Final report of 128345 project

Fungi in the anaerobic degradation of biomass for biogas production

The project was planned to be carried out over three years (01.10.2018 – 30.09.2021), but because of my pregnancy the project was postponed by one year (01.08.2019. – 31.07.2020.).

Theoretical background

The Earth has limited resources of farmland, nutrients, potable water, and fossil energy. Emission of greenhouse gases (GHG) will have to be reduced significantly in the coming years to help abate climate change. In combating these global problems biogas is considered as one of the most important renewable energy sources. Biogas technology reduces the release of organic wastes, pathogenic microorganisms and the GHG CO_2 emission. Degradation of lignocellulose-rich material into biogas is an attractive strategy to face growing energy demands and moderate greenhouse gas emissions from the exploitation of fossil energy resources. Lignocellulosic residues (e.g. crop residues, green waste, mill waste) are highly frequent (1), they are easily accessible, cheap and do not require additional land to grow on in this way do not trigger "food or fuel" conflicts. This biomass is composed of interwoven cellulose and hemicellulose, coated by recalcitrant lignin (2). This is the explanation why bacteria and archaea in the biogas reactor are not efficient in disintegration of the lignin, leaving a considerable portion of the more easily convertible sugars untouched. Current strategies to release this carbon rely on expensive enzyme cocktails and physicochemical pre-treatment, producing inhibitory compounds that hinder subsequent microbial bioproduction. Microbial pre-treatment utilizing the fibre degrading potentials of aerobic fungi may be a much cheaper and therefore more attractive alternative but there are some drawbacks e.g. loss of carbohydrates by respiration and biomass build-up and the requirement of long pre-treatment periods (3). Anaerobic fungi (AF) from the phylum Neocallimastigomycota are natural inhabitants of the digestive tract of herbivorous animals (4), which decompose a big share of the ingested forage. AF attach to the plant material and crack the fibres mechanically by growth and expansion of their rhizoids or bulbous holdfasts (5). These fungi are an appealing solution as they hydrolyze crude, untreated biomass at ambient conditions into sugars that can be converted into value-added products by partner organisms.

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. The cellulosomes contain a multitude of lignocellulolytic enzymes. 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 (6).

The hydrolysis of both cellulose and hemicellulose are rate-limiting steps in all anaerobic fermentation processes of biomass designed to date (7). The main function of rumen fermentation is to provide VFAs from plant polysaccharides (8). The close association of anaerobic fungi with methanogens is well known (9, 10), with inter-species hydrogen transfer leading to both methane production and also more efficient re-generation of oxidized cofactors

(NAD+, NADP+) (10). The anaerobic fungus – methanogen interaction is, however, 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, formiate). Acetate is the preferred carbon and energy source for the acetoclastic methanogens (10, 11). 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 (12). Most of the related studies has been based on in vitro co-cultures, that may not completely reflect conditions of whole rumen or biogas reactor consortia and therefore more research is needed in this field.

A commonly encountered issue during anaerobic digestion is limited degradability of plant biomass, i.e., 40–60% of organic carbon remains unused (13). This problem is due to the physical structure and the recalcitrant chemical nature of these polymers (14, 15). A promising strategy is the use of microorganisms, which are able to successfully perform such complicated degradation processes in their natural environment (16-19). Herbivores evolved the "methodology" of involving fungal symbionts for this purpose and natural selection has created a highly specialised and niche specific community of anaerobic fungi over thousands of years.

Applied methods

Rumen samples were collected from beef steers farms in Csongrád and Bács-Kiskun County, Hungary. These areas have temperate continental climate and are located in South-East Hungary. Rumen contents were collected from 6 healthy adult steers directly from the rumen sac after slaughtering. The samples were transported in an anaerobic container and stored at 37°C.

Fresh fecal samples were collected from fallow deer species and mouflons housed in the ZOO of Szarvas (Körösvölgyi Látogatóközpont), other animals were housed in Szeged ZOO (Szeged, Hungary). A list of each animal and their digestion types is given in Table 1.

Pretreatments and biogas production

The fungal pretreatments lasted for 10 days in 100mL batch reactor vessels. All pretreatments and anaerobic digestion (AD) experiments were carried out under mesophilic conditions at 37°C, with manual mixing 3 times daily. Samples were taken from the liquid phase of the pretreatment reactors for HPLC and enzyme activity assays on days 0, 3, 6, and 9. Total cellulase activity was determined in filter paper (FPase) activity assay system by using Whatman No. 1 filter paper as substrate (20). Endoglucanases randomly cleave β-1,4-glycosidic linkages on the amorphous part of cellulose away from chain ends and was determined by using carboxymethyl cellulose (CMC) as substrate. Exoglucanases produce cellobiose by attacking cellulose from reducing and non-reducing chain ends while β-glucosidase converts cellobiose into glucose (21). Bio-methane concentrations in the headspace were measured on a daily basis with an Agilent 7880 Gas-chromatograph (GC), on a HP Molesieve column, with a length of 30m and an inner diameter of 0.53mm, equipped with a Thermal Conductivity Detector (TCD).

All experiments were performed in triplicates. Standard deviations of mean values were calculated using Microsoft Office Excel 2007 function and the Sigma Plot (USA) software was used for statistical significance analyses.

	Name	Latin name	Digestion type
1	Indian rhinoceros	*Rhinoceros unicornis*	hindgut fermenter
2	Asiatic elephant	*Elephas maximus*	hindgut fermenter
3	Tammar wallaby	*Macropus eugenii*	hindgut fermenter, with two stomach chambers
4	Red deer	*Cervus elaphus*	ruminant
5	Fallow deer	*Dama dama*	ruminant
6	Giraffe	*Giraffa camelopardalis rothschildi*	ruminant
7	Mouflon	*Ovis aries orientalis*	ruminant
8	Bactrian camel	*Camelus bactrianus*	pseudo-ruminant
9	Pygmy hippopotamus	*Choeropsis liberiensis*	pseudo-ruminant
10	Llama	*Lama glama*	pseudo-ruminant
11	Guanaco	*Lama guanicoe*	pseudo-ruminant
12	Vicuna	*Vicugna vicugna*	pseudo-ruminant
13	Alpaca	*Vicugna pacos*	pseudo-ruminant

Table 1. Herbivorous animals involved in this study and their digestion types

DNA extraction from stool samples and next generation sequencing

For total community DNA isolation 200 mg of stool samples were used from each animal. DNA extractions were carried out using the Zymo Research Fecal DNA kit (D6010, Zymo Research, Irvine, USA). After lysis (bead beating was performed by Vortex Genie 2, bead size: 0.1 mm; beating time: 15 min, beating speed: max), the Zymo Research kit protocol was followed. The quantity of DNA was determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, USA).

The recommendations of the Illumina sequencing platform were closely followed (Illumina Inc., USA). DNA samples were used to in vivo sequence preparation applied by the NEBNext Ultra II Library Prep Kit. The metagenomics sequencing was performed by Illumina with MiSeq chemistry (MiSeq Reagent kit v2).

Metagenome data processing and statistical analysis

Galaxy Europe server was employed to pre-process the raw sequences (i.e., sequence filtering, quality checking and assembly) (22). Low quality reads were filtered out by TrimGalore! (min. length 100; automatic adapter sequence detection; max error rate 0.1) and dereplication was performed by VSearch. Filtered sequences were checked with FastQC. Taxonomic and functional profiling of metagenomics reads was performed using Kraken2 and Humann2, respectively. Kraken 2 provides microbial taxonomic profile allowing the quantification of individual taxa across metagenomics samples. MEGAN6 was used to investigate microbial communities and export data for statistical calculation (23). Statistical Analysis of Metagenomics Profiles (STAMP) was used to calculate principal component analysis (PCA) and calculate significantly different taxa (two sided T-test; p-value: 0.05). For microbial alpha-diversity (Shannon index) and core calculation MetaCoMET (Metagenomics Core Microbiome Exploration Tool) web tool was employed (24). The distribution of core microbial taxa presented with Krona (25) and Circos (26).

Functional profiling was performed using HUManN2. Briefly, HUManN2 construct a sample-specific reference database from the pangenomes of the subset of species detected in the given sample by Kraken2 (ChocoPhlan: species' pangenomes are precomputed, reduced representations of the protein-coding sequences from isolates of a given species). HUManN2 pipeline than maps sample reads against this database (by Bowtie2) to quantify gene presence and abundance. The remaining unmapped reads are further mapped by translated search against UniRef50 protein sequence catalogue. The gene families quantified at both the nucleotide and protein levels, HUManN2 reconstructs pathways from the functionally characterized subset and assesses community total, species-resolved pathway abundances (Reads Per Kilobase per Million mapped reads: RPKM). Because of the sequencing depth and coverage, the presented data are containing genus level functional data. In the last step, the output of gene family abundances grouped to GO slim terms, which is a subset of the whole Gene Ontology to get a broad overview of the ontology contents (27).

Results

1. *Metagenomic survey - Comparison of anaerobic fungi composition of cattle rumen and other herbivorous animal fecal samples and biogas reactor*

The results of this part of the project will be presented on 28th International Symposium on Analytical and Environmental Problems (ISAEP 2022) to be held online, November 14-15. 2022.

Microbiome composition at the genus level

The Illumina sequencing approach provides the potential for analyzing the bacterial community at a higher resolution. The most abundant genera were *Clostridium*, *Bacteroides*, *Ruminococcus*, *Prevotella* and among methanogens *Methanobrevibacter* (Fig. 1).

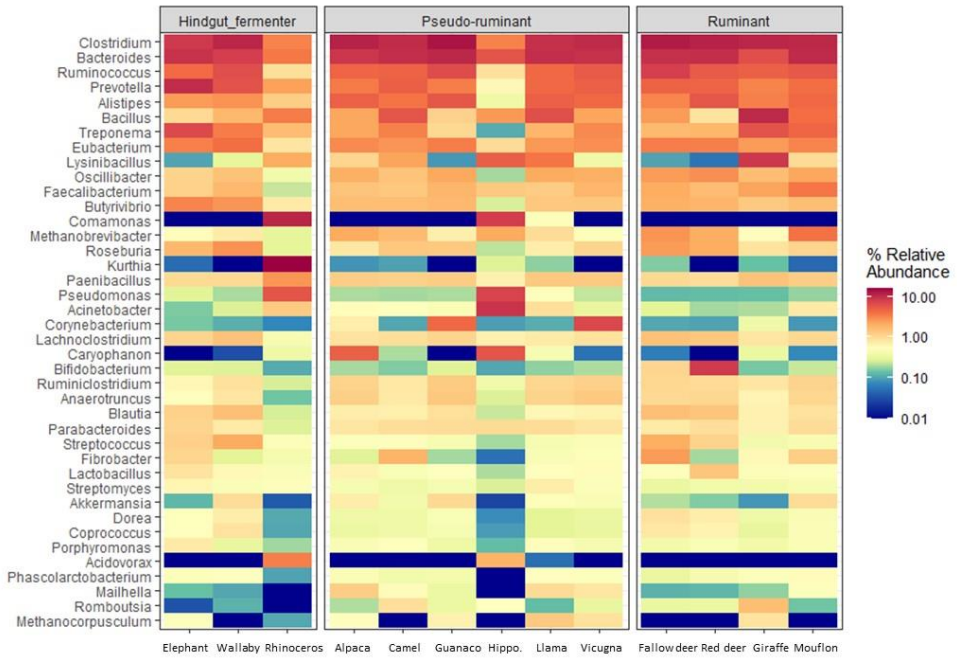

Figure 1. Abundance of genera in various animal digestive gut systems.

The observed differences in the dominant phyla and genera among the tested animals may be multi-factorial and include the distinct diets, original geographic locations, PCR amplification bias or due to the DNA extraction methods employed (28-31). The ruminant digestive tract and its microbiota have evolved to degrade the fibrous plant material consumed (32, 33). Many genera have been identified as rumen-associated bacteria involved primarily in, but not restricted to, the digestion of plant polysaccharides. Important plant polysaccharide-associated degrading bacteria include *Ruminococcus*, *Prevotella* and *Butyrivibrio* (34, 35) all of which were identified in this study as well.

My study demonstrated that the herbivorous animals, possessing various digestion tract anatomy and associated microbial communities, shared a common fecal microbiota but some genera were associated with particular digestion types only. The high bacterial numbers (10^{14}) within the colon of animals gives credence to the use of fecal material in these studies. Fecal sampling serves as an alternate for more laborious and – not negligible - invasive sampling not just from domesticated, farm animals but wild or ZOO housed herbivores.

In conclusion, in our study, we have shown that **the hindgut fermenting, ruminant, and pseudo-ruminant microbiota share ~50% of their phyla and ~50% of their genera in their fecal microbiota**. This degree of overlap between the microbiota of the 14 animal species may suggest that these genera are essential for all herbivorous fibrous polysaccharide-consuming animals. Host phylogeny and digestion method were shown to be potential determinants of bacterial diversity in the domesticated herbivores.

Currently, a manuscript is under preparation and is expected to be submitted to an internationally relevant scientific journal in the near future.

2. *Isolation and determination of culture conditions, enzyme assay*

The results of this part of the project is published in E. Kovács et al. Enhancing methane production from lignocellulosic biomass pre-treated with anaerobic fungi. 6th CEFORM 6th Central European Forum for Microbiology October 13–15, 2021. Kecskemét, Hungary, E. Kovács et al. Improving methane production from lignocellulosic biomass pre-treated with anaerobic fungi. A poster was presented at the 27th International Symposium on Analytical and Environmental Problems (ISAEP 2021), November 22-23, 2021. Szeged, Hungary and E. Kovács et al. Cellulose degradation by anaerobic fungi. Straub Days, May 25-27, 2022. Szeged, Hungary.

The objective of this study was the application of two newly isolated 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 sheep (*Ovis aries*) and Asian elephant (*Elephas maximus*) (Fig. 5.). The effects on biogas production of anaerobic fungi from both animal species were assessed in two step batch experiments, comprised by a hydrolytic/acidogenic stage, followed by a methane production stage. Checking the enzyme activity in hydrolytic stage, beta-glucosidase activity was measured by p-nitrophenyl-β-D-glucopyranoside, for the endoglucanase concentration DNSA-method was used (3,5-dinitrosalicylic acid). The produced organic acids were measured by HPLC. Additionally, gas composition was analysed by GC during the methane production stage.

In this study, treatment with anaerobic fungi cultures increased the total biomethane yield during the experimental period of 20 days. After the pretreatment the medium was taken apart into liquid and solid phases, which were treated separately. Columns show the difference between the pretreated and non-treated data (Fig. 2.).

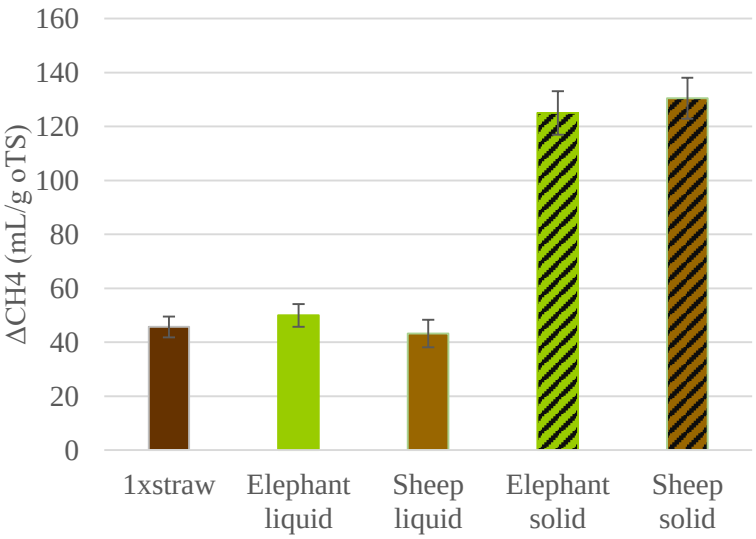

Figure 2. Biomethane yield after the anaerobic fungal pre-treatment.

Pretreatment with anaerobic fungi significantly improved the degradability of straw. This is evident by the fact that about 2.5 times more biomethane was produced from the elephant solid and sheep solid samples during the experiment than from the untreated straw (Fig. 2.).

The results for the elephant fluid and sheep rumen fluid samples correlated well with the organic acid concentrations measured by HPLC (Fig.4.) and the high endoglucanase and beta-glucosidase results measured during the tests (Fig. 3). From these results it should be concluded that anaerobic fungi degraded the substrate efficiently during the 15-day long treatment. As a result of the pre-treatment, acetic acid, lactic acid, glucose and cellobiose were produced in the significant amounts. These products could be used by methane-producing Archea for generation of methane during biogas fermentation. The efficiency of biogas fermentation was well characterized by the amount of biomethane produced as well as the concentrations of organic acid measured by HPLC (Fig.4.), which show that methanogens with otherwise slow metabolism were able to use the products of anaerobic fungi from the solution, so inhibition did not occur.

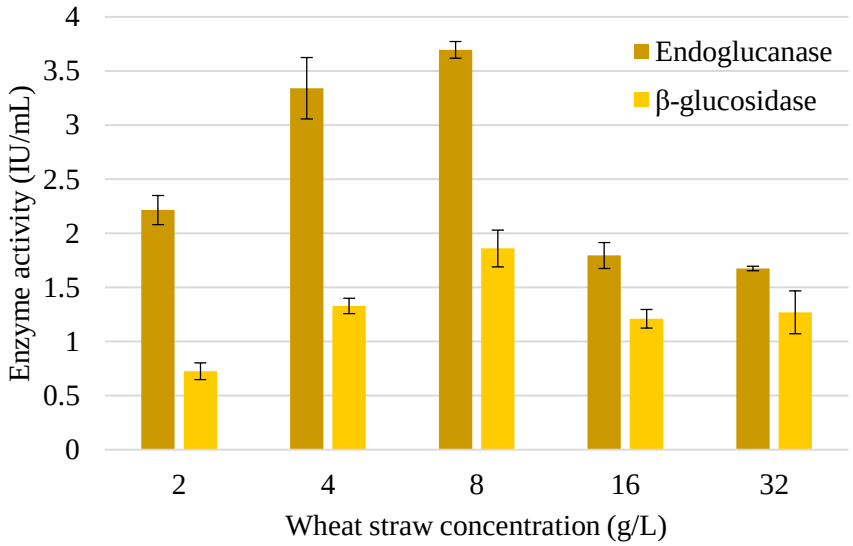

Figure 3. Enzyme activities during wheat straw fermentation using sheep-AF isolate.

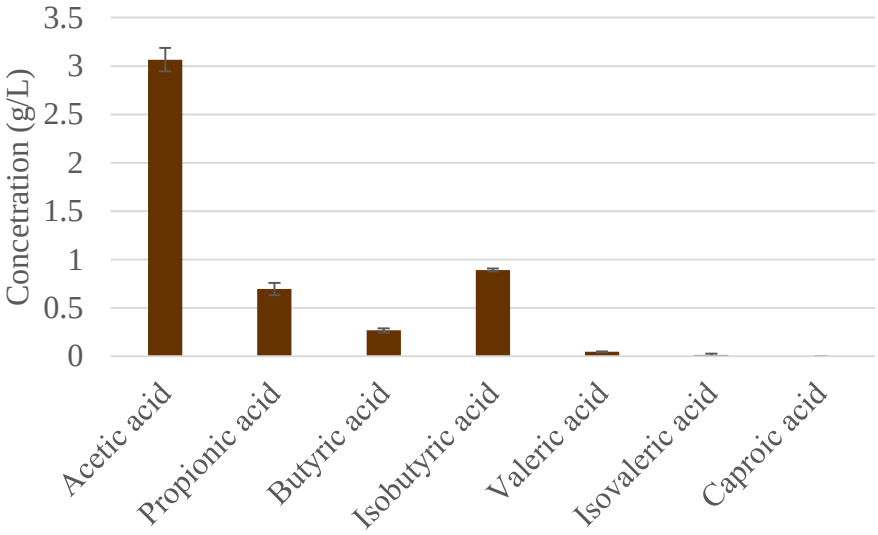

Figure 4. Organic acid concentration at the end of the biogas fermentation using anaerobic fungi from sheep.

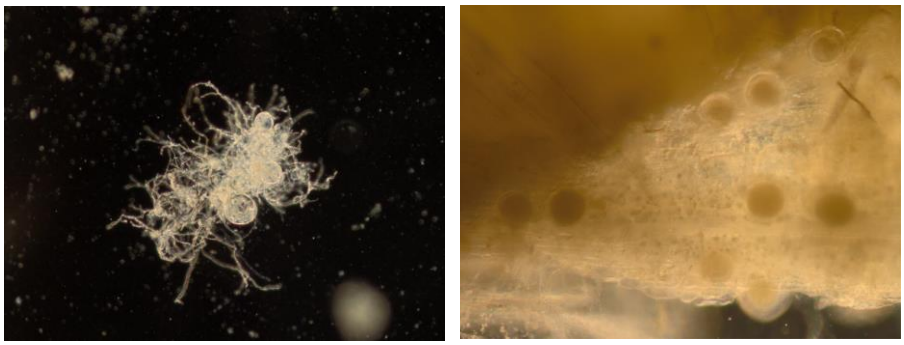

Figure 5. AF isolate from sheep (sheep-AF) on soluble carbon substrate (A) and mass of AF isolated from elephant (elephant-AF) on insoluble carbon substrate (B).

Currently, a manuscript is under preparation and is expected to be submitted to an internationally relevant scientific journal in the near future.

I would like to point out that, as far as I know, there is no other person/group in Hungary that has succeeded in **isolating and maintaining Neocallimastigomycota** fungi. Their characterization and maintenance requires a special technique, which was developed during this tender.
Another positive point is that we managed to get into the "bloodstream" of the international anaerobic fungi community. As a result, a **close collaboration was established with Prof. Michael K. Theodorou** (Department of Agriculture and the Environment, Harper Adams University, Newport, Shropshire TF10 8NB, UK) who is a renowned anaerobic fungi researcher. This cooperation was sealed by an agreement between the University of Szeged and British Harper Adams University.
Further **collaborations began with Michelle A. O'Malley** (Department of Chemical Engineering, University of California, Santa Barbara, CA 93106, USA), and **Yanfen Cheng** (Laboratory of Gastrointestinal Microbiology, National Center for International Research on Animal Gut Nutrition, Nanjing Agricultural University).

3. *Examination of the most effective cellulose-degrader anaerobic fungal species*

The results of this part of the project is published in the following scientific article: E. Kovács et al. (2022) Pretreatment of lignocellulosic biogas substrates by filamentous fungi. J. Biotech. https://doi.org/10.1016/j.jbiotec.2022.10.013 (IF: 3.595) (36) and Cs. Szűcs et al. Enhancing biogas production from agroindustrial waste pre-treated with filamentous fungi. Biologia Futura, 72 (2021), pp. 341-346, 10.1007/s42977-021-00083-3. (IF: 1.069) (37).

After the publication of these articles, I was contacted by Thomas Petit (Department of Health, Safety and Environment, University of La Réunion) with whom we exchanged information regarding the enzyme production of anaerobic fungi.

In these studies, *Penicillium aurantiogriseum* (our filamentous fungal isolate from cattle rumen), and other lignocellulose degrader fungi (*Rhizopus miehei*, *Gilbertella persicaria* and *Trichoderma reesei*) effectively assisted the deconstruction of all tested plant biomass, i.e. wheat straw, corn stover and willow chips. Each fungal strain showed intensive growth under the applied conditions, although they revealed noticeable differences in the production of the tested hydrolases.

The results indicated that fungal pre-treatment could be a useful strategy in industrial scale biogas fermentation to avoid the accumulation of undigested biomass and gain a higher biogas potential from lignocellulosic materials. Production of the fungal hydrolytic enzymes facilitated the breakdown of cell wall structure. The fungi increased the surface area of exposed lignocellulose, hence the pretreatment promoted contact for other microbes and their enzymes.

Conclusion

Aerobic fungi (AeF), as main decomposers of plant biomass in nature, and anaerobic fungi (AF), as key fiber degraders in the ruminants´ digestive tract (38), have great potential to facilitate the deconstruction of lignocellulose-rich biomass due to their mechanical fiber penetration and lignocellulolytic capabilities.

Anaerobic fungi isolated from sheep and elephant are excellent candidates for the conversion of agricultural waste products to biofuels. The two tested isolates efficiently pretreated the hard-to-degrade straw substrate and produced significant amounts of acetic acid, lactic acid, glucose, and cellobiose. The kinetics of the degradation were optimal for the slow metabolism of methanogenic microbes, which were thus able to efficiently utilize the aforementioned by-products and produce biomethane from them.

Based on these results AF isolates were effective in enhancing cellulose degradation and successfully increased biogas production.

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