The effect of the source of microorganisms on adaptation of hydrolytic consortia
dedicated to anaerobic digestion of maize silage

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Abstract
The main aim of this study was to evaluate the effect of the source of microorganisms on the
selection of hydrolytic consortia dedicated to anaerobic digestion of maize silage. The
selection process was investigated based on the analysis of changes in the hydrolytic activity
and the diversity of microbial communities derived from (i) a hydrolyzer of a commercial
agricultural biogas plant, (ii) cattle slurry and (iii) raw sewage sludge, during a series of 10
passages. Following the selection process, the adapted consortia were thoroughly analyzed for
their ability to utilize maize silage and augmentation of anaerobic digestion communities.

The results of selection of the consortia showed that every subsequent passage of each
consortium leads to their adaptation to degradation of maize silage, which was manifested by
the increased hydrolytic activity of the adapted consortia. Biodiversity analysis (based on the
16S rDNA amplicon sequencing) confirmed the changes microbial community of each
consortium, and showed that after the last (10th) passage all microbial communities were
dominated by the representatives of Lactobacillaceae, Prevotellaceae, Veillonellaceae.

The results of the functional analyses showed that the adapted consortia improved the
efficiency of maize silage degradation, as indicated by the increase in the concentration of
glucose and volatile fatty acids (VFAs), as well as the soluble chemical oxygen demand
(sCOD). Moreover, bioaugmentation of anaerobic digestion communities by the adapted
hydrolytic consortia increased biogas yield by 10-29%, depending on the origin of the
community. The obtained results also indicate that substrate input (not community origin) was
the driving force responsible for the changes in the community structure of hydrolytic
consortia dedicated to anaerobic digestion.

1. Introduction

Over the past decades the biogas production technology has been focused on the
development and optimization of systems characterized by high rate of anaerobic digestion of
energy crops and solid agro-industrial wastes [1]. The anaerobic digestion process consists of
two phases. In the first one, the substrate is converted mainly to soluble organic compounds
such as volatile fatty acids (VFAs), which are then used in the second phase by methanogens
to produce biogas [2]. These two phases can be carried out simultaneously in a single
bioreactor or separately, in a two-step system, which consists of two connected bioreactors.
Due to their simplicity, many full-scale biogas plants in Europe utilize single-step systems.
However, two-step technologies may ensure optimal conditions for each of the processes,
which is especially crucial, as hydrolysis of complex polysaccharides such as cellulose or
lignocellulose is often the rate-limiting step of the entire biogas production process. The
production of VFAs proceeds much faster than the conversion of VFAs to methane, thereby
caus[ing acid accumulation], often leading to a drop in pH below 6, and consequently, to the
inhibition of methanogenesis [3].

Two-stage anaerobic digestion allows to overcome the problem of imbalance between
the acidogenesis and methanogenesis processes. Moreover, the two-stage technology enables
the separation of solid and liquid phases and thus maintaining of a high rate of biogas
production. Therefore, the use of the two-stage systems may lead to a higher overall reaction
rate and biogas yield, and for this reason they are considered more effective than the
conventional single-step technologies [4,5]. Moreover, some authors reported that splitting
and separate optimization of hydrolysis/acidogenesis and methanogenesis could enhance the
overall reaction rate, maximize biogas yields, and make the process easier to control, both
under mesophilic and thermophilic conditions [4,5].

One of the most important problems encountered at commercial agriculture biogas
plants is the start-up of new anaerobic reactors. According to Ahring [6], without efficient
hydrolytic microbial consortium, the start-up period of thermophilic anaerobic digestion can
be prolonged to one year before it enters a steady state. Selection of microorganisms
responsible for different stages of anaerobic digestion, from the initial inoculum may be very
long when is carried out during the start-up of anaerobic bioreactors. This often generates
high operating costs for biogas plants. Therefore, the search for efficient consortia sources,
their selection and adaptation, as well as the choice of the main substrate, are the most
important factors affecting the successful start-up of anaerobic digestion.
The substrate used for anaerobic digestion is vary between the biogas production plants.
Maize silage is one of the most popular energy crops and is widely used in agricultural biogas
facilities as a substrate for the biogas production, and it represents 73% mass of the plant
biomass processed in the biogas plants [7]. To increase biogas production from maize silage,
various methods can be used, including chemical, physical and biological pretreatment.
Biological methods are a good alternative to the physical and chemical methods as they are
cost-effective and allow for lower energy use [8]. In nature, plant biomass is degraded by
hydrolytic enzymes produced by microorganisms, including bacteria and fungi. Biologically
stable and controllable microbial consortia with a high hydrolytic activity isolated from such
environments, seem to be more valuable and effective than individually strains [9].

Many studies have shown that hydrolytic microbial consortia are very often isolated
from different natural environments. Wongwilaiwalin et al. [10] developed stable
thermophilic, lignocellulose-degrading microbial consortium (MC3F) from sugarcane bagasse
compost, which can degrade up to 75% of rice straw, 70% of corn stover, and 60% bagasse in
7 days. Haruta et al. [11] obtained a consortium (MC1) from composting materials (sugarcane
dregs, chicken feces, dried straw, pig feces, and cattle feces). These stable community
degraded 60% of rice straw, 88% of cotton, 70 % of corn stalk and 79% of filter paper. The
biodiversity and varying hydrolytic efficiency of bacterial consortia isolated from different
environments indicates the importance of the initial inoculum composition and its effect on
the entire biogas production process.

Additionally, natural consortia are able to survive under a wide range of
environmental conditions. Nowadays, remains a challenge to recognize and optimize
degradation of maize silage by natural mesophilic bacterial consortia. Natural consortia are
very diverse, both in terms of microbial biodiversity and cellulolytic activity [12]. Thus, the
selection, adaptation, stabilization and characterization of the natural consortia may play a key role in improving the degradation of the substrate and create an efficient way leading to the enhancement of biogas production during anaerobic digestion.

Although a number of studies have addressed to hydrolytic microbial consortia used for enhancing the mesophilic anaerobic digestion of maize silage, only few have focused on the actual changes in hydrolytic activity and biodiversity of microorganisms during the selection and adaptation processes.

The purpose of this study was to evaluate the influence of the source of microbial community on the selection and stabilization of hydrolytic consortia designated for utilization of maize silage. Additionally, the effect of bioaugmentation of a stable methanogenic community with the adapted hydrolytic consortia on biogas production from maize silage was investigated.

2. Materials and Methods

2.1. Source of microorganisms and substrate

Hydrolytic microbial consortia were isolated from: (i) two mixed hydrolyzers of an agricultural biogas plant located in Miedzyrzec Podlaski (Poland), where maize silage is used as a substrate, (ii) cattle slurry from farms in Trzebieszow Pierwszy (Poland) and (iii) raw sewage sludge from the wastewater treatment plant "Czajka" in Warsaw (Poland).

Methanogenic consortium for anaerobic digestion was obtained from the fermenter tank of an anaerobic digester from the biogas plant in Miedzyrzec Podlaski (Poland).

Maize silage obtained from a farm in Mikanow (Poland) was homogenized in a blender, mixed with low-mineral water (Eden Springs, Poland) and used as a substrate for the selection of hydrolytic consortia and analysis of anaerobic digestion process.

2.2. Experimental set-up for the selection of hydrolytic microbial consortia
The selection of hydrolytic microbial consortia was carried out in 500 mL bottles. These lab-scale hydrolyzers were filled with 0.5% (v/v) of total solids (TS) of the aforementioned samples, 3% (v/v) of TS maize silage. Mineral water was added up to the final volume of 400 mL. The selection process consisted of a series of 10 passages. Hydrolysis of maize silage in each passage lasted for 72 hours and was carried out at 30 °C. Samples for the estimation of hydrolytic activity were taken at the end of each passage. Samples for microbial community analysis were taken at the end of the passages: 1, 3, 6 and 10 and stored at -20 °C until DNA extraction.

2.3. Estimation of the hydrolytic activity

Semi-quantitative analysis of the hydrolytic activity, was determined by the modified method by Teather and Wood [13] using Congo-Red Dye as an indicator of hydrolytic (cellulolytic activity) of microorganisms. Supernatants from the cultures (8000 rpm, 10 min), obtained after each passage, were injected into metal cylinders placed on CMC-Congo Red agar plates (containing 1% Congo Red Dye) and incubated for 96 hours at 30 °C. A clear zone around a metal cylinder indicated the hydrolytic activity of the tested consortium.

The hydrolytic activity of the consortia after each passage was determined based on the assessment of the amount of reducing sugars (equivalent of glucose) generated during the enzymatic hydrolysis of insoluble cellulose (from maize silage). Reducing sugars concentration was estimated spectrophotometrically, by measuring the absorbance at 540 nm after the addition of 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of the centrifuged cultures [14].

2.4. Hydrolysis of maize silage

Laboratory-scale degradation of maize silage by the adapted ABH, CS and WTP consortia was carried out in 1 L bottles GL 45 (Schott Duran, Germany). The reaction mixture containing 3% TS maize silage was inoculated with 10% (v/v) of the selected consortia. The
pH was adjusted to 7.2 using sodium bicarbonate. The hydrolysis was carried for 120 hours at 30 °C. Physical and chemical analyses were performed at the beginning of the experiment and every 24 hours until 120 hours. The following parameters were assessed for each sample: soluble chemical oxygen demand (sCOD), volatile fatty acids (VFAs) concentration and the total reducing sugar (glucose) released due to the hydrolytic activity of the consortia.

2.5. Batch assay of the anaerobic digestion

The effect of bioaugmentation of anaerobic digestion by the adapted hydrolytic consortia was investigated in laboratory-scale anaerobic batch experiments, which were performed in reactors consisting of 1 L glass bottles GL 45 (Schott Duran, Germany) connected with Dreschel-type scrubbers. To each reactor, a 1 L Tedlar gas bag (Sigma, Germany) was attached to collect biogas. The bioreactors were filled with the liquid phase from the fermenter tank of an anaerobic digester containing the methanogenic consortium inoculate [18 g volatile solids per liter (g vs L⁻¹)], maize silage (9.6 g vs L⁻¹) and supplemented with 100 mL of the adapted hydrolytic consortia. Low-mineral water was added up to the final volume of 900 mL. The pH in each bioreactor was adjusted to 7.2 using sodium carbonate. Anaerobic batch assays were run at 37 °C for 21 days without refeeding. Physical and chemical analyses were carried out at the beginning of the experiment and after 7, 14 and 21 days. The experiment was performed in triplicate.

2.6. Analytical methods

To monitor the hydrolysis of maize silage and the anaerobic digestion process, the following parameters were determined: sCOD, VFAs concentration, the total reducing sugar (glucose) concentration and volume of the biogas produced. The VFAs concentration and sCOD were determined using Nanocolor® kits (Machery-Nagel GmbH, Germany). The total concentration of reducing sugar was determined by the method developed by Miller (1959).
The volume of the produced biogas was monitored by Milligascounter MGC-1 (Ritter, Germany).

2.7. DNA extraction and PCR amplification

Total DNA from the selected consortium was isolated using the method described by Dziewit et al. [15]. Briefly, the samples were centrifuged (8000 rpm, 4 °C, 15 min), pellet was resuspended in lysis buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% (w/v) CTAB] and the cells were disrupted in a 5-step bead-beating protocol, supported by freezing and thawing procedure. Final DNA purification from protein, humic substances, etc., was carried by cesium chloride density gradient ultracentrifugation. The concentration and quality of the purified DNA was estimated by means of the NanoDrop 2000 instrument (NanoDrop Technologies) and gel electrophoresis.

The whole community DNA was used as a template for amplification of bacterial hypervariable V3–V4 regions of the 16S rDNA with the primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21, described by Klindworth et al. [16]. The reaction mixture (50 µL) contained 100 ng of the template DNA, primers and 0.02 U of Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Bacterial 16S rDNA fragments were PCR amplified in a thermocycler (Biorad) with 20 cycles. PCR conditions were as follows: initial denaturation (5 min at 96 °C), cycles consisting of denaturation (30 s at 96 °C), annealing (50 s at 58 °C), extension (25 s at 72 °C), and a final extension step (5 min at 72 °C). The PCR products were analyzed by horizontal gel electrophoresis (2% agarose with ethidium bromide in 1x TAE) and then purified with Agencourt AMPure XP beads (Beckman Coulter).

2.8. Sequencing library preparation and amplicon sequencing

To prepare the libraries for sequencing, approximately 250 ng of amplified DNA (pooled from the PCR replicates) was used with the Illumina TruSeq DNA Sample Preparation Kit.
omitted. Libraries were verified using the 2100 Bioanalyzer (Agilent) High-Sensitivity DNA Assay and KAPA Library Quantification Kits (Illumina).

Amplicon DNA sequencing was performed using the Illumina MiSeq Platform (MiSeq Illumina Kit V3) with a 300 bp read length. Computational analyses were performed in a similar manner as described by Nelson et al. [17], using a local computing environment with the Quantitative Insights in Microbial Ecology (QIIME, v1.9.0) pipeline [18]. Briefly, raw sequences were processed with the Cutadapt software enabling trimming of the nucleotides corresponding to the sequence of adapters and primers used for PCR amplification and library preparation. In next step, the sequences were merged and combined into a single fastq file, in order to ensure an even treatment and comparison for QIIME analyses. This resulted in the generation of 2.4 mln sequences with the mean length of 422 nucleotides. Chimera detection was performed using usearch61 [19] with subsequent filtering from sequences. Operational taxonomic units (OTU) were de novo assigned with uclust [20], clustered at 97% similarity against the SILVA version 111 reference OTU alignment [21]. A representative sequence for each OTU was selected and then the taxonomic assignment was made using the RDP Classifier v2.2 [22]. Additional filtering for sequence errors was performed with filter_otus_from_otu_table.py script by removing OTUs appearing in fewer than 3 samples and represented by less than 0.0005% of the total sequences.

Taxonomic structures were prepared based on OTU tables specific for bacterial amplicons, with a family level default. Sequences that were not assigned at the family level were named in accordance with the lowest possible taxonomy. A principle coordinates analysis (PCoA) plot was constructed to visualize the dissimilarity of samples at different stages of selection.

Raw sequences of 16S rDNA amplicons obtained in this study (15 libraries) were deposited in the SRA (NCBI) database under Bioproject accession number PRJNA350818.
3. Results and discussion

Hydrolytic microbial consortia were obtained after successive subcultivations of various environmental sources of microorganisms: (i) an effluent from a hydrolyzer tank from an agricultural biogas plant (ABH), (ii) cattle slurry (CS) and (iii) raw sewage sludge from a wastewater treatment plant (WTP) on medium containing maize silage. Selection and adaptation of the hydrolytic consortia was monitored during a series of 10 passages carried out every 72 hours at 30 °C on a fresh portion of maize silage medium. The performed experiments were aimed to find out: (i) the effect of the source of microorganisms on the hydrolytic activity of the selected consortia, (ii) biodiversity changes within these consortia and (iii) the effect of the selected consortia on degradation and anaerobic digestion of maize silage.

3.1. The effect of the source of microorganisms on the hydrolytic activity of the adapted consortia

To determine the changes in the hydrolytic activity of the analyzed consortia during each step of adaptation (each passage), basic physiological tests on the CMC-Congo Red medium agar plates were performed. After 96 hours of cultivation, the level of cellulolytic (hydrolytic) activity of studied the consortia was semi-quantitatively assessed by measuring the diameter of the produced clear zones (Fig. 1A).
Fig. 1. Hydrolytic activity of the consortia during the selection process. Analysis of the changes: clear zones diameter on CMC-Congo Red agar plates (A) and concentrations of the released glucose (B).

The initial diameter of the clear zones after first passage (P1) was 18-19 mm for all consortia. The size of the clear zones has increased and reached the maximum diameter of
29.5 mm, 26.5 mm and 25.0 mm for ABH (after P6), CS (after P10) and WTP (after P7), respectively. These results indicate that all the selected consortia had hydrolytic (cellulolytic) activity and it was higher than that of the initial consortium after adaptation for used substrate. Literature data on the Congo red clearing zone assay, describes the results only for pure strains of bacteria. The results obtained in this study indicated that the level of hydrolytic activity of the analyzed consortia, corresponds to that described in the literature as a high for the pure strains. For example, Gupta et al. [23] obtained 28-50 mm clear zones for cellulolytic bacteria isolated from guts of termite, caterpillar, bookworm and snail, and Liang [24] – 20-30 mm clear zones for organic-rich soil isolates. Our earlier studies on hydrolytic strains (belonging to the following genera: *Bacillus, Ochrobactrum, Providencia*), which produced clear zones reaching from 15 to 44 mm in diameter. These strains were isolated from a hydrolyzer tank from an agricultural biogas plant, cattle slurry and manure and were dedicated for degradation of maize silage [25].

As previously stated, the diameter of the hydrolyzing zone produced by bacteria on CMC-Congo Red plates it is only semi-quantitative method for assessing the hydrolytic activity of bacteria and it may not accurately reflect their actual hydrolytic activity. For this reason the hydrolytic activity of the selected consortium was evaluated by analyzing the changes in the concentration of glucose released during maize silage degradation. Cleavage of the β-1, 4-glycosidic bonds by microbial enzymes leads to the hydrolysis of cellulose, resulting in the release of glucose or oligosaccharides. The amount of reducing sugars produced during the degradation of maize silage indicates the pretreatment effectiveness [26,27]. For the studied consortia, the initial concentrations of glucose in the first passage of all cultures were similar (1.13-1.19 g/L). During the selection process, the concentration of glucose gradually increased, reaching the highest value of 1.50 mg/mL, 1.58 mg/mL and 1.47 mg/mL in the passage 10 for ABH, WTP and CS, respectively (Fig. 1B). The increase was equivalent to the release of 26% (ABH), 37% (WTP) and 29% (CS) more glucose molecules.
compared to first passage and indicates a higher hydrolytic activity of the selected consortia. Additionally, the fastest increase in the concentration of glucose was observed for CS (1.34 g/L after the third passage (P3)). CS had a higher increase at the beginning, which means it reached its peak reducing sugar yield earlier than the other two consortia. This trend was also observed in the clearing zone assay and thus suggested that cattle manure can be a good source of microorganisms for fast-acting and stable hydrolytic consortia.

Moreover, another aspect of the source of hydrolytic microorganisms are the nutrients it contains. The micronutrients contained in the source can enhance the metabolism of microorganisms and hydrolytic activity, and consequently, biogas yield [28,29]. However, the concentrations of micronutrients in different sources of microorganisms were rarely mentioned. In the case of using maize silage as a substrate, the concentration of nitrogen could be low with simultaneous excess of carbon, what may result in lower biogas production [30]. When cattle manures is used as source of microorganisms, extra nitrogen, present in the initial sample, is provided to meet the needs of the hydrolytic consortium. However, after the first passage, cattle slurry no longer constitutes a source of nutrients, what may have caused further decrease in the biodiversity of the consortium.

The results of CMC-Congo Red agar plates assay as well as the changes in concentrations of glucose released indicate that hydrolytic activity increased during experiments for all consortia and suggest the selection of specialized bacteria and adaptation for enhanced degradation of maize silage.

3.2. The effect of the source of inoculum on the diversity changes during the adaptation of the hydrolytic consortia

Biodiversity of the microbial consortia was analyzed based on high throughput gene sequencing amplicons of hypervariable fragment V3-V4 of bacterial 16S rDNA. In the studied samples, 3 to 8 bacterial phyla constituting at least 1% of whole microbial community
were detected. The most abundant phylum was *Firmicutes* with the average count 60% (ranging from 6% to 93%) followed by *Bacteroidetes* with 24% (ranging from 1% to 64%) of total microbial sequences. Members of the *Firmicutes* and *Bacteroides* phyla play the most important role in the hydrolysis of the plant biomass and in the secondary fermentation [31].

Considering the microbial structure at family level, the results clearly shows the adaptation of the microbial communities (Fig.2).

**Fig. 2. Bacterial community structure dynamics.** The bar chart shows the relative abundance of bacterial families with abundance > 5% in at least one variant. Inoculum communities were denoted with 0 while the numbers 1, 3, 6, 10 represent the subsequent passages as described in the Materials and Methods section. ABH – agricultural biogas hydrolyzer, CS – cattle slurry, WTP – wastewater treatment plant.

The number of families (with >1% abundance) present in the inoculum samples were reduced from 17 (CS), 12 (WTP) and 10 (ABH) to 4-5 families in each sample in the last passage. For both ABH and WTP samples, a decrease in the dominating families (in the percentage content of the families dominating in the initial sample) was already seen after the first passage. For the remaining consortium CS, the reduction to 4-5 families occurred at passage 3. This quick alteration of the microbial community structure can indicate a fast response of the microorganisms to the changes in conditions and selection of specialized best-adapted bacteria. Confirmation of the adaptation of microbial communities was presented by
Principal Coordinates Analysis (PCoA) of the Bray-Curtis dissimilarity indices (Fig. 3). These data indicate diversity differences between the communities and also within the consortia after successive passages. The original sources of ABH, CS and WTP were significantly different, while the consortia adapted after 10 passages have a very similar structure (Fig. 3).

![Fig. 3. Principal Coordinates Analysis (PCoA Bray-Curtis) of differences in bacterial diversity observed during the selection of microbial communities.](image)

Spheres representing each sample were colored according to their origin as follows: agricultural biogas hydrolyzer, ABH – green, cattle slurry, CS – red, wastewater treatment plant, WTP – blue. The intensity of colors increases with the passage number.

**3.2.1. Agricultural biogas hydrolyzer (ABH) community**

The inoculum originating from the agricultural biogas hydrolyzer was dominated by the representatives of *Prevotellaceae* (39%), *Veillonellaceae* (28%) and *Lactobacillaceae* (11%) families. These groups of bacteria are characterized by a strong hydrolyzing activity towards polysaccharide compounds resulting in high production of volatile fatty acids (VFA).
Since the ABH inoculum was obtained from a full-scale biogas plant utilizing maize silage as a substrate, the initial microbial community was already adapted to maize degradation and was characterized by a strong hydrolyzing activity. During the cultivation experiments, at the next two analyzed time-points, we observed shifts between the dominating bacteria *Lactobacillaceae* and *Prevotellaceae* with the relative abundance of 52% and 27% at passage 1 and 15% and 64% at passage 3, respectively. Additionally, the family *Clostridiaceae* emerged from below 1% to 18% (ABH1) and 14% (ABH3). Members of this family are well-known for their high cellulolytic activity [34] and thus release of simpler sugars from polysaccharides, which eventually leads boosted the growth of *Lactobacillaceae* (72%) and *Bifidobacteriaceae* (15%) in our study. Bacteria belonging to these families are known to produce lactic acid as one of the main products of carbohydrate fermentation [35,36]. Interestingly at the end of the experiments, the abundance of dominant bacterial families, *Prevotellaceae* (45%), *Veillonellaceae* (36%) and *Lactobacillaceae* (13%), almost reflected that of inoculum but with reduced importance of low abundant families.

### 3.2.2. Cattle slurry (CS) community

Most of the sequences identified within the CS inoculum were affiliated with non-cellulolytic *Pseudomonadaceae* (21%), *Carnobacteriaceae* (12%), *Campylobacteriaceae* (8%) and *Moraxellaceae* (7%), as well as microorganisms capable of fermentation of carbohydrates: *Porphyromonadaceae* (12%), *Lachnospiraceae* (5%) and probably Family XI Incertae Sedis (7%) [37,38,39]. After the first passage a drastic (1100-fold) increase in the abundance of *Lactobacillaceae* to the level of 44% of all the bacterial sequences was observed. Second most abundant family became *Veillonellaceae* (27%) with over 90-fold greater coverage in microbial structure. Moreover, all of the previously dominating bacteria (except *Moraxellaceae*) were diminished to the level below 0.5%. This drastic shift indicates very fast changes in bacterial community structure in response to substrate pressure. In the passage 3,
hemicellulose- and pectin-degrading *Prevotellaceae* (49%) gain dominance, which is in accordance with the results of a previously showed in a high-concentrate diet experiment [33]. As a result of the improved cellulose degradation, an increase in abundance of fast growing carbohydrate fermenters, which often produce lactic acid, namely *Lactobacillaceae* (33%) and *Bifidobacteriaceae* (7%) was observed after the passage 6. Similarly to the ABH community, after the passage 10 the microbial community structure was again dominated by *Prevotellaceae* (51%) and *Veillonellaceae* (31%), but instead of lactobacilli, *Bifidobacteriaceae* (11%) remained the third most abundant family. Moreover, in the case of the CS consortium thereafter the passage 3 we observed the most stable microbial structure (calculated as the smallest difference between the dominant families in the P3-P10 passages) of all studied consortia. This observation can explain more stable performance of CS community in the CMC-Congo clearing zones measurement and glucose concentration.

3.2.3. Wastewater treatment plant (WTP) community

The inoculum originating from the wastewater treatment plant was composed of many non-cellulolytic families such as *Campylobacteraceae* (34%), *Aeromonadaceae* (11%), *Moraxellaceae* (9%), and *Leptotrichiaceae* (9%). The families which could be affiliated to polysaccharide and VFA utilization included *Porphyromonadaceae* (8%) and *Bacteroidaceae* (5%) [38]. In the selection experiment after substrate change to maize silage, we observed the dominance of *Lactobacillaceae* (57%) with approximately 440-fold increase compared to the initial community. The change in the bacterial community structure was similar to that detected in the previously described variants. However, the second and the third most abundant families became *Clostridiaceae* (19%) and *Veillonellaceae* (15%) which can explain differences in CMC-Congo clearing zones and glucose concentration tests. In the next analyzed step, the selection of bacteria also occurred in a slightly different way. After passage 3, members of *Veillonellaceae* (52%), *Lactobacillaceae* (31%) and *Acetobacteraceae* (12%)
dominated in the community. Members of the genus *Acetobacter* primarily contribute to acetate production [40]. After passage 6, members of *Lactobacillaceae* again outcompeted other bacteria with the relative abundance of 71%, but at the end of experiment their number was reduced to 12%. Passage 10 was dominated by *Veillonellaceae* (46%) and *Prevotellaceae* (36%).

In the above experiments, adaptation of three communities towards consortia highly specialized in degradation of maize silage was observed. Although the community structure of the initial consortia was very different, from the third passage the dominance of bacteria belonging to six families: *Lactobacillaceae, Prevotellaceae, Veillonellaceae, Clostridiaceae, Bifidobacteriaceae* and *Acetobacteraceae* was observed. This could be an indicator of selection and qualitative stabilization of the bacterial communities but, on the other hand, the relative abundance of the above-mentioned bacterial families varied between the passages, excluding quantitative stability. In the adapted consortia, members of the families *Lactobacillaceae, Prevotellaceae, Veillonellaceae* were the most important as they were characterized by the highest average count (Fig. 2). In all the variants, adaptation the selection process began with an increase in the relative abundance of fast growing *Lactobacillaceae*, specialized in oligosaccharide degradation and production of short chain fatty acids (SCFA), mainly lactic acid [35]. Enrichment of the consortia with members of this family was previously observed in studies where agricultural materials were used for anaerobic digestion [41,42]. Wirth et al. [43] obtained interesting results which reported that indigenous microorganisms of maize silage include representatives of the genera *Lactobacillus* and *Acetobacter*.

Our results of selection process of the analyzed consortia was driven by substrate input rather than the composition of the initial inoculum, which differed with the origin of the sample. After cultivation on maize silage, the multiple families present in sources samples
(even very low abundant <0.1%) in the subsequent passages emerged 3-6 families involved in maize silage degradation. De Francisci et al. [44] also observed that selection process caused changes in the bacterial population by substrate input but not the sample origin. In turn, Porsch et al. [45] indicated that only the initial enrichment step significantly influence the adaptation and changes of microbial community structure of wheat straw. The subsequent passages did not affect the structure and activity of the community.

3.3. Functional analysis of the adapted consortia

3.3.1 Hydrolysis of maize silage

To analyze the hydrolytic potential of the adapted consortia, 120-hour anaerobic batch cultures on maize silage were performed. The control variant consisted of maize silage/low mineral water medium, which was treated exactly as the culture variants, except for the inoculation with hydrolytic consortia. The efficiency of maize silage hydrolysis was expressed as a function of concentration of the produced (released) glucose and VFAs, as well as the level of sCOD (Fig 4.).
Fig. 4. Solubilization of maize silage by the adopted hydrolytic consortia. Changes in the concentration of: glucose (A) and VFAs (B), as well as the level of sCOD (C) were analyzed.
The concentration of glucose increased from 1.12 g/L to 1.37 g/L in the control variant, from 1.38 g/L to 1.85 g/L for ABH, from 1.37 g/L to 1.97 g/L for CS and from 1.36 g/L to 1.75 g/L for WTP after 72 hours. These were the highest glucose concentrations obtained during the experiment. Longer hydrolysis resulted in a decrease in glucose concentration in all the cultures, what is probably caused by the consumption of sugars by microorganisms (Fig. 4). The concentration of VFAs was increased in all variants during 120 hours. In the control increased concentrations of VFAs from 0.99 g/L to 2.24 g/L were observed. in the variants where adopted consortia were added the concentrations of VFAs increased from 1.08 g/L to 2.99 g/L for ABH, from 1.17 g/L to 2.78 g/L for CS and from 1.15 g/L to 2.77 g/L for WTP. The obtained results showed an increase in the amount of reducing sugars (glucose) and VFAs formed/released during the degradation of maize silage. The production of volatile fatty acids may also play an important role in enhancing the degradation of maize silage. Zhang and co-workers [29] suggested that mild acids can loosen the structure of maize silage leading to an improved overall rate of hydrolysis due to increased accessibility of the substrate for hydrolytic enzymes which would further benefit production of biogas.

The initial value of sCOD in all the cultures was similar (2.2-2.3 g/L) and it increased throughout the experiment. The highest level of sCOD was observed after 72 hours for ABH (6.05 g/L), and 120 hours for CS and WTP (5.6 g/L and 5.7 g/L, respectively). In the control, the highest level of sCOD was obtained after 96 hours (only 4.7 g/L) and then it decreased. Faster increase in sCOD maybe an indicator of the accelerated maize silage degradation.

The observed increase in sCOD as well as glucose and VFAs concentrations probably resulted from the metabolic activity of the indigenous microbiota of maize silage. Ensiling is a well-known technology for preserving lignocellulosic material. Microbial silage starters (lactic acid bacteria) are very often added during the process in order to ensure rapid acidification or the formation of specific metabolites which inhibit the growth of other organisms, which may cause spoilage of plant matter [46,47].
Cultivation of hydrolytic consortia in natural media has been previously described by many researches. In 2010, Guo et al. [48] obtained a lignocellulose-degrading composite microbial system (XDC-2) from soil amended with composted agricultural and animal waste. The concentration of reducing sugars obtained after the degradation of rice straw and corn stalk was 1.3 g/L and 2.4 g/L, respectively. Yuan et al. [49] showed that the VFAs concentration and sCOD increased rapidly during 4 days (120 hours) of pretreatment of corn stalk using the MC1 consortium, and their values differed depending on the initial concentration of the substrate.

Our results also indicated an increase in VFAs and glucose concentrations, as well as the level of sCOD during hydrolysis. This was mainly due to the degradation of insoluble macro-molecular organic compounds of maize silage by hydrolytic activity of analyzed consortia. The intermediate products of hydrolysis, could probably be utilized by other groups of microorganisms during the next phase of anaerobic digestion.

3.3.2. The influence of the adapted consortia on degradation and anaerobic digestion of maize silage

The selection process of microbial communities to degrade maize silage was confirmed both in experiments testing hydrolytic activity and bacterial community structure based on 16S rDNA survey. In the next step of our experiments we used the adapted consortia in order to verify if and how the hydrolytic ABH, CS and WTP consortia may affect the activity of methanogenic community of biogas plant fermenter tank and ultimately its efficiency of biogas production.

To determine the effect of augmentation of the anaerobic digestion communities by ABH, CS and WTP consortia on the efficiency of maize silage anaerobic digestion (biogas production), a batch cultures experiment was carried out. The analyzed hydrolytic consortia were added to the anaerobic digester only once at the beginning of the experiment. The liquid
phase from the fermenter tank of anaerobic digester from a biogas plant was used as a the
source of methanogenic consortium. During 21 days simulation of maize silage anaerobic
digestion process, VFAs concentration, sCOD and the daily biogas production were
measured. The physical and chemical parameters affect the anaerobic digestion process. The
first phase of simulation of anaerobic digestion was hydrolysis. According to the literature,
hydrolysis does not take more than 5 days, so it was decided to determine VFAs, and sCOD
only after 7 days, and at the end of entire process (21 days). The obtained results show that
VFAs concentration and sCOD increased during the anaerobic digestion in all batch assays
(Tab. 1). The concentration of VFAs increased from 5.88 g/L to 10.29 g/L, from 5.61 g/L to
10.06 g/L, and from 6.12 g/L to 10.11 g/L , for the ABH, CS and WTP variants respectively),
after 7 days. In the control variant VFAs concentration increased from 5.37 g/L to 9.33 g/L
during the same time, and then decreased to 8.96 g/L after 21 days.. It was observed that
VFAs concentrations in culture ABH, CS and WTP, remained at high level even after 21
days, and amounted 10.37 g/L, 12.06 g/L and 10.71 g/L, respectively. The initial value of
sCOD (18.43 – 18.80 g/L) was increased (to 22.50 g/L for ABH, 23.47 g/L for CS and 23.20
g/L for WTP, after 7 days. After 21 days, the values of this parameter had increased to 24.93
g/L for ABH, 25.90 g/L for CS and 26.10 g/L for WTP. In the control, sCOD was increased
from 18.63 g/L to 20.83 g/L after 7 days and to 24.63 g/L after 21 days. Concentrations of
VFAs were higher by 10%, 48%, 8% after 21 days for ABH, CS and WTP, respectively,
compared to the control. The level of sCOD was higher by 4%, 6% and 9% after 21 days, for
ABH, CS and WTP, respectively, than in the control. Our results suggest that
bioaugmentation of maize silage by hydrolytic consortia contributes to an improvement in
decomposition (liquefaction) of this substrate during anaerobic digestion. The analysis to the
daily and cumulative biogas production (CBP) revealed that the hydrolytic consortia (ABH,
CS and WTP) increased the efficiency of biogas production from maize silage (Fig. 5A and
Fig. 5B).
Fig. 5. The efficiency of biogas production during anaerobic digestion of maize silage: A) daily biogas production and B) cumulative biogas production.
CBP for anaerobic digestion of maize silage with the addition of hydrolytic consortium was 150 dm$^3$/kg of VS and 166.5 dm$^3$/kg of VS and 141.8 dm$^3$/kg of VS for ABH, CS and WTP, respectively. The production of biogas in the experiment without bioaugmentation (control) was 128 dm$^3$/kg of VS. The results showed that the high production of biogas was maintained for 7 days. After this time, in the variants with hydrolytic consortia, biogas production was higher by about 10%, 43% and 15% (for ABH, CS and WTP, respectively) than in the control variant. This was probably caused by the increased efficiency of maize silage hydrolysis related to the presence of the analyzed consortia during the entire anaerobic digestion process.

The obtained results indicate that biogas production increased by 16%, 29% and 10% after bioaugmentation of the methanogenic consortia with ABH, CS and WTP, respectively. The results of physical and chemical analyses show that the accumulation of intermediate products (VFAs) and soluble substrate (as indicated by the increased level of sCOD) after hydrolysis facilitated by the hydrolytic consortia ABH, CS and WTP, may be beneficial for the anaerobic digestion process and it was correlated with the improved efficiency of biogas production (Fig. 5B). Moreover the addition of the adapted hydrolytic consortia to the anaerobic digestion process increased the activity of methanogenic consortium. Furthermore, effective hydrolysis means a short digestion time and this may increase the overall biogas production efficiency [50]. This could bring significant economic benefits for increasing the biogas production efficiency or treatment capacity of anaerobic digesters because of the shortened digestion time.

4. Conclusions

In this study the effect of the source of microorganisms on the selection and adaptation of hydrolytic microorganisms was analyzed. The obtained results showed that subsequent passages of hydrolytic consortia using maize silage as substrate led to the selection of the
representatives of three families of bacteria: *Lactobacillaceae, Prevotellaceae* and *Veillonellaceae*, which dominate in the investigated communities after the adaptation process. This indicates that the substrate, not the community origin was the main driving force in the adaptation of hydrolytic consortia.

Following adaptation, hydrolytic capabilities of all three consortia have improved, as indicated by the increased concentration of the released glucose, VFAs, and the level of sCOD in the culture. Moreover, bioaugmentation of anaerobic digestion using the adapted hydrolytic consortia increased the efficiency of biogas production by up to 29%. This proves that subsequent passaging of hydrolytic consortia on an appropriate substrate, such as maize silage, prior to their application in biogas plant hydrolyzers, may facilitate their adaptation and increase their hydrolytic activity.

Future work should involve the selection and characterization of the key hydrolytic microorganisms found in the analyzed consortia, as well as investigation of the mechanism of their activity, which leads to the enhancement of biogas production during anaerobic digestion.

**Author contributions**

KP: planned and performed the selection, hydrolysis and anaerobic digestion experiments, as well as most of the chemical analyses, DNA isolation and wrote the manuscript; AP: was involved in planning of the metagenomics approach, performed the deep sequencing and bioinformatics analysis, and was involved in writing of the manuscript; ASob and LL: designed and supervised the metagenomics and bioinformatics approaches; ASklo: is the head of a group, and was involved in consultation and article correction; LD: is the head of a project; planned and directed the studies and was involved in consultation and article preparation.
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References


### Table 1. The physical and chemical characteristics of digester during anaerobic digestion.

<table>
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<tr>
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<th>VFAs [g/L]</th>
<th>sCOD [g/L]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 days</td>
<td>7 days</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>5.37±0.82</td>
<td>9.33±0.43</td>
</tr>
<tr>
<td><strong>ABH</strong></td>
<td>5.88±0.19</td>
<td>10.29±0.74</td>
</tr>
<tr>
<td><strong>CS</strong></td>
<td>5.61±0.13</td>
<td>10.06±0.13</td>
</tr>
<tr>
<td><strong>WTP</strong></td>
<td>6.12±0.11</td>
<td>10.11±0.46</td>
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