



Use of ensiled green willow biomass in biogas fermentation

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Abstract

The biggest challenges of our era include climate change and the global fossil energy problem. Extensive utilization of renewable energy sources should be a part of the solution for both these problems. Biogas is a versatile renewable energy carrier that has the potential to substitute fossil fuels. The most frequently utilized substrates for the anaerobic digestion (AD) process include maize silage today, but there is an increasing demand for second-generation biomass sources, which are cheaper and do not interfere with the cultivation of food production. Green biomass from short rotation coppice willow (GWB) may be a promising alternative. However, to ensure feedstock quantity and quality all year round, a preservation method has to be developed. We attempted to ensilage the biomass and subsequently utilized the resulting willow-silage in batch fermenters. Various mixtures of lactic acid bacteria were employed to facilitate ensiling by inoculation of the substrate in anaerobic jars for 60 days. During the ensiling analytical investigations, (HPLC, pH, oTS/TS%) were carried out in order to follow the build-up of fermentation products. AD fermentations were assembled from the ensilaged biomass and the methane production was measured for 56 days. The total methane yields of the ensilaged biomass were 8–15% higher than that of the fresh biomass and methane production rates were also improved. Our findings suggest that ensiling is not only an excellent preservation method for willow biomass, but also stimulates its AD.

Keywords Iomass · Biogas · Ensiling · Second-generation substrates · Willow · Anaerobic digestion

Introduction

The rapidly increasing energy demand (IEA, Enerdata: 515–576 EJ/year) is satisfied today primarily by fossil fuels, however, this leads to dramatic elevation of greenhouse gas levels in the atmosphere. This process makes the global climate change inevitable (Holm-Nielsen et al. 2009). Importance of renewable energies, such as wind, solar or biomass utilization, are rapidly growing (REN21 2016). Biogas (produced by anaerobic fermentation of organic substances) will have an important role, not only because it is a versatile renewable energy carrier, but it can be also produced from many types of biomass and organic wastes (Weiland 2010).

It is a mixture of various gases (CH₄, CO₂ and minor components) produced under anaerobic conditions by multifarious microbial community (Mao et al. 2015; Gerardi 2003). Currently, maize is the most popular energy crop for biogas production, but there is an increasing demand for second-generation lignocellulosic energy plants (Chandra et al. 2012; Zheng et al. 2014; Sindhu et al. 2016). A suitable energy plant is the short rotation coppice willow (GWB), because it has a remarkable biomass yield (11 t oTS ha⁻¹ year⁻¹); it does not need to occupy food or feed producing agricultural land, it can be cultivated on marginal lands; its cultivation costs are far lower than that of traditional energy crops', and willow plantations could play a key role in the global CO₂ cycle (Serapiglia et al. 2013). Thermal energy can be produced by direct burning of wood chips and this has been practiced for many years. Biogas production from woody substrates is problematic, mainly because of the recalcitrant nature of the lignocellulosic components and the susceptibility of wood chips to form floating layers and thus inhibiting efficient biogas release (Alexandropoulou et al. 2017). In order to avoid these drawbacks, we utilize the green shoots of short rotation coppice willow (GWB) that is the biomass

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harvested only after a few months of growth (Dudits et al. 2016). GWB requires a suitable preservation method if the AD reactors are to be fed with this substrate all year round. One promising possibility for this task is ensiling (Vasco-Correa et al. 2016), which has been applied to preserve animal forage for centuries although this method has not been tested for GWB before to the best of our knowledge. During the process, a certain fraction of the water-soluble carbohydrates (WSCs) is fermented by epiphytic lactic acid bacteria (LAB) to lactic acid, which reduces the pH of the plant material (Ohshima et al. 1997). This process preserves the biomass during long-term storage (Haag et al. 2015). The ensiling process can be divided into four stages: (1) anaerobic phase, (2) fermentation phase, (3) stable phase and (4) feed out phase (Driehuis and Elferink 2000). There are several advantages of ensiling as follows: (i) ensiling can optimize the partial deconstruction of plant polysaccharides, (ii) ensiling facilitates the storage of biomass and (iii) ensiling may replace expensive chemical and physical pretreatment methods cost effectively (Herrmann et al. 2011; Kreuger et al. 2011; Franco et al. 2016). The main aim of the present study was to examine whether GWB can be ensiled in order to store the biomass all year round, and additionally to investigate whether this treatment stimulates biogas fermentation.

Materials and methods

The workflow of the experimental protocol was as follows

GWB was placed into glass containers, inoculated with various LABs and ensiled anaerobic conditions at 30 °C. Ensiling was carried out for 60 days, following the literature recommendations (Ohshima et al. 1997; Driehuis and Elferink 2000; Mohd-Setapar et al. 2012). During the ensiling procedure, samples for analytical examinations (pH, HPLC, see below) were withdrawn. After ensiling, AD reactors were assembled and inoculated with industrial biogas plant effluent. Methane production was followed by gas chromatography.

Willow plants (*Salix viminalis*, Strain Energo)

The willow plants (GWB) were collected from plantation. Harvest was carried out in August, 2018, after 6 months of growth. The shrubs were mechanically chopped to ~2 cm long pieces, frozen and stored at – 20°. The total solids (TSs), organic total solids (OTSs) contents were determined and found to be there were 47% and 95%, respectively. Woody willow biomass was also used in comparative examinations, and the OTS of woody biomass was 98%.

Sorghum silage and other substrates (distillery sludge, cow manure)

Sorghum silage is frequently used as biogas substrate in biogas plant, which could be substituted by GWB silage. Therefore, we fermented fresh and ensiled GWB, sorghum silage and other substrates (distillery sludge, cow manure) alone and in co-fermentation. These materials were obtained from Agricultural-Biogas Plant of Kaposzsekcső Ltd., Kaposzsekcső, Hungary. Analytical parameters (pH, TS%/OTS%, HPLC) were also carried out on these substances (Table 1, Fig. 1).

Used LABs and bacterial inocula

The ensiling containers were supplied with *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* pure cultures grown in MRS broth (De Man et al. 1960) at 30 °C for 24 h. 1% of inoculum was used (0.4 ml inoculum, 40 ml MRS broth). They were used alone (S4 and S5), in combination (S6), or with one of the probiotic *Lactobacillus* mixtures: Lalsil (S1), Adisil (S2), Lacto7(S3) (for their composition see Table 2). The probiotic mixtures were also cultivated in MRS broth (40 ml) at 30 °C for 24 h. For determination of required amounts of cultures (v_1 , in 10 ml final concentration) and distilled water (v_{water}), we measured the optical density of cultures at 600 nm (Table 3) (Zhang and Hoshino 2013). These cultures were added to 100 g substrates in four doses (2.5 ml/25 g).

Ensiling procedure

8 sets of ensiling experiments were assembled (S1–S8). 6 of these were treated with various LABs and there were two control silos. 3 of 6 the treated ones were inoculated with commercially available probiotic *Lactobacillus* mixtures (S1: Lalsil, S2: Adisil, S3: Lacto7), the other 3 received pure *Lactobacillus* cultures (*L. acidophilus*, *L. delbrueckii*), administered alone (S4 and S5) and in combination (S6). One of the controls contained equal volume of distilled water and the another was supplied with physiological saline solution. 100 g of thawed GWD were packed into the 350 ml glass containers in anaerobic

Table 1 pH, TS% and oTS% of biogas substrates used in co-fermentations

	pH	TS (%)	oTS (%)
Sorghum silage (SORSIL)	3.7	23.0	93.3
Distillery sludge (DS)	3.5	6.8	90.9
Cow manure (CM)	9.3	16.3	76.4

Fig. 1 Concentrations of organic acids in substrates used in co-fermentation

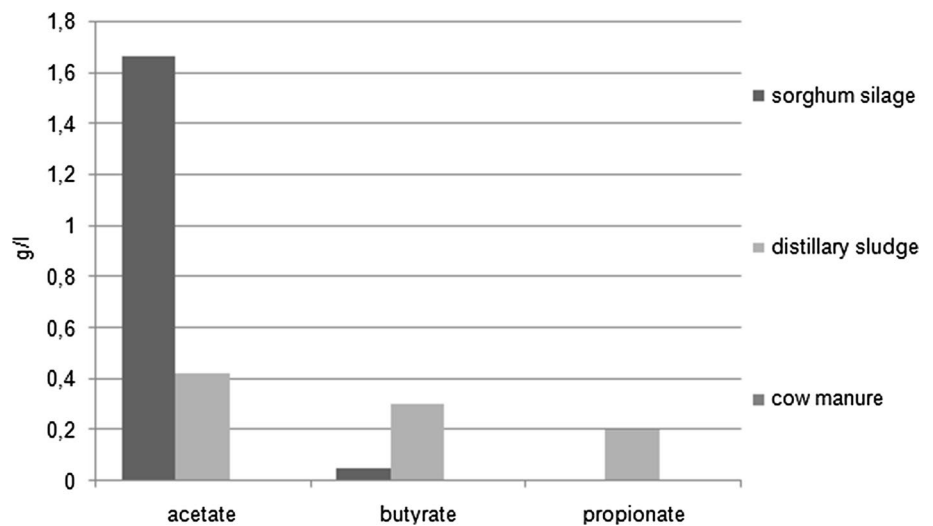


Table 2 Composition of bacterial inocula used for ensiling

Name	Microorganism/additive	Amount
Lacto 7	<i>L. acidophilus</i>	1,2–2,4 × 10 ⁵ CFU/g
	<i>L. rhamnosus</i>	1,2–2,4 × 10 ⁵ CFU/g
	<i>L. plantarum</i>	1,2–2,4 × 10 ⁵ CFU/g
	<i>L. casei</i>	1,2–2,4 × 10 ⁵ CFU/g
	<i>L. reuteri</i>	0,8–1,6 × 10 ⁵ CFU/g
	<i>Bifidobacterium longum</i>	1,2–2,4 × 10 ⁵ CFU/g
	<i>Streptococcus thermophiles</i>	1,2–2,4 × 10 ⁵ CFU/g
Adisil	<i>L. plantarum</i>	> 1 × 10 ¹¹ CFU/g
	<i>L. paracasei</i>	> 1 × 10 ¹¹ CFU/g
	<i>Pediococcus acidilactici</i>	> 1 × 10 ¹¹ CFU/g
Lalsil	<i>L. buchneri</i> NCIMB 40,788 (1k20739)	7,50 × 10 ¹⁰ CFU/g
	<i>Pediococcus acidilactici</i> CNCM MA 18/5 M (1k2104)	5 × 10 ¹⁰ CFU/g
	Beta – glucanase— <i>Aspergillus niger</i> MUCL 39,199 (EC 3.2.1.6) (1 k)	5700 IN/g
	Xylanase – <i>Trichoderma longibrachiatum</i> MUCL 39,203 (E.C. 3.2.1.8) (1 k)	3000 IN/g
	Lactose; natrium – aluminosilicate (2%) – additive	Complete to 100/200 g net mass

Table 3 Compile of used lactobacillus cultures

Name	C1 (A)	v1 (ml)	v2 (ml)	V _{water}		
Lalsil	2138	10	4677	5323		
Adisil	2399	10	4168	5832		
Lacto7	2116	10	4726	5274		
<i>L. acidophilus</i> (L.a.)	1950	10	5128	4872		
<i>L. delbrueckii</i> (L.d.)	1873	10	5339	4661		
L.a. + L.d	1950	1873	10	2564	2670	4766

chamber (ShelLab Bactron Anaerobic Chamber), The stem to leaf ratio was 60: 40.4 × 25 g of biomass have been packed into the glass container. 10 ml of prepared LAB culture has been added to biomass in four doses (2.5 ml/ 25 g). The packing and squeezing were done by hands,

the substrate and inoculum have been mixed by spoon. The containers were placed into anaerobic jars (Oxoid™ AnaeroJar™ 2.5 L and anaerobized using was Oxoid™ AnaeroGen™ 2.5 L Sachet) and were kept at 30 °C for 60 days. Silage samples were taken at 8–15 day intervals.

Assembling AD reactors to monitor methane production (BMP test)

After 60 days of ensilaging, 32 AD fermenters were assembled as summarized in Table 4. The biogas substrate samples were placed into hypovial bottles of 150 ml, which contained 60 ml liquid phase and 90 ml headspace. The batch reactors were complemented with appropriate amounts of biogas inoculum sludge following the VDI 4630 standard procedure (VDI 2006). The biogas inoculum sludge originated from the mesophilic industrial biogas facility of Zöldforrás Ltd., Szeged. According to the standard VDI protocol, 1 g of volatile solids (VS) in the biogas inoculum sludge is mixed with 0.5 g of lignocellulosic substrate VS in the batch reactor. This is termed as VDI=1, while VDI=2 equals to adding 1 g of substrate to each gram of sludge inoculum. The vials were sealed, anaerobized with nitrogen (Messer, industrial nitrogen 4.5) and incubated at 37°C for 40 days. During this period, methanogenic activity in each vial was monitored by regular measurement of the volumetric gas production and gas composition using an Agilent 6890 gas chromatograph, equipped with a Mole sieve column and a TCD detector. Argon was supplied as carrier gas. Gas samples from the vials were injected manually into the gas chromatograph with a pressure-lock type Hamilton syringe. Total methane yields (TMYs) were calculated by dividing the total cumulative methane production with the VS of the lignocellulosic substrate, in mL CH₄/g oTS.

Analytical measurements

pH values

Samples for pH measurements were withdrawn in anaerobic chamber into 15 ml tubes at day 1 and day 60. The samples were diluted with saline solution 4 times and vortexed rigorously. HANNA HI4522 instrument was used for PH measurements (Behnood et al. 2016).

Determination of TS%/OTS%

The TS%/OTS% protocols followed the VDI recommendations (VDI 2006): the wet samples were dried at 105 °C for 24 h. After this, we measured their masses and calculated TS%. The samples were subsequently burnt at 550 °C for 2 h. During this procedure, all organic compounds became fully oxidized allowing the determination of OTS% from the remained ash.

HPLC

Silage samples were taken for determination of concentration of organic acids (acetate, butyrate, propionate and lactate) in anaerobic chamber into 15 ml tubes at day 1; 8; 20; 35; 48; 60. This silage samples were diluted with saline solution four times, vortexed rigorously and centrifuged at 5000 rpm for 10 min. 1.5 ml of supernatants were centrifuged at 13 000 rpm for 10 min and 400 µl of supernatant was filtered through PES centrifugal filter at 14 000 g for 15 min. 200 µl of filtrate was pipetted into HPLC cuvettes. The concentrations of organic acids (acetate, butyrate, propionate and lactate) were assessed using a Hitachi LaChrome Elite HPLC equipped with refractive index detector L2490. The separation was performed on an ICsep ICE-COREGEL-64H column. The temperature of the column and detector was 50 °C and 41 °C, respectively. The eluent was 0.01 M H₂SO₄ (0.8 mL min⁻¹).

Results and discussion

Ensilaging experiments

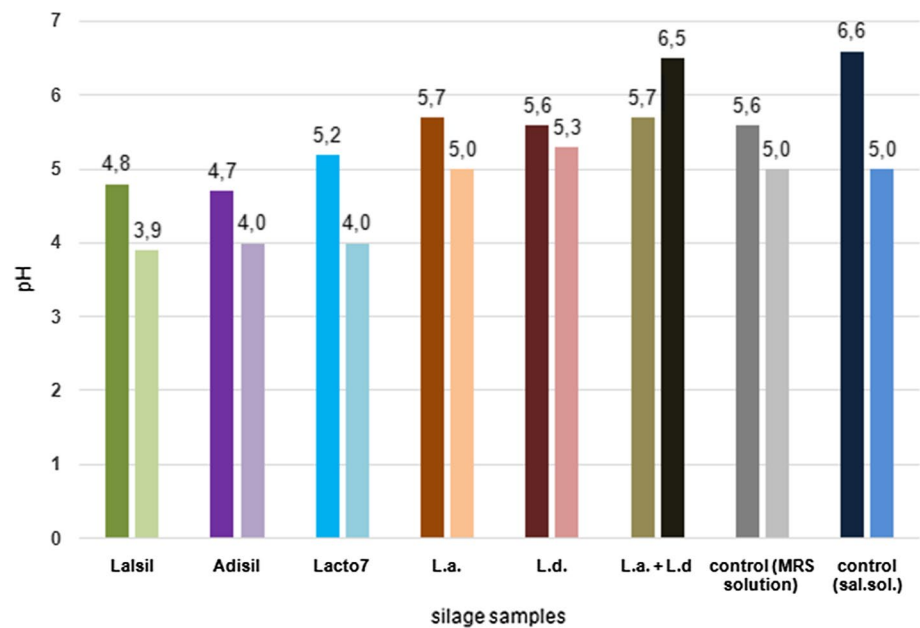
pH of silage samples

A substantial pH decrease was observed in several samples (mainly in Lalsil; Adisil; Lacto7) (Fig. 2). (In these cases,

Table 4 Inocula, substrate to inoculum ratio (VDI) of AD reactor samples and type of fermentation (SGWB = ensilaged GWB)

Sample No	VDI	Type of fermentation	Substrate [and inocula]
1–3	1	Monofermentation	Fresh GWB
4–6	1	Monofermentation	Cow manure (CM)
7–9	1	Monofermentation	Sorghum silage (SORSIL)
10–12	1	Monofermentation	Distillery sludge (DS)
13–15	1	Monofermentation	Express chips (EC)
16–18	1	Co-fermentation	DS + CM + SORSIL
19–21	1	Co-fermentation	DS + CM + SGWB [Lalsil based]
22–24	2	Co-fermentation	DS + CM + SGWB [Lalsil based]
25–27	1	Co-fermentation	DS + CM + EC
28–30	1	Monofermentation	GWB silage (SGWB) [Lalsil based]
31–32	–	–	Control reactors

Fig. 2 pH of silage samples inoculated with various LAB preparations on day 1 (left columns) and day 60 (right columns) of ensiling. (sal. sol. = physiological salt solution)



the “substantial pH decrease” is meaning drop of pH to or below pH 4.). A drop of pH from around 5.0–4.0 clearly indicates pretreatment efficiency of the LAB mixtures (There was observed on average 0.9 unit decrease.). Surprisingly, the pH drop in the ensiling reactors loaded with lignocellulosic substrates and pretreated with *L. acidophilus* or *L. delbrueckii* was very limited (on average 0.5 unit) and addition of both *Lactobacillus* strains even elevated the pH in time. These results need further investigations. The epiphytic lactic acid bacteria, present in the lignocellulosic materials, grew in the control samples (last two column pairs), which resulted in the pH drop observed and these could become the predominant LABs in the control ensiling experiments (Fig. 2). The most effective lactic acid fermentation and coupled pH decrease was achieved with the complex bacterial inocula.

Concentration of organic acids in silage samples

The six samples taken from each ensiling tests on days 1; 8; 20; 35; 48; 60, were analyzed for the amount of produced lactate and VFA components (acetate, butyrate, propionate, lactate). Lactate was of particular interest because higher and faster lactate production is beneficial for the ensiling process. Compared with the probiotic *Lactobacillus* mixtures pretreatments the very low or zero lactate concentrations and elevated yields of other organic acid components (mainly butyrate and propionate) in the controls and experimental samples inoculated with pure LAB cultures indicated the low efficiency of these treatments. Elevated butyrate (3.8 g/L) in L.d. and propionate content (3.6 g/L) in L.a+L.d. inoculated samples were detected (Fig. 3a). The former may indicate

the presence of *Clostridia* predominance, and the latter may point at higher *Propionibacter* abundance. Initially, the lactate content was highest in Adisil treated silages (22.0 g/L), but the difference between the three probiotic based samples was not significant. The lactate concentrations somewhat decreased by the second sampling point (day 8) (Fig. 3b), probably due to rearrangements in the fermentation regimes. The almost complete disappearance of lactate from the pure LAB treated silages was remarkable and correlated with the low pH changes (Fig. 2). The lactate concentration was highest in Adisil treated silages (28.2 g/L) on day 20 (Fig. 3c.). The concentration of propionate was reduced by then (averaging 1.8 g/L) in the pure LAB treated and control silages. The silage treated with Lacto 7 showed the most pronounced increase (averaging 18.9 g/L) and the samples inoculated with pure LAB cultures apparently recovered by day 35 (Fig. 3d.). This trend reversed at the fifth sampling point (Fig. 3e).

Batch biogas production from ensilaged biomasses

After ensiling, the treated biomass was subjected to batch AD. Since in industrial biogas plants the organic loading rate values are much higher than $VDI = 1$, we also examined the effect of an increased substrate input, i.e. $VDI = 2$. The total normalized methane production results are summarized in Fig. 4. In substrate performance evaluation, the experimental methane yield (EXMY), which is equal to the TMY is distinguished from the “theoretical” methane yield (THMY), which is the sum of methane yields (mL CH_4/g oTS) of the individual substrate components (Nielfa et al. 2015), assuming that they participate in the biogas process

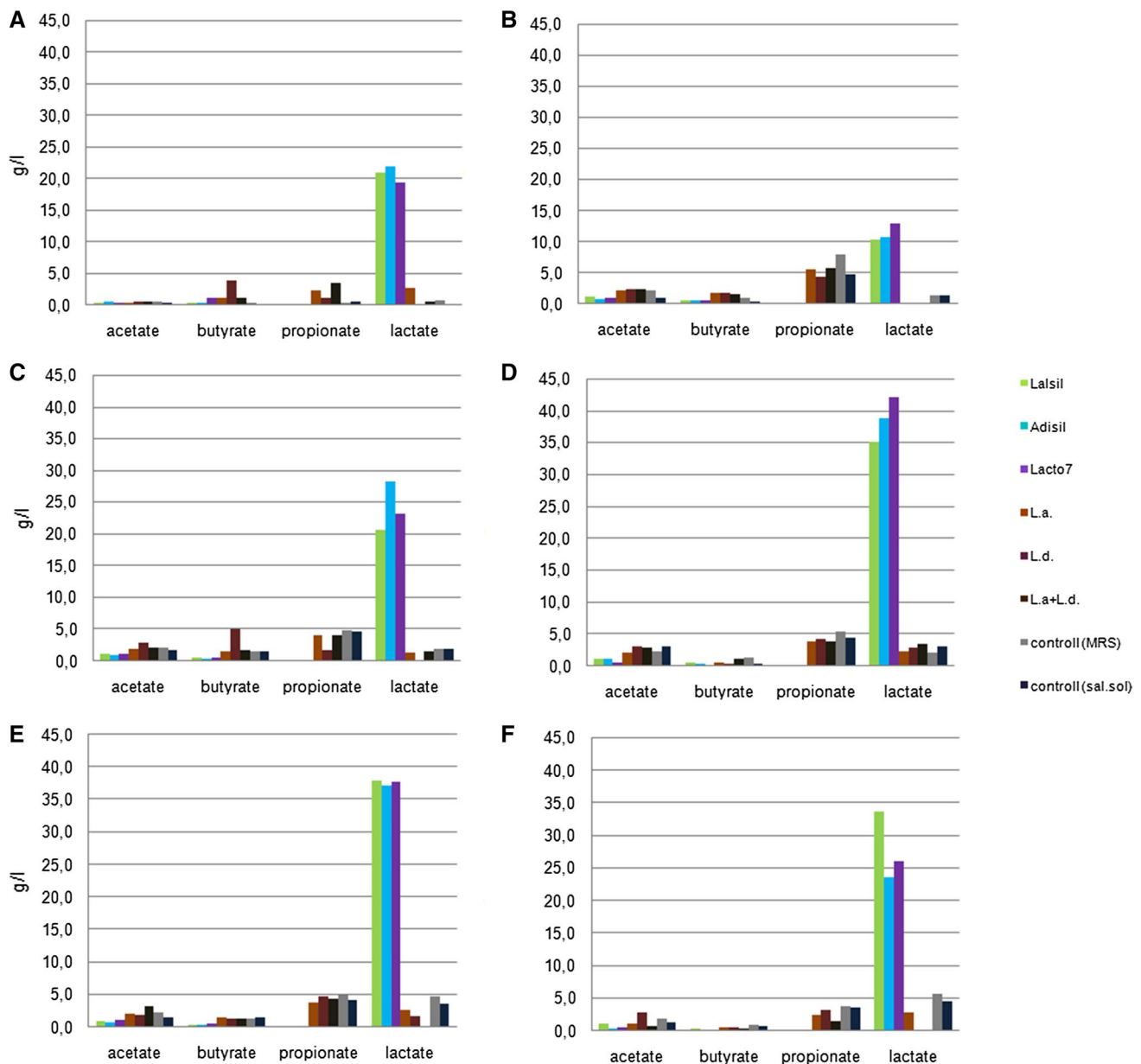


Fig. 3 HPLC results: Concentrations of lactate and VFA on **a:** day 1; **b:** day 8; **c:** day 20; **d:** day 35; **e:** day 48; **f:** day 60

independently. Among monofermentations the distillery sludge (DS) had the highest methane yield (on average 287.9 ml CH₄/g oTS; the dispersion was 14.9 ml CH₄/g OTS [2–7%]) due to its high carbohydrate content. The cow manure (CM) produced lower, but considerable methane yield (on average 130.6 ml CH₄/g oTS, the dispersion was 13.8 ml CH₄/g OTS [8–11%]), close to that of sorghum silage. It is apparent that Lalsil treated silages (marked as *GWB_Silage* in Fig. 4) produced 15% higher methane than that of untreated *GWB* (marked as *GWB* in Fig. 4). The

results confirm that in addition to preservation, ensiling also stimulate biogas fermentation and has positive impact on AD. Overall, the results indicate that ensilaged *GWB*, as well as woody willow biomass (*Express_chips* in Fig. 4) are appropriate substrates for anaerobic biogas production and can substitute other first generation substrates, e.g. sorghum silage. It is particularly noteworthy to emphasize that ensilaged *Express_chips*, i.e. woody willow chips, in co-fermentation with DS and CM produced 39.7% higher methane yield than *Express_chips* in monofermentation.

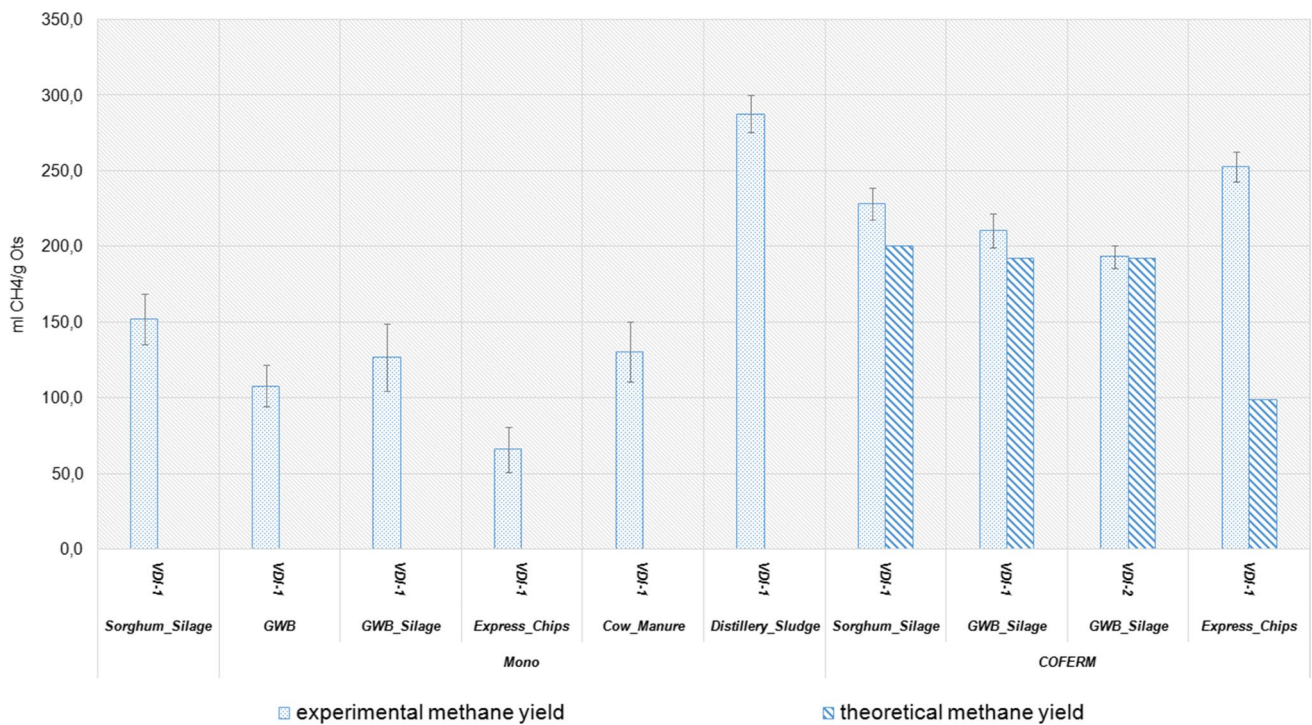


Fig.4 Overall methane fermentation production from various substrates in mono- or co-fermentations

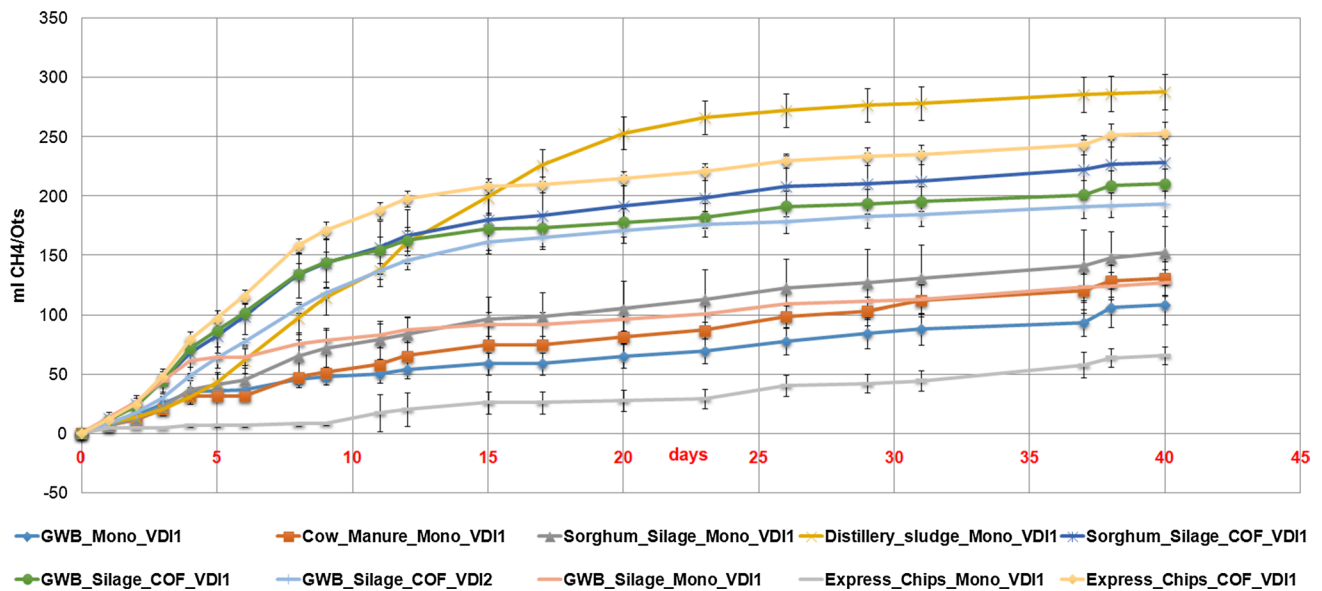


Fig.5 Running of methane fermentation

The striking difference between EXMY and THMY in these biogas fermentations underscores the importance of co-fermentation and the optimal choice of co-substrates.

The cumulative methane yields are summarized for various substrates in Fig. 5. We observed a relatively rapid AD in case of monofermentations of DS and GWB and co-fermentation of GWB, DS and CM at VDI 2.

Conclusions for future biology

Our results indicate that GWB is an appropriate substrate for biogas production, generating around 110 mL CH₄/g oTS as harvested. Therefore, it can substitute other first generation energy plant substrates. GWB requires a suitable preservation method if the AD reactors are to be provided with this substrate all year round. One promising possibility for this task is ensiling. Our ensiling results highlight three main conclusions: (1) The use of LAB inocula is advantageous for the preservation of high quality silages of the plant biomasses in general (Filya et al. 2000) and this is valid also for green woody material. (2) The pure LAB cultures did not facilitate highly successful ensiling, this observation calls for further studies. (3) The highest lactate concentration was reached at day 35 under the employed conditions, indicating that < 60 days are enough for proper preservation of high quality silage, in line with (Mohd-Setapar et al. 2012). Lalsil appeared as the best LAB composite for ensiling biomasses having high lignocellulose content.

Ensilaged willow biomass, in co-fermentation with distillery sludge and cow manure, had 39,7% higher methane yield than in monofermentation. It indicates the topmost importance of co-fermentation and choice of appropriate co-substrates. Ensiling has an effect not just on methane fermentation but also on the rate and yield of methane formation.

Fermentation of willow biomass is promising, as near same amount of energy can be produced by methanization of this substrate at a much profitable manner comparing other commonly used biomasses, since willow plantations require considerably lower costs to up keep. As the need for novel substrates is growing, we need to develop new strategies. Green willow biomass can be a viable alternative, as willow, besides its other advantages, can be cut-back every year, providing stable substrate source in each year.

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Author contributions JNy, with the help from BK and KKK, designed and performed the experiments and contributed to the evaluation of the data. KKK conceived the project and participated in its design. JNy, ZB and KKK drafted the manuscript. GR critically evaluated the manuscript. All the authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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