

## Results of batch anaerobic digestion test – effect of enzyme addition

Teresa Suárez Quiñones<sup>\*1</sup>, Matthias Plöchl<sup>2</sup>, Jörn Budde<sup>1</sup>, Monika Heiermann<sup>1</sup>

(1. *Leibniz Institute for Agricultural Engineering Potsdam-Bornim, Germany;*  
2. *BioenergieBeratungBornim GmbH, Germany*)

**Abstract:** The hydrolysis of lignocellulose is assumed to be the rate-limiting step in the anaerobic fermentation process. A fungal hydrolytic enzyme mixture was used to assess the enzymatic impact on different feedstocks for biogas production. The optimal conditions for enzymatic hydrolysis of rye grain silage, maize silage, grass silage, feed residues and solid cattle manure were determined in lab-scale experiments. Finally, the effects of enhanced hydrolysis on anaerobic digestion were investigated in batch digestion tests. Enzyme treatment of substrate showed Michaelis-Menten-like behavior and reached maximum values after 3 hours for reduced sugars as a product of hydrolysis. Methane production potential was determined for specific feedstock mixtures without enzyme, with inactivated enzyme and with active enzyme (with and without buffer). The results obtained show a clear increase in methane production after enzyme application for solid cattle manure (165 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup> to 340 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup>), grass silage (307 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup> to 388 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup>; enzyme plus buffer), feed residue (303 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup> to 467 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup>), maize silage (370 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup> to 480 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup>) and a lower increase for rye grain silage (355 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup> to 413 L<sub>N</sub> CH<sub>4</sub>·kg<sub>ODM</sub><sup>-1</sup>). The ratios of heating values from methane yields to heating values from the dry materials ranged between 0.3 and 0.7 for the untreated feedstock and increased to levels between 0.6 and 0.9 after the different forms of enzyme application.

**Keywords:** Anaerobic digestion, energy crops, solid manure, feed residue, hydrolytic enzymes

### 1 Introduction

The growing number of biogas plants is placing an increasing strain on the availability of suitable feedstock. Use of energy crops especially has sparked critical discussion on the competition between food and fuel. Although this mainly addresses the field of liquid biofuels such as bioethanol and biodiesel, the use of crops for anaerobic digestion is also being questioned. To prevent a discouraging outcome of this discussion it is worth increasing the number of suitable feedstocks for anaerobic digestion and improving their digestibility (Heiermann et al., 2009).

In general, every organic material is suitable for anaerobic digestion as long as the lignin, hemi-cellulose and cellulose fractions are small. Wood and straw are considered to be less degradable under anaerobic conditions (El Bassam, 1998). Lignocellulose-rich feedstock needs to be decomposed by pretreatment to improve its digestibility. Numerous physical methods known from other fields of preparing crops for material use and relying on mechanical or

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\* **Corresponding author's email:** tsuarez@atb-potsdam.de

thermal treatment to destroy cell structures might be applicable to biogas technology (Budde et al., 2008). These physical methods can also be combined with subsequent chemical treatment, for instance acidifying or alkalizing. However, the effect of pretreatment depends greatly on the biomass composition and operating conditions. All these pretreatments have their advantages and disadvantages and more research is needed to optimize methods (Hendriks and Zeeman, 2009).

Increasing interest is also being shown in using biological alternatives like enzymes to pretreat feedstock. Enzymes are naturally occurring compounds which are biodegradable and therefore environmentally-friendly. One of the promising options seems to be the application of hydrolytic enzymes to the feedstock. Enzymatic hydrolysis leads to higher yields of monosaccharides, because cellulases catalyze only hydrolysis reactions without further sugar degradation reactions (Palmqvist and Hahn-Hägerdal, 2000).

A number of studies have examined the conversion of cellulose and hemi-cellulose from bagasse (sugar cane), rice hulls and rice straw by enzymatic treatment to improve the performance of bioethanol production (Karimi et al., 2006; Kim and Dale, 2004; Palmqvist and Hahn-Hägerdal, 2000; Saha and Cotta, 2008; Schwarz, 2001; Saha et al., 2005). Other studies have looked at improving bioethanol and biogas production from winter rye, oilseed rape, and faba bean (Petersson et al., 2007). The authors used either single enzymes such as cellulases, hemi-cellulase, xylanases, xylan esterases, pectinase, glucosidases, etc. or combinations of these. In general, combinations of up to six different enzymes provided evidence of improved performance compared with single enzyme application (Eun et al., 2006). As hydrolysis is the rate-limiting step in anaerobic digestion of lignocellulosic biomass (Noike et al., 1985; Zhang et al., 2007; Zhang and Cai, 2008), it appears evident that enzyme application is the best way to enhance methane formation. The same breakdown pathway of plant material for the bioethanol production applies for biogas production as well. Enzymatic pretreatment promotes the hydrolysis of lignocellulose, breaking it down to lower molecular weight substances ready for use by the archaea. The hydrolysis of cellulose is a sequential breakdown of the linear glucose chains, whereas hemi-cellulases must be capable of hydrolyzing branched chains containing different sugars and functional groups (Jørgensen et al., 2007).

The mixture of fungal hydrolytic enzymes used here is commonly available and is produced by solid state fermentation. This mixture seems to be a good substitute for expensive conventional enzymes, particularly as enzymes mixtures performed better than single enzymes (Eun et al., 2006). The formation of reduced sugars from different feedstocks and the relationships of enzyme-substrate concentration, temperature and time of enzymatic hydrolysis to determine the effectiveness of the enzyme preparation were investigated. We also explored the effects of enzymatic treatment on anaerobic digestion in batch digestion tests. In these tests, we distinguished between the variant without enzyme, which can be considered as a control variant, and three variants with enzymes applied. As the enzyme mixture manufacturer considers that the enzymes add biomass to the process, which is digested as well, we considered a variant with inactivated enzymes. However, it can also be assumed that the inactivated enzymes display additional effects. We therefore included this variant as well. Finally we also wanted to see whether buffering the systems produced additional effects and tested two variants of enzyme application: with and without acetate buffer. In order to evaluate the effects of enzyme application on the methane production, we compared the heating values of the methane produced in each variant with the heating value of the particular dry materials. From our experiments, we deduced a method for transferring enzyme application into practice and continuous digestion that differs from the mode of application recommended by the manufacturer.

## 2 Materials and methods

### 2.1 Feedstock for batch digestion test

Experiments were conducted with rye grain silage, maize silage, grass silage, feed residues (random mixture of rye-, maize-, and grass silage not eaten by the cattle) and solid cattle manure originated from the biogas plant Fehrbellin, Germany. For silage preparation Biosil<sup>®</sup> was used as biological silage additive with an application amount of 100 g per 200 Mg harvest. All materials were mixed with inoculum for batch digestion tests. The inoculi 1 and 2 used were the mixtures of several digestates and inoculum 3 was provided directly from the biogas plant Fehrbellin, Germany.

All materials were analyzed for their chemical and physical properties according to standard analytical methods (*cf.* section 2.3). Parameters analyzed are shown in Table 1.

**Table 1 Chemical and physical properties of feedstock and inoculum for batch digestion tests**

Feedstock	pH	EC	DM	ODM	Volatile org. acids	NH <sub>4</sub> -N	N <sub>tot</sub>
		mS·cm <sup>-1</sup>	g·kg <sub>FM</sub> <sup>-1</sup>	g·kg <sub>FM</sub> <sup>-1</sup>	g·kg <sub>FM</sub> <sup>-1</sup>	g·kg <sub>FM</sub> <sup>-1</sup>	g·kg <sub>FM</sub> <sup>-1</sup>
Rye grain silage	6.2	0.8	808.1	766.2	1.27	0.1	15.4
Maize silage	3.8	1.5	308.8	285.1	3.4	0.3	4.2
Grass silage	5.3	3.7	366.3	321.4	5.1	0.9	9.5
Feed residue	4.7	2.9	415.4	385.2	2.2	0.5	9.7
Solid cattle manure	8.8	2.1	250.7	227.7	0.6	0.4	3.4
Inoculum 1	8.5	18.0	38.2	23.4	1.3	1.3	2.9
Inoculum 2	8.2	28.8	57.5	37.4	1.9	3.2	5.1
Inoculum 3	7.7	19.2	46.0	32.8	2.3	2.0	3.7

EC = electric conductivity; DM = dry matter; ODM = organic dry matter; NH<sub>4</sub>-N = ammonium nitrogen; N<sub>tot</sub> = total nitrogen  
FM = fresh matter

### 2.2 Enzyme preparation

The fungal hydrolytic enzyme mixture, which was used to improve biogas production of feedstock, is a mixture of commercially available fermentation product. The enzyme-rich fermentation product is a particulate solid brown powder. The product density is approx. 304 g·dm<sup>-3</sup> and the moisture content approx. 50 g·kg<sub>FM</sub><sup>-1</sup>. The product can be completely suspended in water. The major components are cellulase, hemi-cellulase, xylanase, pectinase, xylan esterase, pectin esterase, lipase, amylase glucosidase and protease. There are also traces of non-identified enzymes. Enzyme concentrations in the product vary depending on the fungi and the substrate used for production. The product also contains substrate residue.

The feedstocks were hydrolyzed using the fungal hydrolytic enzyme mixture under anaerobic condition in a 250 ml stoppered Erlenmeyer flask. The effect of enzyme concentration (0.02 and 0.04 g enzyme·g<sub>ODM</sub><sup>-1</sup> substrate), temperature variants (40 and 60°C), pH value (4.6 (0.1 M

acetate buffer) and 5.4) was studied to determine the optimal condition for enzymatic pretreatment.

### 2.3 Analytical methods

Feedstock material was analyzed using the standard analysis methods of Leibniz Institute for Agricultural Engineering Potsdam-Bornim (ATB) and the Association of the German Agricultural Investigation and Research Institutions (VDLUFA). The analyses include pH-value, conductivity, dry matter, organic dry matter, ammonium-N, total-N, volatile organic acids, crude fat and crude fiber (fractions of NDF, ADF, ADL).

The pH-value and electric conductivity (EC) were measured with the Sen Tix 41 (WTW) measuring electrode after homogenizing 10 g of sample FM with 100 ml distilled water for a period of 20 minutes. EC is measured with a resistance in line (VDLUFA, 1997; DIN EN 27888, 1987).

Dry matter content (DM) of fresh material (FM) and silages was investigated by drying the material at 105°C until the sample weight remained constant. As silages contain more components that volatilize during drying, DM was corrected depending on the pH-value of the material (VDLUFA, 1997; Weissbach and Kuhla, 1995).

Organic dry matter (ODM) was measured by determining the ash content of dry samples in a muffle furnace at 550°C (VDLUFA, 1997).

The ammonium nitrogen content (NH<sub>4</sub>-N) was converted to ammonia by adding magnesium oxide, then distilled in a boric acid solution using a Vapodest 20 (GERHARDT). The ammonium nitrogen content was finally quantified by back titration with sulfuric acid (VDLUFA, 1997).

The total nitrogen content (N<sub>tot</sub>) was determined using an elemental analyzer (vario EL, Analysensysteme GmbH) operating on the principle of catalytic combustion with oxygen supply and high temperatures. Elemental analysis was conducted using the DUMAS method. Crude protein content can be calculated by multiplying N<sub>tot</sub> by a factor of 6.25 (DIN EN ISO 16634, 2006).

Volatile organic acids were determined by adding 85 % phosphoric acid and distillation using Vapodest 20 (GERHARDT). The acid content was presented as acetic acid equivalent after sodium hydroxide titration (DIN 38409 H21, 1987).

Crude fat was measured gravimetrically after extracting the sample with a SOXHLET extractor according to the VDLUFA method (VDLUFA, 1997; Lengerken and Zimmermann, 1991).

Crude fiber, acid detergent fiber (ADF) and neutral detergent fiber (NDF) were analyzed according to the AOCS Standard methods described by Ankom Technology using the ANKOM A<sup>2000</sup> Fiber Analyzer system. In order to measure the acid detergent lignin content (ADL) chilled 72 % sulfuric acid was added to the frit from ADF analysis for three hours and then removed. ADL content was determined gravimetrically after drying the frit at 105°C and ashing the sample in a muffle furnace at 550°C. The difference between the NDF and ADF values was calculated as hemicellulose fraction; the cellulose fraction results from the difference between ADF and ADL (VDLUFA, 1997).

### 2.4 Activity of enzyme preparation

The enzyme activity was determined in a quick test by means of HPLC and enzymatic bioanalysis. The results of the enzymatic bioanalysis were similar to the results of the HPLC analysis and are not shown here. The activity was calculated as a percentage of polysaccharide

utilization in relation to the polysaccharide content of the untreated feedstock (Iyer and Lee, 1999; Lee et al., 2009; Petersson et al., 2007):

$$\text{hydrolysis rate (\%)} = \frac{0.9 \times \text{reducing sugars (g)}}{\text{polysaccharides (g)}} \times 100 \quad (1)$$

Experiments were conducted in three replicates. Standard deviations of assays were in the range of 0.1%-0.4 %.

### 2.4.1 HPLC analyses

In order to confirm enzymatic bioanalysis tests, sugar contents (cellobiose, glucose, arabinose, xylose and ribose) were also analyzed with a high performance liquid chromatography (Ultimate 3000 Inc. DIONEX) equipped with a Eurokat H column (KNAUR, 300 mm × 8 mm). The chromatograph operated with 0.01 N H<sub>2</sub>SO<sub>4</sub> as a solvent at a flow rate of 0.8 mL·min<sup>-1</sup>. A refractive index detector RI 101 (Inc. SHODEX) was used.

### 2.4.2 Enzymatic bioanalysis test

The amount of glucose produced after hydrolysis was determined using enzymatic bioanalysis. This enzymatic bioanalysis comprised different test-combinations to determine glucose, fructose, saccharose, galactose, arabinose etc. in food and other materials.

Glucose was phosphorylated to D-glucose-6-phosphate by hexokinase (HK) and adenosine-5-triphosphate (ATP) with simultaneous formation of adenosine-5-diphosphate (ADP).

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by NADP to D-gluconate-6-phosphate with the formation of reduced NADPH.

The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose and was measured with a UV/VIS Spectrophotometer CADAS 2000 at 340 nm.

## 2.5 Batch digestion tests

### 2.5.1 Conducting batch digestion tests

Samples of feedstock material were stored at 3-4°C with carbon dioxide snow for analysis and batch anaerobic digestion tests. Batch anaerobic digestion tests were conducted according to German Standard Procedure VDI 4630 (VDI, 2006). For this, 2-liter vessels were filled with 1.5L inoculum and approximately 50 g feedstock material. The actual mixture was balanced, the ODM<sub>Feedstock</sub> to ODM<sub>Inoculum</sub> ratio being equal to 0.5 as required by VDI 4630.

The reactors were incubated under mesophilic conditions at a temperature of 35°C. The vessels were shaken once a day to re-suspend sediments and scum layers.

The biogas produced was collected in scaled wet gas meters over a period of approximately 30 days and was measured daily. The actual duration of the test, fulfilling the criterion for terminating batch anaerobic digestion experiments stated in VDI 4630, was determined by the time at which the daily biogas rate became equivalent to 1 % of the total volume of biogas produced up to that time.

Methane (CH<sub>4</sub>), carbon dioxide (CO<sub>2</sub>), oxygen (O<sub>2</sub>) and hydrogen sulfide (H<sub>2</sub>S) content were determined at least eight times during the batch fermentation test, using infrared and chemical sensors (ANSYCO GA 2000 Plus).

In addition to anaerobic digestion of the untreated feedstock, biogas production was also recorded for feedstock added with inactivated enzyme, enzyme and enzyme plus acetate buffer (Figure 1). Each experiment was performed in three replicates. In addition to the samples with inoculums and feedstock, a sample with inoculum only was tested in each set of experiments.

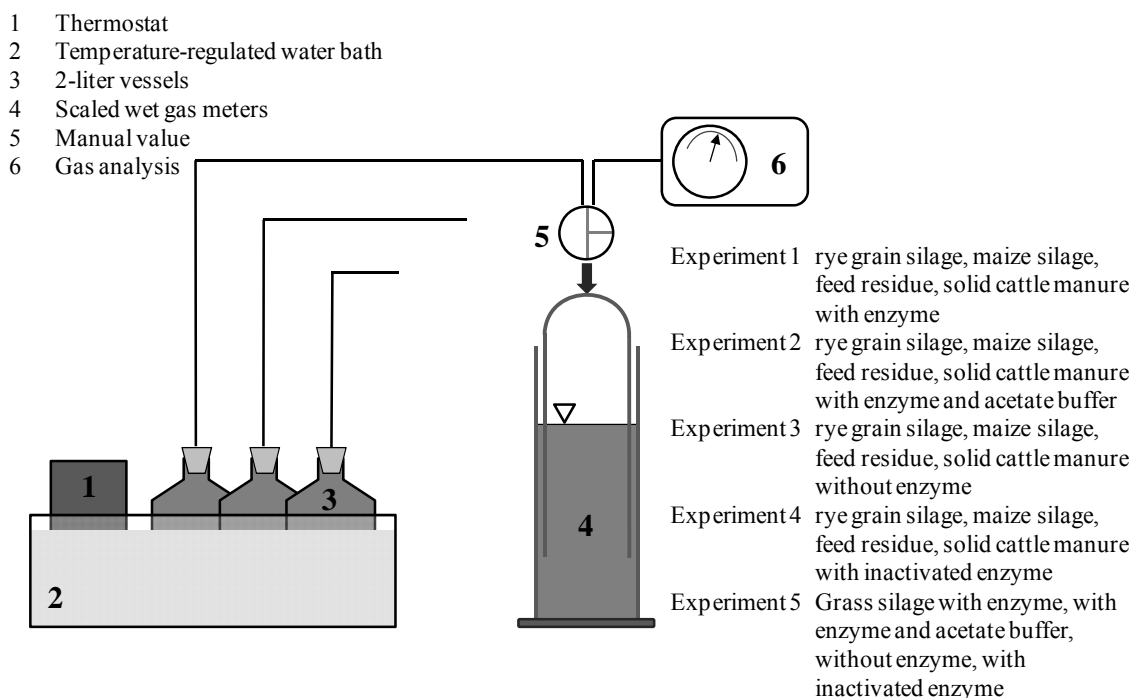


Figure 1 Design of batch digestion tests conducted under mesophilic conditions

Batch digestion tests 1 and 2 were conducted with inoculum 1. Inoculum 2 was used for batch digestion tests 3 and 4. Batch digestion test 5 was conducted with inoculum 3. Quantitative evaluation of the results of batch anaerobic digestion tests included:

- normalizing the volume of biogas to standard conditions: dry gas,  $t_0=273$  K,  $p_0=1013$  hPa
- correcting the methane and carbon dioxide content to 100 % (headspace correction, VDI 4630)
- subtracting the volume of biogas produced by the inoculum from the biogas volume produced in the batch anaerobic digestion test with feedstock and inoculum

### 2.5.2 Adaptation of experimental results with the hill equation

According to VDI 4630 (2006), batch digestion tests are interrupted when the daily biogas production is less than 1 % of the total sum of biogas formed. In some cases the potential biogas formation is expected to exceed this value considerably. It therefore appears crucial for evidence to adapt the sum curve with a sigmoid function, like the Hill equation. The results shown below are obtained from the Hill regression function associated with SigmaPlot (Version 10.0 by Systat Software Inc., [www.sigmaplot.com](http://www.sigmaplot.com)). The Hill regression produces a Michaelis-Menten-like curve with an initial acceleration phase. The function describes the maximum value ( $Y_{max}$ ),

which might be reached in infinity ( $t = \infty$ ), and a time constant ( $K_M$ ) at which half of this maximum value is reached;  $b$  is a fitting parameter:

$$Y(t) = \frac{Y_{\max} \times t^b}{K_M^b + t^b} \quad (2)$$

### 3 Results

#### 3.1 Specific composition of feedstock

The results of proximate analysis are displayed in Table 1. The pH-values of silages reveal the success of ensiling, with values below 4.0, indicating good quality silage (Heiermann et al., 2009). The preservation effect of rye grain silage is based more on the presence of carbon dioxide than on acidification, as can be seen from the pH of 6.2. NDF, ADF and ADL as well as other values are often related to the dry matter fraction.

The values of crude fiber, NDF, ADF and ADL are summarized in Table 2. NDF is composed of degradable compounds and a less degradable ADF fraction. ADF is almost equivalent to crude fiber. A sub-fraction of ADF is ADL, the least degradable compound identical with lignin.

**Table 2 Crude fat and crude fiber composition of feedstock before enzyme preparation**

Feedstock	Crude fat %DM	Crude fiber %DM	NDF %DM	ADF %DM	ADL %DM
Rye grain silage	1.2	4.0	19.1	5.9	1.8
Maize silage	2.9	19.7	35.9	23.3	3.0
Grass silage	2.9	33.7	56.6	43.2	18.6
Feed residue	2.9	24.6	46.3	30.6	5.6
Solid cattle manure	2.0	45.6	73.9	57.3	12.7

NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; DM = dry matter

The NDF fraction varied from 19.1 % DM for rye grain silage to 73.9 % DM for solid cattle manure. The remaining organic fraction is totally degradable. ADF values and crude fiber values were comparable with approximately 20 % higher values for ADF, which ranged from 5.9 % DM for rye grain silage and 57.3 % DM for solid cattle manure. While maize silage had considerable NDF and ADF fractions of 35.9 % DM and 23.3 % DM, respectively, its ADL value was almost as low as that of rye grain silage (3.0 % DM and 1.8 % DM, respectively). The maximum value of 18.6 % DM was for grass silage, while feed residue and solid cattle manure had medium ADL fractions of 5.6 % DM and 12.7 % DM, respectively.

As NDF and hence ADF as well as ADL are related to the dry matter of the material, and also because of the decomposition of dry matter as well as the continuous addition of cattle slurry as basic feedstock during anaerobic digestion, it is very difficult and not really informative to compare these values (NDF, ADF, ADL) of the digestate with the feedstock values.

## 3.2 Activity of enzyme preparation

### 3.2.1 Effect of enzyme concentration on the enzymatic hydrolysis

The effects of enzyme concentration ( $0.02$  and  $0.04 \text{ g enzyme} \cdot \text{g}_{\text{ODM}}^{-1}$  substrate) on enzymatic hydrolysis are shown in Figure 2 - mean values of three replicates with a variation coefficient less than  $0.094$ . Hydrolysis increases with higher enzyme concentration and reaches a maximum value after three hours in both variants. The hydrolysis rate (of three hours in sugar produced per cellulose and hemi-cellulose available) of the substrates was  $69.0 \%$  w/w for rye grain silage,  $19.7 \%$  w/w for maize silage,  $17.9 \%$  w/w for grass silage,  $18.3 \%$  w/w for feed residue and  $6.1\%$ w/w for solid cattle manure.

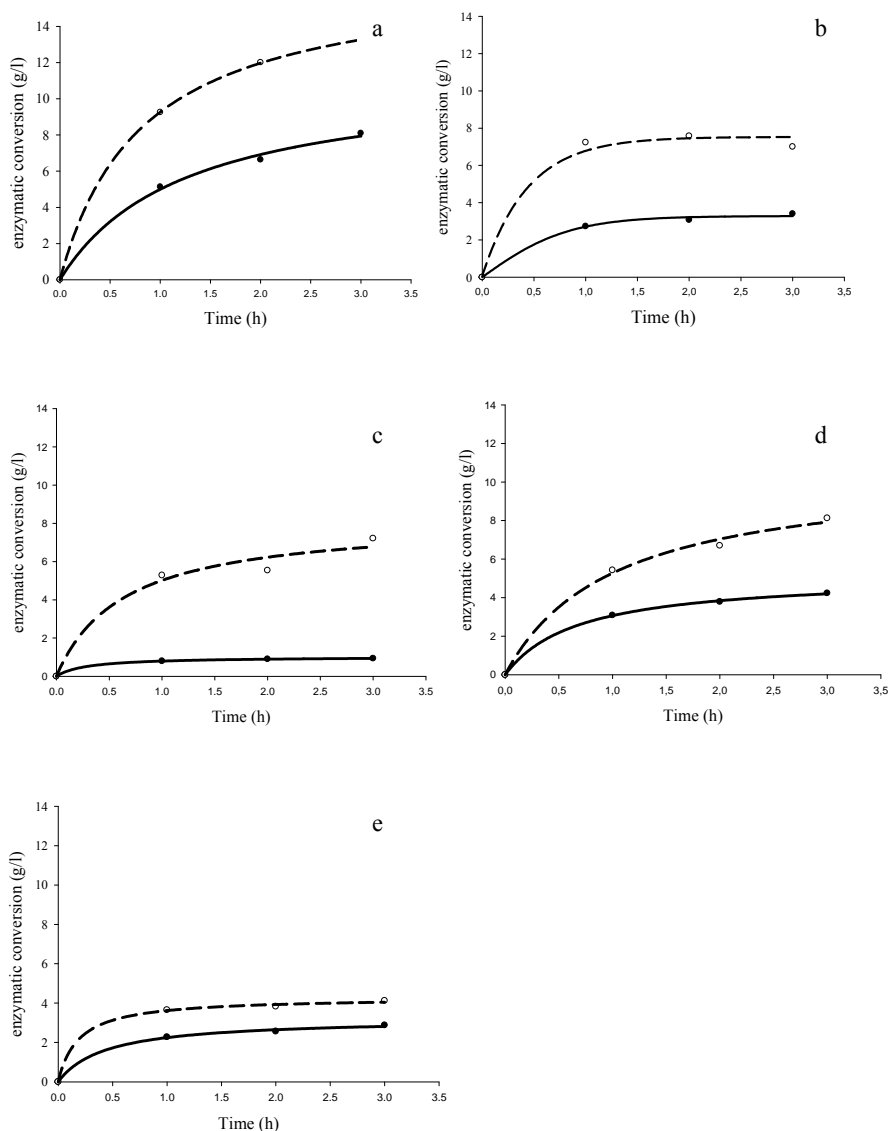
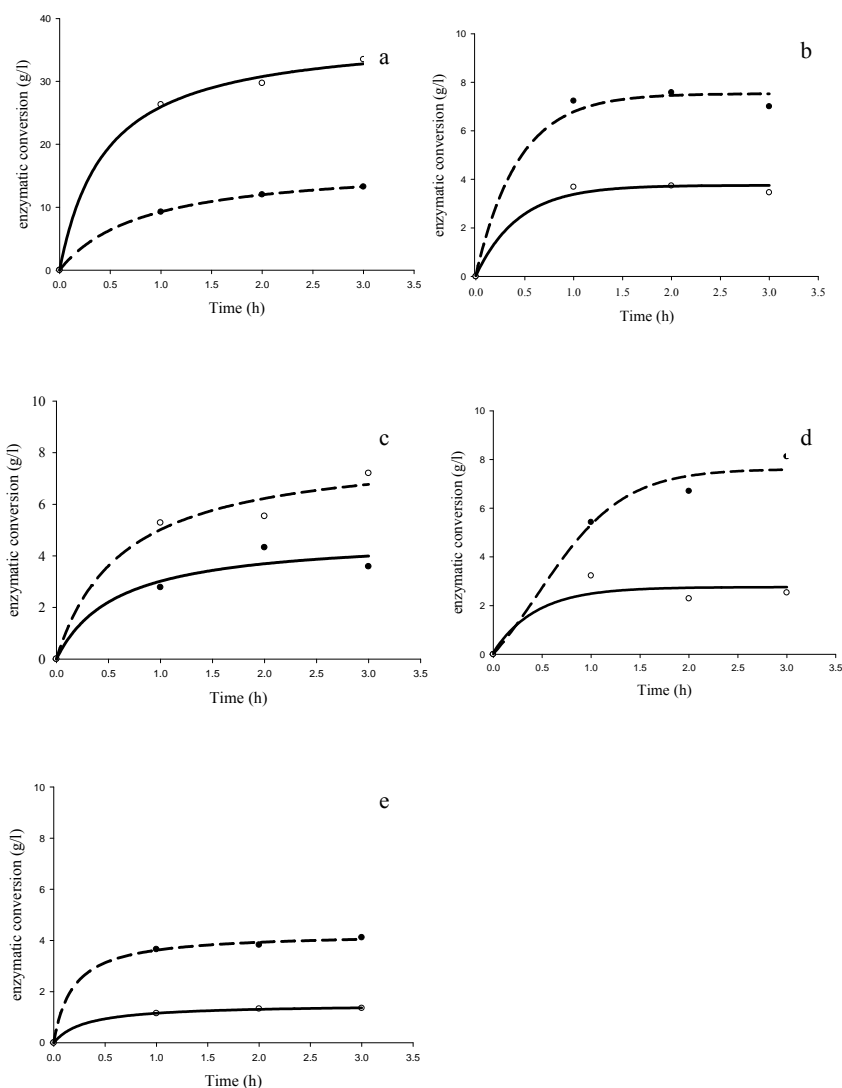


Figure 2 Effect of enzyme concentration on sugar formation of different feedstock  
a) rye grain silage, b) maize silage, c) grass silage, d) feed residue, e) solid cattle manure; solid line  $0.02 \text{ g enzyme} \cdot \text{g}_{\text{ODM}}^{-1}$  feedstock, dashed line  $0.04 \text{ g enzyme} \cdot \text{g}_{\text{ODM}}^{-1}$  feedstock. (Values shown here are means of three replicates with variance coefficient less than  $0.094$ )



### 3.2.2 Effect of temperature on the enzymatic hydrolysis

The hydrolysis of feedstock was carried out at 40 and 60 °C (Figure 3 - mean values of three replicates with a variation coefficient less than 0.074). The initial hydrolysis rate increased with rising temperature, and a higher hydrolysis rate was observed at 60 °C in rye grain silage and at 40 °C in the other feedstock.



a) rye grain silage, b) maize silage, c) grass silage, d) feed residue, e) solid cattle manure; solid line 60°C, dashed line 40°C. (Values shown here are means of three replicates with variance coefficient less than 0.074)

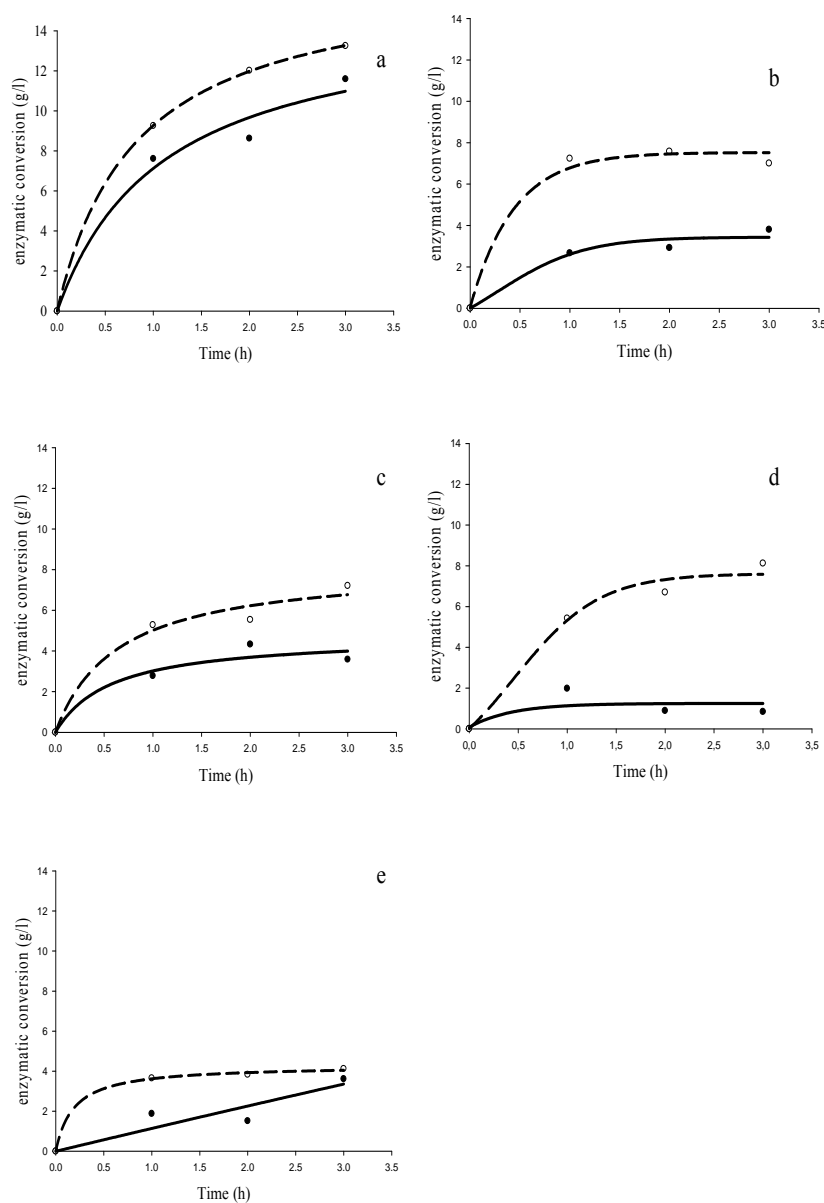
Figure 3 Effect of temperature on the enzyme concentration of different feedstock

The hydrolysis rate decreased when the temperature exceeded 60 °C (not shown). This result could be attributed to thermal inactivation of enzyme preparation.

These results indicate that further experiments could best be conducted at a temperature of 40°C.

### 3.2.3 Effect of pH on the enzymatic hydrolysis

The effect of pH on enzymatic hydrolysis of rye grain silage, maize silage, grass silage, feed residue and solid cattle manure is shown in Figure 4 (mean values of three replicates with a variation coefficient less than 0.179). At a pH value of 5.4, a clear increase in hydrolysis could be observed for all feedstock compared with a pH of 4.6 obtained in 0.1 M acetate buffer. It can be seen from this that buffer application is not necessary if the pH of the feedstock is close to 5.4.



a) rye grain silage, b) maize silage, c) grass silage, d) feed residue, e) solid cattle manure; solid line pH 4.6 dashed line pH 5.4. (Values shown here are means of three replicates with variance coefficient less than 0.179)

Figure 4 Effect of pH on the enzyme hydrolysis of different feedstock

### 3.3 Batch digestion tests

#### 3.3.1 Experimental results

In Table 3 the results are displayed of the mesophilic anaerobic conversion of rye grain silage, maize silage, feed residues and solid cattle manure to methane determined without enzyme, with enzyme (with and w/o acetate buffer) and with inactivated enzyme. The results are the mean values of three replicates. Standard deviation of the methane values obtained after approx. 30 days ( $Y_{30}$ ), showed excellent to acceptable congruence between the single experiments.

After enzyme application without buffer, a clear increase in methane production could be detected for rye grain silage (Figure 5) and for maize silage (Figure 6, Table 3). Feed residue (Figure 8) and solid cattle manure (Figure 9) showed a very clear increase with almost doubling of the values. Grass silage showed a strong rise in methane production after enzyme application, but after the 30-day period the total methane production was equivalent to the value of untreated grass silage (Figure 7). Maize silage and solid cattle manure show a lower increase after enzyme application with buffer, whereas rye grain silage and feed residue show even higher values after enzyme application with buffer than without buffer. Here, too grass silage shows a different picture with a clear increase in methane production after enzyme application with buffer (Table 3).

The application of inactivated enzyme exceeded the methane production of the control variant in all cases except for grass silage (Table 3). Hence in general, inactivated enzymes display an additional effect above the higher methane production due to the biomass added. While the increase is moderate for maize silage and solid cattle manure, the effect of inactivated enzyme is considerable for rye grain silage and feed residue.

**Table 3 Methane yield ( $Y_{30}$ ) with standard deviation from experiments of selected feedstock**

Feedstock	$Y_{30}$ $L_N CH_4 \cdot kg_{ODM}^{-1}$	$Y_{30}$ var. coeff.	$Y_{max}$ $L_N CH_4 \cdot kg_{ODM}^{-1}$	$K_M$	$R^2$	$\eta^\ddagger$	$\eta^\ddagger_{LHV}$
<b>Rye grain silage</b>							
without enzyme	355.4	0.29	329.8	9.4	0.9912	0.74	0.77
with inactivated enzyme	363.2	0.02	373.0	4.5	0.9962	0.76	0.89
with enzyme	412.6*	0.16	432.5	3.4	0.9923	0.86	0.79
with enzyme + buffer	432.2*	0.05	435.2	2.4	0.9966	0.90	0.93
<b>Maize silage</b>							
without enzyme	370.3	0.10	356.5	9.9	0.9948	0.74	1.05
with inactivated enzyme	354.9	0.07	379.9	6.4	0.9979	0.71	1.36
with enzyme	480.6*	0.29	541.3	5.2	0.9947	0.96	1.00
with enzyme + buffer	410.7*	0.29	423.0	3.4	0.9978	0.82	1.16
<b>Grass silage</b>							
without enzyme	306.9	0.12	317.9	6.3	0.9978	0.58	0.75
with inactivated enzyme	295.1	0.23	304.8	6.8	0.9974	0.56	0.73
with enzyme	297.1	0.08	300.4	6.3	0.9966	0.56	0.72
with enzyme + buffer	387.9*	0.17	417.0	7.5	0.9966	0.74	0.95
<b>Feed residues</b>							
without enzyme	302.6	0.11	295.6	6.1	0.9943	0.61	0.74
with inactivated enzyme	327.5	0.07	358.1	6.6	0.9978	0.66	1.15
with enzyme	467.2*	0.48	540.4	6.1	0.9979	0.94	0.52
with enzyme + buffer	477.5*	0.06	513.0	4.0	0.9966	0.96	0.97
<b>Solid cattle manure</b>							
without enzyme	165.5	0.21	189.9	10.8	0.9966	0.33	0.56
with inactivated enzyme	154.4	0.18	184.2	9.4	0.9989	0.31	1.14
with enzyme	340.0*	0.42	577.6	17.9	0.9968	0.68	0.52
with enzyme + buffer	289.5*	0.16	364.4	8.4	0.9975	0.58	0.97

Note: Maximum values ( $Y_{max}$ ) and Michaelis-Menten equivalent constant ( $K_M$ ) are obtained with Hill regression (SigmaPlot),  $R^2$  refers to quality of regression

\* variant significantly ( $\alpha < 0.05$ ) different to control variant

$\ddagger$   $\eta$  is the energy efficiency calculate using  $Y_{30}$  ( $L_N CH_4 \cdot kg_{ODM}^{-1}$ ), the heating value of methane (39.96 MJ·m<sup>-3</sup>; DIN 51850, 1980), the heating value of dry biomasses (18.1 MJ·kg<sub>DM</sub><sup>-1</sup> for rye grain and solid manure and 18.5 MJ·kg<sub>DM</sub><sup>-1</sup> for the other material; Kaltschmitt and Hartmann, 2001) and the ODM/DM ratio.  $\eta_{LHV}$  2440 kJ·kg<sup>-1</sup> as latent heat of vaporisation of water is used.

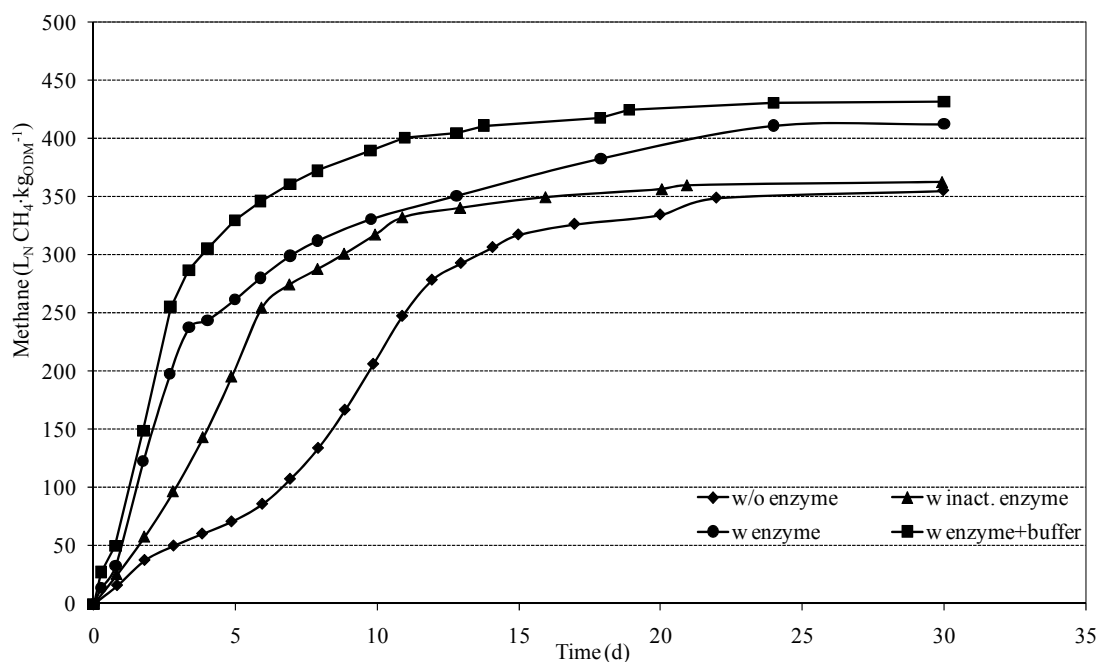


Figure 5 Methane production from rye grain silage, without enzyme application and with application of inactivated and active enzyme, the latter without and with buffer (Values shown here are means of three replicates, for variance coefficient and significance *cf.* Table 3)

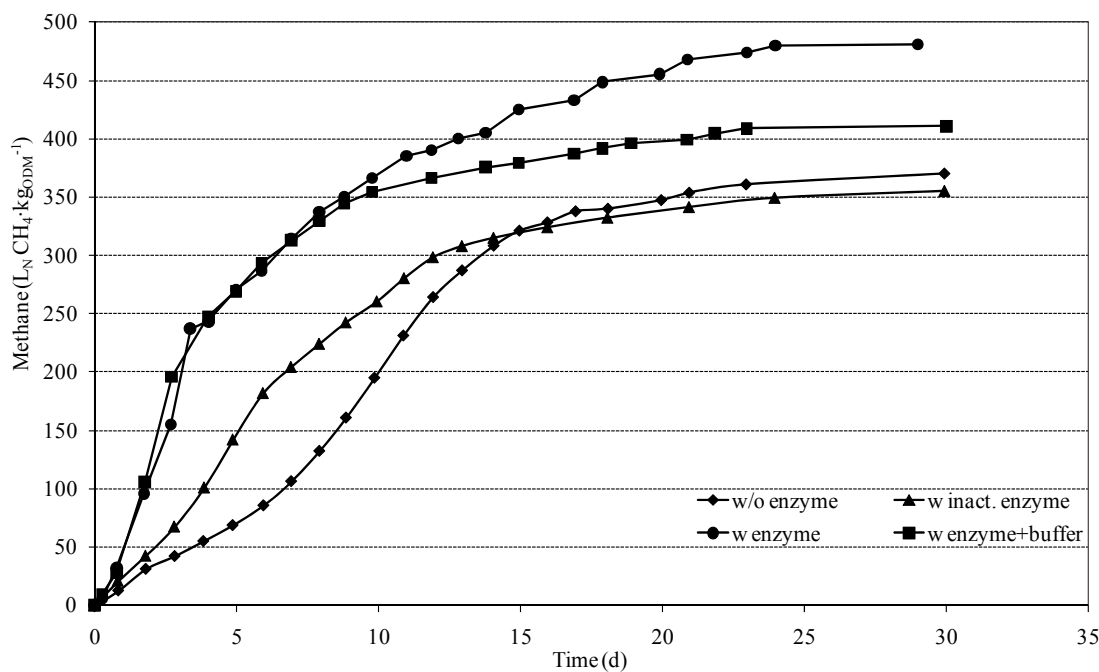


Figure 6 Methane production from maize silage, without enzyme application and with application of inactivated and active enzyme, the latter without and with buffer (Values shown here are means of three replicates, for variance coefficient and significance *cf.* Table 3)

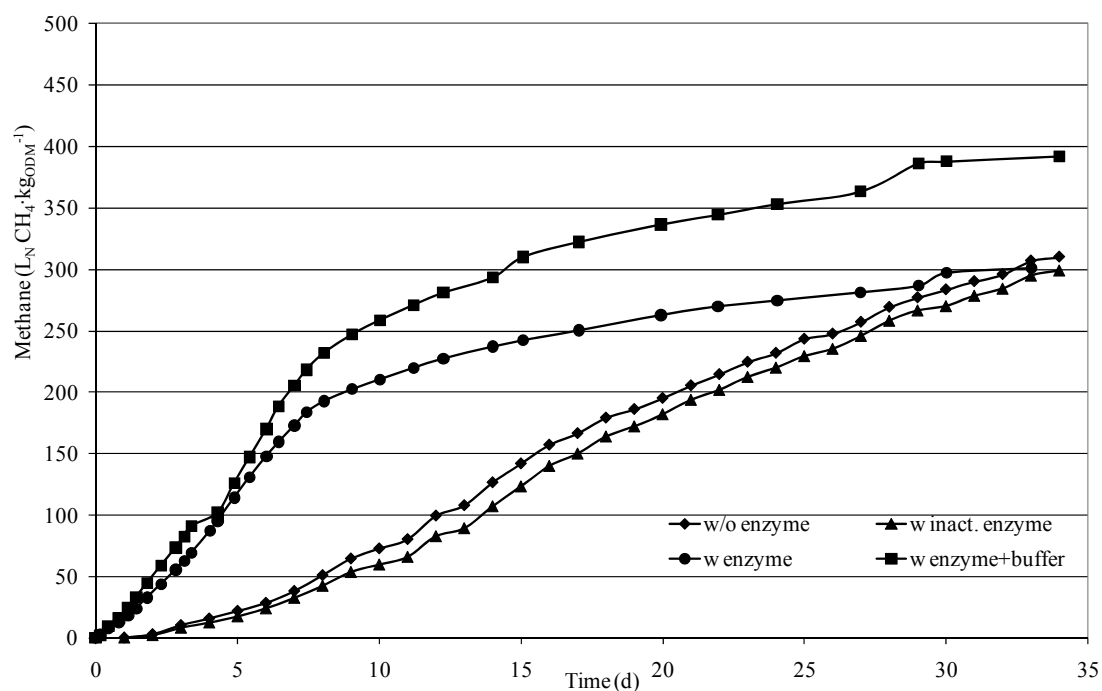


Figure 7 Methane production from grass silage, without enzyme application and with application of inactivated and active enzyme, the latter without and with buffer (Values shown here are means of three replicates, for variance coefficient and significance *cf.* Table 3)

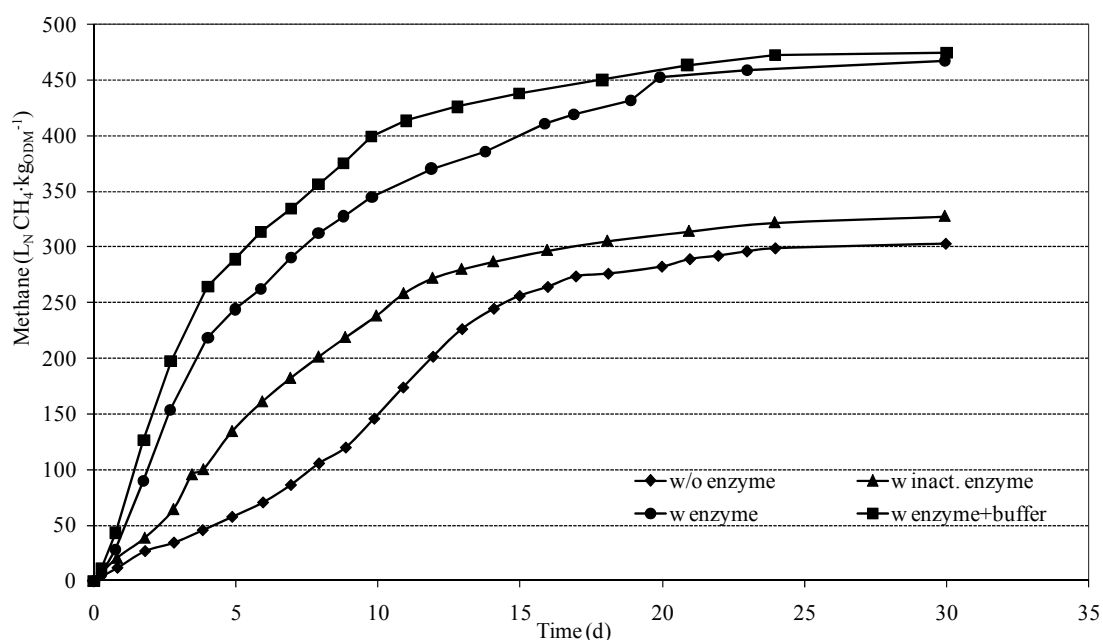


Figure 8 Methane production from feed residue silage, without enzyme application and with application of inactivated and active enzyme, the latter without and with buffer (Values shown here are means of three replicates, for variance coefficient and significance *cf.* Table 3)

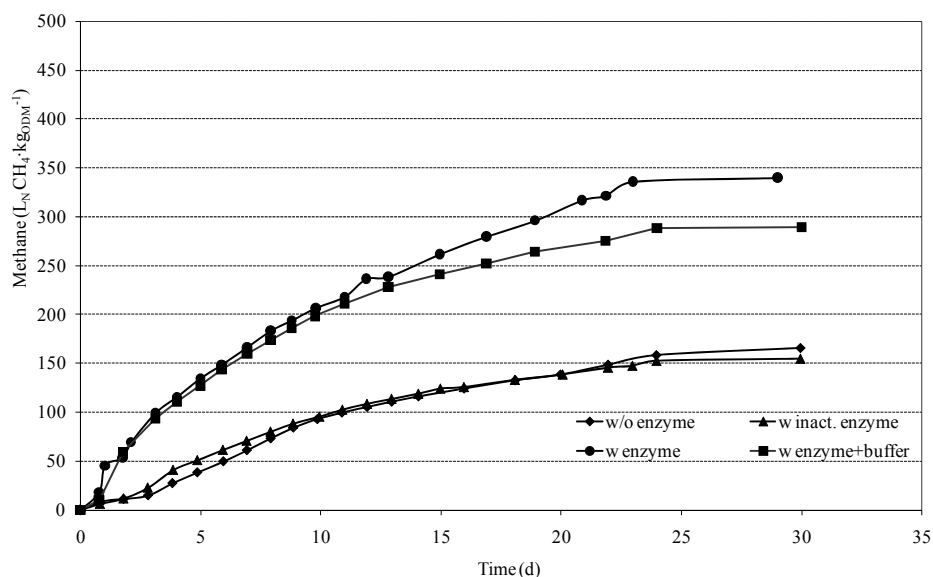


Figure 9 Methane production from solid cattle manure, without enzyme application and with application of inactivated and active enzyme, the latter without and with buffer (Values shown here are means of three replicates, for variance coefficient and significance *cf.* Table 3)

The application of enzymes, without and with buffer led to an increase in biogas methane content of 5% to 10% (Figure 10), whereas after applying inactivated enzymes an increase of less than 5% or even a slight decrease (rye grain silage) was reached.

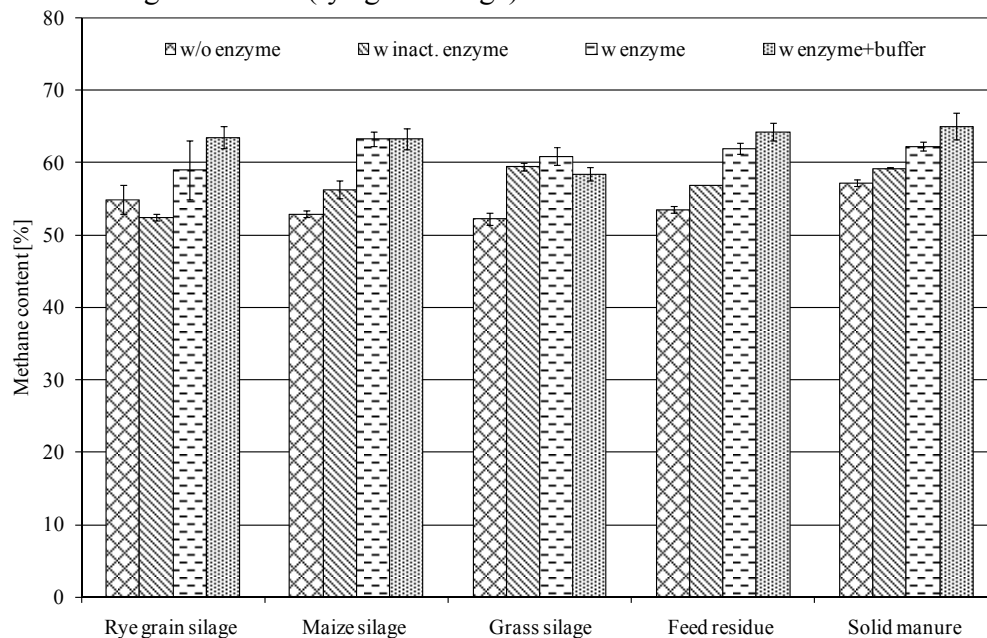


Figure 10 Methane content of biogas produced from different feedstock without enzyme application and with application of inactivated and active enzyme, the latter without and with buffer (Error bars indicate standard deviation of three replicates)

The ratios of heating values of the methane produced in each variant and of the heating value of the particular materials are given in Table 3. Generally, enzyme application increased these ratios from values between 0.33 and 0.74 to 0.58 and 0.96. Nevertheless, it must be considered that burning these materials would mean that considerable amounts of water have to be evaporated. Thus the heating values of the fresh materials are much lower than the heating values of the dried materials. It could be stated that (especially after enzyme application) the biogas process provides up to 36 % more energy (in the form of a high valuable energy carrier) than burning these materials if the latent heat of water vaporization is taken into consideration (see Table 3;  $\eta_{\text{LHV}}^{\dagger}$ )

### 3.3.2 Results of mathematical approximation

The Hill equation delivers an appropriate approximation of the experimental results, as can be seen from  $R^2$ -values not lower than 0.991 (Table 3). The approximation confirms the experimental results of a clear increase in methane production after enzyme application. In the case of solid manure, the Hill approximation indicated that after enzyme application the methane yield would triple compared with the control variant if only there were enough time for digestion. As the  $Y_{\text{max}}$  values of the control variants are in general much smaller than the  $Y_{30}$  values of the enzyme variants, it is also quite obviously that the enzyme application has a significant impact on the digestibility of the feedstock.

The application of enzyme with buffer always has an accelerating effect on the conversion of feedstock, i.e. the  $K_M$ -value of the enzyme application is always smaller than the  $K_M$ -value of the control variant. By contrast, after application of enzyme without buffer, feed residue and solid cattle manure show decelerated conversion, as can be concluded from the higher  $K_M$  values. However as already mentioned, the latter showed higher or even much higher  $Y_{\text{max}}$ -values.

## 4 Discussion and conclusions

The application of hydrolytic enzymes of fungal origin to selected feedstock considerably enhances the hydrolysis of cellulose and hemi-cellulose. By contrast with other experiments conducting enzymatic hydrolysis over 48 hours and more, we obtained considerable effects already after 3 hours under mild conditions. From our experiments we concluded that an application of fungal enzymes to the kind of feedstock we used will be optimal if treated for 3 hours, at pH from 5 to 6, at approx. 40 °C and at a concentration of  $0.04 \text{ g}_{\text{enzyme}} \cdot \text{g}_{\text{ODM, feedstock}}^{-1}$ . Under these conditions approx. 20 % of the available cellulose and hemi-cellulose could be converted to reduced sugars in the silages of maize and grass and feed residue. In rye grain silage 69 %, in solid cattle manure 6 % of the cellulose and hemi-cellulose were converted. The low value of cellulose and hemicelluloses conversion in solid cattle manure may be due to high straw content. Values may be different in other solid manures, i.e. also a high NDF content.

The enhanced hydrolysis also has a considerable effect on the following methane production in batch digestion tests. Feedstock with large fractions of crude fiber and ADL, feed residue and solid manure, show the largest increases in methane production (50 % and 100 %, respectively). Easy degradable feedstock shows only moderate increases in methane production after enzyme application (rye grain silage 15 % increase and maize silage 30 % increase). Grass silage showed a very individual picture after enzyme application, as it differs extremely depending on whether buffer is applied or not. In both cases methane production increased during the first days, but while the application without buffer did not reached higher values than the grass silage without enzyme application, the application with buffer exceeded this value by approx. 70 %.



If the potential maximum methane production is calculated from the measured values using a sigmoid regression function according to Hill, the values for feed residue and solid cattle manure are even doubled or tripled. In addition to the amount of methane formed, the share of methane in the biogas also increased after enzyme application. In general, we saw an increase of 5 % to 10 % in the methane concentration of the biogas.

Batch digestion tests deliver the maximum yield of methane or biogas respectively under laboratory conditions. A considerable amount of biogas would be obtained if the experiment were continued infinitely, as can be seen from the mathematical approximation. Hence if enzyme application increases the methane yield, it can be concluded that there is a remarkable fraction of less digestible material would be made accessible by applying hydrolytic enzymes. Nevertheless, these results are not transferable to continuous-flow, i.e. full-scale commercial biogas plants, as here other factors such as the actual component retention time play an important role. Therefore continuous-flow experiments are needed to assess the enhancing effects of enzyme application.

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