

Anaerobic digestion of Green Willow Biomass, a
novel lignocellulosic substrate, and the evaluation of
H₂-induced stress in anaerobic bioreactors by
genome-resolved metatranscriptomics

PhD dissertation

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1. Background

The share of fossil fuels in the increasing global energy demand is still about 80%. Their combined usage of renewable energies is required to provide green-house-gas-mitigation. Biomass is a ubiquitous renewable energy source that can be harnessed in many ways. Because its many advantages, one very promising technology for alternative energy generation from biomass is the anaerobic digestion. In the context of climate change, the generation of biogas as a renewable energy form has become popular and was intensively examined over the last few decades. In the course of my doctoral work, I aimed to contribute to the following two major challenges that the biogas industry faces:

1.) To find novel substrates that have high biogas production potential, yet cheap to produce and do not come into contradiction with the food vs feed debate, as traditional substrates, such as maize do. Willow biomass may be a promising choice, since producing it has many advantages, including low production costs and the possibility of growing it on marginal lands, but the high amounts of lignin in the woody biomass hindered its usage as a substrate for biogas generation until now. To this end, I utilized a novel lignocellulosic biogas substrate: the young shrubs of the short-rotation coppice willow (*Salix spp.*), which I termed Green Willow Biomass (GWB).

2.) The coupling of other renewable sources, such as photovoltaics and wind energy, with biogas to harness their excess energy produced when the energy consumption rate is lower than its generation. This

can be achieved with process called a power-to-methane (P2bioCH₄), wherein the excess energy is used to generate H₂ via water electrolysis, which in turn is fed to an anaerobic digester to increase the methane concentration of the produced biogas. To better understand this *in situ* biological biogas upgrading process (BBU), I investigated the near-immediate effects of hydrogen-addition (a form of environmental stress) on the biogas producing microbial community, in the methane-producing *Archaea* in general, via a combined metagenomic (MG) and metatranscriptomic (MTR) approach.

2. Materials and methods

2.1. Anaerobic Digestion of Green Willow Biomass (GWB)

An experimental plantation of *Salix viminalis* diploid Energo (EN) cultivar and its tetraploid PoliPlus (PP) forms (Dudits et al., 2016) was harvested in three occasions during 2018: June, August and October. The collected biomass, termed green willow biomass (GWB), was weighed, volatile solid contents were assessed, and fiber analyses were carried out according to the Van Soest method. Subsequently the GWB was used as a substrate in batch anaerobic digesters according to VDI 4630. Leaves and stems were measured separately and in proportions equal to the ratio that was observed in the original harvest as well. Methane production was monitored via gas chromatography (GC) and the fermentation parameters (maximal methane yields, K , and highest methane production rates, μ_{max}) were

estimated via fitting the modified Gompertz model to the experimental data. Metataxonomic analyses using the V3-V4 region of the 16S rRNA gene were conducted via an absolute-sequence variant-based method on the fermentor samples taken at the end of the process.

2.2. Metatranscriptomic analysis of the H₂-induced response of a biological biogas upgrading system

Anaerobic digestions were carried out in a 5 L continuously stirred tank reactor (CSTR), fed with alpha-cellulose at a loading rate of 1 g oDM/L/day, operated under mesophilic conditions, at 37° C. The first set of samples were taken when the reactor operation was stabilized under N₂ in the headspace (day 15). 2 mL of reactor content was withdrawn, then, the digesters were flushed with pure H₂ gas for 10 min. Two hours after flushing the second set of samples were taken. The headspace was then replaced with N₂ and the reactors were run under the same conditions as before. After 2 months the complete H₂ treatment procedure was repeated to test the reproducibility of the setup. Immediately after the samplings, total RNA for metatranscriptome analysis and total DNA for metagenome analysis were isolated. cDNA was generated from the total RNA, and both it and the gDNA were sequenced on an Illumina MySEQ platform.

The resulting MG reads were analyzed with Anvi'o, DASTools and SqueezeMeta to generate and analyze metagenome-assembled genomes (MAGs). The downstream analysis was carried out by a custom bioinformatic pipeline to find differentially expressed MAGs,

ORFs and pathways between the N2 and H2 MTR samples. This workflow relies heavily on the following R packages: Clusterprofiler and DESeq2.

3. Results

3.1. Evaluation of Green Willow Biomass (GWB) as potential alternative lignocellulosic substrate for biogas production

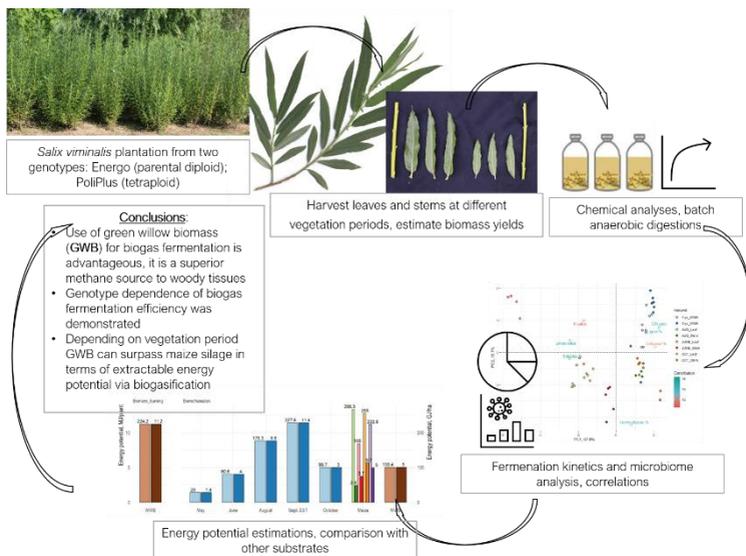


Figure 1. Experimental workflow, part I.

In the first part of my research, I evaluated a novel substrate, the juvenile stems and leaves of short rotation coppice willow, which I

termed Green Willow Biomass (GWB), for biogas production (Figure 1.). The analyses of fermentation efficiency, based on parameters as maximal methane yields (K) and highest methane production rates (μ_{max}), showed that both the commercial willow genotype (EN) and the novel tetraploid variant (PP) can be viable alternative AD feedstocks. My results showed that:

1. The best fermentation parameters can be expected from mid-summer harvests (June in my experiments)
2. Low lignin and high “solubles” are key factors for efficient AD of GWB, similarly to other previously characterized substrates.
3. Leaf biomass is beneficial, as its values were higher than the stem’s.
4. PP is slightly better than EN in this context.
5. Higher Leaf-to-Stem (L/S) ratios were shown to be positively correlated with μ_{max} values, suggesting that PP plants harvested in June are optimal substrates for AD.
6. Compared to WWB, GWB has better fermentation parameters, even more so when considering CSTR fermentations and industrial-scales operations
7. In further research, breeding can improve biomass characteristics.

In addition to the laboratory experiments, I estimated the biomass yields (t/ha) based on a small-scale field experiment to compare GWB with alternative feedstocks. This showed that:

8. EN plants harvested in August produced VS amounts that was comparable to maize silage.
9. Compared to WWB the yields were higher because the large amount of leaf biomass.
10. An extrapolation suggested that September harvest could result in even higher yields.
11. If we consider fermentation parameters and biomass yields, a late August-early September can be the choice for harvest. This is because the extractable energy potential (GJ/ha) in August – September harvests of GWB reaches and can even surpass the corresponding yields of either maize silage or combusted WWB.

Energy willow shrub plantations, besides other advantages, can be harvested annually thereby reducing biomass production costs considerably. The annual cutbacks likely won't affect substantially the yearly biomass production. Ensiling may help to store and/or improve the quality of the green biomass for AD. Further studies, spanning several years and hectares are required however to validate the possibility of this scenario, e.g. annually harvesting the GWB substrate, ensiling it and using it CSTR reactors.

These findings help optimizing the GWB-based technology by using different genotypes, harvesting time, different lignocellulose composition and anaerobic microbial community. Since cultivating willow biomass has several advantages both from an economical and from an ecological point of view, anaerobic digestion of GWB has the potential to produce biogas for a lower cost and with more benefits than many other feedstocks. Therefore, the presented data and

calculations provide substantial support to promote and recommend green willow biomass (GWB) as valuable feedstock for biomethanation.

3.2. Genome-centric metatranscriptomic analysis of H₂-induced stress on anaerobic digestion community

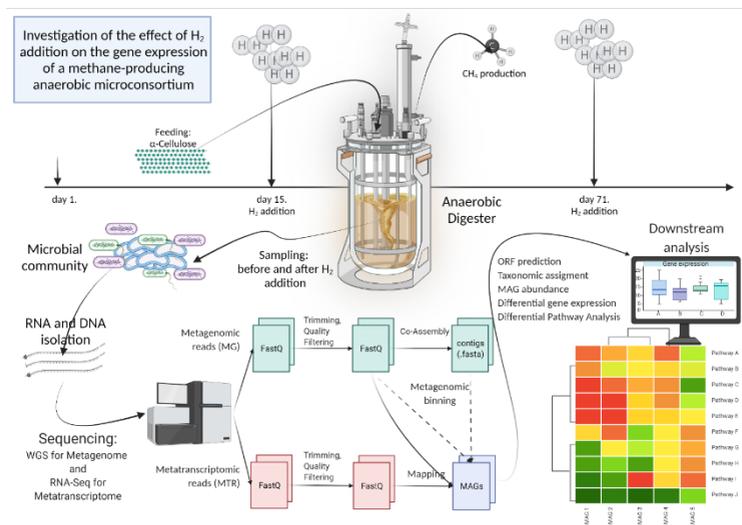


Figure 2. Experimental workflow, part II.

Within the course of the second part of my thesis, I examined the behavior of the biogas producing microbial community in a suggested P2bioCH₄ process on the DNA and mRNA levels. More precisely, I examined the early response of the mixed biogas microbial community to the presence of saturating amount of H₂. Genome-centric

metatranscriptomics could expand our current understanding about the early response of the microbial community and thus contribute to manage the turn-on and turn-off steps of the P2bioCH₄ process. Thus, I carried out metagenomic and metatranscriptomic analyses to determine the changes of the expression levels of the various genes of metagenome assembled genomes (MAGs), with a focus to those related to methanogenesis. These investigations enabled the distinction of the activity of each individual MAG and the identification of the key and most sensitive members of the community. My main findings were the following:

1. The microbial community responded practically instantaneously (2 hours after the induced H₂) to the changed environment.
2. The activity of pluripotential methanogens, mainly *Methanosarcina* MAGs, reduced significantly, while the hydrogenotrophic *Methanobacter* MAGs increased.
3. In addition, the metabolic activity of numerous bacterial strains changed substantially as a response to H₂.
4. Clearly, the excess H₂ does not only affect the methanogenesis pathways in Archaea, rather the microbial community responds with a multifarious gene expression profile change, which seems to be rather selective. This indicates a more global regulatory role of H₂ in the life of anaerobic communities than assumed earlier.
5. The syntrophic interactions contribute to the stability and metabolic activity of the hydrogenotrophic methanogens.

6. This, together with the non-sterile operation conditions and continuous supply of inexpensive catalyst, underlines the benefits of using mixed communities in the P2bioCH₄ process instead of pure hydrogenotrophic cultures.
7. The metatranscriptomic responses to the H₂ treatments separated 2 months apart were very similar to each other indicating not just the robustness of the employed methods, but also that the metabolic pathways could be flexibly restored after switching on and off the P2bioCH₄ operational mode.

This thorough analysis of the differences between the control and H₂-treated metatranscriptomes identified the early events in the microbial communities brought about by the H₂ addition, contributing to our understanding of the BBU mechanism in a P2bioCH₄ system.

4. Co-author waiver

I hereby certify that I am familiar with the thesis of the PhD applicant Balázs Kakuk. Regarding our jointly published results that form part of this PhD dissertation, I declare the followings:

The applicant's contribution was prominent in obtaining the results and I did not and will not use these results in getting an academic research degree.

Regarding the following publications, the contribution of the applicant and me is indistributable.

Kakuk, B., Bagi, Z., Rákhely, G., Maróti, G., Dudits, D., Kovács, K.L., 2021a. Methane production from green and woody biomass using short rotation willow genotypes for bioenergy generation. *Bioresour. Technol.* 333. <https://doi.org/10.1016/j.biortech.2021.125223>

Kakuk, B., Wirth, R., Maróti, G., Szuhaj, M., Rákhely, G., Laczi, K., Kovács, K.L., Bagi, Z., 2021b. Early response of methanogenic archaea to H₂ as evaluated by metagenomics and metatranscriptomics. *Microb. Cell Fact.* 20. <https://doi.org/10.1186/s12934-021-01618-y>

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