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Accelerated Biomethane Potential assay for straw with artificially flocculated sludge and defined 'synthetic manure'

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ABSTRACT

A Biomethane Potential (BMP) assay for lignocellulosic material was elaborated with milled wheat straw (WS) as reference substrate. The generally used cow manure as co-substrate was replaced with a buffering salt solution having trace elements called 'synthetic manure'. The inoculum sludge was artificially flocculated and thickened to accelerate the BMP assay and to favour microbial syntrophy. The chemical stoichiometric formula for WS was estimated to be $C_{3.71}H_{6.04}O_{2.79}N_{0.044}S_{0.005}$ and this was used to calculate the theoretically possible 100% methane yield being 293.4 mL_{STP} CH_4 g_{VS}^{-1}, after the non-degradable lignin portion (23.05%) was subtracted. Experimentally, an average specific methane yield of 287.1 mL_{STP} CH_4 g_{VS}^{-1} was obtained at about 98%. This result indicated a 100% degradation, if the microbially generated biomass during the BMP assay was considered. Methanization was completed after 15 days only, instead of mostly 60 days as shown by novel in-situ methane sensors.

1. Introduction

Lignocellulosic residues, such as wheat straw (WS) (wheat = *Triticum aestivum L.*) or other straw varieties, are considered as strong candidates to substitute energy crops for low-cost, sustainable biogas and energy production, without competing with food/feed or land-use change (Ma et al., 2020; Meyer et al., 2018). About 30 million metric tons of cereal straw with 0.8 kg straw/kg grain is produced annually in Germany, which represents 14% of the total amount of agricultural residues (Andersen et al., 2020). In China, the total output of rice, wheat and corn straw is about 764 million tons (Cheng et al., 2020), which is mostly unused or even burnt in the fields. Meanwhile, anaerobic sludge of straw digestion may be returned to the fields as valuable inorganic and organic fertilizer to produce humus. A reference Biochemical Methane Potential (Chynoweth et al., 1993) or Biomethane Potential test (BMP), which could ultimatively reveal methane yields of straw within a short time, so far does not exist, but has been demonstrated in this study. The BMP is a well-known, reliable, quick and inexpensive batch fermentation for determining the potential and rate of conversion of biomass and organic waste to methane. Interlaboratory BMP studies hitherto focused on the improvement or repeatability/reproducibility of BMP assays and to ascertain the most vulnerable test parameters. A selection of reviews have been done by the authors such as Angelidaki et al. (2009); Müller et al. (2004); Raposo et al. (2011); VDI.4630 (2016); Hafner et al. (2020); Holliger et al. (2016); Hülsemann et al. (2020); Filer et al. (2019); Koch et al. (2019).

The most recommended inoculum, according to literature, is the anaerobic digestate from municipal wastewater treatment plants (WWTP) due to the presence of a full range of diverse and active microorganisms (Filer et al., 2019; Raposo et al., 2011). Also, Hülsemann et al. (2020) through microbiome analysis confirmed that, the highest biodiversity can be found in sludge obtained from a WWTP. However, the inoculum sludge should be fresh and ought to have minimum gas production, in order to obtain low blank value of gas production in the absence of the test substrate, as recommended in (VDI.4630 (2006) and summarized by Angelidaki et al. (2009) and Koch et al. (2019). In addition, Koch et al. (2017) found that the choice of inoculum had no significant impact on the specific methane yield of the substrates they tested. Similar results were reported by Hülsemann et al. (2020), which showed no differences between five different inocula and four different BMP measurement devices, with coefficient of variation of <4.8%. Nevertheless, the speed of degradation could be enhanced by adapted inocula, whereas possible lag phases and the time of incubation could be reduced, as confirmed by the reviews by Koch et al. (2019, 2017) and

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Filer et al. (2019).

Also the structure of inoculum sludge is critical when considering the syntrophic methanogenic conversion of complex substrates. It should be a dispersed or granular sludge as well. In granular sludge, the cells are close to each other, which is a precondition for syntrophic growth or interspecies hydrogen transfer 'IHT' (Schink, 1997) or even direct interspecies electron transfer 'DIET' (Xu et al., 2020). This is further outlined under Results and discussion. De Vrieze et al. (2015), studied four different types of seed sludge (biowaste plant, manure plant, biogas plant with manure & energy crops, natural granular sludge from a brewery WWTP) and four test substrates. Interestingly, the authors found that the granular sludge was superior in all cases. Nonetheless, a flocculation technique for BMP- seed sludge, which would favour syntrophy and shorten the digestion time is so far not known. Therefore, in this work, a method for preparing artificially flocculated inoculum sludge has been presented.

A high concentration of seed sludge is defined by a high inoculum-VS to substrate-VS ratio (ISR). The right and high ISR-value of BMP assays guarantees adequate equilibrium between the first step of hydrolysis with acid production and the subsequent conversion to CO_2 and CH_4 as well as a sufficient buffering capacity. The ISR should generally be higher than 2 (Raposo et al., 2011). Hashimoto (1989) tested the influence of different ISR-values on BMP assays using ball-milled straw and manure and found a clear relationship between low ISR values and an insufficient buffering capacity. Hashimoto (1989) found that the initial pH of 7.6 in the assay was stable during incubation with an ISR of 10.9, but with an ISR of 1.2, the pH was unstable and decreased slightly to 7.2. With an ISR of 0.19, the pH further dropped to 4.95. Therefore, a buffering agent is prerequisite for BMP assays as recommended by Raposo et al. (2012, 2020). Raposo et al. (2020) found in their review, that only 3 of 26 BMP related publications considered the addition of buffer.

Manure provides a source of buffer and nitrogen, because lignocellulosic substrates alone have an unfavourable Carbon/Nitrogen (C/N) ratio of greater than 70. However, the manure, which is mostly used, would introduce other inorganic and organic material as well as microbial-based effects to the WS substrate. Also 'synthetic manure' has not been described in any literature so far. Therefore, a defined salt solution based on analyses of cow manure, was developed in order to provide a suitable C/N ratio, sufficient buffering capacity and optimum concentration of trace element ions. The manure substitute was termed 'synthetic manure'. But the aim was not to investigate the essential or minimum constituents of manure, as that would have gone beyond the scope of this work. Methane yields were measured with a modern automated methane potential test system (AMPTS) and milligascounters® of the "Bergedorfer fermentation test" cited in VDI.4630 (2016), which was equipped with in-situ infrared methane sensors as additional novelty. Meanwhile, automation was not a precondition for accurate measurements as shown by Hülsemann et al. (2020) with four different instrumental setups including a Milligascounter® station and cellulose as calibrating substrate. The minimum test duration should be 25 days according to the German guidelines for BMP assays and 60 days for residual gas potentials of already digested material (VDI.4630, 2016) or if the gas production falls below 0.5% of cumulative net production for at least 3 consecutive days. Astals et al. (2020) and Hafner et al. (2020) used the term "1% net 3 d duration" with a limit of 1% cumulative net gas production on 3 consecutive days.

Straw was considered as a reference material for lignocellulosic substrate, because it consists mainly of cellulose (35–40%), hemicellulose (25–30%) and lignin (15–30%). So far, the lignocellulosic complex of WS is known to be highly recalcitrant to biodegradation and lignin is even regarded as non-degradable under strict anaerobic conditions (Frigon and Guiot, 2010; Jimenez et al., 1990; Triolo et al., 2012). In addition, Jimenez et al. (1990) found a 'blocking effect of lignin' on the methanization process of lignocellulosic substrates. Steffen et al. (2016) also discovered that, the impact of lignin-rich fiber fines had a more severe effect on the biodegradability than inorganic fine particles, such as $CaCO_3$. Therefore, the lignin content should be quantitatively taken into account in BMP assays with lignocellulosic substrates as proposed by Raposo et al. (2020) in their review. Raposo et al. (2020) also summarized several publications that predicted methane potential by estimating the biochemical constituents and by regression models. In this present research, the extended elemental Buswell formula of Boyle was used to calculate the possible methane yield of WS as outlined by Achinas and Euverink (2016). If the predicted biogas of WS is obtained, the targeted 100% conversion of straw should be ascertained. Therefore, the lignin chemical formula shown in Crestini and Argyropoulos (1997), should to be subtracted from the total elemental formula of WS to obtain the fermentable portion of WS. This was similar to the approach used by Triolo et al. (2012), which was used to calculate the theoretical BMP by estimating the main biochemical constituents of herbaceous substrates, and generated an elemental formula for each and then subtracted the lignin formula, which was the non-degradable fraction. The simplified approach used in this work here was also to ascertain whether the estimation of BMP would still be accurate if only the lignin portion is subtracted.

2. Materials and methods

2.1. Analytical methods

The pH measurement was done with Calimatic 761 pH meter (Knick, Berlin). Total Solids (TS) were determined by drying the sample for 8 h at 105 °C to constant mass, according to the standard method DIN/EN12879 (2001) using ED 115 (115 L) oven (WTB Binder). Volatile Solids (VS) or ash-free organic dry matter was estimated by heating the sample for 5 h at 540 °C to constant mass, according to the standard method DIN/EN12880 (2001) using Heraeus M104 Muffle furnace with muffle furnace tongs. TS was determined and the VS was determined. The alkalinity was measured using a 2-step titration method (McGhee, 1968) as described in Scherer et al. (2021). Ammonium (NH_4^+) and phosphate (PO_4^{3-}) were determined using Merck cuvette tests (test numbers 1.00683 and 1.14729, respectively) (Merck, Darmstadt). Compositional analysis, including determination of cellulose, hemicellulose, lignin and sugar contents of the WS, was carried out at the Department of Wood Science, Institute for Chemical Wood Technology, University of Hamburg, according to Willför et al. (2009) and Steffen et al. (2016). Elemental analysis (content of C, H, N, and S) of the samples was performed with the Elementar Vario EL cube at 1150 °C in triplicates (Elementar Analysensysteme GmbH, Hanau, Germany) as outlined by Steffen et al. (2016).

2.2. Characterization of wheat straw

The WS used was obtained from a single charge and harvest (Table 3). The dry WS was milled with a special industrial air miller at 50 °C, which was used for milling dried herbal spices (Goergens GmbH, Dormagen, Germany).

2.3. Characterization of inoculum

The inoculum sludge used was anaerobic digestate obtained from a municipal wastewater treatment plant (WWTP) with anaerobic technology, located in Geesthacht, a town in Germany. The WWTP plant had a hydraulic retention time of 50 days with a temperature of 35 °C. This was a compromise for large scale plants between rate of gas production and energy costs, but a temperature of 41 °C was used in the assays, as many agro-based biogas plants in Germany are mostly operated at 38–45 °C through self-heating. The sludge was stored at room temperature of 19–22 °C and used within 5 days after collection.

In this study, as a precaution, the pH was checked, in all assays before and after the incubation period. The new flocculation procedure for inoculum sludge and the use of the 'synthetic manure' made it possible to prepare a distinct and high ISR value without changing the amount of the substrate. Additionally, it eliminated the effect of declining pH. The polymeric flocculant used was cationic with acrylic base. Polymerized acrylic is presumed to be biologically inert and non-toxic. Such cationic flocculants are widely used in WWTPs to dewater sewage sludge before further treatment in a sludge press or centrifuge. An amount of 3 g of flocculant was dissolved in 1 L tap water (35 °C) and stirred continuously for 30 min (Synthofloc 5840 VS, Venator Water Chemistry GmbH, 47181 Duisburg, Germany). However Superfloc 49-4 from Kemira, Helsinki could also be used as the flocculant. The resulting flocculant solution was added to 20 L raw sewage sludge (1 L was taken to be equal to 1 kg) in a plastic barrel and mixed thoroughly with a paddle. Thus, a final flocculant concentration of about 0.015% was obtained. The mixture was allowed to stand overnight at room temperature to enable gentle flocculation. Subsequently, the supernatant was separated from the mixture by sieving it through a polyester cloth (sieve size 0.25 mm, example given, disposable lab coat).

Furthermore, the sufficient buffer capacity and the influence of the incubation procedure on the BMP assay were also evaluated, Table 1 and Fig. 3. The calculation of the different ISR values in the range between 1.45 and 2.44, was based on the dry mass of VS in the WS (90.96%) as presented in Table 3.

The flocculated sludge was transferred by squeezing through a 'piping bag' into the assay vessels and weighed. Each assay vessel was filled with 50 mL of the 'synthetic manure' salt solution (Table 1) to obtain 168.3 g, after which 1.7 mL trace elements solution was added to obtain a final mass of 170.0 g. This resulted in the VS content of the flocculated seed sludge per assay to be 3.88%. The flocculated sticky sludge, was carefully mixed (usual source of error) with the substrate to obtain a homogeneous distribution before the incubation and to enable syntrophic conversion. However, no further mixing was further required for the process. Also, there was no need to flush the head space of the assay vessels with an inert gas as recommended by Amodeo et al. (2020).

For series A, B, F and G, the WS substrate suspension was prepared on the day that the experiment commenced, as shown in Table 1 and Fig. 3. The BMP tests of all seven series were started at the same time and with the same seed sludge by increasing the temperature from ~21 °C (room temperature) to incubation temperature 41 °C, Fig. 3, Table 1. The gas volumes were continuously corrected to the German or European standard temperature of 0 °C and of atmospheric pressure (STP) (VDI.4630, 2016). This was accomplished with the automated temperature and pressure sensors in the gas flow measurement set-up, which were verified also manually.

Table 1
Experimental design for the series A–G of anaerobic batch assays with wheat straw as substrate (10% suspension) to test the optimum pre-incubation conditions and general suitability of the defined salt solution ('synthetic manure') as manure substitute (C–G). Additionally, pre-incubation of straw (10% suspension) was performed with water (A, B). Each series was performed with five replicate assays (n = 5).

Series	Mass of WS (g)	Incubation medium for WS	Inoculum-to-substrate ratio ISR (based on VS)	Preparation condition
A	3	Water	2.44	Freshly prepared
B	4	Water	1.82	Freshly prepared
C	4	Chemical salt solution	1.82	Pre-incubated for 10 days at 20 °C
D	5	Chemical salt solution	1.45	Pre-incubated for 10 days at 20 °C
E	4	Chemical salt solution	1.82	Pre-incubated for 10 days at 4 °C
F	4	Chemical salt solution	1.82	Freshly prepared
G	5	Chemical salt solution	1.45	Freshly prepared

2.4. *Chemical composition of 'synthetic manure' for BMP assays*

The high C/N ratio of 71.8 of the WS (Table 3) would require a nitrogen-rich co-substrate to prevent process imbalance. Speece (1996) found that the C/N ratio between 10 and 30 was a prerequisite for stabilisation of sufficient alkalinity and pH-buffering during anaerobic digestion. Therefore in this work, a defined chemical salt solution, based on the chemical analysis of cattle manure, was created (Table 2). It was termed 'synthetic manure'.

For direct comparison, the analysis of the original cattle manure is presented in the last column (Table 2). The alkalinity of the cattle manure was in the range of 8000–12,000 mg $CaCO_3$ equivalents L^{-1} being typical for biogas plants (Maus et al., 2017), whereas the alkalinity of 'synthetic manure' was 10,900 ± 200 mg $CaCO_3$ eq. L^{-1} (Table 2). Furthermore, optimized trace elements solution was supplemented in order to avoid a trace elements deficiency. The developed formulation of the trace elements solution was based on a novel method for quantifying the uptake of dissolved, active ions during growth of methanogens (Arthur and Scherer, 2020). An amount of 1.7 mL of a stock solution of trace elements (100 fold) was added to the 168.3 g material in each 250 mL BMP assay vessel. Sodium polyphosphate $(NaPO_3) \times$ (1 mM, 100 mg L^{-1}) and Na-EDTA (86 μM) were used as complexing agents for the trace elements, but also as a new bioavailable phosphate source. The BMP assay also contained boric acid (H_3BO_3, 0.1 μM), copper ($CuCl_2$ * 2 H_2O, 1 μM), selenium (Na_2SeO_3 * 5 H_2O, 1 μM), tungsten (Na_2WO_4 * 2 H_2O, 1 μM), manganese ($MnCl_2$ * 4 H_2O, 2 μM), molybdenum (Na_2MoO_4 * 2 H_2O, 2 μM), cobalt ($CoCl_2$ * 6 H_2O, 3 μM), nickel ($NiCl_2$ * 6 H_2O, 3 μM), zinc ($ZnCl_2$, 10 μM) and iron ($Fe(III)Cl_3$ * 6 H_2O, 20 μM).

2.5. *Measurement of biogas volume and methane content with an automated methane potential test system (AMPTS)*

Quintuplicate assays were simultaneously performed for each test variation, together with five calibrating assays with 3 g of pure cellulose (particle size ~20 μm, Aldrich, number 31.069-7) instead of the WS. Also, five blank values of the inoculum sludge (without test substrate) were assayed for each series. The blank values were subsequently subtracted from the corresponding gross values obtained from substrate in order to obtain the net gas yields of the substrate. In general, one-way vessels for medical application, having a volume of 1 L or even 2 L, could be used for incubation. However, commercially available borosilicate glass vessels of 250 mL were adapted and used as digestion vessels (Fig. 1C), because a single seed sludge charge of 30 L was used for 120 simultaneous assays, as well as space availability. In the process, 20 of the borosilicate glass vessels (4 of which had methane sensors) were placed in one BD115 incubator (WTB Binder GmbH, Tuttlingen, Germany), Fig. 1A–C. The volume of

Table 2
Chemical composition of the mineral solution, which was used as manure substitute and designated as 'synthetic manure'.

Chemical salt solution 'synthetic manure'				Natural cattle manure[a]
Composition (Salt, gram-molecular weight)		Main ion [mmol L^{-1}]	Weighted salt [g L^{-1}]	[mmol L^{-1}]
$KHCO_3$	(100.115)	50.1 K^+	5.52	28.1–94.6 K^+
K_2CO_3	(138.205)	10.2 K^+	0.705	
NH_4HCO_3	(79.055)	138.6 NH_4^+	10.96	83.2–166.3 NH_4^+
$CaCl_2$ * $6H_2O$	(219.076)	6.2 Ca^{2+}	1.36	3.0–11.2 Ca^{2+}
$MgCl_2$ * 6 H_2O	(203.303)	3.7 Mg^{2+}	0.752	1.2–6.2 Mg^{2+}
$Fe_2(SO_4)_3$	(399.878)	0.116 Fe^{3+} 0.174 SO_4^{2-}	0.023	0.02–0.11 Fe^{3+} 2.6–3.7 SO_4^{2-}
Na_2SO_4	(142.042)	0.20 Na^+ 0.10 SO_4^{2-}	0.142	17.4–52.2 Na^+
$NaHCO_3$	(84.007)	30.9 Na^+	2.596	

[a] Long term analysis for several years of the chemical composition of cattle manure from the biogas plant of a dairy farm in D-23845Seth, Germany (for further details see Materials and methods).

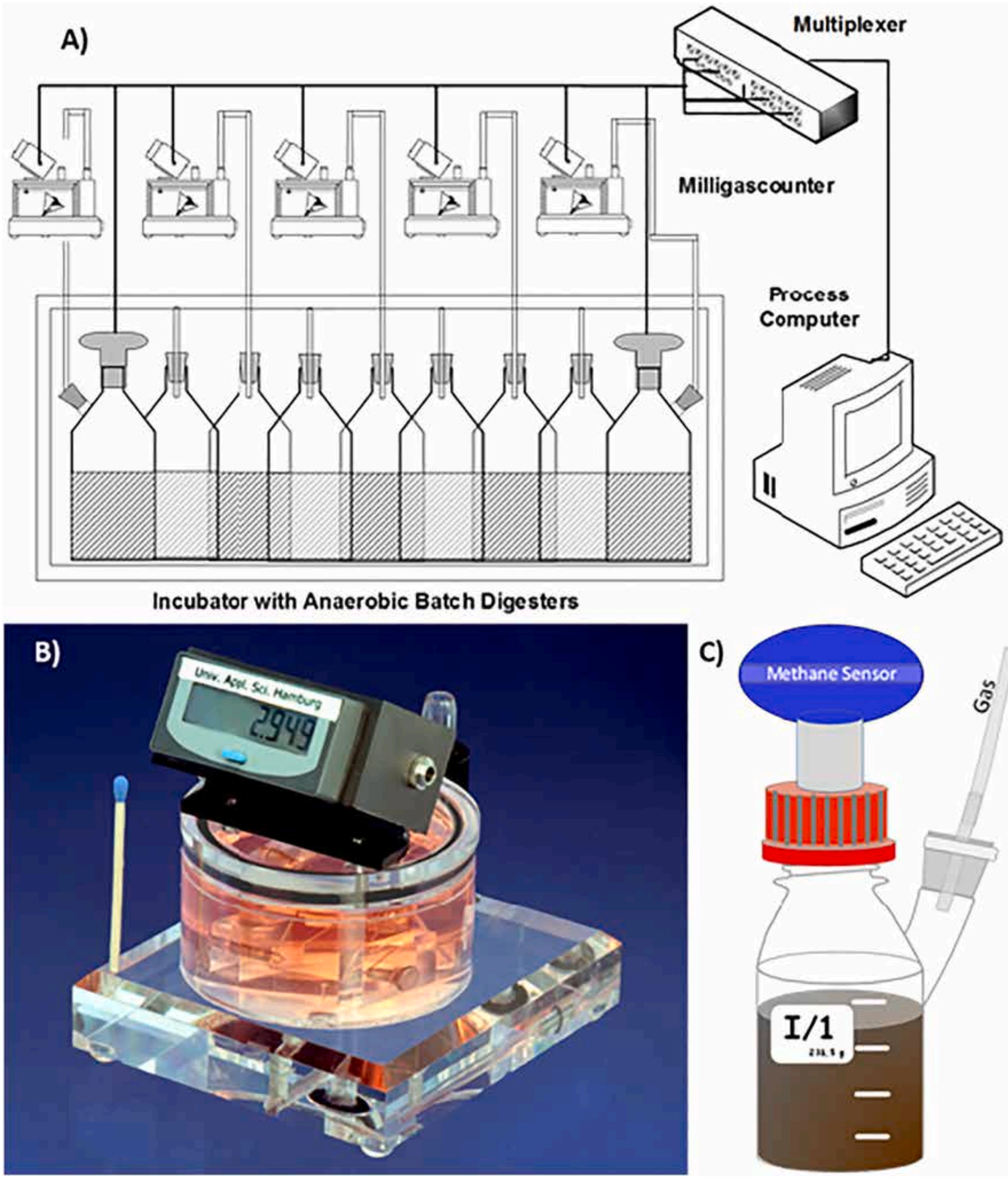

Fig. 1. **A)** General, experimental design of the used automated Milligascounter® (MGC)-station (AMPTS) for batch assays to obtain specific methane yields per gram VS. **B)** Detail photo of the used MGC. The counter with display on the top enables a standalone operation, the data port at the side was connected for online recording with a data logger and a PC. **C)** View of the in-situ methane analyzer on the top of the 250 mL assay vessels. For details see Materials and methods.

biogas being produced during anaerobic digestion process was recorded online with a Milligascounter® (MGC). The setup of the instrument is schematically shown in Fig. 1 A–C.

The MGC-technology, including data ports with analogous/digital converters, was developed at the Hamburg University of Applied Sciences (HAW) on the campus 'Bergedorf' and mentioned as 'micro gas meter' in the 'Bergedorf fermentation test' (VDI.4630, 2016). But the complete BMP assay with the MGC-station is currently available with some modifications from Dr. Ritter GmbH and Co KG, Bochum, Germany, Fig. 1B, as tested by Hülsemann et al. (2020). The measurement cell of the MGC consists of a flipping gas collection chamber with a triangular geometry (original version), which is separated by a central wall into two chambers of 1 mL each and fixed at the tip-side by an axis on the base (Fig. 1B). The separate gas collecting chambers were alternatively filled from a precise gas outlet under the divided chamber by rising very slowly. When a gas collecting chamber was filled by the small gas bubbles (about 30 μL each) arising from the gas outlet in the bottom, the buoyancy of the full chamber causes the measurement cell to abruptly tip over to provoke the opposite measuring chamber to be filled. The volume is measured in discrete steps by counting the tilts of the measurement cell. A small overpressure in the MGC system was due to the short (5 cm) height. Therefore, nearly no surface effects could occur in contrast to BMP systems which use pressure measurements (Casallas-Ojeda et al., 2020). The MGC was originally filled with a non-toxic silicone oil as barrier liquid. But, it was replaced with 0.1 M HCl, because it was found that the silicon liquid could absorb CO_2 (Wedlake and Robinson, 1979) resulting in about 10% lower gas yields (Walker et al., 2009).

Further details of the MGC can be found on www.milligascounter.de or by the distributor, www.ritter.de. The methane concentrations were determined in-situ inside the BMP assay vessels with novel infrared IR-analysers obtained from BlueSens GmbH, Herten, Germany (www.bluesens.com). They were mounted on the 250 mL assay vessels and were held in place with a screw cap and rubber gasket. PVC tubings with a high wall thickness of 1.5 mm were used to guarantee gas tightness between vessels, IR-analysers and the MGC. A 7 mm (o.d.) glass tubing and a 27 mm (o.d.) grey rubber stopper were connected to the side port of the glass vessels. Vessels without infrared analysers needed no side port (Fig. 1C). In this case a 34 mm (o.d.) rubber stopper with glass tubing was used on the top as connector. Alternatively, the Ritter company offers a complete BMP-system to measure the methane content of the biogas produced and having only CO_2 absorbing vessels. The data from the MGC and methane analysers were collected every 10 min via a BACCom unit ("multiplexer") through a BACVis software (Blue Sense GmbH) and the average was determined as the daily value.

2.6. Theoretical Biomethane Potential (BMP) with the extended Buswell equation of Boyle

The maximum possible CH_4 yields were calculated using the chemical formula, $C_{3.71}H_{6.04}O_{2.79}N_{0.044}S_{0.005}$, which was based on the elemental analysis of the WS used (Table 3). The percentage content of the elements (Table 3) was divided by the corresponding atomic number of C, H, O, N and S, as simplified in Eqs. (2) and (3) to obtain the index values of the formula. The established formula was applied according to the modified Buswell equation by Boyle as described by Achinas and Euverink (2016) to accurately predict the possible theoretical methane yield. It also includes the possible production of NH_3 and H_2S during the degradation of a protein-containing substrate. The protein content of WS was found to be 3.39% (Table 3).

$$C_aH_bO_cN_dS_e + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4} + \frac{e}{4}\right) \times H_2O \rightarrow \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8} + \frac{e}{4}\right) \times CO_2 + \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} + \frac{3d}{8} - \frac{e}{4}\right) \times CH_4 + d \times NH_3 + e \times H_2S \quad (1)$$

Based on the elemental substrate composition ($C_aH_bO_cN_dS_e$) of Eq. (1), the Eq. (2) becomes

$$BMP_{Th} = \frac{\left[\left(\frac{a}{2}\right) + \left(\frac{b}{8}\right) - \left(\frac{c}{4}\right) - \left(\frac{3d}{8}\right) - \left(\frac{e}{4}\right)\right] \times 22400}{(12a + b + 16c + 14d + 32e)} \left[\frac{mL}{g_{VS}}\right] = \frac{\left[\left(\frac{3.71}{2}\right) + \left(\frac{6.02}{8}\right) - \left(\frac{2.786}{4}\right) - \left(\frac{3 \times 0.044}{8}\right) - \left(\frac{0.005}{4}\right)\right] \times 22400}{(12 \times 3.71 + 6.02 + 16 \times 2.79 + 14 \times 0.044 + 32 \times 0.005)} \left[\frac{mL}{g_{VS}}\right] = 442.3 \left[\frac{mL}{g_{VS}}\right] \quad (2)$$

Table 3
Chemical composition of the milled wheat straw used.

Parameter	Wheat straw (WS)
Dry substance (TS)	96.9
Water content [% TS]	3.10
Organic dry matter (VS) [%TS%]	90.10
Ash content or loss by ignition [% TS]	6.80
Particle size [mm]	0.13
Total carbon [% VS]	44.51
Total N [% VS]	0.62
C/N ratio	71.8
Cellulose[a] [% TS]	36.73
Hemicellulose[b] [% TS]	25.26
Lignin (Klason) [% TS]	23.05
Protein[c] [% TS]	3.40
Fat[d] [% TS]	1.67
Total hydrogen [% VS]	6.02
Total oxygen [% VS]	44.80
Total sulfur [% VS]	0.16
Total phosphorus [% VS]	0.09
Glucose[a] [% TS]	37.72
Xylose[b] [% TS]	20.8
Arabinose[b] [% TS]	2.89
Galactose[b] [% TS]	0.89
Mannose[b] [% TS]	0.45
Rhamnose[b] [% TS]	0.18

The percentage of elements like C, H, N, O, S, P were related to VS. C/N = Carbon/Nitrogen, Quotient. Biochemical constituents were related to TS.

[a] Cellulose was calculated by determination the glucose content (HPLC).

[b] Hemicellulose was estimated by the sum of xylose, arabinose, galactose, mannose and rhamnose (HPLC).

[c] Protein = Total nitrogen × 5,46 (N estimated by elementary analysis).

[d] Fat was regarded as the remaining part of VS, if all other biochemical constituents were subtracted. The following trace elements were quantified as mg/kg dry matter of WS with TXRF (Arthur and Scherer, 2020): Mn 19.9, Fe 110, Zn 11.0, Cu 2.06, Ni 0.33. Se. The elements Co and W were not detected (<0.005 mg/kg).

$$BGP_{Th} = \frac{442.3 \left[\frac{mL}{g_{VS}}\right]}{51.8\%} = 853.3 \left[\frac{mL}{g_{VS}}\right] \quad (3)$$

Using Eqs. (2) and (3), a BMP of 442.3 mL_{STP} g_{vs}^{-1} and a biogas potential (BGP) of 853.3 mL_{STP} biogas g_{vs}^{-1} was obtained for the digested WS. Using the WS chemical formula and Eqs. (1)–(3), the biogas composition would be 2.04 CH_4 + 1.94 CO_2 + 0.044 NH_3 + 0.005 H_2S. Accordingly, the experimentally obtained CH_4 concentration was 51.5%, which was almost equal to the theoretical value of 51.75%. But the VS content includes a varying percentage of non-degradable lignin, which has to be corrected.

2.7. Correction of the theoretical Biomethane Potential (BMP) by a lignin factor or 'fermentable organics' VS_{fs}

As outlined in the introduction, there is no evidence to suggest that the lignin fraction of milled WS with non-degradable poly-phenolic chains could be degraded under strict anaerobic, methanogenic conditions (Triolo et al., 2012). In view of that, the BMP part of non-degradable lignin content *w* (*lignin*) was subtracted from the BMP of WS in order to obtain the possible true theoretical gas yield.

$$BMP_{Th} = BMP_{Th\ Straw} - BMP_{Th\ lignin} \times w(lignin) \quad (4)$$

The $BMP_{Th,straw}$ of Eq. (4) is the expression for the theoretical BMP of WS and included the BMP of the non-degradable Klason-lignin content, which was found to be 23.05% (Table 3). The Klason lignin was preferred to correct the methane yield instead of the Van Soest procedure for lignin estimation (Hatfield et al., 1994). It has been shown that the Klason Lignin values are higher and more accurate compared with the Acid Detergent Lignin – fraction ADL by Van Soest. As an example, Klason Lignin values of grass samples were 200 to 300% higher for forage plants than ADL-fraction lignin. The higher values of Klason Lignin from grass were not based on protein contamination or incomplete hydrolysis of carbohydrates, but probably a consequence of the solubilisation of lignin components of the ADL treatment (Hatfield et al., 1994; Steffen et al., 2016). The lignin content of WS varies widely between 10 and 30% and mainly depends on the time of harvest and location.

The theoretical BMP of lignin ($BMP_{Th,lignin}$) would be the calculated as pseudo-BMP of the lignin-fraction of WS using the lignin molecular formula $C_9H_{9.1}O_3(OCH_3)_{1.0}$, as proposed by Crestini and Argyropoulos (1997). Using this lignin chemical formula, the theoretical pseudo-methane yield of lignin would be comparatively high, being 645.9 mL g_{vs}^{-1}. The pseudo-CO_2-volume would be 515.3 mL g_{vs}^{-1} and the theoretical pseudo-biogas potential of WS, including lignin, would become 1161.2 mL g_{vs}^{-1}. The resulting pseudo-methane content of lignin could theoretically be 55.62%.

In view of that, the term 'fermentable organics' was used to compare of BMP values obtained from literature to relate the CH_4 yields on the basis of only the bio-degradable lignin-free portion of WS, which may be called 'holo-cellulose' (Raposo et al., 2020). Therefore, the specific gas yields of WS were calculated based on only the 'fermentable organics' by subtraction the lignin portion (23.05%) from the total organics of WS on the basis of the VS-content of the fermentable substrate (fS), Eqs. (5) and (6).

Furthermore, in determining the degradable part of a biomass, the dissolved Volatile Fatty Acids (VFA) should be considered, as they could equally contribute as a microbial substrate, Eqs. (5) and (6).

$$VS_{fS} = m_{substrate} {}^* \left(VS_{Substrate} - VS_{Lignin} + VS_{VFA}\right) \tag{5}$$

$$sGPR_{f,Methane} = \frac{V_{Methane}}{VS_{fS}} \quad or \quad sGPR_{f,Biogas} = \frac{V_{Biogas}}{VS_{fS}} \tag{6}$$

Therefore, Eqs. (5) and (6) could also be used to estimate the specific biogas or methane yield in relation to 'fermentable organics' of the WS, which is less the Klason lignin, but possible including dissolved VFA in the case of a liquid silage of forage plants (inapplicable here with the dry WS).

As shown by the extended Buswell formula Eq. (3), the specific 100% BMP of WS was calculated to be 442.3 mL_{STP} g_{vs}^{-1} (7) for methane and 853.3 mL_{STP} g_{vs}^{-1} for biogas including the pseudo $BMP_{Th,lignin}$ part of lignin.

However, after excluding the Klason lignin content of WS (23.05%) with its corresponding pseudo-BMP and using Eqs. (5) and (6), the corresponding 100% biogas potential of the 'fermentable organics' of WS reduced to 566.4 mL_{STP} g_{vs}^{-1} and the theoretical, maximum CH_4 yield reduced to 293.4 mL_{STP} g_{vs}^{-1} (with theoretical CH_4 concentration of 51.75% for WS), Eq. (7).

$$\begin{aligned} BMP_{Th} &= 442.3\left[\frac{mL}{g_{VS}}\right] - 645.9\left[\frac{mL}{g_{VS}}\right] \times 23.05\% \\ BMP_{Th} &= 442.3\left[\frac{mL}{g_{VS}}\right] - 148.9\left[\frac{mL}{g_{VS}}\right] \\ BMP_{Th} &= 293.4\left[\frac{mL}{g_{VS}}\right] \end{aligned} \tag{7}$$

The analyses have shown that WS contains only small amounts of proteins and lipids (Table 3). Therefore, the degradable, lignin-free part of the WS could be represented as cellulose or 'holo-cellulose'. Based on the chemical formula of pure cellulose $C_6H_{10}O_5$, the theoretical biogas yield is 747 NmL_{STP} or 343.5 NmL_{STP} CH_4 per gram cellulose (Lübken et al., 2010; Raposo et al., 2011; Wang et al., 2014). If 1 g VS of a suggested holo-cellulose compound would be reduced by 23.05 %, corresponding pseudo-BMP of lignin according to Eqs. (5) and (6), then the theoretical methane yield of 'holo-cellulose' would decrease by 23.05% to 574.8 mL $_{STP}$ g_{vs}^{-1}. This value is almost equal to the BMP_{Th}-value of WS as shown previously with 566.4 mL_{STP} g_{vs}^{-1} reflecting the presence of the small protein content as the difference. Thus, it confirms the suggestion that the BMP_{Th}, using Eqs. (4)–(6) for the 'fermentable organics' of WS, is almost identical with pure cellulose.

2.8. Mathematical procedures

The calculated average values of the specific biogas yield and CH_4 yield of all the experiments were subjected to Analysis of Variance (ANOVA). The ANOVA procedure was carried out to determine the significant differences in the experimental results (Raposo, 2016).The term significant was used only when a statistical test was performed using a $p < 0.05$.

Moreover, the modified Thompson Tau Technique was used to eliminate outliers in the results of the BMP series (Dieck, 1997). s = $\sqrt{\text{variance}}$ = standard deviation = variance around an arithmetic mean value, RSD = relative standard deviation = $\sigma/\bar{x}$ ($\bar{x}$ = average value for n = 5),RR = relative range or span length (x_{max}–x_{min}).

3. Results and discussion

3.1. Composition of wheat straw

Analysis of the particle size distribution of the milled WS showed a geometric mean diameter of 0.13 mm. The particles were of uniform size with a 'coefficient of uniformity' (CU) <4.0. The TS and VS content were 96.9 and 90.1%, respectively. It was used as a 10% suspension either in water or 'synthetic manure' as indicated. The properties of the WS used are also presented in Table 3.

The chemical formula of WS as a lignocellulosic reference substrate was determined through elemental analysis (Table 3). It was a prerequisite to enable the calculation of the theoretically possible methane yield according to Buswell and Boyle as outlined by Achinas and Euverink (2016). If the experimentally estimated methane yield is equal to the calculated of WS, then a 100% conversion of straw must has been verified. The elemental composition of the biochemical constituents have been presented in Table 3. Also the average particle size of the WS used was found to be 0.13 mm. The following fractions were quantified: carbon 44.51%, hydrogen 6.02%, oxygen 44.80%, nitrogen 0.62%, sulfur 0.16% and phosphorus 0.09% of the VS-content, with the respective elemental formula of $C_{3.71}$ $H_{6.04}$ $O_{2.79}$ $N_{0.044}$ $S_{0.005}$ and a C/N ratio of 71.8. Compositional analyses of WS showed 23.05% Klason-lignin, 36.73% cellulose and 25.26% hemicellulose of the VS, respectively. Also the protein content was calculated by multiplying the total nitrogen with 5.46, according to Mosse (1990), and was found to be 3.40% of the VS content of the WS. Fat was considered as the residual part of VS, if all other biochemical constituents were subtracted and was found to be 1.67%, Table 3. Trace elements in the dry matter were also estimated, Table 3.

3.2. Effect of flocculation of sludge characteristics

The inoculum was artificially flocculated to obtain a granular sludge which favours a complete and syntrophic bioconversion. The TS and VS were increased by a factor of about 4, which corresponded to an increase from 2.09 to 8.76% TS and from 1.28 to 6.60% VS, respectively. Each 250 mL assay vessel contained 120 g of the flocculated seed sludge (TS adjusted to 8.76%) and 3, 4 or 5 g of WS in 30 mL of a 10% aqueous suspension in water (incubation series A and B) or in 30 mL 'synthetic manure' (incubation series C–G) to determine the best ISR for WS as lignocellulosic reference substrate.

As has been mentioned earlier, in granular sludge, the cells are located close to each other, which is a precondition for syntrophic growth or interspecies hydrogen transfer 'IHT' (Schink, 1997) or even direct interspecies electron transfer 'DIET' (Xu et al., 2020). The distance between bacteria should be as close as 5–8 μm, being typical for a disperse microbial suspension to enable the necessary exotherm reaction

Table 4
Analysis of the raw and the artificially flocculated seed sludge used for the different series of anaerobic digestion batch assays with wheat straw WS as substrate.

Parameter	Unit	Raw digester sludge WWTP	Flocculated seed sludge WWTP
pH[a]	–	7.71	7.92
TS	%	2.27	10.47
VS	%	1.39	6.36
VS/TS relation	%	61.23	60.74
Alkalinity equivalents	mg $CaCO_3$ L^{-1}	5190	7260
Ammonium	mg L^{-1}	1184	1724
Phosphate	mg L^{-1}	147	152
VFA	Mg L^{-1}	50–60	250–280

[a] Parameters were measured before and after thickening with a flocculating agent (see Materials and methods) to get a distinct ISR value.

during IHT. This has already been calculated by McCarty and Smith (1986) and Schink and Thauer (1988). A study by Schink and Thauer (1988) assumed a microbial suspension of 10^{-9} cells/mL and found that the process would improve by a factor of 10–100 if an aggregated structure is present with a microbial distance of only 0.08 μm. Accordingly, a microbial cell density of 10^{-10} was reported for high performance agricultural biogas plants. That correlated with a Total Solids (TS) content of 7.5–12.5% (Maus et al., 2017). From this point of view, artificial granular sludge is a precondition for a quick and complete anaerobic conversion. Flocculated sludge would also prevent the risk of evolving unused H_2 in the headspace of BMP assays as a result of an incomplete IHT. As already described, the new sludge flocculation procedure for BMP assays was transferred from the practice on WWTPs, for dewatering sewage sludge with a flocculant before pressing or centrifuging.

Results of the analysis of the raw sewage sludge showed a low TS and VS content, being 2.09 (2–2.5%) and 1.28%, respectively. Further details of the raw and artificially flocculated inoculum sludge are shown in Table 4.

Natural manure, which is often used in BMP tests in combination with WS, was replaced with a defined salt solution. The undefined organic and inorganic constituents of livestock manure mostly influence the gas yields of the blank assay or the assay with WS (Kafle and Chen, 2016). These effects were minimized by using the 'synthetic manure' (Table 2) and a control assay, being without substrate ('blank', n = 5 per series), under the same conditions as the flocculated seed sludge and synthetic manure. The yield from the blank was subtracted from the gross gas yields of assays, which had substrate, to obtain the net gas yields as shown in Fig. 2 A.

3.3. Effect of pre-incubation conditions on methane yield

An appropriate pre-incubation method for the WS suspension, such as temperature, duration of storage and medium (pure water or buffered) was ascertained, as WS is water repellent. Thereby, the best ISR value for WS was evaluated. Figs. 2, 3 and Table 1 show the assays performed in this study. The estimated relative standard deviation (RSD) in Fig. 3 was 1–9.8%, with the best being 1% for 5 g WS and an ISR of 1.45.

The specification of 40 days (Fig. 2A) refers to average degradable substrates. The actual degradation of cellulose and WS under the optimized BMP conditions lasted for only 10 or 15 days, respectively, as seen in Fig. 2A and B. However, overall incubation period was extended to 30–40 days per assay for research purposes.

Apparently, there were no significant differences in the BMP, irrespective of the ISR value (1.45, 1.82 and 2.44) in this work (Fig. 3). These results are in congruence with Holliger et al. (2016), who suggested that an ISR value of around 1 should be sufficient for BMP assays with respect to lignocellulosic substrates. The relative range (RR) was 2%, as there were no outliers, series D, Figs. 2A and 3. However, an amount of 3 g WS per 170 g assay (250 ml total volume) is recommended for better handling (series A). The variation D is with a RSD of 1% (Fig. 3) better than most results found in literature. Hülsemann et al. (2020), found a good overall coefficient of variation (CV) of <4.8% of the results obtained from their studies. However, they investigated five different substrates, as well as five different inocula and the effect of four different BMP measuring devices for BMP experiments. The obtained CV-values of the different inocula were in the range of 1.8–4–9%. It

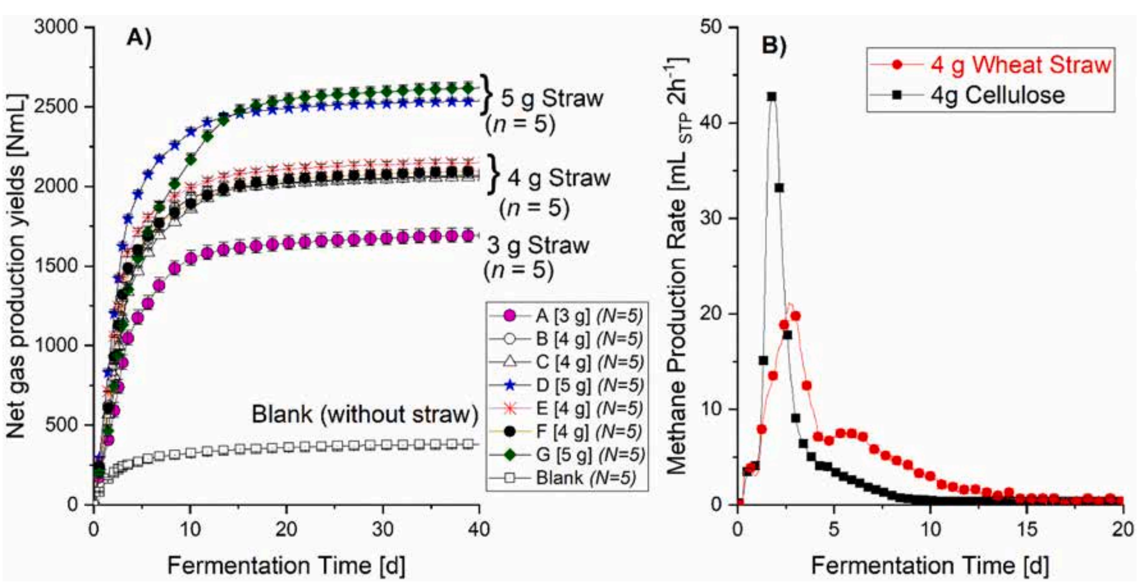

Fig. 2. A) Cumulative net biogas yield curves with WS as sole substrate. The gas yields paralleled the inserted amounts of WS per assay. Different incubation procedures and inoculum to substrate ratios were adjusted to ascertain possible impacts of the created 'synthetic manure' on the BMP assay (for ISR-values see Table 1). Biogas yields (NmL_{STP}) of blank assays being 371.2 NmL/gvs and of 204.4 Nml CH_4/gvs, without added substrate, were subtracted from the gross gas yields of assays with added substrate to get the net gas yields. For further details of series A–G, see Table 2 and Fig. 3. **B)** Original graph of an in-situ methane analyzer during anaerobic digestion of pure cellulose or wheat straw in a 250 mL incubation vessel. The main peak from the methane production of straw appeared after the single peak of cellulose, but two additional CH_4 peaks of straw could be seen. For details see Materials and methods, and Fig. 3.

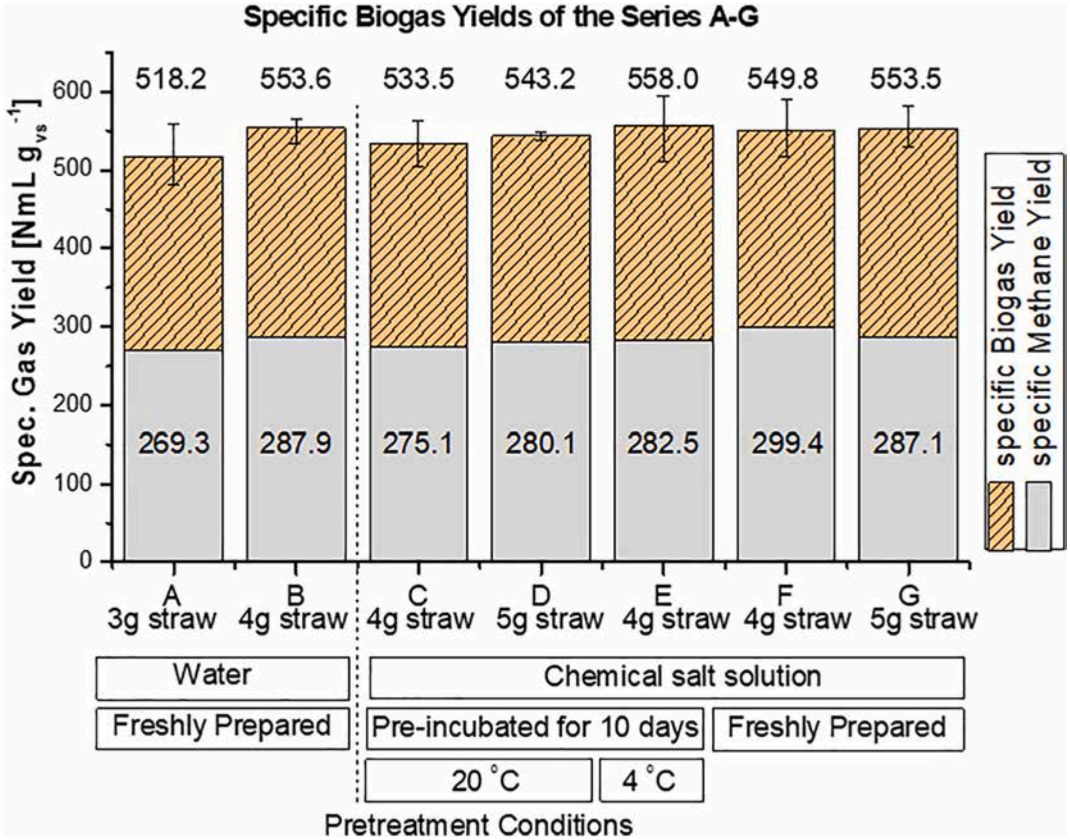

Fig. 3. Different pretreatment conditions were tested for milled WS as substrate: Specific biogas yields of the series A–G using wheat straw pre-incubated in water or in a defined salt solution ('synthetic manure') as substrate (n = 5 per series). Assays were prepared based on different storage time and temperature conditions as given in Table 1. Values of gas yields are average values (mL_{STP}) after the blank values, without substrate were subtracted. RSD (±): A ± 7.2%, B ± 3.5%, C ± 8.3%, D ± 1.0%, E ± 9.8%, F ± 8.1%, G ± 4.4%. RR (B–G) = 8.1%.

shows, that a high reproducibility is possible. But the best inter-laboratory reproducibility was achieved in a study involving 22 to 36 participating laboratories and a RSD of 7.5–24.3% with a RR of 31 to 130% (Hafner et al., 2020). In a German study led by KTBL/VDLUFA, a RSD value of ±14% was obtained from 30 laboratories, as reviewed by Holliger et al. (2016). Additionally, the inter-laboratory study led by Raposo et al. (2011)involving 19 laboratories revealed a RSD of 8–11% after outliers were eliminated. Otherwise, the standard deviation would be 15% with cellulose and 37% with gelatine (Raposo et al., 2011). In series S2 of the international study by Hafner et al. (2020), wheat straw was also tested, in comparison with 'animal feed' and pure microcrystalline cellulose, as reference substrate. But the apparent variation of their BMP values for WS, with an average gas yield of 279 mL_{STP} CH_4 g_{VS}^{-1}, revealed a RSD-value of 17.3% and a RR-value of 128%. A similar RSD was achieved with WS (series 1) in the study by Ribeiro et al. (2020) involving 11 French laboratories.

Furthermore, the need for a pre-incubation time for the WS particles (0.13 mm) was not ascertained from the CV-values of this study. That means, there was no significant difference ($p < 0.05$) in the gas yields after storing WS at 4 °C or at 20 °C, freshly prepared or stored for 10 days in water or in 'synthetic manure', Fig. 3, Table 5. It suggests that incubating the substrate for a period of time before commencing the experiments would not improve the biogas yields. Based on the high methane yields, it also confirmed that storing the seed sludge at room temperature for not longer than 5 days was important. Such inoculum could be regarded as 'fresh' (Angelidaki et al., 2009) and active as well.

The rate of hydrolysis and acidogenesis must be equal during anaerobic digestion in order to avoid an increase in acid concentration, which could reduce the pH to less than 7. Before the commencement of the experiments, the pH values of the batch assays were found to be in the range of 7.40–8.10, except for the 4 g substrate assay with water as storage medium, which had an initial pH of 6.98 (Figs. 2A, 3, series B). At the end of the experiments, the pH of all assays increased by 0.3–0.4 units to uniform levels of pH 7.8–8.2. This was exactly the same range of typical pH values as seen for agro-biogas plants with good performance (Maus et al., 2017). The slight increase in pH value during digestion period could be attributed to the consumption of the remaining VFA by the microorganisms in the seed sludge (Table 4), as was similarly observed by Chandra et al. (2012). The buffering capacity of the 'synthetic manure' being nearly 11,000 mg $CaCO_3$ equivalents L^{-1} and a buffer molarity of 200 mmolar (Table 2), was apparently more than enough to ensure process stability. Raposo et al. (2012) had even postulated that only 2500–5000 mg $CaCO_3$ equivalents L^{-1} would be sufficient for BMP assays. However, the highly buffered 'synthetic manure' cannot be compared with the low buffered mineralic medium of Angelidaki et al. (2009) and another inter-laboratory study by Ribeiro et al. (2020), in series 1 of the their work. The low buffered medium (1.5 mmol phosphate) supplemented with vitamins, reducing agents such as cysteine and resazurin as oxygen indicator, was similar to a general culture medium for anaerobes by the German Culture Collection DSMZ for microorganisms (www.dsmz.de) and pure cultures of methanogens, such as presented in Scherer and Sahm (1981). Later, in their series 2, the authors Ribeiro et al. (2020) modified the incubation medium by adding bicarbonate buffer (33 mmol $NaHCO_3$).

Figs. 2A and 3 show the average specific biogas yields and specific CH_4 yields of the different sample preparation methods tested (n = 5), which showed minor differences between the final biogas or CH_4 volumes of the different BMP series A–G. The volume of biogas and methane were in the range of 518–558 mL_{STP} g_{VS}^{-1} and 269–299 mL_{STP} CH_4 g_{VS}^{-1}, respectively (Fig. 3). But the average biogas and methane yield

Table 5

Average specific methane yields of wheat straw from this work were compared with results obtained from the literature 2–10. All values are related to the $VS_{substrate}$. The theoretical, maximum possible methane yield of 293.4 mL_{STP} was based on the extended chemical Buswell formula of Boyle (Achinas and Euverink, 2016), see Materials and methods. Similar results were obtained in this work (row 1) and by references 6, /7B-10. Also the specific methane yields of the 'fermentable organic portion' or 'holo-cellulose' of wheat straw (excluding lignin) are presented. The Klason-lignin content for reference 2–10 was subtracted if available ($VS_{substrate} - VS_{Klason\text{-}Lignin}$). The buffering sources, pretreatment methods and particle size of WS are also shown.

Ref.	Specific methane yield of wheat straw	Specific methane yield of "fermentable organics"	Source of buffer and nutrients	Pretreatment method	Particle size (μm)
	Average ± SD[a] [$mL_{STP} g_{VS}^{-1}$]	Average ± SD [$mL_{CH4\ STP} g_{FVS}^{-1}$]			
This work	287.1[d]	352.5	Defined chemical salt solution + trace element solution	Mechanical	130
1	165.9	f	Urea	Chemical	–
2	210.0	244.4[e,f]	Seaweed as co-substrate	Physicochemically	(Powder)-
3	208.0[c]	f	Cow manure	Not reported	–
4	249.0[b] ± 0.97	f	Cow manure	Mechanical	88
4	248.0[b] ± 0.91	f	Cow manure	Mechanical	400
4	241.0[b] ± 0.29	f	Cow manure	Mechanical	1000 (1 mm)
4	227.0[b] ± 1.17	f	Cow manure	Mechanical	6000 (6 mm)
4	162.0[b] ± 2.97	f	Cow manure	Mechanical	30,000 (30 mm)
5	254.0 ± 19.49	317.0[e]	Cow manure	Mechanical	200
6	281–291[b]	346.2[e]	'Macro-elements' + phosphate buffer solution	Mechanical	48–759
7A	234.0[b]	f	Cow manure, digested food waste	Mechanical	600–5700
7B	277.8[b]	f	Cow manure, digested food waste	Mechanical	600–5700
8	302[b]	354.5[e]	Sewage sludge	Mechanical	5000
9	276[b]	f	Agricultural biogas plant	Mechanical	500–1000
10	279	f	Not specified	Mechanical	Not specified

1: Chandra et al. (2012), **2:** Nkemka and Murto (2013), **3:** Döhler et al. (2013), **4:** Sharma et al. (1988), with a too high methane content of "around 60%" (according to the chemical composition of straw it had to be only 50.75%). **5:** Heiske et al. (2013). **6:** Dumas et al. (2015). CH_4 content 47–59% (actually 50.75%), but due to the varying CH_4 content, the true methane yields of Dumas et al. remain somewhat uncertain. **7A:** Sapci (2013) (summer wheat SW), **7B:** Sapci (2013) (winter wheat WW, SW and WW) with only 8.6 and 8.0% ADL lignin respectively, according to AOC or Van Soest as cited in Sapci (2013) and Raposo et al. (2020), see also Results and discussion. **8:** Tong et al. (1990). **9:** Bauer et al. (2010). **10:** Hafner et al. (2020), mean RSD 17.3%, RR 128%.

[a] SD: standard deviation.

[b] Results were based on TS, otherwise related on VS.

[c] Average from general practice associated with agricultural biogas plants in Germany as reported by Döhler et al. (2013).

[d] Average of specific methane yields for the complete wheat straw of the present study and of reference.

[e] Klason lignin value was subtracted on VS basis according to Jung et al. (1997).

[f] No Klason lignin value was specified.

of series A, being 518 mL_{STP} and 269 mL_{STP}, respectively, appeared to be outliers as 2 values out of the quintuplicate assays did not fit properly. In view of that, only the average values of the biogas yields of the remaining six series B–G (275.1–299.4 $mL_{STP}\ g_{VS}^{-1}$) were used to estimate the average methane yield, 287.1 $mL_{STP}\ g_{VS}^{-1}$ for WS (Fig. 3).

The AMPTS setup used (Fig. 1 A–C) enabled the direct measurement of methane in-situ in the BMP vessels, of which an example has been shown in Fig. 2 B. Thus, the methane content of biogas determined by in-situ CH_4 analysers was found to be 51.5% value for WS (Fig. 2 B), which was almost identical with the theoretical value of 51.75%, as derived from the molecular formula described by Achinas and Euverink (2016). The pure cellulose, which was used as the reference substrate, showed only one distinct methane peak with 49.8% CH_4 in the biogas, which was almost equal to the theoretically calculated value of 50.0%. Apparently, the degradation of pure cellulose was completed in 10 days. However, the main peak of the three CH_4-peaks of WS appeared after the CH_4 -peak of pure cellulose and was almost completely anaerobically degraded after 15 days (Fig. 2B). This is an indication that the WS was more complex and exhibited rate limiting biodegradation, more than pure cellulose with only one sharp peak. Also the amount of WS-substrate per assay influenced the peak width and degradation behaviour. Wider peaks seem to indicate a slowed degradation. Therefore, in-situ estimation of CH_4 in the BMP batch digesters could give valuable information about the best ISR value, the optimum substrate mixture and could track the possible loading rate for a full-scale biogas plant.

Sapci (2013) reported a straw BMP incubation period of 60 days for nearly complete degradation, Kang et al. (2021) obtained from wheat straw with their best pretreatment method (addition of sewage sludge) 112.6 mL/g TS methane in 70 days at 37 °C, whereas Tong et al. (1990) found for wheat straw 302 ml/gVS-1 in 45–60 days at 35 °C. Gallegos et al. (2017) obtained 195 mL methane/g_{VS}^{-1} in 80–110 days at 35 °C for 1 mm WS, whereas Hashimoto (1989) required 60–150 days for WS degradation in BMP assays at the same temperature. But, precise information about the needed BMP incubation time for a complete substrate degradation is rare in literature. Besides this, the anaerobic degradation period could be even halved and predicted with a >5% certainty through modeling as has also been described by Strömberg et al. (2014). We used the same modeling approach, but with the empirical Hill kinetics by Nakhla et al. (2006). This allowed predictions of cumulative sigmoidal gas plots with a lag phase (unpublished results). It is noteworthy, that the WS used was subjected to a gentle, temperature regulated mechanical pretreatment leading to an average particle size of 0.13 mm. Therefore, gas yields cannot be compared with gas yields of physico-chemically pretreated WS. For example, the authors Ferreira and Taherzadeh (2020) improved the methane yield of WS by 40% from 233 to 296 mL CH_4/gVS_{added}, through thermal steam explosion for 5 min at 200 °C. Vásquez et al. (2015) also incubated WS with NaOH to separate the lignin-cellulose-hemicellulose bond and measured specific CH_4 yields of 400 mL for WS in the presence of NaOH, which was 30% higher than specific CH_4 yields found in other literature (Table 5).

Most of the gas yields published in literature, as showed in Table 5, were performed with cow manure, whereas in the present study, 'synthetic manure' was used. An average methane yield of 287.1 mL $CH_4\ g_{VS}^{-1}$ was found in this study (Table 5), which was comparable with the CH_4 yields of 281–291 mL $CH_4\ g_{VS}^{-1}$ obtained by Dumas et al. (2015) and 302 mL $CH_4\ g_{VS}^{-1}$ obtained by Tong et al. (1990). If the CH_4 yields of Dumas et al. (2015) were corrected using the Klason Lignin approach to obtain the 'fermentable organics' or straw as 'holo-cellulose', the resulting average CH_4 yield would be 346.2 mL CH_4 $g_{VS\text{-}fS}^{-1}$ (Table 5). Tong et al. (1990) would have found 354.4 mL CH_4 g_{VS}^{-1} methane yield of the 'fermentable organics'. Such values are comparable with the CH_4 yield of 'fermentable organics' or 'holo-cellulose' found in our study with 352.5.1 mL CH_4 $g_{VS\text{-}fS}^{-1}$ and a content of 23.05% Klason lignin. Thus, the results confirm the importance of considering the Klason lignin in order to obtain a simple value for 'fermentable organics' and to harmonise reports on CH_4 yields of lignocellulosic compounds (Table 5). It is worth noting, that the results obtained by Dumas et al. (2015) indicated that the maximum methane production is not significantly different when the particle size is reduced from 0.759 mm to 0.048 mm (Table 5, line 6). This is in contrast to the findings of Ferreira and Taherzadeh (2020), who concluded that 3–5 cm size of wheat straw gave 5–13% higher methane yields than milled WS with a particle size < 1 mm. On the contrary, Andersen et al. (2020) concluded in their recent review that hammer milling to a size of 1 mm would be most suitable. However, Sharma et al. (1988) found the highest methane yield with a straw particle size of 0.088 mm and 0.400 mm. This is congruent with the results in the present study with 0.13 mm particle size (Table 5). But milled particles having small size and a large surface generally provide an accelerated biogas production rate due to the improved accessibility of microorganisms to the 'fermentable organics' (Dumas et al., 2015). Similar observations were also made when comparing reed straw (5.2% lignin) with different particle sizes (Mahmoud et al., 2021). The authors observed, that methane yields did not significantly change ($p > 0.05$), but particle size of 0.13 mm was more positively correlated with fast degradation rates than reed straw with particle size 0.6–0.9 mm. Ribeiro et al. (2020), in their inter-laboratory study on wheat straw, obtained a mean BMP of 267 mL for series 1 and 277 mL_{STP} CH_4 g_{VS}^{-1} for series 2, respectively (not shown in Table 5). Nevertheless, if different straw varieties are compared, the typically varying ash content has to be considered as well (Antongiovanni and Sargentini, 1991).

In addition to the main test series A–G with WS (Fig. 3), the CH_4 yield of 3 g pure cellulose was experimentally determined as a control with 'synthetic manure' to be 363.5 mL CH_4 g_{VS}^{-1}. This yield was slightly lower (2.7%) than the theoretical 100% value derived from the chemical formula $C_6H_{10}O_5$ for cellulose (373.5 mL CH_4 g_{VS}^{-1}. But VDI.4630 (2016) suggests 372,5 mL_{STP} CH_4 g_{VS}^{-1}. This value was almost the same as the highest achieved methane yield of 366 mL CH_4 g_{VS}^{-1} found by Wang et al. (2014). The authors compared 4 different instruments and experimental setups of BMP tests by using pure cellulose as their sole test substrate (1 g) and found 50.0% CH_4 content for cellulose (theoretical value) and a biogas yield of 680 NmL_{STP} ± 18 NmL_{STP} biogas and 732 NmL_{STP} ± 5 NmL_{STP} biogas or 366 NmL_{STP} CH_4 per 1 g VS. That means, that 98.0% of the theoretical maximum CH_4 yield of cellulose, being 373.5 mL_{STP} CH_4 g_{VS}^{-1}, was experimentally recovered. In the inter-laboratory study by Hafner et al. (2020), with up to 36 laboratories, a mean BMP value of cellulose was revealed with 346–365 mL_{STP} CH_4 g_{VS}^{-1} and a RSD of 7.7–11.4%. On the other hand, Raposo et al. (2011) found 340 mL_{STP} CH_4 g_{VS}^{-1} with a RSD of 8% and a SD of ±58 NmL_{STP} for 1 g cellulose in an inter-laboratory study involving 19 participants. Hülsemann et al. (2020) obtained a methane yield for pure cellulose of the same category around 370 mL_{STP} CH_4 g_{VS}^{-1} as found here. However, the CH_4 yields generated from WS should be somewhat higher than pure cellulose, as WS contains small amounts of lipids and protein (Table 3). Incidentally, the estimated specific methane yield of the 'fermentable organics' of WS (Table 5) was with 352.5 mL_{STP} CH_4 g_{VS}^{-1} around 95% of the theoretically calculated maximum methane yield of pure cellulose. Again, it shows that the gas yield is dependent on the lignin content and that the cellulose/hemicellulose part of WS could be completely converted as 'holo-cellulose' or 'fermentable organics' to methane under the defined conditions, as showed in this work.

The estimated mean methane yield of WS in this work was 287.1 mL_{STP} CH_4 g_{VS}^{-1}, which was only slightly influenced by the pre-incubation of WS, variation 8.1% of RR (Fig. 3, Table 5, row 1). The theoretical, maximum methane yield of WS was found to be 293.4 mL_{STP} g_{vs}^{-1} based on the chemical formula of $C_{3.71}H_{6.04}O_{2.79}N_{0.044}S_{0.005}$ as estimated here according to Achinas and Euverink (2016). However, the estimated average methane yield being 287.1 mL_{STP} g_{VS}^{-1} was 98% of the theoretical value, which would mean a WS degradation rate of 100% under the BMP test conditions, if a correction factor of 2–3% is considered for the generation of new microbial biomass. The authors Wang et al. (2014) and Filer et al. (2019) assumed in their reviews, that up to 10% of the substrate is used for biomass growth during anaerobic digestion and heat transfer. However, the authors of VDI.4630 (2016) assume 5% substrate usage at the fermentative stage and 3% for the methanogenic stage, making a total of 8% for newly generated microbial biomass in the overall process. But the assumptions are derived from earlier energetic considerations with pure cultures. An amount of 1–4.5 mol ATP per mole glucose can be generated by fermentative bacteria, where 6–12 g biomass is assumed per mole ATP (Stouthamer and Bettenhaussen, 1973). However, aceticlastic methanogens may have less than 1 mol ATP per mole of consumed acetate available, in which case less biomass would be produced (Schink, 1997). But, to the best of our knowledge, only one paper makes reference to the study regarding newly generated microbial biomass during a BMP test by using spent grain as substrate. The authors found through DNA analysis, that 2.3% microbial biomass was formed during the BMP assays (Scherer et al., 1990). The BMP experiments, based on the chemical formula of WS of this study, will not make explicit contribution in this direction, but exactly confirm the 2% biomass factor and therefore support the suggestion, that 2% newly generated microbial biomass was formed during BMP assay with WS as lignocellulosic compound.

4. Conclusions

Special features of this BMP approach, with milled WS as reference substrate, were the use of 'synthetic manure' and artificial flocculation of the seed sludge to favour synthrophic growth and to allow accelerated and complete conversion of WS in only 15 days. Under these defined conditions, the specific methane yield of WS was found to be 287.1 mL_{STP} CH_4 g_{VS}^{-1}, which is 98% of the theoretically possible yield (293.4 mL_{STP} CH_4 g_{VS}^{-1}), based on the estimated molecular formula $C_{3.71}H_{6.04}O_{2.79}N_{0.044}S_{0.005}$, for WS. But the results suggest that there was almost a 100% biodegradation if the 2% microbial biomass, which is known to be formed during the BMP assay, is included. It also validates the concept of comparing specific methane yields of straw as lignocellulosic reference material by estimating the Klason lignin and the 'fermentable organics'. Furthermore, the reliability of the innovative AMPTS-apparatus with milligascounters® and in-situ infrared analysers was confirmed.

CRediT authorship contribution statement

Paul A. Scherer: Conceptualization, Supervision, Funding acquisition, Project administration, Resources, Writing – review & editing. **Richard Arthur:** Investigation, Methodology, Data curation, Validation, Writing – review & editing. **Sebastian Antonczyk:** Investigation, Methodology, Data curation, Validation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

A.1. Calculation of methane concentrations with in-situ methane analysers inside of batch BMP assays

Generally, the methane concentration of batch digesters is measured behind the gas outlet, but by this novel method, infrared IR-analysers (BlueSens GmbH, Herten, www.bluesens.com) were directly inserted inside the assay vessels. They were mounted on the 250 mL incubation vessels and were held in place with a screw cap and rubber gasket. For further details see Materials and methods, Fig. 1C. The methane content in-situ was recorded every 10 min as it increases with the CO_2-content consistently and replaces the air of the assay vessels with incubation time as a sign of biodegradation. Therefore, first the final recorded CH_4 percentage value of the in-situ methane analysers have to be summarized and cannot be directly used. The average methane value were derived automatically from the 10 min measuring intervals and calculated as 2 h-values. The mean value of all the averaged 2 h-values of one assay vessel was calculated at the end of the entire measurement period. Furthermore, the progressive displacement of air in the head space of the batch fermentations-were calculated to achieve the true final CH_4 percentage (Koch et al., 2015). This is unlike the simple measurement of the cumulative biogas volume with the milligascounter® MGC, which can be regarded as a final value at the end of the digestion period. Eqs. (A.1)–(A.7) shows the complete procedure. The sum of recorded CH_4 volumes ($\Sigma\ V_{Methane}$) from the substrate was obtained using the estimated volume at the time of the measurement (volume produced at time t, V_t), every two hours. The individual changes of the CH_4 concentrations were multiplied by the measured volumes (V_1–V_0, etc.), as shown in Eqs. (7) and (A.1). The final CH_4 content φ_{tc} in % (t = time, c = concentration) of the produced biogas was obtained after eliminating the headspace error at the end, using Eq. (A.7).

$$\sum_{t=0}^{t=n}(V_t - V_{t-1}) \times \varphi_{Bt_c} = (V_1 - V_0) \times \varphi_{Bt_c1} + (V_2 - V_1) \times \varphi_{Bt_c2} + \ldots = \sum V_{Methane} \tag{A.1}$$

A similar equation was applied by Kafle and Chen (2016) to combine the summarized online BMP biogas volumes as recorded by an automated gas chromatography system. Afterwards, the CH_4 concentration ($\varphi_{Methane}$ in %) was calculated with Eq. (A.1) using the ratio of CH_4 volume ($\Sigma\ V_{Methane}$) according to Eq. (A.1) and the sum of the cumulative online values of biogas volume at standard conditions STP ($\Sigma\ V_{Biogas}$), which was automatically obtained with the MGC-station. Finally, the methane content was expressed as percentage.

$$\varphi_{B_{Methane}} = \frac{\sum V_{Methane}}{\sum V_{Biogas}} = \frac{V_{Methane}}{V_{Biogas}} \times 100\% \tag{A.2}$$

In order to eliminate additional CH_4 content obtained only from the inoculum, the CH_4 content of the blank reference assays with inoculum (n = 5), were also taken into consideration and subtracted as shown in Eq. (A.3).

$$\varphi_{B_{Methane}} = \frac{\sum V_{Methane_{Sample,Corrected}} - \sum V_{Methane_{Blank,\ \ Corrected}}}{\sum V_{Biogas_{Sample}} - \sum V_{Biogas_{Blank}}} \times 100\% \tag{A.3}$$

Therefore, using Eq. (10), the experimental CH_4 content of biogas from the wheat straw WS as substrate was obtained. A record of the in-situ measured methane production of WS and pure cellulose as reference substrate during anaerobic digestion is shown in Fig. 2B.

A.2. Error of methane concentration caused by air in the headspace of incubation vessels

Flushing the headspace with an inert gas was omitted, because it was observed that flushing with N_2/CO_2 or N_2/O_2 (air) had no effect on methane yields. Also Raposo et al. (2012) concluded, that there is no difference between the gas yields of BMP assays with N_2/CO_2 or N_2/O_2 (air), as was observed in an inter-laboratory study (Raposo et al., 2011). But Koch et al. (2016, 2015) found 20–30% increase in methane yields after using N_2 and CO_2 as inert gas. However, their experiments were performed with only sludge blank values without any substrate, whereas in the study by Raposo et al. (2011) the equally treated blank values were subtracted from the simultaneously produced gas values from sludge plus added substrate. Subtracting the yields of the blank assays, with air in the vessels, apparently eliminated this type of error. But at the commencement of the batch assays of this study, a mixture of air and biogas was in the head space of the incubation vessels, which could falsify the in-situ methane measurement. It is a continuous process, as the air is displaced by the production of biogas during anaerobic digestion of a substrate. The same is evident when an inert gas is used instead of air. The volume of air or inert gas in the digestion apparatus should be determined and subtracted from the gas yields. The headspace had a volume of 122 mL and the volume of the gas tube was about 42 mL. About 98% of the air would be driven out after about 500 mL of biogas is produced. This was based on the assumption that the total headspace of a single incubation vessel had a total volume of 164 mL, including 42 mL by the gas tube of the MGC, Fig. 1A and C. It was further assumed, that the biogas produced by the anaerobic microbes did not contain any air and that the biogas produced was an ideal gas. As a result, the volume in percentage of a recently produced biogas volume, should be equal to the quotient of the difference between the biogas volume produced at the time t (F_t) and the standard time interval of two hours of the online recording at $t_1 = t_{-1}$ ($V_t - V_{t-1}$). This estimated biogas volume ($V_t - V_{t-1}$) was related to the volume of the headspace V_{HS} expressed as a percentage, as indicated by Eq. (A.4).

$$F_t = \frac{V_t - V_{t-1}}{V_{HS}} \times 100\% \tag{A.4}$$

However, Eq. (A.4) does not consider the presence of biogas/air mixture at the beginning t_1, in the headspace prior to the production of any new volume produced biogas. Therefore, Eq. (A.5) was introduced to determine the proportion β_t of biogas in the headspace at any given time.

$$\beta_t = F_t + \beta_{t-1} - \frac{\beta_{t-1} \times F_t}{100} \tag{A.5}$$

At the beginning of the experiment t = 1, there was no biogas in the headspace, before the first biogas was produced. Consequently, substituting t = 1 into Eq. (A.5), it follows $\beta_{1-1} = 0$. Therefore, Eq. (A.5) could be simplified to become $\beta_1 = F_1$ (volume fraction), see Eq. (A.4). This implies that the biogas concentration is generally equal within any part of the incubation vessels, because the term '$-(\beta_{t-1} \times F_t / 100)$' in Eq. (A.5) represents the gas output from the headspace.

Eq. (A.6) was used to correct the measured CH_4 content (φ_{tc}) in the headspace. The quotient of the measured CH_4 concentration (φ_{tm}) at any time t in the vessels and the corresponding proportion β_t of biogas at the same time t in the headspace were used.

$$\varphi_{tc} = \frac{\varphi_{tm}}{\beta_t} \times 100\% \tag{A.6}$$

By substituting Eq. (A.5) into Eq. (A.6), the resulting Eq. (A.7) was used to obtain the corrected headspace CH_4-concentrations (φ_{tc}) of the in-situ measurement in the BMP incubation vessel at any given time, t:

$$\varphi_{t_c} = \frac{\varphi_{t_m}}{F_t + \beta_{t-1} - \frac{\beta_{t-1} \times F_t}{100}} \times 100\% \varphi_{t_c} = \frac{\varphi_{t_m}}{F_t + \beta_{t-1} - \frac{\beta_{t-1} \times F_t}{100}} \times 100\% \tag{A.7}$$

To obtain the volumetric CH_4 yields for the substrate WS, the CH_4 concentration (φ_{tc}) of Eq. (A.7) was inserted into Eq. (A.1) with the summarized volumes. This was done for each point of the recorded values of the BMP estimation.

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