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D5: Standardised test procedures for assessing kinetic constants and evaluating reaction rates

1. Kinetic constants and reaction rates in anaerobic digestion

Knowledge on kinetics allows for optimization of performance, a more stable operation and a better control of the anaerobic digestion process. Various steps are involved in the anaerobic digestion process, as presented by Pavlostatis and Giraldo-Gomez (1991) these include:
(a) the hydrolysis of complex, particulate organic material,
(b) fermentation of aminoacids and sugars,
(c) anaerobic oxidation of long-chain fatty acids and alcohols,
(d) anaerobic oxidation of intermediary products (such as short-chain fatty acids),
(e) homoacetogenesis, and
(f) methanogenesis (aceticlastic and reductive).

In general, the methane production rate resembles the methanogenic capacity of the reactor. However, depending on the characteristics of the substrate to be digested (soluble vs. particulate, and its chemical composition) different steps from the above list should be considered for a complete description of the anaerobic digestion process. The digestion of plant material and agro-residues is characterized by the digestion of complex particulate matter. Hence, all steps mentioned play a role.

Given that different steps involve different bacterial populations, all of them exhibiting different optimum growth conditions and different kinetic constants, there is a need to simplify the kinetics describing the overall process. For this reason, the rate limiting step approach is generally used. This approach is based on the recognition that in a multi-step process, one step is usually slower than the others. The slowest step in such a reactions sequence is therefore named as the rate-limiting step (Pavlostathis et al., 1991). The rate limiting step approach leads to simplification of mathematical descriptions needed for reactor design, as assessing the reaction rate of the rate limiting step will provide enough information about the reaction rate of the whole process. In anaerobic digestion the rate-limiting step is related to the nature of the substrate, process configuration, temperature and loading rate (Speece 1983 in (Pavlostathis et al., 1991)).

2. Hydrolysis as the rate limiting step in anaerobic digestion of plant material

Hydrolysis can be defined as the breakdown of organic substrate into smaller products that subsequently can be taken up and degraded by bacteria (Morgenroth et al 2002 in (Angelidaki et al., 2004)). In the hydrolysis step, complex suspended compounds and colloidal matter are converted into their monomeric or dimeric components, such as aminoacids, single sugars and long chain fatty acids (LCFA). The many intervening factors and the complex nature of the substrate makes the hydrolysis process a complex one (Mata-Alvarez et al., 2000).

When digesting lignocellulosic material, hydrolysis of the complex organic matter can be regarded as the rate-limiting step (Hobson, 1983; Noike et al., 1985). Therefore, understanding the hydrolysis process and assessing properly the implied parameters is of crucial importance for proper process design. Deliverable D27 further discusses the complexity of hydrolyses kinetics as well as difficulties that may arise from interpreting data from batch tests.

3. Important factors intervening in the hydrolysis rate assessment

When striving for a standardized test for assessing kinetic rates, it is important to consider how different factors can influence the outcome of the test. Hence, emphasis can be put in the control of the most important ones for an optimum experimental set-up.

3.1. Substrate pre-treatment

The hydrolysis constant is very much related to the particle size distribution of the substrate. Therefore, literature values can only be used for reactor design when sufficiently detailed data in this aspect is presented. It is clear that particle size pre-treatment of plant material constitutes a main factor to consider when striving for a standardized protocol for kinetic constant assessment.

The influence of substrate pre-treatment in biodegradability has been previously reported as well. Total biogas production of hay was found to increase up to 20% by comminution (Palmowski et al., 2000), a similar finding to that of Chynoweth and Jerger (1985) who found an approx. 18% increase in the final methane yield of hybrid poplar when reducing particle size from ≤ 8 mm to ≤ 0.8 mm. Sharma et al (1988) found an even greater influence of 48% in maximum methane yield of wheat straw samples when diminishing particle size from 30 mm to 1 mm. However, Perez Lopez et al. (2005) found no major differences in degradation of corn grains when comparing samples treated at 10mm and 1mm, while for whole maize silage only 9% higher degradation was found . The effect of particle size reduction below 1mm on the maximum achievable BMP seems to be negligible as judging from the results of Sharma(1988) and Chynoweth and Jerger (1985).

3.2 Inoculum type and amount

Origin of the inoculum used for the hydrolysis assessment is a major factor to consider (Pavlostathis et al., 1991). In fact, although at full bacterial colonization of the substrate the hydrolysis rate is constant per unit surface area basis, the rate of microbial attachment depends on the type of micro-organism (Angelidaki et al., 2004). In addition, the type of inoculum, after full colonization has occurred, will still affect the reaction rate. In experiments carried out with pure cellulose, the hydrolysis rate constant once the surfaces were saturated with biofilm, was found to be two times higher with a rumen inoculum compared to a digester leachate inoculum (Song et al., 2005). As well, the rate reached a constant value one day after inoculation with rumen fluid and 3 days after inoculating with digester

leachate. The influence of the microbial community in the assessment is, in fact, not surprising if considering that microbial hydrolysis proceeds through complex enzyme systems comprising few to 20 or more enzymes, all of which will hydrolyze a specific substrate (Warren, 1996).

With respect to the substrate to inoculum ratio, in regards to hydrolysis, it is expected that this plays a major role in soluble substrates than in particulate ones. Hydrolysis of complex substrates is a surface related process. While in the case of particulate substrates, the hydrolysis rate will be only dependent on the surface area available, in the case of dissolved substrates, the available amount of surface corresponds to the total amount of substrate available and it is very likely that the amount of enzymes is rate limiting (Sanders, 2001).

In the case of particulate matter, the hydrolysis rate can only be described by first order kinetics after full colonization of the substrate has occurred (Song, 2003). Some evidence shows that the colonisation process proceeds very fast though. In experiments of Ten Brummeler (1993) it was shown that acid formation from organic solid waste typically started 2 hours after anaerobic conditions are established, making unnecessary the additional inoculation of the substrate for hydrolysis rate assessment. Also Hobson (1983) mentioned that in the case of rumen digestion, colonisation occurs in a matter of minutes after the matter enters the rumen, so it is not a rate-limiting factor in the degradation. In experiments of Veeken and Hamelers (1999) the hydrolysis rate could be excellently described by first order kinetics, which as reported by the authors, might have been due to the initial presence of hydrolytic enzymes on the surface of the biowaste components or to the fast growth of hydrolytic bacteria.

An additional factor playing a role in the assessment is the balance of acidogenic and methanogenic bacteria populations. Conversion and growth rates of hydrolyzing-fermentative bacteria are about 5 times faster than that of methanogens. Since anaerobic digestion is the result of a careful balance between different processes producing and consuming intermediates, the presence of enough quantities of all populations is crucial to avoid failure. Acidification of the reactor is of special importance since the absence of sufficient methanogens will lead to the accumulation of acids followed by a decrease in pH, both affecting the hydrolysis rate as explained in the following section.

From the previous follows that hydrolysis rate assessment, optimal for reactor design, will ideally require a fully balanced, well adapted inoculum. In addition the inoculum concentration should as well be considered as far as the rapid colonization of the particle surface is desired, the soluble substrate present in the plant material will degrade proportionally to the amount of active enzymes present, and enough amounts of methanogens should be guaranteed to avoid accumulation of intermediates.

3.3 pH and concentration of intermediates

If care is not taken in choosing a good balanced inoculum, intermediates can accumulate, which implies changing process conditions during the assessment of the hydrolysis rate. Llabres-Luengo and

Mata-Alvarez (1988) postulated that the concentration of intermediates can affect the hydrolysis rates and proposed a model in which the hydrolysis rate was inversely proportional to the VFA concentration.

With respect to pH, Dinamarca et al. (2003) found no major influence of the pH in the hydrolytic stage of anaerobic digestion (AD) of the organic fraction of municipal solid waste (OFMSW) when working in the range 6 to 8. However, Zhang et al. (2005) found an improvement in hydrolysis and acidogenesis rate when pH was adjusted to 7 in comparison to pH values of 5, 9 and 11.
However, since VFA concentrations and pH changes are strongly related, the influence of both needs to be examined. In experiments carried out with OFMSW Veeken et al. (2000), found that the hydrolysis rate constant was pH dependent and not so much related to the total and undissociated VFA concentrations when working at 3-30 gCOD/l. The observed empirical relation between Kh and pH could be described by equation 2.

$$k_h = 0.048\,pH - 0.172 \quad (2)$$

The optimum value of hydrolysis kinetics at pH 7 is in fact related to optimum values for enzyme activity which are as well in the neutral range as presented in the bell shaped curve. Still, considering that different enzymes play a role in anaerobic digestion, the effect of pH on the first order hydrolysis constant is a complex one as it will depend on the characteristics of the substrate as well as on the enzymes involved present (Sanders, 2001).

3.4 Temperature

In their experiments, Veeken and Hamelers (1999) found that the hydrolysis rate constants increased at higher temperatures for all six biowaste components evaluated. They conclude that if the enzyme concentration is not rate limiting, the rate of hydrolysis as a function of temperature can be described by the Arrhenius equation for enzyme catalysis.

Temperature effect in hydrolysis is the result of combined effect of enzyme kinetics, bacterial growth and solubility of the substrate. In general, the overall process rates double for every 1^{o}C increase in operating temperature (Harmon et al 1993 in (Angelidaki et al., 2004)), and optimum microbial growth can be found close the thermophilic ranges.

3.5 Reactor type

It has been noted before that the differences in the digestion conditions of different studies reporting hydrolysis rates, particularly reactor configuration, makes interstudy comparison very difficult (Pavlostathis et al., 1991).

Commonly two different experimental set ups are used to assess kh, i.e. batch or continuous experiments (Eastman et al., 1981; Veeken et al., 1999):

- In the batch approach, the substrate is incubated at a specific temperature with or without an amount of seed sludge. After incubation the degree of hydrolysis is assessed via the methane production and in most cases also the concentration of volatile fatty acids and/or soluble COD in the batch reactor. In some cases the degree of hydrolysis is assessed by analysis of the depletion of the substrate and/or the measurement of free NH_4^+/NH_3 (protein conversion).

- The continuous set up uses completely stirred tank reactors (CSTR) operated at a specific temperature and at least three different hydraulic retention times. The CSTR reactors are operated until steady state conditions. Subsequently the degree of hydrolysis of the influent substrate is assessed via methane production and effluent quality (viz. volatile fatty acids, soluble COD and possibly substrate and/or reaction product concentration).

Both experimental approaches have advantages and disadvantages. The approach using CSTR systems is much more time consuming than the batch approach and it requires higher amounts of substrate. Nevertheless the CSTR seems to have more resemblance to reality, as the sludge produced in the CSTR reactors is adapted to the substrate, unlike the batch approach in which mostly digested manure or sewage sludge is used as seed sludge. Therefore it is unclear if the k_h values assessed from batch experiments can be used to design CSTR systems.

In both set-ups intermediates can accumulate possibly influencing the assessment, thus in the batch set-up care must be taken in choosing an adapted inoculum to prevent the previous. In the CSTR case, choosing appropriate HRT for a balanced digestion is important; however as for the hydrolysis rate assessment at least 3 HRTs are required, different amount of intermediates will accumulate in the three reactors.

3.6 Dilution

Full scale digesters work at a TS content of 10-12% in the case of CSTR systems, and up to 20-25 % in the case of plug flow systems. Plants operated in batch mode digesting OFMSW have shown feasible to increment the solids concentration up to 35% TS (Ten Brummeler, 1993).

Incrementing the concentration of solids provide a more effective use of available volume, however the stability of the process will be affected and other operational conditions like the mixing and loading-unloading of the digester will become an issue. Therefore, for a standardized test providing maximum conversion and easiness of follow up, the set-up should be adjusted to dilutions suitable to avoid unacceptable accumulation of intermediates and facilitate mixing.

4. Experimental Findings

The literature research previously presented showed as pertinent to study in more detail the influence of the substrate treatment, the type of inoculum, the substrate to inoculum ratio, and the effect of different treatment systems (batch-CSTR) in the anaerobic digestion of plant material. Following results concerning our experiments are shown.

4.1 Influence of substrate treatment

When assessing methane potential of lignocellulosic material some choices need to be made about the treatment of the substrate, especially regarding storage and particle size. Samples can be digested fresh, frozen or dried, also blended, grinded or cut in pieces of desired sizes. Restrictions imposed by time, available experimental set-up and sampling procedures play an important role in the decision, while the choice will in turn influence the comparability of the results to real full scale applications, test reproducibility, test duration, sample representativeness and easiness of the procedure.

Figure 1 presents the condensed results of the laboratory digestion experiments of the three selected lignocellulosic materials under different treatments. The test was carried at a S/I of 0.4 (VS basis) using digested primary sludge as inoculum. From the results, it can be observed that freezing and blending did not exert an important influence in the final methane production of any of the samples. Drying and grinding samples influenced the assessment of all the species except carrot, being it incremented in 44, 25 and 43% for mustard, endive and green beans, respectively.

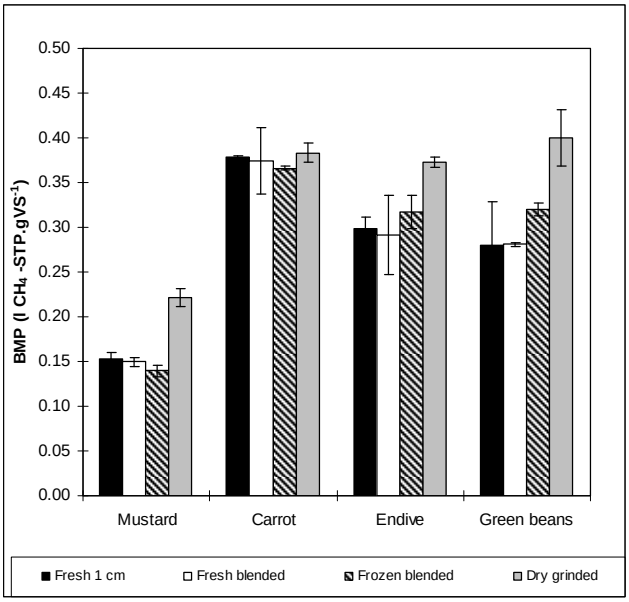


Figure 1. Biodegradability results from sample treatment experiments

Considering the results for the four substrates researched higher BMP values were achieved for species showing less fibre content. In this respect our findings are consistent with previous research showing that structured materials with high lignocellulose content are only partially anaerobically biodegradable (Chandler, 1980; Tong et al., 1990).

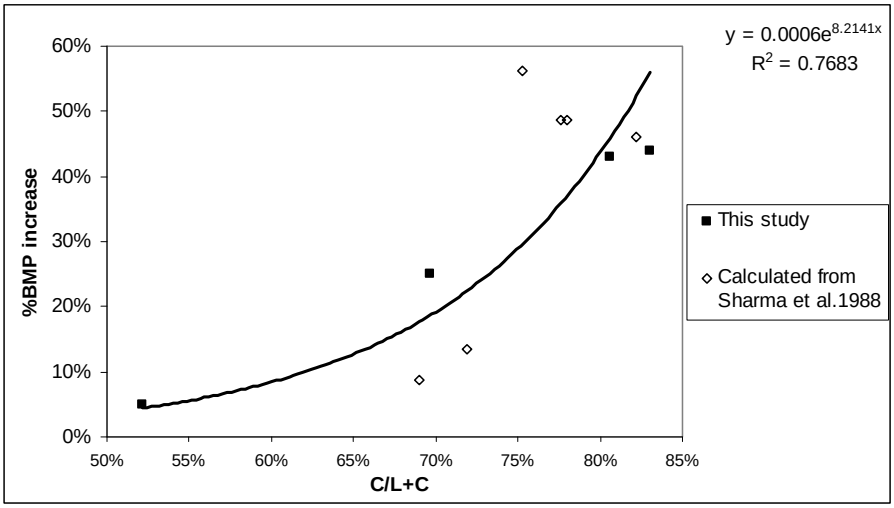


Figure 2. Increase in biodegradability of dry grinded samples in relation to the relative content of cellulose in the sum lignin plus cellulose
(C: Cellulose;L:Lignin)

Furthermore, our results also show that the increase in the relative BMP value and COD conversion increase was higher for species showing a higher proportion cellulose in relation to the sum lignin plus cellulose *(Figure 2*). It is known that the availability of cellulose for bacterial attack depends on the structure in which it is embedded, especially in relation with its cristallinity and lignin content, while lignin is being very resistant to degradation by anaerobic bacteria (Jimenez et al., 1990; Reid, 1989; Tong et al., 1990; Turick et al., 1991). Considering that the cellulose content in mustard is higher in relation to the other samples. The higher increase in biodegradability achieved for this sample could be related to the increased availability of cellulose for biodegradation. The non-increase in biodegradability in the case of carrot would be related to the low content of cellulose in relation to the other plant samples. Data from Sharma et al.(1988) show a similar trend, as the percentual BMP increase was higher for the samples with higher cellulose content and low lignin content.

In all cases, the hierarchy of the materials in terms of their methane potential remained unchanged, which is also the case in the results of Sharma et al.(1988). This could mean that although particle size can lead to different biodegradability results, the influence of the particle size reduction is not a limitation for material screening as long as the same size is kept for all samples.

Oven drying of lignocellulosic samples can alter their chemical composition. As reported by Parissi et al. (2001) this procedure can induce non enzymatic browning, which is the result of heat treatment causing polymerization of sugars with aminoacids resulting in a brown complex similar to lignin. The

so called artifact lignin can block the accessibility of the substrate for digestion reducing its digestibility. Temperature, moisture, non-structural plant carbohydrates and time exposed to heat influence the extent to which non enzymatic browning can occur (Broesder et al., 1992).

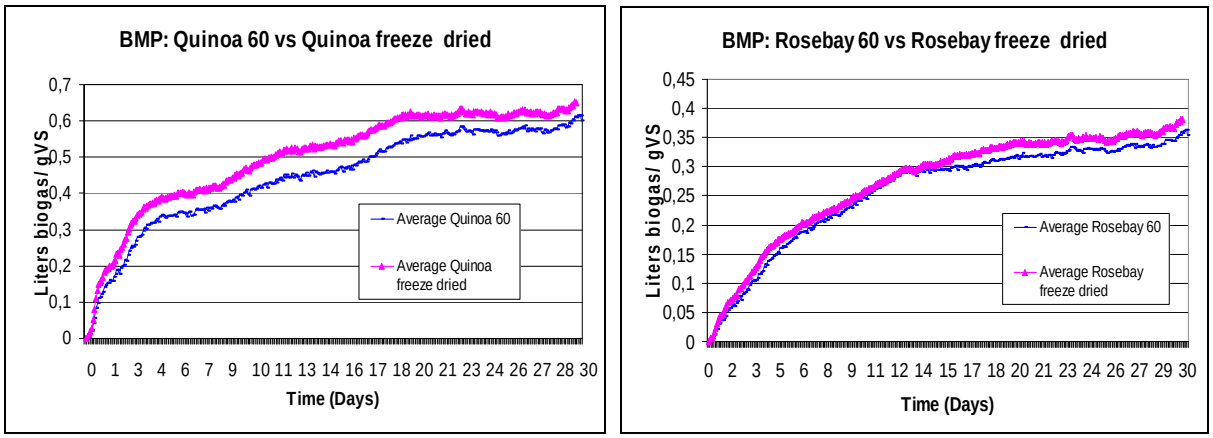


Figure 3. Influence of drying method in biodegradability and biodegradation rate of plant material

Samples of quinoa and winter bean were selected for carrying out a comparison of BMP and rate assessment under different type of drying method. Representative samples were dried at 60°C and freeze dried and digested at the same conditions. The results shown in Figure 3, demonstrate a small effect of the drying method in the final biodegradability, but no effect in the rate of biodegradation, corresponding with the hydrolysis rate.

4.2 Influence of inoculum type and ratio

Mesophilic granular and digested primary sludge were used in the experiment conducted to assess the influence of sludge type in the BMP of green beans frozen blended samples. As shown in Figure 4, BMP assessed with the Digested Primary Sludge (DPS) and the granular sludge (GS) did not show significant variation. The mixture of sludges gave a higher BMP, equivalent to 16% more than DPS or GS alone, still the standard deviation does not allow to conclude that the effect is significant. Rates of biogas production did not vary substantially among the fresh DPS, the granular sludge and the sludge mixture, being 0.17, 0.14 and 0.18 liters biogas/g VS, respectively. The biogas production rate of the DPS that has been stored for more than two months was much lower than the others, 0.04 liters/g VS, a lag phase being evident in the biogas production curve.

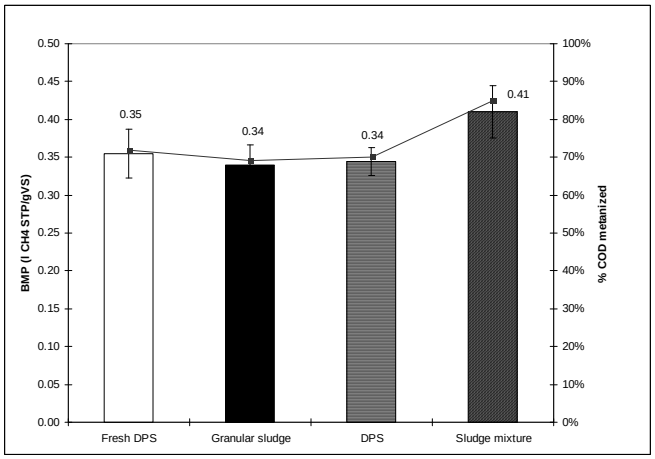


Figure 4. Influence of inoculum type in biodegradability of green beans blended samples

Figure 5 shows the conversion rate evolution for the four sludge types. The initial substrate contained sCOD (approx 40%) which in all cases was readily utilized for methane production, with the exception of the stored DPS which acidified the substrate effectively but was much slower in its transformation to methane. Contrary to what was expected, the fresh DPS did not hydrolyze more substrate as compared to the others. Still, the synergetic effect of mixing the sludges was evident in the amount of hydrolyzed products, which after the second day was already 30% higher than the amounts hydrolyzed by the individual sludges.

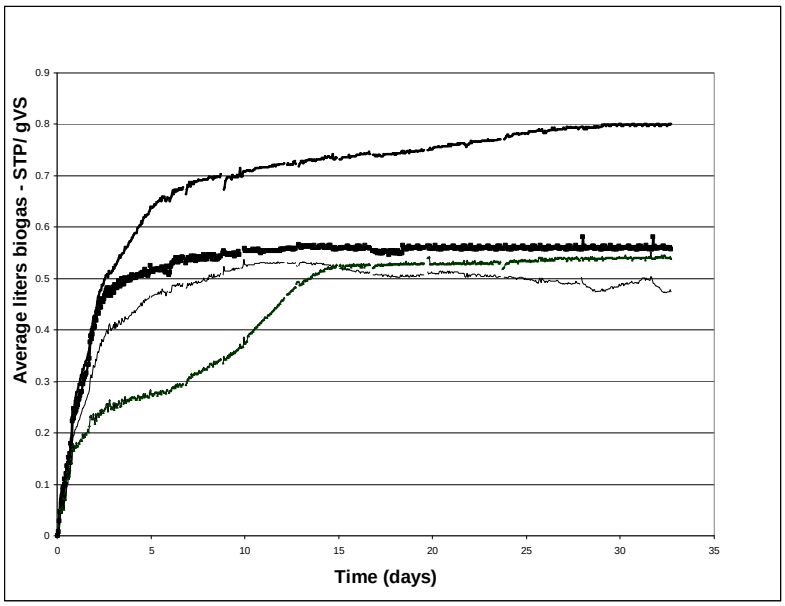


Figure 5. Influence of inoculum type in biogas production curves of green beans blended samples (Lines from top to bottom: sludge mixture, granular sludge, DPS, stored DPS)

For the different inocula the Specific Methanogenic Activity (SMA) test was assessed using acetate and glucose. It is clear that sludges differ substantially in terms of their acetate conversion capacity with granular sludge having the highest SMA. The stored DPS showed a very poor performance compared to fresh DPS, implying that methanogenic populations decayed during the storage period.

The influence of Substrate to Inoculum ratio was assessed for the sludge mixture and the granular sludge, results are shown in Figure 6. Apparently the ratio affected both DPS and GS, the biodegradability decreasing inversely to the ratio variation. Nonetheless, the rank of variation was so small in the case of the granular sludge that it could fall within the experimental error. A higher influence for the sludge mixture in which a difference of 24% in the COD methanized was found when going from a S/I ratio of 0.5 to 3. It is also interesting to notice that the BMP value assessed for the sludge mixture and the granular sludge at the highest S/I ratio was the same.

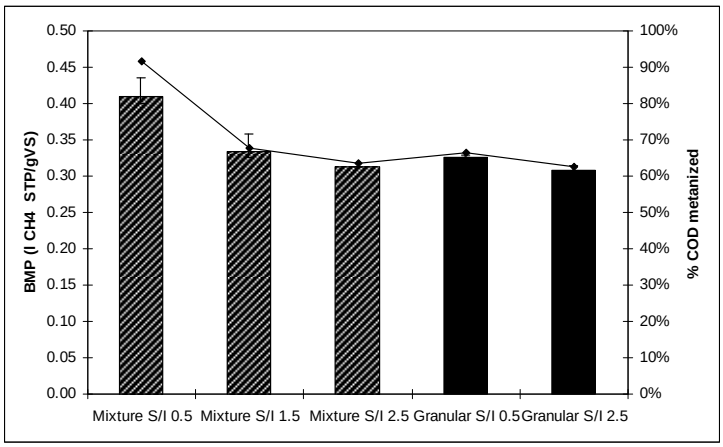


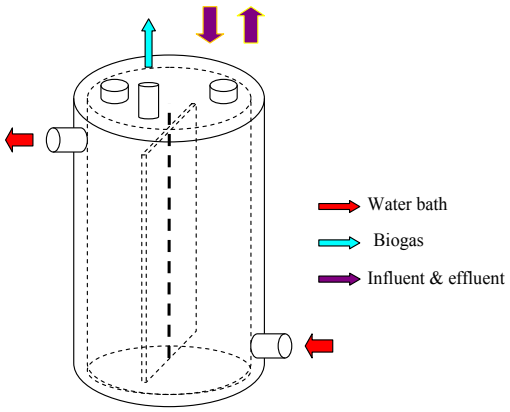
Figure 6. Results from variation in substrate to inoculum ratio

It has been presented elsewhere that the substrate to inoculum ratio affects the evaluation of methane production rates resulting in substantial VFA accumulation at a higher ratios (Chynoweth et al., 1993).

4.3 Comparison batch-CSTR assessment

Experiments were conducted to assess the hydrolysis rate in batch and CSTR systems using model substrate compounds. Both batch and CSTR experiments had the same influent and process conditions to ensure the comparability of results.

Three eight-litre CSTR reactors were operated at 12, 15 and 20 days retention time and at 35°C (Figure 7a). The reactors were fed with a suspension of cellulose (1 g COD/l), casein (1 g COD/l), macro nutrients (2 ml/l), trace elements (1 ml/l) and yeast extract (0.2 g/l). The estimated times needed for the 3 reactors to reach steady state were 36, 45 and 60 days, respectively. Due to experimental practicality, each CSTR was fed manually every weekday. The temperature was controlled by a water bath. A schematic drawing of the reactors is presented in figure 7b.



Figures 7a and b. CSTR reactors: experimental set-up and schematic representation

The experiments for the calculation of the hydrolysis rate using a batch system were conducted using a multiple flask approach. In this way a better assessment of the intermediates concentration can be performed. Six multiple flask batch experiments were performed for assessing hydrolysis constants. Sludge adapted to casein and cellulose, from the CSTR experiments was used as inoculum. For three of the six multiple flask batch experiments the substrate used was cellulose (1g COD/l), while for the other three it was casein (1g COD/l). Figure 8 shows the set-up of the multiple flask batch experiment. The dark bottles with the OxiTop system were used for biogas measurement and the translucent bottle with silver cover for hydrolysis products sampling.

Figure 8. Multiple flask batch experiment

Frequent samples were taken and measured for soluble COD, VFA, pH and methane production in both systems. Calculations of the hydrolysis rate for batch system were done according to the procedure presented in section 5.5. Calculations of the hydrolysis rate using the CSTR systems were performed once they have reached steady state. From equation 1, the relationship for the hydrolysis constant, digestion time and effluent concentration for CSTR can be derived (eq 3). For estimation of the first order hydrolysis rate constant, equation 3 can be linearised to give equation 4.

$$X_{ss,eff} = \frac{X_{ss,\inf} \cdot f_h}{(1+\theta \cdot k_h)} + X_{ss,\inf} \cdot (1-f_h) \quad (3)$$

$$\theta = (f_h \cdot X_{ss,\inf})(\frac{\theta}{X_{ss,\inf} - X_{ss,eff}}) - \frac{1}{k_h} \quad (4)$$

Where:

Xss,eff Concentration of total substrate in the effluent (Biodegradable + non biodegradable) (g/l),

Xss,inf Concentration of total substrate in the influent (Biodegradable + non biodegradable) (g/l),

f_h Biodegradable fraction of substrate, 0<fh<1

k_h First order hydrolysis constant (d^{-1})

θ Hydraulic retention time of reactor (d)

Effluent and influent concentrations of substrates *Xss,eff* and *Xss,inf* are calculated by subtracting the VFA concentrations and methane production from the total COD_{in}. K_h and f_h can be determined when plotting θ against $\theta/(X_{ss,inf}-X_{ss,eff})$. The hydrolysis constant can be determined from the intercept of the line with the y-axis and the biodegradability from the slope of the line (Eastman and Ferguson, 1981).

Results of the multiple flask experiments are presented in Table 1, while Figure 9 shows the plotting for the calculation of the hydrolysis rate constant for the CSTR system.

Table 1 Hydrolysis constant from multiple flask batch experiments

Experiment	*Substrate*	*Inoculum*	*Kh (d^{-1})*
1	cellulose	Sludge from CSTR at 12 d HRT	0.28
2	cellulose	Sludge from CSTR at 15 d HRT	0.26
3	cellulose	Sludge from CSTR at 20 d HRT	0.19
4	casein	Sludge from CSTR at 12 d HRT	0.52
5	casein	Sludge from CSTR at 15 d HRT	0.29
6	casein	Sludge from CSTR at 20 d HRT	0.63

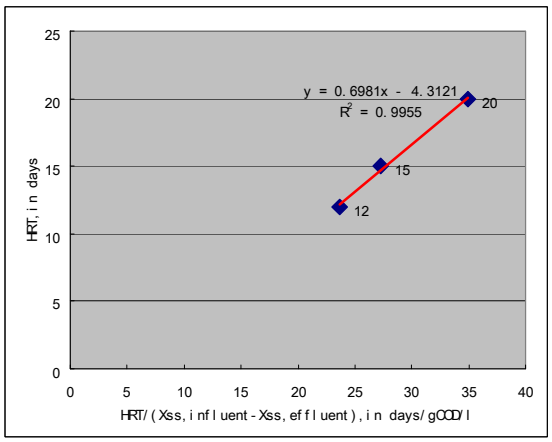


Figure 9. Calculation of hydrolysis rate for the CSTR system

The first order hydrolysis rate constants obtained in batch systems for cellulose vary from 0.19 - 0.28 d^{-1}, while those for casein varied between 0.29-0.63 d^{-1}. Average values were 0.24 and 0.48 d^{-1} respectively. Since theoretical values calculated via modelling showed hydrolysis constants of 0.2 and 0.4 d^{-1}, respectively, the results of the batch assays are very much in agreement. In the CSTR system, the value for the hydrolysis rate constant of carbohydrate and protein was calculated to be 0.23 d^{-1} this is in fact lower to the one calculated via batch experiments but still in the same order of magnitude.
Theoretically CSTR experiments would be more accurate to assess the hydrolysis rate constant due to the existence of adapted inoculum and other conditions similar to reality. However since these types of experiments are too laborious, it would be advantageous if the same hydrolysis rate constant could be assessed by batch experiments. Based on the k_h comparison between CSTR and batch experiments, we can conclude that values are in the same order of magnitude and do not deviate a lot from each other. Further research should be performed with optimized operational conditions, using plant material as substrate and bigger range of HRTs.

5. Proposed protocol for biodegradability and kinetic rate assessment of plant material

Main points coming from the literature review and performed experiments, being of key importance for the protocol definition, are summarized in the subsequent list. Following those findings in the next subsections the proposed protocol is presented.

- Sample treatment affects biodegradability of plant samples; therefore for comparability of results samples should follow the same preparation procedure.
- Different objectives can influence the choice for a specific treatment for the hydrolysis rate assessment. If comparability of results with amounts attained in reality is desired, samples of 1 cm particle size (or bigger according to the kind of agricultural pre-treatment applied in practice) should be favoured. Freezing samples in principle does not significantly affect the biodegradability. If the maximum conversion rate and biochemical methane potential needs to be assessed, drying and grinding give the highest results. In addition, reproducibility and replicability of the test is enhanced with drying and grinding, due to the fact that a more homogeneous sampling procedure is made possible, which also allows using less replicates without compromising accuracy. Not to underestimate are also the additional practical advantages like the fact that samples can be stored for a longer time, space required for storing is diminished, particle size can be better assessed via screening of the sample and dried samples can be used as well for COD, calorimetry and fiber analysis determination. With respect to the drying method to utilize, freeze drying is recommended in order to avoid the non-enzymatic browning effect. Blending the samples could be faster than drying and grinding, however taking a representative sample is more difficult.
- When working with a mixture of hydrolytic and methanogenic sludges, in the adequate ratio, the presence of the optimum bacteria required for the process to proceed can be ensured.
- An optimal degradation and a faster test were found when using substrate to inoculum ratio close to 0.5 (VS basis).

- In experiments not described in this document, the influence of buffer molarity was assessed. Results show that phosphate buffer can be applied until 20 mM while carbonate buffer can be applied up to 50 mM. A 20 mM phosphate buffer solution has shown to be enough to achieve a constant pH in the neutral range during the duration batch experiment. It is not recommended to exceed this concentration since inhibition of methanogenesis has been identified.
- Batch systems are easier to operate and allow to screen many samples at the time. When using well adapted inoculum, batch systems are in principle able to give results close to those achieved in CSTR systems.
- The Oxitop system® has proved to be a reliable method for gas monitoring in batch systems. Releasing pressure from the measuring bottles does not affect their gas composition.

5.1 Sample preparation

Fresh plant sample preparation

- Divide the sample in its different constituent parts (according to expected different lingo-cellulose content) and weight each of them.
- Calculate percentual composition of the material and calculate the amount to add of each part in each bottle according to the total amount of sample to be added (see 5.3).
- Cut each constituent part in pieces of 1cm approx. using gardening scissors. (Figure 10)


Figure 10. Achieving a representative fresh plant sample

- It is recommended to assess kinetic rates in relation to the particle size of the sample, therefore if necessary to take account of the exact particle size, place a representative sample against a white background and ruler and take a digital picture of the sample (Figure 11). The image can later be analyzed with proper software like Image Tool ®.

Figure 11. Sample for assessment of particle size distribution

- Weight the samples to add in the bottles (Figure 12).Weight 7 samples per test substance (3 for each bottle, 2 for the crucibles and 2 extra to keep in the fridge for two days in case test has to be repeated. Afterwards the two extra samples are to be placed in the freezer).

Figure 12. Weighed fresh plant samples ready to add in the batch bottles

Blended sample preparation

- Weight the empty blender and take note (a)
- Add a representative sample to the blender (according to the weight of each constituent part –see fresh plant sample preparation), weight and take note (b)
- Add just enough demiwater to make the blender run, so then a "yogurt type" mixture is resulting. Weight again and take note (c). Let the blender run for 1-2 minutes, longer blending should be avoided as warm-up of the sample acting as an extra pre-treatment can occur.
- Put the resulting mixture in a storing bottle. Then place the blender in the scale again, make zero and slowly add demiwater, just enough to clean the borders of the blender. Take note of the weight of the water added (d) and add the mix to the plastic bottle. Mix vigorously. Repeat this step if needed until the vase is left almost clean.
- Calculate the "soup" concentration in g substrate per g soup: $Soup.conc\ (g/g) = (b-a) / (c+d-a)$
- Measure TS/VS of the resulting mixture for necessary calculations.
- If necessary to freeze the resulting mixture, use ice cubetes adding to each cube a known amount

of sample equivalent to the amount that would be added in fresh weight. Representative sample has to be taken by continuous mixing before adding the mixture in the cubete.

Dry sample preparation

- Take a representative sample of plant material by following the first step in the procedure for fresh plant sample preparation (see above).
- Put the complete plant sample to dry at 40^{o}C for one night or preferably freeze dry it.
- Grind the sample using 1mm mesh.
- Measure TS/VS of the resulting powder.
- Weight necessary amounts to fill in the bottles. (Figure 13)

Figure 13. Weighed dry grinded plant samples ready to add in the batch bottles

5.2 Inoculum selection

When selecting appropriate inoculum for the test, the hydrolytic and methanogenic capacity of the sludge are of importance. Having an inoculum adapted to the digestion of complex material like digested primary sludge, can resemble a fully adapted inoculum. DPS however, needs to be collected fresh, since it is methanogenic capacity is negatively affected after storage at 4^{o}C as shown in our experiments. If sludge needs to be stored for subsequent assessments, it is our recommendation to use a mixture of DPS and GS, the last being characterized by a good methanogenic capacity, less likely to be lost after long periods of storage.

5.3 Experimental set-up

The BMP test is performed at 35^{o}C in 500 ml batch bottles of which the exact total volume is known. The bottles should have a sampling port. The experiment is performed in triplicate; if the substrate is homogeneous enough (like in the case of dry grinded material) duplicates can be used. For each test run 4 additional bottles are required (2 for control, 2 for blanks). Blank bottles are to be filled with exactly the same media as regular bottles, except no plant sample is added. Using a dry grinded plant material for control bottles is recommended.

The set-up used for our experiments relies on the OxiTop system® for biogas increase monitoring. The OxiTop system ® is a pressure monitoring system originally developed for BOD measurements. The system comprises the measuring heads and a controller, and uses an infrared interface for data transfer. The OxiTop measuring head contains a pressure sensor and a data memory being able to store up to 360 data sets depending on running time. The data collected can be called up at anytime and graphically displayed on the controller (Figure 14). In principle other gas monitoring methods by pressure increase or volume displacement could be used, however specific precautions related with the reliability of the methods need to be undertaken.

Figure 14. Oxitop system for pressure monitoring.

Two experimental set-ups are shown in tables below. In set-up 1 the amount of plant sample is kept below oxitop limit so pressure does not need to be released during the test (Table 2). In set-up 2 the amount of sample is increased allowing for better sample representativeness (Table 3). In this case, pressure needs to be followed and released as needed, and calculations should be adjusted taking into account the residual amount of methane remaining after the opening.

Table 2. Set-up 1- Fixed to oxitop limit

Input	*Amounts*				*Concentrations*	
Plant sample	0.75	g	0.19 (0.16)	gCOD (gVS)	1.2 (1)	gCOD/l (gVS/l)
Sludge mixture total	10.5	g	0.3	gVS	2	gVS/l
- Granular sludge	*3*	*g*	*0.15*	*gVS*		
- Digested primary sludge	*7.5*	*g*	*0.15*	*gVS*		
Macronutrients *	0.4	ml			2.5	ml/l
Trace elements *	0.2	ml			1.25	ml/l
Phosphate buffer *	8	ml			20	mM
DemiWater	140	ml				
Total liquid volume	160	ml				

Table 3. Set-up 2- For a more representative sample

Input	*Amounts*				*Concentrations*	
Plant sample	1.3	g	0.34 (0.27)	gCOD (gVS)	2.0 (1.6)	gCOD/l (gVS/l)
Sludge Mixture Total	17.5	g	0.5	gVS	3	gVS/l
- Granular sludge	*5*	*g*	*0.25*	*gVS*		
- Digested primary sludge	*12.5*	*g*	*0.25*	*gVS*		
Macronutrients *	0.4	ml			2.5	ml/l
Trace elements *	0.2	ml			1.25	ml/l
Phosphate buffer *	8	ml			20	mM
DemiWater	140	ml				
Total liquid volume	167	ml				

** Indications on the composition of medium solutions including macro nutrients, micronutrients and phosphate buffer are provided in annex 1.*

IMPORTANT NOTE: *Amounts shown above are based on average plant composition. From our experience in the analysis of 40 plant samples, average values for plant material composition are: 1.35 gCOD/gVS, 0.26 gCOD/g and 0.21 g VS/g.*
However great variation among COD and VS content of plant samples was found:
- Minimum values: 0.68 gCOD/gVS, 0.09 gCOD/g, 0.08 g VS/g;
- Maximum values 1.8 gCOD/gVS, 0.65 gCOD/g, 0.68 g VS/g).
Therefore, according to VS (and COD) content of the material the amount to add in grams should be adjusted.

The bottles should be filled by adding first the demiwater and medium solution, followed by the sludges. Finally when all bottles are prepared the plant sample is to be added. After completing the procedure bottles should be flushed with nitrogen gas for approx 30 seconds and covered with the oxitop head equipped with a septum rubber ring to avoid leakages. The bottles are to be provided with adequate shaking (100 rpm) and mesophilic temperature conditions (35^{o}C) for the duration of the experiment (Figure 15). It is recommended to start up oxitop heads after the bottles are in mesophilic conditions to avoid interference of temperature increase in the measurements.

Figure 15. Anaerobic test initialized.

5.4 Test monitoring

In order to calculate the biodegradability and degradation rates of the plant samples proper monitoring should be guaranteed. Since the set-up proposed departs from the existence of a sufficient amount of methanogenic bacteria able to convert all intermediates, calculation of the hydrolysis rate could be done directly from the methane production values. However, considering that the hydrolysis rate can considerably vary for different substrates (see figure 19), it is important to calculate the amount of inoculum to add considering a high kh value that allows for sufficient methanogenic activity in the case of an easily degradable substrate. To secure that indeed the previous is the case, it is recommended that during a first test trial with such an easily degradable substrate intermediates (soluble COD and VFA) are also monitored simultaneously with the methane gas sampling. The sampling of liquid can be done via the same port used for gas sampling, using a syringe and taking 3 ml liquid sample each time.

When using fresh plant samples of 1 cm particle size or more, monitoring should be done once per day during the first two weeks and once per week, thereafter. In the case of dry grinded samples, more intensive sampling is to be applied during the first week, since faster degradation will occur.

In both cases when the test approaches its end, meaning methane plateau is achieved, sampling of gas should be done at least three times during a week time frame in order to ensure a good average value for BMP calculation.

5.5 Calculations

5.5.1 Biodegradability

Anaerobic biodegradability of organic material is usually expressed in litres methane per gVS sample added. The litres methane are to be calculated by subtracting from the assessment bottle the amount of

methane produced by the blank bottle, in order to correct from the organics present in the inoculum and its decay. Equations 5 and 6 present the formulas used for calculations:

$$BMP(lCH_4/gVS) = \frac{Max\ \ liters\ \ CH_4(l) - Max\ \ liters\ \ CH_4 blank(l)}{VS\ \ plant\ \ material\ \ in\ \ bottle\ \ (gVS)} \qquad (5)$$

$$Max\ \ liters\ \ CH_4\ \ (l) = \left(\frac{(P_{atm} + MaxP) \cdot V_{headspace}}{R \cdot T} \right) \cdot \%CH_4 \cdot 22{,}4(l/mol) \qquad (6)$$

With:

P_{atm}: atmospheric pressure (Pa)

Max P: Maximum value of pressure obtained in the oxitop (Pa)[1]

$V_{headspace}$: Volume of the headspace of the bottle (m^3)

R : Ideal gas constant (Pa m^3/mol K)

T: Temperature of the experiment (^oK)

5.5.2 Hydrolysis rate

As described previously the hydrolysis rate in anaerobic systems can be described as first-order with respect to the concentration of degradable particulate organic matter (equation 1).The calculation of the hydrolysis rate in batch reactors is done using equation 8 which relates the first order hydrolysis constant, the digestion time and effluent concentration.

$$X_{ss,t=t} = X_{ss,t=0} \cdot (1 - f_h) + f_h \cdot X_{ss,t=0} \cdot e^{-kh \cdot t} \qquad (8)$$

For the estimation of the first order hydrolysis rate constant, equation 8 can be linearised to give equation 9.

$$\ln\left(\frac{X_{ss,t=t} - X_{ss,t=0} \cdot (1 - f_h)}{X_{ss,t=0} \cdot f_h}\right) = -k_h t \qquad (9)$$

[1] Oxitop measurements are expressed in HPa, therefore the value should be multiplied by 100

Where $X_{ss,t=t}$ is the concentration of particulate substrate in the bottle at time t (Biodegradable + non biodegradable) (gCOD/l), $X_{ss,t=0}$ is the concentration of particulate substrate at time t=0 (Biodegradable + non biodegradable) (gCOD/l), f_h is the biodegradable fraction of particulate substrate, $0<f_h<1$, k_h is the first order hydrolysis constant (d^{-1})and t is the batch digestion time (day). In all cases, calculation of Xss is done by substracting the total COD solubilised (soluble COD plus COD methanized at time t) from the total plant COD added at the start up.

The hydrolysis rate should be assessed correcting for the non degradable portion of the substrate, as omitting this step will lead to erroneous reduced rates (Pavlostathis et al., 1991). That means that biodegradability of particulate material fh should be calculated for hydrolysis experiments by following input and output concentrations, output concentrations in this case referring to those assessed when final biodegradability is achieved. Calculation is presented in eq 10.

$$f_h = \left(\frac{COD_{methane,t=\infty} + COD_{s,t=\infty} - COD_{s,t=0}}{gCODin - COD_{s,t=0}} \right) \qquad (10)$$

Where: $COD_{methane,\ t=\infty}$: COD equivalent of methane produced at final digestion time

$COD_{s,\ t=\infty}$: soluble COD at final digestion time

$COD_{s,\ t=0}$: soluble COD at time t=0

g CODin: initial amount of COD in the influent

In order to calculate the previous equations two methods could be used, either measurement of the direct fraction of particulate amounts in effluent and influent or, its calculation. Given that measuring the real concentration of particulate matter at a certain time is difficult at lab scale, calculation of these fractions is performed. Calculation of Xss is done by subtracting the total COD solubilised (soluble COD plus COD methanized at time t) from the total COD measured at the start up. The solubilised COD can be assumed to be equal to the methanized one only when methanogenesis is not rate limiting which implies having a well-balanced microbial population in the inoculum.

Both biodegradability and rates are affected by the concentration of intermediates in the blank, therefore for all calculations presented net values should be used, that is after subtraction of the blank values.

6. Results using the protocol

Cropgen plant species were analyzed for their biodegradability and kinetic rates using the protocol presented. Samples were analyzed using two different sample treatment methods, frozen plant samples cut in 1 cm particle size and freeze dried grinded samples grinded and sieved through a 0.2 mm mesh.

Results of the achieved biodegradabilities are shown in D27. For the freeze grinded material maximum and minimum BMP achieved was 0.17 and 0.37 l CH_4/gVS, the majority of the species being in the range between 0.25 and 0.33.

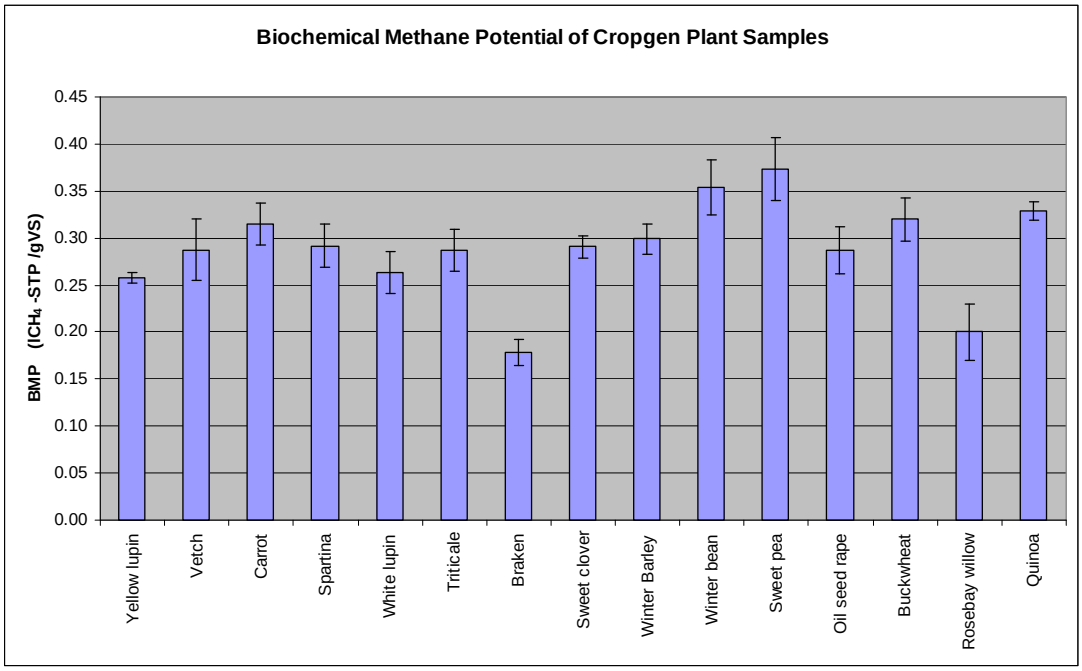


Figure 16. Biochemical Methane Potential of freeze dried grinded Cropgen plant samples

Some of the species were also tested at 1 cm particle size, our results indicate that the production of methane is faster for grinded material, as well the duration of the test is shorter compared to samples of 1 cm particle size (Figure 17). In order to reach 80% the maximum pressure, the test using dry grinded material required 8-12 days, while in the test performed with 1 cm particle size 28-45 days were needed.

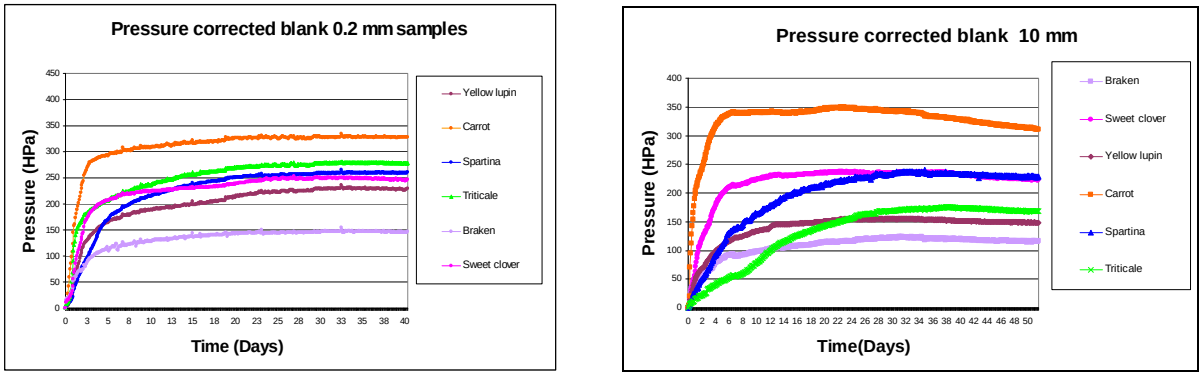


Figure 17. Pressure evolution Cropgen species using the optimized protocol and two different particle sizes

It is clear from the outcomes of our experiments that the BMP test using grinded material can be reduced to a few days without majorly affecting the final outcome in terms of categorization of the plant species according to their biodegradability.

From the results of these experiments also hydrolysis rates were calculated. The rates obtained could be excellently described by first order kinetics (Figure 18).

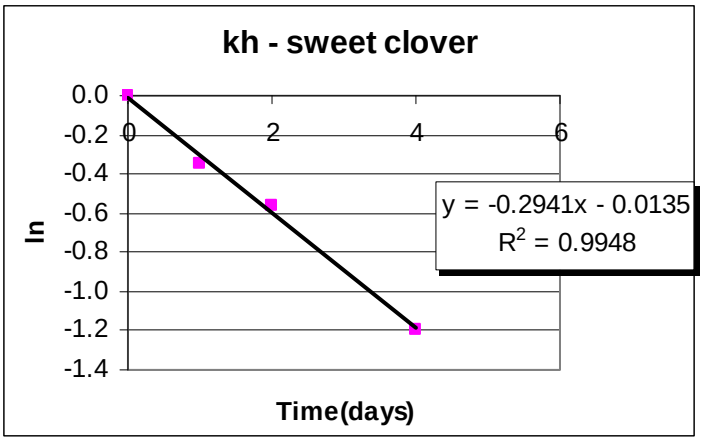


Figure 18 . Hydrolysis constant assessment

For the freeze dried grinded material, the hydrolysis constant range was between 0.22 and 0.72 d^{-1}. In general the hydrolysis constant values for 1 cm samples were found to be considerably lower than the one assessed for dry grinded material. This was in fact expected since it is known that the rate of hydrolysis increases for small particle sizes, as it has been previously reported by Hills and Nakano (1984) and Sanders (2001). Since the effect of the particle size in the hydrolysis rates is more significant compared to the effect in the final BMP achieved, for reactor design purposes it is more convenient to use the particle size closest to reality.

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Annex 1. Nutrient and buffer stock solutions*

Solution 1: Macronutrient solution, dose 2 ml l^{-1}	
Nutrient	**Amount (g $.l^{-1}$)**
NH_4Cl	170
$CaCl_2.2H_2O$	8
$MgSO_4.4H_2O$	9

Solution 2: Trace elements solution, dose 1 ml l^{-1}	
Nutrient	**Amount (g $.l^{-1}$)**
$FeCl_3.4H_2O$	2
$CoCl_2.6H_2O$	2
$MnCl_2.4H_2O$	0.5
$CuCl_2.2H_2O$	0.03
$ZnCl_2$	0.05
H_3BO_3	0.05
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.09
$Na_2SeO_3.5H_2O$	0.1
$NiCl_2.6H_2O$	0.05
EDTA	1
HCl 36%	1 (ml $.l^{-1}$)
Resazurin	0.5

Solution 3: Phosphate buffer**, dose 40 ml l^{-1}		
Solution	**Amount (g $.l^{-1}$)**	**Demiwater (g $.l^{-1}$)**
Primary Solution KH_2PO_4	43.08	956.92
Secondary solution $Na_2HPO_4.2H_2O$	88.86	911.14

**Solution 1, 2 and 3 after Sjon Kortekaas. It differs from the original recipe as phosphate amounts in the macronutrient solution are not added (a separate phosphate buffer solution is prepared). The proposed dosage is related to 2- 4 g VS l^{-1} sludge concentration.*

***For a phosphate buffer of pH .7.0 the primary and secondary solutions should be mixed in equal amounts. The dosing can be changed according to buffer capacity of inoculum, however the resulting molarity when adding the recommended dose should be equal or below 20 mM P. Less amount can be added to the blank bottle to avoid inhibition.*