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Abstract:

TITLE

Development of an enzymatic assay for the determination of cellulose bioavailability in Municipal Solid Waste

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KEYWORDS

biodegradation, cellulosic compounds, enzymatic hydrolysis test, biochemical methane potential assay, municipal solid waste

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 monosaccharids 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 monosaccharids could be related to the biodegradability of the cellulosic material contained in the samples. This enzymatic cellulose degradation test was assayed on 26 samples originating from two 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.

ABREVIATIONS

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 pretreatments, 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; Parkin, G.F. & Owen, W.F., 1986; Metcalf & Eddy Inc., 1991; 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 and 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 (Shelton & Tiedje, 1984; Bogner, 1990; Wang et al., 1994; Stinson & Ham, 1995; Eleazer et al., 1997) or by an incubation test (Binner et al., 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

69 oxygen consumed or the carbon dioxide produced during a respiration test (Pagga et al.,
70 1995; Binner et al., 1999).
71 Whilst different methods offer certain advantages, they also suffer from certain
72 limitations. For instance, chemical parameters such as COD and TOC do not take into
73 account the biodegradable fraction of the organic matter. Spectroscopic methods require
74 sophisticated equipment and are limited to the study of chemical transformations.
75 Anaerobic tests need to be run for several months and respiration tests simulate aerobic
76 conditions that do not prevail into the landfill.
77 The organic fraction of MSW is made up of 30-50 % of cellulosic substances that can
78 undergo biological degradation (Rees, 1980; Barlaz et al., 1989; Eleazer et al., 1997).
79 Cellulose and hemicellulose are therefore the most significant carbon source for
80 methanogenesis in landfills as their degradation contributes to 90% of the total methane
81 produced (Barlaz et al., 1989). However, the biodegradation of cellulosic substrates,
82 such as paper, cardboard, wood and textile, is very slow with a half-life of about 15
83 years (Gendebien et al., 1992) and therefore represents one of the limiting steps of the
84 biological processes occurring in MSW landfills.
85 Our study focused on the first stage of the bioconversion process, *i.e.* the enzymatic
86 hydrolysis step. In this work, a new test allowing a reliable and rapid evaluation of the
87 enzymatic cellulose bioavailability was developed. This test was based on enzymatic
88 hydrolysis of residual cellulosic material to quantify the biodegradability with
89 subsequent measurement of the quantity of sugars liberated. This enzymatic cellulose
90 degradation test (ECD) has been performed on refuse samples originating from various
91 layers of two different landfills and results were compared with those obtained from
92 BMP assays realised in parallel.
93

MATERIAL AND METHODS

Sample preparation

Waste refuses were collected from boreholes (up to 35 m-depth) made in two Belgian landfills L1 and L2. 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 while the remaining refuse materials were shredded with a cutting mill to a particle size of ≤ 5 mm and homogenised. Samples containing 3 to 35 % cellulosic material were then dried at 105°C for 24 h.

Chemical analysis

Cellulosic materials were analysed according to a HPLC method adapted from Pettersen & Schwandt (1991). 300 mg 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 1h. 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 x 7,8 mm, Supelco, Bellefonte, PA.) and quantified using standards. All samples were filtered through 0,2 µm Minisart Syringe filter (Vivascience, Hannover, Germany) prior to analysis.

Lignin was determined gravimetrically following extraction with triethylene glycol as described by Edwards, 1972 and after a clean-up procedure of the waste material with a modified neutral detergent fibre (NDF) pre-treatment (Rowland and Roberts, 1999).

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 1.5L and Viscozyme L were dialysed overnight in the same buffer using nitrocellulose membranes with a cut-off of 10 kD (Sigma-Aldrich, S^t 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 1.5L to 350 ml of 0.1 N phosphate buffer-0.05 % $NaNO_3$ at pH 5.5.

Determination of enzyme activities

The filter paper assay (FPase activity) was used for cellulase activity determination (Mandels et al., 1976). Endoglucanase (CMCase) and β-glucosidase activities were measured after incubating 200 µl of enzymatic solution with respectively 1500 µl of

carboxymethylcellulose 1 % and 1500 µl cellobiose 1 % (adapted from Miller et al., 1960 & Gordon and Phillips, 1989), both prepared in the same buffer as mentioned above before being heated for 2 min at 100°C to stop the reaction**.** Hemicellulase (xylanase) activity was determined by using oats spelt xylan (Sigma-Aldrich, S^t Louis, USA) following the procedure for filter paper assay.

All activities were calculated after 1 hour at pH 5.5 and 40 °C. In all cases, one enzyme unit was defined as the quantity of micromoles of monosaccharid liberated per minute. According to the technical data given for each enzyme, pH and temperature values were fixed so as they cover the range allowing an optimal activity.

The cellulase and hemicellulase activities of Celluzyme, Celluclast 1.5L and Viscozyme L were first tested in order to determine the best compromise to use them in a mixture. 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.

Kinetic of enzymatic hydrolysis

Cellulase and hemicellulase-mediated hydrolysis were performed either with each enzyme (Celluzyme, Celluclast 1.5L and Viscozyme L) preparation or with a mixture of all three. For hydrolysis, 1000 mg of sample were mixed with 30 ml of an enzymatic solution for 40 hours at 40 °C. The biodegradability of refuse samples is evaluated by the mass of monosaccharids liberated reported to the total mass of sample hydrolysed.

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). 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 x 0,32 mm,) (Alltech, Deerfield, USA) coupled to a CP-Carboplot P7 column (27,5 m x 0,53 mm, Varian, Middelburg, The Netherlands). Helium N45 (Air Liquide, Liège, Belgium) was used as carrier and reference gas. Calibration was performed using gas mixtures standards purchased by Air liquide (Liège, Belgium). Equation 1 (Parkin and Owen, 1986) was used to calculate the theoretical methane potential of monosaccharids when converted to methane.

$$C_nH_aO_b + [n - (a/4) - (b/2)]H_2O \rightarrow [(n/2) - (a/8) + (b/4)]CO_2 + [(n/2) + (a/8) - (b/4)]CH_4 \quad (1)$$

RESULTS

Cellulolytic and hemicellulolytic activities of enzymes used

Cellulase (FPase) and xylanase activities of the different commercial products (Celluzyme, Celluclast and Viscozyme) 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 and Celluclast

193 diluted 10 and 20 times respectively and 10 g/l of Celluzyme. The resulting activities of
194 the mixture show a FPase activity of 350 mIU/ml and a xylanase activity of 420
195 mIU/ml. The FPase and xylanase activities measured for the enzymatic mixture were
196 close to the sum of each enzyme activity. Moreover, specific CMCase and cellobiase
197 assays made sure that this mixture had endoglucanase (30 mUI/ml) and β-glucosidase
198 (540 mUI/ml) activities. A lack of β-glucosidase activity would lead for example to an
199 accumulation of cellobiose that is known for its feedback effect on cellulases. An
200 efficient β-glucosidase activity is also essential in order to degrade cellulose completely
201 to monomeric sugars that will be quantified by HPLC.
202
203 **Enzymatic hydrolysis of cellulosic substrates**
204
205 In a next step, enzymatic hydrolysis was performed on cellulosic (Whatman n°1 paper),
206 and hemicellulosic (xylan from oat spelts) substrates in order to investigate the time
207 needed to reach the end of the kinetic phase and to determine the concentrations of
208 glucose and xylose associated with the decrease of the enzymatic activity. The
209 hydrolysis associated with the degradation of 500 mg of these substrates was followed
210 for 30 hours. Each enzyme and the enzyme mixture was tested in triplicate (figure 1).
211 For both substrates, the rate of hydrolysis was higher during the first five hours of
212 incubation and decreased after 20 hours (beginning of the stationary phase). With
213 respectively 80 and 50 % of cellulose and xylan hydrolysed after 30 hours, the mixture
214 of enzymes increased significantly the hydrolysis yield in comparison with each enzyme
215 tested alone. This degradation of cellulose and xylane was associated with an
216 accumulation of glucose and xylose that reached respectively 15 and 10 g/l in the
217 media. This gives an indication of the concentration of monosaccharids that could be

obtained when other cellulosic substrates are degraded without being interpreted as a limiting enzymatic activity if the concentrations reached are lower.

Enzymatic hydrolysis was also performed on spruce wood (figure 2), containing 51 % cellulosic materials and 29 % of lignin. Wood was tested because their cellulosic compounds are closely linked to lignin, limiting therefore the bioavailability of these polysaccharids. Results showed a lower percentage of hydrolysis compared to those obtained with substrates such as pure cellulose and xylan (figure 1). The level of degradation induced by the mixture of cellulases was similar to that observed with celluclast as only 0.6 g/l of monosaccharids was released into the medium. This relatively low yield of hydrolysis led to the question of whether enzyme inhibition or bioavailability was limiting cellulose/hemicellulose conversion to glucose/xylose. To address this, a cellulose spike (100 mg of Whatman n°1 filter paper) was added to the enzymatic medium for 14 hours after 30 hours of incubation. The medium spike recovery was 78 % of the glucose expected from filter paper addition. These data suggest that there was not an environmental condition within the enzymatic cellulose degradation (ECD) test that limited cellulose conversion to glucose, but rather the bioavailability of the cellulose.

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 two different MSW landfills (L1 and L2). 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

243 monosaccharids or methane respectively released were reported to the mass of the
244 sample in order to describe the potential of biodegradation of cellulosic substances in
245 MSW samples.
246 The Figure 3 shows the correlation between the total specific amount of sugars liberated
247 after 48 hours of enzymatic hydrolysis and the total specific volume of methane
248 produced after 100 days of anaerobic degradation. The two measures appear to be
249 significantly correlated (calculated with a Student test, P = 0.05) both for samples from
250 L1 (r^2 = 0.87) and L2 (r^2 = 0.65). However, the regression lines have different slopes
251 although there is still a globally significant correlation (r^2 = 0.46) when all the 26
252 samples from L1 and L2 are considered together. On the other hand, the volumes of
253 methane experimentally measured for samples L1 are close to those theoretically
254 produced if all the sugars released during the ECD test were converted to methane
255 (figure 3). This is not the case for samples L2 where experimental methane potential is
256 higher than the theoretical methane potential of the sugars released by the ECD test
257 suggesting that MSW samples were more completely degraded by the anaerobic
258 biomass.
259
260 **Assessment of the enzymatic hydrolysis**
261
262 Further experiments have been carried out to validate the enzymatic test and particularly
263 to achieve a complete hydrolysis of the cellulose bioavailable The samples submitted to
264 the enzymatic hydrolysis were dried at 50 °C to constant weight and then submitted to a
265 second, and in the same way, to a third hydrolysis. The figure 4 shows the average
266 proportion of each hydrolysis compared to the total percentage of cellulose hydrolysed.
267 The first hydrolysis degraded on 83 % of the total amount of the cellulose bioavailable

after three hydrolysis. The second and the third hydrolysis degraded respectively 11 and 6 %. In the case of the samples coming from L1, the correlation coefficient between the total specific amount of monosaccharids 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 0.69 to 0.64 and to 0.47 for samples coming from L2.

Anyway, the low concentrations measured after the first hydrolysis in most of the samples suggest that one hydrolysis is sufficient enough 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 two different landfills. Other works also compared the results of anaerobic tests to other assays based on respiration activity or volatile solids measurements (VS), Binner et al. (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.

293 However, the biodegradation potential evaluated by respiration assays or by some
294 chemical analysis (TOC and VS) do not take into account the non biodegradability of
295 some organic compounds under the anaerobic conditions taking place in landfills. For
296 example, lignin that is intimately associated with cellulose in woody tissues and plants,
297 is only slowly degradable under anaerobic conditions (Young and Frazer, 1987;
298 Colberg, 1988). Therefore, its resistance is thought to delay strongly the biodegradation
299 of the cellulosic material (Crawford, 1981) due to a lack of cellulose availability. On the
300 other hand, the main disadvantage of anaerobic tests, such as a BMP assay, is that they
301 must be carried out over a very long period (more than 100 days). In this context, the
302 ECD test we report here is more appropriate as it assesses the fraction of cellulose that
303 is readily available without changes of the lignin properties. Results from ECD test and
304 BMP assay applied to 26 samples from two Belgian landfills showed a significant
305 correlation. However, the regression slopes between ECD and BMP results were quite
306 different in the two considered landfills. The lower slope of the regression line L2
307 (figure 3) implies that MSW samples were more completely degraded by the anaerobic
308 biomass, suggesting that cellulose was more available for the anaerobic microflora than
309 for the enzymatic mixture even if this mixture was active enough to degrade all the
310 cellulose contained in the samples. The presence of other carbon sources (proteins,
311 lipids) as substrates for the anaerobic microflora in the BMP assay or as a barrier
312 limiting cellulose bioavailability for enzymes in the ECD test might also explain the
313 variations observed between L1 and L2 samples. However, protein and lipids respective
314 contents are usually not higher than 5-6 % in fresh MSW (Rees, 1980; Barlaz et al.,
315 1989) and 5-8 % of the TOC in old waste Bäumler et al. (2001). Moreover, Gendebien
316 et al. (1992) considered that food waste, that is mainly composed of proteins and lipids,
317 which have a relatively short half-life time of 1 year.

Nevertheless, our results show that the ECD test combined with the BMP assay could highlight a different trend between samples coming from two different landfills. Such a differential bioconversion behaviour of cellulosic substances to methane reinforces the need for parameters evaluating the biodegradation potential instead of, or in combination with, other chemical measurements like TOC, VS, COD...

In fact, the study of MSW with combined tests gives a good idea of the methane potential still expected from the mass of enzymatically degraded (hemi)cellulose. Moreover, limit values can be recommended, as suggested by Binner et al. (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; Pacey, 1990; Gendebien et al., 1992, Binner et al., 1999), a limit value of 10 % of this potential (10-20 Nl/kg) could be considered as acceptable to classify waste samples as sample with a low methane potential. The correlating values with the ECD when the 26 samples of L1 and L2 are considered together ranges between 10 and 20 g of monosaccharids / kg of waste.

CONCLUSIONS

In this paper, a new and rapid enzymatic test using a 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 two 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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434

434 **Figure Legend**

435

436 **Fig.1** Cellulose or hemicellulose hydrolysed when Whatman n°1 paper (A) and Xylan
437 from oats spelt (B) are degraded for 30 h with Viscozyme (10 fold diluted) (▲), with
438 Celluzyme (10 g/l) (■), with Celluclast (20 fold diluted) (♦) and with the enzymatic
439 mixture (●). Concentrations of glucose (A) or xylose (B) released during the
440 degradation of Whatman n°1 paper and Xylan with the enzymatic mixture.

441

442 **Fig.2** Cellulosic substances (cellulose and hemicellulose) hydrolysed when spruce wood
443 are degraded for 30 h with Viscozyme (10 fold diluted) (▲), with Celluzyme (10 g/l)
444 (■), with Celluclast (20 fold diluted) (♦) and with the enzymatic mixture(●).
445 Concentrations of monosaccharids released during the degradation of the spruce wood
446 with the enzymatic mixture.

447

448 **Fig.3** Relationship between the total specific amount of monosaccharids (and the
449 corresponding methane potential) liberated by the enzymatic test after 48 h and the total
450 specific volume of methane produced by a 100 days-BMP test. The 26 samples tested
451 are originating from different layers of two different Belgian landfills called L1 (♦) and
452 L2 (□).

453

454 **Fig. 4** Proportion of the total percentage of cellulose and hemicellulose hydrolysed after
455 each of the 3 hydrolysis. The 26 samples tested are originating from different layers of
456 landfills L1 and L2.

457

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

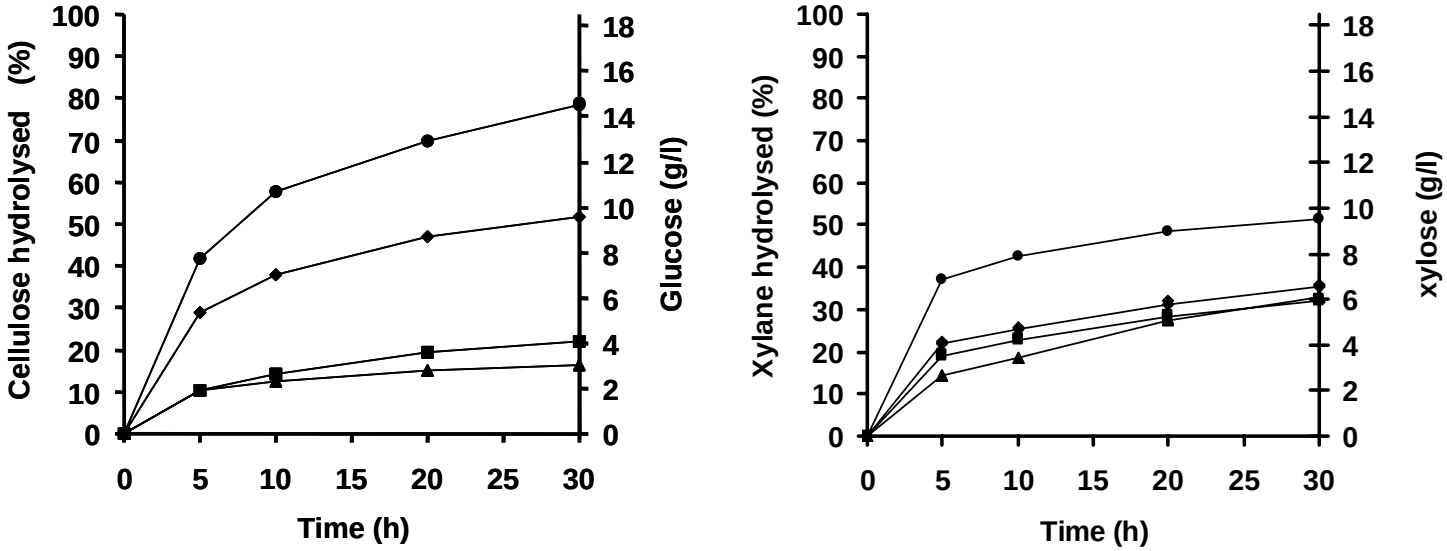
Enzymes	Concentration (g l^{-1}) dilution factor	FPase activity (mUI ml^{-1})	Xylanase activity (mUI ml^{-1})	Background (g l^{-1})
Celluzyme [a]	2.5	39	nd	0.073
	5	60	nd	0.111
	10	85	200	0.15
	20	121	nd	0.319
Celluclast [b]	20	176	135	0
	50	109	84	0
	100	68	53	0
Viscozyme [b]	10	62	100	0,23
	50	nd	35	0
	100	nd	32	0
Enzymatic mixture[c]	-	350	420	nd

nd: non determined

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

465 **Figure 1**

466



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468 **Figure 2**

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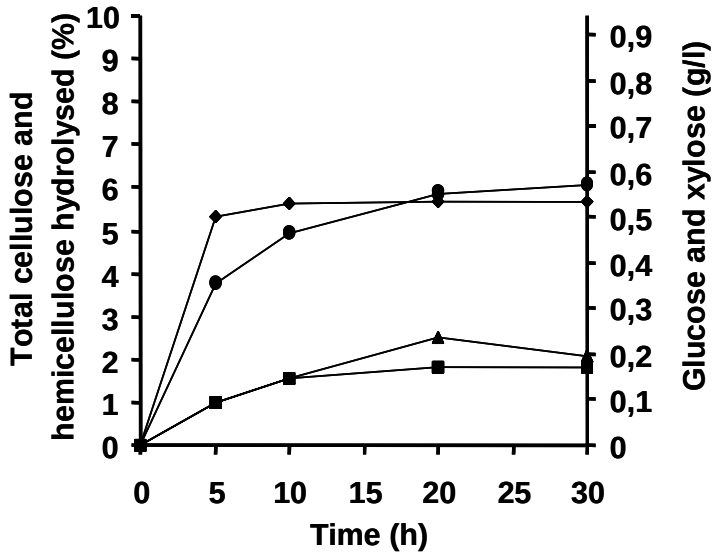

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475 **Figure 3**

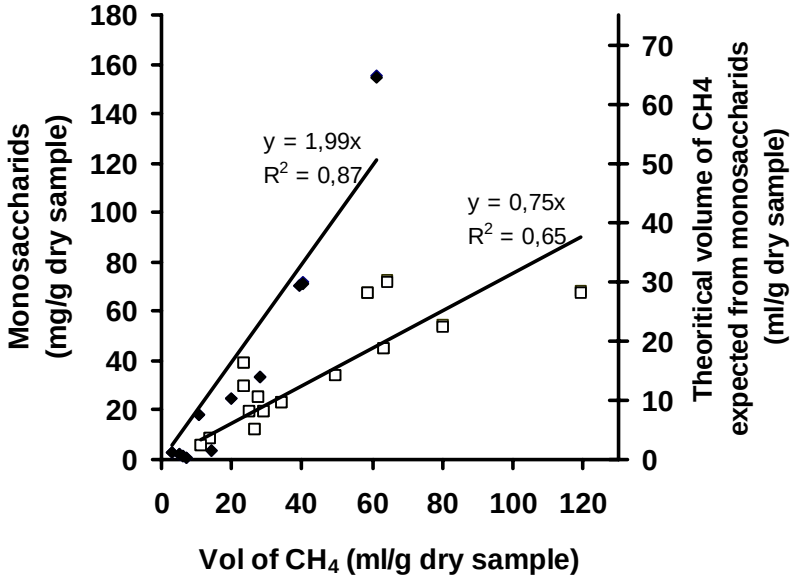

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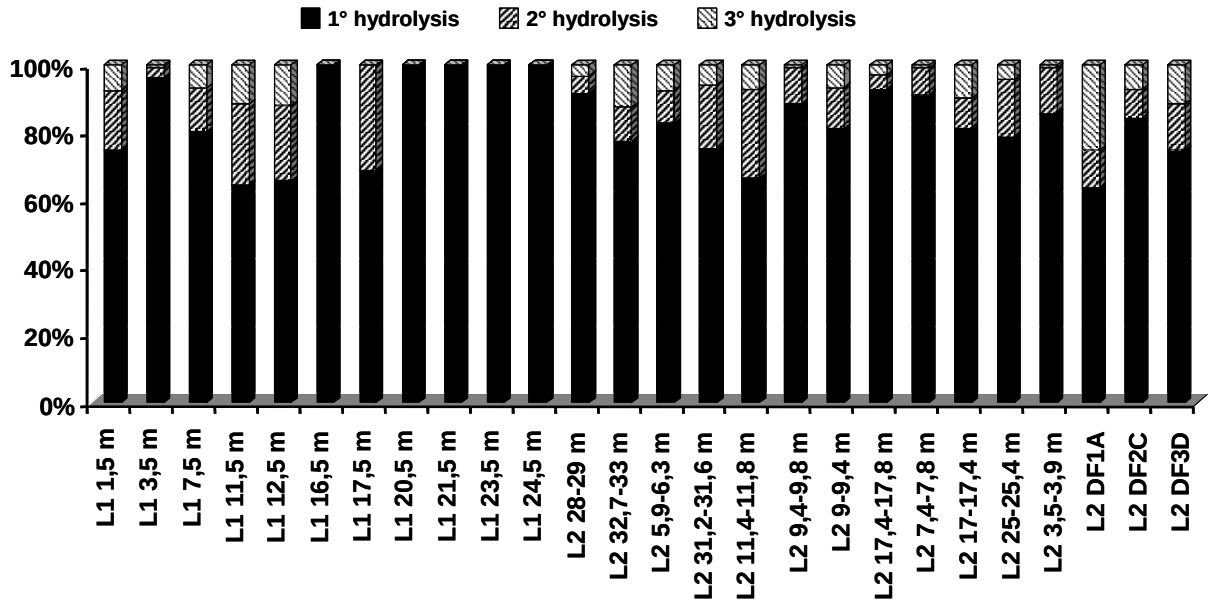
Figure 4


1° hydrolysis
2° hydrolysis
3° hydrolysis
100%
80%
60%
40%
20%
0%
L1 1,5 m
L1 3,5 m
L1 7,5 m
L1 11,5 m
L1 12,5 m
L1 16,5 m
L1 17,5 m
L1 20,5 m
L1 21,5 m
L1 23,5 m
L1 24,5 m
L2 28-29 m
L2 32,7-33 m
L2 5,9-6,3 m
L2 31,2-31,6 m
L2 11,4-11,8 m
L2 9,4-9,8 m
L2 9-9,4 m
L2 17,4-17,8 m
L2 7,4-7,8 m
L2 17-17,4 m
L2 25-25,4 m
L2 3,5-3,9 m
L2 DF1A
L2 DF2C
L2 DF3D