Economical production of algae-based biomass and its utilization in renewable energy production

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I. Background

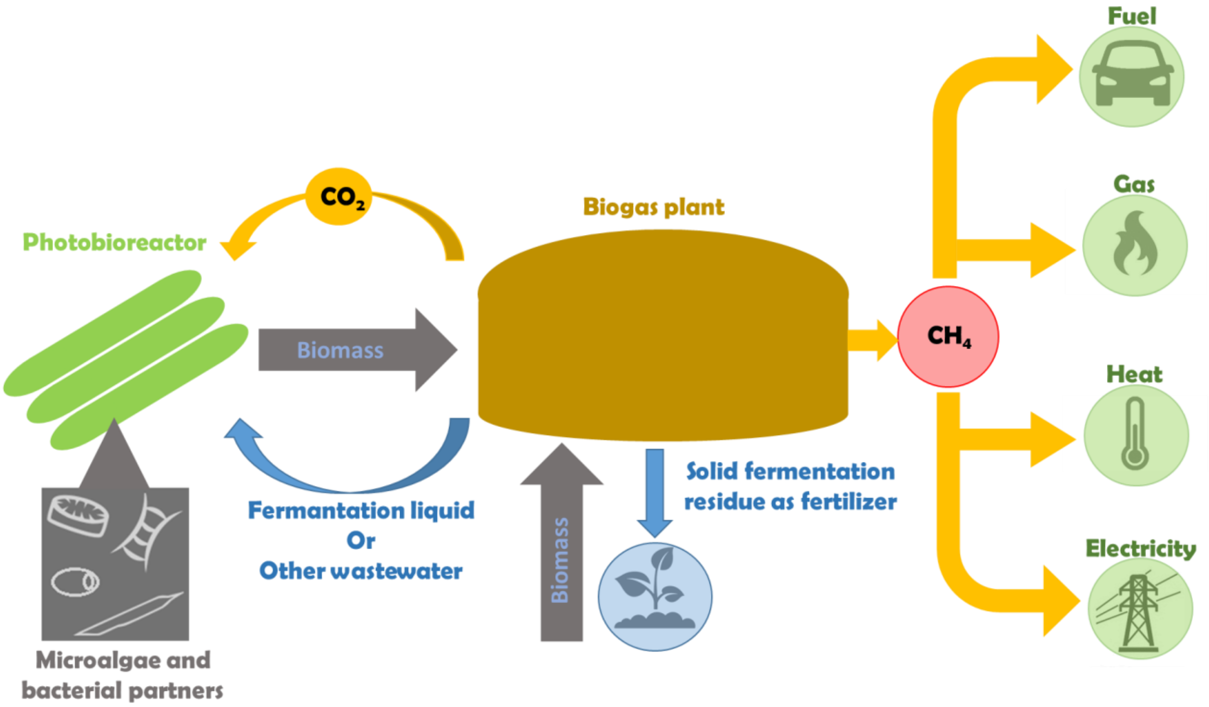
Alternative bioenergy production is one of the hot topics in both basic and applied research. Microalga based bioenergy production is a promising field with regard to the wide variety of algal species. Extensive previous studies employed microalgae for biohydrogen and particularly for biodiesel production, but relatively little attention has been paid at their utilization as biogas substrate (Wirth et al., 2018). The algal biomass grown under less stringent conditions and therefore containing symbiotic bacterial biomass as well can be utilized in the biogas reactor and the total biomass is degraded and converted by complex biogas producing microbial community (Sapp et al., 2007; Seymour et al., 2017). The critical elements of energetic use of microalgal biomass are the high cultivation cost, and cultivation efficiency (Klassen et al., 2015; Morales-Sánchez et al., 2015). In this study, simple and inexpensive cultivation methods had been tested by waste streams containing high organic material and open pond like systems applied. *Chlorella vulgaris* green microalgae will be used in these experiments because this is a widespread strain in natural waters and is therefore easily accessible. The product containing bacterial and *C. vulgaris* biomass had been investigated for its biogas potential and for its anaerobic degradation process stability. A potential problem in anaerobic digestion of microalgal biomass may be due to the low C/N ratio as increasing ammonium content in the fermentation liquid has a negative effect on methanogens (Mussgnug et al., 2010a; Yen and Brune, 2007). Therefore, alternative co-substrates are tested to make the process stable and more effective (Wirth et al., 2015a, 2015b). Metagenomic investigations are implemented to identify microbes that are beneficial for the cultivation and the anaerobic digestion process for possible bioaugmentation.

In the research plan of the present proposal, the following project goals have been assigned:

1. **Develop a sustainable algal biomass cultivation method.**
2. **Anaerobic digestion of *Chlorella vulgaris* mixed biomass**
3. **Anaerobic digestion of photoautotrophically grown *Chlorella vulgaris***
4. **Increase biogas productivity of microalgal biomass with co-fermentation**
5. **Metagenome research**

II. Project goals and achievements

In the focus of our project, there is a zero carbon-dioxide production-and-use cycle and sustainable circular economy system concept. In this concept, the photoheterotrophically grown mixed microalgal bacterial biomass is cultivated in wastewater and the produced biomass is used as a biogas substrate. Because of the nature of the mixed microalgal-bacterial biomass, other alternative substrates should be co-digested, which are grown in marginal lands and fed with the fermentation residues of the fermentation effluent. The produced biomethane than can be converted to heat and electricity or is suitable for every function for which fossil natural gas is used today (Figure 1).



1. Figure: The concept of the circular economy based alternative substrate utilizing biogas producing system.

A review has been published, which focuses on microalgal biomass and its use for biological hydrogen and methane production. Microalgae offer several advantages compared with terrestrial plants. Strategies to maintain an anaerobic environment for biohydrogen production have been summarized:

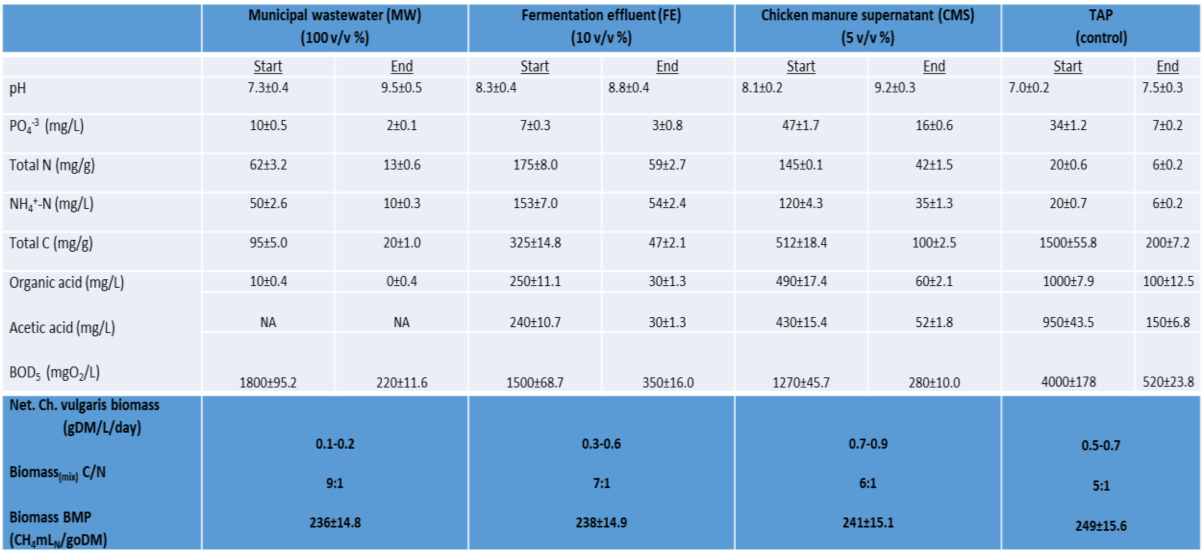
Wirth, Roland; Lakatos, Gergely ; Böjti, Tamás ; Maróti, Gergely ; Bagi, Zoltán ; Rákhely, Gábor ; L. Kovács, Kornél Anaerobic gaseous biofuel production using microalgal biomass – A review. ANAEROBE 52 pp. 1-8. (2018) (IF: 2.7; number of independent citations: 8).

1. Development of a sustainable algal biomass cultivation method

The bioremediation and cultivation efficiency of *Chlorella vulgaris* microalga and its phycosphere was characterized by carbon, nitrogen, phosphate and BOD removal capability in three biological replicates. Microalgal-bacterial (MABA) dry biomass profiles were monitored in three liquid waste types i.e., municipal wastewater (MW), fermentation effluent (FE) and chicken manure supernatant (CMS) over four days. The origins of wastewaters were as follows: MW: Wastewater plant of Szeged, FE: Zöldforrás biogas plant, CMS: our laboratory experiment: liquid residue of water pre-treatment of solid chicken manure (Böjti et al., 2017). The light transmittance of the cultivating medium is key in microalgal biomass cultivation. These wastes are typically dark liquids, therefore different dilutions were examined in order to increase the transparency of the cultures (Otondo et al., 2018). I have characterized and determined the most effective varieties of growth conditions selected by MABA biomass productivity. The control *C. vulgaris* pure strain was maintained and cultivated on TAP (TRIS-Acetate-Phosphate) plates, then grown in TAP liquid medium (1 L). The TAP plates and liquid media were incubated under 50 µmol m^{-2} s^{-1} light intensity at 25°C. The microalgal stock solutions were distributed into 45 mL Falcon tubes at a final optical density (OD_{750}) of 4 (±0.1). Microalgal biomass was separated by centrifugation from the media and used to inoculate the waste water samples (microalgal dry mass content: ~100 mg/L). Cultivation was performed in 250 ml serum bottles (Wheaton glass serum bottle, WH223950) with a liquid volume of 200 mL and stirred with a magnetic stirrer. Bottles were sealed with paper plugs. Tris-acetate phosphate medium was used as control.

Substantial nutrient removal was observed in all three wastewaters indicating a high efficiency of N, P and BOD uptake by MABA. We observed a relationship between the available nutrient and biomass growth efficiency. The undiluted municipal wastewater (MW) contained the lowest concentration of nutrients and hence the lowest optical density (OD_{750}=0.02), relative to the 10 v/v % fermentation effluent (FE) (OD_{750}= 0.72) and the 5 v/v % chicken manure supernatant (CMS: OD_{750}:=0.25) (Table 1). The removal efficiency of phosphate (P) and total nitrogen (N) (mostly ammonium) seemed to depend on the capability of light penetration. The highest P removal efficiency was observed in MW (80%), while in FE and in CMS 57% and 66% loss was measured, respectively. The monitored nutrient consumption in liquid wastes was comparable with that of the control TAP medium, except for P, which was higher in TAP (80%). Moreover, in all media, the MABA more effectively removed N, than P. Total N removals were 79% in MW, 67% in FE and 72% in CMS, respectively. Similar values were observed in the case of ammonium content (MW: 80%, FE: 65% and CMS: 71%). Substantial carbon (C) uptake by MABA was observed in every liquid wastes, i.e. C removal efficiency was over 80% in CMS, FE and in MW. Likewise, a considerable organic acid decrease was monitored through the experiment (FE: 90%, CM and MW: 88%). Moreover, the high C utilization ability of MABA correlated with the BOD loss, i.e CMS: 78%, FE: 77% and MW: 88%. During cultivation a pH increase was observed, which may have been due to formation of hydroxide from the reduction of oxygen molecules produced in photosynthesis (Table 1). The dry mass of the MABA biomass was the highest in CMS: 0.7-0.9 gDM L^{-1} day^{-1}, while in FE it was 0.3-0.6 gDM L^{-1} day^{-1}. The lowest MABA biomass was measured in MW (0.1-0.2 gDM L^{-1} day^{-1}). The MABA carbon to nitrogen ratio in MW, FE, and CMS was 9:1, 7:1 and 5:1, respectively. The biological methane potential (BMP) of MABA biomass samples had negligible differences (maximum 7%) compared to the control (TAP: 249±15 CH_4 mL_N $goDM^{-1}$). The measured BMP from MABA cultivated on MW was 236±14

CH_4 mL_N $goDM^{-1}$, on FE was 238±14 CH_4 mL_N $goDM^{-1}$, and on CMS was 241±15 CH_4 mL_N $goDM^{-1}$, respectively.



	Municipal wastewater (MW) (100 v/v %)		Fermentation effluent (FE) (10 v/v %)		Chicken manure supernatant (CMS) (5 v/v %)		TAP (control)	
	Start	End	Start	End	Start	End	Start	End
pH	7.3±0.4	9.5±0.5	8.3±0.4	8.8±0.4	8.1±0.2	9.2±0.3	7.0±0.2	7.5±0.3
PO_4^{-3} (mg/L)	10±0.5	2±0.1	7±0.3	3±0.8	47±1.7	16±0.6	34±1.2	7±0.2
Total N (mg/g)	62±3.2	13±0.6	175±8.0	59±2.7	145±0.1	42±1.5	20±0.6	6±0.2
NH_4^+-N (mg/L)	50±2.6	10±0.3	153±7.0	54±2.4	120±4.3	35±1.3	20±0.7	6±0.2
Total C (mg/g)	95±5.0	20±1.0	325±14.8	47±2.1	512±18.4	100±2.5	1500±55.8	200±7.2
Organic acid (mg/L)	10±0.4	0±0.4	250±11.1	30±1.3	490±17.4	60±2.1	1000±7.9	100±12.5
Acetic acid (mg/L)	NA	NA	240±10.7	30±1.3	430±15.4	52±1.8	950±43.5	150±6.8
BOD_5 (mgO_2/L)	1800±95.2	220±11.6	1500±68.7	350±16.0	1270±45.7	280±10.0	4000±178	520±23.8
Net. Ch. vulgaris biomass (gDM/L/day)	**0.1-0.2**		**0.3-0.6**		**0.7-0.9**		**0.5-0.7**	
$Biomass_{(mix)}$ C/N	**9:1**		**7:1**		**6:1**		**5:1**	
Biomass BMP (CH_4mL_N/goDM)	**236±14.8**		**238±14.9**		**241±15.1**		**249±15.6**	

1. Table: Summary of *Chlorella vulgaris* microalgal biomass bioremediation and cultivation efficiency on different types of wastewaters.

MABA effectively reduced the C, N, P content and the BOD. The N and P uptake was presumably caused by microalgal activity. The BOD content of liquid wastes was utilized by both the microalga and its bacterial partners, although the microalga seemed to have limited importance in this activity. The phycospheric bacteria produced CO_2 from organic acids consumed by microalgae. The uptake of CO_2 was pH-dependent for microalgae, therefore affected the dissolved oxygen content, which had an impact on the bacterial partners. Based on the microalga dry mass production, CMS was the most efficient, while FE had medium and MW has the lowest growth medium efficiency. MW had the lowest bacterial biomass content in its dried biomass. The biogas potential of MABA biomass cultivated on liquid wastes was comparable with the pure algal biomass grown on TAP medium.

A longer manuscript is being prepared from the above-mentioned results. The tentative title is *Chlorella vulgaris and its phycosphere in action: microalga-bacteria interactions in nutrient removal from different type of wastewaters*. Target open access journals: *Biotechnology for Biofuels (IF:5.20), Frontiers in Microbiology (4.07).*

Additional article published related to this task:

Shetty Prateek, Boboescu I.Z., Pap B., Wirth R; Kovacs K.L., Biro T.; Futo Z., White R.A. III, Maroti G. Exploitation of algal-bacterial consortia in combined biohydrogen generation and wastewater treatment FRONTIERS IN ENERGY RESEARCH 7 Paper: 52 , 13 p. (2019) (CS: 3.3, Number of independent citations: 3)

The results were presented at a conference organized by the Hungarian Microbiology Society.

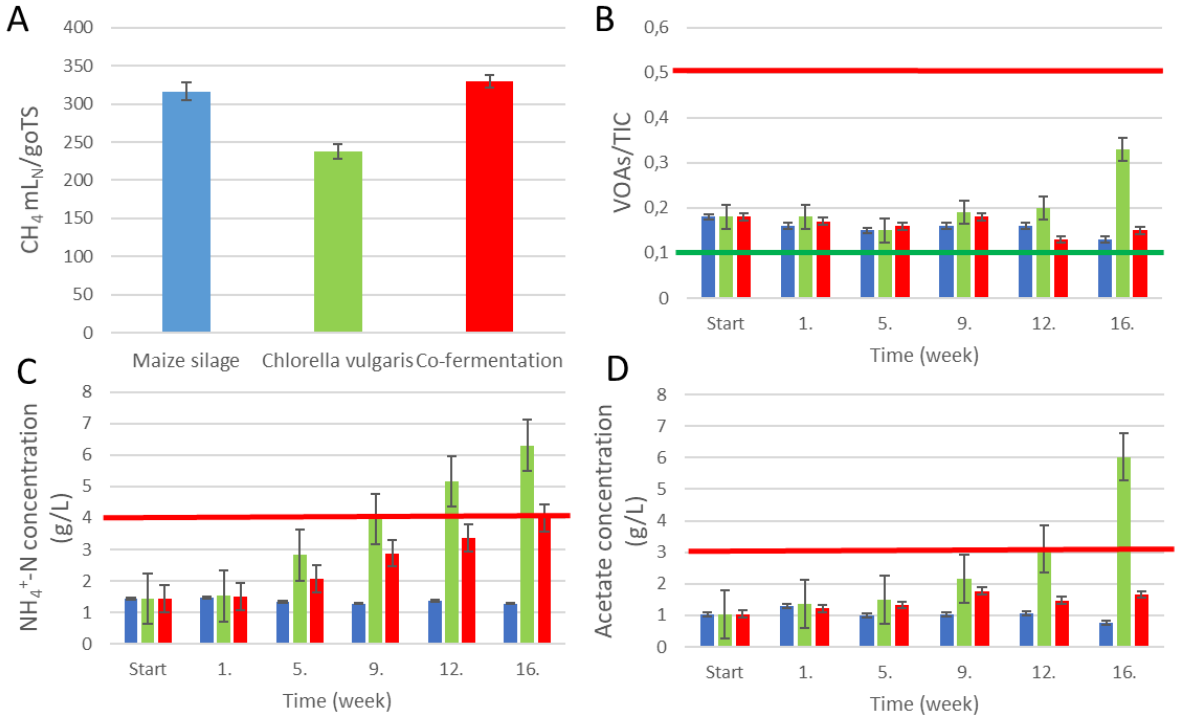
Wirth R.; Böjti T., Maróti G., Bagi Z., Rákhely G., Kovács K.L. Cultivation of algal-bacterial biomass for renewable energy production In: Tamás László; Zelenyánszki, Helga (edited) Fiatal Biotechnológusok Országos Konferenciája "FIBOK 2018": Abstract Book Szeged, Magyarország : JATEPress Kiadó, (2018) pp. 36-36. , 1 p.

2. Continuous AD of photoautotrophically grown *Chlorella vulgaris* biomass

Specially designed experimental 5L semi-continuous biogas reactors have been developed in our laboratory (Kovács et al., 2013). The generated biogas is measured by special software connected to the fermenters. Standardized methods are used to determine other parameters like biogas composition and process parameters (pH, volatile fatty acid and puffer capacity, ammonium content, organic acid measurement) during the experiment. AD reactors were fed with photoautotrophically grown *Chlorella vulgaris* pure microalgal biomass, maize silage and the mixture of the two feedstocks. Although microbiologically pure algal biomass is certainly a more expensive biogas substrate than an algal-bacterial mixture (MABA), it was selected for this study so that the bacterial components, other than the constituents of the microalgal phycosphere, would not distort the core AD microbiome and the degradation process.

During the anaerobic degradation of the various substrates, temperature (37±0.5°C), mixing speed (10 rpm min^{-1}) and pH (7.5–8.3) were continuously monitored by software. The redox potential was below −500 mV throughout the experiment. By the end of the “start-up” period, the residual biogas production ceased completely. Gas production data were collected during weeks 1 through 16 in every 4 h and the cumulative CH_4 productions are plotted in Figure 2A. The CH_4 content in the evolved gas from *C. vulgaris* and from maize silage was 57–67 and 50–52%, respectively, these were compared with previously reported data (Mussgnug et al., 2010b). Co-digestion of *C. vulgaris* and maize silage yielded an intermediate CH_4 value of 57–59%.

The VOAs/TIC (Volatile Organic Acids/Total Inorganic Carbon) ratio is a reliable indicator of a stable digestion process (Lienen et al., 2013). The optimal ratio ranges between 0.1 and 0.5 (Leite et al., 2015). Most of our values fell within this range, due to the low organic loading rate (OLR), indicating a stable and balanced operation in all three reactors, although toward the end of the experimental period the VOAs/TIC in the reactor digesting *C. vulgaris* alone started to increase (Figure 2B).



2. Figure: Biogas digestion process parameters. The feedstocks: blue columns: maize silage green columns: *Chlorella* biomass; red columns: maize silage + *Chlorella* biomass. (A) Specific CH_4 yields from various biomasses. (B) Weekly measured VOAs/TIC ratios. The area between the green and red lines indicates the optimum range. (C) Weekly measured NH_4^+ concentrations. The red line indicates the inhibitory concentrations. (D) Weekly measured acetate concentrations. The red line indicates the inhibitory concentration.

The amount of NH_3-N, conveniently measured as NH_4^+-N, is also a critical indicator of the stability of the biogas-forming process (Yenigün and Demirel, 2013). Levels above 4 g NH_4^+-N L^{-1}, corresponding to about 0.15 g NH_3-N L^{-1} under the current experimental conditions, apparently have a negative effect on the methanogenic community (Chen et al., 2008; Nielsen and Angelidaki, 2008). The inhibitory concentrations reported in earlier studies varied, depending on the feedstock, inoculum, environmental conditions, and acclimation (Chen et al., 2008; Yenigün and Demirel, 2013). In the reactor fed with *C. vulgaris*, the NH_4^+-N content tended to increase, and by week 9, approached, and later exceeded the limiting inhibitory concentration (week 12: 5.16 g NH_4^+-N L^{-1} or 0.26 g NH_3-N L^{-1}) (Figure 2C). It is noteworthy that co-digestion showed balanced digestion and approached the limiting NH_4^+-N values (3.99 g NH_4^+-N L^{-1} or 0.15 g NH_3-N L^{-1}) only in week 16. The acetate concentrations (Figure 2D) exhibited a tendency similar to that of VOAs/TIC (Figure 2B). The acetate concentration in the *C. vulgaris* reactor reached the inhibitory limit of 3 g L^{-1} (Siegert and Banks, 2005) by week 12. Ammonia and acetate accumulation was due to the low C/N ratio of the microalgal biomass, although the pH remained stable in the *C. vulgaris* reactor because of the high TIC.

Results are summarized and published in an open-access journal.

Wirth R., Böjti T., Lakatos G., Maróti G., Bagi Z., Rákhely G. Kovács K.L. Characterization of core microbiomes and functional profiles of mesophilic anaerobic digesters fed with Chlorella *vulgaris green microalgae and maize silage FRONTIERS IN ENERGY RESEARCH 7 Paper: 111 , 18 p. (2019) (CS: 3.3)*

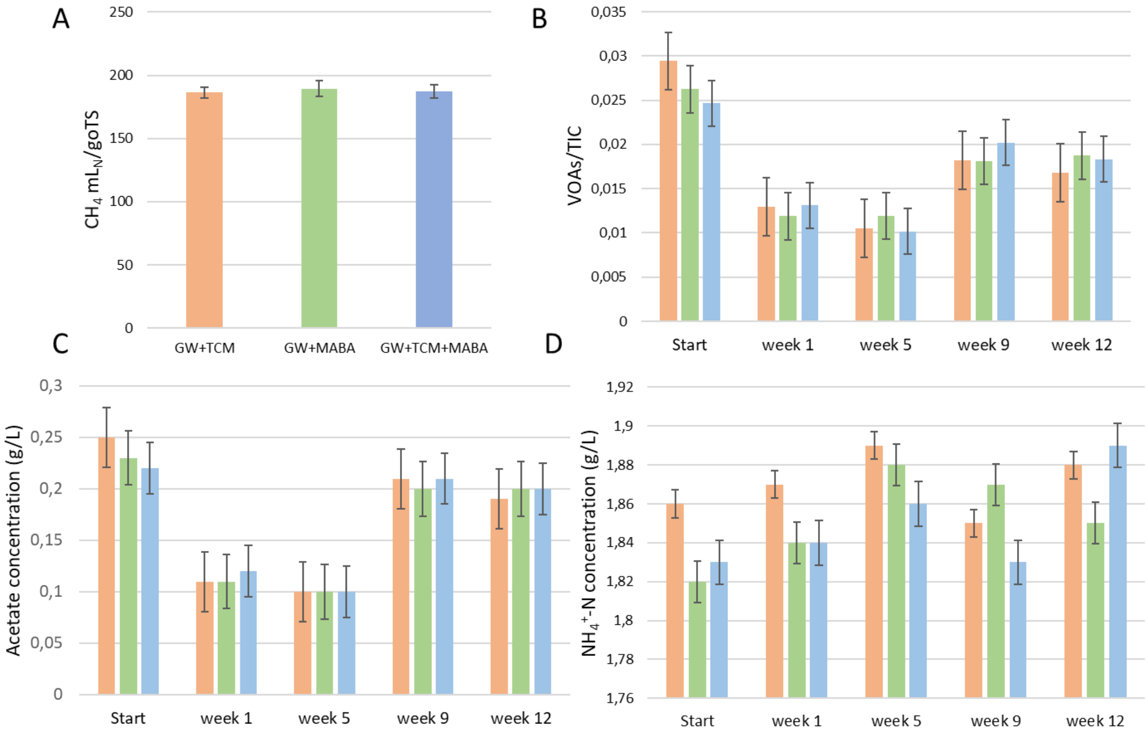
The results were presented at a conference organized by the Hungarian Microbiology Society.

Wirth R., Böjti T., Lakatos G., Maróti G., Bagi Z., Rákhely G., Kovács K.L. The core microbial populations and co-occurrence patterns of microalga-fed biogas digesters ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 64 : Suppl. 1. pp. 191-192., 2 p. (2017)

3. Anaerobic digestion of mixed microalgal-bacterial biomass and biogas productivity enhancement with co-digestion

Our previous study demonstrated that algal-bacterial (MABA) biomass grown on TAP (Tris-acetate-phosphate) is suitable for anaerobic biogas production (Wirth et al., 2015b). In the first section of present project achievements, we demonstrated that different types of wastewater are suitable for *Chlorella vulgaris* microalgal-bacterial biomass cultivation and wastewater bioremediation. Based on the microalga dry mass production, cultivation in CMS was the most efficient. The BMP tests showed that the alternatively cultivated biomass biogas potential is comparable to that of the pure *Chlorella vulgaris* biomass (Section 1). The CMS is produced through the water extraction of soluble molecules of chicken manure, where the treated chicken manure (TCM) is the end product. Therefore we incorporated TCM in our circular economy based biogas production system. However, the problem was that both TCM (C/N: ~15) and the MABA had a low C/N ratio compared with terrestrial plants, therefore as discussed above ammonium inhibition can be expected during the fermentation process in case of these biomasses in time. Maize silage can be used as co-substrate (Wirth et al. 2015a,b), although in this project we attempt to completely replace the maize silage in the biogas reactors. Therefore, an other alternative substrate, having high C/N ratio, had been tested, i.e. energy willow. Energy willow is a rapidly growing and sustainable alternative biomass, which can be easily inserted in the circular economy concept. In this section, the results of the co-digestion of grinded woody willow (GW), and nitrogen-rich materials, TCM and microalgal-bacterial biomass (MABA) are summarized.

Our 5L semi-continuous biogas reactors have been used as described in the previous section. The AD reactors were fed with the substrates in 1 goTS L^{-1} day^{-1} OLR. In this case, the C/N ratio was set to 22 (within optimum range of C/N ratio). The CH_4 content of the evolved gas from the co-digestions were between 42 and 52%, and the yields were 186-189 mL_N/goTS respectively. We observed that, the methane yield was 20% higher compared to the methane potential of GW biomass alone in mono-digestion based on its BMP tests (~150 mL_N/goTS). The gas results indicated that, because of its high lignocellulosic content, the co-digested GW degraded slowly, although TCM and MABA had synergistic effect. The slow degradation of willow biomass were observed previously (Estevez et al., 2012). The fermentation parameters also indicated a slow degradation process, NH_4^+-N and acetate concentrations were far from inhibitory levels (Figure 3C and D). As in the previous experiment (section 2) the acetate concentrations (Figure 3D) exhibited a tendency similar to that of VOAs/TIC (Figure 3B). These results show a similar biogas production efficiency and fermentation parameters like co-digestion of steam-exploded willow and cow manure (Estevez et al., 2014).



3. *Figure:* Biogas digestion process parameters. The feedstocks: orange columns: grinded willow and treated chicken manure, green columns: grinded willow and microalgal-bacterial biomass; blue columns: grinded willow + treated chicken manure + microalgal-bacterial biomass. (A) Specific CH_4 yields from various biomasses. (B) Weekly measured VOAs/TIC ratios. (C) Weekly measured NH_4^+ concentrations. (D) Weekly measured acetate concentrations.

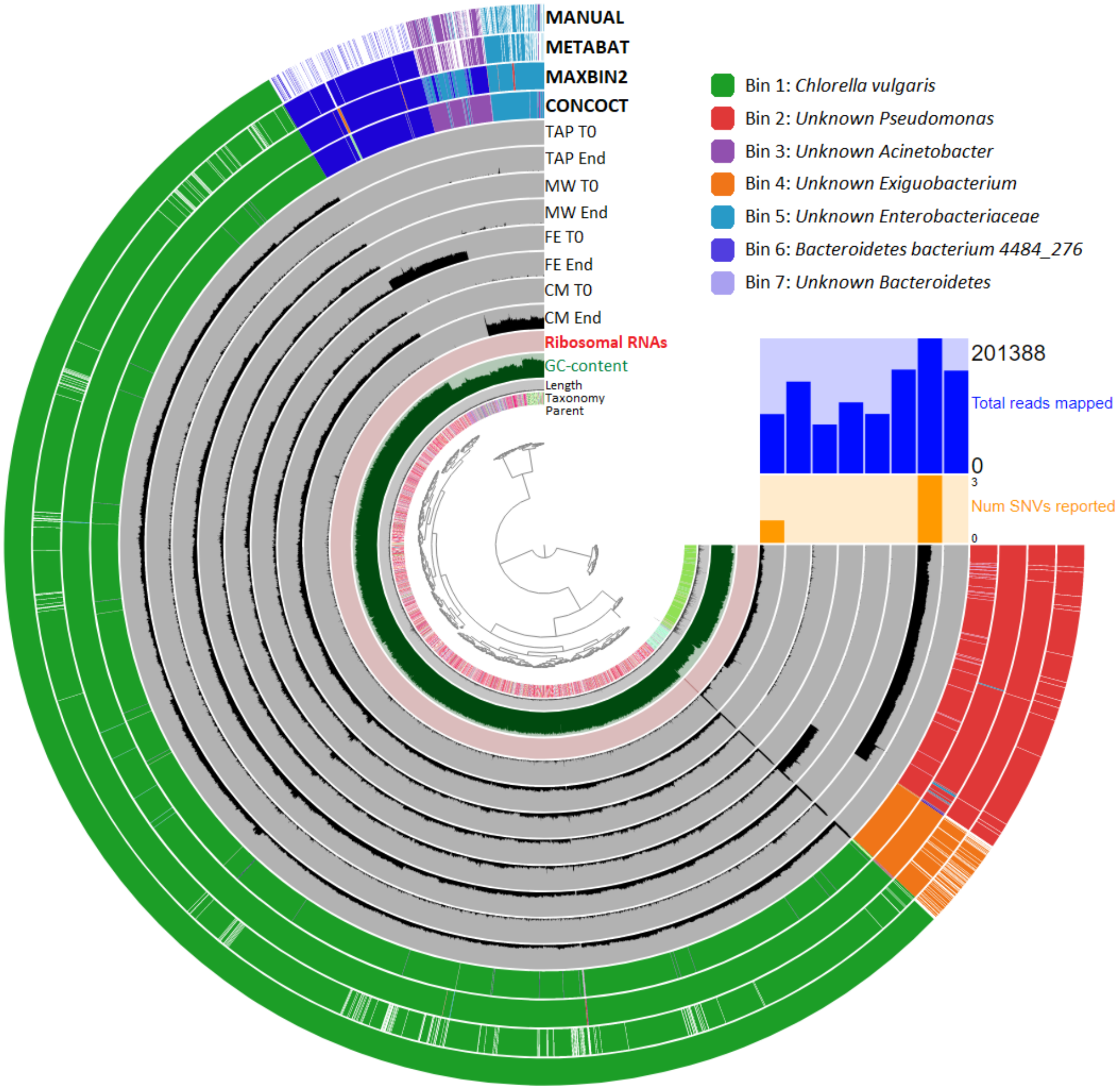
4. Metagenome research

During the cultivation and the AD process, the MABA community has been investigated by high-throughput next-generation sequencing (Ion Torrent and Illumina). With these approaches, we gain insight into the microbial community during cultivation and follow the alterations in the microbial community of the microalgal AD process. Understanding of the organization and behaviour of biogas producing community and inter-kingdom connections in cultivation are crucial for the optimization of their performance and attainment of the stable operation of the process. Advanced bioinformatics tools were employed in read-based and genome-centric metagenomics approaches to identify the different microbial ecosystems and gain insight into the complex microbial relationships. Digesting microbial communities can be more precisely identified by the combination of read-based and genome-centric metagenome techniques (Ács et al., 2019; Campanaro et al., 2019; Maus et al., 2016; Nolla-Ardèvol et al., 2015; Treu et al., 2016; Wirth et al., 2019)

4.1 Genome-centric metagenomic investigation of MABA communities during the cultivation in various wastewaters

The human-guided binning approach by Anvi'o resulted from one medium (Bin 2) and six low-quality MAGs (Bin 1, 3-7) based on the standard Minimum information about Metagenome-Assembled Genome (MIMAG) initiative (Bowers et al., 2017). These bins were identified as one eukaryotic assembly (Bin 1) and six bacterial MAGs (Bin 2-7). The *unknown Pseudomonas* (Bin 2), *unknown Acinetobacter* (Bin 3) and *unknown Enterobacteriaceae* (Bin 5) belong to the class *Gammaproteobacteria* belonging in the phylum *Proteobacteria*. Two bins were found as representatives of the phylum *Bacteroidetes*, i.e. the *Bacteroidetes bacterium 4484-246* (Bin 6) and *unknown Bacteroidetes* (Bin 7), while the *unknown Exiguobacterium* belonged to the phylum *Firmicutes* (Figure 4). To predict metabolic pathways, the translated protein-coding sequences created by Prokka were further analyzed to calculate the module completion ratio (MCR) by MAPLE 2.3.2 using the Kegg database.

In the genom-centric examination of the phycosphere, we found the representatives of *Proteobacteria* (Bin 2, 3 and 5) and *Bacteroidetes* (Bin 6, 7), which are also known as plant growth-promoting bacteria. One of the identified bacterial bins had medium, while the other 5 had low genome completeness. From MAGs of the *unknown Pseudomonas* (Bin 2) and *unknown Acinetobacter* (Bin 3) were detected in all examined growth media, while *unknown Enterobacteriaceae* (Bin 5) was found in some of the alternative cultivation media, i.e. CMS, FE, MW. The representatives of *Bacteroidetes* (Bin 6, 7) were observed only in FE, and the *unknown Exiguobacterium* (Bin 4) only in MW. Therefore, we concluded that; *unknown Pseudomonas* (Bin 2) and *unknown Acinetobacter* (Bin 3) have a close interaction with *Chlorella vulgaris*. Furthermore, the *unknown Enterobacteriaceae* (Bin 5), *unknown Exiguobacterium* (Bin 4) and the representatives of *Bacteroidetes* are (Bin 6, 7) wastewater-specific microbes, that are able to live together with microalgae. Taking into account the functional profiles, *unknown Pseudomonas* (Bin 2) had the highest genome completeness, they are oxidizing organic acids and produce CO_2 for its microalgal partner. In addition, this microbe is capable of synthesizing vitamin B derivatives (B_1, B_7, B_{12}), which may have a great impact on *C. vulgaris* growth and lipid production. The *unknown Acinetobacter* (Bin 3) and *unknown Enterobacteriaceae* (Bin 5) presumably oxidize organic acids, like the *unknown Pseudomonas* (Bin 2) does, while the *unknown Exiguobacterium* (Bin 4) and the representatives of *Bacteroidetes* (Bin 6, 7) can utilize carbohydrates, although knowledge of their detailed metabolic functions is limited.



4. Figure: Visualization of the genome-centric metagenomics data. In the center of the picture is the hierarchical dendrogram of the contigs based on its tetra-nucleotide frequency. Above that the taxonomy of individual contigs (color-coded). In the next level is the length, GC content, and the presence of ribosomal RNAs of contigs is displayed. The inner circles show the frequency of the contigs in each wastewater type (CM: chicken manure supernatant, FE: fermentation effluent, MW: municipal wastewater) in time (T0, End). The outer layer shows the CONCOCT, MAXBIN2, METABAT2 and manual binning results.

A longer manuscript is being prepared from the above-mentioned results. The planned title is: *Chlorella vulgaris and its phycosphere in action: microalga-bacteria interactions in nutrient removal from different types of wastewaters*. Target open access journals: *Biotechnology for Biofuels (IF:5.20), Frontiers in Microbiology (4.07)*

4.2 Read-based and genome-centric metagenomic investigation of continuous AD of photoautotrophically grown *Chlorella vulgaris* biomass

In this experiment, the aim was to determine the response of the core biogas producing microbial community in time, to the feedstock, i.e., *Chlorella* algal biomass. The microbial community and the metagenomic-based functional profile, derived from these data, were monitored during the process by using high-throughput sequencing technology (Ion Torrent PGM™). The read-based core microbiome consisted of 92 genera from which 60 abundant taxa were directly associated with the microbial methane-producing food chain. The data-set was also analyzed in a genome-based approach. Sixty-five bins were assembled, 52 of them belonged in the core biogas producing genera identified by the read-based metagenomes. The read-based and genome-based approaches complemented and verified each other. The functional profiles indicated a variety of glycoside hydrolases. Substantial rearrangements of the methanogen functions have also been observed. Co-fermentation of algal biomass and plant biomass can be carried out for an extended period of time without process failure. The microbial members of the inoculum are well-conserved, feedstock composition changes caused mostly relative abundance alterations in the core microbiome.

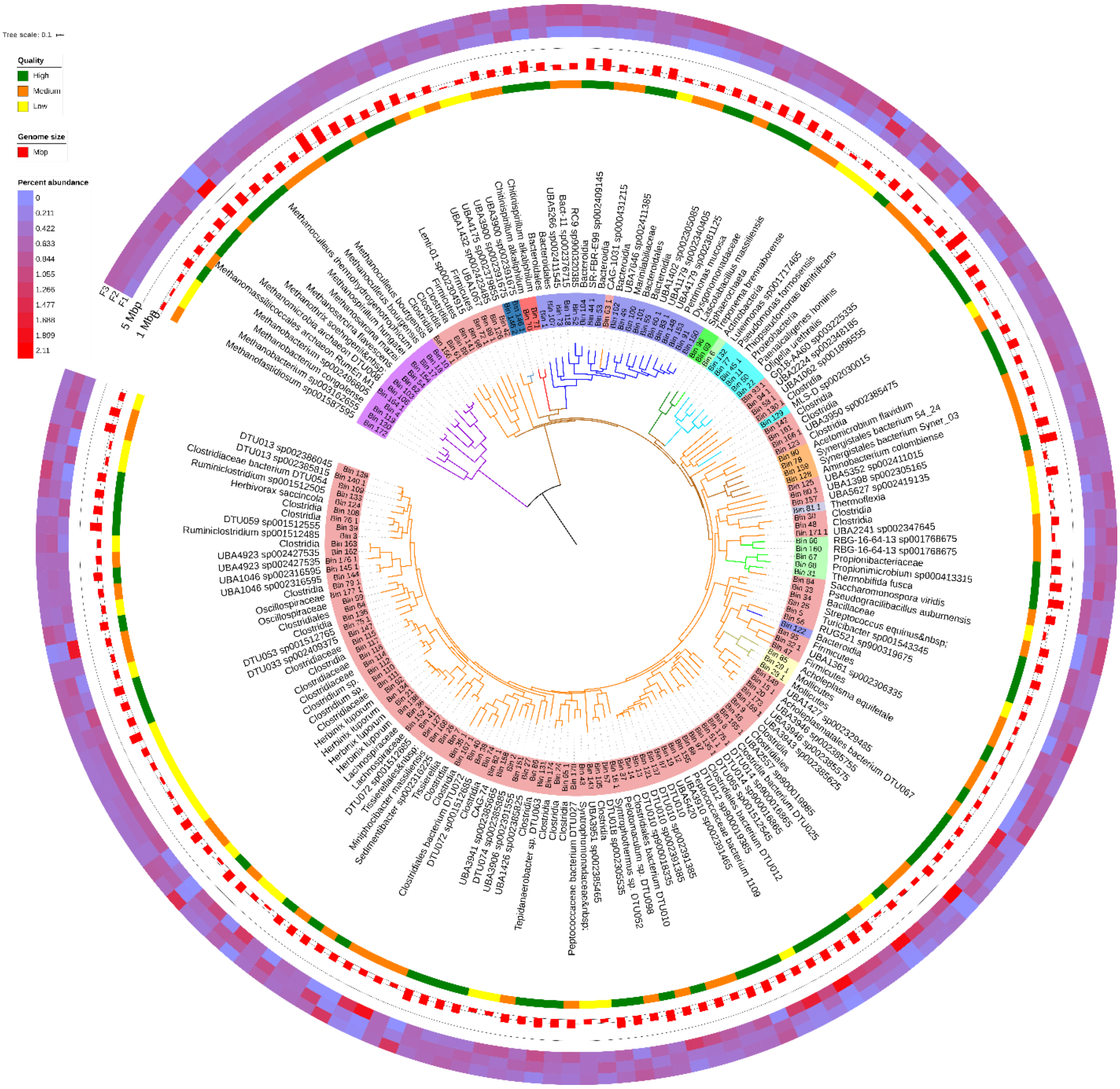
Detailed results have been published in:

Wirth R., Böjti T., Lakatos G., Maróti G., Bagi Z., Rákhely G. Kovács K.L. Characterization of core microbiomes and functional profiles of mesophilic anaerobic digesters fed with Chlorella vulgaris green microalgae and maize silage FRONTIERS IN ENERGY RESEARCH 7 Paper: 111 , 18 p. (2019) (CS: 3.3)

4.3 Read-based and genome-centric metagenomic investigation of continuous AD of mixed microalgal *(Chlorella vulgaris)*-bacterial biomass

The microbial alterations have been monitored during the AD process of grinded woody willow (GW), TCM and MABA biomass by high throughput next-generation sequencing (Illumina NextSeq) at five time points, similarly to the previous experiments. After quality checking and trimming of raw sequences, read-based and genome-centric metagenomics investigations were performed. Read-based metagenomics resulted around 88% *Bacteria* and 12% *Archaea* present in the AD fermenters in average over time. Among *Bacteria* the phylum *Firmicutes* (47%), *Actinobacteria* (12%), *Proteobacteria* (8%) and *Bacteroidetes* (8%) predominated. Mostly hydrogenotrophic methanogens like the genera *Methanoculleus* (4%) and *Methanobacterium* (4%) were present from the domain Archaea. The statistical calculation on the microbiome of different AD reactors over time resulted in significant differences primarily among the rare microbes (at the level of genera). Thus, the microbial content of MABA and TCM did not cause remarkable changes in the AD microbial community.

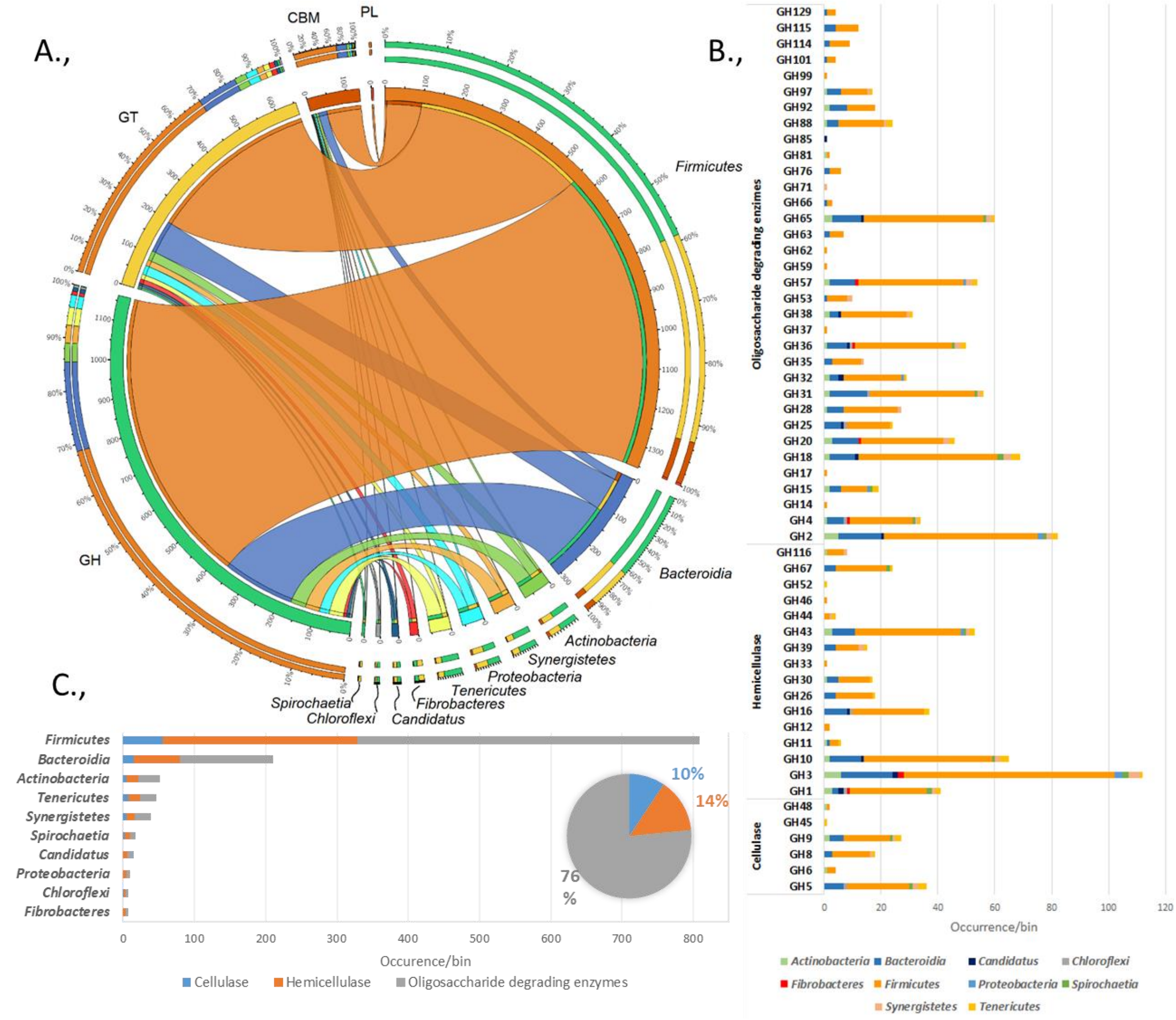
The combination of automated and human-guided genome-centric metagenomic approach resulted in 178 bins (reconstructed genomes), from which there were 75 high, 64 medium and 39 low quality bins, based on the MIMAG initiative (Bowers et al., 2017). For taxonomic assignment of bins three different genomic databases were used: GTDB, Progenomes2 and Miga. Consensus results are used to name the specific bins. The reconstructed bins were also compared to the Biogasmicrobiome database in Miga (Campanaro et al., 2019). Based on taxonomic assignment 13 bins belongs to the domain *Archaea* and 165 bins to the domain *Bacteria* (Figure 5).



5. *Figure:* Phylogenetic tree of the reconstructed genomes. The background colour of bin names shows the phylum where the bin belongs. Above the bin names can be found the quality and the size of the bins (in Mbp). The heat map ring shows the relative abundances of the specific bins.

According to the read-based metagenomic results, most of the reconstructed genomes belong in the phylum *Firmicutes* (117 bins). Within this phylum, 4 high quality bins coded newly discovered microbes based on Miga Biogasmicrobiome database (AAI similarity: <60%). Moreover, two bins identified candidate phyla radiation (CPR) and the *Candidatus Cloacimonas Sccharibacteria* taxon. These results, combined with functional profiles indicate a high hydrolytic potential microbiome, which has unique members. In order to find the species involved in complex carbohydrate decomposition, bins featuring high enrichments in CAZymes

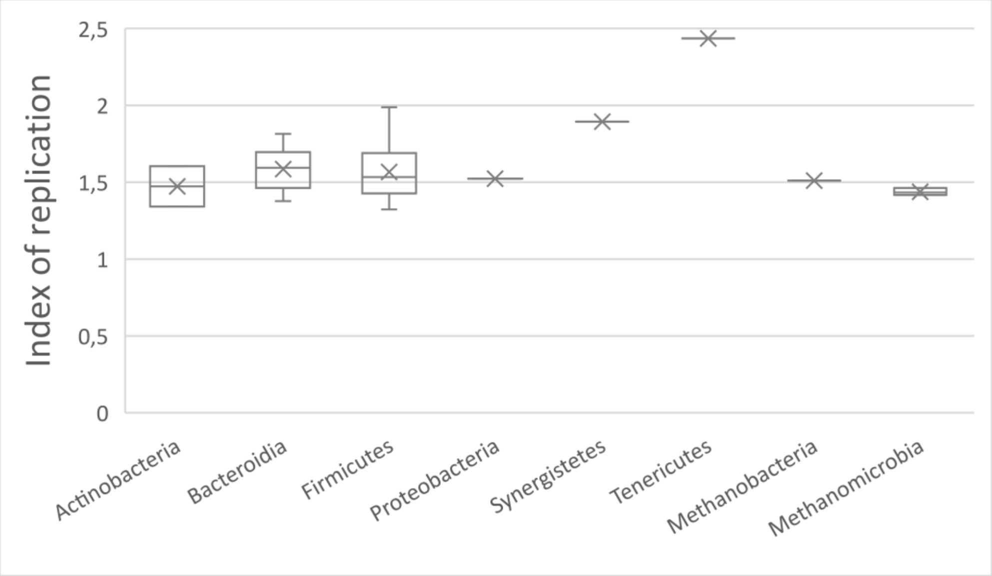
(Carbohydrate-active enzymes) have been selected for further analysis. Globally, 165 bins (92% of the total) are enriched in one or more CAZymes classes, evidencing that polysaccharide degradation is one of the most widespread functional activities in the co-digesting AD system (Figure 6). Although polysaccharide degraders are frequently associated with *Firmicutes* (117 bins) and *Bacteroidetes* (21 bins), many other taxa were found CAZyme enriched, and an involvement in polysaccharide degradation can be hypothesized for members of these taxa. For example, all bins belonging to the *Actinobacteria* (6 bins), the *Tenericutes* (3 bins) and the *Synergistetes* (4 bins) are potentially involved in this process. The detected CPR taxa are also predicted as associated to carbohydrate degradation.



6. Figure: The distribution of CAZymes. A., The relative distribution of CAZyme categories: GH: glycoside hydrolases, GT: glycosil transferases, CBM: carbohydrate-binding modules, PL: polysaccharide lyases. B., The list of the identified GH genes categorized by its activity and ordered to taxa. C., The occurrence of GH genes in the different taxa and relative abundance percentage of GH category.

A tentative estimation of the relative impact of the polysaccharide degradation process was obtained by considering the relative abundance of bins encoding genes for a specific function (e.g., GH, GT, or PL) (Figure 6). The AD community is dominated by polysaccharide hydrolysing bins, most probably because they were fed with substrates rich in cellulose, while generally the fraction is lower than 2% (Figure 5). This indicates that, despite the number of bins involved in polysaccharide degradation being high, the relative abundance of most species is low. This can be due to the presence of relative minor players in terms of abundance, but having high transcriptional activity. If they are highly active, they can enhance or trigger the metabolic processes of dominant members, but this needs additional verification. However, previous results showed that co-digesting of microalgae with carbohydrate containing substrates a synergistic effect was observed parallel with *Firmicutes* abundance and effective gasification of biomass occurred (Wirth et al. 2015a, 2019).

Analysis of bins provides insights into the genetic composition of non-cultivable biogas community members and enhances our understanding of their contribution to the AD process. Such analysis is able to provide knowledge related to the replication capacity of certain biogas-producing members. To determine the replication index of reconstructed genomes across multiple samples, the sequencing coverage resulting from bi-directional genome replication was used to calculate the index of replication (iRep) (Brown et al., 2016). From 178 bins 112 are passed the precondition of iREP calculation. Results were obtained for 6 phyla evidencing that some of the representatives of *Firmicutes* (Bin 32_1, Bin 65_1, Bin 47 and Bin 123), *Synergystetes* (Bin 90) and *Tenericutes* (Bin 28_1) have on average high median iRep values (iREP 1.88, 1.89 and 2.43 respectively) (Figure 7).



7. Figure: Box plots reporting the index of replication for selected taxonomic groups.

In the domain *Euryarchaeota* the replication index was calculated around 1.45 on average for 5 bins indicating their slow growing nature as well as having different abilities in substrate utilization. Calculation of iRep values performed independently for bins encoding different functions revealed that most of the bins involved in polysaccharide degradation had quite low iRep values. This is evident for microbes that are attached to plant fibers. These species represent the slow-growing cellulolytic microflora. Species involved in “carbon and nitrogen fixation” (e.g., protein degraders, VFA producers) have higher values, but methanogenic *Archaea* also creates a slow growing group.

A longer manuscript is being prepared from the above-mentioned results. The planned title is: *Genome-centric microbial characterization of willow, treated chicken manure and* *alternatively cultivated microalgal biomass anaerobic digestion.* Target open access journals: *Biotechnology for Biofuels (IF:5.20) Frontiers in Microbiology (4.07).*

III. Planned publications

On average, a 1-year lag period may occur between data collection and publication, due to the submission and reviewing procedure. Accordingly, the experimental data have been determined, which cover the whole scope of this project, although not all the results are published yet. Results about cultivation of MABA biomass in various waste streams are summarized above and the manuscript has been prepared from the results (Section 1 and 4.1). We plan to publish this publication this year. The next article will report on the results of AD of the co-digestion of willow, TCM and MABA biomasses. These experiments are done and the data are collected (Section 3 and 4.3). The manuscript is under writing and is planned to be published this year. The titles and target journals are the follows:

Chlorella vulgaris and its phycosphere in action: microalga-bacteria interactions in nutrient removal from different types of wastewaters. Target open access journals: *Biotechnology for Biofuels (IF:5.20), Frontiers in Microbiology (4.07)*

and

Genome-centric microbial characterization of willow, treated chicken manure and alternatively cultivated microalgal biomass anaerobic digestion. Target open access journals: *Biotechnology for Biofuels (IF:5.20) Frontiers in Microbiology (4.07).*

IV. Additional publications related to this project

My PD-16 OTKA program is closely related to other works in our team and all publications mentioned in this section include reference to this project. These publications are briefly presented as follows.

1. We reviewed results in three areas of anaerobic microbiology in which methane formation and utilization plays central part. a.) Bio-methane formation by reduction of carbon dioxide in the power-to-gas process and the various possibilities of improvement of the process. b.) Bio-methane as a platform chemical in various chemical and biochemical synthetic processes. c.) Methane-consuming reactions and methane emission mitigation with special emphasis on anaerobic microbiology of ruminants and approaches to diminishing methane emissions by ruminant animals.

Bagi Z., Ács N., Böjti T., Kakuk B., Rákhely G., Strang O., Szuhaj M., Wirth R., Kovács K.L. Biomethane: The energy storage, platform chemical and greenhouse gas mitigation target ANAEROBE 46 pp. 13-22. , 10 p. (2017) (IF: 2.7; number of independent citers: 8)

2. Proper DNA isolation method is crucial for the investigation of complex microbiota such as the biogas producing anaerobic community. Nevertheless, it is essential for metagenomic research to collect all the DNA from cells without contamination and fragmentation in high quantity and in a reproducible way. In the past 8 years we have developed a DNA purification method, which is suitable for application to wide variety

of samples, e.g. different types of soil, rumen content or anaerobic sludge. This method combines chemical and physical lysing and a commercially available DNA purification kit (Zymo Research fecal DNA kit). This method was utilized in the evolutionary analysis of antimicrobial peptides (AMPs) in soil. These peptides are potent, broad spectrum antibiotics which demonstrate potential as novel therapeutic agents. Soil is an ancient reservoir of antibiotic resistance genes and it has been shown that antibiotic resistance genes can be exchanged between soil dwelling and pathogenic bacteria. For all these reasons, we consider soil as a relevant source environment to study the mobilization potential of AMP-resistance genes. The results were presented in:

Spohn R., Daruka L., Lazar V., Martins A., Vidovics F., Grezal G., Mehi O., Kintses B., Szamel M., Jangir P.K., Csörgő B., Györkei Á., Bódi Z., Faragó A., Bodai L., Földesi I., Kata D., Maróti G., Pap B., Wirth R., Papp B., Pál Cs. Integrated evolutionary analysis reveals antimicrobial peptides with limited resistance NATURE COMMUNICATIONS 10 Paper: 4538, 13 p. (2019) (IF:11.8 number of independent citations: 4)

3. There are two possible bioinformatics approaches to analyse metagenomic results: the read-based and genome-centric metagenomics. We developed workflows to analyse metagenomics data in both ways in high quality. The results have been summarized in:

Wirth R., Kádár Gy., Kakuk B., Maróti G., Bagi Z., Szilágyi Á., Rákhely G., Horváth J., Kovács K.L. The planktonic core microbiome and core functions in the cattle rumen by next generation sequencing FRONTIERS IN MICROBIOLOGY 9 Paper: 2285 , 19 p. (2018) (IF:4.2 number of independent citations: 3)

Ács N., Szuhaj M., Wirth R., Bagi Z., Maróti G., Rákhely G., Kovács K.L. Microbial community rearrangements in power-to-biomethane reactors employing mesophilic biogas digestate FRONTIERS IN ENERGY RESEARCH 7 Paper: 132 , 15 p. (2019) (CS:3.3)

4. As mentioned in Section 3, the CMS used to grow MABA biomass was produced via water extraction of chicken manure, where the treated chicken manure (TCM) is the end product. The related experiments have been reported in:

Böjti T., Kovács K.L., Kakuk B., Wirth R., Rákhely G., Bagi Z. Pretreatment of poultry manure for efficient biogas production as monosubstrate or co-fermentation with maize silage and corn stover ANAEROBE 46 pp. 138-145. , 8 p. (2017) (IF: 2.7; number of independent citations: 11)

V. Scientific work of students and collaborations

One student (Schoblocher Dzsenifer) in our Department actively takes part in the experiments related to the present proposal. She summarizes the data about alternative growing of MABA biomass and utilizing it in an AD system. The planned title of her diploma work is: "Alternative cultivation of microalgal-bacterial (MABA) biomass and its utilization in anaerobic system".

There are numerous domestic and international scientific collaborations research and industrial partners, which are related to the present project, e.g. Institutes of Biochemistry and Plant Biology, Biological Research Centre, Szeged; University of Veszprém; NAIK, Herceghalom; BOKU Tulln (AT); Indian Institute of Technology, DELHI-New Delhi (India);

Güssing Energy Technologies (AT); Skupina Fabrika (SLO); R2Gas Research Centre, Wien (AT); Dongguk University, Seoul (KR); Helvécia Kft., Szeghalom, Zöld Forrás Energia Kft., Szeged; Pilze-Nagy Kft., Kecskemét; Cyclolab Kft., Budapest; Agrár-Béta Zrt., Dombovár.

VI. Connections of present project with other projects

The project titled “Economical production of algae-based biomass and its utilization in renewable energy production” is connected the following ongoing or closed projects:

- GINOP-2.2.1-15-2017-00081: Utilization of short-cut energy plantations with innovative biogas co-fermentation and phytofermentation technologies.
- GINOP-2.2.1-15-2017-00033: Development of combined quantitative diagnostic procedures for the rapid detection of food counterfeiting.
- EFOP-3.6.2-16-2017-00010: Developing a thematic network for sustainable commodity management - RING 2017.
- FK123899: Molecular exploring of inter-kingdom algae-bacterial interactions and utilizing its mechanisms to identify novel antimicrobial agents.
- GINOP-2.3.2–15–2016–00014: Evolutionarily optimized antibacterial foldamer: from chemical building blocks to system biology EVOMER.
- GINOP-2.3.2–15–2016–00020: Molecular mapping of genomic instability and carcinogenesis, MolMedEx TUMORDNS

References:

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