An OxiTop® protocol for screening plant material for its biochemical methane potential (BMP)

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ABSTRACT

A protocol was developed for determining the biochemical methane potential (BMP) of plant material using the OxiTop® system. NaOH pellets for CO_2 absorption and different pretreatment methods were tested for their influence in the BMP test. The use of NaOH pellets in the headspace of the bottle negatively affected the stability of the test increasing the pH and inhibiting methanization. Sample comminution increased the biodegradability of plant samples. Our results clearly indicate the importance of test conditions during the assessment of anaerobic biodegradability of plant material, considering BMP differences as high as 44% were found. Guidelines and recommendations are given for screening plant material suitable for anaerobic digestion using the OxiTop® system.

Key words | anaerobic biodegradability, BMP test, energy crops, NaOH pellets, pretreatments

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INTRODUCTION

Production of biogas and valuable digestate from materials of plant origin through anaerobic digestion (AD) is an interesting alternative to add value to different biomass chains, to minimize environmental problems related to inadequate management of residues, and to provide an alternative use for land for energy generation purposes. Given the immense diversity of lignocellulosic materials that can be converted to methane, an accurate and simple method is needed to screen for those materials best suited for digestion. Within the EU Project 'Cropgen', in which the potential of European crops and agro-residues for methane production was studied, the revision and simplification of the biochemical methane potential (BMP) test was prioritized. To do so a BMP protocol was developed, adapted for the use of the OxiTop® pressure monitoring system (WTW, Giessen, Germany).

The OxiTop® system is a pressure monitoring device originally developed for biochemical oxygen demand (BOD) measurements. The system comprises the measuring heads and a controller, and uses an infrared interface for data transfer. The main advantage of the system is the possibility of carrying out up to 100 measurements in parallel minimizing human interference. The pressure data are collected automatically at time intervals defined by the user, it can be graphically displayed on the controller at any time and can be downloaded to the computer in Excel format for analyzing the results. However, the OxiTop® system has as a major limitation the pressure limit of the measuring head, i.e. 0.30 atm which causes restrictions on the amount of sample that can be used in the experiments. Consequently, achieving sufficient sample representativeness from non-homogeneous material such as crops and agro-residues can be problematic. In addition, by measuring pressure, changes in the CH_4/CO_2 ratio are not determined, possibly affecting the results. Within the CROPGEN project, priority was given to overcome this limitation by studying the impact of the use of NaOH pellets for CO_2 capture and different pretreatment/storage methods to homogenize substrates.

As the produced overpressure in the batch bottle consists mainly of CH_4 and CO_2, absorbing the CO_2 would permit doubling of the sample amount and allow for test simplification as the need for gas analyses for assessing energy content could be avoided. Rudrum (2005) used the OxiTop® system for composting stability measurements using NaOH pellets as CO_2 absorbent. In anaerobic measurements the use of NaOH pellets has been studied

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by Souto *et al*. (2009) reporting only a minor influence in the specific methanogenic activity (SMA) test.

Although it is ideal to test substrates in a form close to reality, pretreatments are used in laboratory tests both to achieve sample representativeness and to cope with the restrictions imposed by the available experimental set-up and the decay of the substrates. Altering the particle size of the substrates is used in the first case, whereas freezing or drying samples is employed to overcome decay limitations. Particle size reduction has been reported to influence anaerobic biodegradability (Chynoweth & Jerger 1985; Sharma *et al*. 1988; Palmowski & Muller 2000), with both the rate and extent of degradation of plant material being affected. Conversely, other authors have reported minor or no influence of the particle size reduction on the anaerobic degradation of paper/cardboard mixtures and industrial wastes (Hansen *et al*. 2007; Pommier *et al*. 2010). Freezing and drying can potentially exert changes on the physical and chemical properties of the material. Freezing adversely affects the texture of nearly all plant tissues, due to cellular dehydration and the accumulation of ice in the intercellular spaces (Thomashow 1998). Oven drying has been reported to alter the chemical composition of plant samples by inducing the loss of energy containing volatile organic matter (Broesder *et al*. 1992), and causing the non-enzymatic browning effect where polymerization of sugars with aminoacids results in a brown complex similar to lignin (Parissi *et al*. 2001). In both cases, cell compounds could be released and/or new surfaces created for biodegradation to take place.

Whereas many necessary guidelines have been released as a response to the need for standardization of the BMP test (Angelidaki *et al*. 2009), the influence of freezing and drying samples in the BMP testing of plant material has been insufficiently reported as well as the combined influence of particle size reduction and storage conditions. Further, the use of NaOH pellets for BMP assessment of complex substrates has not been sufficiently reported. Hence, within this research, the study of those factors under the conditions of an Oxitop test was undertaken.

MATERIALS AND METHODS

Substrates and pretreatments

Four substrates were used in this study and their characteristics are summarized in Table 1.

Two of the substrates were homogeneous in nature, i.e. green beans and endive, and two less homogeneous, i.e. mustard and carrots. The substrate samples were collected fresh, divided into their constituent parts and weighed separately. The total amount of sample was divided into four fractions following the proportions of their constituents: one portion used fresh cut in 1 cm pieces, a second was blended using a 40% dilution with demineralized water and being further divided into two fractions, one to be used fresh and the other to be frozen at −18 °C. A fourth fraction was dried at 65 °C during one day and ground to pass through a 1 mm mesh.

For drying plant samples a WTC Binder Labortechnik (Tuttlingen, Germany) oven was used at 65 °C, samples being left overnight. Freeze drying was performed in liquid nitrogen in a GRI 20–85 MP freeze drier (Wijk bij Duurstede, The Netherlands) equipped with two condensers. Comminution was performed in a Retsch BV grinder (Haan, Germany) equipped with a 1 mm sieving device, and blending was performed in a Turrax commercial laboratory blender.

Inocula

Digested primary sludge (DPS) originating from a sewage treatment plant (Ede, The Netherlands) was used as the main source of inocula. Characteristics of the inocula in terms of volatile solids (VS), chemical oxygen demand (COD) and SMA in acetate and glucose, were: 0.2 gVS g^{-1}, 1.64 gCOD gVS^{-1}, 34.03 ± 8.0 mgCOD gVS^{-1} d^{-1} and 86.98 ± 3.7 mgCOD gVS^{-1} d^{-1}, respectively. A S/I ratio of 0.4 (VS basis) was used to provide appropriate working conditions as described in the calculations section below.

Table 1 | Substrate characteristics[a]

Substrate	TS gDM g^{-1}	VS %DM	Total COD gCOD gVS^{-1}	NDF g gVS^{-1}	Lignin g gVS^{-1}	Hemi-cellulose g gVS^{-1}	Cellulose g gVS^{-1}	TKN G gVS^{-1}
Mustard	0.098	92	1.21	0.52 ± 0.00	0.06 ± 0.00	0.13 ± 0.00	0.33 ± 0.01	0.052
Carrot	0.132	92	1.36	0.17 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.07 ± 0.00	0.022
Endive	0.069	81	1.42	0.22 ± 0.01	0.05 ± 0.02	0.04 ± 0.00	0.13 ± 0.02	0.040
Green beans	0.079	93	1.41	0.19 ± 0.00	0.03 ± 00	0.05 ± 0.00	0.11 ± 0.00	0.058

[a]Mustard – *Brassica juncea*, Carrot – *Daucus carota*, Endive – *Cichorium endivia*, Green beans – *Phaseolus vulgaris*. TNK-Total Kjeldahl Nitrogen.

Experimental set-up

The set-up was a modified version of the method described by Owen *et al.* (1979). A schematic representation of the employed set-up is presented in Figure 1.

All the experiments were carried out under the addition of nutrients, using 500 ml serum bottles (600 ± 10 ml working volume), the contents occupying 150 ± 10 ml. Serum bottles were filled starting with the medium solution and demineralized water, followed by the addition of the inoculum and substrate. A phosphate buffer solution was used at a 20 mM concentration. When using NaOH pellets, they were dosed inside a plastic pellet holder with aeration holes just underneath the screw-cap for closing the vial at the top. Thereafter, they were flushed with N_2 gas for 1 minute and tightly sealed. The bottles were incubated at 35 °C and shaken at 120 rpm during the first 8 days of the assay, afterwards they were shaken occasionally. Biogas production was measured as pressure increase at constant volume, using the Oxitop® system. Biogas composition was measured daily during the first week and thereafter weekly until no more significant biogas production was observed, meaning pressure was not varying more than 5 HPa. The final BMP was calculated as the average of three consecutive daily measurements. Blank bottles, containing all additions except substrate were used to correct for inoculum methane production. Samples of green beans were used as a control to ensure optimal inoculum performance during digestion of complex plant substrate.

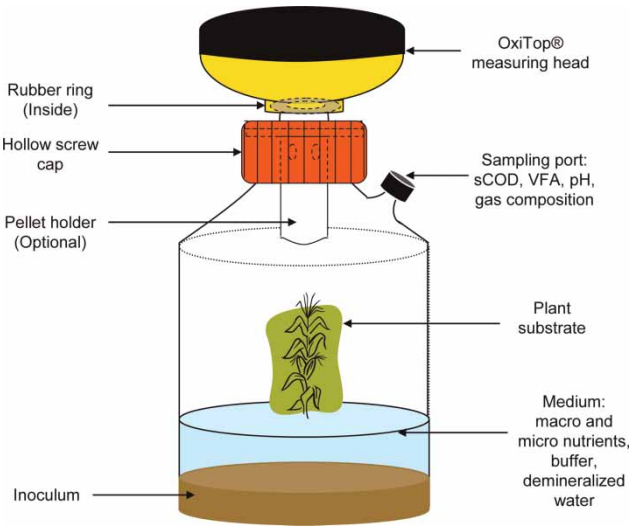

Figure 1 | Schematic representation of the batch set-up employed.

Experimental design

Two types of experiments were performed as shown in Table 2. In experiment 1 different amounts of NaOH pellets were tested in duplicate: 0, 0.25, 0.5, 1, 2 and 5 g, using endive as substrate. In a second set of experiments, combined sample storage and particle size reduction was studied using a minimum of three replicates with substrates undergoing four treatments: fresh 1 cm pieces, freshly blended, frozen blended and dry ground samples to pass a 1 mm mesh.

Analytical methods

Total solids (TS) and VS analyses were performed for the characterization of the substrates and sludges according to standard methods (APHA 1998). Total COD was measured by oxidizing a sample of suspended plant material, 20 g plant per L demineralized water, and using potassium dichromate under acidic conditions and using Ag+ as catalyst. The excessive amount of dichromate is determined through titrimetric analysis with Mohr's salt. Fiber analysis was performed according to the method of van Soest *et al.* (1991), briefly, 1 g of dried sample was analyzed using the crucible system. Reagents such as sodium lauryl sulfate, sulfuric acid and alfa-amylase were used. The sequential system was selected to determine neutral detergent fiber (NDF), acid detergent lignin (ADL) and neutral detergent acid detergent fibers (NDADF). Nitrogen analysis was performed according to a modified Kjeldahl method in which the sample is digested using sulfuric acid and hydrogen peroxide and copper sulfate as catalyst. All nitrogen is converted to ammonium sulfate, and ammonium is determined by

Table 2 | Outline of the experiments performed for optimization of an OxiTop® protocol for BMP determination

Experiment	Treatment	Type of substrate[a]
1. NaOH pellets	Amount of NaOH absorbent, i.e. 0, 0.25, 0.5, 1, 2 and 5 g	Endive – *blended and frozen*
2. Sample treatment	No pellets added Sample storage and comminution	Mustard, carrot, endive, green beans –*different pretreatments as described in text*
	No pellets added Drying samples at 65 °C	Mustard, Green beans – *fresh and dried*

[a]Mustard – *Brassica juncea*, Carrot – *Daucus carota*, Endive – *Cichorium endivia*, Green beans – *Phaseolus vulgaris*.

adding an excess of sodium hydroxide and by distilling the liberated ammonia. This free ammonium is collected in boric acid solution and titrated with hydrochloric acid solution (NEN 7434 1998). Volatile fatty acid (VFA) was analyzed in a Hewlett Packard 5890A gas chromatograph, the sample preparation and equipment settings used have been described in detail in Pabón Pereira *et al.* (2009).

Calculations

Preliminary calculations

The calculation of the amount of substrate to add in relation to the amount of inocula was performed considering: (1) the OxiTop® pressure limitation, (2) a minimum substrate concentration of approximately 1 gCOD L^{-1} to avoid mass transfer limitations, and (3) the recommendations provided by Angelidaki & Sanders (2004) of keeping a maximum S/I ratio proportional to the hydrolysis constant and the SMA of the inoculum.

Calculations for our set-up were performed as follows. The maximum pressure increase allowed by the OxiTop® measuring head is 0.3 atm. Hence, following the ideal Gas law, assuming a 50:50 CH_4:CO_2 gas composition and a liquid volume of 150 ml in the case of our set-up, the maximum allowed gas production at 35 °C is 0.0054 mol. If the CO_2 is not removed from the headspace 0.0027 mol CH_4 or 0.043 gCH_4 are expected to be produced. As stoichiometrically 4 gCOD are reduced per gCH_4, the maximum amount of substrate to add is 0.17 gCOD meaning a substrate concentration of 1.14 gCOD L^{-1} (Equation (1)):

$$S[\mathrm{gCOD}] = 32\left[\mathrm{gCOD}\cdot \mathrm{mol}_{\mathrm{CH_4}}^{-1}\right] \times 50\% \times \left(\frac{0.3[\mathrm{atm}]\times 4.5\times 10^{-1}[\mathrm{L}]}{0.08206\left[\mathrm{L}\cdot\mathrm{atm}\cdot\mathrm{mol}^{-1}\cdot\mathrm{K}^{-1}\right]\times 308.16[\mathrm{K}]}\right) \quad (1)$$

Departing from this figure a S/I ratio of 0.4 (VS basis) was selected to appropriately perform the batch tests considering a typical first-order hydrolysis constant for 1 cm particle size plant material of 0.2 d^{-1} and a SMA of the digested sludge used of 0.1 gCOD gVS^{-1} d^{-1}.

BMP calculations

BMP, expressed as liters methane at STP of 273 °K and 10^5 Pa per amount of VS added (lCH_4-STP gVS^{-1}), is calculated from the maximum methane production measured as volume increase in the sample bottle corrected by the maximum methane production of the blank bottle (Equation (2)):

$$\mathrm{BMP} = \frac{\left[\left[\frac{(P_s + P_{atm})*V_s}{R*T}\right]*\frac{\%CH_{4s}}{100}\right] - \left[\left[\frac{(P_{bl}+P_{atm})*V_{bl}}{R*T}\right]*\frac{\%CH_{4bl}}{100}\right]}{S_o} * 22.4 \quad (2)$$

where P_s is the pressure in the sample bottle (atm), P_{atm} is the atmospheric pressure (atm), P_{bl} is the pressure in the blank bottle (atm), V_s is the headspace volume of the test bottle (L), V_{bl} is the headspace volume of the blank bottle (L), T is the temperature (308.16 °K), R is the universal gas constant (0.08206 L atm mol^{-1} $°K^{-1}$), $\%CH_{4s}$ is the percentage methane in the test bottle, $\%CH_{4bl}$ is the percentage methane in the blank bottle, 22.4 is a conversion factor (L STP mol^{-1}) and S_o is the amount of substrate added (gVS).

RESULTS AND DISCUSSION

Effect of NaOH pellets in the BMP assessment

In the presence of the NaOH pellets, a concomitant accumulation of VFA and increase in pH occurred resulting in a lower CH_4 recovery. After one day incubation all treatments with pellets addition showed a sudden increment of the pH, which rose from 7.2 to 8.2–8.8 and remained high until the end of the treatments except in the bottles with 0.25 g pellets in which the pH was gradually reduced towards the neutral range.

After 32 days, 91, 36, 22, 18 and 18% of the biogas production relative to the bottle without pellet addition was recovered in the bottles with 0.25, 0.5, 1, 2 and 5 g pellets, respectively (Figure 2). The low methane production was accompanied by VFA accumulation (Figure 3). When correcting the total CH_4 production for that in the inhibited blank bottles overestimation of the net CH_4 production results. Overestimation of the BMP was also the result of incomplete absorption of CO_2 in the treatment with 0.25 g pellet addition. Due to this fact, biodegradability misinterpretation was strikingly evident in the 0.25 g pellet treatment, where the calculated BMP was 220% of that in the treatment with no pellet addition.

Results of the assessment of other plant materials performed as part of the CROPGEN Project showed similar results, i.e., the majority of the treatments performed using

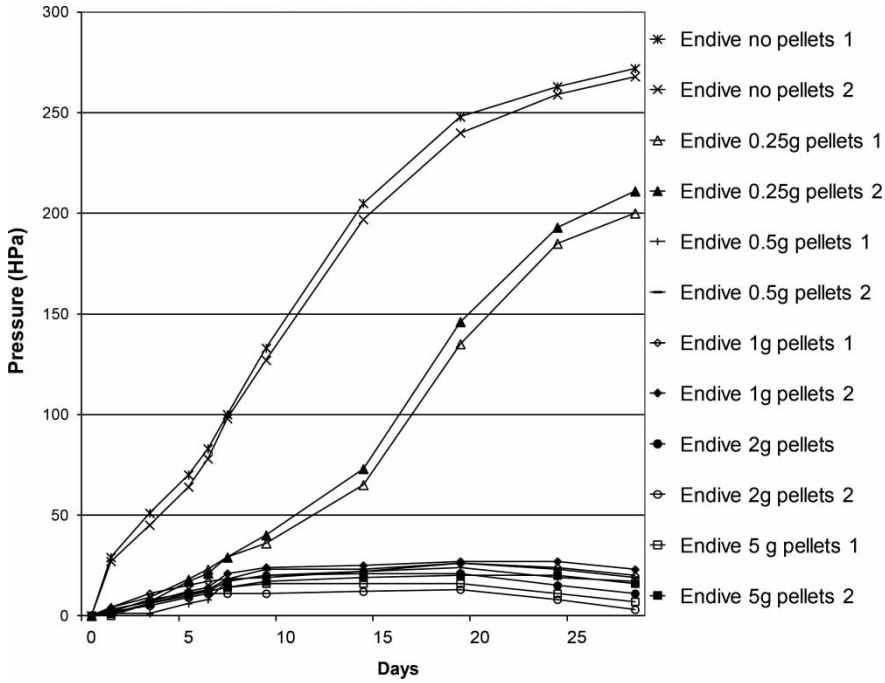

Figure 2 | Cumulative biogas production measured as pressure increase of samples treated with different amounts of NaOH pellets in the headspace.

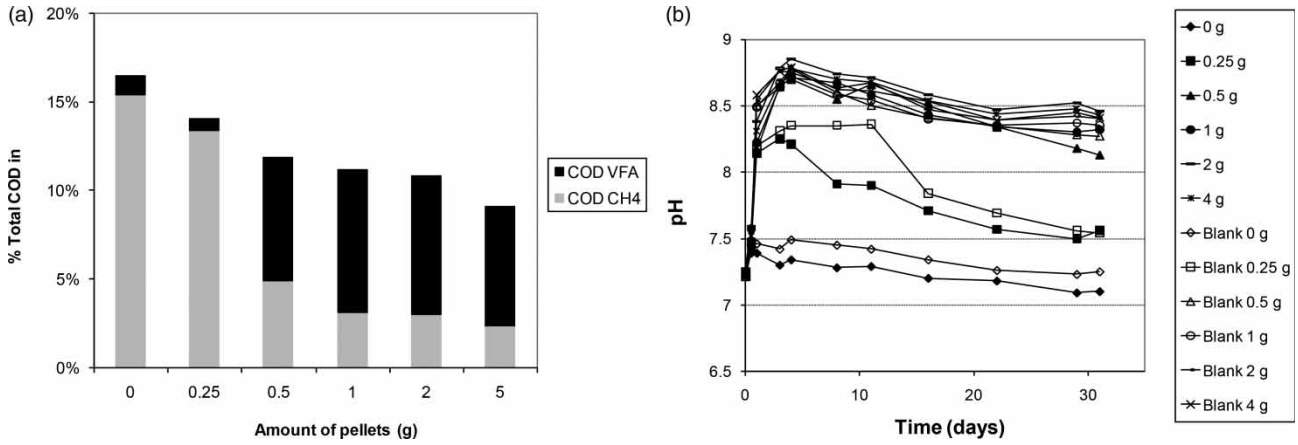

Figure 3 | Effect of NaOH pellets in COD conversion (a) and pH (b) during BMP assessment of frozen blended endive.

NaOH pellets showing 70–90% lower BMP than that assessed under non-inhibiting conditions (data not shown). Apparently, the CO_2 absorption capacity of the pellets at all the tested concentrations negatively impacted the carbonate system in the liquid phase. Indeed, when calculating the concentration of bicarbonate based on the pH and CO_2 concentration in the gas phase, a 4-fold lower concentration of bicarbonate is found in the bottles with pellet addition. In additional experiments it was also found that acetate and propionate were the main VFAs present and that the H_2 concentration severely incremented in the bottles with pellets addition, indicating retarded acetogenesis, and a possible negative feedback to hydrogenotrophic methanogenesis. Probably, in our research, the NaOH pellets captured the CO_2 present in the gas phase forcing the bicarbonate and the carbonic acid in the liquid phase to dissociate and disappear from the liquid phase. Under low concentrations of CO_2 in the liquid phase, hydrogenotrophic methanogenesis cannot proceed resulting in an increase in the H_2 partial pressure, which in turn will

negatively impact both acidogenesis and acetogenesis reactions. Acidogenesis under high pH_2 will lead to the formation of more reduced intermediates such as propionate, butyrate, and lactate. Finally, the resulting alkaline conditions also inhibit acetoclastic methanogenesis.

Although results are in disagreement with previous reports (Souto *et al.* 2009), our evidence strongly ratifies the negative influence of NaOH pellets in BMP assessment under our test conditions.

Effect of pretreatments in BMP assessment of plant material

Figures 4 and 5 present the condensed results of the laboratory digestion experiments of plant material undergoing different pretreatments. As can be observed, blending did not exert an important impact on the BMP value but particularly influenced the experimental replicability of the carrot and endive samples as shown by the standard deviation. Similarly, freezing and blending did not particularly influence the BMP of the materials whereas drying and grinding influenced the BMP assessment of all species except carrot, resulting in a BMP increase of 44, 25 and 43% for mustard, endive and green beans, respectively, as compared with the fresh 1 cm samples. Our findings agree with those from Palmowski & Muller (2000), Chynoweth & Jerger (1985) and Sharma *et al.* (1988) who showed total biogas production of different samples to increase by comminution up to 20, 18 and 56%, respectively. On the other hand, they are in disagreement with those of Pommier *et al.* (2010) who showed that for paper and cardboard samples reducing the particle size did not result in any particular improvement in the BMP.

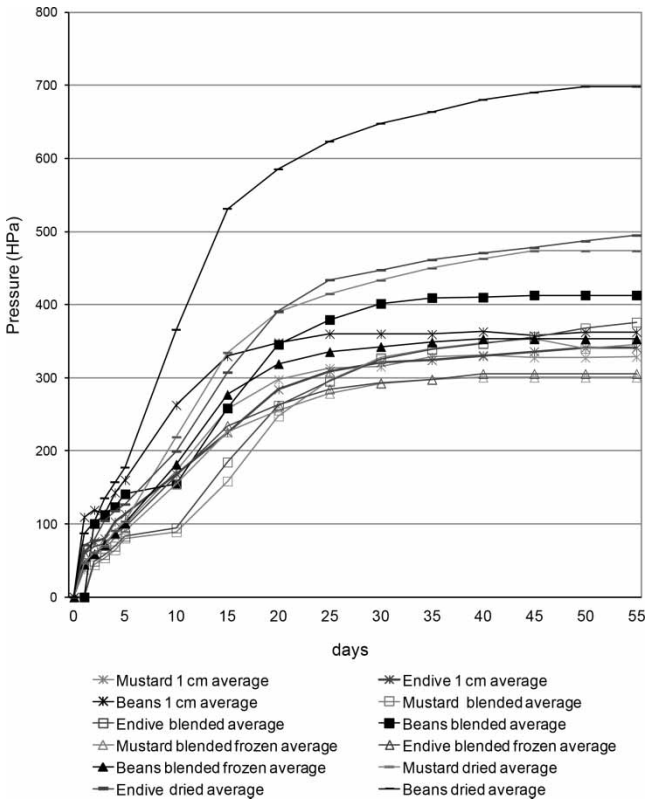

Figure 4 | Cumulative biogas production measured as pressure increase in samples of mustard, endive and green bean following different pretreatments.

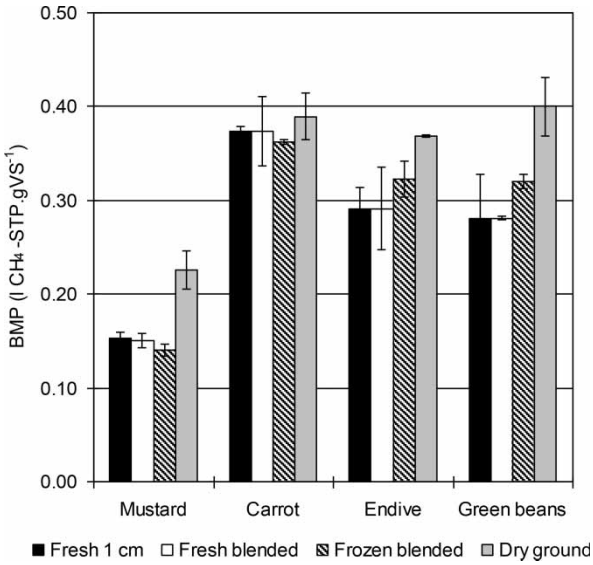

Figure 5 | Effect of sample treatment in the BMP of mustard, carrot, endive and green bean samples.

It is hypothesized that the lower content of lignin with respect to NDF in samples of mustard and green beans is related to a higher increase in biodegradability as compared with endive samples. In fact, a similar reason could explain why other authors like Pommier *et al.* (2010) did not find a significant increase in BMP of paper material, in this case the high lignin content would be blocking cellulose accessibility. The fact that biodegradability of carrot is not affected can be explained by its low content of cellulose in relation to lignin, and its high proportion of soluble matter as compared with the other plant samples.

Although cellulose and hemicellulose are considered to be fully anaerobically biodegradable their availability for bacterial attack depends on the structure in which they are embedded, especially in relation to the lignin content (Reid 1989; Tong *et al.* 1990). Furthermore, it is known that the rate and extent of hydrolysis correlates with the available surface sites for bacterial attack (Hills & Nakano 1984; Sanders *et al.* 2000). Therefore, an increase in comminution,

which leads to an increase in the suitable sites for enzymatic attack, is expected to exert a higher influence in samples containing more biodegradable particulate material than in those with higher amounts of non-biodegradable material, i.e. lignin, and/or insignificant amounts of particulate in relation to soluble matter. Despite our data points being insufficient to provide full proof of this we believe this hypothesis deserves further research.

The effect of drying the samples at 65 °C was assessed in a separate experiment using 1 cm particle size samples of green beans and mustard. Both substrates were selected due to their higher nitrogen content to assess the possible effect of the aforementioned enzymatic browning on the BMP. While the biogas evolution in time was similar, a slightly lower BMP value of dried samples in comparison to frozen samples was found in both cases, giving a difference of 9 and 13% relative to the value found with the frozen samples (Figure 6). However, given the important impact previously found when drying and comminuting vs. blending samples, the drying method does not seem to be particularly significant as compared with the particle size.

RECOMMENDATIONS FOR AN OXITOP® PROTOCOL FOR THE BMP ASSESSMENT OF PLANT MATERIAL

The main goal in our present study was to develop a simple and reliable test for screening multiple plant samples with limited labor input. The use of the OxiTop® protocol appears to be very convenient but has several pros and cons. Using NaOH pellets as a CO_2 scavenger is not advisable, thus headspace analysis for CH_4 content is still needed. The reproducibility of the OxiTop® results is in direct relation with the limited sample representativeness imposed by the pressure limit of the OxiTop® head. Under the conditions of the set-up employed a maximum 0.2 g COD biodegradable substrate can be added. A higher amount of sample is only possible with periodic biogas release, complicating the procedure. Regarding sample preparation, cutting in 1 cm pieces, blending and freezing samples produce similar results, whereas drying and grinding exerts an effect on samples in relation to their fiber composition. It should be stressed that none of these procedures simulates substrates used in full scale applications where variable and bigger sizes can be expected. From our perspective, for a BMP test used for screening purposes, freeze drying and grinding should be favored as maximum conversion, reproducibility and interlaboratory comparability is desired. Such a procedure allows for higher homogeneity and storability in time plus it can be easily incorporated in a protocol. The additional practical advantages should not be underestimated, like the fact that dried ground samples can be stored for a longer period of time in a reduced space and can be used for COD, calorimetry and fiber analysis determination as well. Still, as already mentioned, awareness should be kept on the fact that results cannot be used directly for estimating the BMP of material of other particle sizes, especially in the case of samples with low lignin content which are expected to be more affected by the pretreatment.

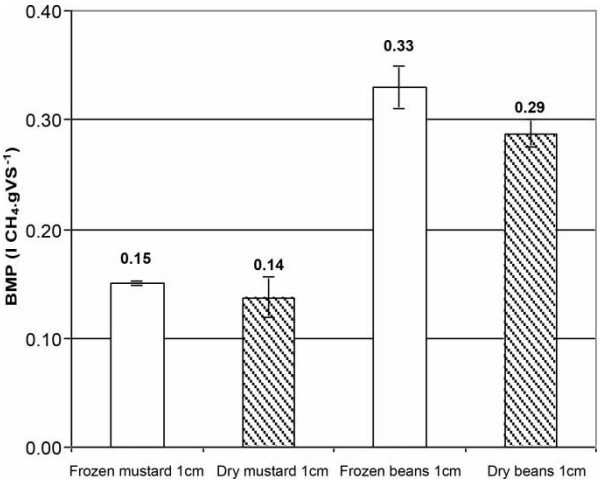

Figure 6 | Effect of drying samples at 65 °C in BMP results using mustard and green beans as substrates.

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