate.

The decoupled pre-treatment approach tested in Chapter 6 thus has a pivotal advantage, as the examined *Neocallimastix frontalis* strains could be offered under more favourable conditions to hydrolyse the supplied hay for their growth and survival without the detrimental effects obviously caused by the conditions in biogas producing anaerobic sludge.

Even if some of the observed issues could be mitigated by separation of a hydrolytic pre-treatment step with anaerobic fungi from the methane production step, some obstacles were still obvious leaving space for improvement. During the hydrolysis of hay, the two *Neocallimastix frontalis* strains produced a variety of VFAs besides hydrolysis gas (Chapter 6). In their natural habitat, the digestive tract of herbivores, VFAs are the nutritional source for the host and are constantly assimilated via its mucosa. In the applied batch fermentation set-up, soluble metabolites were not removed from the reactors, leading to accumulation of VFAs. Recent publications on the regulation of enzyme expression in anaerobic fungi suggest that many CAZymes are subject to catabolite suppression (Solomon *et al.* 2016; Henske *et al.* 2018). Transcriptomic analysis confirmed that CAZyme expression and activity in *Piromyces finnis*, *Anaeromyces robustus* and *Neocallimastix californiae* was repressed by the application of glucose, an end-product of fiber degradation. Similar regulation patterns seem to be induced by the accumulation of VFAs, as it was been shown that saturated fatty acids inhibited cellulose degradation by *Neocallimastix frontalis* strain C5-1 (Ha *et al.* 2001). Two

strategies are conceivable to overcome the issue of end-product inhibition and thus bolster the degradation efficiency of anaerobic fungi:

One option is to utilize syntrophic co-cultures instead of pure anaerobic fungal isolates. Just like syntrophic bacteria and methanogenic archaea interacting in biogas production, anaerobic fungi in the rumen live in close synergistic relationship with methanogenic archaea (Joblin and Williams 1991; Cheng *et al.* 2009; Leis *et al.* 2014; Sun *et al.* 2014; Wei *et al.* 2016; Li *et al.* 2017). Anaerobic fungi generate H_2, CO_2, formate, acetate, lactate, and ethanol as main fermentation end products. Their methanogen partners, most commonly *Methanobrevibacter* sp. and *Methanobacterium* sp. (Cheng *et al.* 2018) transform H_2, CO_2 or formate to methane, altering the fungal metabolism to a more ATP gaining route and mitigating end product inhibition (Li *et al.* 2019). Such interactions lead to more efficient substrate utilization by the anaerobic fungi coupled to higher gas yields and faster gas production. Further, co-cultures are supposedly more robust, allowing long-term *in-vitro* cultivation (200 days to 10 years) and growth of a higher diversity of anaerobic fungi (Cheng *et al.* 2018). The application of more stable and more efficient lignocellulolytic co-cultures instead of pure anaerobic fungal cultures might be an alternative way to realize their utilization for biogas production.

Alternatively, continuous cultivation systems may help to remove fermentation end-products, mitigating inhibition. Such systems were hitherto only applied to study growth habits and fiber degradation of anaerobic fungi (Zhu *et al.* 1996; Zhu *et al.* 1997) or to harvest their lignocellulolytic enzymes (Teunissen *et al.* 1992). Similar to the process in herbivore digestive tracts, spent medium is removed and replaced by fresh medium during (semi)-continuous cultivation from time to time or constantly, depleting anaerobic fungal metabolites in the reaction volume and thereby mitigating end-product inhibition. Anaerobic fungal enzyme production and substrate degradation was improved in such cultivation systems (Zhu *et al.* 1997). However, semi-continuous cultivation has only been applied in laboratory scale (100 ml to 1.5 L). Scaling up would be necessary prior to industrial use.

Summarized, hydrolytic pre-treatment with anaerobic fungi as exemplified by the application of the two *Neocallimastix frontalis* strains (Chapter 6) can be a starting point to improve the degradation of fibrous residues for biogas production. A separate pre-treatment unit operated at optimum conditions for the applied fungus might also be more universally applicable besides biogas production e.g. for sugar extraction in bioethanol production processes (Ranganathan *et al.* 2017). Utilization of anaerobic fungi with their superior set of CAZymes might outcompete other microbial pre-treatment set-ups e.g. with aerobic fungi since these possess a much smaller enzymatic arsenal (Seppälä *et al.* 2017). Some hurdles must still be overcome before a pre-treatment approach with anaerobic fungi becomes applicable in industrial scale. Better knowledge on essential growth requirements of anaerobic fungi must be gathered, and based on this knowledge, industrial applicable standardized media may be developed. Cultivation techniques must be improved to allow working with bigger culture volumes in order to produce reasonable amounts of seed inocula. Further, the pre-treatment stage should be transformed from a batch into a (semi)continuous system sustaining long-term growth and activity of the anaerobic fungal work horses.

Chapter 8
Conclusion and outlook

The first aim of this Ph.D. thesis was to find suitable methods for the detection of anaerobic fungi in biogas processes. With the development of the three PCR based assays AF-SSU, AF-LSU and AF-Endo in this work, this task was accomplished. The tools allow quantifying anaerobic fungi by their 18S rRNA gene copy numbers, examining their community composition, and measuring their transcriptional activity by specific quantification of GH5 endoglucanase transcripts. Re-evaluation of the primer pairs and the 5'-hydrolysis probe further showed that they are able to detect also the known strains of all recently discovered anaerobic fungal genera. However, the approach to apply three different assays is tedious and might be simplified by the combination of analyses.

The developed methods were applied in a study screening ten agricultural biogas plants for the presence, the activity and the community composition of anaerobic fungi. Anaerobic fungal 18S rRNA copies were detected in seven of the ten tested biogas plants, all operated with a high share of cattle manure and slurry. Low transcriptional activity of anaerobic fungi was detected only in two of the positive tested biogas plants. These results, together with results from the literature, suggest that anaerobic fungi are transferred into biogas plants with the animal derived substrates but quickly were inactivated under the present conditions. Further, experiments trying to adapt anaerobic fungi to a digestate medium showed that concentrations of digester sludge over 6 % had a detrimental effect on the tested strains of anaerobic fungi and concentrations over 16 % inhibited the growth, probably leading to death of the fungi.

Thus, for the second aim of this Ph.D. thesis to improve biogas production from lignocellulosic residues by the application of anaerobic fungi, direct implementation of anaerobic fungi into biogas digesters was ruled out. Instead, a separate hydrolytic pre-treatment of hay was performed with two different *Neocallimastix frontalis* strains. Both strains, compared to inactivated controls, accelerated the initial biogas production from hay, coupled to increased dry matter degradation and VFA production. The successful pre-treatment with the two *Neocallimastix frontalis* strains is a good basis for future practical applications. However, for realization in practice, more knowledge on the essential growth requirements of anaerobic fungi needs to be gathered, and based on this knowledge, suitable media and reactors for continuous growth of stable anaerobic fungal cultures should be developed.

Chapter 9
Publication list and author contributions

Publication 1: Anaerobic Fungi and Their Potential for Biogas Production

Dollhofer V, Podmirseg SM, Callaghan TM, Griffith GW, Fliegerová K (2015). Anaerobic fungi and their potential for biogas production. In: Guebitz G (ed) Biogas Science and Technology, vol 51. Advances in Biochemical Engineering/Biotechnology. Springer, pp 41-61. doi:10.1007/978-3-319-21993-6_2

Authors contributions: Veronika Flad drafted the outline and the main text body of the manuscript, further she gathered and coordinated the authors team. Dr. Sabine Marie Podmirseg, Dr. Tony Martin Callaghan provided the text passage on cultivation of anaerobic fungi. Dr. Gareth Wyn Griffith drafted the section on anaerobic fungal taxonomy. Dr. Katerina Fliegerovà drafted the main part of the section on enzymatic capacity of anaerobic fungi. All authors read and approved the final manuscript.

Publication 2: Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi

Dollhofer, V., Callaghan, T.M., Dorn-In, S., Bauer, J., Lebuhn, M. (2016). Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. Journal of Microbiological Methods, 127, 28-40.

Author contributions: Veronika Flad did the bioinformatics work, designed primers and probes, performed the experiments, analyzed the data and drafted the manuscript. Dr. Tony Martin Callaghan helped to develop system AF-LSU for phylogenetic analysis of anaerobic fungi and the comparison to ITS based data. Dr. Samart Dorn-In provided the samples necessary for specificity testing. Prof. Dr. Johann Bauer and Dr. Michael Lebuhn supervised the work, helped with data analysis and structuring of the manuscript. All authors read, revised and approved the final manuscript.

Publication 3: Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants

Dollhofer, V., Callaghan, T.M., Griffith, G.W., Lebuhn, M., Bauer, J. (2017). Presence and transcriptional activity of anaerobic fungi in agricultural biogas plants. Bioresource Technology, 235, 131-139.

Author contributions: Veronika Flad performed and designed the experiments, analyzed the data and drafted the manuscript. Dr. Tony Martin Callaghan supervised the isolation of anaerobic fungi and helped with the phylogenetic analysis of anaerobic fungi. Dr. Gareth Griffith classified the isolated *Piromyces* sp. and helped with phylogenetic analysis of anaerobic fungi. Dr. Michael Lebuhn and Prof. Dr. Johann Bauer supervised the work, helped

with data analysis and structuring of the manuscript. All authors read, revised and approved the final manuscript.

Publication 4: Accelerated biogas production from lignocellulosic biomass after pre-treatment with *Neocallimastix frontalis*

Dollhofer, V., Dandikas, V., Dorn-In, S., Bauer, C., Lebuhn, M., Bauer, J. (2018). Accelerated biogas production from lignocellulosic biomass after pre-treatment with *Neocallimastix frontalis*. Bioresource Technology, 264, 219-227.

Author contributions: Veronika Flad performed and designed the molecular biological experiments, analyzed the resulting data and drafted the manuscript. Dr. Vasilis Dandikas designed and performed the batch fermentation experiments and analyzed the resulting data. Dr. Christoph Bauer performed the statistical analysis. Dr. Michael Lebuhn and Prof. Dr. Johann Bauer supervised the work, helped with data analysis and structuring of the manuscript. All authors read, revised and approved the final manuscript.

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