Anaerobic Fungi and Their Potential for Biogas Production

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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 hydrolysing 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

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production from recalcitrant biomass. This chapter presents an overview about their biology and their potential for implementation in the biogas process.

Keywords Anaerobic fungi · Neocallimastigomycota · Phylogeny · Cellulosomes · Biogas process improvement · Recalcitrant cellulosic substrates

Contents

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 [1, 2]. Recent studies indicate their appearance in herbivorous reptiles like the green iguana [2] and termites [3] 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 [4]. The Neocallimastigales are 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 [5].

Anaerobic fungi are the only fungi which possess cellulosomes. These extraordinary features are presented in more detail in Sect. 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 [6]. Such phylogenetic determinants and unique taxonomy of anaerobic fungi are discussed in the following Sect. 1.1.

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 [7], Phycomycetes [8] and Chytridiomycetes [9, 10] the anaerobic fungi were finally placed into the distinct phylum Neocallimastigomycota [11]. The phylum contains only one order (Neocallimastigales) and one family (Neocallimastigaceae) within which eight genera are currently described: The monocentric rhizoidal genera *Neocallimastix*, *Piromyces, Ontomyces* and *Buwchfawromyces*, the polycentric rhizoidal genera *Anaeromyces* and *Orpinomyces,* and the two bulbous genera, monocentric *Caecomyces* and polycentric *Cyllamyces*, respectively [12–14].

The genera are defined on the basis of 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 7–20 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 [15].

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 [16]. 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 [17].

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 [2, 15, 18]. Several of these novel clades are now recognized from sequences of cultured fungi [15], while other clades still consist of environmental nucleic sequences (ENAS) only.

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

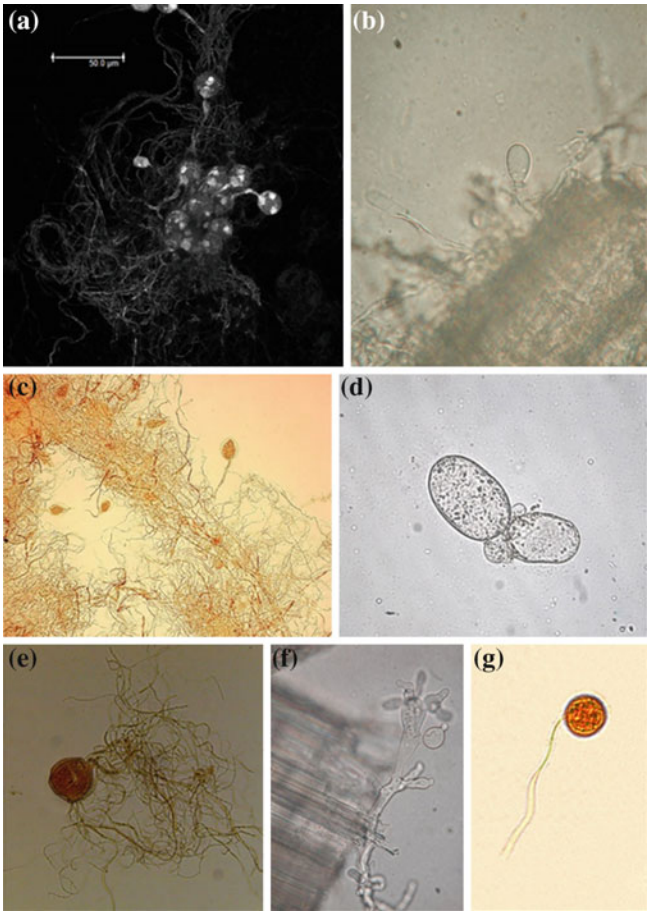

Fig. 1 Different culture morphologies of anaerobic fungi: **a** *Neocallimastix* sp. sporangia and rhizomycelium (CLSM: superimposed z-stacks (26.7 µm total depth) showing culture auto fluorescence (excitation at 561 nm and emission from 570 to 620 nm); **b** *Piromyces* sp. light microscopy of native preparation; **c** Rhizoid of *Anaeromyces mucronatus* with apical sporangia. Light microscopy of lugol-stained preparation (×200); **d** Bulbous species *Caecomyces communis*. Light microscopy of native preparation (×400); **e** *Neocallimastix frontalis* sporangium and rhizoid. Light microscopy (×400); **f** *Orpinomyces* sp. with sporangia and rhizoid. Light microscopy of native preparation; **g** Light microscopy of a biflagellated zoospore of *Piromyces* sp. (×1000)

material and fruiting bodies known as sporangia (Fig. 1) [13]. Flagellate zoospores (see Fig. 1g) released from mature sporangia actively swim towards freshly ingested plant tissues using chemotactic response to soluble sugars and/or phenolic acids [19]. 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*) [20].

The rhizoidal system penetrates the plant tissue by a combination of enzymatic activity and hydrostatic pressure using appressorium-like penetration structures [21, 22]. In the non-rhizoidal bulbous species (*Caecomyces, Cyllamyces*), the expanding holdfast formed within the substrate causes a splitting of the plant fibers [23–25]. Sporangium maturation and release of asexual zoospores can occur as quickly as eight hours after encystment [26, 27].The complete life cycle, is conducted within 24–32 h [25]. Propagules of the anaerobic fungi are known to survive up to and probably over a year in feces [28] and have also been found to be transferred to neonatal hosts through saliva [29]. Putative aero-tolerant survival structures have been observed only rarely [14, 30, 31] and many questions as to the formation of these structures and their occurrence in the various genera of anaerobic fungi remain to be answered.

1.3 Anaerobic Fungi and Their Interactions with Methanogens and Bacteria

Close association of anaerobic fungi with methanogens is well known [23, 32], 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 [33]. 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 [32, 33]. 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 [34].

Syntrophic interactions between acetogenic bacteria and methanogens are well known to occur in the biogas biocoenosis [35]. 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 [36], who tested the degradation efficiency in different culture combinations of three anaerobic fungi 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 [36]. This improved degradation was also confirmed when testing the contribution of different microbial groups (fungi, bacteria, protozoa) on orchard grass decomposition [37]. 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.

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: hydrolysing 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 wastes 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 [38, 39]. A promising strategy is the use of microorganisms, which are able to successfully perform such complicated degradation processes in their natural environment [40, 41]. Herbivores as biogas reactors evolved the need for fungal symbionts for this purpose and over millions of years natural selection has created a highly specialised 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.

2.1 Lignocelluloytic Enzymes of Anaerobic Fungi and Their Potential Use

Lignin-embedded cellulose and hemicellulose [42] represent a physical barrier against microbial and enzymatic attack. Known as the primary digesters of plant biomass in the rumen anaerobic fungi [37] 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 [5]. 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 [43]. The residual cellobiose is then hydrolyzed to glucose by β-glucosidases (EC 3.2.1.21) [40, 44]. Auxiliary enzymes like the recently discovered lytic polysaccharide mono-oxygenases (LPMO) (family AA9) have been reported to enhance or complete the utilization of cellulose in many fungal species [45]. 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 [46]. But it seems that basal fungal groups including the anaerobic fungi lack those enzymes [45].

All three major cellulase types have been reported for the Neocallimastigomycota ([5, 47, 48, 49, 50] 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 reesei* or many bacterial species. Thus costly addition of β-glucosidase becomes unnecessary [51].

Due to the heterogeneous 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 [52]. In contrast to aerobic higher fungi (Dikarya), anaerobic fungi lack the enzymatic machinery to catabolise lignin. The enzymatic reaction to cleave the aromatic ring requires oxygen and can therefore not take place in an anaerobic environment [53]. 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 [54]. Additional feruloyl (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 [55].

2.1.1 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* [56] 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 [57]. Up to now cellulosomes have been described for species of *Piromyces* [58, 59], *Orpinomyces* [48], and *Neocallimastix* [52, 60]. 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 [55]. Unfortunately the molecular structure of the anaerobic fungal cellulosome is still unclear and miscellaneous theories exist (see [61] 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 [62].

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 [63]. 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 [43]. 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 [64]. Additionally it could be shown that a double-dockerin domain and a β-glucosidase enzymatic subunit from glycosyl hydrolase family 3 (GH 3), both belonging to one fungal species, could bind to each other [58, 61]. 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 the anaerobic fungi to use their cellulolytic enzymes in a synergistic and more efficient way, unequalled by individually secreted enzymes [61]. It also provides protection against proteases from the surrounding environment in the form of a serine protease inhibitor named celpin [65].

2.1.2 Substrates Utilized by Anaerobic Fungi

In addition to municipal solid waste (MSW) and animal wastes, lignocellulose-rich materials potentially useful for biogas production are by-products of various industrial processes, including agriculture, forestry, pulp-, paper- and food

Table 1 Examples for lignocellulosic residues degraded by anaerobic fungi

Lignocellulosic residue	Lignin content % [66]	Organism	Reference
Wheat straw	16-21	*Neocallimastix frontalis*	[67]
Coastal Bermuda grass	6.4	*Piromyces* MC-1, *Orpinomyces* PC-1-3, *Neocallimastix* MC-2	[49]
Sugar cane bagasse	19-24	*Piromyces* strain E2	[68]
Hard wood	18-25	*Neocallimastix* sp.	[69]
Rice straw	18	*Piromyces* M014, *Orpinomyces* GSRI-001, *Neocallimastix* T010	[3]

production [51, 66]. 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.

2.1.3 Production of Recombinant Enzymes

One strategy to overcome the bottleneck 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 [70]. 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 *P. methanolica*) was low or else the recombinant proteins were not catalytically active [71–74]. Genetic modification of *S. 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 [75–77] represents at this time the most promising technique for improving the industrial production of ethanol [78] and several patents have been filed so far [79]. 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 [66]. 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 [80]. 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.

2.2 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 remains unused [81]. 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 [82], thermal [83, 84], chemical [85], oxidative [86] or ultrasonic [87, 88] pre-treatment.

However, in the rumen the natural biogas system these techniques 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 ± 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 [89] 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 [90] applied mesophilic *Enterobacter cloacae* and thermophilic *Caldicellulosyruptor 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 *C. 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 [91, 92].

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 [3]. 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 [34]. 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 [81, 93]. A recent study showed that bioaugmentation with anaerobic fungi did not increase the overall methane yield, but that it speeds up initial gas production and thus may help to reduce retention time [94]. 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.

3 Anaerobic Fungi: Methodological State of the Art

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 [95]. 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 [96] and Orpin [97]. 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, autofluorescence of plant material over a wide wavelength range clearly impedes distinct identification of fungal structures. Staining with Calcofluor white [98] or the more recently proposed stains Solophenyl Flavine 7GFE 500 and Pontamine Fast Scarlet 4B [99] 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. [34], an approach originally used to bring out plant root fungi, e.g. arbuscular myccorhizas.

Measurement of fungal abundance with culture-dependent techniques i.e. thallus forming units (TFU) is generally performed through the most probable number (MPN) method [29, 100] and by using the roll-tube method as described by Joblin [101]. 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 [102].

Anaerobic fungi produce a wide range of potent enzymes, e.g. cellulase, endoglucanase, xylanase or amylase amongst others, that help to degrade plant material [93, 103, 104]. 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 [93].

Another very promising approach that has yet to be tested for anaerobic fungi is the raising of enzyme-specific antibodies. Li and coworkers [103] 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 anaerobic fungi detection and community analysis but also quantification through qPCR are the small ribosomal subunit (18S rRNA gene) and the internal spacer (ITS) region [15, 32, 95, 105–109]. However, both gene regions also bear certain drawbacks that should be considered and are discussed in [13]. 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 [110], and the ITS region, despite its prevalent utilization in fungal phylogeny [111], 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 [112], indicating that each chosen DNA region 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.

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 fungi 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. [93] 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 [113]. 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.

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 there cohorts is needed.

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