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# Development of an enzymatic assay for the determination of cellulose bioavailability in municipal solid waste

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**Abstract**

As there is a constant need to assess the biodegradation potential of refuse disposed of in landfills, we have developed a method to evaluate the biodegradability of cellulosic compounds (cellulose and hemicellulose) in municipal solid waste. This test is based on the quantification of monosaccharides released after the hydrolysis of solid waste samples with an optimised enzyme preparation containing commercially available cellulases and hemicellulases. We show that the amounts of monosaccharides could be related to the biodegradability of the cellulosic material contained in the samples. This enzymatic cellulose degradation test was assayed on 37 samples originating from three Belgian landfills and collected at different depths. As results correlated well with those obtained with a classical biochemical methane potential assay, this new and rapid test is sufficiently reliable to evaluate cellulose bioavailability in waste samples.

## Introduction

Municipal solid waste (MSW) has been disposed of in landfills for several decades. The organic matter contained in the landfill body is degraded microbiologically generating leachate and biogas that have to be managed for several years. There is thus a constant need to assess the biodegradability of buried MSW in order to evaluate the efficiency of different MSW pre-treatment, to predict the duration of the aftercare period or to estimate the remaining potential for landfill gas production.

The gas potential can be indirectly determined via stoichiometric and empirical equations from the determination of total organic carbon (TOC), chemical oxygen demand (COD) and other specific parameters such as cellulose and lignin contents (Chandler et al. 1980; Metcalf & Eddy Inc. 1991; Parkin & Owen 1986; Wang et al. 1997). It is also possible to measure the calorific value ($H_0$) describing the potential amount of energy that will be gained in an incineration process. Alternatively, powerful analytical methods such as NMR and FT–IR spectroscopy have been developed to monitor the changes in the chemical structure of MSW during composting (Pichler et al. 2000; & Smidt et al. 2002). Some biological tests based on aerobic and anaerobic assays have also been developed to evaluate the biodegradability of MSW and the gas generating potential. At the same time, several workers have estimated the biodegradability of solid waste components by the use of a biochemical methane potential (BMP) assay (Bogner 1990; Shelton & Tiedje 1984; Stinson & Ham 1995; Wang et al. 1994) or by an incubation test (Binner & Zach 1999). Both assays

are based on the measure of methane gas produced by a methanogenic biomass degrading the organic matter in anaerobic conditions. Other tests evaluate the biodegradability of organic polymers and residual wastes by measuring the oxygen consumed or the carbon dioxide produced during a respiration test (Binner & Zach 1999; Pagga et al. 1999).

Whilst different methods offer certain advantages, they also suffer from certain limitations. For instance, chemical parameters such as COD and TOC do not take into account the biodegradable fraction of the organic matter. Spectroscopic methods require sophisticated equipment and are limited to the study of chemical transformations. Anaerobic tests need to be run for several months and respiration tests simulate aerobic conditions that do not prevail into the landfill.

The organic fraction of MSW is made up of 30–50% of cellulosic substances that can undergo biological degradation (Barlaz et al. 1989; Eleazer et al. 1997; Rees 1980). Cellulose and hemicellulose are thus the most significant carbon source for methanogenesis in landfills as their degradation contributes to 90% of the total methane produced (Barlaz et al. 1989). However, the biodegradation of cellulosic substrates, such as paper, cardboard, wood and textile, is slow representing therefore one of the limiting steps of the biological processes occurring in MSW landfills.

Our study focused on the first stage of the bioconversion process, *i.e.* the enzymatic hydrolysis step. In this work, a new test allowing a reliable and rapid evaluation of the enzymatic cellulose bioavailability was developed. This test was based on enzymatic hydrolysis of residual cellulosic material to quantify the biodegradability with subsequent measurement of the quantity of sugars liberated. This enzymatic cellulose degradation test (ECD) has been performed on refuse samples originating from various layers of three different landfills and results were compared with those obtained from BMP assays realised in parallel.

## Material and methods

### *Sample preparation*

Waste refuses were collected from boreholes (up to 40 m-depth) made in three Belgian landfills called in this work L1, L2 and L3. Waste was extracted from a borehole and separated into samples corresponding to 1 m intervals. Large glass pieces, stones, plastics and metal pieces were removed manually. After sorting, the remaining refuse materials were dried for 5 days at 105 °C before being shredded with a cutting mill to a particle size of $\leq$5 mm and homogenised. All the data were reported to the grindable fraction. Samples containing 3–35% cellulosic material were then dried at 105 °C for 24 h to constant weight before each experimental trial.

### *Chemical analysis*

Cellulosic materials were analysed according to a HPLC method adapted from Pettersen & Schwandt (1991). Three hundred milligrams of each MSW sample was hydrolysed with 3 ml of 72% $H_2SO_4$ for 1 h at 30 °C. The samples were then diluted to 2.5% $H_2SO_4$ with distilled water and autoclaved at 120 °C for 1 h. Samples were run in triplicate and D (+) Fucose (Fluka, Buchs, Switzerland) was used as standard to correct for further hydrolysis due to the autoclave operation. Samples were analysed by HPLC on an Agilent 1100 series apparatus (Agilent Technologies, Massy, France) equipped with a refractometric detector. Sugars were separated on a C-610-H ion exchange column (300 mm $\times$ 7.8 mm, Supelco, Bellefonte, PA.) and quantified using standards. All samples were filtered through 0.2 $\mu$m Minisart Syringe filter (Vivascience, Hannover, Germany) prior to analysis.

## Enzymatic hydrolysis test

### *Enzymes*

The enzymes used for the hydrolysis test were all purchased from Novo Nordisk (Bagsvaerd, Denmark). Viscozyme L® and Celluclast 1.5L® are liquid cellulolytic preparations and Celluzyme® is a solid cellulolytic preparation.

Celluzyme solutions were prepared by dissolving the commercial product in 0.1 N phosphate buffer at pH 5.5 to which 0.05% $NaNO_3$ was added to prevent microbial growth. The solutions were then filtered on a GF/C membrane (Whatman, Maidstone, England). Celluclast 1.5 L and

Viscozyme L were dialysed overnight in the same buffer using nitrocellulose membranes with a cut-off of 10 kD (Sigma-Aldrich, St. Louis, USA). One litre of the working enzymes mixture was obtained by adding 500 ml of Celluzyme 20 g/l, 100 ml of dialysed Viscozyme L and 50 ml of dialysed Celluclast 1.5 L to 350 ml of 0.1 N phosphate buffer-0.05% $NaNO_3$ at pH 5.5.

*Determination of enzyme activities*

The cellulase and hemicellulase activities of Celluzyme, Celluclast 1.5L and Viscozyme L were first tested in order to determine the best dilution to use them in a mixture. One enzyme unit was defined as the concentration of enzymes liberating one micromole of monosaccharide per minute. All activities were tested during 1 h at pH 5.5 and 40 °C in 0.1 N phosphate buffer-0.05% $NaNO_3$. After incubation, the samples were heated for 2 min at 100 °C to stop the reaction. The filter paper assay (FPase activity) was used for cellulase activity determination (Mandels et al. 1976). Hemicellulase (xylanase) activity was determined by using oats spelt xylan (Sigma-Aldrich, St Louis, USA) following the same procedure as the filter paper assay.

Celluzyme activities were tested at 5, 10 and 20 g/l. Celluclast and Viscozyme activities were tested after being respectively diluted 20, 50, 100 times for the Celluclast and 10, 50, 100 times for the Viscozyme. For each dilution, controls were made to measure the background of sugars already present in Novo Nordisk enzymatic preparations. Each enzyme and the enzyme mixture were tested in triplicate.

Specific endoglucanase (CMCase) and $\beta$-glucosidase activities of the final mixture of enzymes were measured after incubating 200 $\mu$l of enzymatic solution with respectively 1500 $\mu$l of carboxymethylcellulose 1% and 1500 $\mu$l of cellobiose 1% (adapted from Gordon & Phillips, 1989; Miller et al. 1960).

*Kinetic of enzymatic hydrolysis*

Cellulase and hemicellulase-mediated hydrolysis were performed either with each enzyme (Celluzyme, Celluclast 1.5 L and Viscozyme L) preparation or with a mixture of all three. For hydrolysis, 500 mg of cellulosic substrates or 1000 mg of MSW samples were mixed with 30 ml of an enzymatic solution for 40 h at 40 °C. Each hydrolysis was performed in triplicate. The mass of monosaccharides liberated is reported to the initial mass of sample hydrolysed in order to assess the biodegradability of refuse samples.

Equation 1 (Parkin & Owen 1986) was used to calculate the methane potential of monosaccharides released by the enzymatic hydrolysis if they are converted into methane.

$$\begin{aligned} C_nH_aO_b + [n - (a/4) - (b/2)]H_2O \\ \rightarrow [(n/2) - (a/8) + (b/4)]CO2 \\ + [(n/2) + (a/8) - (b/4)]CH_4 \end{aligned} \quad (1)$$

*Biochemical methane potential (BMP) assay*

The BMP assay and the volumes of methane produced were determined following the procedure described by Wang et al. (1994). Each assay was performed in triplicate. The concentrations of methane and carbon dioxide in the biogas produced in a BMP assay were measured on a gas chromatograph (Hewlett Packard 5890 series II) equipped with a thermal conductivity detector (TCD) using a GasPro GSC column (30 m $\times$ 0.32 mm,) (Alltech, Deerfield, USA) coupled to a CP-Carboplot P7 column (27.5 m $\times$ 0.53 mm, Varian, Middelburg, The Netherlands). Helium N45 (Air Liquide, Liège, Belgium) was used as carrier and reference gas. The GC was calibrated with external standards (Air Liquide, Liège, Belgium).

## Results

*Cellulolytic and hemicellulolytic activities of enzymes used*

Cellulase (FPase) and xylanase activities of the different commercial products (Celluzyme, Celluclast 1.5L and Viscozyme L) and the content of sugars already present in these preparations (background) were measured at various concentrations (table 1). All three original enzyme solutions had both xylanolytic and cellulolytic activities. These results enabled the determination of the best compromise between a high enzymatic activity and a low background, *i.e.* a mixture containing Viscozyme L and Celluclast 1.5 L diluted 10 and 20 times respectively and 10 g/l of Celluzyme. The resulting activities of the mixture show a

*Table 1.* FPase and xylanase activities of Celluzyme, Viscozyme, Celluclast and enzymatic mixture measured at different concentrations or dilutions of the commercial products. Background of sugars measured in the different enzymatic preparations

| Enzymes | Concentration ($gl^{-1}$) | Dilution factor | FPase activity (mIU/ml) | Xylanase activity (mIU/ml) | Background ($g\ l^{-1}$) |
|---|---|---|---|---|---|
| Celluzyme | 2.5 | – | 39 | nd | 0.073 |
| | 5 | – | 60 | nd | 0.111 |
| | 10 | – | 85 | 200 | 0.15 |
| | 20 | – | 121 | nd | 0.319 |
| Celluclast 1.5L | – | 20 | 176 | 135 | 0 |
| | – | 50 | 109 | 84 | 0 |
| | – | 100 | 68 | 53 | 0 |
| Viscozyme L | – | 10 | 62 | 100 | 0,23 |
| | – | 50 | nd | 35 | 0 |
| | – | 100 | nd | 32 | 0 |
| Enzymatic mixture[a] | – | – | 350 | 420 | nd |

[a] For 1 l: 500 ml of 'Celluzyme' 20 g/l, 100 ml of dialysed 'Viscozyme L' and 50 ml of dialysed 'Celluclast 1.5L' and 350 ml of 0.1 N phosphate buffer-0,05% $NaN_3$ at pH 5.5.

FPase activity of 350 mIU/ml and a xylanase activity of 420 mIU/ml. The FPase and xylanase activities measured for the enzymatic mixture were close to the sum of each enzyme activity. Moreover, specific CMCase and cellobiase assays confirmed that this mixture had endoglucanase (30 mIU/ml) and $\beta$-glucosidase (540 mIU/ml) activities. A lack of $\beta$-glucosidase activity would lead for example to an accumulation of cellobiose that is known for its feedback effect on cellulases. An efficient $\beta$-glucosidase activity is also essential in order to degrade cellulose completely to monomeric sugars that will be quantified by HPLC.

*Enzymatic hydrolysis of cellulosic substrates*

Kinetic studies performed on cellulosic (Whatman n°1 paper) and hemicellulosic (xylan from oat spelts) substrates revealed that the use of the mixture of enzymes increased significantly the hydrolysis yield compared to each enzyme tested alone (Figure 1). This degradation of cellulose and xylan was associated with an accumulation of glucose and xylose that reached respectively 14 and 10 g/l in the medium upon the use of the enzyme mixture. This gives an indication over the concentration of monosaccharides that could be reached without

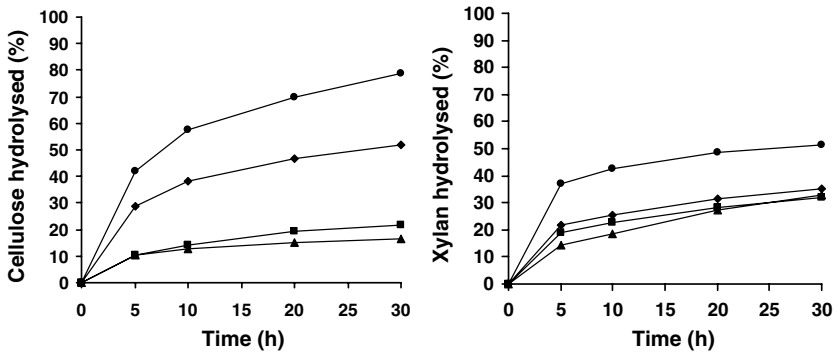


*Figure 1.* Cellulose or hemicellulose hydrolysed when 500 mg of Whatman n°1 paper. (A) and Xylan from oats spelt. (B) are degraded for 30 h with Viscozyme L (10 fold diluted); (▲), with Celluzyme (10 g/l) (■), with Celluclast 1.5L (20 fold diluted) (◆), and with the enzymatic mixture (●).

affecting the enzymatic activity by the so-called feedback effect of glucose and xylose if other cellulosic substrates are degraded. The effect of lignin, another possible inhibitor, was also investigated. Indeed, further experiments performed on wood (spruce wood), in which cellulosic compounds are closely linked to lignin, showed that the level of hydrolysis (about 6%) was lower than the one obtained for pure cellulose and xylan. When filter paper was added to the resulting enzymatic medium, 78% of the initial mass of cellulose was converted into glucose. This strongly suggests that wood cellulose degradation was limited by its bioavailability rather than by any other environmental condition affecting the ECD test.

### *Comparison of ECD and BMP assays on MSW samples*

The BMP assay, which involves an anaerobic process close to the one taking place in a landfill, was compared to the ECD test. Both tests were performed on waste samples collected from various layers of three different MSW landfills (L1, L2 and L3). Therefore, the selected samples had distinct chemical compositions (from 3 to 35% of cellulosic material) and different disposal times (from several months to more than 20 years). The monosaccharides or methane respectively released were reported to the initial mass of the sample in order to describe the potential of biodegradation of cellulosic substances in MSW samples.

The Figure 2 shows the correlation between the total specific amount of sugars liberated after 48 h of enzymatic hydrolysis and the total specific volume of methane produced after 100 days of anaerobic degradation. According to the correlation coefficients calculated from the 3 sets of data, i.e. L1 ($r^2 = 0.87$), L2 ($r^2 = 0.65$) and L3 ($r^2 = 0.79$), it can be assumed that both measures are significantly correlated (Student's *t*-Test for a 99% confidence interval and $p < 0.01$). A significant correlation ($r^2 = 0.55$) is still observed when all the 37 samples from the three landfills are considered together (Student's *t*-test for a 99% confidence interval and $p < 0.01$). It should be mentioned that the volumes of methane experimentally measured for samples L1 are close to those produced if all the sugars released during the ECD test were converted to methane (Equation 1). This is not the case for samples L3 and L2 where experimental methane potential is about

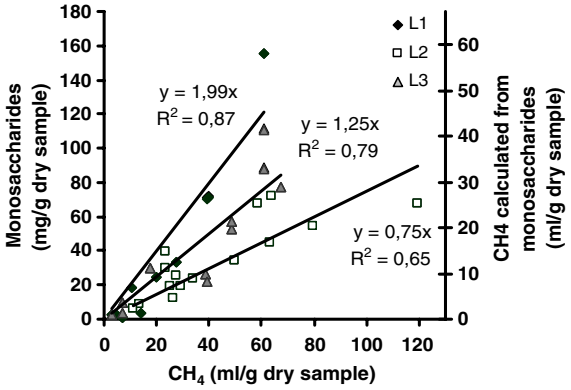


*Figure 2.* Relationship between the total specific amount of monosaccharides (and the calculated methane potential of these monosaccharides on the second y-axis) liberated by the enzymatic test after 48 h and the total specific volume of methane produced by a 100 days-BMP test. The 37 samples tested are originating from different layers of three different Belgian landfills called L1, L2 and L3.

twice and four times higher than the methane potential of the sugars released by the ECD test. This suggests that MSW samples were more completely degraded by the anaerobic biomass.

### *Assessment of the enzymatic hydrolysis*

Further experiments have been carried out to validate the enzymatic test and particularly to achieve a complete hydrolysis of the cellulose bioavailable. The samples L1 and L2 submitted to the enzymatic hydrolysis were dried at 50 °C to constant weight and then submitted to a second, and in the same way, to a third hydrolysis. The figure 3 shows the average proportion of each hydrolysis compared to the total percentage of cellulose hydrolysed. The first hydrolysis degraded 83 ± 13% of the total amount of the cellulose hydrolysed after three steps. The second and the third hydrolysis degraded respectively 11 ± 9% and 6 ± 6%. Regarding the samples coming from L1, the correlation between the total specific amount of monosaccharides liberated by the enzymatic test and the total specific volume of methane produced by the BMP test rises from 0.87 to 0.91 after the second hydrolysis and to 0.92 after the third one. However, this correlation coefficient decreases from 0.79 to 0.64 and to 0.47 for samples coming from L2.

Anyway, the low concentrations measured after the second and third hydrolysis in most of the

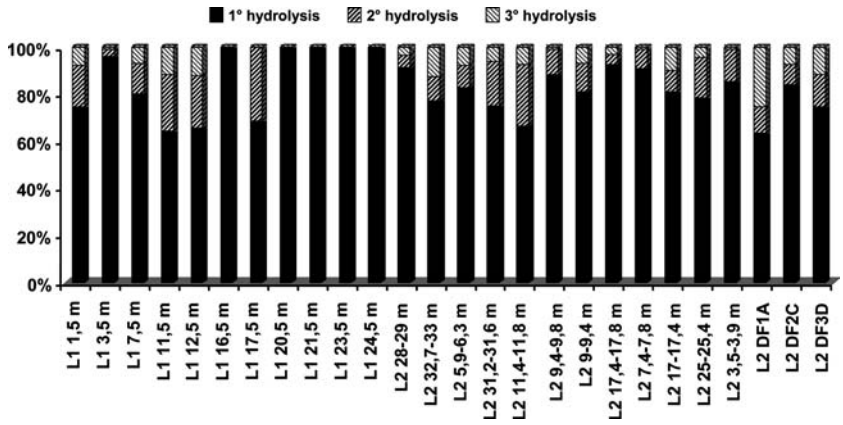


*Figure 3*. Proportion of the total percentage of cellulose and hemicellulose degraded after 3 successive hydrolysis steps. The 26 samples tested are originating from different layers of landfills L1 and L2.

samples suggest that one hydrolysis is sufficient to calibrate the test with a BMP assay.

## Discussion

The results presented in this paper show that the ECD test describes as well as the anaerobic BMP assay the degradation potential of MSW samples collected at various depths in three different landfills. Other works also compared the results of anaerobic tests to other assays based on respiration activity or volatile solids measurements (VS). Binner & Zach (1999) showed a good relationship between results from a 7 days respiration assay and an anaerobic assay running over 90 days when both were applied to 23 MSW samples coming from different mechanical biological pre-treatment plants. They also showed that the respiration activity was related to the mass lost by the samples after ignition at 1000 °C (Ignition Loss) but the correlation was only significant for the samples coming from the same treatment plant. By comparing different stability criteria for mechanical biological pretreated waste, Cossu et al. (2001) also showed a relationship between a respiration activity and an anaerobic fermentation test but only 6 samples were considered in this case.

However, the biodegradation potential evaluated by respiration assays or by some chemical analysis (TOC and VS) do not take into account the non biodegradability of some organic compounds under the anaerobic conditions taking place in landfills. For example, lignin that is intimately associated with cellulose in woody tissues and plants, is only slowly degradable under anaerobic conditions (Colberg 1988; Young & Frazer 1987). Therefore, its resistance is thought to delay strongly the biodegradation of the cellulosic material (Crawford 1981) due to a lack of cellulose availability. On the other hand, the main disadvantage of anaerobic tests, such as a BMP assay, is that they must be carried out over a very long period (100 days in our case). In this context, the ECD test we report here is more appropriate as it assesses the fraction of cellulose that is readily available without changes of the lignin properties. Results from ECD test and BMP assay applied to 37 samples from three Belgian landfills showed a significant correlation. However, the regression slopes between ECD and BMP results were quite different in the three considered landfills. The lower slopes of the regression lines L2 and L3 (Figure 2) imply that MSW samples were more completely degraded by the anaerobic biomass, suggesting that cellulose was more available for the anaerobic microflora than for the enzymatic mixture even if this mixture was active enough to degrade all the cellulose contained in the samples. The presence of other carbon sources (proteins, lipids) as substrates for the anaerobic microflora in the BMP assay or as a barrier limiting cellulose bioavailability for enzymes in the ECD test might also explain the variations observed between L1, L2 and L3 samples. However, protein and lipids respective contents are usually not higher than 5–

6% in fresh MSW (Barlaz et al. 1989; Rees 1980) and 5–8% of the TOC in old waste (Bäumler et al., 2001). On another hand, beside the potential of the enzymatic test used alone for direct assessment of cellulose bioavailability, our results also show that this ECD test combined with the BMP assay could be useful to highlight different biological reactivities between samples coming from different landfills. In fact, this study of MSW coming from 3 landfills with combined tests allows to estimate the methane potential still expected from the mass of enzymatically degraded (hemi)cellulose by ECD using the equation of conversion into BMP. Moreover, limit values can be recommended, as suggested by Binner & Zach (1999), in order to define MSW with a low biodegradation potential. For example, assuming that gas generating potential of fresh MSW ranges between 100 and 200 Nl/kg MSW (Barlaz et al. 1990; Binner & Zach 1999; Gendebien et al. 1992; Pacey 1990), a limit value of 10% of this potential produced by waste samples using a BMP assay could be considered as acceptable to classify these ones as samples with low methane potential. The correlating values with the ECD when the 37 samples of L1, L2 and L3 are considered together ranges between 10 and 20 g of monosaccharides released / kg of waste.

## Conclusions

In this paper, a new and rapid enzymatic test using an optimised mixture of cellulases/hemicellulases has been compared to a classic 100 days-BMP assay in order to assess the cellulose degradation of MSW. Both methods have been performed on three sets of MSW samples under suitable conditions for biodegradation *i.e.* no limiting moisture content, optimal pH and temperature. The results show a good correlation between the two assays. As it allows a large set of trials with reduced incubation time, this enzymatic test is a promising tool to study the biodegradation potential of cellulosic material in MSW samples. Moreover, it simulates the microbial degradation of cellulose in the presence of the lignin barrier using high activities of (hemi)cellulolytic enzymes. It may thus assess rapidly the methane potential of waste refuses and may point out different behaviours of bioconversion when combined with methanisation tests.

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