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Comparing methods for measuring the digestibility of miscanthus in bioethanol or biogas processing

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Abstract

Lignocellulosic biomass is a candidate for future renewable energy resources. Choice of optimum biomass types and biological conversion techniques requires well-founded assessment of the digestibility determining the conversion efficiency. The aim of this study was to investigate and evaluate the digestibility of miscanthus samples that were tested using three methods: 3,5-dinitrosalicylic acid assay (DNS), anaerobic batch digestion test, and high-throughput pretreatment and hydrolysis method, including a grinding and hydrothermal pretreatment prior to the analysis (HTPH). The miscanthus samples were expected to have different digestibilities due to maturity stage, dry matter content and the implementation of extrusion as a mechanical pretreatment. The results of the DNS and the biogas batch test methods were highly correlated (R^2 between 0.75 and 0.92), but not with the results of the HTPH method. The DNS and biogas batch test showed that digestibility differed between samples, probably due to the degree of lignification and content of soluble sugars. For the HTPH method, the digestibility for biorefining was the same irrespective of the variation in the other analyses. The HTPH method had higher biomass use efficiency, closely followed by the biogas batch test running for 91 days on the mechanically pretreated biomass. The HTPH method provided information on the overall quantity of carbohydrates that can be made available from a given biomass. Additionally, DNS and biogas batch test visualize the variation in digestibility between biomass types caused by lignification and particle. The study concludes that the choice of evaluation method for miscanthus will depend on the bioenergy conversion method used and that important information on the interaction between physio-chemical pretreatment and biological accessibility of the biomass can be obtained by comparing the methods. This information will enable sound decisions on the future choice of bioenergy conversion technologies.

Keywords: 3,5-dinitrosalicylic acid assay, energy crop, enzymatic saccharification, harvest time, hydrolysis, methane yield

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Introduction

The transition from using fossil to renewable resources has advanced the research on the processing of renewable biomass resources into fuels and chemicals. However, the need for a parallel supply of food and fuels has shown that the biomass supply should preferably be based on nonedible lignocellulosic biomass. A number of lignocellulosic residues and crops are available or have high yields and as such are attractive feedstocks (Bentsen & Felby, 2012), but compared to starch-based feedstocks lignocellulose is highly recalcitrant and thus more difficult to process. Utilizing the carbon from such recalcitrant lignocellulosic structures for energy carriers can provide renewable and storable carbon-based fuels for the transportation sector which are difficult to produce from other renewable sources such as the sun, wind and hydropower. The main component of biorefining is the sustainable processing of lignocellulose-derived sugars in a cascade of processes transforming them into a spectrum of biobased products and fuels (Jungmeier *et al*., 2013; Parajuli *et al*., 2015). In this context, digestibility, that is the ease by which the processed biomass is converted into fermentable sugars, has been the subject of a number of studies related to the biorefining process for bioethanol or biogas production (Hendriks & Zeeman, 2009; Weiland, 2010; Lindedam *et al*., 2012). Both bioethanol and biogas are important bulk products in biorefining processes and are consequently the focus of the present study. Bioethanol is a liquid fuel which can be directly blended

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into petrol, whereas biogas (methane) can replace natural gas used in heat and power plants, for transportation or as a feedstock in the petrochemical industry. As a fuel, bioethanol has an advantage over biogas in that it can easily be incorporated in the existing transportation systems and partly or fully substitute fossil fuels. The technology of biogas production is established and can be implemented in simple set-ups for single households or small communities in developing countries (Katuwal & Bohara, 2009) or as part of bigger complex plants feeding a larger gas grid (Berglund & Borjesson, 2006). Biorefineries that convert lignocellulosic biomass to bioethanol are technically more complex and have only recently been established on a commercial level (Somerville *et al*., 2010). Moreover, the biomass retention time in the bioethanol process is usually shorter than in the biogas process – an important factor determining the capacity of the processing plant. The more readily the lignocellulosic biomass can be degraded by enzymes or microorganisms, the higher the rate of conversion of lignocellulosic carbon into energy carriers such as biogas and bioethanol (Hendriks & Zeeman, 2009). Higher digestibility and conversion rates will decrease the retention time as well as the capacity of the process plants by producing more energy per production unit.

Digestion of recalcitrant biomass is performed by complex interactions of enzymes and microorganisms, and many factors affect the digestibility. The factors can be divided into chemical factors, such as content and structure of the polymers cellulose, hemicellulose and lignin, and physical factors, such as particle size and surface area. Furthermore, the organization of the polymers in the cell wall matrix further affects the availability and digestibility of the cellulose and hemicellulose as particularly the lignin presents a physical barrier to microorganisms and enzymes (Fu *et al*., 2011).

Plant species vary in their suitability for biological conversion, that is due to variations in digestibility (Karp & Shield, 2008; Somerville *et al*., 2010), just as the digestibility varies between the components of a given plant (Hayes, 2013; Zhang *et al*., 2014b). Miscanthus is the focus of this study because this perennial, rhizomatous, C_4 crop is one of the highest-yielding energy crops in Europe (Hastings *et al*., 2009) which combines with a low environmental impact (Hamelin *et al*., 2012). The composition of leaves and stems from miscanthus differs (Hodgson *et al*., 2010; Hayes, 2013; Wahid *et al*., 2015b), and the lower stem part of miscanthus has a higher lignin content than more juvenile plant parts (Huyen *et al*., 2010; Hayes, 2013). The composition of the plant will change with the maturing of the plant from autumn to late winter by, for example, increasing the lignin content (Jørgensen, 1997; Hayes, 2013) and decreasing the ash content (Lewandowski *et al*., 2003).

The lignified fibres and crystalline cellulose are difficult to degrade biologically, but the crystallinity does not *per se* affect the digestibility (Caulfield & Moore, 1974). Decreasing the crystalline particle size and increasing the surface area of the cellulosic biomass are known to improve the enzymatic accessibility of the biomass and the hydrolysis of the lignocellulosic content (Caulfield & Moore, 1974; Hendriks & Zeeman, 2009; Surendra & Khanal, 2015). Lignocellulosic biomass is recalcitrant to degradation, which means that lignocellulosic biomass, due to its low digestibility, needs a chemical or physical pretreatment to saccharification in the biorefining process (Hendriks & Zeeman, 2009).

As the conversion of biomass is controlled by a number of factors and their interactions, an integrated approach is needed when evaluating digestibility. However, normally just one methodology is used to evaluate biomass convertibility: for example, measuring the convertibility of biomass to ethanol using different thermo-chemical pretreatments (Jørgensen *et al*., 2007), measuring the amount of sugars enzymatically hydrolysed from the biomass (Wahid *et al*., 2015a) or using biogas batch tests (Wahid *et al*., 2015b). In the current study, three methods were used in parallel as the combined information was hypothesized to provide additional information. The three methods included a biogas batch test measuring the methane yield, an enzymatic saccharification followed by quantification of total sugars using a spectrophotometer, and a hydrothermal pretreatment followed by enzymatic saccharification and quantification of glucan, xylan and arabinan using HPLC (Zhang *et al*., 2014a). Table 1 shows the details and a comparison of the methods.

The overall objective of this study was to evaluate the digestibility of biomass from miscanthus using three different methods and measuring the conversion into either biogas (methane) or fermentable sugars. The biomass samples were chosen to cover the biological variation induced by different harvest times and the altered particle size caused by mechanical extrusion.

Materials and methods

Biomass

Miscanthus (*Miscanthus* × *giganteus*) was established in field experiments at Aarhus University in Foulum, Denmark (56.49N, 9.55E), in 2001, and the field has been maintained and harvested annually during winter with the last harvest in 2012. Biomass for this experiment was harvested early, intermediate and late in the harvest season 2013–2014 using a forage harvester. Samples were stored at −18 °C before further analysis.

Table 1 Comparison of experimental conditions for the three methods

	Biogas batch test	DNS	HTPH
Condition of input biomass	As received	As received	Dried (40 °C) and ground
Experimental pretreatment	None	None	Hydrothermal (190 °C, 10 min)
Enzymes	Inoculum	Cellulase	Cellulase
		Mannase	β-Glucosidases
			Hemicellulase
Temperature	35 °C	50 °C	50 °C
Time	91 days	72 h	72 h
Measured variables	Methane yield	Reducing sugars	Glucan
	Methane concentration in the gas		Xylan
			Arabinan

After thawing at room temperature, the samples were shredded twice (Untha RS 30 4-2, Kuchl, Austria) through a 4-cm sieve to eliminate long stem and leaf parts. The majority of particles were then shorter than 4 cm with an average of approximately 1 cm.

Harvest time changes both the composition and dry matter content of biomass, and to differentiate the effects, the late harvest was randomly subdivided into three samples. One sample was kept at its original dry matter concentration, while the dry matter content of the other two samples was modified by the addition of water to achieve low, medium and high (the original) dry matter concentrations. Water and biomass were blended using a concrete mixer, and the biomass and water mixture was then left for 24 h at 4 °C to let the water saturate the biomass. Samples from early and intermediate harvests were used in their original dry matter concentrations.

Mechanical pretreatment

Subsamples were taken from all biomass types and pretreated mechanically by extrusion to introduce more variation into the digestibility. The extrusions were performed by a corotating twin-screw extruder (PSHJ-65, Xinda Corporation, Jiangyin, Jiangsu, China, barrel length: 2.84 m) with 340 mm of kneading followed by reverse kneading (56 mm). The kneading causes a particle size reduction, and the reverse kneading forces the biomass to change direction, resulting in a build-up of biomass inside the barrel; more force is needed to move the compact biomass and the close contact with the barrel and extruder screw increases the effect due to the build-up, and the barrel heats up due to the friction. The temperature measured was 20 °C at the feeding point and increased steadily to 100–105 °C after 1.42 m. In the rest of the extruder, the temperature was stable (±5 °C), and the last element of the barrel was cooled down to avoid evaporation of water, which would bias the mass balance.

3,5-Dinitrosalicylic acid assay – sugar availability measure

Enzymatic saccharification using 3,5-dinitrosalicylic acid (DNS) assay (Selig *et al.*, 2008) and spectrophotometric quantifications of the total amount of sugars enzymatically hydrolysed from the biomass (Adney & Baker, 2008) were carried out. Hydrolysis was performed in 100-ml blue-cap bottles. Biomass corresponding to 3.5 g dry matter was mixed with 0.928 g cellulase (Celluclast 1.5L; Novozymes, Bagsværd Denmark) and 0.208 g mannanase (Novozym 51054; Novozymes), and to prevent unwanted growth of microorganisms, 1 ml 2% sodium azide was added. To this was added a 50 ml citrate buffer (pH = 4.8) and finally demineralized water to obtain a total of 100 g. Hydrolysis ran for 72 h at 50 °C in a shaking incubator (185 rpm). All samples including enzyme and cellulose controls were analysed in triplicate.

The quantification of sugars was performed by DNS assay. The bottles were shaken and a sample was extracted and centrifuged, after which an aliquot was extracted from the supernatant and diluted (1 : 9 and 3 : 7 in the samples with high and low sugar yields, respectively) with demineralized water. Glucose standards (concentration of 0 to 1.00 g glucose l^{-1}) were made and analysed with the samples to obtain a standard curve for evaluation of the samples. One millilitre of 3,5-dinitrosalicylic acid solution was added to the diluted samples, and colour was developed during 5 min of boiling (Miller, 1959). The samples were homogenized by shaking, and 280 μl was extracted and transferred to a microtiter plate for spectrophotometric measurement at 538 nm. All measurements were corrected for blanks, and the sugar contents were calculated based on the standard curve.

Biogas batch test

The anaerobic biogas production was measured in a batch test using inoculum from Bånlev biogas plant, Aarhus, Denmark. The plant primarily uses pig manure as a feedstock, but also deep litter, slaughterhouse residues and industrial wastes (mainly lipids). The inoculum was stored in mesophilic conditions (35 °C) for 14 days to halt the biogas production from the inoculum. The properties of the inoculum after storage were 3.06% dry matter and 1.62% volatile solids. A 200-g inoculum and biomass mixture (in a substrate/inoculum ratio of 1 : 1 based on volatile solids) was put in 1-l glass bottles. All biomass types were analysed in triplicate and the experiment also included a control containing inoculum only. The bottles were

closed with butyric rubber stoppers and a metal seal. Oxygen was removed from the bottle by flushing with N_2 for 2 min, and the bottles were shaken and stored in an incubator under mesophilic conditions (35 °C). During the following 91 days, the volume of produced biogas (mixture of methane and carbon dioxide) was frequently recorded using an acidified water displacement method, and the methane-to-carbon dioxide ratio was measured using a gas chromatograph (Gas Chromatograph System 7890A, Agilent Technologies, Santa Clara, CA, USA, with Agilent Technologies GC sampler 80) equipped with a thermal conductivity detector and a flame photometric detector, and the methane yield was corrected for the methane yield from the inoculum.

High-throughput pretreatment and hydrolysis method

The high-throughput pretreatment and hydrolysis set-up (HTPH) for evaluation of the conversion efficiency of the biomass into sugars was set up at University of Copenhagen as described by Zhang *et al*. (2014a) including an automated plant material grinding and dispensing set-up from Labman Automation Ltd (Stokesley, North Yorkshire, UK). The biomass was ground to powder and 0.028 g was dispensed into a 96-well aluminium plate, 422 μl sodium citrate buffer (pH = 4.8) was added and the plate was closed with a Teflon plate and a clamp. The plate was heated to 190 °C for 10 min, acting as a hydrothermal pretreatment. Subsequently, the plate was cooled, enzymes (Cellic Ctec, 20 FPU) were added and the hydrolysis ran for 72 h at 50 °C in a shaking incubator (600 rpm; Heidolph Titramax, Schwabach, Germany). The samples were filtered and the contents of glucan, xylan and arabinan measured using a Dionex Ultimate HPLC system (Sunnyvale, CA, USA). A schematic overview and comparison of the three methods can be found in Table 1.

Calculations and statistical analysis

Data on glucan, xylan and arabinan from the HTPH analysis and sugar contents from the DNS analysis were analysed using ANOVA and linear models to evaluate the effect of harvest time, pretreatment (with or without extrusion of biomass) and their interaction. When no interaction was significant, the significance of the fixed effects was analysed using the 'general linear hypotheses' of the 'multcomp' package. The effect of three dry matter concentrations at the late harvest was evaluated with a linear model that contained the three dry matter levels of the late harvest, pretreatment (with or without mechanical pretreatment of biomass) and their interaction. The analyses of data with interaction were computed using the least squares means of the 'lsmeans' package in R. All statistic computations were performed using R version 3.1.3.

The methane yield was calculated in normalized litres (l_N) by correction to 0 °C and 1.013 bar and converted into g CH_4 and g CO_2 as a percentage of biomass using the ideal gas law and the methane-to-carbon dioxide ratio measured in the biogas batch test.

Results

3,5-Dinitrosalicylic acid assay – sugar availability measure

DNS assay was able to discriminate between pretreated and nonpretreated samples and only the early harvest yielded significantly more sugar than the other four biomass types (Fig. 1a). Comparing only samples from different harvest dates (all original dry matter concentration) or only late harvest with different dry matter contents, the amount of total enzymatically hydrolysed sugars from the DNS method depended significantly on the dry matter concentration (of the late harvests), the harvest time (comparing biomass types with their original dry matter concentration) and the use of pretreatment. The results show that the more lignified, mature miscanthus is more inaccessible to the enzymes.

However, the mechanical pretreatment was able to reduce the particle size and break the fibre structures whereby the sugar yields were higher than from the nonpretreated miscanthus samples.

High-throughput pretreatment and hydrolysis method

A significant positive effect on glucan yield from the mechanical pretreatment was found on the two biomass types (intermediate harvest, and late harvest with medium dry matter content). In contrast, there was a significant negative effect of the mechanical pretreatment on the late harvest with a high dry matter content. Where samples were not pretreated, there was no effect of biomass. For the pretreated samples with a high dry matter content, the glucan yield of the late harvest was significantly lower than early, intermediate and late harvest with medium dry matter concentrations. The HTPH analysis, on the other hand, revealed no clear pattern for the xylan yield from treatment, dry matter concentration or harvest time (Table 2 and Fig. 1b).

Biogas batch test

Pretreatment resulted in a significant positive effect on all biomass samples at all sampling dates from day 24. The early harvest gave the highest methane yield and methane + CO_2 (biogas) yield compared to all other investigated samples ($P < 0.05$). The pretreatment had a positive effect at all harvests (Fig. 1c,d). The later the biomass harvest, the lower the methane yield, starting at day 18 of the biogas batch test measurements, with significant differences among early, intermediate and late harvests with the original dry matter concentration.

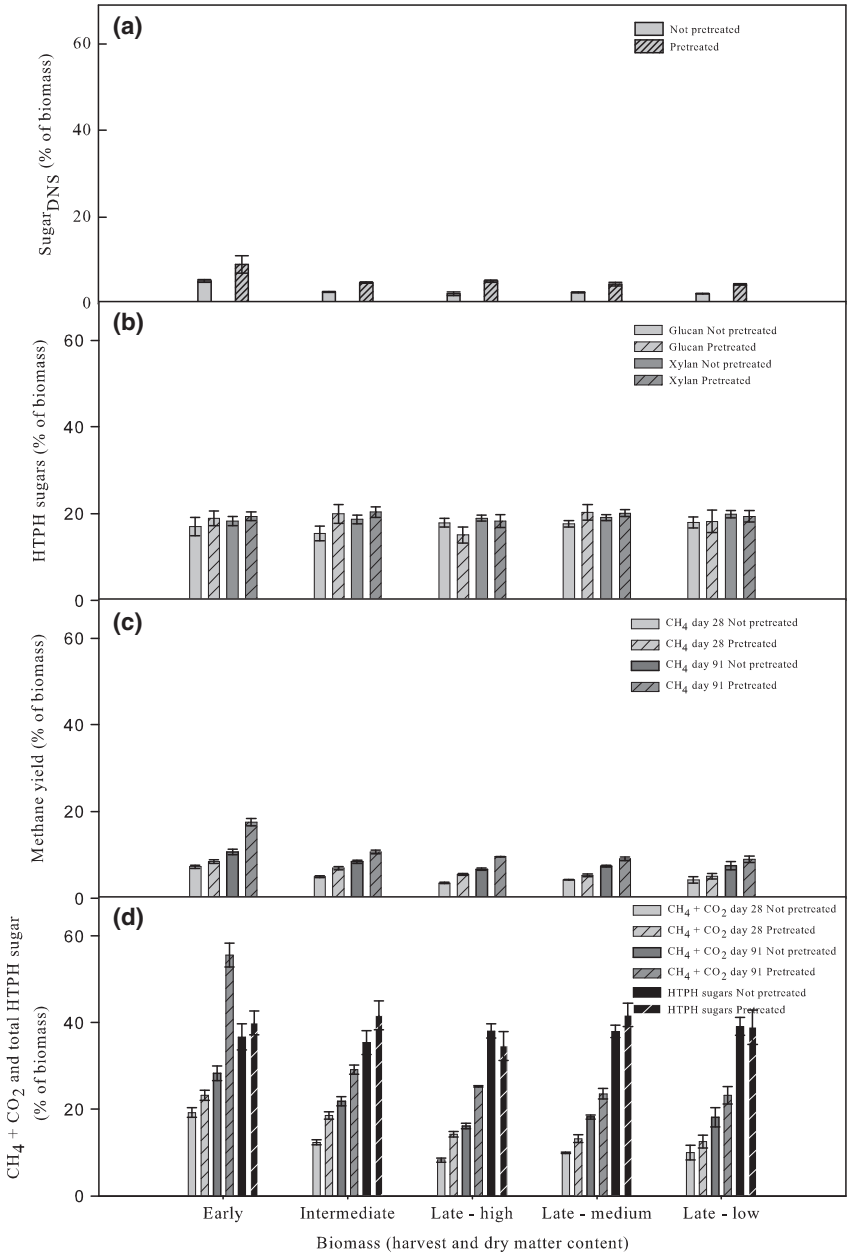

Fig. 1 Yield of enzymatically hydrolysed sugars, methane and biogas at different harvest times and dry matter contents (late harvest). (a) Total amount of sugars hydrolysed in the DNS experiment as a percentage of the biomass; amount of (b) glucan (light grey) and xylan (dark grey) hydrolysed by the HTPH method; (c) methane (CH_4) yield after 28 days (light grey) and 91 days (dark grey) and (d) sum of produced biogas (methane and carbon dioxide) after 28 (light grey) and 91 (dark grey) days and sum of hydrolysed sugars from the HTPH method (black), shown as percentages of the biomass. Hatched bars show extruded biomass whereas bars not hatched show the biomasses that are not pretreated.

The artificial modification of dry matter concentration had no effect on the methane yield of the late harvest for either the extruded or untreated biomass. The effects of harvest time and pretreatment on methane yields at day 91 showed significant interactions (Table 2).

Correlation between methods

The DNS method and methane data correlated well, the correlation coefficient (R^2) being 0.92 at day 91 of methane yield measurement and 0.82 at day 28 (Table 3). The coefficient for earlier methane yields was

Table 2 Significance levels of experimental parameters (dry matter concentration (DM), extrusion, harvest and interactions) for total sugar content (DNS), glucan, xylan and arabinan measured after high-throughput pretreatment and hydrolysis and methane (CH_4) yield after 28 and 91 days. The upper part compares biomass types from the late harvest with different dry matter contents (Late-low, Late-medium and Late-high) and in the lower part are biomass types with the original dry matter content compared (Early, Intermediate and Late-high)

	DNS sugars	Glucan	Xylan	Arabinan	CH_4 day 28	CH_4 day 91
DM	***	0.12	0.18	***	0.60	***
Extrusion	***	*	0.12	**	***	***
DM*Extrusion	0.15	**	0.11	0.08	0.09	***
Harvest	***	0.24	0.25	***	***	***
Extrusion	***	0.12	0.13	*	***	***
Harvest × Extrusion	0.31	**	0.11	*	0.10	***

Indicated P-values: ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

Table 3 Correlation coefficient (R^2) of DNS sugars, glucan, xylan, arabinan, methane yield and $CO_2 + CH_4$ yield, the latter two after 28 and 91 days

	DNS	Glucan	Xylan	Arabinan
Glucan	0.01			
Xylan	0.00	0.76		
Arabinan	0.89	0.12	0.01	
Methane day 28	0.82	0.04	0.00	0.77
Methane day 91	0.92	0.05	0.00	0.91
$CO_2 + CH_4$ day 28	0.81	0.04	0.00	0.75
$CO_2 + CH_4$ day 91	0.91	0.05	0.00	0.90

0.75 for day 18, and increasing until the end of the experiment at day 91, although data are only shown for day 28 and 91. Contrastingly, the correlations between DNS, biogas or methane and glucan or xylan from the HTPH method were poor (Table 3). Arabinan from the HTPH method showed a high correlation with DNS ($R^2 = 0.89$), methane at day 28 and 91 ($R^2 = 0.77$ and 0.91, respectively) and biogas at day 28 and 91 ($R^2 = 0.75$ and 0.90, respectively), whereas the correlations with glucan and xylan from the HTPH method were poor ($R^2 = 0.12$ and 0.01, respectively).

Discussion

The DNS and biogas batch test methods were able to document the expected change in digestibility of biomass types due to a reduction in particle size (Caulfield & Moore, 1974; Hjorth *et al.*, 2011; Surendra & Khanal, 2015). Both the DNS and the biogas batch methods showed a significant positive effect of pretreatment, and the glucan yield of HTPH revealed a difference between the pretreatments for three of the five biomass types. Moreover, DNS and methane yields decreased with later harvests, as also shown by Wahid *et al.* (2015b). In contrast, the sugar yield from HTPH was not related to harvest times, which is inconsistent with the findings of Hayes (2013) who modelled an increased yield of ethanol per tonne of miscanthus when harvests from November to April were compared.

The starting condition for the HTPH method is dried and ground biomass, which is likely to change its digestibility as particle size influences digestibility (Caulfield & Moore, 1974), and this processing will minimize the physical difference between pretreated and nonpretreated biomass. Secondly, HTPH quantifies the sugars hydrolysed by enzymes after opening of the fibres by hydrothermal pretreatment, redistributing lignin and hemicellulose and even removing hemicellulose (Jørgensen *et al.*, 2007; Hendriks & Zeeman, 2009), and in this way hiding the physical difference between the pretreated and nonpretreated biomass. The DNS method, on the other hand, analysed the raw miscanthus samples with intact fibre structures only exposed to cutting and possibly extrusion. The hydrothermal pretreatment was very effective in degrading fibres of green and mature lignified biomass (Fig. 1b). The result was a 3–15-fold higher sugar yield compared to DNS sugar yields (Fig. 1a and d).

The DNS and HTPH methods both involve enzymatic hydrolysis of the biomass, although different enzymes were used for the two methods whereas duration and temperature were similar (Table 1). The inoculum used in the biogas batch test was a liquid consisting of a cocktail of cellulytic and other enzymes and microbes adapted to the environment in the biogas tank. This cocktail is able to degrade the substrates normally available in the inoculum, providing energy sources for the methanogens on which methane yield depends. The inoculum in this experiment was adapted to industrial and slaughterhouse waste and lignocellulosic material (wheat straw from deep litter). The microbiota therefore needed some time to adapt to the new miscanthus feedstock, which resulted in a slight delay in biogas production compared to what would be expected if the inoculum had already been adapted to the substrate.

The methane yields in the current study were 50–123 l_N CH_4 kg^{-1} VS at day 28 and 95–258 l_N CH_4 kg^{-1} VS at day 91, which was lower than those found by Wahid *et al.* (2015b) of approximately 200 and 300 l_N CH_4 kg^{-1} VS at days 30 and 90, respectively. The biomass converted by Wahid *et al.* (2015b) was green and the inoculum used was better adapted to lignocellulosic material (Moset *et al.*, 2015), explaining the higher methane yields. The biogas batch test and DNS method were apparently able to quantify the digestibility of the existing fibre composition and to discriminate among different pretreatments.

Another important difference between the methods was that the DNS and HTPH methods measure sugars from which a theoretical bioethanol or biogas output can be calculated. The biogas batch test, on the other hand, quantifies the output of the energy carrier, methane. Comparing the total output from the HTPH method (sum of the sugars) and the biogas batch test (sum of methane and CO_2) reveals that the HTPH technique facilitates a more efficient conversion of the biomass (Fig. 1d). A 100% fermentation of the hydrolysed sugars into ethanol is impossible to obtain and consequently the proportional ethanol yield from the biomass is expected to be slightly smaller than the proportional sugar yield from the biomass (Hayes & Hayes, 2009).

The DNS and biogas batch test results are well correlated (Table 3) and similar to the findings of Wahid *et al.* (2015a) after 28 days of methane production (R^2=0.70). This underlines that enzymes used in the DNS method and the mixture of microbiota and enzymes in the inoculum of the biogas batch test have a comparative ability of degrading the biomass. By contrast, methane, biogas and sugar yields from the DNS were not correlated to glucan or xylan (Table 3) due to the difference between the methods (Table 1).

The HTPH set-up is designed to evaluate biomass for the biorefining to ethanol in the Inbicon plant design (Larsen *et al.*, 2012). In Inbicon, hydrothermal pretreatment is chosen because of and efficiency. Modification of the HTPH pretreatment such as lowering the temperature or duration of the treatment could allow a range of physiologically different samples to be produced from which the biomass type (e.g. harvest time) with the lowest pretreatment requirements (and thus lowest costs) relative to its energy yield could be identified. Alternatively, at a given price you could choose the most cost-effective pretreatment for a given biomass to fit the bill.

The coherence between the DNS and the biogas batch test results suggests that the DNS method can be used as a rapid test method, as it allows comparisons of relative methane yields of a certain biomass in only 72 h compared to the 91 days for the batch test. DNS may also be a more accurate method as the exact methane yields from batch tests will depend on the variation in inoculum.

The HTPH is a model system for the biorefining, and our results revealed that the variation in quality of miscanthus for enzymatic hydrolysis was not important if a severe hydrothermal pretreatment was applied and that the convertibility did not depend on, for example, harvest time. For biogas production, on the other hand, the quality of miscanthus was highly dependent on the timing of the harvest.

In conclusion, the methane measurements from the biogas batch test and the DNS method were highly correlated (R^2 = 0.75 at day 18 of the biogas batch test and increasing at every sampling date to R^2 = 0.92 on day 91) because both methods are based on similar physical biomass input conditions. The HTPH method measures glucan and xylan which did not correlate well with the results of the biogas batch test or the DNS method due to the hydrothermal pretreatment prior to the enzymatic hydrolysis in HTPH. The hydrothermal pretreatment very efficiently breaks and opens the lignocellulosic structures and the differences between the miscanthus samples are thereby eliminated. As a result, HTPH provides information on the overall quantity of carbohydrates that can be made available from a given biomass. Additionally, the DNS and biogas batch test methods visualize the variation in direct digestibility between biomass types caused by lignification and particle size, dependent on harvest time, extrusion and dry matter concentration. Thus, a combination of methods (e.g. DNS plus HTPH) can provide a more complete picture of potential and actual accessibility of lignocellulosic material for biological conversion. This is helpful when choosing the most cost-efficient combination of biomass production (e.g. harvest time), pretreatment (e.g. hydrothermal or extrusion) and final conversion method.

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