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Mathieu Brulé

The effect of enzyme additives on the anaerobic digestion of energy crops

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University of Hohenheim
Institute of Agricultural Engineering
Department of Livestock Systems Engineering
Prof. Dr. Thomas Jungbluth
and
State Institute of Agricultural Engineering and Bioenergy
Dr. Hans Oechsner

The effect of enzyme additives on the anaerobic digestion of energy crops

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Mathieu Brulé
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Supervisor and reviewer:	Prof. Dr. Thomas Jungbluth
Co-reviewer:	Dr. habil. Jean-Philippe Steyer
Additional examiner:	Prof. Dr. Lutz Fischer
Head of the Examination Committee:	Prof. Dr. Ralf T. Vögele

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Stuttgart, November 2014 Mathieu Brulé

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List of abbreviations

ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
BMP	Biochemical Methane Potential
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
FW	Fresh Weight
HBT	Hohenheim Biogas Test
HRT	Hydraulic Retention Time
n	Number of repetitions in the analysis
NDF	Neutral Detergent Fiber
OLR	Organic Loading Rate
p	Probability of the statistical hypothesis not being valid
SD	Standard Deviation
TS	Total Solids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acid
LCFA	Long-Chain Fatty Acid
VS	Volatile Solids

List of units

°C	Degree Celsius
d	Day
Da	Dalton (a unit of molecule size)
g	Gram
kg	Kilogram
hPa	Hectopascal
K	Degree of Kelvin
kg	Kilogram
L	Litre
m^3	Cubic meter
mL	Millilitre
µL	Microlitre
min	Minute
M	Molarity (mole per litre)
ppm	Part Per Million
rpm	Revolutions Per Minute
v/v	Volume-to-volume
w/w	Weight-to-weight

1 Introduction

Germany developed an energy policy aiming at reducing its dependency on conventional energy sources, which allowed the country to emerge as the leading producer of biogas from energy crops in Europe. The increasing demand for biomass as an energy source leads to increasing competition with other biomass uses, namely food and fodder or raw material (Thrän and Kaltschmitt 2007). However, biomass shortage can be counterweighed by bringing fallow land surfaces back into use (Weiland 2003). German on-farm biogas plants co-digesting manure with energy crops help to reduce the energy dependency of the country while ensuring an additional source of income for farmers (Weiland 2006).

A critical issue related to the use of fiber-rich energy crops or crop fractions (e.g. maize straw) in biogas digesters is that a substantial fraction of the substrate (consisting mainly of cellulose, hemicellulose, and lignin) is slowly or incompletely degraded by anaerobic bacteria. To cope with that problem, some on-farm biogas plants rely on the addition of fibrolytic enzymes in order to reach higher conversion rates and biogas yields from the energy crops (Gerhardt et al. 2007).

There is a need for scientific information regarding the efficiency of enzyme addition in on-farm biogas plants. Gathering this information is the target of this thesis, which combines both a literature review to understand the principles of enzyme action and laboratory experiments.

The approach followed in this thesis focused on the following targets:

1. Evaluate the degree of efficiency of fibrolytic enzyme additives in anaerobic digestion processes;
2. Draw hypotheses about conditions to be fulfilled to ensure optimal efficiency of fibrolytic enzyme additives in anaerobic digestion processes.

2 Objectives

The objective of this thesis is to investigate the potential of commercial fiber-degrading enzyme products as additives to increase the efficiency of the anaerobic degradation of energy crops. Laboratory-scale assays were performed under different conditions so as to discover the parameters that allow maximal efficiency of enzyme additives.

The work is supported by four complementary approaches:

1. Literature review of critical factors that affect the efficiency of enzyme additives in industrial processes and in anaerobic digestion processes.
2. Laboratory evaluation of the efficiency of several enzyme products for sugar formation from plant material in microbe-free enzymatic hydrolysis assays performed in a watery buffer medium.
3. Laboratory evaluation of the effects of enzyme additives on end product formation during anaerobic digestion of energy crops. Two different anaerobic digestion processes were considered: methane production and acidogenic fermentation.
4. Critical discussion of the results in the light of present knowledge and hypotheses about the conditions required for optimal efficiency of enzyme additives. The discussion also includes suggestions to adjust experimental protocols and research strategies, based on both practical and theoretical findings.

The present work should support further research on the improvement of biological processes by the addition of exogenous fibrolytic enzymes, providing a better knowledge about fundamental requirements for enzyme efficiency.

Furthermore, theoretical and experimental approaches to investigate the effects of enzyme additives are critically evaluated, and recommendations are made for their improvement.

3 Literature review

3.1 Anaerobic digestion

3.1.1 Microbiological processes

The process of anaerobic digestion may be divided into two subsequent steps (Cohen et al. 1979; Hobson and Wheatley 1993; Sahm 1981; Shin and Song 1995; Weiland 2001):

1. Hydrolysis and acidification;
2. Acetogenesis and methanogenesis.

Hydrolysis and acidification

Native macromolecules of the substrates are cleaved and decomposed into smaller molecules, oligomers and monomers through hydrolysis, which generates LCFA, glycerol, peptides and oligosaccharides. Acidogenesis further converts the latter, smaller molecules into VFA and alcohols (Hobson and Wheatley 1993).

Acetogenesis and methanogenesis

Acetogenesis

VFA and alcohols are converted both into acetate, and into the gases CO_2 and H_2 by acetogenic bacteria (Hobson and Wheatley 1993). Under certain conditions, bacterial strains named homoacetogenic bacteria are also able to carry out a reverse reaction and generate acetate from CO_2 and H_2 (Diekert and Wohlfarth 1994). Depending on substrate composition, the anaerobic mineralization of organic matter may also release inorganic compounds, such as ammonia (NH_3), phosphates (PO_4^{3-}) and hydrogen sulphide (H_2S) (Hobson and Wheatley 1993).

Methanogenesis

Methane can be formed via two competing pathways (Stams 1994):

- Acetoclastic methanogens convert acetate into CH_4 and CO_2;
- Hydrogenotrophic methanogens convert H_2 and CO_2 into CH_4 and H_2O.

H_2 and CO_2, which are substrates for hydrogentrophic methanogens, are released during the acetogenesis of VFA and alcohols. However, acetate-oxidizing bacteria can also generate H_2 and CO_2 from acetate oxidation. These gazes can subsequently be turned into CH_4 by hydrogenotrophic methanogens (Karakashev et al. 2006; Zinder and Koch 1984). Hence, when coupled with acetate oxidation, hydrogentrophic methanogenesis can provide a competing pathway to acetoclastic methanogenesis. In agricultural biogas plants, hydrogenotrophic methanogens may dominate over acetoclastic methanogens (Weiland 2010). Therefore, H_2 consumption may dominate over acetate consumption as a CH_4 production pathway. However, under certain conditions, shifts of bacterial populations towards a dominance of acetoclastic methanogens may occur (Delbès et al. 2001).

Synthrophic associations

Acetogenic bacteria that convert VFA and alcohols into acetate generate high amounts of H_2. However, the concentration of dissolved H_2, i.e. H_2 partial pressure, should be kept low for these reactions to remain thermodynamically feasible. Hence, there is a close association between acetogenic bacteria (hydrogen-producing) and hydrogen-utilizing bacteria such as methanogens. This close association forms granules of bacterial biomass (Stams 1994; Verstraete et al. 1996).

Energy balance and bacterial growth

The energy yield of oxygen-free oxidation performed by anaerobic bacteria is 20-fold lower than the energy yield of aerobic bacteria in the presence of oxygen. Low energy gain results in low biomass generation. Microbial biomass generation in anaerobic processes uses only 3-10% of the carbon contained in the substrates, compared to 50% for aerobic composting. The low energy gains of bacteria induce lower growth rates. The residence time in the reactors must be high enough in order to maintain sufficient amounts of bacteria to ensure substrate degradation. Low growth rates of methanogenic bacteria (5-10 days) may be the main limiting factor in reactor design. Moreover, methanogens are more sensitive to acidification than other bacterial strains (Weiland 2001). The mass generation of anaerobic bacteria through carbohydrate degradation may be threefold higher than from the degradation of protein and lipid-rich substrates (ATV 1990). However, by increasing the residence time of bacteria in the reactors, a fraction of the energy trapped in bacterial biomass generation may be recovered for biogas production due to bacterial decay (Tong et al. 1990).

3.1.2 Degradability of substrates

Degradation products of hydrolysis and acidification

The products of hydrolysis and acidification reactions (VFA, alcohols and H_2) can be converted into biogas by synthrophic associations of acetogens and methanogens at a very fast rate, but for this purpose bacterial populations should be present in sufficient amounts and well balanced so as to avoid substrate inhibition, especially from VFA and H_2 (Stams 1994; Weiland 2001).

Soluble substances

Organic acids, oligosaccharides and peptides can dissolve in the aquous medium. Bacteria can access and degrade these products easily since their enzymatic degradation involves monophasic (liquid-liquid) reactions, i.e. bacterial enzymes do not encounter the difficulty of accessing the solid phase (Hobson and Wheatley 1993).

Pure substances

The degradation of pure substances of higher molecular size mainly depends on their accessibility to the liquid phase. Pectin, which is highly hydrophilic, can be degraded at a very fast rate. On the opposite, cellulose, which is hydrophobic, is degraded more slowly (Hobson and Wheatley 1993). The hemicellulose digestion rate can vary within a wide range depending on its composition (Jung and Engels 2002).

Aromatic and polyaromatic molecules

Lignin, which is a polymer macromolecule composed of phenolics, is not degradable under anaerobic conditions (Tong et al. 1990). However, smaller phenolic molecules may be degraded. In spite of their small size, aromatic molecules such as phenols are difficult to degrade and may also be strongly inhibiting to the anaerobic digestion process (Hobson and Wheatley 1993).

Cell wall

Cell walls define cell shape and fix cell position within tissues (Cosgrove 2005; Smith 2001). They make up 30 to 70% of the plant's organic matter. Their share and composition varies largely depending on tissue type and localization in the plant (Cosgrove 2005; Knox 2008). Plant tissue bear different cell walls depending on their function. Thick-walled sclerenchyma ensure structural support of the plant, xylem vessels conduct water and minerals, while phloem transports sugars (Vermerris 2008). Cell wall concentration generally increases during the maturation of plant tissues (Jung and Casler 2006b; Jung and Engels 2002). Cell wall structure and its relation to plant tissues and developmental stages will not be further investigated in this thesis, though these factors may have a tremendous influence on plant degradability.

Cell walls are mainly formed of lignocellulose, a network composed of lignin, cellulose and hemicellulose that surrounds plant cells and protects them against biodegradation (Tong et al. 1990). As an additional component, cell wall proteins, also named structural proteins, make up 2-10% of the cell wall (Caffall and Mohnen 2009; Harris and Stone 2009). Pectin, which fills the space between adjascent cells, may also be considered as a cell wall component. When integrated into the lignocellulosic complex of cell walls, cellulose is much less degradable than as a pure substance (Angelidaki and Sanders 2004; Tong et al. 1990). Lignocellulose not only restricts the degradation of cellulose and hemicellulose, but may also hamper the degradation of other components, such as pectin and proteins (Jung and Engels 2002).

Lignin is a polymer formed of three different phenylpropanoid alcohols (or monolignols): coniferyl alcohol (or guaiacyl propanol, G), coumaryl alcohol (or hydroxyphenyl propanol H) and sinapyl alcohol (or syringyl propanol, S). The monolignols are derived from the aromatic amino-acids Phenylalanine (Phe) and Tyrosine (Tyr) (Davin and Lewis 2005; Ralph 2010). They are bound to each other through multiple C-C and C-O-C bounds, resulting in a complex structure (Jeffries 1994; Ralph 2010; Taiz and Zeiger 2006).

Lignin plays the main role in limiting degradability, acting in two ways (Tong et al. 1990):

- **Shielding effect:** encapsulation of other molecules that can not be accessed by bacteria and enzymes.
- **Cross-links:** linkages to other cell wall fractions, mainly via ether and ester linkages mediated by ferulic acid, that bind the lignocellulose complex together.

Cellulose microfibrils of land plants are usually about 10 µm width and consist of 30-36 parallel linear glucan polymers tightly associated to each other in a crystalline structure through noncovalent hydrogen bounds along the chains components (Somerville et al. 2004). The arrangement of microfibrils may determine cell shape and the direction of cell growth (Carpita and Gibeaut 1993; Darley et al. 2001). Hemicellulose polymers become entrapped in cellulose microfibrils as they form (Darley et al. 2001), resulting in a loss of crystallinity of the microfibrils and disordered regions (Cosgrove 2005). Furthermore, cellulose microfibrils are linked to hemicellulose and pectin through hydrogen bonds along some areas of the polysaccharide chains (Cosgrove 2005; Darley et al. 2001).

Hemicellulose is a generic term that covers mainly four classes of polymers: heteroxylans, xyloglucans, mixed-linkage glucans and heteromannans. Their properties, share and composition depends on plant type and maturity stage (Ding and Himmel 2009).

Pectin typically form gels, i.e. loose networks of highly hydrated polymers. Pectin chains may be connected to each other by covalent glycosidic linkages, noncovalent calcium ion bridges, and covalent ester linkages, e.g. through ferulic acid (Taiz and Zeiger 2006; Willats et al. 2001). They are mainly formed of linear or branched homogalacturonan and rhamnogalacturonan polymers (Caffall and Mohnen 2009; Willats et al. 2001).

Structural proteins are formed of repeating units of about 2-11 amino-acids and also contain a high share of sugars (Iiyama et al. 1993). They are divided into three classes (Iiyama et al. 1993; Jamet et al. 2006; Keller 1993; Showalter 1993): hydroxyproline-rich glycoproteins (HRGP), glycine-rich proteins (GRP), and proline-rich proteins (PRP). Structural proteins are bound to polysaccharides and lignins, and may be cross-linked by di-isodityrosine bonds (Jamet et al. 2006; Keller 1993; Saulnier et al. 1995). Ionic interactions with pectins may also play a role in proteins bonding (Showalter 1993).

Cuticle

In addition to cell walls, that protect the inner structure of plant tissues against degradation, epidermial cells at the outer surface of plant stems possess a coating of lipidic compounds and long-chain linear compounds with alkane, alkene, and alcohol functions, that is highly hydrophobic and forms an effective barrier against degradation. This outer protection is designated as cuticle and the mixture of compounds forming it as cutin. Cutin is very recalcitrant to bacterial and enzymatic degradation (Dutta et al. 2009; Kolattukudy 1980; Kolattukudy 1981; Pollard et al. 2008; Samuels et al. 2008).

Summary of substrate biodegradability

The biodegradability of organic substances depends on molecule size and on the type of molecular bounds involved. The greater molecule size, the more insoluble or hydrophobic, and the longer will be the duration required for anaerobic degradation by microorganisms (Hobson and Wheatley 1993). The composition and properties of pure substances are summarized in **Table 1**. When molecules are integrated into the matrix of lignocellulose from plant cell walls, the biodegradability is reduced compared to pure substances. Even easily degradable molecules can become encapsulated into lignocellulose, restricting their accessibility and degradability (Jung and Engels 2002; Tong et al. 1990).

Tong et al. (1990) found an inverse linear relationship between lignin content and volatile solids (VS) degradation of selected substrates in batch digestion trials, also designated as BMP (Biochemical Methane Potential) assays. However, the relationship was weak. The authors hypothesized that the rate of degradation may be affected by other factors: the structure of the lignocellulosic complex and lignin cross-linking with other fractions. Buffiere et al. (2006) found a much better inverse linear relationship between COD (Chemical Oxygen Demand) degradation rate and ADF (Acid Detergent Fiber) fraction (i.e. cellulose + lignin) while comparing a wide range of substrates.

Figure 1 shows a sorting of molecules according to their expected rate of degradation. Lignin is excluded from the classification because it is not degradable under anaerobic conditions (Tong et al. 1990).

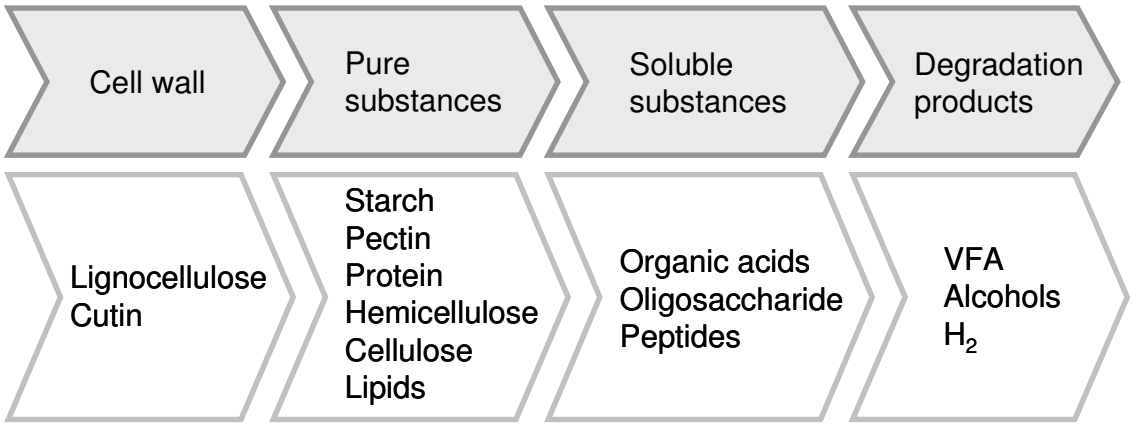


Figure 1. Classification of molecules in increasing order of degradability [Compiled after Hobson and Wheatley (1993), Tong et al. (1990), Jung and Engels (2002)].

Table 1. Chemical structure of compounds found in natural organic matter [Compiled after Bekker (2006), Hobson and Wheatley (1993), Jeffries (1994), Saha (2003), Friedrich (2010)].

Compound	Composition	Linkages	Properties
VFA	Short-chain fatty acids	Monomers	Soluble
Alcohols	Short-chain alcohols	Monomers	Soluble
Sugars	Glucose, xylose, fructose, arabinose, galactose, mannose,…	Monomers	Soluble
Amino-acids	-	Monomers	Soluble
Oligosaccharides	Sucrose, cellobiose, xylobiose,..	α-(1-4) β-(1-4),..	Soluble
Fructan	Fructose	β-(1-2)	Soluble
Starch	Glucose	α-(1-4)	Granules 2-150 μm in diameter
Peptides	Amino-acids	Oligomers	
Cellulose	Glucose	β-(1-4)	Linear chains associated side-by-side through hydrogen bonds Insoluble, hydrophobic
Pectin	Glucoronic acid, rhamnose	α-(1-4)	Forms gels Highly hydrophilic
Hemicellulose	Xylose, arabinose, glucose, mannose, galactose, anhydrouronic acid, glucoronic acid,…	β-(1-4) β-(1-3),…	Branched chains with side-chains Insoluble, hydrophilic
Proteins	Amino-acids, lipids	Polymers	Insoluble
Lipids, LCFA	Long-chain fatty acids	Monomers	Hydrophobic
Phenols, polyphenols	Ferulic acid Coumaric acid p-hydroxybenzoic acid,…	Monomers, oligomers	Soluble
Lignin	p-coumaryl alcohol Coniferyl alcohol Sinapyl alcohol	Polymers	Insoluble, hydrophobic

3.1.3 Reaction conditions

Reaction rate

The rate of anaerobic degradation of the molecules increases together with temperature until 65-70°C, but the increase is not linear. Scientists usually distinguish between three different temperature ranges: psychrophilic (0-15°C), mesophilic (15-45°C) and thermophilic (45-70°C). The optimum temperature for mesophilic digestion is thought to be ~35°C. The optimum temperature for thermophilic digestion is ~55-60°C (Hobson and Wheatley 1993). Generally, temperature should affect reaction kinetics, but not the ultimate methane yield of substrates (Kusch et al. 2008; Veeken and Hamelers 1999).

Role of bacterial supports

Bacteria associate with each other to form bacterial granules or biofilms which provide a higher digestion efficiency. The anaerobic oxidation of butyrate and propionate into acetate by acetogenic bacteria generates hydrogen, but can run only at low hydrogen concentrations. Bacterial biofilms and granules provide the close cell-to-cell contact required for the inter-species hydrogen transfer between acetogenic bacteria and methanogenic bacteria. Methanogenic bacteria maintain low hydrogen partial pressure of ~10^{-5} atm by taking up hydrogen produced by acetogens and converting it into methane (Verstraete et al. 1996).

The presence of particles or a support where the bacteria can deposit greatly increases the reaction rate of acetogenesis and methanogenesis. Designing the reactors in such a way that bacteria are kept inside the reactor for a longer time through their fixation to a support, or through decantation enhance the reactor-specific methane production rate as well as the affordable loading rates (Lettinga 1995; Rajeshwari et al. 2000). However, bacterial supports may improve process efficiency only in the case when the degradation of soluble substrates is considered. Contrary to the degradation of soluble substrates, the degradation of insoluble substrates requires direct attachment of bacteria onto the substrate, in a process which is described later (cf. ***3.2.7 Microbial degradation versus enzymatic hydrolysis***).

Reactor technologies

Within enhanced reactor technologies, one may distinguish between reactor systems based on biomass granulation (UASB - Upflow Anaerobic Sludge Blanket, EGSB - Expanded Granular Sludge Blanket, IC - Internal Circulation), on the fixation with a floating support (fluid bed, gas-lift reactors) or on the fixation with a static support (anaerobic filters). In the case of processes based on biomass granulation (i.e. close association of numerous bacteria into granules), bacterial granulation occurs naturally through the selection pressure in the reactors, where only granulated bacteria are retained through granules decantation and non-granulated bacteria are washed out. Granules formation can be favored by divalent ions Mg^{2+} or Ca^{2+}, organic / inorganic nuclei (e.g. clay) or surfactants (Verstraete et al. 1996). Fixed biomass systems rely on the implementation of supports like nylon sponges, plastics, clay (Yadvika et al. 2004), sand (Verstraete et al. 1996), and straw bed (Andersson and Björnsson 2002; Svensson et al. 2006). Research recently concentrated on porous materials which offer a higher surface area for the growth of microorganisms (Ward et al. 2008).

Enhanced reactor systems based on improved biomass retention are generally not applicable to substrates containing a high share of particulate matter (Verstraete et al. 1996). The anaerobic digestion of energy crops in Germany relies generally on Continuously Stirred Tank Reactor (CSTR) systems that do not have features allowing enhanced retention of bacterial biomass (Fischer 2002; Weiland 2006). Nevertheless, in CSTR systems, it is recommended to limit turbulence and abrasion force in the reactor in order to avoid disrupting synthrophic bacterial associations involved in inter-species hydrogen transfer (Sahm 1981).

Nutrients and micronutrients

According to the literature, the ratio of macronutrients C:N:P:S should be ~600:15:5:1 (Fricke et al. 2007; Weiland 2010). Other macronutrients (K, Na, Ca, Mg), as well as trace metals, also designated as mineral trace elements (Fe, Mn, B, Co, Zn, Ni, Cu, Mo, Se, Al, W, V) are also required to support microbial growth (Kayhanian and Rich 1995; Lettinga 1995; Oleszkiewicz and Sharma 1990; Scherer et al. 1983; Zandvoort et al. 2006). Trace metals are naturally present in some substrates, like fodder beet silage. For this reason fodder beet silage can be digested as a sole substrate even at high loading rates (Demirel 2009).

Nevertheless, the mono-digestion of fodder beet was improved upon supplementation of NH_4^+, Na^+, K^+, PO_4^{3-} and SO_4^{2-} (Demirel and Scherer 2009; Demirel and Scherer 2008; Scherer et al. 2009).

The bioavailability of trace metals is controlled by precipitation and chelation mechanisms. Sulfide (S^{2-}), carbonate (CO_3^{2-}) and, to a lesser extent, phosphate (PO_4^{3-}) are able to precipitate metal ions (Callander and Barford 1983). Metals may also form organic complexes with humic acids and certain amino-acids. If trace minerals supplementation is intended, it is recommended to determine the concentrations in soluble trace metals in order to adjust the doses (ATV 1990). This may be done by measuring the acid-soluble fraction of trace metals, including not only soluble metals, but also complexed metals that may be available to bacteria (Richards et al. 1991). In-situ chemical desulfuration with high amounts of iron oxides, iron chloride or iron hydroxide is usually performed prior to or simultaneously with trace minerals addition (Güßbacher 2007; Lemmer et al. 2010; Naegele et al. 2013; Oechsner et al. 2011; Vintiloiu et al. 2012). The availability of trace metals may be enhanced upon addition of chemical chelators (Hu et al. 2008; Vintiloiu et al. 2013). The digesting organic matter might also contain chelating agents that mobilize trace metals (Peiffer et al. 1994). Additionally, organic trace elements such as vitamins might also have a stimulatory effect on anaerobic digestion (Aquino and Stuckey 2003; Burgess et al. 1999).

Manure is known to be a good provider of trace metals for co-digestion processes (Angelidaki and Sanders 2004; Lemmer et al. 2010; McBride and Spiers 2001; Vintiloiu et al. 2012; Weiland 2006). Preißler et al. (2007a; 2007b) found that replacing the manure component with water while performing semi-continuous digestion of ensiled maize in laboratory CSTR-reactors led to the collapse of the biological digestion process, and imputed this to trace metal deficiency. Nordberg et al. (2007), dealing with the anaerobic digestion of alfalfa silage as a sole substrate faced the same issue, and tried to solve this problem through recirculation of process water. In the case of nutrients or micronutrients deficiency or excess related to an unbalanced substrate mix, the best way to deal with the unbalance might be to change or complete the substrate mix so as to reach better balanced nutrients and micronutrients contents. Alternatively, mineral additives have to be purchased or the system should be operated at lower loading rates (ATV 1990; Richards et al. 1991; Weiland 2001).

In the German context, a simple way of optimizing the anaerobic digestion process was found to be the co-digestion of manure with energy crops (Oechsner et al. 2003). Manure supplies essential trace elements to the process, while energy crops addition usually balances the C:N ratio (Carbon to Nitrogen ratio) of the feedstock (Lehtomäki et al. 2007). Moreover, sulfur contents may become lower, so that fewer H_2S gas is produced from feedstock digestion, thus alleviating the issues related to H_2S removal that has to be applied prior to energy conversion of biogas.

Toxicity and inhibitory compounds

According to the basic principle of toxicology, the toxic effect of substances depends on their concentration in the medium (FNR 2010). Even substances that are usually considered non-toxic may reveal inhibiting effects when present in excessive amounts. Furthermore, biodegradability and toxicity are not necessarily linked. For example, VFA are easily degradable, but turn out to be highly inhibiting when present in high concentrations. In addition to the substance's concentration, physical conditions of the medium (pH, temperature) strongly affect the extent of inhibition. VFA inhibition strongly increases with decreasing pH because the inhibition is related to the protoned (acid) form of the VFA (He et al. 2006). Inhibition through free ammonia increases with higher pH and with higher temperatures (Fricke et al. 2007; Hobson and Wheatley 1993), and can be reduced upon addition of zeolithe, which captures ammonia (Kotsopoulos et al. 2008; Milán et al. 2001; 2003).

Reaction stability

Although the reaction rate of thermophilic digestion (~55°C) is much higher, mesophilic digestion (~35°C) is often preferred because of its higher stability (Wandrey and Aivasidis 1983). The lower inhibition of ammonia makes mesophilic digestion more suitable for the conversion of nitrogen-rich substrates, which usually contain a high share of proteins (Angelidaki and Ahring 1994; Sung and Liu 2003). Slower reaction rates make the mesophilic process easier to control. In mesophilic anaerobic digestion, higher contents of soluble carbon dioxide in the liquid phase compared to thermophilic anaerobic digestion, may increase bicarbonate concentrations, thus providing a stronger buffering against pH changes, resulting in enhanced process stability (McCarty 1964; Murray and Riley 1979; Musvoto et al. 2000).

Digesting a high share of lipids may cause process instability because they produce high amounts of organic acids as intermediate degradation products (Weiland 2001). Maintaining a well-balanced substrate mixture, constant feeding rate, appropriate nutrient and micronutrient contents, and constant temperature may be the best ways to ensure stable anaerobic digestion. Parameters that are often considered while adjusting the substrate mix are C:N ratio and trace metals contents (Richards et al. 1991).

3.2 Enzymatic and microbial degradation of lignocellulosic substrates

3.2.1 Structure and function of enzymes

Composition of enzymes

Enzymes are protein-based molecules produced by living organisms that, due to their specific conformation, are able to act as catalysts, i.e. to reduce the activation energy required to perform a biochemical reaction. Enzymes have an active site (catalytic domain) in which the reaction is performed and a substrate-binding site aimed at ensuring contact to the substrate. Fibrolytic enzymes are formed of proteins, which may be glycosylated (i.e. comprise oligosaccharide residues) and contain metal ions, generally calcium (Dashtban et al. 2009; Gilbert 2010). In addition to the substrate-binding site located in close vicinity to the catalytic domain, many fibrolytic enzymes possess one or several carbohydrate-binding modules (CBM) that favor attraction and contact to the substrate.

Definition of enzyme activity

For convenience, enzymes are generally named after their enzymatic activity, i.e. after the substrate upon which they are active and the type of reaction which they catalyze. However, enzymatic activities do not strictly define the mode of action of enzyme proteins. A single enzymatic activity may involve several enzymes acting synergistically, and a single enzyme may display multiple enzymatic activities, for example some β-xylanases also cleave β-glucan (Banerjee et al. 2010; Collins et al. 2005). Enzyme activity and substrate-specificity are determined by the size and shape of the substrate binding domain. A larger substrate binding domain allows action against long-chain, insoluble polymers, while a shorter binding domain is more appropriate for the hydrolysis of short-chain, soluble oligomers (Collins et al. 2005; Zhao et al. 2012). Some enzymatic proteins may contain several catalytic domains, which provide them with the ability to catalyze several enzymatic reactions (Maki et al. 2009).

Carbohydrate-binding module (CBM)

Enzymes that catalyze reactions on polysaccharides face the challenge of acting at the solid-liquid interface in order to access insoluble substrates (Chundawat et al. 2012). Therefore, their substrate-binding site is often complemented with a carbohydrate binding module (CBM), which supports the attachment of the enzyme at the polysaccharide surface, presumably via hydrophobic interactions. CBMs can be found in cellulases, xylanases, mannanases, and amylases (Jørgensen et al. 2007). Though the presence of a CBM is not a prerequisite to enzymatic hydrolysis, it may improve the efficiency of enzyme towards insoluble substrates. Some enzymes may even have multiple copies of a CBM in order to magnify its effect (Gilbert 2010; Subramaniyan and Prema 2002).

Free enzymes versus complexed enzyme systems

Enzymes produced by microorganisms for lignocellulose degradation may be attached to microbial cells (cell-bound) or released into the medium (free enzyme). Enzymes that are attached to microbial cells and perform the degradation of cellulose or xylan can be associated into large complexed enzyme systems (>1MDa) named cellulosomes. Cellulosomes contain several enzymes in close association, which act synergistically to each other (Gilbert et al. 2008; Yang et al. 2011). Comparatively, the size of free enzymes may range between 5 and 300 kDa (Subramaniyan and Prema 2002). Current commercial enzyme products are prepared with free enzymes, since engineering of cell-free cellulosomes is more complicated (Ding et al. 2008).

Classification systems for fibrolytic enzymes

Enzymes that contribute to lignocellulose degradation may be classified according to different systems (Sweeney and Xu 2012):

1. International Union of Biochemistry and Molecular Biology: classification according to enzyme activity, e.g. glycosidases, lyases, esterases, peroxidases, carbohydrate oxidases, phenol oxidases;
2. CAZY database (Carbohydrate-Active Enzymes): classification according to amino-acid sequence similarity between enzymes, which is related to coding gene sequence of the enzyme-producing microorganism;
3. FOLY database (Fungal Oxidative Lignin Enzymes): classification system specific to fungal enzymes contributing to lignin degradation, also according to amino-acid sequence similarity.

Non-hydrolytic enzymes: expansin and swollenin

Expansin and swollenin are designated as non-hydrolytic enzyme or, confusingly, as non-enzymatic protein, and do not cleave covalent linkages. Expansins are active in plant cell walls and disrupt noncovalent hydrogen bonds between cellulose and hemicellulose so as to catalyze cell wall extension (Darley et al. 2001). The use of expansins to degrade plant biomass synergistically with cellulases has been patented (Cosgrove 2001; Vermerris 2008). Expansins have homologs in fungi which are termed swollenins (Banerjee et al. 2010).

Parameters for selecting enzyme products

The majority of enzymes developed for lignocellulose degradation are from fungi, which are easy to cultivate. Hydrolytic activity and ease of production should not be the sole factors applied to enzyme selection. The parameters of thermal stability, optimal pH range, resistance to proteases, potential to act synergistically with other enzymes, and possession of low product inhibition should also be considered (Banerjee et al. 2010; Menon and Rao 2012).

3.2.2 Factors affecting enzyme efficiency

Substrate-related factors

Substrate loading

In most enzymatic processes, high solids loadings of more than 15% (w/w) Total Solids (TS) can not be applied, since they render mixing difficult due to increased viscosity of the medium (Jørgensen et al. 2007; Menon and Rao 2012). Another drawback of high substrate loadings is increased product inhibition.

Solubility

Enzymatic hydrolysis occurs faster on more soluble substrates, which are more easily accessible than insoluble substrates. Hence, cellulose hydrolysis is slow, owing to its insoluble, semi-crystalline nature (Knauf and Moniruzzaman 2004). Crystalline cellulose is formed from tightly packed cellulose chains linked via hydrogen bonds along the chains. Crystalline cellulose hydrolysis is ~3 to 30 times slower than the hydrolysis of amorphous regions of cellulose, which are less compact (Zhang and Lynd 2004; Zhao et al. 2012). A higher water content of biomass increases the degree of swelling of cellulose and reduces its crystallinity, resulting in a higher degradability (Jeoh et al. 2007). In purified "crystalline cellulose", a share of amorphous regions of 30-50% still remains. Part of the cellodextrin molecules generated after cleavage of covalent bonds do not migrate into the liquid phase, and are still retained on insoluble cellulose via hydrogen bonding. The hydrolysis of soluble cellodextrins present in the liquid phase and of cellobiose occurs at a much faster rate than the insoluble fraction of cellulose and cellodextrins. Starch is a branched polysaccharide which is not tightly packed. It is more soluble than cellulose and degraded more rapidly (Jeoh et al. 2007; Zhang et al. 2012). Enzymatic hydrolysis of cellulose requires ~100-fold more enzyme than starch hydrolysis (Yang et al. 2011). The activity of xylanase acting on xylan is 2-3 times greater than the activity of cellulases acting on crystalline cellulose (Subramaniyan and Prema 2002).

Available surface area

Lignin, and, to a lesser extent, hemicellulose, act as physical barriers preventing the access of cellulose and reducing enzymatic hydrolysis rates. The Accessible Surface Area (ASA) of cellulose depends on particle size, porosity and pore volume. Particle size reduction increases the Specific Surface Area (SSA), expressed in m^2/g or in m^2/m^3. Physico-chemical pretreatment enhances porosity and pore volume. Both effects significantly improve ASA. Alternately, wet pressing of the substrate reduces porosity, water content and ASA. Substrate drying reduces pore size within the substrate and generates "hornification", i.e. enhanced hydrogen-bonding due to the removal of water. These factors diminish ASA and reduce the efficiency of enzymatic hydrolysis (Jeoh et al. 2007; Zhao et al. 2012). On the opposite, prolonged contact with water can increase the hydration of cellulose, allowing higher rates of enzymatic hydrolysis (Ovando et al. 2005).

ASA may have a stronger effect on the hydrolysis rate of lignocellulosic substrate than the degree of crystallinity of cellulose (Zhang and Lynd 2004). Cellulose crystallinity is not correlated with the efficiency of enzymatic hydrolysis of biomass (Zhao et al. 2012). However, crystallinity can only be efficiently measured on pure cellulose. No reliable method exists for measuring cellulose crystallinity within a lignocellulosic matrix (Chundawat et al. 2012; Zhang and Lynd 2004).

Non-productive binding

Non-productive binding can occur when an enzyme binds to another polymer instead of its normal substrate, so that no cleavage reaction occurs. If the enzyme is not able to desorb this polymer, inhibition is irreversible.

The most potent inhibitor of lignocellulose degradation is lignin, which induces non-productive, irrevesible binding of enzymes, and can cause a tremendous reduction of enzymatic hydrolysis rates (Berlin et al. 2006; Berlin et al. 2005; Eriksson et al. 2002; Zhao et al. 2012). Enzymes acting on polysaccharides can be inhibited by other saccharides as well. Cellulases are inhibited by xylan and its degradation products, namely xylooligomers and derived monosaccharides (Van Dyk and Pletschke 2012; Ximenes et al. 2010; Zhang et al. 2012). The productive binding of cellulases is also inhibited by acetyl groups of heteroxylans (Zhao et al. 2012).

Steric hindrance

Enzymes acting on polysaccharides can be inhibited by steric hindrance from residues or side chains along the main chain. For example, acetyl side chains of xylan hamper its degradability by xylanase (Van Dyk and Pletschke 2012). In a similar manner, processive cellobiohydrolases reaching steric obstacles along a cellulose chain become deactivated (Chundawat et al. 2012).

Substrate protection

When adsorbed onto their substrates, enzymes may show a higher resistance to inactivation (e.g. against thermal inactivation) than in a free state, a phenomenon designated as "substrate protection" (Nath and Rao 1995).

Molecular and macromolecular structure of substrate

Many substrate features, both at molecular and macromolecular level, may affect enzymatic hydrolysis. At molecular level, these features include hydrophobicity, extent of hydrogen-bonding and ionic bridges between linear chains of sugar polymers, such as cellulose, hemicellulose, pectin and glycoproteins (Cassab 1998; Ebringerová 2006; Ebringerová et al. 2005; Keller 1993; Showalter 1993). At macromolecular level, physical entanglement may occur when the net of polymers prevents access to a specific substrate (Darley et al. 2001), and enzymes may become entrapped into pores (Zhang and Lynd 2004). At cell level, layers with a lower degradability can prevent access to easily degradable cell contents. A similar phenomenon can occur at tissue level, where hardly degradable plant tissues can prevent access to easily degradable regions (Engels and Jung 1998; Engels and Schuurmans 1992; Grabber et al. 2002; Jung and Casler 2006a; Jung and Casler 2006b; Jung and Engels 2002; Jung and Engels 2001; Wilson 1993). These parameters, which are well-known in the field of animal nutrition, have been poorly studied in relation to enzymatic hydrolysis. This deficit is possibly due to the harsh nature of physicochemical pretreatments usually applied prior to enzymatic hydrolysis of lignocellulosic plant material, as these pretreatments completely disrupt the original structure of the plant (Banerjee et al. 2010).

Enzyme-related factors

Enzyme loading

Higher enzyme loading increases hydrolysis rate. However, at very high enzyme loadings, enzymes may compete with each other for the attachment to available substrate binding sites, resulting in decreased hydrolysis efficiency (Van Dyk and Pletschke 2012; Zhang and Lynd 2004).

Enzyme stability

Enzyme stability depends on medium-related factors such as pH, temperature, shear stress, and protease activity (Morgavi et al. 2001; Van Dyk and Pletschke 2012). An improper pH range may result in unfolding of the proteins constituting the enzyme. Changing the shape of the molecule may not only deactivate the enzyme, but also reduce its resistance to degradation. Enzyme stability is an issue if enzymatic reactions are performed at long residence times. For such reactions, most stable enzymes should be selected (Eriksson et al. 2002).

Enzyme stability is related to protein composition and structure. For example, thermal stability can be enhanced by salt bridges, hydrogen bonds, internal packing of amino-acids, charged amino-acids at the surface of the protein, disulphide bridges, and hydrophobic interactions between aromatic amino-acids at the surface of the protein. Stability under acidic conditions may be conferred by the concentration of acidic amino-acids such as aspartate or glutamate at the surface of the protein, while an increased number of arginine amino-acids may provide stability under alkaline conditions (Collins et al. 2005).

Synergy between enzymes

Enzymes act synergistically to degrade lignocellulosic substrates. Several types of synergy can be distinguished: homeosynergy, heterosynergy, antisynergy and removal of physical barriers or steric hindrance of other polymers (Van Dyk and Pletschke 2012; Zhao et al. 2012).

Homeosynergy occurs when several enzymes act on one single substrate. For example, the degradation of cellulose requires synergistic action of cellobiohydrolase, endoglucanase and β-glucosidase. Cellobiohydrolase, also named exoglucanase, acts specifically either at the reducing or non-reducing end of crystalline cellulose regions to release cellobiose. Cellobiose residues are produced continuously as cellobiohydrolase moves along the cellulose chain until it reaches obstacles. Hence cellobiohydrolase is classified as a processing enzyme. Endoglucanase cleaves cellulose at random sites in amorphous regions, creating new chain ends on which cellobiohydrolase can attach (Malherbe and Cloete 2002; Walker and Wilson 1991; Zhang and Lynd 2004). Moreover, endoglucanase may prevent cellobiohydrolase processing on crystalline cellulose from stalling in amorphous cellulose regions (Jalak et al. 2012; Malherbe and Cloete 2002). β-glucosidase, also named cellobiase, cleaves cellobiose and cellodextrins (DP<7) to release glucose. Cellobiose inhibits cellobiohydrolase via product inhibition. By removing cellobiose, β-glucosidase reduces inhibition of cellobiohydrolase and improves hydrolysis efficiency (Walker and Wilson 1991; Zhao et al. 2012). For efficient cellulose hydrolysis, excess β-glucosidase is recommended, the optimal cellulase:β-glucosidase ratio being 1:2 (Van Dyk and Pletschke 2012). Hence commercial *Trichoderma reesei* cellulase is often supplemented with β-glucosidase from *Aspergillus sp.* for optimal hydrolysis efficiency (Sun and Cheng 2002; Van den Brink and De Vries 2011). A similar type of synergy may occur between xylanase and β-xylosidase acting on xylan (Van Dyk and Pletschke 2012).

Heterosynergy involves enzymes acting on different molecules of a same polymer, commonly enzymes acting on main chain and side chains of a polysaccharide. In xylans, heterosynergy can occur between main-chain xylanase and enzymes acting on side chains (also named debranching enzymes) such as acetyl xylan esterase, α-arabinofuranosidase, and feruloyl esterase (Saha 2003; Van Dyk and Pletschke 2012). Similarly, arabinofuranosidase and galactosidase removing side chains of polygalacturonans enhance pectin degradation by enzymes acting on the main chains (Sweeney and Xu 2012).

Antisynergy occurs when an enzyme inhibits another enzyme. For example, a debranching enzyme can remove a substituent which is required for the action of a main-chain cleaving enzyme. Hence the action of debranching enzymes is not necessarily beneficial to enzymes acting on main chains of polysaccharides (Van Dyk and Pletschke 2012).

Removal of physical barriers or steric hindrance from other polymers is often quoted as a synergy. For example xylanases improve cellulose hydrolysis performed by cellulases by removing the hemicellulose coating surrounding cellulose (Zhao et al. 2012).

Improvement of enzyme products

Industrial enzyme products are generally improved compared to enzymes produced by microorganisms in their natural state. Industrial modifications of enzyme systems may include the following processes (Galante and Formantici 2003; Howard et al. 2003; Maki et al. 2009):

1. Strain improvement: selection and modification of the most efficient enzyme-producing microorganisms via genetic engineering methods.

2. Overexpression: expression of the gene sequence of enzymatic proteins in another microorganism that is easier to cultivate, in order to mitigate enzyme production costs.

3. Enzyme engineering: modification of the amino-acid sequence of enzymatic proteins, so as to change their properties (increased activity, increased stability). This can be achieved by altering the coding gene sequence of the microorganisms accordingly.

Medium-related factors

Physicochemical conditions

Enzyme efficiency can be optimized by controlling medium conditions. Optimal conditions are specific to each enzyme. The most important parameters include pH, temperature, ionic strength, mixing, protease activity, and inhibitors concentration. When these parameters are not within the optimal range, a decrease or even a complete removal of enzyme activity may result (Van Dyk and Pletschke 2012; Walker and Wilson 1991). It is often thought that the conditions for highest enzyme activity are also optimal conditions with regards to enzyme stability. This is not always the case. For example, at pH 4 and a temperature of 50°C, which are optimal for the activity of fungal pectinases from *Aspergillus sp.*, enzyme stability may be lower than at 20°C and pH 7 (Akao et al. 1992). Hence, depending on reaction time, optimal medium conditions may reflect a compromise between the requirements for enzyme activity and enzyme stability. Enzymes from mesophilic fungi such as *Trichoderma reesei* may become unstable at temperatures higher than 50°C (Hari Krishna et al. 2000). Such enzymes may be inactivated to ~40% when being maintained for 15 days at 50°C (Kirsch et al. 2011).

Protease inhibition

Proteases are enzymes that cleave proteins, and have the ability to degrade other enzymes. An increase in protease concentration is correlated to a decrease in enzyme stability (Morgavi et al. 2001). The presence of proteases is such an issue that enzyme suppliers have removed the genes encoding proteases from *Trichoderma reesei* strains via genetic engineering so as to generate protease-free enzyme extracts with increased stability (Banerjee et al. 2010). Enzyme inactivation through proteases becomes problematic when exogenous enzymes have to act within a biological medium, for example enzymes used as feed supplements in animal nutrition (Marquardt and Brufau 1997; Morgavi et al. 2001).

Reactor mixing

Reactor mixing favors the access of enzymes to insoluble substrates, but intensive mixing may also induce shear stress and reduce enzyme stability. Hence a compromise should be found between the positive effects of intensive mixing on hydrolysis rate and the requirements for enzyme stability (Van Dyk and Pletschke 2012).

Product inhibition

Enzymes are often inhibited by their own reaction products. Product inhibition has been reported for cellobiohydrolase, endoglucanase, β-glucosidase, endoxylanase, β-xylosidase and feruloyl esterase (Maki et al. 2009; Saha 2003; Van Dyk and Pletschke 2012; Walker and Wilson 1991). Regarding β-glucosidase, product inhibition is so intense that the enzyme must generally be added at a higher loading than cellulase to perform complete hydrolysis of cellulose into glucose. Hence selecting for β-glucosidases that are less inhibited by glucose, is an important research target (Jørgensen et al. 2007). Product inhibition can be alleviated by the removal of degradation products via ultrafiltration in membrane reactors (Ding et al. 1998; Jørgensen et al. 2007).

Inhibitors

Cellulases can also be inhibited by many compounds, including formic acid and lactic acid (Jørgensen et al. 2007), the metal ions Hg^{2+} and Ag^{2+} (Walker and Wilson 1991) and ethanol (Sun and Cheng 2002). Phenols also deactivate cellulases, but the inhibition is reversible (Zhao et al. 2012). Xylanases are inhibited by aromatic compounds, such as vanillic acid, syringic acid and syringaldehyde (Jørgensen et al. 2007).

Additives

Enzymatic hydrolysis can be significantly improved by additives. Non-ionic surfactants (e.g. Tween 20) and proteins (e.g. BSA, Bovine Serum Albumine) bind to lignin via hydrophobic interactions and repel enzymes from the lignin surface. Hence non-productive binding of enzymes on lignin is reduced. Of course, the effects of surfactants and proteins are significantly lower on delignified substrates (Eriksson et al. 2002; Sun and Cheng 2002; Zhao et al. 2012).

Hydrolysis duration

The extent of product formation increases together with the duration of enzymatic hydrolysis. The duration to be applied to reach significant hydrolysis rates depends on substrate properties, enzyme loading and temperature. Recalcitrant substrates, low enzyme loadings and low temperatures require a longer hydrolysis period (Jørgensen et al. 2007; Menon and Rao 2012).

3.2.3 Enzymatic degradation of plant material

The lignocellulosic matrix of the cell wall is highly interlinked. Therefore, lignocellulose hydrolysis requires multiple enzyme activities, not only on cellulose and hemicellulose, but also on pectin, proteins, lignin and on the plant cuticle. **Table 2** classifies these activities according to the plant component and the polymers to be degraded. Some plant components, like hemicellulose, comprise a wide range of polymers of widely different properties. For each polymer described in the table, core activities operate against the backbone, while accessory activities operate against the side chains.

Since hemicellulose composition varies between plant species, the optimal enzyme mixture should be well adjusted to the plant material (Jørgensen et al. 2007). Glycosidases hydrolyze glycosidic bonds, esterases hydrolyze ester bonds, and lyases cleave glycosidic bonds, i.e. contrary to hydrolysis the reaction occurs without hydration of reaction products (Sweeney and Xu 2012). Most enzymes retain the conformation of sugars after cleavage, but some enzymes also have an inverting effect, changing the conformation of sugars after cleavage (Gilbert et al. 2008; Sweeney and Xu 2012).

Table 2. Enzyme activities required for hydrolysis of lignocellulose [Compiled after Dutta (2009), Gilbert (2010), Menon and Rao (2012), Van den Brink and De Vries (2011), Van Dyk and Pletschke (2012). [1] In commelinid monocots (e.g. grasses). [2] In non-commelinid monocots and in dicots (e.g. legumes)].

Component	Polymer	Core activity	Accessory activity
Cellulose	-	Cellobiohydrolase Endoglucanase β-glucosidase	Extensin Swollenin
Hemicellulose	Heteroxylan	Exo-xylanase Endo-xylanase β-xylosidase	α-L-arabinofuranosidase α-glucuronidase Acetyl xylan esterase Feruloyl esterase [1] p-coumaric acid esterase
	Xyloglucan	Endo-xyloglucanase α-xylosidase β-glucosidase	α-fucosidase α-glucuronidase β-galactosidase
	Mixed-linkage glucan	β-glucanase Mixed-linked glucanase Lichenase	
	Heteromannan	Endo-mannase β-mannosidase β-glucosidase	α-galactosidase Acetyl mannan esterase
Pectin	Homo-galacturonan	Polygalacturonase Pectin lyase Pectate lyase	Pectin methylesterase Pectin acetylesterase
	Rhamno-galacturonan I	Rhamnogalacturonase Rhamnogalacturonan lyase α-galactosidase	α-arabinase β-galactanase β-galactosidase α-rhamnosidase Rhamnogalacturonan acetyl esterase Pectin acetylesterase Feruloyl esterase [2]
Proteins	-	Proteases	α-Arabinase β-Galactase
Lignin	-	Laccase Manganese peroxidase Lignin peroxidase	
Cuticle	-	Cutinase Lipase Esterase	

3.2.4 Enzyme-mediated oxidative degradation of lignin and cellulose

Lignin in its polymeric form can not be degraded by microorganisms under anaerobic conditions (Tong et al. 1990). A positive redox potential and the presence of oxygen are a prerequisite for bacterial or enzymatic lignin degradation (Call and Mucke 1997; Hofrichter 2002). Certain microorganisms, including white-rot fungi and some soil fungi, are able to degrade lignin in an aerobic environment (Hofrichter 2002; Pérez et al. 2002). This reaction is mediated by laccases and peroxidases. These oxidative enzymes generate strong oxidants, which are able to modify lignin structure, in a process named “enzymatic combustion” (Pérez et al. 2002).

However, laccases and peroxidases alone do not degrade lignin, and may even have the opposite effect by catalyzing lignin polymerization (Grönqvist et al. 2005; Ward et al. 2001). The addition of chemical mediators, such as phenolics or aromatic amines, is required to effectively perform lignin depolymerization. Microorganisms are able to produce chemical mediators naturally through the action of reductive enzymes, such as sugar oxidases (e.g. cellobiose dehydrogenase), aryl alcohol oxidases, and aryl alcohol dehydrogenases. The chemical mediators first react with oxidizing enzymes. In a second step, mediators in their oxidized state attack lignin structure (Call and Mucke 1997; Henriksson et al. 2000; Hofrichter 2002; Pérez et al. 2002).

In addition, cofactors and other products facilitating the reaction are required for oxidizing enzymes to function properly. These products include veratryl alcohol and hydrogen peroxide for lignin peroxidase (Ward et al. 2001), manganese, hydrogen peroxide, oxygen, organic chelatants for manganese peroxidase (Hofrichter 2002; Pérez et al. 2002), and oxygen for laccase (Call and Mucke 1997). Lignolytic fungi are able to generate hydrogen peroxide naturally from oxygen through the action of specific enzymes such as glyoxal oxidase, glucose oxidase, veratryl alcohol oxidase, and methanol oxidase (Howard et al. 2003; Sweeney and Xu 2012).

Oxidative enzymes can be inhibited by various products. Excessive amounts of hydrogen peroxide reversibly inhibit peroxidases, but this inhibition can be avoided by the addition of veratryl alcohol. The enzyme can also be deactivated by its own reaction products. Gelatine or polyethylene glycol added into the reaction medium can alleviate this problem by competitivey binding to these reaction products (Call and Mucke 1997; Hofrichter 2002; Ward et al. 2001).

While microorganisms produce these compounds naturally and perform lignin degradation at a very slow rate, in the industry, these additives are very costly and the reaction needs to be optimized in order to occur at a much faster rate. An oxygen pressure of several atmospheres is necessary for efficient lignin degradation with laccase (Call and Mucke 1997).

Recent advances indicate that the process of "enzymatic combustion" utilized by some microorganisms for lignin degradation under aerobic conditions, may also apply to cellulose degradation in aerobic environments. Oxidative enzymes such as copper monooxygenase may be involved in this process (Horn et al. 2012).

3.2.5 Applications of enzymatic hydrolysis of plant biomass

Bioethanol process

Origin of the enzymatic process

In the middle of the 20th century, industrial processes have been implemented to extract sugars from lignocellulosic feedstocks on industrial scale through acid hydrolysis, either with dilute acid at high temperature or with concentrated acid at low temperature, before subsequent fermentation of the sugars into ethanol (Möller 2006; Peters 2006). These harsh chemical treatments did not necessitate any enzymatic hydrolysis step since sugars were already generated in their monomeric form. Disadvantages of acid hydrolysis processes were the degradation of part of the monosaccharides released during the process, together with high energy and maintenance costs. In the 1970s, the engineering of the aerobic fungi *Trichoderma reesei* for cellulase production gave rise to the idea of incorporating an enzymatic hydrolysis step into the process of glucose production. The implementation of enzymatic hydrolysis may alleviate the drawbacks of chemical pretreatments by reducing their severity (Howard et al. 2003; Möller 2006; Yang et al. 2011). However this process is not mature and enzyme suppliers themselves confess that current commercial cellulases are still inadequate for cost-effective biomass processing (Knauf and Moniruzzaman 2004). High enzyme costs and the requirement for high enzyme dosage are the main hurdles to the economic viability of lignocellulosic ethanol (Menon and Rao 2012).

Process conditions

Enzymatic hydrolysis of lignocellulose without pretreatment is usually ineffective, because in its native state the complex formed by lignin, cellulose and hemicellulose is recalcitrant to enzymatic attack. Enzymatic hydrolysis of native lignocellulose solubilizes less than 20% of sugars (Zhang and Lynd 2004). Research focuses on pretreatments preceding enzymatic hydrolysis in the context of second-generation ethanol production, i.e. ethanol derived from lignocellulosic feedstocks. Pretreatments for bioethanol production can be divided into two classes: physical and chemical or physicochemical. Physical (or mechanical) pretreatment involves particle size reduction via grinding, milling, shearing, extrusion, and is usually performed prior to physicochemical pretreatment because it makes it more effective (Saha 2003; Van Dyk and Pletschke 2012).

Chemical or physicochemical pretreatments separate major biomass components or solubilize part of the biomass. The liquid fraction usually contains a share of degraded hemicellulose or lignin (Menon and Rao 2012; Möller 2006; Taherzadeh and Karimi 2008). Processing substrates under acidic conditions (low pH) tends to solubilize hemicellulose, while preserving lignin and cellulose in the solid fraction. Treating substrates under alkaline conditions (high pH) tends to remove lignin or at least to suppress ester linkages between lignin and hemicellulose (Carvalheiro et al. 2008; Knauf and Moniruzzaman 2004). Pretreatments with dilute acid, steam explosion and hot water are acid, while pretreatments with lime, ammonia and oxidants (e.g. hydrogen peroxide) are performed under alkaline conditions (Chundawat et al. 2012; Saha 2003).

Pretreatment steps

Efficient pretreatment often requires a dilution of the substrate with large amounts of water or solvent (Taherzadeh and Karimi 2008). Moreover, pretreated biomass is often washed prior to enzymatic hydrolysis and ethanol fermentation in order to remove undesirable inhibitory products generated by the pretreatment process (Sun and Cheng 2002).

The typical pattern of an ethanol production process via dilute-acid pretreatment of corn stover includes the following steps: mechanical particle size reduction, treatment with 1% sulphuric acid at 121-160°C for 30 min, washing of pretreated substrate, enzymatic hydrolysis at 50°C for 48-72h, ethanol fermentation, and ethanol distillation (Aden et al. 2002; Sun and Cheng 2002; Taherzadeh and Karimi 2008).

SSF process

Enzymatic hydrolysis and ethanol fermentation can be performed simultaneously as Simultaneous Saccharification and Fermentation (SSF). In the latter case, temperature should meet the requirements of ethanol fermentation. Optimal temperature is ~45-50°C for enzymatic hydrolysis and 28-35°C for ethanol fermentation (Schober 2008; Sun and Cheng 2002). The future development of thermotolerant yeast strains may solve the problem of these different temperature optima (Menon and Rao 2012). The advantage of SSF is profiting from the synergy between enzyme and microbe. The microbe improves enzymatic hydrolysis by removing inhibitory hydrolysis products from the medium (Schober 2008; Van Dyk and Pletschke 2012). On the other hand, ethanol produced by yeast fermentation inhibits cellulase (Sun and Cheng 2002).

Extending hydrolysis and fermentation to other substrate fractions

In the past, objectives of pretreatments were to remove hemicellulose from the solids in order to make cellulose accessible to enzymatic hydrolysis, because the valorization of pentose sugars by ethanol-producing microorganisms was problematic. For this purpose, most reseach focused on enzymatic hydrolysis of dilute-acid-pretreated substrate. Hence the knowledge about hemicellulose hydrolysis is less extensive compared to cellulose hydrolysis. The new trend is to maximize sugar recovery from both cellulose and hemicellulose, so that other pretreatments and enzyme mixtures should be applied (Banerjee et al. 2010; Gilbert et al. 2008; Jørgensen et al. 2007; Van Dyk and Pletschke 2012).

Removing the physicochemical pretreatment step

Schober (2008) followed an innovative approach. Instead of resorting to physico-chemical pretreatments, she selected an easily hydrolyzable subtrate: whole crop ensiled maize. Since ensiled maize contains high amounts of organic acids (pH 4), buffer addition was not required. Particle size reduction via ball milling was applied prior to enzymatic hydrolysis. For enzymatic hydrolysis a mixture of 66% GC 880® from Genencor and 33% Novozym 188® from Novozymes was applied at an enzyme:substrate ratio of 0.5 g protein / g substrate dry mass. Enzymatic hydrolysis performed at 55°C for 50 h resulted in monosaccharide release to the extent of 48% of total sugar content. Novozym 188®, besides its β-glucosidase activity, was the main supplier for α-amylase activity.

Unfortunately Novozym 188® was applied at a low share in the enzyme mixture owing to the high costs of this commercial enzyme product. Therefore, one may hypothesize that resorting to commercial amylases, hemicellulases and pectinases may further improve enzymatic hydrolysis of whole crop ensiled maize.

Consolidated bioprocessing

Prospective studies indicate that lignocellulosic bioethanol may evolve towards consolidated bioprocessing (CBP) processes in the future. In CBP the severity of chemical pretreatment will be reduced, or the chemical pretreatment step will be removed, while enzymatic hydrolysis will be replaced with microbial fermentation, so as to carry out all treatment steps within one vessel. Genetically engineered microbes should be able to degrade substrate macromolecules and to produce ethanol, solvents, or organic acids (Lynd 1996; Lynd et al. 2005; Menon and Rao 2012; Olson et al. 2012). To carry out both substrate degradation and fermentation, yeasts may be replaced with anaerobic bacteria, which would hold the advantage of requiring no oxygen addition and generating limited overgrowth: bacterial biomass generation per unit ethanol produced would be less, allowing higher ethanol yields to be reached (Chundawat et al. 2012; Maki et al. 2009).

Industrial processes

Lignocellulose-degrading enzymes are used in various industrial processes (**Table 3**). Laccase and peroxidase originate from lignolytic fungi (Pérez et al. 2002), but may be overexpressed in other organisms for easier cultivation (Galante and Formantici 2003). Cellulase, xylanase and pectinase operating at low pH originate from aerobic fungi such as *Trichoderma reesei* and *Aspergillus niger*, while enzymes with similar activities operating at neutral pH originate from aerobic bacteria such as *Bacillus subtilis* (Beg et al. 2001; Maki et al. 2009; Subramaniyan and Prema 2002; Van den Brink and De Vries 2011).

Contrary to acid cellulases from *Trichoderma reesei*, neutral cellulases selected for industrial applications have few or no cellobiohydrolase activity. The main application of neutral cellulases is the cleaning or finishing of fabrics. For such applications cellobiohydrolase activity is undesirable because it is active on crystalline cellulose which forms the inner structure of tissues, and would damage them. Hence, in these enzyme formulations only the endoglucanase component is maintained and the cellobiohydrolase component is removed so as to degrade solely amorphous parts of cellulose fibers (Galante and Formantici 2003).

Table 3. Some industrial applications of fibrolytic enzymes [Compiled after Beg et al. (2001), Bhat and Bhat (1997), Collins et al. (2005), Galante and Formantici (2003). * Contains only endoglucanase activity and no cellobiohydrolase].

	Acidic pH			Neutral pH		
	Cellulase	**Xylanase, pectinase**	**Laccase**	**Cellulase ***	**Xylanase**	**Peroxidase**
Detergents				×	×	×
Prebleaching of paper pulp					×	
Enzymatic stone washing, finishing of fabrics				×		
Decolourization of dyes			×			
Bakery, extraction of juices, beer filtration		×				
Digestibility of animal feed, silage additive	×	×				

3.2.6 Microbial strategies for plant degradation

Specialization on plant degradation

Filamentous fungi are well suited for the production of lignocellulose-degrading enzymes since they have a lifestyle involving the degradation of plant biomass. These fungi have different enzyme sets depending on their specialization. Lignolytic fungi such as *Phanerochaete chrysosporium* have an oxidative enzyme system which is efficient against lignin. *Trichoderma reesei* is specialized in cellulose degradation, and *Aspergillus sp.* has many enzymes acting against pectin (Van den Brink and De Vries 2011).

Microbial degradation in anaerobic environments

While aerobic bacteria and fungi generally produce free enzymes, anaerobic bacteria and fungi follow a different strategy for lignocellulose degradation and build up cellulosomes, releasing only small amounts of free enzymes (Banerjee et al. 2010; Malherbe and Cloete 2002).

Nevertheless, many anaerobic organisms having cellulosomes also release free enzymes. Hence, one may hypothesize that a synergism exists between cellulosome and free enzymes (Yang et al. 2011; Zhao et al. 2012).

Since catabolic reactions are less favorable in the reduced oxidation potential prevailing in anaerobic environments, microorganisms gain less energy from lignocellulose degradation compared to aerobic conditions. The lower energy gain under anaerobic conditions may not allow the waste of resources in the form of enzymes being released into the medium, so that most enzymes remain cell-bound in form of cellulosomes (Lynd and Zhang 2002; Malherbe and Cloete 2002). Besides the catabolism of monosaccharides, anaerobic microorganisms get significant amounts of energy from the depolymerisation of soluble oligosaccharides such as cellodextrins and cellobiose, via specific enzymes with phosphorylase activity producing glucose monophosphate (Bhat and Bhat 1997; Maki et al. 2009). These microorganisms may also have β-glucosidases for cellobiose and cellodextrin degradation as well, but these enzymes may be regulated via product inhibition (Bhat and Bhat 1997).

Bacterial attachment and cellulosome system

In anaerobic microorganisms, bacterial attachment and direct physical contact to the substrate are essential for polymer hydrolysis, while that may not be the case of aerobic microorganisms. In anaerobic bacteria, attachment to substrate is ensured through the excretion of substantial amounts of glyocalyx (Lynd and Zhang 2002; Malherbe and Cloete 2002; McAllister et al. 1994). In the cellulosome system, the amount of enzyme required per molecule degraded is minimized in several ways: optimization of synergism between enzymes acting in close vicinity to each other, limitation of non-productive binding, optimal spacing of enzymatic components, avoidance of enzyme competition for binding sites (a phenomenon which is designated as competitive binding) and easier transfer of soluble degradation products into the cells (Maki et al. 2009).

Particle size reduction

The waxy cuticle forming the external protective layer of the plants prevents the access and degradation of the interior of leaves and stems. Plant-degrading organisms such as ruminants and termites apply particle size reduction in order to overcome these barriers, while anaerobic fungi physically disrupt tissues with their hyphae (Malherbe and Cloete 2002; McAllister et al. 1994; Schober 2008).

3.2.7 Microbial degradation versus enzymatic hydrolysis

Advantages of microbial degradation over enzymatic hydrolysis

Cell-free enzymatic hydrolysis differs from in-vivo microbial degradation of biomass (Banerjee et al. 2010; Lynd et al. 2005). Microorganisms may outperform enzyme products for the following reasons:

1. Microorganisms can develop by exploiting biomass degradation products and invest a high share of their own energy into enzyme production (Lynd and Zhang 2002);
2. Microorganisms assimilate hydrolysis products of enzymes, removing product inhibition which affects separate enzymatic hydrolysis (Schober 2008; Sun and Cheng 2002).

Energy investment into substrate degradation

Microorganisms have to invest much energy into lignocellulose degradation. The specific activity of cellulases being lower than for most catabolytic enzymes (e.g. ~100-fold lower than for amylase), the synthesis of an efficient cellulase system requires tremendous amounts of energy. The aerobic fungi *Trichoderma reesei* spends approximately half of its energy gain on the synthesis of cellulases (Lynd and Zhang 2002). Cellulolytic fungi may secrete ~70% cellobiohydrolases, ~20% endoglucanases, and ~1% β-glucosidases (Sweeney and Xu 2012).

Intracellular degradation of hydrolysis products

Current processes of enzymatic hydrolysis for ethanol production require complete hydrolysis of polysaccharides into monomers, which can be used by yeasts as an energy source for ethanol production (Chundawat et al. 2012). Alternately, living cells produce cell-bound or free extracellular enzymes which transform polymers into soluble oligomers that are absorbed by cells, and part of the final hydrolysis steps may occur intracellularly, with enzymes being either cell-bound or active inside the cell (Gianfreda and Rao 2004; Subramaniyan and Prema 2002).

3.3 Enzyme additives in anaerobic digestion processes

3.3.1 Fields of application

Enzyme additives in animal nutrition

Animal digestion may have many similarities to the biogas process, especially ruminant digestion. Interestingly, Marquardt and Brufau (1997) stated that enzyme additives may be more effective in monogastric animals (pigs, chicken) that have shorter intestines than ruminant animals, and only achieve a poor rate of fiber degradation. In these animals, some fiber fractions may coil around easily degradable substrate fractions and reduce their availability to digestive processes in a phenomenon termed "cage effect" in fermentation studies for animal nutrition (Aulrich and Flachowsky 2001; Simon 2000).

Enzyme additives in the biogas process

There are several ways for enzyme additives to be used in the biogas process (Heiermann et al. 2010; Heiermann et al. 2011):

1. **Addition to fresh energy crops prior to ensiling** to improve the digestibility or the stability of ensiled material.
2. **Addition in a separate pretreatment step** upstream of the biogas reactor. The pretreatment step may occur in a dilute aqueous medium complemented with a chemical buffer to maintain the pH at a certain value, considered optimal for enzyme action. Alternately, the pretreatment step may be enriched with bacterial biomass and constitute an acidogenic fermentation reactor (commonly designated as hydrolysis step or acidification step). In the latter case, interactions can occur between added enzyme and acidogenic bacteria.
3. **Direct addition into the biogas reactor**.

In order to narrow the scope of this thesis, enzyme addition to fresh energy crops prior to ensiling was not reviewed. The conditions of success for enzyme addition prior to ensiling may differ dramatically from enzyme addition into the biogas process. The latter topic is mainly addressed by animal nutrition experts. Hence a separate investigation would be required to address this topic.

3.3.2 Action of enzyme additives on the biogas process

Process parameters affected by enzyme additives

The effect of enzyme addition on the biogas process has already been reviewed by several authors (Binner et al. 2011; Heiermann et al. 2010; Heiermann et al. 2011; Koch et al. 2010; Parawira 2012; Suárez Quiñones et al. 2012b).

Added enzymes can have several effects in the biogas process:

1. Increase of methane yield to the end of the digestion period;
2. Acceleration of methane production rate;
3. Reduction in viscosity of reactor contents and reduction of the formation of floating layers in the reactors.

Effects of enzyme additives on viscosity of fermenting substrate

Effects on viscosity have been described by other authors (Binner et al. 2011; Junne et al. 2010; Plöchl et al. 2009). From an economical point of view, a reduction in viscosity and an improvement in the consistence and homogeneity of fermenting substrate in biogas reactors can be very profitable. Along with reduced energy consumption of stirrers, wear of equipment can be mitigated and clogging of pipes can be avoided (Heiermann et al. 2010; Koch et al. 2010; Suárez Quiñones et al. 2012b). This topic is not further developed in this thesis because the research focus is on enzyme effects on product formation in anaerobic digestion.

Types of enzyme additives used

Enzyme additives currently used on the biogas process in both research and commercial applications in Germany may be classified into three categories:

1. Extracted, purified and stabilized enzymes from pure fungal culture in solid or liquid form which are mixed with conservatives in order to retain their activity (IZMB 2006; Schimpf et al. 2012b);
2. Dried residue from solid-state fermentation of mixed yeast cultures (Demmig et al. 2010; IZMB 2006);
3. Dried residue from the cultivation of edible mushrooms (IASP Berlin and Schulz 2010; Schimpf et al. 2011b; Schimpf et al. 2012a; Schimpf et al. 2012b).

3.3.3 Effects of enzyme additives reported in the literature

Evaluations of enzyme addition in the biogas process by research groups are compiled in the following tables. In order to narrow the results, only the enzyme doses and mixtures showing maximal effects or considered optimal by the authors are shown.

The effects of enzyme additives presented in this review are split as follows:

1. Batch digestion with energy crops (**Table 4**);
2. Continuous digestion with energy crops (**Table 5**);
3. Batch and continuous digestion with organic wastes (**Table 6**);
4. Enzymatic pretreatment upstream of anaerobic digestion (**Table 7**);
5. Direct enzyme addition into acidogenic fermentation processes, including dark fermentation processes (**Table 8**).

The wide range of effects obtained suggest that no definitive statement can be made concerning the effect of enzyme additives on the biogas process. Some authors obtained positive effects which turned out to be below the level of statistical significance (Romano et al. 2009; Stenströmer Moglia 2008; Wulf and Clemens 2006). Interestingly, some authors noticed an increase in the methane conversion rate in the course of batch digestion assays, while the curves of cumulated methane yields converged to the end of the digestion period, and final methane yields were similar (Stenströmer Moglia 2008; Wulf and Clemens 2006).

On the other hand, there is some evidence suggesting that added enzyme may be quite influential on the acidogenic digestion processes, increasing substrate degradation as well as both VFA and H_2 production (**Table 8**). However, effects on a downstream biogas process become weaker (**Table 7**). One may hypothesize that much of the gain in initial fermentation velocity is lost with downstream methane production occurring at a much higher retention time.

Table 4. Effect of enzyme additives on batch digestion of energy crops [Compiled after Telschow (2006) [1], Kaiser (2004) [2], Rieker and Wittmann (2007) and Wulf and Clemens (2006) [3], Amon et al. (2007) [4], Schimpf and Valbuena (2009) [5], Romano et al. (2009) [6], Schimpf et al. (2011b; 2012a; 2012b) [7]].

Process conditions	Enzyme and dosage	Substrate	Effect
HRT 30 d 37°C [1]	Cellulase, hemicellulase 0.1 g/kg TS	Ensiled maize	Methane yield **+14%** 404 → 460 L/kg VS
HRT 25 d 38°C [2]	Cellulase, hemicellulase 0.2 g/kg TS	Ensiled maize	Methane yield **+14%** 429 → 488 L/kg VS
HRT 42 d 38°C Daily enzyme addition [3]	Dried yeast culture broth 10 g/(kg VS × d)	Ensiled maize	No effect on methane yield ~ 300 L/kg VS
HRT 42 d 38°C [3]	Dried yeast culture broth 10 g/(kg VS × d)	Rye silage	Slight increase of methane production rate, no effect on final methane yield ~ 350 L/g VS
		Grass silage	No effect on methane yield ~ 300 L/g VS
HRT 47 d 38°C [4]	Cellulase, hemicellulase 0.2 g/kg TS	Reed silage	Methane yield **+19%** 187 → 223 L/kg VS but not significant
HRT 35 d 38°C [5]	Cellulase, pectinase, laccase 1 g/kg FW	Rye silage 8 mm fiber length	Methane yield **+9%** 258 → 282 L/kg VS
HRT 14 d 50°C [6]	Neutral cellulase 25 g/kg VS	Milled wheat grass (0.28-0.33 mm)	No effect on methane yield 160 L/kg VS
HRT 30 d 38°C [7]	Residue from edible mushroom cultivation 10 g/kg FW	Hay and wheat straw 1:1 w/w	Methane yield **+11%** 169 → 187 L/kg VS

Table 5. Effect of enzyme additives on continuous digestion of energy crops [Compiled after Telschow (2006) [1], Lebuhn et al. (2010) [2], Binner (2011) [3], Rieker and Wittmann (2007) and Wulf and Clemens (2006) [4], Schimpf et al. (2011a) [5]].

Process conditions	Enzyme and dosage	Substrate	Effect
HRT 22 d 37°C OLR 3 kg VS/(m^3 × d) [1]	Cellulase, hemicellulase 1 g/kg TS	Dairy manure (80% VS) Maize silage (20% VS)	Methane yield **+27%** 223 → 283 L/kg VS
37°C OLR 1 kg VS/(m^3 × d) [2]	Cellulase, amylase, β-galactosidase 1 g/kg VS	Maize silage	Methane yield **+13%** 300 → 340 L/kg VS
OLR 3 kg VS/(m^3 × d) [3]	Cellulase, hemicellulase 0.8-2 g/(m^3 × d)	Maize silage	No effect on biogas yield 750 L/kg VS
HRT 25 d 38°C OLR 2 kg VS/(m^3 × d) [4]	Dried yeast culture broth ~10 g/kg VS	Maize silage	Methane yield **+0.4%** ~ 420 L/kg VS but not significant
		Rye silage	Methane yield **+0.5%** ~ 420 L/kg VS but not significant
		Grass silage	Methane yield **+3%** ~ 400 L/kg VS but not significant
Full-scale biogas plant HRT 63 d OLR 5 kg VS/(m^3 × d) [5]	Pectinase 0.1 g/kg TS	Maize silage, Rye silage, maize corn, sweet sorghum, rye	Decrease in viscosity of **6-18%**

Table 6. Effect of enzyme additives on batch and continuous digestion of organic wastes [Cammarota et al. (2001) [1], Sri Bala Kameswari et al. (2011) [2], Cail et al. (1986) [3], Roman (2006) [4], Beijer (2008) [5], Rintala and Ahring (1994) [6], Lagerkvist and Chen (1993) [7]].

Process conditions	Enzyme and dosage	Substrate	Effect
Continuous digestion in UASB - 1 d - 30°C OLR 4 kg COD/(m^3 × d) [1]	Lipase-rich residue of fungi cultivation 10 g/L effluent	Dairy wastewater	Increase of COD removal rate **+80%** 50% → 90%
Batch digestion 42 d [2]	Lipase 100 g/kg VS	Tannery waste	Biogas yield **+16%** 379 → 440 L/kg VS
Continuous digestion in SBR - 3 d - 35°C OLR ~10 kg VS/(m^3 × d) [3]	Cellulase 10 g/L effluent	Wool scouring effluent	Increase of COD removal rate **+14%** 59% → 67%
Batch digestion 5 d [4]	Cellulase, protease ~5 g/kg TS	Pre-digested primary sewage sludge	Total Suspended Solids (TSS) reduction **+300%** 20% → 80%
Batch digestion 33 d - 37°C [5]	Cellulase, xylanase, lipase, amylase 19 g/kg TS	Mixed sewage sludge + 0.3 g sodium citrate per g sludge VS	Methane yield **+19%** 290 → 346 L/kg VS
Batch digestion 15 d - 55°C [6]	Neutral xylanase, protease, lipase ~1.6 g/kg VS	Source-sorted household solid waste (biowaste)	Methane yield **+19%** 588 → 607 L/kg VS but not significant
Batch digestion in simulated landfill reactor 300 d – 30°C [7]	Cellulase, hemicellulase, amylase, protease ~1g/kg FW	Municipal Solid Waste	VS conversion rate **+12%** 52% → 58%

Table 7. Effect of enzyme pretreatment on batch and continuous digestion of all substrates [Compiled after Steinströmer Moglia (2008) [1], Suárez Quiñones et al. (2009; 2012a) [2], Suárez Quiñones et al. (2011) [3], Romano et al. (2009) [4], Schieder et al. (2009) and Ellenrieder et al. (2010) [5]].

Process conditions	Enzyme and dosage	Substrate	Effect
Pretreatment 3 d - 37°C - pH 5 Batch digestion HRT 23 d - 37°C [1]	Cellulase, hemicellulase 20 g/kg TS	Wheat (90% VS) Distillers waste (10% VS)	Slight increase of methane production rate, no effect on final methane yield 306 L/kg VS
Pretreatment 3 h - 40°C - pH 5-6 Batch digestion HRT 30 d - 35°C [2]	Dried yeast culture broth 40 g/kg VS	Cattle manure	Methane yield **+106%** 165 → 340 L/kg VS
		Rye silage	Methane yield **+16%** 355 → 412 L/kg VS
		Grass silage	Methane yield **-3%** 307 → 297 L/kg VS
		Maize silage	Methane yield **+30%** 370 → 480 L/kg VS
Pretreatment 3 h - 40°C - pH 5-6 Continuous digestion HRT 40 d - 38°C OLR 3 kg VS/(m^3 × d) [3]	Dried yeast culture broth 40 g/kg VS	Rye silage	Biogas yield **+6%** 620 → 660 L/kg VS
		Maize silage	Biogas yield **+12%** 600 → 670 L/kg VS
		Solid dairy manure	Biogas yield **+3%** 390 → 400 L/kg VS
Pretreatment in aqueous buffer 7d - 50°C - pH 7 Batch digestion 14 d - 50°C [4]	Neutral cellulase 25 g/kg VS	Milled wheat grass (0.28-0.33 mm)	Slight increase of methane production rate, no effect on final methane yield 160 L/kg VS
Acidogenic step 7 d - 50°C Batch digestion 14 d 50°C [4]	Acidic cellulase + β-glucosidase 25 g/kg VS	Milled wheat grass (0.28-0.33 mm)	Methane yield **+32%** 220 → 290 L/kg VS
Pretreatment in water 1 d - 50°C - pH 3-4 Continuous digestion 37°C - 25 d OLR 2 kg VS/(m^3 × d) [5]	Pectinase	Maize silage	Methane yield **+3%** 410 → 400 L/kg VS Increase of VFA concentration
	Amylase	Maize silage	Methane yield **-7%** 410 → 380 L/kg VS Increase of VFA concentration

Table 8. Effect of enzyme additives on acidogenic fermentation [Yang et al. (2010) [1], Quéméneur et al. (2012) [2], Kim et al. (2006) [3], Akao et al. (Akao et al. 1992) [4], Lagerkvist and Chen (1993) [5]].

Process conditions	Enzyme and dosage	Substrate	Effect
Batch Acidogenic digestion 12h - 50°C [1]	Amylase, protease 60 g/kg FW	Secondary sewage sludge (filtered 0.71 mm)	TS reduction **+580%** 10% → 68%
Batch Dark fermentation 10d - 37°C [2]	Cellulase, hemicellulase 5 g protein/kg FW	Wheat straw	H_2 yield **+82%** 11 → 20 L/kg VS Acetate yield **+146%** 13 → 32 g/kg VS Butyrate yield **+169%** 16 → 43 g/kg VS
Pretreatment 1d - 45°C – pH 6.5 Batch Acidogenic digestion 35°C - 10d [3]	Cellulase, protease, lipase ~1 g/kg FW	Food waste	VFA release **+200%** 2000 → 6000 mg COD/L
Batch Acidogenic digestion 2d – 30°C [4]	Pectinase 6.2 U/g VS (Enzyme Unit)	Mandarin peels	Substrate fraction passing through 200-mesh filter **+42%** 60% → 85%
Batch Acidogenic digestion in simulated landfill reactor 300 d - 30°C [5]	Cellulase, hemicellulase, amylase, protease ~1g/kg FW	Municipal Solid Waste	VS conversion rate **+12%** 34% → 38%

4 Materials and methods

4.1 Overview of the experimental design

Classification of the experiments

The experiments carried out in this thesis were divided into the following groups, as shown in **Table 9**:

1. **Enzymatic hydrolysis assays:** the conditions required to maximize enzymatic hydrolysis of maize straw were determined in a controlled water bath system in which no significant interference through bacterial development should occur.
2. **Batch digestion trials:** the efficiency of enzyme additive to increase methane production was tested on plant substrates in batch reactors in presence of a digestion medium and anaerobic bacteria.
3. **Batch digestion following enzymatic hydrolysis step:** anaerobic digestion took place after a separate enzymatic hydrolysis step (pretreatment) in which conditions were optimized for maximal efficiency of enzymatic hydrolysis of substrate.
4. **Batch digestion of effluent from a biogas plant:** the effluent of an on-farm digester was supplemented with enzyme additives and further digested in batch reactors to evaluate their effects on methane production under practical conditions.
5. **Semi-continuous acidogenic digestion:** an enzyme additive was tested in an acidogenic fermentation process operating at a lower pH than the biogas process. Acidogenic fermentation generated biohydrogen, organic acids and alcohols as end products instead of biogas.

Table 9. Overview of the experimental targets and parameters.

Method	Substrate	Parameters	Information
Enzyme activity assay	Finely chopped maize straw	Reducing sugars	Enzyme efficiency on the substrate under controlled experimental conditions
Batch digestion trials	Finely chopped maize straw Finely chopped maize corn Coarse rye silage Grass silage	CH_4 yield	Effect of enzyme additives on CH_4 yield in anaerobic digestion
Batch digestion following enzymatic hydrolysis step	Finely chopped ensiled maize straw	Monosaccharides CH_4 yield	Effect of enzyme additives on CH_4 yield in anaerobic digestion following optimized enzymatic pretreatment
Batch digestion of effluent from a biogas plant	On-farm biogas plant effluent	CH_4 yield	Effect of enzyme additives on CH_4 yield in anaerobic digestion under practical conditions
Semi-continuous acidogenic digestion	Coarse maize silage	pH VFA Lactic acid Alcohols COD CO_2 content of gas Gas yield H_2 yield	Effect of enzyme additives on acidogenic fermentation products

Implementation of the experiments

Laboratory experiments took place at the State Institute of Agricultural Engineering and Bioenergy at the University of Hohenheim. The Department of Biotechnology and Enzyme Science of the Institute of Food Science and Biotechnology at the University of Hohenheim (Director: Prof. Dr. Lutz Fischer) offered technical support and advice for the enzymatic hydrolysis assays. The Microbial Biotechnological Center of the University of Bucharest, Romania (Prof. Dr. Stefana Jurcoane) provided the original protocol of enzymatic hydrolysis trials, and sent Dr. Camelia Diguta and Dr. Anca Vintiloiu as gast scientists to perform the assays. Furthermore, one batch digestion experiment was performed in cooperation with Dr. Ulrike Schimpf, from the Institute of Agricultural and Urban Ecological Projects (IASP) of Humboldt University, Berlin.

4.2 Enzymatic hydrolysis assays

4.2.1 Assay protocol

Principle of enzymatic hydrolysis trials

Enzymatic hydrolysis trials involved the microbe-free enzymatic hydrolysis of maize straw at controlled pH and temperature in a watery buffer medium, generally performed at an enzyme dosage of ~30 g/kg of substrate VS for a duration of 24 hours and a substrate:water ratio of 1:6. After completion of the hydrolysis period, samples were removed and analyzed to evaluate the amount of sugars released during hydrolysis.

Possible biases of enzymatic hydrolysis assays

Substrate-water ratio

The efficiency of enzymes is diminished by the presence of their products in the medium (Maki et al. 2009; Saha 2003; Van Dyk and Pletschke 2012; Walker and Wilson 1991). A substrate:water ratio of 1:6 was applied in the trials. This ratio was assumed to provide sufficient dilution, that limit the extent of enzyme inhibition due from sugar release.

Substrate concentration

Substrate concentration should be high enough to provide potential binding sites for enzyme action in sufficient amounts (Bailey 1988). The hydrolysis trials generally involved 1800 mg FW (i.e. 385 mg VS) of fresh substrate (maize straw) added in 10 mL of buffer solution with an enzyme dosage as low as ~3 % of substrate VS weight.

Effect of the water balance on the measurements

In enzymatic hydrolysis trials, the water balance was neglected while measuring sugar release. Nevertheless, the water balance of the hydrolysis process may induce the following biases:

Water uptake during hydrolysis: when cellulose is hydrolyzed to glucose, water is absorbed. Assuming a complete degradation of cellulose to glucose, the original cellulose weight would be 0.9-fold the weight of glucose generated (Hari Krishna et al. 2000) i.e. glucose mass would be 1.111-fold higher than the original cellulose weight (Zhang et al. 2007). The same consideration applies to xylan degradation to xylose, although the factor for weight increase would be different.

Water release from the fresh substrate: water contained in maize straw (substrate) could introduce a bias by providing additional water that would dilute the medium. This bias was not taken into account because the extent to which water was not bound to the substrate and could dilute sugars generated during substrate hydrolysis was not known.

Measurement of reducing sugars

Principle of reducing sugars measurement

All sugars containing a free aldehyde or ketone group adjacent to a hydroxyl group have reducing properties and can be oxidized easily, thus they can be designated as reducing sugars. All monosaccharides are reducing sugars. Some polysaccharides are reducing sugars (e.g. maltose), while some other are not (e.g. sucrose) because all free aldehyde or ketone groups were condensed for binding the sugars to form the molecule (Triebold 1946). For linear oligosaccharides, only one chain end may have a free aldehyde or ketone group and thus reducing properties. Measurement methods for reducing sugars are color-forming oxidation reactions that evaluate photometrically the number of reducing chain ends. The nature and degree of polymerization of sugars, as well as the presence of interfering substances, may have an influence on the intensity of the color reaction, creating biases to the quantification. The degree of interference depends on reagents, reaction conditions and degree of purity of the medium. Two methods are most frequently used for the determination of reducing sugars (Marais 2008): the DNS assay (Miller 1959) and the Nelson-Somogyi assay (Nelson 1944; Somogyi 1952). The DNS method is often preferred because of its simplicity (Breuil and Saddler 1985). Nevertheless, it is more sensitive to interferences from other compounds, such as phosphates (Dighe et al. 1985) and metal ions (Forouchi and Gunn 1983; Sinegani and Emtiazi 2006). Moreover, the response factor of the DNS reaction increases with increasing degree of polymerization of the oligosaccharides (Breuil and Saddler 1985; Jeffries et al. 1998; Robyt and Whelan 1972; Schwald et al. 1988). Robyt and Whelan (1972) suggested that this effect would be due to alkaline erosion of oligosaccharide which would compete with DNS oxidation. Alkaline erosion would split oligosaccharide into shorter fragments, generating more reducing ends before DNS oxidation could take place. This issue could be prevented by lowering the heating temperature to 50°C and increasing the cooking time up to 55 min. In the Nelson-Somogyi assay, alkaline erosion would not take place because the oxidation reaction occur much faster than alkaline erosion.

Principle of the DNS reaction

Sugar chains having reducing ends (reducing sugars) react with 3,5-dinitrosalicylic acid (DNS) to form brown-colored 3-amino-5-nitrosalicylic acid. The intensity of the color is proportional to the sugars concentration (Miller 1959). Taking glucose as an example, the reaction runs as presented in **Figure 2**.

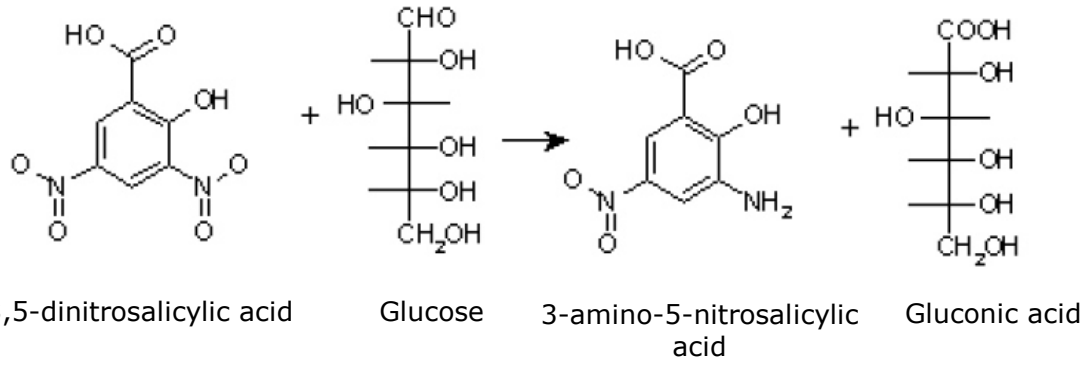


Figure 2. Reaction of glucose with dinitrosalicylic acid.

Preparation of the DNS reagent

DNS reagent was prepared according to the formulation of Wood and Bhat (1988). 10 g dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulfite and 200 g potassium sodium tartrate tetrahydrate were diluted successively in a beaker containing 500 mL of 2% (w/v) NaOH solution. The mixture was heated to ~50°C in a water bath to favor dissolution of the reagents. The DNS solution was brought in a brown flask and stored in a dark place for approximately 2 weeks before use. This delay was found to be required for stabilizing the color-yielding potential of the mixture (König et al. 2002). The amount of DNS solution prepared per batch was limited to 1 L in order to restrict the storage time because the atmospheric oxidation of bisulfite could damage the reagent. To solve this problem the original method advised adding bisulfite shortly before using the reagent (Miller 1959), but most methodical standards established afterwards did not follow this recommendation (Ghose 1987; Ghose and Bisaria 1987; König et al. 2002; Wood and Bhat 1988).

Absorbance measurements

After completion of enzymatic hydrolysis, samples were passed through filter paper (Whatman 602 H 1/2) and 0.4 mL of each sample were diluted to 20 mL with redistilled water in volumetric flasks (dilution 1:50). 2 mL of each filtrated and diluted aliquots were added into 3 mL DNSA solution in a test tube. A blank was generated by replacing aliquots with redistilled water. The tubes were heated in a boiling water can on a heating plate at ~95°C for exactly 15 min and brought in cool water immediately afterwards. Although shorter cooking periods have been reported in the literature (Ghose 1987; König et al. 2002; Wood and Bhat 1988), the original publication of the method specifies that a longer cooking time of 15 min should be preferred (Miller 1959). Following a 30 min waiting time for color stabilization, absorbance of the samples was measured with a photometer (UV Mini 1240 UV-VIS-Spectrophotometer, Shimadzu, Kyoto, Japan) at 640 nm (Ghose and Bisaria 1987; Tjerneld et al. 1991).

Glucose calibration curve

For each new DNS reagent the corresponding glucose calibration curve was generated with increasing glucose concentrations between 0 and 1 g/L at 0.1 g/L intervals by diluting glucose in citrate buffer 0.1 M (pH 5). For experiments at neutral pH, glucose was diluted in phosphate buffer 0.1 M (pH 7). The glucose dilution series was carried out in 1 mL aliquots. 1 mL redistilled water was eventually added to the aliquots to give a total volume of 2 mL before DNS addition. The glucose concentration in g/L (x) was plotted against the absorbance (y). The extinction of glucose standards ranged between 0.0 and 0.5.

Glucose calibration curves for experiments run at neutral pH under the application of sodium azide were performed in the range 0.1-1 g/L at increments of 0.1 g/L with 0.1 M citrate (pH 6) or phosphate (pH 7) buffers, as well as sodium azide. No deviation from linearity was noticed. Linearity coefficients were r^2=0.9998 and r^2=0.9995 for the citrate-buffered and phosphate-buffered calibration curve, respectively. The linear coefficients of the citrate-buffered calibration curve was applied to the samples tested at pH 6-6.5. The linear coefficients of the phosphate-buffered calibration curve was applied to the samples tested at pH 7-8.

Verifying the validity of the measurement wavelength

In most publications absorbance measurement with the DNS assay took place at 530 (Colombatto et al. 2003), 540 (Ghose 1987; Wallace et al. 2001), or 575 nm (Breuil and Saddler 1985; Dighe et al. 1985; Forouchi and Gunn 1983). The absorbance of a glucose standard series was tested at different wavelengths (**Figure 3**). Meur et al. (1977) stated that yellow unreacted DNS reagent would cause an interference with the red-brown reaction product 3-amino-5-nitrosalicylic acid. According to spectral analysis, this interference would be minimal at a wavelength of 530 nm. However, measurements of the glucose standard series did not reveal any loss of linearity at higher wavelengths compared with lower wavelengths. Hence, the linearity of the method was not affected by wavelength. However, the sensitivity increased at shorter wavelength. In practice, the selected wavelength of 640 nm seemed to provide the highest reliability of reducing sugar measurements (data not shown), presumably because of lower interference from compounds contained in either the substrate or the medium.

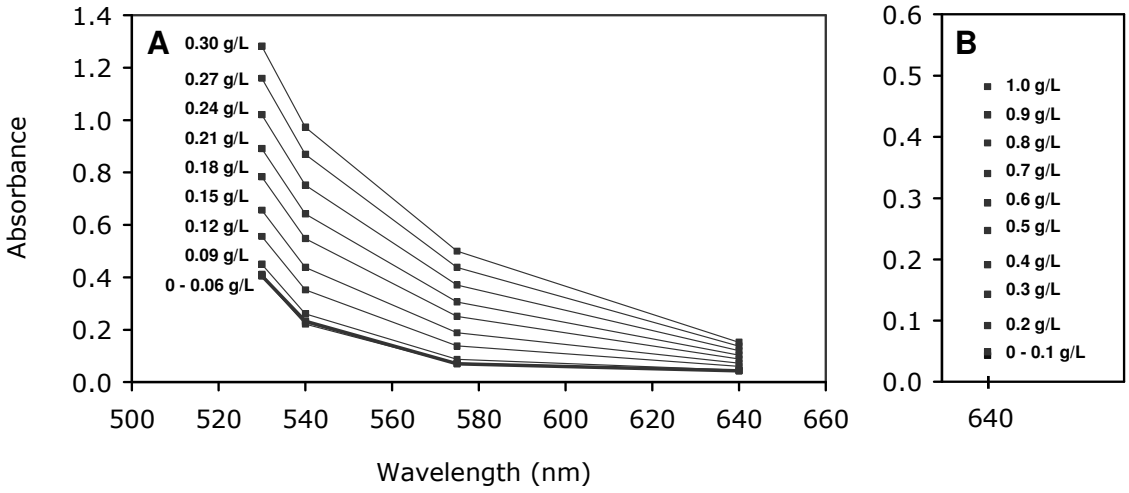


Figure 3. Absorbance of glucose standard series at different wavelengths [Absorbance of glucose standard series in the range 0-0.3 g/L in 0.03 g/L increment measured at 530, 540, 575 and 640 nm (A) and in the range 0-0.1 g/L in 0.01 g/L increment measured at 640 nm (B)].

Sensitivity of the DNS method

Regardless of wavelength, absorbance values for glucose concentration of 0.09-0.1 g/L were almost similar to lower concentrations and to the zero value. Hence the DNS-method was not sensitive at glucose concentrations below 0.1 g. Resolving this issue by adding of 0.1 g glucose to each sample before determination would be practicable (König et al. 2002; Wood and Bhat 1988).

Calculation of reducing sugars concentration

Since enzyme concentrations applied in the assay were low, sugars contained in added enzyme products could be neglected, and in this case the systematic generation of a blank containing only enzyme product and no substrate could be spared (Ghose 1987). Measured extinctions of diluted hydrolysis samples ranged between 0.0 and 0.3, and thus were probably in the linearity range. Sugar concentrations of hydrolyzed maize straw as percent of substrate VS were calculated according to formula (1).

$$T = \left(\frac{E - B}{A} \right) \times D \times \frac{V}{m_S} \times \frac{100}{K_{TS}} \times \frac{100}{K_{VS}} \times \frac{10}{2} \qquad \textbf{(1)}$$

E Measured extinction

A Constant of the glucose standard curve (y = Ax + B)

B Constant of the glucose standard curve (y = Ax + B)

D Dilution factor (D = 50)

V Sample volume (V = 0.01 L)

m_S Substrate weight (g)

K_{TS} Total solids content of the substrate in percentage (K_{TS} = 22.79% FW)

K_{VS} Volatile solids content of the substrate in percentage (K_{VS} = 93.96% TS)

The factor 10 stood for the conversion into percentage. The factor 2 accounted for the fact that glucose standard curve had been generated on 1 mL aliquots, which were further diluted by addition of 1 mL distilled water prior to analysis, while diluted samples of hydrolyzed substrate were directly measured on 2 mL aliquots.

Control: Comparison of reducing sugars with glucose measurement

In order to confirm the validity of reducing sugars measurement to quantify the release of sugars in the medium after enzymatic hydrolysis of maize straw, an enzymatic measurement method based on the enzyme glucose oxidase was applied as a tool for comparison on a set of samples. The results are shown in the Appendix ***A 2 Reducing sugars against glucose determination***.

Control: Glucose standard solution

In order to check the consistency of the reducing sugar measurement method, a glucose solution of defined concentration (1 g/L) was prepared and 2 mL-aliquots were put into a freezer. One aliquot was unfrozen shortly before each trial. Aliquots were diluted 6-fold and analyzed together with the samples investigated in the respective measurement batches. The measured concentrations (g/L) of the glucose standard solution were calculated with formula (2).

$$C = \left(\frac{E - B}{A} \right) \times \frac{D}{2} \qquad \textbf{(2)}$$

E Measured extinction

A Constant of the glucose standard curve (y = Ax + B)

B Constant of the glucose standard curve (y = Ax + B)

D Dilution factor (D = 6)

The factor 2 accounted for the fact that glucose standard curve had been generated on 1 mL aliquots that were further diluted by addition of 1 mL distilled water, whereas diluted samples of glucose standard solution were directly measured on 2 mL aliquots.

The target value was 1.000 g/L (**Figure 4**). The maximal value was 1.087 g/L (**trial 12**) and the minimal value was 0.961 g/L (batch number 6). Thus, the maximal deviation to the average was 0.087 g/L, i.e. 8.7% of the target value. The standard deviation of the concentrations of glucose aliquots over the whole trial period was 0.024 g/L, i.e. 2.4% of the target value. In order to counteract the high uncertainty of the method, enzyme effects were usually tested thrice in three successive batches. For hydrolysis trials assessing temperature effects, this approach was not feasible since in this case incubation temperature was specific to each batch, and the triplicates had then to be grouped into the same batch.

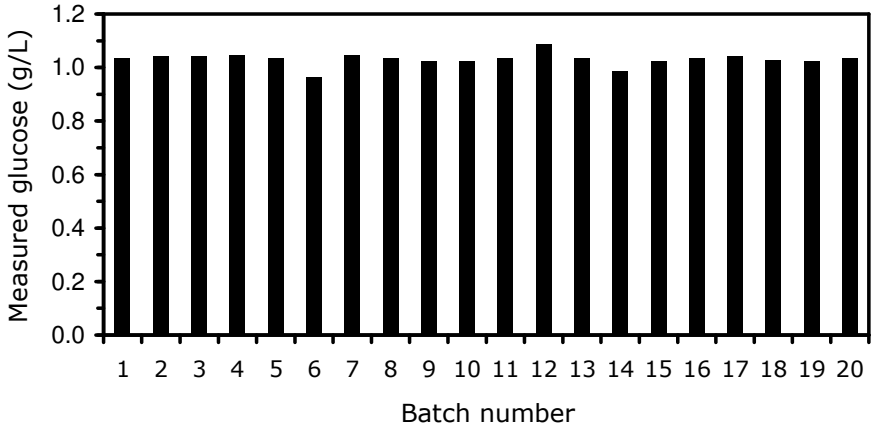


Figure 4. Measured concentrations of glucose standards.

Control: Internal standards

The use of internal standards is recommended to quantify the effects of interfering substances in the substrate (Miller 1959). The internal standard is a pure product of defined composition (here: glucose) which is added to the substrate investigated (here: maize straw) in order to check for positive or negative interferences. 1800 mg of finely chopped maize straw was brought together with 10 mL of 0.1 M citrate buffer (pH 5) in glass flasks, and glucose was added at concentrations of 5, 7 and 10 g/L. For each glucose concentration level, one blank was run without substrate addition. After an incubation time of 24 hours at 50°C, samples were measured on the photometer and glucose concentrations with and without substrate addition were compared (**Table 10**). Measured values for glucose samples without substrate addition accounted for 95.9 to 98.5% of the added glucose. The underestimation of glucose concentration might be due to a delay between generating glucose calibration curve for the DNS solution and performing the assay. The recovery rate was defined as the ratio of measured glucose concentration without substrate addition to measured glucose concentration with substrate addition for the same amount of added glucose. Recovery rates ranged between 96.8 and 97.0, showing that ~3% of the glucose may have been lost because of a negative interference with the substrate.

Table 10. Determination of the recovery rate of glucose internal standard [Incubation of maize straw with glucose solution in 10 mL of 0.1 M citrate buffer solution (pH 5) at 50°C for 24 hours. Average values ± SD. n=2].

Concentration of glucose added (g/L)	Concentration of maize straw added (g/10 mL)	Total glucose measured (g/L)	Measured concentration of glucose added (g/L)	Ratio of measured glucose to added glucose (%)	Glucose recovery rate after substrate addition (%)
5	-	4.9 ± 0.0	4.9 ± 0.0	98.5	-
7	-	6.7 ± 0.0	6.7 ± 0.0	95.9	-
10	-	9.8 ± 0.3	9.8 ± 0.3	97.7	-
-	1.8	9.6 ± 0.1	-	-	-
5	1.8	14.3 ± 0.4	4.8 ± 0.4	95.3	96.8
7	1.8	16.1 ± 0.3	6.5 ± 0.3	92.9	96.9
10	1.8	19.0 ± 0.1	9.5 ± 0.1	94.8	97.0

HPLC analysis of monosaccharides

HPLC analysis of monosaccharides released by enzymatic hydrolysis of maize straw was carried out by the Department of Biotechnology and Enzyme Science of the Institute of Food Science and Biotechnology at the University of Hohenheim (Director: Prof. Dr. Lutz Fischer).

Samples for HPLC determination were taken in 5 mL plastic syringes, and filtered by plugging 150 µm filtering units onto the syringes. In order to protect the HPLC-device (Urginovits 1980), protein removal with perchloric acid was carried out: For each sample 0.5 mL aliquots were brought together with 1 mL of 1M potassium perchloric acid ($KClO_4$) in centrifuge tubes, which were homogenized in a vortex, and centrifuged at 14,000 rpm for 2 min at 4°C (centrifuge type 5417R, Eppendorf, Hamburg, Germany). Subsequently, 1 mL aliquots of the supernatant were transferred into 5 mL glass flasks and 0.217 mL of 2 M potassium hydroxide (KOH) were added for neutralization, pH was checked with paper strips (Alkalit, Merck, Darmstadt, Germany) and additional 0.1 M KOH was dosed if required to reach pH-neutrality. Glass flasks were briefly agitated and put into an ice bath for 20 min. Finally, 1 mL aliquots were taken from the flasks with plastic syringes and filtered through 0.45 µm filtering units (Multoclear-25, Chromatographie Service, Langerwehe, Germany).

Purified samples were analyzed in duplicates in a Rezex RCM-Ca^{2+} column of 300 mm length, 8 mm internal diameter with 8 µm filling material as described by Schober (2008). The mobile phase was water, temperature was set to 85°C and pressure to 55 bar. The retention times were 8.5 min for glucose, 12.5 min for xylose, galactose, mannose, and 13.5 min for arabinose. This method had two main limitations:

1. Xylose, galactose and mannose, as well as fructose and arabinose, came in the same peaks and could not be quantified separately. This issue is related to the type of column used for HPLC determination. Using, for example, a Pb-form column instead of a Ca^{2+} column, would have resulted in a better separation of the different monosaccharides (Buchert et al. 1993).
2. Oligosaccharides composed of 2 to 5 monosaccharide units, which can also be important hydrolysis products, were not quantified. It would be feasible to assess oligosaccharide release through either performing a secondary enzymatic hydrolysis turning oligosaccharides into glucose (Buchert et al. 1993), or adapting HPLC device operation as well as purchasing and testing pure oligosaccharide standards for calibration (Jeffries et al. 1998).

Glucose generated a separate peak and did not interfere with other sugars. The other monosaccharides, which had each a slightly different response factor, could not be quantified individually. This created a bias in the assessment of sugar weights through the discrepancy in the response factors. In order to evaluate this bias, monosaccharide standards as well as their mixtures were tested separately in the HPLC device (**Table 11**). Dilutions having total sugar concentrations of 1.000 ± 0.002 g/L in redistilled water were prepared in 10 mL glass flasks. For each sugar sample, two injections were performed to account for the HPLC device's internal deviation. The maximal deviations between monosaccharide response factors were of 4.5% between xylose, galactose mannose and of 3.8% between fructose and arabinose, respectively. This bias was judged acceptable and the response factors of galactose and fructose were used arbitrarily for the peaks of xylose / galactose / mannose and of fructose / arabinose mixtures, respectively.

Table 11. Response factors of monosaccharide and their mixtures in HPLC [Average values ± SD. n=2].

Sugar type	Retention time (min)	Response factor (Peak area/g)	Maximal deviation of the response factors (%)
Xylose	12.3 ± 0.052	1 330 619 ± 21 498	4.5
Galactose	12.4 ± 0.033	1 384 203 ± 20 350	
Mannose	12.8 ± 0.001	1 340 066 ± 5 879	
Xylose + Galactose + Mannose (33% w/w each)	12.5 ± 0.002	1 324 360 ± 17 082	
Fructose	13.4 ± 0.026	1 343 101 ± 38 438	3.8
Arabinose	13.8 ± 0.001	1 293 773 ± 34 432	
Fructose + Arabinose (50% w/w each)	13.7 ± 0.016	1 299 580 ± 8 979	

Commercial liquid enzyme preparations usually contain high amounts of soluble sugars, which are aimed at stabilizing enzyme activity, providing storage stability to the product (Nieves et al. 1998). Although enzyme dosages used were relatively high, experimental results show that the sugar content of enzyme products did not seem to interfere with the monosaccharide concentration of samples.

4.2.2 Description of the experiments

Goal of the experiments

The goal of enzymatic hydrolysis trials was to better understand the mechanisms of action of enzyme additives on fresh maize straw. The trials were performed in a watery buffer medium to provide a microbe-free environment for enzyme action.

Enzyme products

Enzyme products used in hydrolysis experiments and are described in **Table 12**. These products were a gift of the supplier Novozymes, Bagsvaerd, Denmark, apart from MethaPlus L100 that was a gift of the supplier DSM Biorpract, Berlin, Germany.

Table 12. Enzyme products used for hydrolysis trials on maize straw [Enzymes characteristics according to manufacturer's information. n.s. = not specified].

Enzyme name	Main activity	Micro-organism	Application	Temperature optimum (°C)	pH optimum
Celluclast 1.5L	Cellulase	*Trichoderma reesei*	Food industry	65	5
MethaPlus L100	Cellulase, xylanase	*Trichoderma reesei*	Biogas	55	4.5 – 5.5
Novozym 188	Cellobiase	*Aspergillus niger*	Food industry	55	5.5
Novozym 342	Cellulase	*Humicola sp.*	Textile	40 - 65	7.5
Ultraflo Max	β-glucanase, xylanase	*n.s.*	Beer filtration	*n.s.*	*n.s.*
Viscozyme L	Arabinase, cellulase, xylanase	*Aspergillus aculeatus*	Food industry	25 - 55	3.3 – 5.5

Enzymatic hydrolysis of maize straw

Enzymatic hydrolysis for evaluation of enzyme efficiency

Fresh finely chopped maize straw for enzymatic hydrolysis trials (particle size <3 mm) was weighed into 30 mL glass vials together with buffer solution and enzyme. Most experiments were carried out with 1800 ± 3 mg of substrate (maize straw) diluted into 10 mL buffer solution, before addition of 10 µL of commercial enzyme preparation. The buffer solution was either citrate buffer 0.1 M (for pH range 3.5 to 6) or phosphate buffer 0.1 M (for pH 7) prepared in redistilled water. Citrate buffer was prepared by mixing 0.1 M citric acid ($C_6H_8O_7 + H_2O$) with 0.1 M trisodium citrate ($Na_3C_6H_5O_7 + H_2O$) at desired proportions to reach target pH value.

Phosphate buffer was prepared by mixing 0.1 M sodium phosphate monobasic ($NaH_2PO_4 + H_2O$) with 0.1 M sodium phosphate dibasic ($Na_2HPO_4 + 2H_2O$) at desired proportions to reach target pH value. Glass vials were then placed in a shacked water bath (Type 1083, GFL GmbH, Hannover, Germany) operated at a shacking velocity of 60 rpm for the duration of hydrolysis, which was carried out at 50°C for 24 hours unless stated otherwise. pH was systematically measured before and after hydrolysis (data not shown). After hydrolysis, the final pH differed from the initial values in less than 0.2 pH units, proving the stability of the buffer.

At pH higher than 5.5, contaminating bacteria developed at increased rates during the hydrolysis assay, consuming sugars and creating a significant interference. In the latter case sodium azide was added as a microbial inhibitor to hamper bacterial development and applied at a dosage of 1 g/L. For this purpose, 1 mL of a 10 g/L sodium azide solution was added into each sample together with 9 mL buffer solution.

Enzymatic hydrolysis as pretreatment step before anaerobic digestion

Enzymatic hydrolysis of ensiled maize straw as a pretreatment step before anaerobic digestion in the HBT process was carried out in 100 mL graduated glass syringes (model Fortuna, Häberle Labortechnik, Lonsee-Ettlenschieß, Germany) brought on a rotating support in an incubator (model INE 700, Memmert, Schwabach, Germany). The buffer solution usually required for sample dilution could be replaced with distilled water since organic acids from the ensiled substrate served as a buffer.

Preparation of enzyme mixtures

In several enzymatic hydrolysis trials, mixtures of enzyme products were tested. For this purpose, enzyme products expected to have high cellulase and xylanase activities (Ultraflo Max, Celluclast 1.5 L and MethaPlus L100) were coupled together with enzyme products having high β-glucosidase activity (Viscozyme L and Novozym 188). Enzyme mixtures ratios were related to the fresh weight of enzyme products. The total fresh weight of enzyme products added was kept constant, at 10 µL (i.e. ~3% of substrate VS).

4.3 Batch digestion

4.3.1 Assay protocol

Routine analysis of total solids (TS) and volatile solids (VS) of the substrate

The routine analysis of the substrate which was performed at the start of each digestion assay was the determination of their total solids (TS) and volatile solids (VS) contents. The total solids (or dry matter) content was evaluated by leaving substrate samples in a dryer at 105°C for more than 24 h and subsequently weighing the dried material. The ratio of dried material to fresh material is the TS content. The organic matter content (VS) was assessed by burning the samples in an oven at 550°C for more than 4 h, and weighing the remaining ash fraction. The organic matter fraction lost upon burning (or volatile solids, VS) was calculated by substracting the ash weight from the dry material weight. The ratio of volatile solids to total solids is the volatile solids content, expressed as % TS. These parameters are used for the calculation of substrate amounts to be fed into the digesters to maintain the desired loading, and of the specific methane yields (m^3/kg VS), which is a convenient measure of the energy density of the substrate, or of its degradability.

Inoculum to substrate ratio

In standard batch biochemical methane potential (BMP) assays designed to measure methane production of substrates, the volatile solids (VS) weight of the substrate should not exceed 50% of the VS weight of the inoculum to prevent overloading of the biological process. This corresponds to an inoculum:substrate ratio of 2:1 (Angelidaki and Sanders 2004; Shelton and Tiedje 1984; VDI 4630 2006). This recommendation was applied to perform standard batch digestion trials. In modified batch digestion trials, this rule was violated on purpose via inoculum dilution or the application of a weakend inoculum, in an attempt to magnify the effects of enzyme additives.

Standard inoculum

Most batch digestion assays were performed using a standard inoculum ("manure inoculum"). This standard inoculum was produced in a laboratory reactor at the State Institute of Agricultural Engineering and Bioenergy of the University of Hohenheim. The reactor was fed daily at an organic loading rate of 0.5 kg VS/($m^3 \times d$) with a mixture comprising predigested dairy manure, i.e. effluent from a manure-fed full-scale biogas plant, as well as maize silage, cereals, rapeseed oil and soybean extract.

This particular procedure was aimed at developing an adapted bacterial population while ensuring a sufficiently low biogas production from the inoculum. Prior to use, the inoculum was passed through a 1 mm sieve to remove the particulate fraction.

Correction of total solids (TS) and volatile solids (VS) contents

Background for the correction of total solids (TS) and volatile solids (VS) contents

Volatile compounds, such as VFA and alcohols, are partly lost upon drying, while contributing the organic matter fraction. An underestimation of the total solids (TS) fraction of the substrate leads to an underestimation of the volatile solids (VS) fraction. On plant silages, this bias can lead to an overestimation of specific methane yields (expressed in m^3/kg VS) as high as 15% (Mukengele and Oechsner 2007). In the field of animal nutrition, methods have been developed to overcome this bias. In this thesis the method of Weißbach and Kuhla (1995) was applied for TS correction of some ensiled substrates.

Formula for correction of the total solids (TS) content

A modified version of the formula of Weißbach and Kuhla (1995) was applied for the correction of the dry matter content of grass silage. The formula was extended to include losses of alcohols during drying. The corrected Total Solids (TS) content was calculated as follows:

$$TS_C = TS_O + k_{VFA} \times C_{VFA} + k_{Lac} \times C_{Lac} + k_{Alc} \times C_{Alc} + k_{NH3} \times C_{NH3} \quad \textbf{(9)}$$

TS_O Original total solids content before correction (% FW, Fresh Weight)

k_{VFA} Correction factor of the VFA content

C_{VFA} VFA content of the substrate (% FW)

k_{Lac} Correction factor of the lactic acid content

C_{Lac} Lactic acid content of the substrate (% FW)

k_{Alc} Correction factor of the total alcohol content

C_{Alc} Alcohol content of the substrate (% FW)

k_{NH3} Correction factor of the NH_3 content

C_{NH3} Ammonia content of the substrate (% FW)

Correction factors applied to rye silage

Volatilization rates (in g/kg) measuring the share of each volatile fraction that is lost upon drying of grass silage at 100°C are reported by Porter and Murray (2001). These values were divided by 1000 to be converted into correction factors that were applied to rye silage:

$k_{VFA} = 0.892$; $k_{Lac} = 0.375$; $k_{Alc} = 0.975$; $k_{NH3} = 0.987$

Correction factors applied to maize silage

The original correction factor of the publication of Weißbach and Kuhla (1995) was applied to k_{VFA}, and the remaining factors were taken from the volatility values of Porter and Murray (2001):

$k_{VFA} = 0.94$; $k_{Lac} = 0.375$, $k_{Alc} = 0{,}975$, $k_{NH3} = 0.987$

Formula for correction of the volatile solids (VS) content

After correcting the TS content of a substrate, a proportion rule was applied to adjust the corresponding VS content. The corrected Volatile Solids (VS) content was calculated as follows:

$$VS_C = 100 - \left((100 - VS_O) \times \frac{TS_O}{TS_C} \right) \quad \textbf{(10)}$$

VS_O Original volatile solids content before correction (% TS)

TS_O Original total solids content before correction (% FW)

TS_C Corrected total solids content (% FW)

Equipment used

Hohenheim Biogas Test (HBT)

The Hohenheim Biogas Test (HBT) is derived from the Hohenheim Feed value Test (HFT) developed by Steingass and Menke (1986) for evaluating the nutritive value of cattle feed with rumen fluid as an inoculum. The adaptation of the former assay for biogas applications has been described by Helffrich and Oechsner (2003) as well as Mittweg et al. (2012) and patented (Helffrich et al. 2005). The assay is one of six different processes described in the German directive VDI 4630 (2006).

In the HBT apparatus, a calibrated glass syringe of 100 mL with a gas outlet served as a reactor (**Figure 5**). The syringe's plug was sealed against the glass syringe by means of a non-biodegradable lubricant (Baysilone, Bayer, Leverkusen, Germany). An outlet pipe closed by a fastening clip was connected to the bored side of the syringe. Through this pipe, gas could be let out of the syringe for measuring the methane content. 129 syringes were fitted inside a motorized rotating support. The continuous rotation of the support ensured the thorough mixing of the substrate. The whole rotating unit was built inside a thermostat-controlled incubator (model INE 700, Memmert, Schwabach, Germany), in which the digesters filled with substrates as well as the generated gas were heated to the desired temperature.

Usually three reactors were fed 50 g of inoculum (inoculum control variant). Three other reactors were fed 30 g inoculum together with 400 mg concentrate feed (standard control variant). The remaining reactors were fed 30 g of inoculum and the desired amounts of substrate and enzyme additive. Substrates and inoculum in the HBT process were weighted at a precision of ± 3 mg and ± 0.3 g, respectively. Recorded weights values were applied for the calculation of the methane yields. After being filled with inoculum and test substrates, syringes were closed with the plugs and put inside the rotating support.

Gas volumes were measured on the graduated scales of the syringes at short time intervals in the beginning of the trial. Measurement intervals became gradually longer throughout the experiment. Gas measurement of a syringe could be performed when the volume of biogas generated went over 20 mL. For this purpose, the volume of each syringe was first read on the calibrated scale. The syringe was then placed vertically, gas being on the top, next to the exhaust pipe and substrate remaining on the bottom. After opening the fastening clip of the exhaust pipe, the piston was pushed upwards and biogas was let out of the syringe, passed through a filter filled with dessicant to remove water and obtain dry gas, and analyzed for its methane content. The remaining volume after emptying the gas from the syringe was also measured.

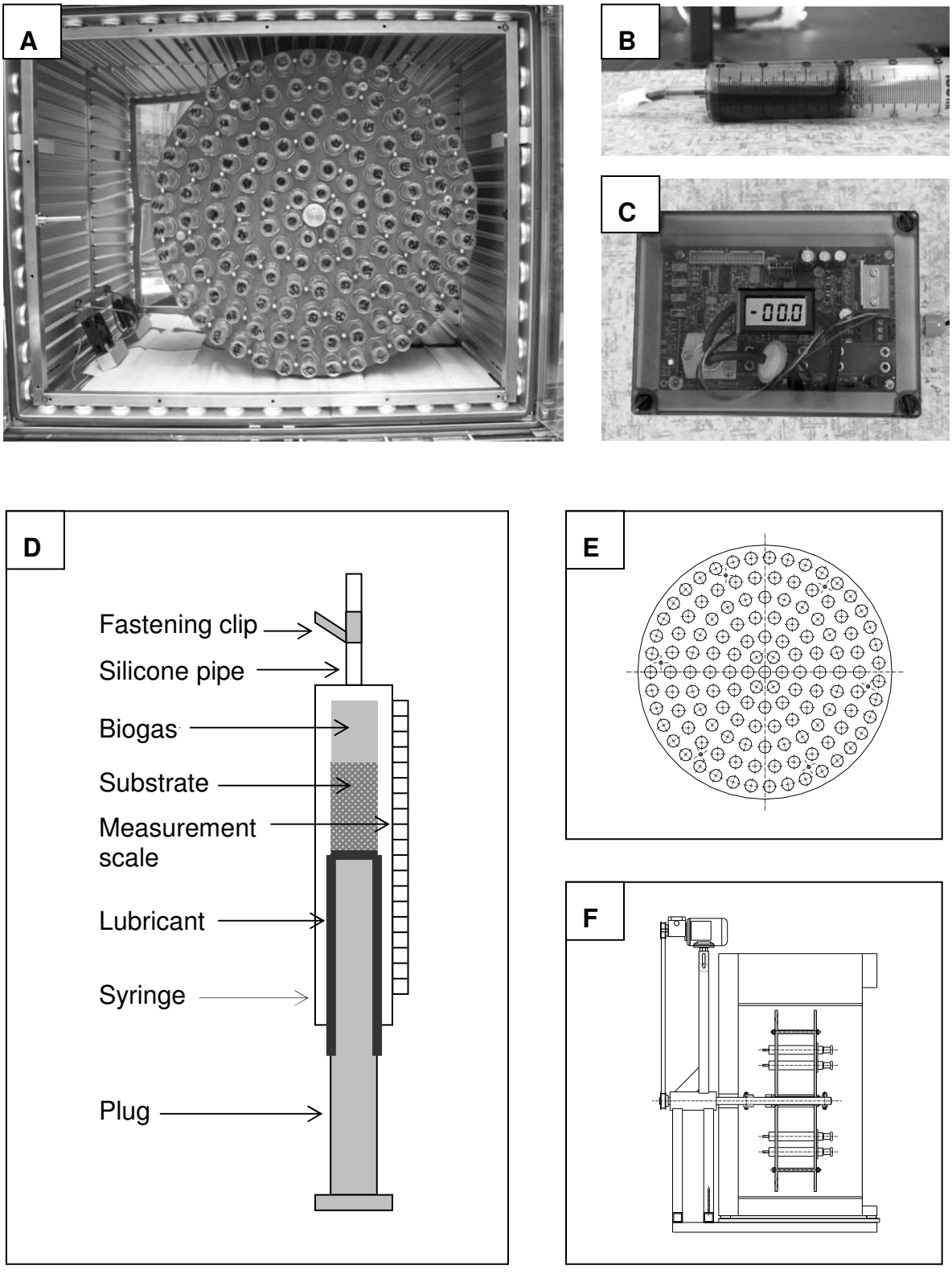


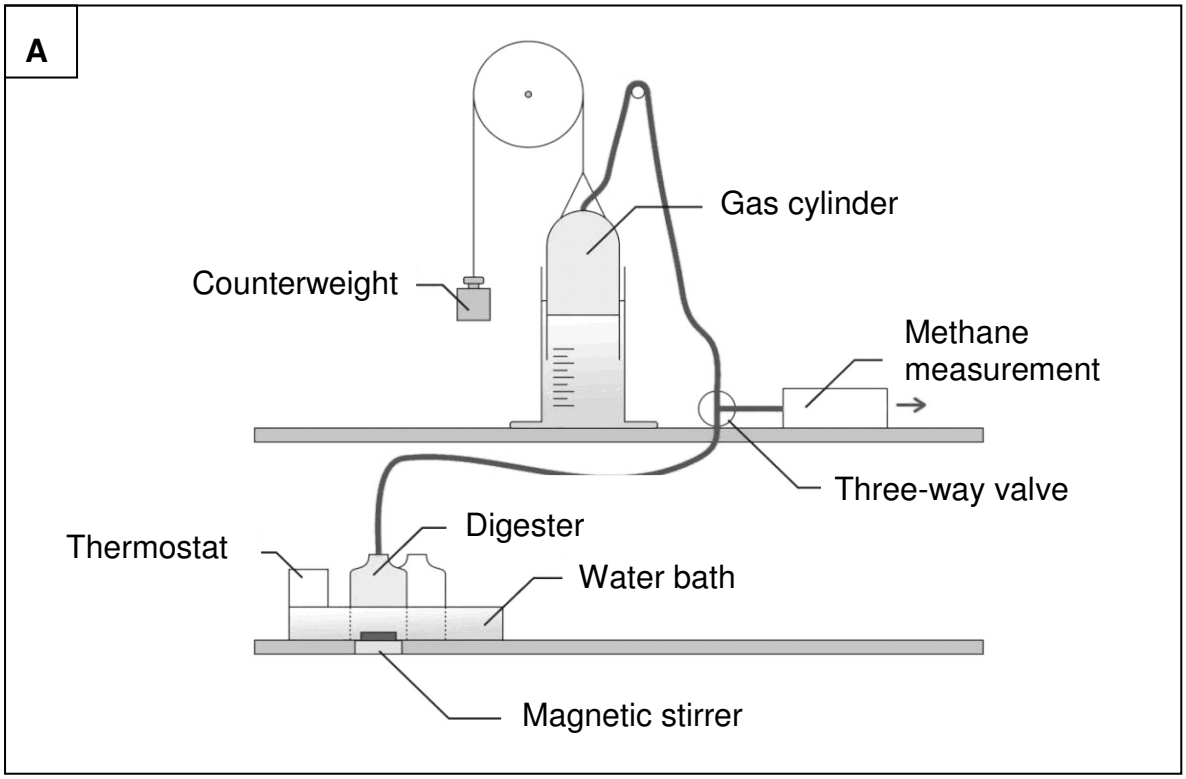
Figure 5. HBT process for batch experiments [A. Incubator with rotating unit bearing syringes. B. Syringe containing biogas and substrate. C. Methane analyzer. D. Syringe. E. Rotating support. F. Incubator with rotating unit bearing syringes].

Stirred glass digesters

The stirred glass digesters apparatus was made of 24 Erlenmeyer flasks, each of 2 L capacity. Each Erlenmeyer flask was covered by a rubber stopper with a gas outlet. Each gas outlet was connected to a 3.2 L transparent cylinder (gasometer) diving into a broader cylinder filled with a barrier solution for gas collection (**Figure 6**). The composition of the barrier solution was taken from ISO 14853 (1997). The barrier solution was prepared by adding 600 g sodium chloride, 15 g citric acid and 4 mg bromophenol blue (colour indicator) successively into 1.5 L distilled water. Erlenmeyer flasks were kept in a thermostat-controlled water bath filled with distilled water at 37°C. A cylindrical magnetic stirrer was placed in each flask. Electric motors fitted with circular plates bearing strong magnets were placed under the water bath, below each flask. The motors rotated the magnetic stirrers for 5 min after a 15 min delay and ensured the mixing of the feedstock. Three-way valves allowed biogas to be directed either from the digesters into the gasometers for storage or from the gasometers into the methane analyzer for gas measurement.

At the start of the experiment glass digesters were fed 1800 g of inoculum as well as the desired amounts of substrate and enzyme additive. Substrates and inoculum were both weighted at a precision of $\pm$ 0.5 g. Recorded weights values were applied for the calculation of the methane yields.

Gas measurements could be performed when the amount of biogas stored in the gasometers exceeded 800 mL. In order to equilibrate the pressure of biogas contained in each gasometer with room pressure, the string of the counterweight was pulled until the levels of the barrier solution in the inner and outer cylinders were equal. The volume of biogas was noted from a measuring scale on the outer cylinder. Following that operation, the three-way valve of each digester was operated in order to connect the outlet of the gasometer with the methane analyzer while closing the outlet of the reactor. The counterweight was hung to bring the gasometer content under pressure and let biogas pass through the analyzer. About 300 mL of biogas were circulated through a filter filled with dessicant to remove water and obtain dry gas, and the analyzer. The remaining gas was disposed. After measurement, the gasometers being emptied, the three-way valves were turned back to restore the digester's connection to the gasometer, while disabling the outlet to the gas measurement.



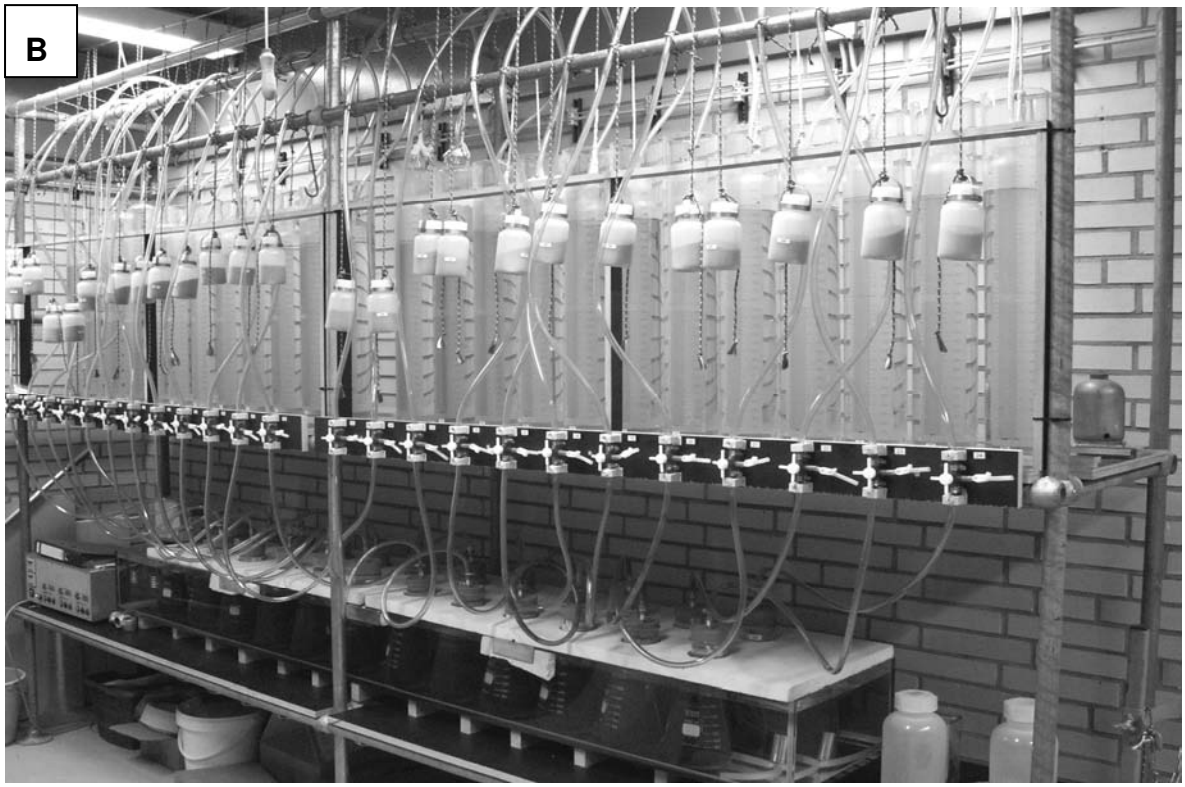


Figure 6. Stirred glass digesters for batch experiments [A. Schematic design of a digester (Barthelmeß 2008). B. Picture of the apparatus].

Conversion of gas production into normal conditions

In order to calculate gas production under normal conditions (i.e. at 0°C and 1013.25 hPa), room air pressure was measured with a manometer (GPB 2300, Greisinger, Regenstauf, Germany) and gas temperature was read on the incubator's display (HBT process) or room temperature was read on a thermometer (stirred gas digesters).

Methane measurement

Biogas was passed through an electronic analyzer with a double-bean non-diffractive infrared sensor (Advanced Gasmitter, Pronova, Berlin, Germany) to determine the percentage of methane in the biogas. The inlet pipe of the device was filled with phosphorus pentoxide as a desiccant (Sicapent, Merck, Whitehouse Station, NJ, USA). The device was calibrated before the experiment using standard gas having a methane content of ~60%. At each measurement, air (0% CH_4) and standard gas (60% CH_4) were injected successively into the measuring device to verify the stability of methane measurements.

Calculation of the methane yield

Normalized gas and normalized methane volumes

For each gas measurement, biogas volume, biogas temperature, room air pressure and CH_4 content were measured. The gas contained water vapor. The water vapor fraction in biogas was supposed to be equal to saturation water vapor pressure in air brought at the same temperature. The estimated water vapor pressures in biogas were 58 hPa at 35°C and 34 hPa at 26°C for the HBT process and for the stirred glass digesters, respectively. For the HBT process, biogas in the syringes was expected to cool of ~2°C (i.e. from 37°C to 35°C) when syringes were taken out of the incubator. The gasometers containing biogas from the stirred glass digesters were expected to be at room temperature.

Volume of dry biogas (corrected from the water vapor) to normal conditions (temperature of 0°C, pressure of 1013.25 hPa):

$$V_N = V_R \times \frac{(p - p_w) \times T_0}{T_R \times p_0} \quad \textbf{(3)}$$

V_N Volume of the dry gas to normalized conditions (L)

V_R Read-off value of the biogas volume (L)

p Pressure of the gas at the time of the reading (hPa)

p_w Water vapor pressure in biogas (hPa)

T_0 Normal temperature; $T_0 = 273$ K

p_0 Normal pressure; $p_0 = 1013.25$ hPa

T_R Temperature of biogas (K)

Headspace correction

Each stirred glass digester had a headspace volume of ~500 mL. The former volume was contained on the top of the Erlenmeyer flasks and within the pipes, and could not be emptied during gas measurement. Therefore, a headspace correction of CH_4 content according to VDI 4630 (2006) was performed. In the HBT process, no headspace correction was carried out because the headspace volume was very low (<0.5 mL) compared to the gas production (>20 mL per measurement) and therefore could be neglected.

Headspace correction of CH_4 content in dry biogas for stirred glass digesters:

$$C_{Kn} = C_n + (C_n - C_{n-1}) \times \frac{V_H}{V_{Bn}} \quad \textbf{(4)}$$

C_{Kn} Corrected CH_4 content of biogas at measurement n (% v/v)

C_n Original CH_4 content of biogas at measurement n (% v/v)

C_{n-1} Original CH_4 content of biogas at previous measurement (n-1) (% v/v)

V_H Headspace volume (L)

V_{Bn} Biogas volume at measurement n (L)

Normalized methane volumes

Biogas was passed through a drying filter before flushing into the methane measuring device, and methane concentrations were measured on a dry gas basis. Methane volumes under normal conditions were obtained by multiplying the normalized dry biogas volumes with the methane contents after headspace correction:

$$V_M = V_N \times \frac{C_K}{100} \quad \textbf{(5)}$$

V_M Normalized CH_4 volume (L)

V_N Volume of the dry biogas to normalized conditions (L)

C_K CH_4 content after headspace correction (% v/v)

Cumulated normalized methane volumes

Normalized methane volumes were added over the whole experimental period to obtain the cumulated methane production:

$$V = \sum V_M \quad \textbf{(6)}$$

V Cumulated CH_4 volume (L)

ΣV_M Sum of normalized CH_4 volumes (L)

Cumulated methane production of the substrate

The cumulated methane volume measured from each digester (containing both substrate and inoculum) was the sum of the methane volumes produced from the substrate and from the inoculum. The control variant containing inoculum only allowed the estimation of the cumulated methane volume of the inoculum, and thus the determination of the cumulated methane volume of substrate:

$$V_S = V_{SI} - \left(V_I \times \frac{W_{II}}{W_{IS}} \right) \quad \textbf{(7)}$$

V_S Cumulated normalized methane volume of the substrate (L)

V_{SI} Cumulated normalized methane volume of the substrate-inoculum mixture (L)

V_I Average cumulated normalized methane volumes of the inoculum variants (L)

W_{II} Average weight of inoculum in the inoculum variants (g)

W_{IS} Exact weight of inoculum in the digester containing substrate and inoculum (g)

Interpolation of cumulated methane production of the inoculum

The inoculum produced few gas and could not be measured at the same intervals as the substrate. In order to draw the course of cumulated methane production from the substrate along digestion time, a linear interpolation of the average cumulated methane production of the inoculum was performed. Subsequently, the interpolated values of inoculum's cumulated methane production were substracted from the cumulated methane production of each digester.

Specific methane yields

The specific methane yields were calculated by dividing the cumulated methane volumes of the substrates by their respective volatile solids weights in the reactors:

$$Y_S = \frac{V_S}{W_{VS}} \quad \textbf{(8)}$$

Y_S Specific methane yield of the substrate (L/g VS or m^3/kg VS)

V_S Cumulated normalized methane volume of the substrate (L)

W_{VS} Exact weight of volatile solids from the substrate brought into the digester (g VS)

4.3.2 Description of the experiments

Batch digestion of finely ground fresh maize

Goal of the experiment

The goal of the experiment was to compare the effects of different enzyme products added in a standard BMP assay and to observe if enzyme additive would behave differently on different plant fractions, i.e. corncob and straw from maize.

Substrates

At harvest, maize (cultivar: Gavott) was separated into two fractions: corncob and straw. Both fractions were ground with a laboratory mixer-grinder (B-400, Büchi Labortechnik AG, Flawil, Switzerland) until a fiber length of ~3 mm was reached. Further substrate characteristics are reported in the section ***4.6 Characteristics of the substrates***.

The total solids (TS) and volatile solids (VS) contents of finely ground fresh maize straw were 22.79% FW (i.e. related to fresh weight) and 93.90% TS (i.e. related to total solids), respectively. TS and VS contents of finely ground fresh corncob were 44.43% FW and 98.30% TS, respectively.

Enzyme products

Four commercial enzyme preparations were tested (MethaPlus L100, Goldferm Mais, Genencor Laminex BG, Novozym 188). MethaPlus L100 and Goldferm Mais were specifically designed by companies to work as additives in biogas plants. The other products (Genencor Laminex BG and Novozym 188) were designed for bioethanol production or for technical applications in the food industry. MethaPlus L100 and Genencor Laminex BG were enzyme mixtures containing cellulases and xylanases originating from the yeast *Trichoderma reesei*. Novozym 188 was a β-glucosidase produced by the yeast *Aspergillus niger*. Goldferm Mais was a mixture of enzyme and microorganisms, with which manufacturer did not include any enzyme extraction step. Hence, in addition to enzymes, Goldferm Mais also contained microorganisms and residue of growth substrate. Genencor Laminex BG (Genencor, Palo Alto, CA, USA) and Novozym 188 (Novozymes, Bagsværd, Denmark) were provided by the Department of Biotechnology and Enzyme Science of the Institute of Food Science and Biotechnology at the University of Hohenheim (Director: Prof. Dr. Lutz Fischer). MethaPlus L100 was a gift of the supplier DSM Biopract GmbH, Berlin, Germany. Goldferm Mais was a gift of the supplier Bioreact GmbH, Troisdorf, Germany.

Design of the experiment

Enzyme products were added in the start of the batch digestion assay, at two different dosages: 0.13 and 1.3 g/kg VS. For each enzyme, an inactivated variant (heating at 95°C during 15 min) was included at the higher enzyme dosage.

Glass syringes of the HBT process were fed either 1800 mg of finely ground fresh maize straw or 900 mg of finely ground fresh corncob. Subsequently 30 g of standard inoculum (“manure inoculum”) were added. Before closing the syringes 0.5 mL of 1000 or 10000-fold diluted enzyme products were added to obtain 0.13 g/kg VS and 1.3 g/kg VS dosage levels, respectively. In this experiment tap water was used for dilution of enzyme products. Anaerobic digestion was operated at 37°C for a duration of 35 days. Each variant was run within three replicates. Substrates were also tested without enzyme supplementation. Those "zero variants" were used as reference for the calculation of the increments of the methane yields obtained through addition of active or inactivated enzyme.

Batch digestion of coarse rye silage

Goal of the experiment

The trial was developed in partnership with the Institute of Agricultural and Urban Ecological Projects (IASP) of Humboldt University, Berlin. Ulrike Schimpf, a scientific assistant and PhD-student at IASP-Berlin, provided rye silage and enzyme preparations, as well as assistance at the start of the experiment with stirred glass digesters of 2 L capacity. IASP-Berlin wanted to check if the positive results they obtained with enzyme additives could be reproduced at the University of Hohenheim.

Substrate

Coarse rye silage was provided by IASP-Berlin. The substrate had a fiber length of ~8 mm Further substrate characteristics are reported in the section ***4.6 Characteristics of the substrates***.

Total solids (TS) and volatile solids (VS) were corrected for the loss of volatile compounds resulting from the determination method, as described previously (*cf.* ***4.3.1 Assay protocol***). The concentrations of volatile compounds in rye silage (in % FW), determined by chemical analysis, were:

$C_{VFA} = 0.40$; $C_{Lac} = 1.84$; $C_{Alc} = 0$; $C_{NH3} = 0$

The absence of alcohols (C_{Alc}) and ammonia (C_{NH3}) from the sample may be related either to the high maturity of the rye silage sample (450 days of ensiling), or to volatilization of these compounds prior to or during the analysis process. The uncorrected TS and VS contents were 42.72% FW and 95.43% TS, respectively. TS and VS contents after correction were 43.68% FW and 95.53% TS, respectively.

Enzyme products

Commercial cellulase, pectinase and laccase were delivered by IASP-Berlin. For confidentiality reasons, IASP did not communicate products and suppliers names. Three enzyme products were used: a cellulase from *Trichoderma reesei*, a pectinase from *Aspergillus niger* and *Trichoderma longibrachiatum*, and a laccase from *Trametes sp.* The cellulase and pectinase were in liquid form, while laccase was in solid form. Ms. Schimpf recommended testing several enzyme combinations: pectinase alone, pectinase and laccase, cellulase together with pectinase and laccase. The former enzyme products, tested by Dr. Schimpf on rye silage in batch digestion trials at IASP-Berlin under quite similar conditions, yielded significant increases of substrate's methane production.

Design of the experiment

The stirred glass digesters of 2 L capacity were used in place of the HBT process for anaerobic digestion assays because application of ungrounded substrate required digesters with a higher capacity. A higher amount of plant material (70 g instead of 1.8 g for HBT) was used in each digester, allowing the analysis of ungrounded material that had a lower homogeneity.

Each product was applied at the start of the batch assay at a dose of 0.07 g/kg relative to rye silage fresh weight of 70 g, corresponding to 0.17 g/kg VS of substrate. For that purpose 49 mg of each enzyme product were diluted in glass flasks with distilled water to 20 mL volume. Subsequently, diluted enzymes (2 mL) were spread upon rye silage with a pipette, before inoculums fluid (1800 g) were added. This procedure was designed to increase contact between enzyme and substrate. Hence total enzyme concentration of binary (pectinase + laccase) and tertiary (cellulase + pectinase + laccase) enzyme mixtures were twice and thrice higher than the added amount of single pectinase, respectively.

Some digesters produced lower methane yields, especially at the end of the digestion period. By setting overpressure to the gasometers after completion of the trials and observing changes in the position of the water column, leakages were detected in 6 digesters out of 24, due to improper sealing at gas pipes junction points and inside valves. The issue was further solved by fixing pipes junctions with hose clamps and spreading silicone paste on the inside of valves. However, repeating the trial was not feasible, so that replicates corresponding to leaky digesters had to be discarded.

Batch digestion of finely ground rye silage

Goal of the experiment

The experiment was run in order to verify results obtained in the previous experiment. It was suspected that the absence of enzyme effects would be due to unfavorable inoculum properties. The standard inoculum produced by the State Institute of Agricultural Engineering and Bioenergy at the University of Hohenheim, which is derived from on-farm biogas plant digestate, clearly differs from inoculum used for anaerobic digestion trials at IASP Berlin, which originates from an anaerobic digester treating municipal sewage sludge.

Substrate

Coarse rye silage, used in previous trials, was ground using a laboratory mortar grinder (KM 100, Retsch, Haan, Germany) to a much finer particle size of ~2 mm. The device was provided by the Department of Biotechnology and Enzyme Science of the Institute of Food Science and Biotechnology at the University of Hohenheim (Director: Prof. Dr. Lutz Fischer). Further substrate characteristics are reported in the section ***4.6 Characteristics of the substrates***.

The high dry matter content of substrate (TS content of 44%) as well as its fibrous structure resulted in warming of substrate and extensive water losses during the grinding process. Dry matter content increased to ~55% after grinding.

The method described previously (*cf.* ***4.3.1 Assay protocol***) was applied to correct TS and VS contents of rye silage for losses of volatile compounds resulting fron the determination method. The same substrate was used for this experiment, in a finely ground state, so that the concentrations of volatile compounds in finely ground rye silage (in % FW) used for the calculation were the same as for the experiment with coarse rye silage:

$C_{VFA} = 0.40$; $C_{Lac} = 1.84$; $C_{Alc} = 0$; $C_{NH3} = 0$

The uncorrected TS and VS contents were 53.87% FW and 95.57% TS, respectively. TS and VS contents after correction were 55.08% FW and 95.67% TS, respectively.

Enzyme products

Enzyme products and combinations used in this trial were similar to the trial with coarse rye silage. Each enzyme product was applied at 0.07 g/kg FW, or 0.13 g/kg VS of substrate. Additionally, a 100-fold higher enzyme concentration was also tested, i.e. 13 g/kg VS of substrate.

Design of the experiment

At the beginning of the trial, 70 mg of each enzyme product was weighted and diluted in 1 L and 10 mL of distilled water to provide simple and 100-fold concentrations, respectively. After weighing 1 g chopped rye silage in the tubes, 2 mL diluted enzyme solution was spread on the substrate with a pipette. Following this, 30 g inoculum fluid were added and the tubes were closed. Anaerobic digestion took place at 37°C for a duration of 35 days.

In order to assess the influence of inoculum source on gas production, sewage sludge inoculum was used as an alternative to the standard inoculum ("manure inoculum"). For the preparation of sewage sludge inoculum, digested sewage sludge (municipal sewage plant of Wansdorf) was provided by the IASP institute and further digested for nine days at room temperature before the start of the digestion trial in order to reduce its own biogas production.

Batch digestion of coarse grass silage with weak inoculum

Goal of the experiment

Enzyme additives had few effects in previous batch anaerobic digestion assays. Possible reasons were high concentrations in fibers and high bacterial activities of inoculums that were not favorable for enzyme additives. Moreover, finely grinding the substrate to a short fiber length prior to its use in the HBT process may result in rapid substrate degradation, that may also hide the effects of enzyme additives that are applied to increase substrate degradation velocity. The goal of the experiment was to verify if resorting to a more dilute inoculum with weaker bacterial activity along with substrate applied at a coarser fiber length would reveal effects of enzyme additives on the methane yield of grass silage.

Substrate

In contrast to previous experiments, where substrate was finely chopped prior to the anaerobic digestion assays, grass silage was used in the HBT process at a coarse fiber length of 0.5-1 cm. The TS content of grass silage was 45.06% FW and its VS content was 90.58% TS. Contrary to previous experiments with ensiled substrates, TS and VS contents were not corrected for losses of volatile compounds upon drying. Further substrate characteristics are reported in the section ***4.6 Characteristics of the substrates***.

Inoculum characteristics

In this experiment, a different inoculum was used, which was both more diluted and less active. The inoculum was taken from a laboratory digester, consisting of a methanogenic fixed bed reactor that was coupled with a leach bed reactor (Zielonka et al. 2007), resembling to the process described by Zhang and Zhang (2002). This inoculum originated from a fixed bed reactor that had been left in dormancy at room temperature for several months, i.e. no substrate feeding was applied over this period. Such a treatment may have resulted in a decrease in bacterial activity. The inoculum had a TS content of 0.7% and a VS content of 35% of the dry weight.

Enzyme product

The enzyme product MethaPlus L100 was tested at a high dosage of 11 g/kg VS of substrate. A control variant was run without enzyme addition.

Design of the experiment

1500 mg of grass silage were mixed with 30 g inoculum. Digestion conditions were similar to previous experiments (35 days at 37°C in the HBT process), but with only 2 replicates per variant. 1 mL of diluted enzyme product was spread onto coarse grass silage prior to addition of inoculum fluid (same protocol as in previous experiments).

Batch digestion of coarse grass silage with diluted inoculum

Goal of the experiment

Results from the precedent experiment suggested that enzyme had a higher effect on methane production when a weak inoculum was used. In this experiment, the standard inoculum ("manure inoculum") consisting in predigested dairy manure was diluted 3, 5 and 10-fold in order to weaken its activity and reach the conditions that may be beneficial to enzyme action. The raw, undiluted inoculum had a TS content of 3.4% and a VS content of 53% of the dry mass. In order to avoid a collapse of methane production due to excessive acidification, a medium containing both buffer agents and mineral nutrients was used to dilute the inoculum.

Substrate

The same substrate was used as in the previous experiment, i.e. coarse grass silage, with the same characteristics.

Preparation of diluted inoculum

The dilution medium was prepared as described by Steingass and Menke (1986). However, some chemicals mentioned in the original publication of these authors were not added. Furthermore, the medium was completed according to the recommendations of DSM Biopract GmbH, Berlin, Germany.

First, three component block solutions were prepared:

1. **Buffer solution:** 35 g ($NaHCO_3$), 4 g $(NH_4)HCO_3$ were diluted successively into ~800 mL distilled water and the volume was then completed to 1 L with distilled water.
2. **Nutrient solution:** 6.2 g KH_2PO_4, 0.6 g ($MgSO_4 + 7H_2O$) were diluted successively into ~800 mL distilled water and the volume was then completed to 1 L with distilled water.
3. **Trace metal solution:** 13.6 g ($CaCl_2 + 2H_2O$), 8 g ($FeCl_3 + 6H_2O$), 10 g ($MnCl_2 + 4H_2O$), and 1 g ($CoCl_2 + 6H_2O$) were diluted successively into ~80 mL distilled water and the volume was then completed to 100 mL with distilled water.

Finally, 356 mL buffer solution, 356 mL nutrient solution, 0.18 mL trace metals solution and 5.8 mL of the commercial trace metal mixture MethaTrace from DSM Biopract GmbH were added successively. The volume was then completed to 2000 mL with distilled water. The final solution was used to perform dilutions of the inoculum.

Enzyme product

The enzyme product MethaPlus L100 was tested at dosages of 0.7 and 7 g/kg VS. Diluted enzyme product was applied using the same protocol as in previous experiments. A control variant was run without enzyme addition.

Design of the experiment

Digestion conditions were similar to previous experiments (fresh substrate: 1500 mg; inoculum: 30 g; temperature: 37°C, 3 replicates per variant in the HBT process). To account for the slower methane production rate, the duration of the assay was extended to 60 days. Due to the low viscosity of the digesting material in syringes of the HBT process, substrate losses occurred in the course of the digestion. This is a weakness of the HBT process, in which the viscosity of digesting material contributes to making the reactor impermeable. Liquid sealing by the digesting material itself complements silicone sealing between the syringe and the plug.

Hence the results from 3 samples differing of more than 3-fold from the standard deviation of the corresponding variant had to be discarded. However, in each case the results of at least 2 samples per variant remained exploitable.

Batch digestion following enzymatic hydrolysis step

Goal of the experiment

It was expected that conditions prevailing in mesophilic anaerobic digestion (i.e. high pH, low temperature, enzyme degradation through bacteria) would not be best for enzymatic hydrolysis from commercial fungal enzymes. In this experiment the enzymatic hydrolysis of substrate was performed in a separate step upstream of the anaerobic digestion step.

Conditions thought to be favorable with regard to enzyme's requirements, i.e. diluted substrate, slightly acidic pH, and thermophilic temperature, were implemented to the enzymatic hydrolysis step.

Substrate

Finely ground ensiled maize straw was used as a substrate. Maize straw was chopped at ~2 cm fiber length after harvest and ensiled in 2 L glass jars (Weck GmbH, Wehr, Germany). After 248 days (8 months) of ensiling, maize straw was taken out of the jars and ground with a laboratory mortar (KM 100, Retsch, Haan, Germany). Further substrate characteristics are reported in the section ***4.6 Characteristics of the substrates***.

The method described previously (*cf.* ***4.3.1 Assay protocol***) was applied to correct TS and VS contents of maize straw for losses of volatile compounds resulting from the determination method. The concentrations of volatile compounds in maize silage (in % FW) used for the calculation, determined by chemical analysis, were:

$C_{VFA} = 0.22$; $C_{Lac} = 1.90$; $C_{Alc} = 0.51$; $C_{NH3} = 0$

The uncorrected TS and VS contents were 21.60% FW and 93.58% TS, respectively. TS and VS contents after correction were 23.02% FW and 93.98% TS, respectively.

Enzymatic hydrolysis step

The enzyme mixture previously showing highest monosaccharide release in preliminary trials (cf. Appendix ***A 2 Reducing sugars against glucose determination***), i.e. Genencor Laminex BG together with Novozym 188, was chosen to build up an enzymatic hydrolysis pretreatment step. The target of enzymatic pretreatment was to improve substrate digestibility in the subsequent anaerobic fermentation step. Enzyme products were applied at concentrations of 1.3 and 13 g/kg substrate VS. Diluted enzyme solutions were prepared by adding 10 or 100 µL enzyme into 10 mL distilled water for low and high dosage, respectively. Inactivated enzymes were produced through autoclaving of diluted enzyme solutions at 121°C for 30 min. The operating mode was similar to the previous experiments: 1.8 g ensiled maize straw, 10 mL water and 0.5 mL of diluted enzyme were put inside glass tubes of the HBT process. The pH level in the watery medium was increased to 4.5 through the addition of 0.1 M NaOH. There was no need of additional buffer system to maintain pH to the former value, since organic acids contained in the silage built up an efficient buffer system. After a 24-hour enzymatic hydrolysis period, pH ranged between 4.45 and 4.62. Experimental results of previous experiments as well as data from the literature (Durand et al. 1984), showed that a pH value in the range 4.0-4.5 would be optimal for the hydrolysis of substrate from fungal enzyme systems of Trichoderma reesei and Aspergillus niger. The hydrolysis duration was 24 hours and temperature was set to 45°C. Monosaccharides released after enzymatic hydrolysis were analyzed via HPLC as described previously (cf. ***4.2.1 Assay protocol***).

Anaerobic digestion step

After completion of the enzymatic pretreatment step, syringes containing water, substrate and enzyme were opened, and 30 g standard inoculum (”manure inoculum”) were added in each tube in order to initiate anaerobic digestion. Subsequently, each tube was closed again and brought back to the incubator, and incubation temperature previously applied for enzymatic pretreatment (i.e. 45°C) was changed to reach the incubation temperature of anaerobic digestion (i.e. 37°C).

Control variants were run without enzymatic pretreatment step (single-step process). For that purpose 30 g manure inoculum and 0.5 mL of diluted enzyme solutions (prepared as described previously) were added directly to 1800 mg ensiled maize straw, before eauch tube was closed and brought back to the incubator.

Batch digestion of effluent from a biogas plant

Goal of the experiment

The experiment was an attempt to evaluate the effect of enzyme additive under conditions that are closer to practice by running batch digestion on reactor samples from a full-scale biogas plant. Moreover, enzymes were added regularly in the course of the digestion process instead of being fed in a single step at the start of the experiment.

Furthermore, it was thought that the slightly alkaline pH level prevailing in the biogas process may be an obstacle to the action of enzymes from acidophilic fungi. Hence, enzyme products designed to function under slightly alkaline conditions were tested and compared to an enzyme product from acidophilic fungi.

The impact of digestion temperature was also tested by running two digestion trials, a first trial at mesophilic temperature, at 37°C, and a second trial at psychrophilic temperature, in the range 9-22°C. While the mesophilic trial simulated digestion in a biogas reactor, the psychrophilic trial simulated digestion in a covered storage tank for digested effluent.

Reactor samples

Samples were taken from the first and second reactor of an on-farm biogas plant consisting of a series of two digesters. The characteristics of the reactors and of the samples are described in ***4.6 Characteristics of the substrates***. Inoculum addition was not required to perform batch digestion because the reactor samples of the full-scale biogas plant were biologically active.

Enzyme products

One enzyme product and one mixture of products were used:

1. MethaPlus L100 (having both cellulase and xylanase activities). The product was a gift of the supplier DSM Biopract, Berlin, Germany;
2. A mixture of Novozym 342 (having high cellulase activity) together with Pulpzyme HC (having high xylanase activity). Both products were mixed at a concentration of 50% in weight. The latter products were a gift of the supplier Novozymes, Bagsvaerd, Denmark.

As shown in enzymatic hydrolysis trials, MethaPlus L100 had maximal activity at low pH (~4.5), while Novozym 342 and Pulpzyme HC were both adapted for use under neutral to alkaline pH conditions.

Enzyme loading

Enzymes were added at two different concentrations: 0.2 and 2 g/kg substrate FW (Fresh weight) of digester samples. Corresponding dosages expressed relatively to substrate VS are shown in **Table 13**. Due to higher TS and VS contents of the sample from the first reactor, enzyme load relative to VS was higher for the second reactor, i.e. 3.2 and 32 g/kg VS compared with 2.5 and 25 g/kg VS for low and high dosages of the first reactor, respectively.

Table 13. Conversion of enzyme dosages from fresh weight to volatile solids weight.

Reactor	Reactor sample content		Enzyme dosage related to reactor sample FW (g/kg FW)		Enzyme dosage related to reactor sample VS (g/kg VS)	
	TS (% FW)	VS (% TS)	Low dosage	High dosage	Low dosage	High dosage
First reactor	10.4	78	0.2	2	2.5	25
Second reactor	8.5	73	0.2	2	3.2	32

Stock solutions of enzyme products

In previous batch digestion trials, enzyme products were always added in a single step immediately at the start of the experiment. This protocol was changed in order to prevent issues related to possible enzyme inactivation in the course of anaerobic digestion, and enzymes were added at regular intervals throughout the batch digestion period. To that end total enzyme doses of 0.2 and 2 g/kg substrate FW were split into 20 equal parts. Diluted enzyme solutions were freshly prepared shortly before each addition to the biogas process. For that purpose, 20 and 200 µL of enzyme products (MethaPlus L100 or of Novozym 342 / Pulpzyme HC mixture) for simple and 10-fold dosage, respectively, were diluted to 20 mL with distilled water. Inactivated enzyme stock solutions were prepared with enzyme solutions containing 10 g/L enzyme products. These enzyme stock solutions were autoclaved in a single step at 121°C for 15 min for inactivation, then split into 2 mL-portions that were placed in a freezer for conservation. Enzyme addition was performed from the start of the experiment with the solutions of active and inactivated enzyme products, and took place at regular intervals as described further.

Batch digestion at mesophilic temperature

Digester samples were homogenized with a laboratory mixer having a capacity of 1 L, that was not meant for particle size reduction, but for mixing the substrate and providing representative samples. At the start of the experiment, after weighing 50 g reactor samples in the tubes, 0.5 mL from the diluted enzyme solutions, prepared as described previously, were added with a pipette and the tubes were closed. Each variant was run with three replicates. Following the first enzyme feeding at the start of the trial, the addition of 0.5 mL of freshly prepared diluted enzyme product was repeated every third day until the 57^{th} day. Anaerobic digestion was performed at 37°C for 87 days.

Batch digestion at psychrophilic temperature

Complementrary to the first digestion trial, performed at mesophilic temperature, at second trial was run at room temperature (psychrophilic temperature). In this trial, incubation temperature was not regulated and was influenced by room temperatures, which ranged from 9 to 22°C. The enzymes previously described (i.e. MethaPlus L100 and Novozym 342/Pulpzyme HC mixture) were employed. Enzyme addition was performed the same way as detailed previously. Enzymes were added only at the higher dosage of 2 g/kg substrate FW. No inactivated enzyme variants were used. The anaerobic digestion process was slower at room temperature (psychrophilic mode) than at 37°C (mesophilic mode). Hence, the duration of the psychrophilic experiment was extended to 180 days instead of 90 days used previously for the mesophilic trials. The same protocol was applied for enzyme addition as in the mesophilic-temperature experiment. While mesophilic temperatures (37°C) simulate the conditions prevailing in an anaerobic digester, psychrophilic (here: room temperature) digestion simulates a digester that is not heated. This is the case for the post-storage of biogas plant effluent in Germany, the capacity of which should be sized for a storage duration of 4 to 6 months according to present agricultural regulations (Weiland 2006). Effluent post-storage usually occurs in open tanks. This experiment was aimed at simulating anaerobic digestion in a post-storage tank, in order to evaluate the efficiency of enzyme additives under such conditions. Open tanks are increasingly converted into impermeable storage in order to capture more methane from feed substrates. In such cases, methane production is expected to show the following characteristics: (1) Occurring at a very low rate (due to prevailing low temperatures of digestion), and (2) Continuing over a very long period (due to a long storage period as well as slow substrate degradation rate).

4.4 Semi-continuous acidogenic fermentation

4.4.1 Assay protocol

Horizontal digesters

The semi-continuous acidogenic digestion experiment was run on eight horizontal digesters with a capacity of 17 L liquid volume each (**Figure 7**). The digesters were designed to operate as continuously stirred tank reactors (CSTR). Each digester was made of long-shaped stainless steel cylinders with a central horizontal agitator. Comparable designs can be found in practice in horizontal plug flow digesters (Fischer 2002; Weiland 2006; Weiland 2003). The long shape of the digesters together with the horizontal mixing device optimized the vertical mixing of the feedstock. Vertical mixing prevents the formation of floating and sinking layers, which are caused by density discrepancies between the individual components of the input substrates. Floating layers are often found in anaerobic digesters fed with energy crops. This issue can be dealt with through reducing substrate particle size and stirring continuously (Lehtomäki et al. 2007).

The digesters were heated by warm water pumped from a thermostat-controlled water bath and circulating inside a water jacket. Each digester was equipped with one feeding pipe and one outlet pipe for the substrates. Inlet pipes were made of stainless steel and integrated into the reactor's structure. Outlet pipes were made of PVC plastic. The height of the outlet pipes could be modified to adjust the bypass limit determining the maximum substrate volume in the digester. Each digester was provided with a stirrer powered by an electric motor. The speed, operating time and frequency of the motors could be adjusted. Since the motors could not stand continuous stirring without warming up, the stirring was operated during one minute every second minute at ~60 rpm.

Exhaust pipes for biogas were placed into gas domes fitted on the top of each digester. The gas domes offered a buffer volume to avoid blockage of the gas pipes while the mixing devices could incidentally spread the digesting substrate on the upper part of the digesters. Removable rubber stoppers were fitted on the top of the gas domes, so that they could be opened if required for removing incrusted substrate. The digesters were maintained under a slight overpressure (a couple of cm water column) through glass wash bottles connected to the gas outlets. Distilled water served as the washing liquid. Due to the semi-continuous design, the important gas production and the saturation of water with bicarbonate, losses of CO_2 in the washing liquid were thought not to be a relevant issue.

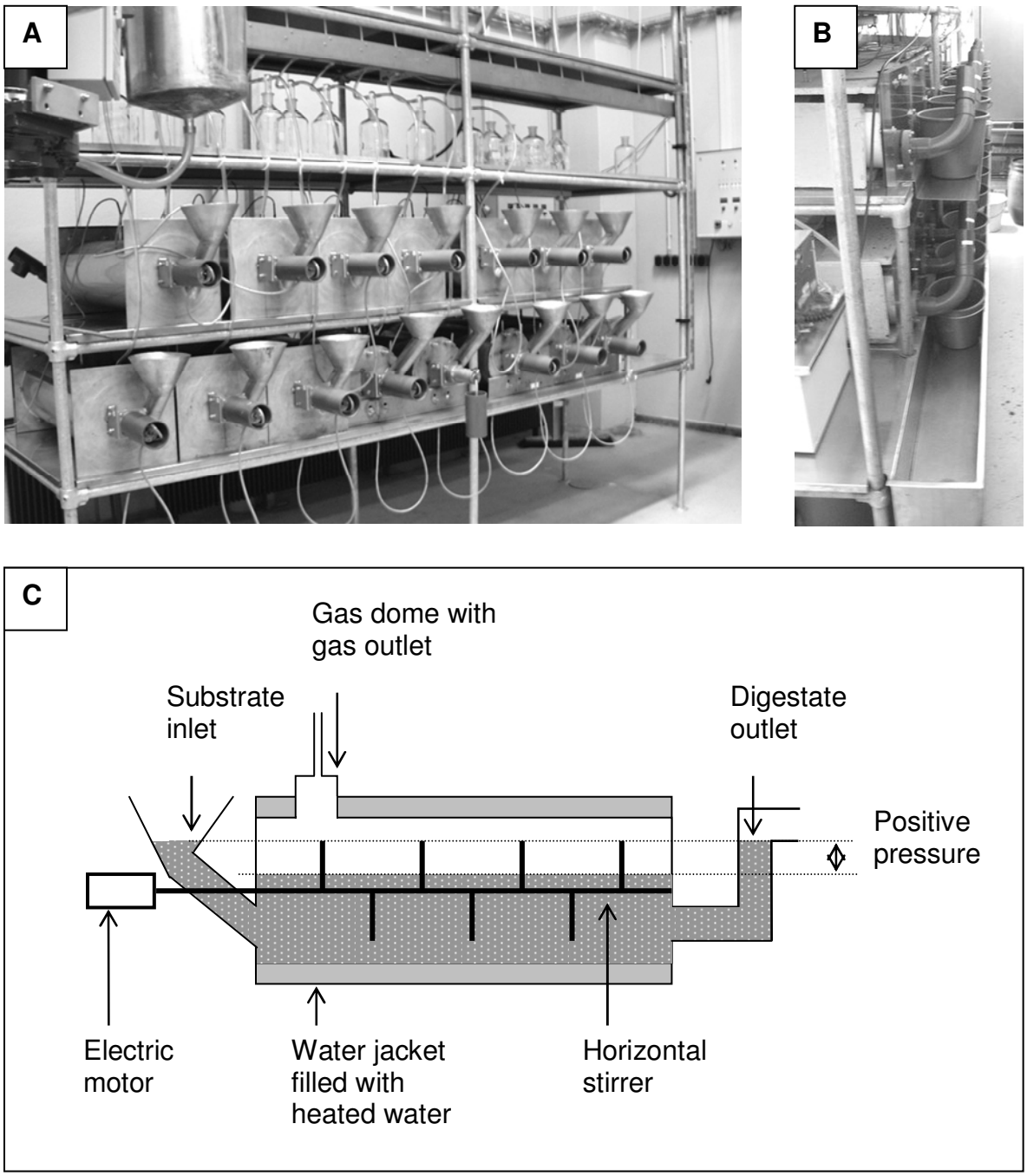


Figure 7. Horizontal digesters for continuous experiments [A. Front side. B. Back side. C. Scheme of a digester].

Gas measurement system

Upcoming gas from acidogenic fermentation of each digester passed through gas wash bottles to fill gas bags (Plastigas, Linde Gas, Pullach, Germany). The gas wash bottles generated a cooling effect, allowing some of the water contained in the gas to condensate. The gas bags were emptied and measured once a day at a pre-set time with an automatic computer-monitored system (**Figure 8**).

Biogas from the gas bags of each digester was measured automatically as follows:

1. Biogas contained in the local gas bag of a digester was pumped into a central gas bag for temporary storage during the gas measurement step. When the local gas bag was empty, the pressure decrease recorded by a differential manometer gave the signal to stop the pump as all its content had been transferred to the central gas bag. On the way from the local gas bag to the central gas bag, gas flow rate was measured with a mass flow meter (EL-Flow Select, Bronkhorst, Enschede, Netherlands) and summed over the pumping time to give the total gas mass.

2. The filled central gas bag was sampled for ~40 s at a lower flowrate by a secondary pump. The biogas sample passed through a gas cooler followed by double-beam NDIR gas sensors for CH_4 and CO_2 (Advanced Gasmitter, Pronova, Berlin, Germany) as well as an electrochemical sensor for H_2S (Pronova, Berlin, Germany). Before H_2S measurement, gas could be diluted through a third pump in order to avoid saturation of the sensor if H_2S concentrations were above the measurement range. After biogas had been flushed into the sensors for ~30 s, three successive values of gas composition were recorded at ~2 s interval and averaged. However, in this experiment, H_2S could not be measured due to the disruption of the electrochemical H_2S sensor from interfering H_2 present in high concentrations in acidogenic fermentation gas. Hence H_2S was not measured, assumed to be present only in low concentrations and neglected.

3. The remaining gas of the central gas bag was pumped away and evacuated through gas pipes outside the laboratory. Simultaneously, room air was injected for cleaning the gas sensors for ~3 min.

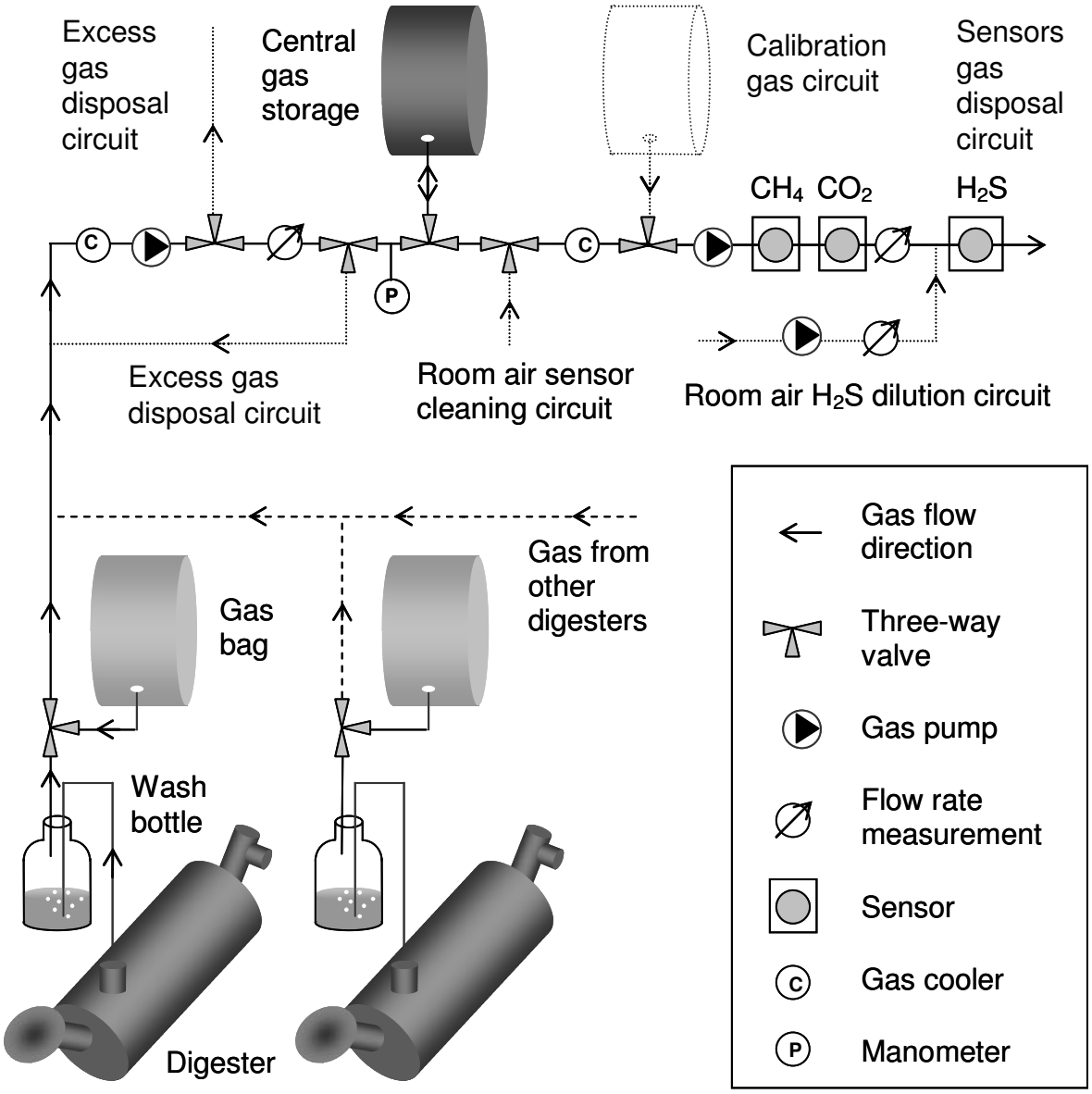


Figure 8. Automatic gas measurement system for continuous experiments.

Calculation of the gas yield

The gas measurement software integrated the mass flow over time as equivalent nitrogen volume and subsequently computed the biogas volume. For this purpose, the nitrogen volume was at first converted to standard conditions:

$$V_{N2} = V_R \times \frac{T_0}{T_R} \quad \textbf{(11)}$$

V_{N2} Normalized nitrogen volume (L/d)

V_R Recorded gas volume (L/d)

T_0 Normal temperature; $T_0 = 273$ K

T_R Temperature of biogas (K)

Following this step, the gas measurement software converted the nitrogen volume into biogas volume by means of correction factors:

$$V_G = \left(K_{CO2} \times R_{CO2} + K_{CH4} \times R_{CH4} + K_{H2S} \times R_{H2S} + K_{N2} \times R_{N2}\right) \times V_{N2} \quad \textbf{(12)}$$

V_G Normalized biogas volume (L/d)

V_{N2} Normalized nitrogen volume (L/d)

R_{CO2} Volume ratio of CO_2 (v/v)

R_{CH4} Volume ratio of CH_4 (v/v)

R_{H2S} Volume ratio of H_2S (v/v), where $R_{H2S} = 0$ (H_2S fraction neglected)

R_{N2} Volume ratio of N_2 and H_2 (v/v), where $R_{N2} = 1 - R_{CH4} - R_{CO2} - R_{H2S}$

K_{CO2} Correction factor for CO_2

K_{CH4} Correction factor for CH_4

K_{H2S} Correction factor for H_2S

K_{N2} Correction factor for N_2 and H_2 where $K_{N2} = 1$

The correction factor of N_2 was also applied to H_2, since H_2 was no measured directly.

Specific gas yield

The specific gas yield of the digesters was calculated as follows:

$$Y_G = \frac{V_G}{W_{VS}} \quad \textbf{(13)}$$

Y_G Specific gas yield (m^3/kg VS)

V_G Normalized gas yield (m^3/d)

W_S Daily fed VS weight of substrate - here: maize silage (g)

Specific biohydrogen yield

The specific biohydrogen (H_2) yield of the digesters was calculated as follows, assuming that only H_2 and CO_2 were contained in the gas in significant amounts:

$$Y_H = Y_G \times \left(\frac{100 - C_{CO2}}{100} \right) \quad \textbf{(14)}$$

Y_H Specific biohydrogen yield (m^3/kg VS)

Y_G Specific gas yield (m^3/kg VS)

C_{CO2} Carbon dioxide concentration (% v/v)

4.4.2 Description of the experiment

The trial involved acidogenic fermentation, which has the following characteristics:

1. Operational conditions chosen were suboptimal for the conversion of substrates that are hardly degradable, i.e. contain a high share of lignocellulose.
2. Methane was not the end product of anaerobic digestion, since anaerobic digestion occurred under acidic conditions and at a short HRT. Organic acids, alcohols and ketones, as well as the gases H_2 and CO_2 were produced instead.

The process conditions chosen were a hybrid between optimal conditions for H_2 production (dark fermentation) and optimal conditions applying to organic acids production (hydrolysis or acidification step). Optimal conditions for dark fermentation are: pH in the range 5-5.5, HRT 10-20 h, temperature 55°C, OLR 10 kg/(m^3 × d), substrate concentration 5-7.5 g/L, and accordingly important water addition to dilute the substrate to the required concentration, and strong, continuous mixing (Bartacek et al. 2007; Fang and Liu 2002; Kyazze et al. 2006; Lay 2000; Logan et al. 2002; Van Ginkel et al. 2001). Optimal conditions for the acidification step are: pH in the range 5.5-6, HRT 3d, temperature 55°C, high substrate concentration, and continuous mixing (Puchajda and Oleszkiewicz 2006; Traverso et al. 2000; Veeken et al. 2000; Yu and Fang 2002; Zoetemeyer et al. 1982a; Zoetemeyer et al. 1982b).

The experiment was run with eight heated horizontal digesters having a capacity of 17 L liquid volume. Acidogenic inoculum was acquired from acidogenic fermentation trials previously taking place at this institute (Preißler et al. 2008). In accordance with these trials, the temperature in the digesters was set to 55°C. Whole crop maize silage (particle size 0.5-3 cm) was put inside the digesters at an OLR of 4 kg VS/($m^3 \times d$). Substrate feeding was not evenly distributed along the time of the experiment, but took place in a single step for a short period every day (semi-continuous operation). During this feeding step quicklime was added in order to maintain pH value between 5.0 and 5.5.

HRT was set to the proper value through water addition. The 10-day HRT previously chosen by Preißler et al. (2008) was first maintained. Unfortunately, clogging of the outlets of the digesters rapidly occurred. In order to cope with this problem, HRT was eventually reduced to 5 days. Following this, the clogging issue was only partially solved, but it was possible to regularly remove substrate caught in digester outlets by means of a flexible plastic stick. Preißler et al. (2008) did not face similar clogging issues because they used a laboratory digester with a 20-fold larger size (i.e. 400 L capacity), and accordingly outlet pipes with higher diameters. Semi-continuous anaerobic digestion trials were run in this institute using the same digesters with maize silage of similar characteristics applied at the same OLR of 4 kg VS/($m^3 \times d$) and a longer HRT of 35 days for methane production. In these trials no blocking of digesters outlets was noticed (Mukengele et al. 2006; 2007). Acidogenic anaerobic fermentation probably results in much lower substrate degradation rates compared with methanogenic anaerobic digestion. Reducing the particle size of the substrate might be a solution to prevent clogging issues.

Contrary to previous experiments involving ensiled plant material, substrate VS content used for OLR calculation was not corrected for losses of volatile compounds resulting from the determination method. Such a procedure may be inaccurate due to the the high variability of substrate dry matter content in this experiment.

The amount of substrate required for daily feeding of each digester was calculated as follows:

$$W_S = \frac{100}{C_{TS}} \times \frac{100}{C_{VS}} \times R \times V \quad \textbf{(15)}$$

W_S Weight of maize silage fed daily (g/d)

C_{TS} TS content of maize silage (% FW)

C_{VS} VS content of maize silage (% TS)

R Organic loading rate (kg VS/($m^3 \times d$))

V Digester total useful volume (L)

HRT was set at the target value through dilution with tap water. The amount of water required in daily feeding to set HRT at the target value was calculated as follows, assuming that the density of the substrate in the digester was equal to 1 g/mL:

$$W_W = \frac{V}{t} - W_S \quad \textbf{(16)}$$

W_W Daily fed weight of water (g/d)

V Digester total useful volume (mL)

t Hydraulic retention time (d)

W_S Daily fed weight of maize silage (g)

Both substrate and tap water were weighed at a precision of ± 0.5 g. Following experiment start-up, the total solids and volatile solids contents of substrate were measured weekly (**Figure 9**). High variability and lack of accuracy was noticed regarding substrate TS content. The highest and lowest substrate TS values along the trial were 27 and 22%, respectively. This represents a 25% difference between lowest and highest value. Alternately, VS contents had a low variability, ranging between 95 and 98% of TS, and could be measured with high accuracy. Maize silage was stored in 60 L-barrels. During storage, part of the liquid fraction of the substrate would percolate to the bottom, creating differences in moisture contents between substrate samples taken from the top and from the bottom of the barrels. Possible ways to prevent such an issue in the future may include: removing excess liquid from the bottom using a tap integrated to the barrel, as usually done for silages, using substrate with a lower moisture content, or mixing substrate regularly while resorting to appropriate storage containers of low volume.

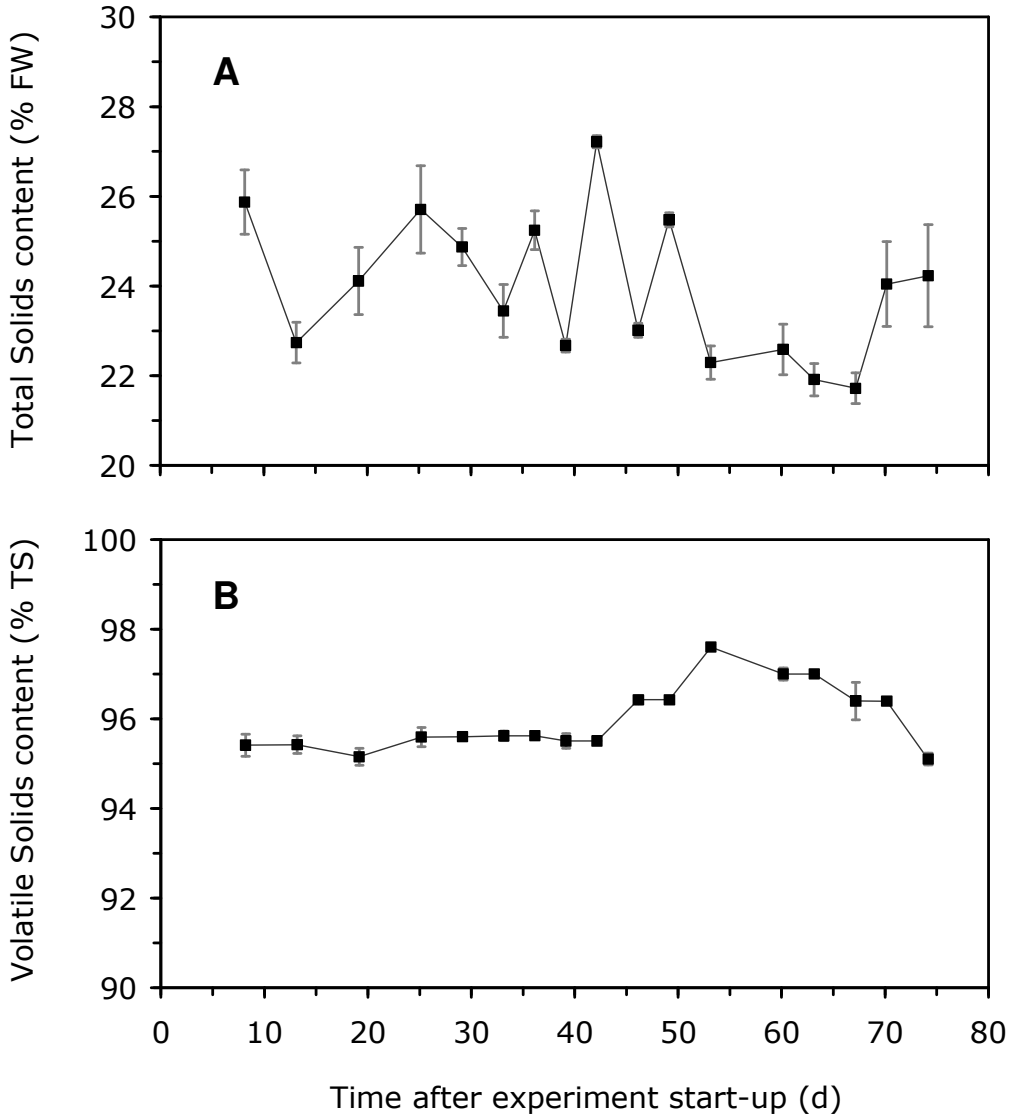


Figure 9. Total solids (A) and volatile solids (B) contents of maize silage samples [Averaged values. n=3. Vertical arrows stand for SD].

Quicklime was first added at a rate of 20 g/d during 4 days (**Figure 10**). Following this, lime concentration was decreased to 5 g/d. Later on, lime doses were slightly increased or decreased in order to maintain pH values in the desired range. The amounts of quicklime added were identical for all digesters. Between days 51 and 80, quicklime addition was stabilized at 5.75 g/d.

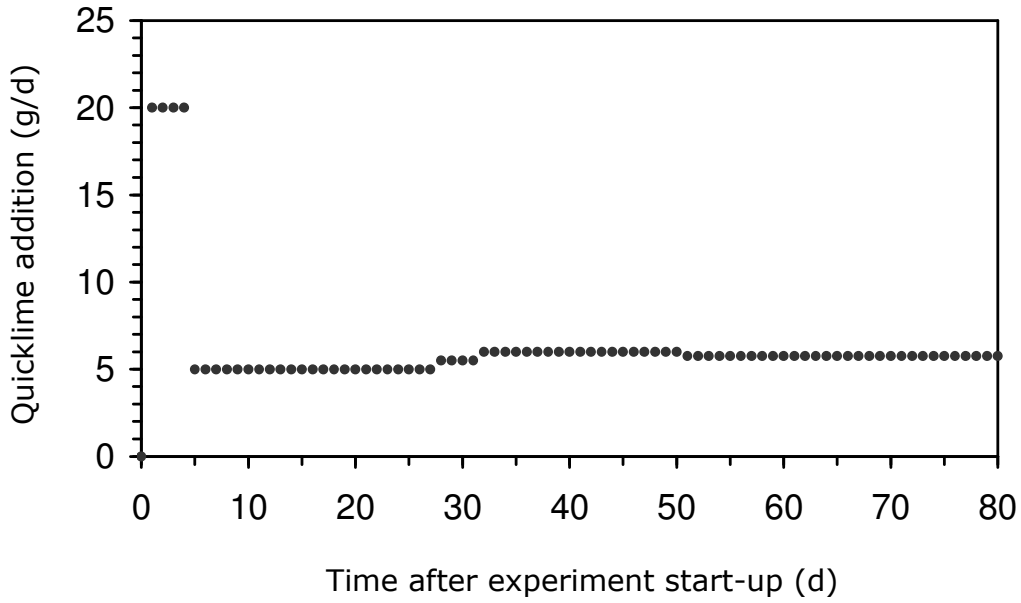


Figure 10. Quicklime addition to semi-continuous acidogenic digesters.

An OLR of 4 kg VS/(m^3 × d) was maintained during the whole trial. From day 0 until 33, HRT was set to 10 days. From day 34 until 80, a reduced HRT of 5 days was applied to cope with clogging issues. From day 51 until 65, the enzyme additive MethaPlus L100 was added at 10 g/kg VS of maize silage in four of eight digesters. In the remaining digesters no enzyme was added (control variant). From day 66 until the end of the trial enzyme addition was reduced to 1 g/kg VS.

The daily feeding of the digesters was run as follows: substrate, lime and water were weighed successively in beakers and placed inside the digesters. The weight of water added daily was ~1400 g at 10 days HRT and ~3000 g at 5 days HRT. The weight of maize added daily was ~300 g. The amounts of water and maize were adjusted weekly according to the results of TS and VS determination of the substrate. The enzyme product was added directly to the water before being poured into the digesters, and amounted to 680 mg at the higher dosage of 10 g/kg VS and 68 mg at the lower dosage of 1 g/kg VS.

The amount of gas produced, as well as its carbon dioxide and methane contents, were measured daily. No methane was detected in the gas. Devices for measurement of hydrogen gas were not available. As previously described (*cf.* ***4.4.1 Assay protocol***), hydrogen content was estimated by difference to carbon dioxide content, based on the assumption that no other gases were present in high amounts. H_2S content in the gas could not be measured.

4.5 Chemical analyses during digestion trials

4.5.1 Liquid reaction products of acidogenic fermentation

Volatile Fatty Acids (VFA)

About 1 g of liquid sample material was weighed in 10 mL Erlenmeyer flasks. 1 mL concentrated formic acid (98 or 100% v/v) and 1 mL n-methyl-valeric acid (internal standard) were added successively. Erlenmeyer flasks were filled to 10 mL with distilled water. 2 mL of diluted sample were centrifuged at 13,200 rpm (5415 D, Eppendorf, Hamburg, Germany). The centrifuged liquid was pipetted and brought into a reagent flask and VFA content was analyzed with a CP-3800 gas chromatograph (Varian, Palo Alto, CA, USA) with a FID-detector and a WCOT fused silica column 50 m × 0.32 mm (Varian, Palo Alto, CA, USA). The injection temperature was 150°C, detection temperature was 280°C. Temperature was held at 60°C for 2 min, raised to 150°C at 30°C/min, then to 240°C at 8°C/min. Helium was used as a carrier gas.

Chemical Oxygen Demand (COD)

COD was measured with a cuvette test (LCK 014, Hach Lange, Köln, Germany). Samples were diluted to match the measurement range of the assay (1000-10,000 mg/L). 0.5 mL sample volume were added into a cuvette containing 65% v/v sulphuric acid, mercury sulphate and potassium dichromate as a reagent. Samples were heated at 148°C for 120 min with a heating plate (Hach Lange, Köln, Germany). After the heating phase, samples were homogenized and CSB was detected with a sensor array photometer (LASA 20, Hach Lange, Köln, Germany).

Lactic acid

Lactic acid was determined enzymatically according to a method developed at the State Institute for Agricultural Chemistry of the University of Hohenheim.

Alcohols

Ethanol, n-propanol, iso-propanol, n-butanol, iso-butanol were analyzed through gas chromatography according to a method developed at the State Institute for Agricultural Chemistry of the University of Hohenheim.

Ketones

Ketones were thought to be generated in semi-continuous anaerobic acidification, but their contents could not be determined because the required knowledge and equipment for analysis was not available.

4.5.2 Substrate composition

Substrate composition was analyzed at the State Institute for Agricultural Chemistry of the University of Hohenheim as listed in **Table 14**. Fiber analysis was based on the Van Soest method (Van Soest et al. 1991), which is widely used for fodder analyses. Most analyses were performed after German standards of VDLUFA method book (Naumann and Bassler 1997). Crude protein, NDF and ADF were performed on plant material (maize and grass), while the alternative methods raw protein, NDForg and ADForg were thought to be more suitable for the analysis of digester effluents. All other methods were carried out on both plant material and digester effluent.

Table 14. Parameters and reference methods for substrate analysis.

Parameter	Description	Method reference	Applied to
TS	Total Solids	71/393/CEE	Plant material and digester effluent
VS	Volatile Solids	71/250/CEE	Plant material and digester effluent
Protein	Crude Protein	93/28/CEE	Plant material
Protein	Raw Protein	VDLUFA MB III 4.4.1	Digester effluent
Lipids	Crude Lipids	98/64/CEE	Plant material and digester effluent
NDF	Neutral Detergent Fiber	VDLUFA MB III 6.5.1	Plant material
NDForg	Organic fraction of Neutral Detergent Fiber	VDLUFA MB III 6.5.1	Digester effluent
ADF	Acid Detergent Fiber	VDLUFA MB III 6.5.2	Plant material
ADForg	Organic fraction of Acid Detergent Fiber	VDLUFA MB III 6.5.2	Digester effluent
ADL	Acid Detergent Lignin	VDLUFA MB III 6.5.3	Plant material and digester effluent

4.6 Characteristics of the substrates

Maize for hydrolysis trials and batch digestion

Maize crops from the cultivar Gavott were harvested on September 8th, 2006 at the beginning of milk-ripe maturity stage (cob content related to the fresh mass was 39 %) at an experimental field of the university of Hohenheim (seeding date, May 3rd, 2006).

Whole crop maize was separated into two maize fractions, which were used for the trials: maize straw and maize corncob. For this purpose, fresh maize plants were cut at the bottom of the stalk, and corncob was manually separated from the stem, i.e. maize straw. Subsequently, maize straw was chopped to 1-2 cm fiber length with harvesting equipment and corncob was cut into slices of ~1 cm with a knife.

Substrate composition is drawn in **Figure 11**. Analysis of both fractions revealed, from top to bottom: lignin, lipids, proteins, hemicellulose (NDF - ADF), cellulose (ADF - ADL), and other compounds. The remaining part of the organic matter, which were not analyzed, corresponded to Non-Fibre Carbohydrates (NFC) or Nitrogen-Free Extract (NFE). This fraction was assumed to be mostly composed of saccharides, i.e. starch and soluble sugars, although it may also comprise other substances (e.g. tannins) in small amounts (Chen et al. 2007). Maize corncob was expected to have a relatively high share of starch, whereas maize straw should contain only soluble sugars from plant sap (Chen et al. 2007). Maximal total sugars contents, including soluble sugars, starch, cellulose and hemicellulose, reached 88% and 86% of substrate VS for maize straw and corncob, respectively.

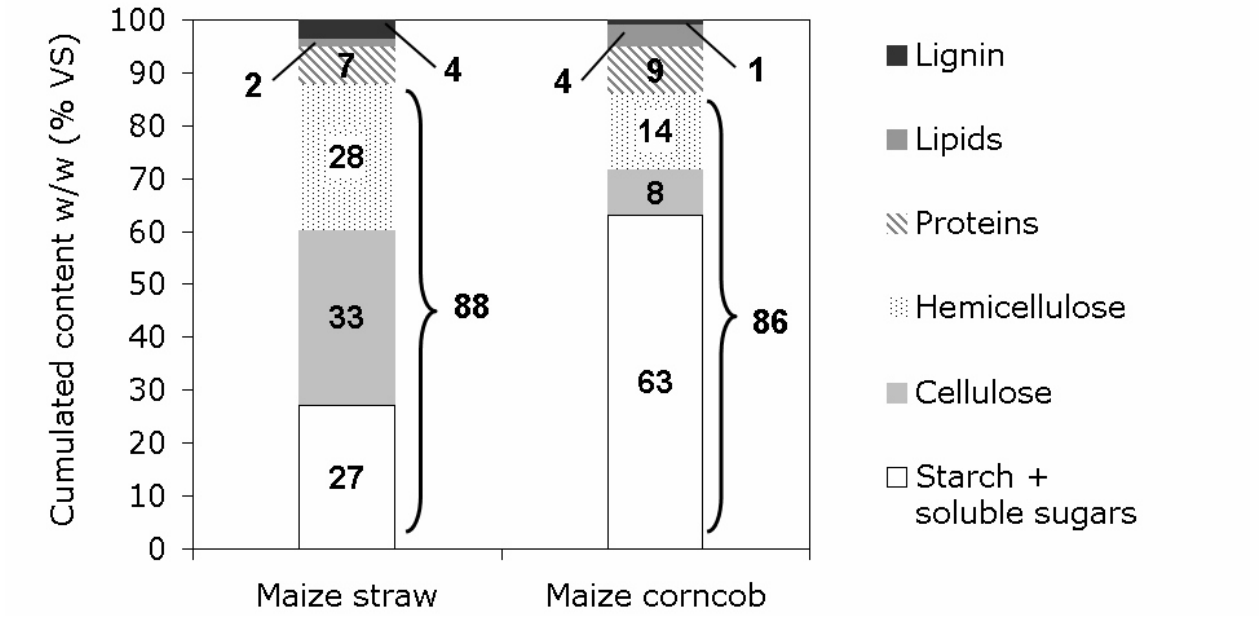


Figure 11. Chemical composition of maize straw and of maize corncob.

Rye silage for batch digestion

Rye (cultivar Vitalis) was harvested on June 28th, 2006 in Dolgelin (located in the region of Brandenburg, near Berlin, Germany). Rye was chopped to a fiber length of 8 mm, ensiled in glass jars at the IASP institute and stored at room temperature for 450 days. A substrate sample of rye silage was ground in a laboratory mill and analyzed for volatile compounds. Lactate and acetate contents amounted for 3.9 and 0.8% of substrate TS, respectively. Other compounds, i.e. formate, 1.2-propanediol, propionate, ethanol, n-butyrate and ammoniac, were not detected. However, the process used in substrate grinding with the laboratory mortar was suspected to generate water loss and to probably volatilize some compounds. Sucrose, glucose and fructose were also determined and amounted for 5.0, 2.4 and 4.9% of substrate TS, respectively.

Data about rye silage composition according to Van Soest analysis was provided by the IASP institute. **Figure 12** shows substrate composition determined on 90-day old silage. The estimated total sugar content reached 85% of substrate VS. Substrate composition after a much longer storage period of 450 days might be different. However, the analysis of 450-day old silage could not be carried out because the amount of substrate available was not sufficient for analysis.

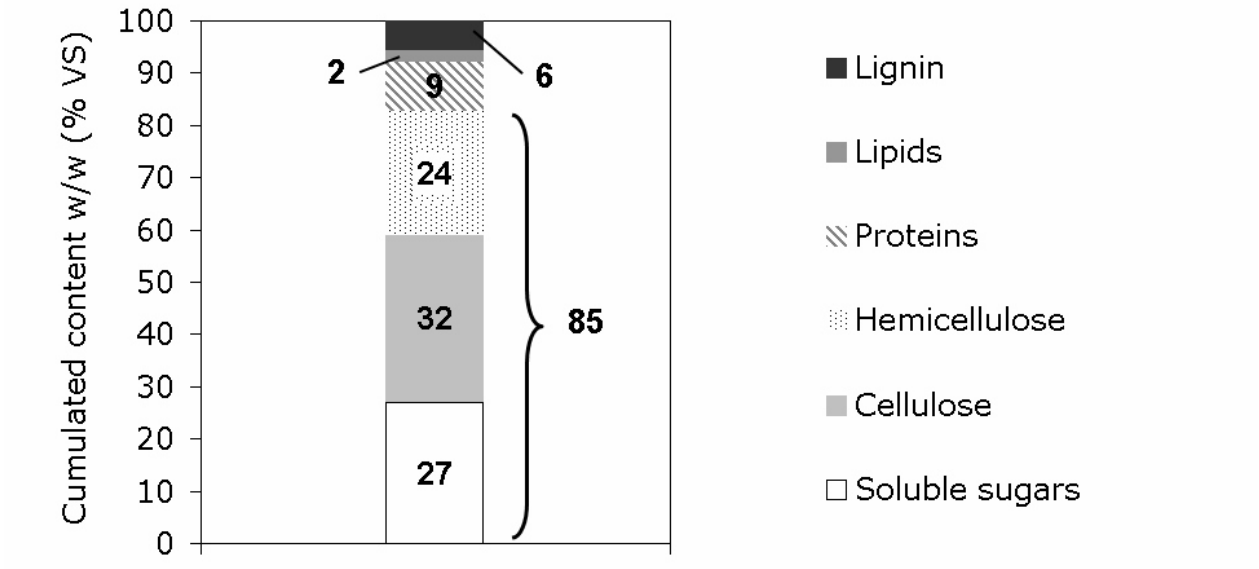


Figure 12. Chemical composition of rye silage.

Grass silage for batch digestion

Grass silage from extensive grassland (round bale silage, 2nd harvest, 2 harvests per year) was used as substrate in this experiment. This material originated from a field of the research station of Unterer Lindenhof, which belongs to the University of Hohenheim, and is located near to the city of Reutlingen in the region of Baden-Württemberg, Germany. It was harvested on September 14th, 2007. The previous harvest was on July 15th, 2007. The original ensiled material had a TS content of 67% and a VS content of 90% of the dry mass. A batch of 490 kg was left for 1 h in a mixing wagon to reduce fiber length from 5-10 cm to 0.5-1 cm. In the course of this process ~200 L of water and 3 L of pure propionic acid were added to ensure optimal conservation of the fine material, which was packed in impermeable plastic drums and stored at 4°C in a cooling chamber. This treatment resulted in a decrease of the TS content to 45%. Substrate composition was analyzed according to the Van Soest method (**Figure 13**). This substrate was considered to be more recalcitrant to anaerobic degradation than most energy crops used in biogas processes.

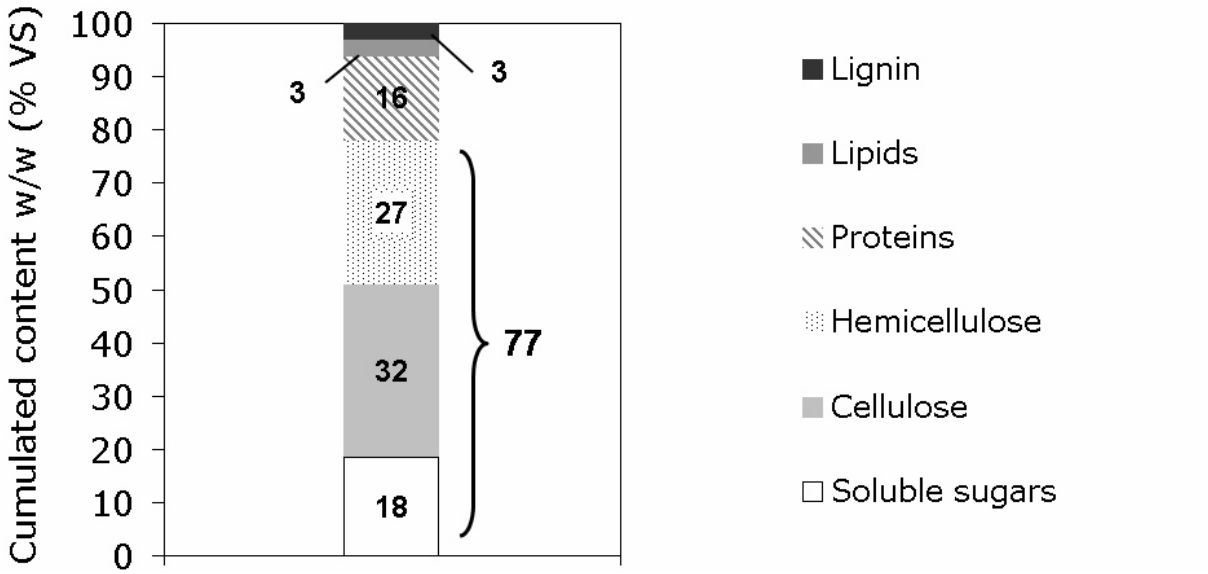


Figure 13. Chemical composition of grass silage.

Maize silage for semi-continuous acidogenic fermentation

Maize silage used in semi-continuous acidogenic fermentation was taken from a full-scale bunker silo at the research farm of Meiereihof at the University of Hohenheim. Maize silage had a particle size of 0.5-3 cm. The chemical composition of this substrate was not determined. The water content of samples was regularly measured and found to be variable between batches, presumably due to inhomogenous maize composition under practical conditions. Moreover, water migrated to the bottom of 30 L-storage containers, so that substrate lying close to the bottom was more wet.

Effluent of a biogas plant for batch digestion

Typical on-farm biogas plants operating in Germany consist of a series of two reactors, the first one converting primarily easily degradable material into methane, the second one digesting the most recalcitrant fraction of the substrate (Weiland 2006). Samples were taken from the first and second reactor of an full-scale agricultural biogas plant. The biogas plant was fed daily with dairy liquid and solid manure together with energy crops (**Table 15**).

Table 15. Substrates fed in the full-scale agricultural biogas plant.

Substrate	Share in digester's feed (% of total fresh weight)
Dairy liquid manure	33
Dairy solid manure	16
Maize silage	27
Grass silage	22
Whole crop silage (i.e. other silages)	3

Both reactors of the on-farm biogas plant were run under mesophilic temperature conditions, at 41°C and 39°C for first and second reactor, respectively (**Table 16**). The first and second reactor were operated in series: feed entered the first reactor while digested effluent leaving the first reactor entered the second reactor, where it was further fermented. Both reactors had similar capacities. HRTs are approximate values, which do not take into account additional volume left free by substrate degradation, which would increase real HRT. Moreover, the OLR value does not allow accurate appreciation of digesters load since the influence of substrate loading on process behavior depends on substrate composition, which is not known with sufficient accuracy.

Table 16. Process parameters of the full-scale agricultural biogas plant.

Reactor	Temperature (°C)	pH value	HRT (d)	OLR (kg VS/(m^3.d))	Reactor volume (m^3)
First reactor	41	7.8	35	6.5	1250
Second reactor	39	8.2	35	3.3	1250

Substrate composition of samples from the first and second reactor was analyzed according to Van Soest method (**Figure 14**). The first reactor contained raw substrate (crops and manure) which would have been anaerobically degraded during ~35 days, a period corresponding to the HRT. The second reactor continued the degradation of digested effluent coming out of the first reactor for another 35 days. The substrate fraction that was not covered by Van Soest analysis was as low as 2% and 0% for first and second reactor, respectively. The lignin content of reactor effluents was much higher than for raw plant substrates, and might be close to values of manure. Lignin is often considered to be non-degradable under anaerobic conditions (Tong et al. 1990).

Lignin enrichment occurred in the digested effluents, while degradable compounds were converted to methane. Comparing the contents of the first and second reactor, one notices that cellulose and hemicellulose degradability should be higher than lipids and proteins degradability. The remaining fraction of lipids and proteins increased from the first to the second reactor, while cellulose and hemicellulose fractions decreased. However, living bacterial biomass, which is not degradable, should account for an unknown share of total proteins. Biomass development in anaerobic digesters is assumed to be as low as 3-10% of the degraded substrate (Weiland 2001). The low share of hemicellulose as well as its ample decrease from first to second reactor tends to prove that part of the hemicellulose from plant fibers should be degraded faster than cellulose. However, hemicellulose is known to form a coating around cellulose fibers (Harris and Stone 2009). Therefore, cellulose and hemicellulose degradation might be interrelated.

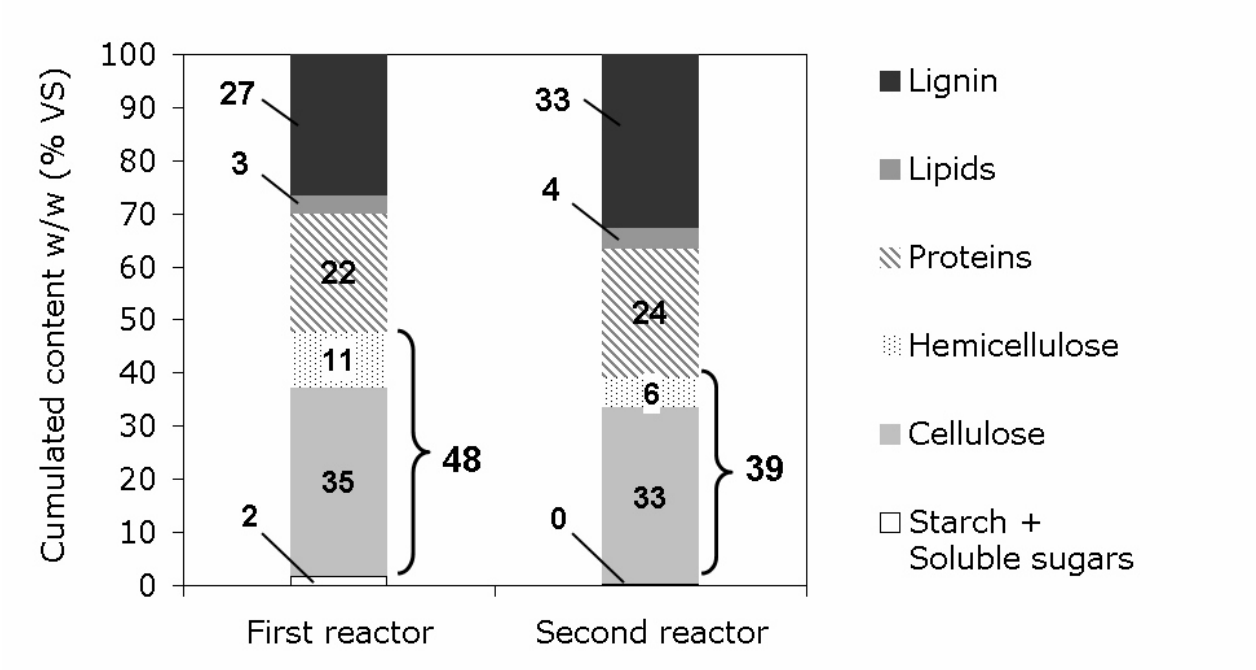


Figure 14. Chemical composition of reactor samples from the agricultural biogas plant.

4.7 Storage and conservation of enzyme and substrates

Substrates for batch experiments were stored at -20°C in freezers. Maize silage used in the continuous acidogenic experiment was manually compressed in impermeable plastic drums of 30 L capacity and kept in a cooling room at 4°C. Active enzyme products were stored at 4°C in a cooling room. Diluted active enzyme products were stored at 4°C and not for a duration longer than one week. Inactivated enzyme products were stored at -20°C in freezers.

5 Results

5.1 Enzymatic hydrolysis assays

pH values

Figure 15 compares reducing sugars contents in the watery medium of a buffer solution after 24-hour enzymatic hydrolysis of fresh maize straw at different pH values. Reducing sugar concentrations without enzyme addition to the substrate are also shown. Reducing sugar concentrations are reported as a percentage of substrate's volatile solids weight (VS) in order to evaluate hydrolysis rates.

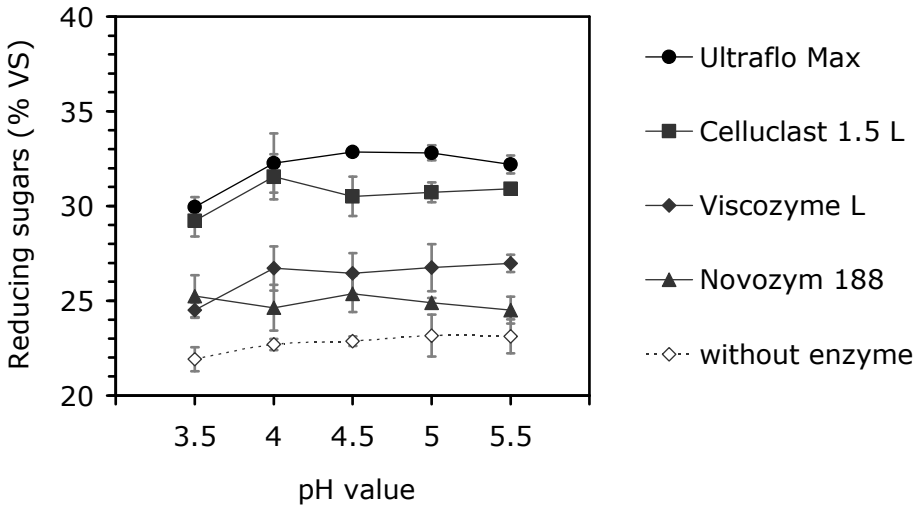


Figure 15. Reducing sugars release from maize straw at slightly acidic pH values [Hydrolysis of 1.8 g maize straw with 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer solution at 50°C for 24 hours. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

The pH range investigated (pH 3 to 5.5) is appropriate to most fungal enzyme systems developed for cellulose and xylan hydrolysis. Ultraflo Max was the most efficient enzyme in the pH range 3-5.5 (**Figure 15**).

The effect of Ultraflo Max and Novozym 342 on the hydrolysis of maize straw was further tested in the higher pH-range 6-8 (**Figure 16**). Ultraflo Max yielded ~35% sugars at pH 6.5. Its efficiency sharply decreased at pH 7, down to the value of the control without enzyme addition. Alternately, high efficiencies were expected from Novozym 342 at neutral pH. The supplementation of Novozym 342 released ~30% reducing sugars at pH 7, and the sugar release from this product rose to 35% at pH 7.5.

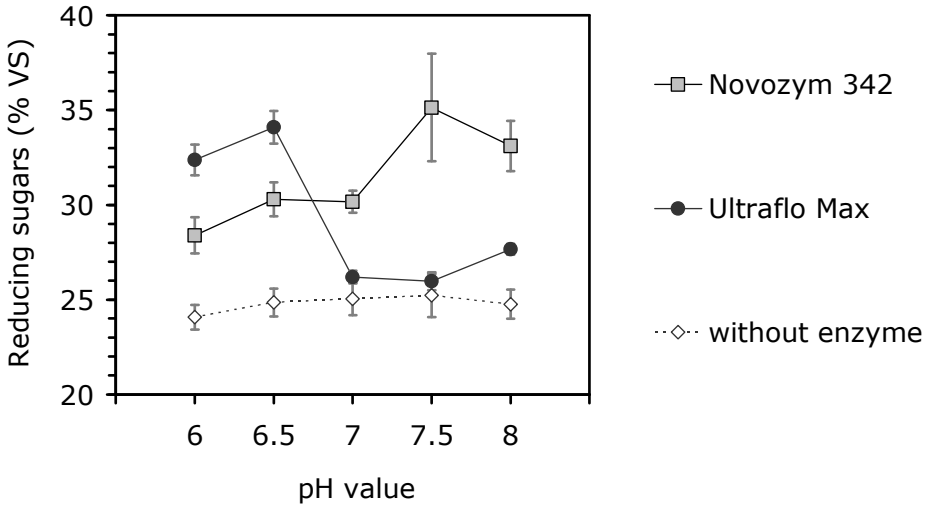


Figure 16. Reducing sugars release from maize straw at neutral pH values [Hydrolysis of 1.8 g maize straw with 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer (pH 6) and phosphate buffer (pH 6.5-8) solution at 50°C for 24 hours. Addition of sodium azide at 1 g/L in each sample for bacterial inhibition. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

Temperature

Figure 17 compares reducing sugars release at different temperatures, pH value being set to 4.5. Highest hydrolysis rates occurred in the range 45-55°C. At lower temperatures of 20 to 35°C, enzymatic activity was halved, but did not disappear. The standard temperature setting of 50°C for enzymatic hydrolysis trials is appropriate, since enzyme activity may show only a slight increase or even a decrease when temperature is increased to 55°C.

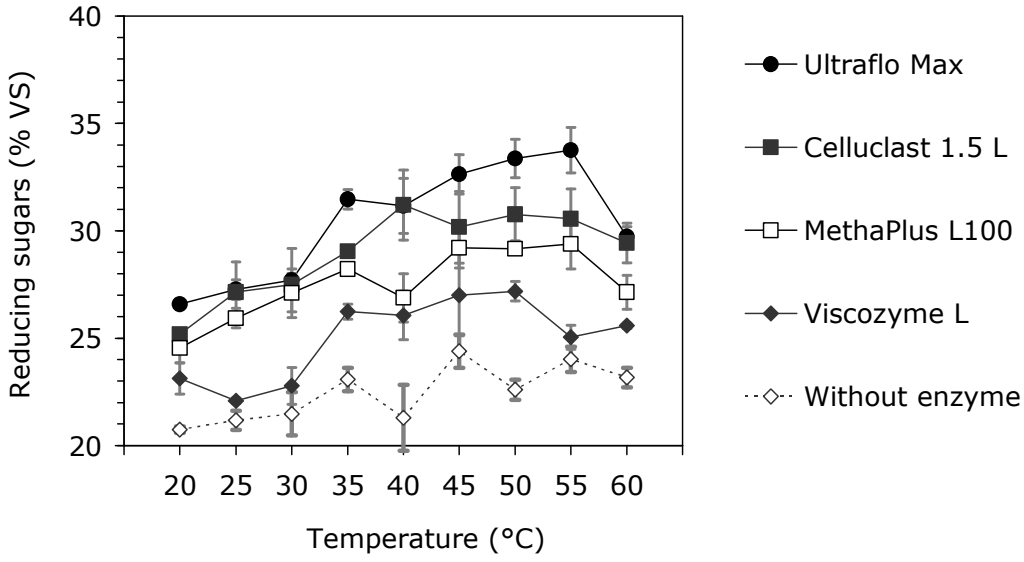


Figure 17. Reducing sugars release from maize straw at different temperatures [Hydrolysis of 1.8 g maize straw with 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer solution at pH 4.5 for 24 hours. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

Optimizing enzyme mixture

The release of reducing sugars remained relatively constant while increasing the share of Viscozyme L or Novozym 188 up to 50% of the total enzyme mix. The sugar yields decreased only when the share of Viscozyme L or Novozym 188 in the mix reached 75 or 100%. The mixture of Ultraflo Max together with Novozym 188, each with 50% in weight, yielded the highest amount of reducing sugars related to substrate VS (i.e. 34%). This mixture was chosen for further hydrolysis trials.

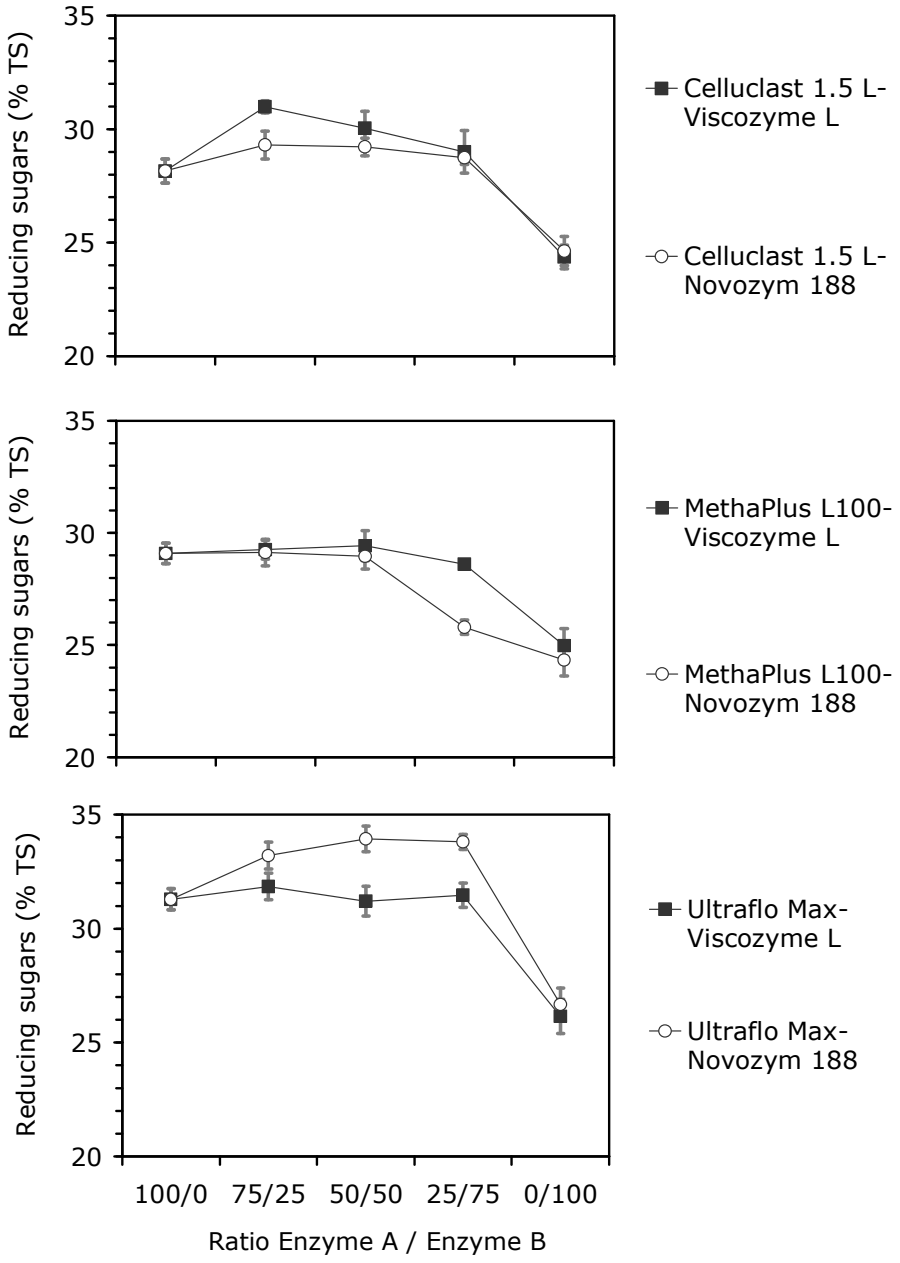


Figure 18. Reducing sugars release from maize straw with different enzyme mixtures [Hydrolysis of 1.8 g maize straw with 10 µL enzyme mixture in 10 mL of 0.1 M citrate buffer solution for 24 hours at pH 4.5 and 50°C. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

Hydrolysis duration

Figure 19 compares reducing sugars release at different hydrolysis durations, pH value being set to 4.5 and temperature to 50°C. The mixture of Ultraflo Max and Novozym 188, (each 50% fresh weight), which was found to be the most efficient in the previous experiment, was used. The curve of the sugar release (continuous line) partially matches a logarithmic correlation (slotted line), its equation and correlation coefficient are given on the chart. The logarithmic model assumes rapid sugar release immediately after enzyme addition and steadily decreasing sugar release velocity along hydrolysis time. Assuming that the logarithmic model is suitable and can be extended to longer hydrolysis times, there would not be much benefit in extending hydrolysis time: reducing sugar content would reach only 42% and 43% of substrate VS after 30 and 60 days of hydrolysis, respectively.

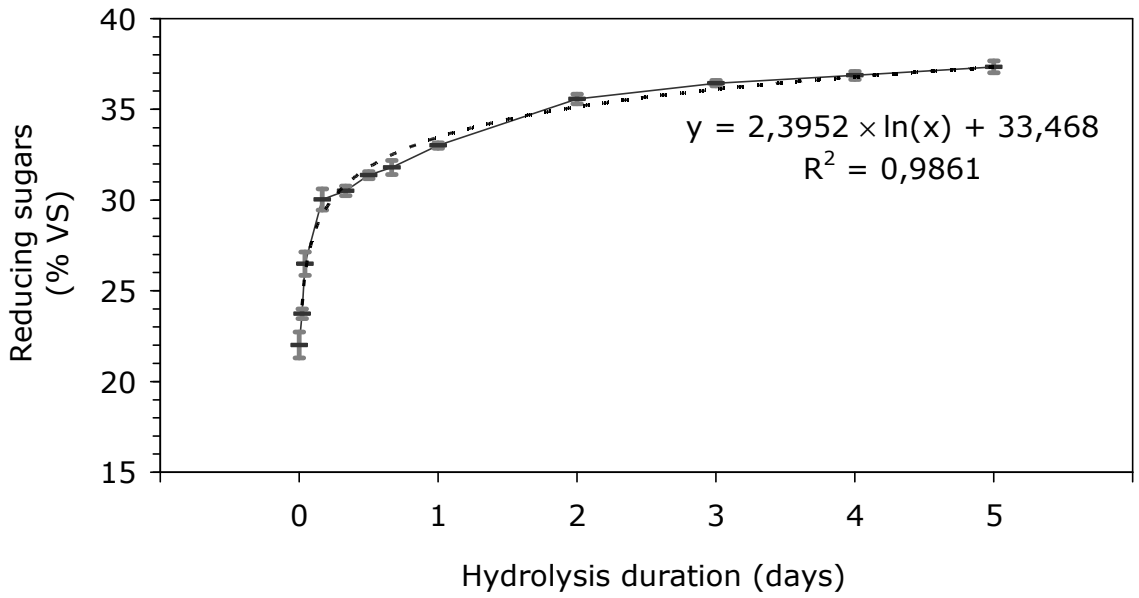


Figure 19. Reducing sugars release from maize straw at different durations [Hydrolysis of 1.8 g maize straw with 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer solution for 24 hours at pH 4.5 and 50°C. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

Enzyme loading

Figure 20 compares reducing sugars release at different enzyme concentrations, pH value being set to 4.5, temperature to 50°C and hydrolysis duration to 24 hours. The optimized enzyme mixture containing Ultraflo Max and Novozym 188, each 50% fresh weight, was used. As before, enzyme concentrations were expressed as fresh weight of enzyme product relative to the VS weight of the substrate.

Reducing sugar release did not increase linearly with enzyme concentrations. With regard to sugar release per unit weight of enzyme product added, lower enzyme concentrations seem to be specifically more efficient than higher ones. Therefore, concentrations higher than these investigated would presumably not lead to much higher sugar release. Taking 0% enzyme addition as reference (zero variant), additional sugar release from enzymatic hydrolysis of the substrate at 0.3% concentration of enzyme product was ~40% of the value reached with 3% of enzyme product, i.e. 10-fold higher concentration.

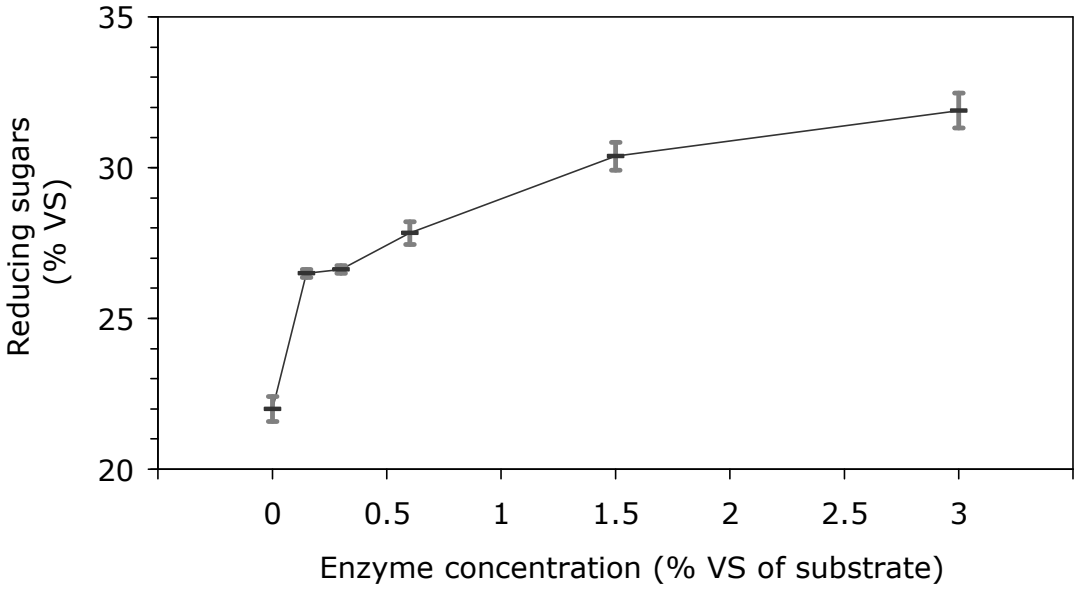


Figure 20. Reducing sugars release from maize straw at different enzyme concentrations [Hydrolysis of 1.8 g maize straw in 10 mL of 0.1 M citrate buffer solution for 24 hours at pH 4.5 and 50°C. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

Substrate loading

Figure 21 compares reducing sugars release at different substrate concentrations, where a fixed enzyme load was maintained. Hence enzyme to substrate ratio decreased with increasing substrate addition. The optimized enzyme mixture, i.e. Ultraflo Max and Novozym 188, each 50% fresh weight, was used. The weight of reducing sugars per unit of substrate VS decreased with increasing amounts of substrate. Therefore, no productivity gain could be achieved by increasing substrate concentration without concurrent increase of the enzyme load. Surprisingly, sugar yields without enzyme addition also decreased together with higher substrate input. This might be due to a saturation of the liquid phase with soluble sugars from the substrate.

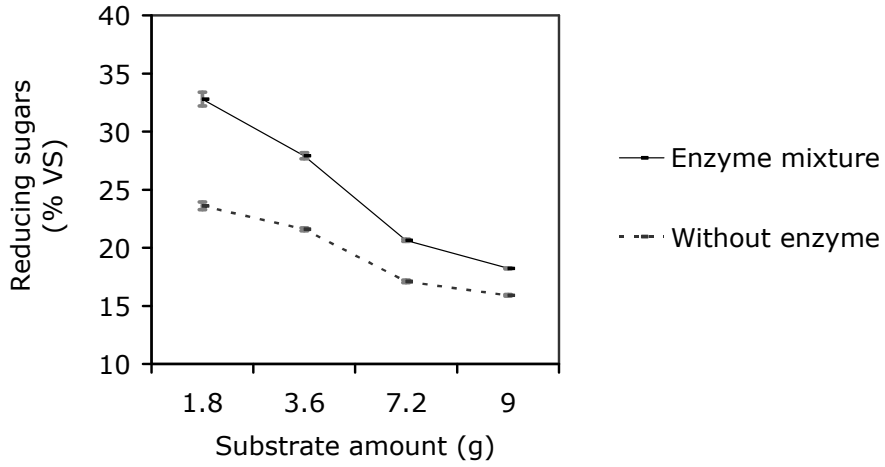


Figure 21. Reducing sugars release from maize straw at different substrate concentrations [Hydrolysis of maize straw with 10 µL enzyme mixture in 10 mL of 0.1 M citrate buffer solution for 24 hours at pH 4.5 and 50°C. n=3. Vertical arrows stand for SD. Numerical values cf. Appendix ***A 1***].

5.2 Batch digestion

Batch digestion of finely ground fresh maize

The course of cumulated methane production from anaerobic digestion of all variants of corncob, maize straw and manure inoculum with and without enzyme addition in the HBT process is represented in **Figure 22**. Enzyme addition at concentrations of both 0.13 and 1.3 g/kg substrate VS had few influence on the patterns of the curves. Hence enzyme addition did not bring any improvement to methane production kinetics.

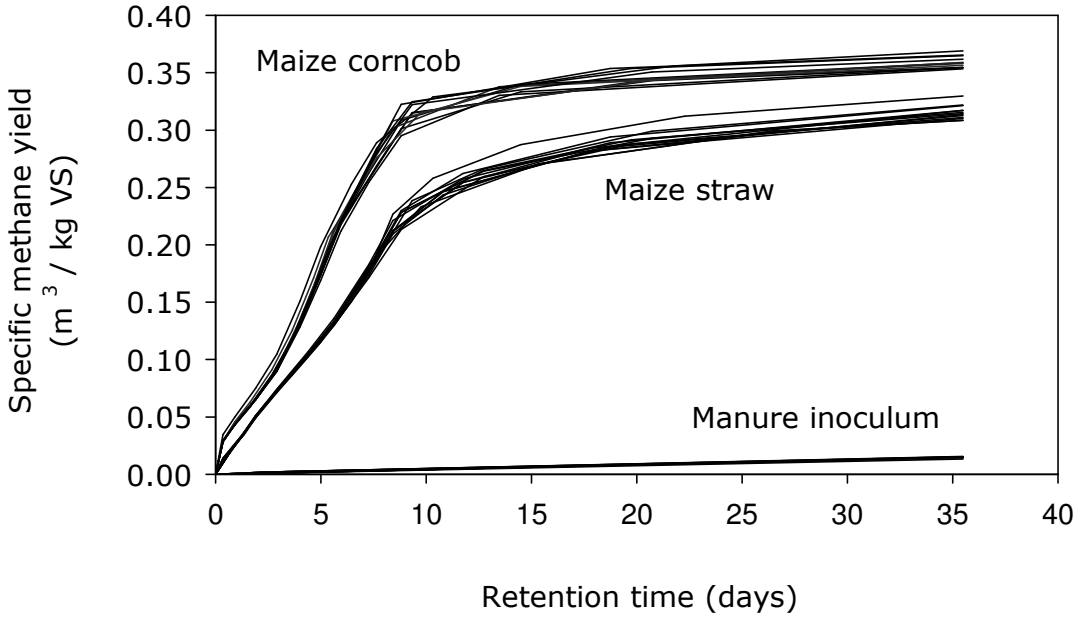


Figure 22. Methane production of corncob, maize straw, and manure inoculum [All variants with and without enzyme addition. Average values. n=3. Digestion temperature 37°C. Numerical values cf. Appendix ***A 4***].

Specific methane yields after 35 days of digestion reached 0.314 m^3/kg VS and 0.353 m^3/kg VS for maize straw and corncob without enzyme addition, respectively. Standard deviations of the methane yields after 35 days were between 0.2 and 3.6% for corncob and maize straw, and between 0.4 and 8.5% for manure inoculum (**Table 17**). There was no important increase of the final methane yields through enzyme addition compared with the variant without enzyme addition. The maximal increase of the methane yield was 5.0% for maize straw and 4.6% for corncob, respectively. Surprisingly, in some cases, the addition of inactivated enzymes seemed to have positive effects on the methane yield. A Student t-test revealed a significant ($p<0.05$), but not very significant ($p<0.01$) difference of some variants with enzyme addition compared with enzyme-free variants.

Due to the slight effect noticed with Goldferm-Mais, a complementary experiment, described in Appendix ***A 5 Complementary experiment with the enzyme product Goldferm-Mais***, was run with this enzyme product. The experiment did not confirm the increase of the methane yield of maize straw initially noticed, and revealed an experimental bias that may have generated this "false positive" result.

Table 17. Effect of enzyme additives on final methane yields of maize straw and corncob [Final values after 35 days of digestion at 37°C. Average values. n=3. * Significant difference to a tolerance degree of 5% ($p<0.05$) compared to the variant without enzyme addition. Numerical values cf. Appendix ***A 4***].

Enzyme dosage	Enzyme added	Change of the methane yield (%)	
		Maize corncob	Maize straw
Without enzyme	-	0.0 - Reference	0.0 – Reference
1.3 g / kg VS inactivated	MethaPlus L100	Not determined	+2.5
	Goldferm-Mais	+3.5	+0.1
	Genencor Laminex BG	+2.5	+2.3
	Novozym 188	+3.4 *	-1.7
0.13 g / kg VS active	MethaPlus L100	+1.6	-0.1
	Goldferm-Mais	+0.4	-1.1
	Genencor Laminex BG	+0.9	+1.0
	Novozym 188	+0.4	-0.4
1.3 g / kg VS active	MethaPlus L100	+1.7	-1.7
	Goldferm-Mais	+4.6 *	+5.0 *
	Genencor Laminex BG	+0.1	+1.0
	Novozym 188	+1.0	+0.5

Batch digestion of coarse rye silage

The course of cumulated methane production of all variants of rye silage with and without enzyme addition, as well as manure inoculum in stirred glass reactors of 2 L within the retention time of 35 days is presented in **Figure 23**.

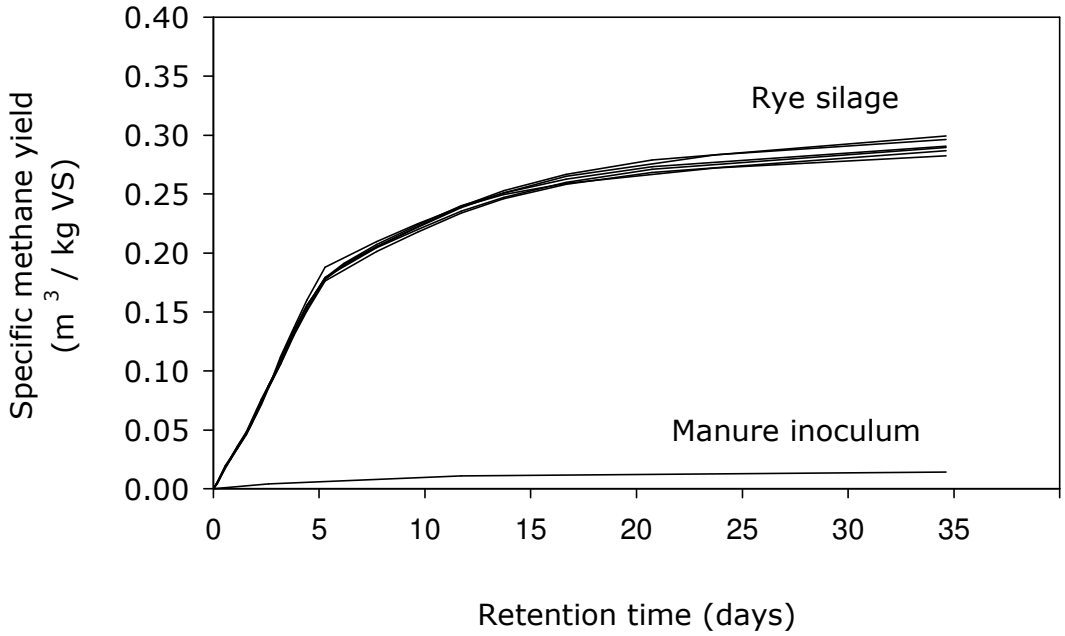


Figure 23. Methane production of coarse rye silage and manure inoculum [All variants with and without enzyme addition. Average values. $n \geq 2$. Digestion temperature 37°C. Numerical values cf. Appendix ***A 4***].

Standard deviations of final methane yields of rye silage after 35 days ranged from 2.6 to 3.4%. No significant increase or decrease of final methane yields was found compared with enzyme-free variants. The extent of increase or decrease of cumulated methane yields is shown in **Table 18**.

Table 18. Effect of enzyme additives on final methane yields of coarse rye silage [Final values after 35 days of digestion at 37°C. Average values. $n \geq 2$. Applied dosage of each component in the mixture: 0.17 g/kg VS of enzyme product. Numerical methane yield values cf. Appendix ***A 4***].

Enzyme combination	Change of the methane yield of rye silage (%)
Without enzyme	0.0 – Reference
Pectinase	-2.9
Pectinase + Laccase	-1.0
Cellulase + Pectinase + Laccase	+1.1

Batch digestion of finely ground rye silage

The course of cumulated methane production of all variants of rye silage in HBT process with two different inoculums (i.e. manure inoculum and sewage sludge) is shown in **Figure 24**. Methane production in the HBT process shows different patterns according to the type of inoculum applied for substrate digestion. After ~20 days, differences in the digestion's behavior between both inoculums tended to disappear. Although sewage sludge itself had higher methane production, this was apparently not correlated to a higher efficiency as an inoculum source. In the beginning of the trial, methane was produced at a faster rate when sewage sludge inoculum was used for substrate digestion, but this tendency reversed after 8 days of digestion.

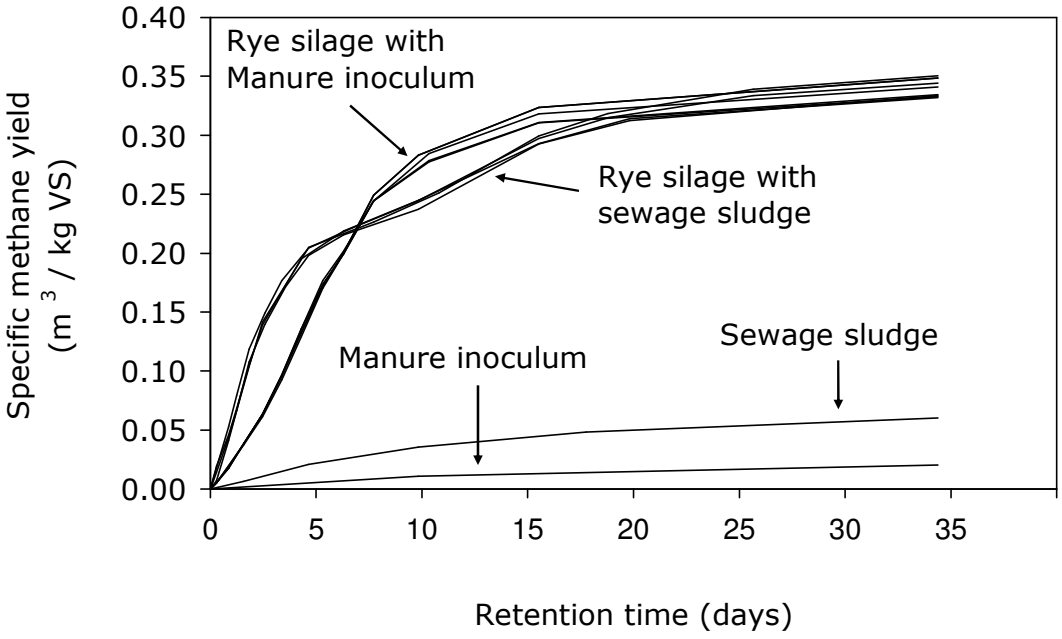


Figure 24. Methane production of finely ground rye silage and manure inoculum [All variants with and without enzyme addition. Average values. n=3. Digestion temperature 37°C].

After 35 days of digestion, final methane yields of manure inoculum and sewage sludge reached 0.020 and 0.087 m^3/kg VS, respectively. Final methane production from finely ground rye silage after 35 days was 0.341 and 0.332 m^3/kg VS with manure inoculum and sewage sludge, respectively. Standard deviations of the methane yields of the replicates of rye silage in the HBT process were between 0.6 and 3.4%.

Table 19 shows the increase in methane yields of rye silage after 35 days of digestion through enzyme addition. A Student test (t-test) of the methane yields did not prove any significant difference (at $p<0.05$) compared with enzyme-free variants. Results bring evidence that no significant effects should be expected from enzyme products under the standard experimental conditions of BMP assays.

Table 19. Effect of enzyme additives on final methane yields of finely ground rye silage [Final values after 35 days of digestion at 37°C. Average values. n=3. Applied dosage: 0.13 g/kg VS of each enzyme product, and 13 g/kg VS for 100-fold concentration. Numerical methane yield values cf. Appendix ***A 4***].

Enzyme state	Enzyme combination	Change of the methane yield (%)	
		Digestion with manure inoculum	Digestion with sewage sludge
	None	0.0 - Reference	0.0 - Reference
Inactivated 0.13 g/kg VS	Pectinase	+0.6	+3.2
	Pectinase + Laccase	+3.2	-1.0
	Cellulase + Pectinase + Laccase	-1.5	+1.0
Active 0.13 g/kg VS	Pectinase	-2.4	+3.6
	Pectinase + Laccase	-1.9	+2.2
	Cellulase + Pectinase + Laccase	-1.9	+0.5
Active 13 g/kg VS	Cellulase + Pectinase + Laccase with 100-fold concentration	+2.3	+5.4

Batch digestion of coarse grass silage with weak inoculum

Methane production of coarse grass silage with weak inoculum started after a lag phase of 7 days, presumably due to the weakness of the inoculum (**Figure 25**). Subsequently, methane production from the variant with enzyme addition became significantly higher than from the variant without enzyme addition until day 25. At this point, both curves converged again. Hence only the initial rate of methane formation was increased by enzyme addition, while final values of the methane yield were similar, at ~0.190 m^3/kg VS. The slope of the curves suggested that maximal values of the methane yield had not yet been reached within the 35-day duration of the experiment.

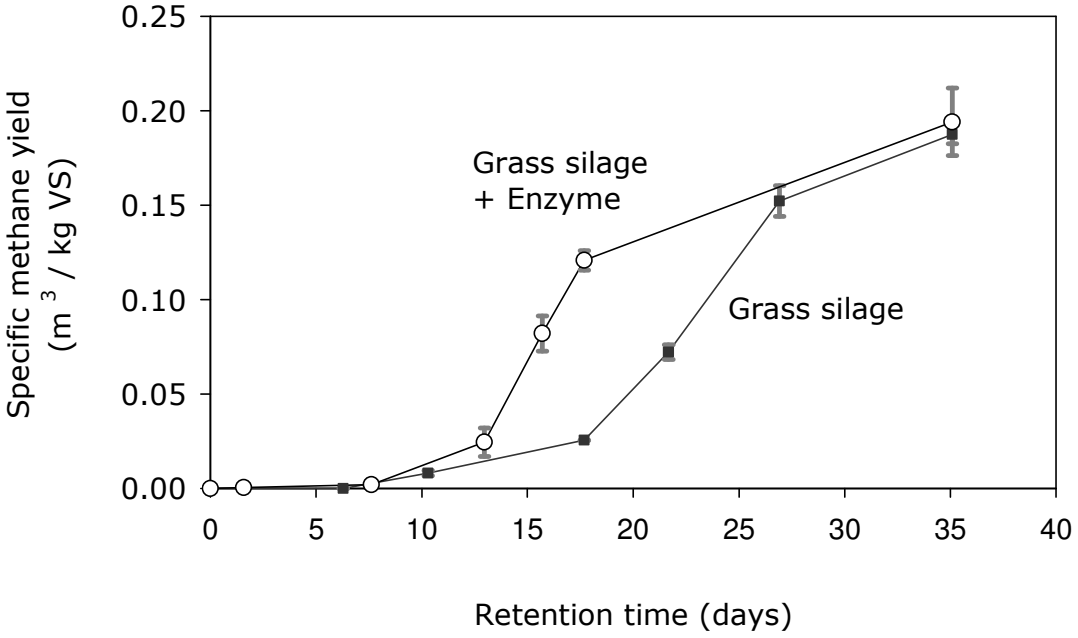


Figure 25. Methane production of coarse grass silage incubated with weak inoculum [Average values. n=2. Vertical arrows stand for SD. Enzyme dosage of MethaPlus L 100 in variant with enzyme addition: 11 g/kg VS. Digestion temperature 37°C. Numerical values cf. Appendix ***A 4***].

Batch digestion of coarse grass silage with diluted inoculum

As shown in **Figure 26**, at both 3-fold and 5-fold dilutions of the standard inoculum, enzyme addition had no significant effect on the methane yield of coarse grass silage, even at the higher enzyme dosage of 7 g/kg VS.

Significant effects of enzyme additives occurred solely at the highest dilution of the inoculum (10-fold). With inoculum applied at the highest dilution, enzyme addition at higher dosage slightly increased methane production velocity at the beginning of the digestion period, while enzyme addition at both low and high doses dramatically increased the final methane yield at the end of the digestion period, from ~0.210 to ~0.290 m^3/kg VS, i.e. ~40% increase. However, due to the unconventional assay conditions employed, the experiment should be repeated in order to confirm these effects.

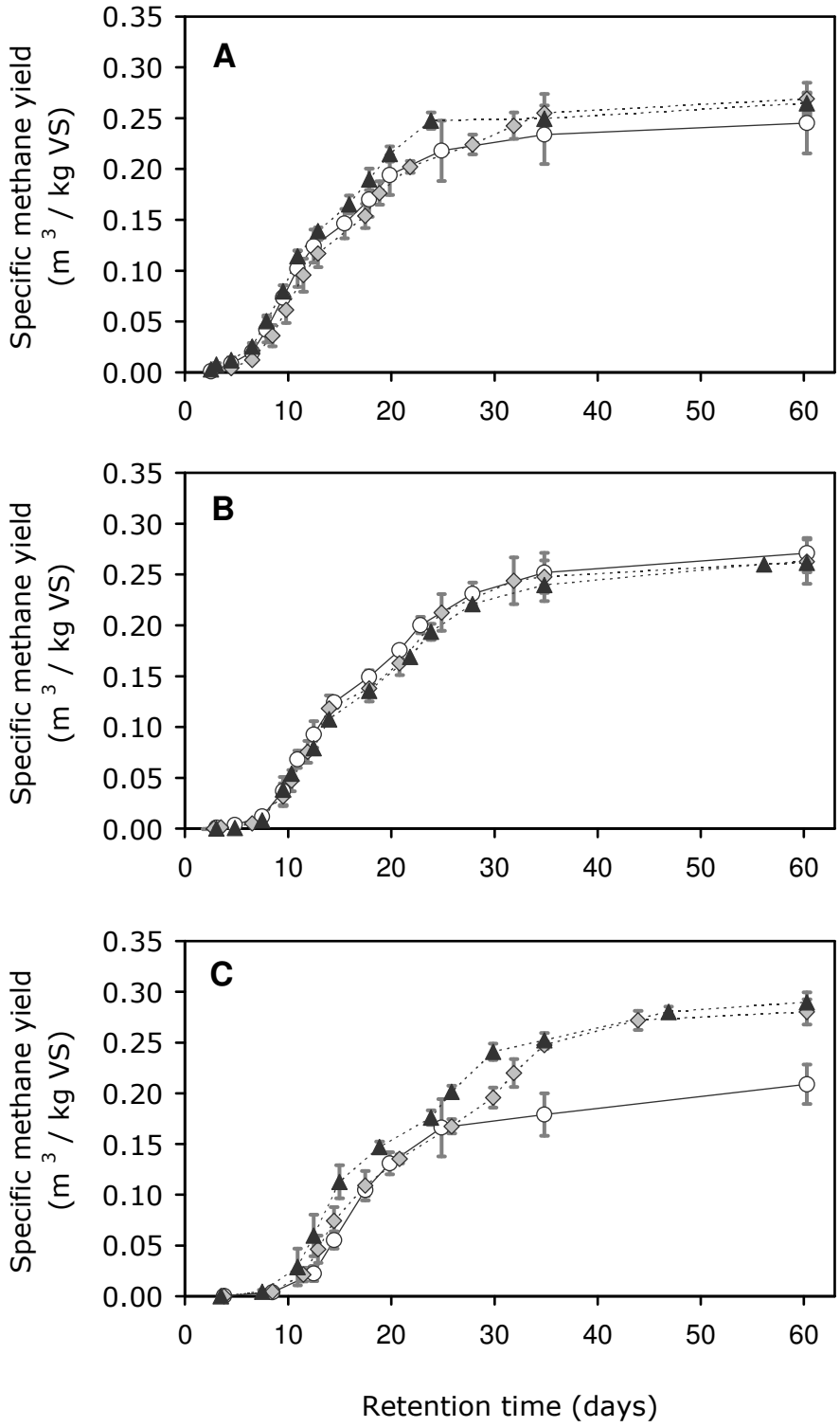


Figure 26. Methane production of coarse grass silage incubated with diluted inoculum [Average values. n=2. Vertical arrows stand for SD. Standard inoculum diluted 3-fold (A), 5-fold (B) and 10-fold (C). Addition of enzyme product MethaPlus L100 at low (0.7 g/kg VS) and high enzyme dosage (7 g/kg VS). Numerical values cf. Appendix ***A 4***].

Batch digestion following enzymatic hydrolysis step

Enzymatic hydrolysis step

The enzyme mixture Genencor Laminex BG / Novozym 188 (80%/20% w/w) was selected. This mixture was found to be the most active in a preliminary experiment (cf. Appendix ***A 3 Experiment to optimize the hydrolysis step***).

The inactivation procedure successfully suppressed enzyme activity. Reducing sugars released from the application of inactivated enzymes were not higher than sugar release without enzyme addition (**Figure 27**). Interestingly, the additional monosaccharide release following enzymatic hydrolysis seemed to be exclusively composed of glucose. Compared to the reference without enzyme addition, no additional release of xylose, galactose, mannose, fructose, or arabinose was noticed.

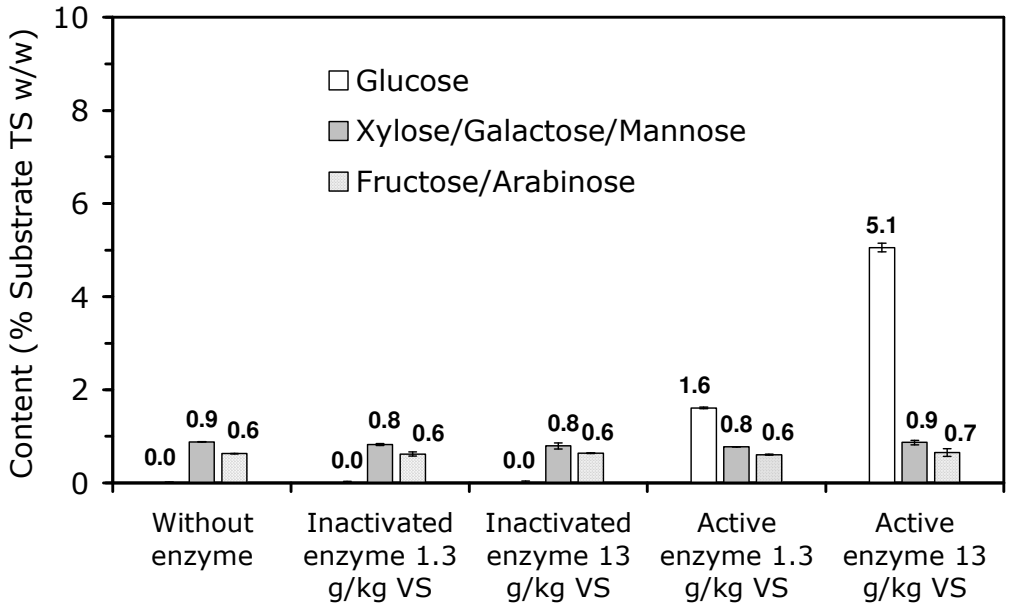


Figure 27. Concentration of soluble monosaccharides after enzymatic hydrolysis of ensiled maize straw as a pretreatment step before anaerobic digestion [Hydrolysis of 1.8 g ensiled maize straw in 10 mL of water at pH 4.5 and 45°C for 24 hours. Application of enzyme mixture Genencor Laminex BG / Novozym 188, respectively 80% and 20% (w/w). n=2. Vertical arrows stand for SD].

Anaerobic digestion step

Final methane yields of ensiled maize straw without enzyme addition after 35 days were 0.354 and 0.340 m^3/kg VS for single-step (enzyme addition directly into the biogas reactor) and two-step process (biogas production following separate enzymatic treatment), respectively. According to Mukengele and Oechsner (2007), methane production obtained from batch digestion trials (BMP assays) with fresh and ensiled plant material should be quite similar following proper application of correction factors. Therefore, a bias either in substrate analysis, in VS value determination or in the correction formula is suspected, since final methane yields in the range 0.340-0.350 m^3/kg VS were much higher than the value of 0.314 m^3/kg VS for fresh maize straw without enzyme addition recorded in previous trials with similar material.

Only slight variations were noticed in the final values of methane yields after 35 days of digestion at 37°C through enzyme addition (**Table 20**). According to a Student t-test, there was no significant difference between final values of methane yields at $p<0.05$.

Table 20. Effect of enzyme additives on final methane yields of ensiled maize straw in single-step and two-step processes [Final values after 35 days of digestion at 37°C. Average values. n=3. Application of enzyme mixture Genencor Laminex BG /Novozym 188 (80% / 20% w/w). Numerical methane yield values cf. Appendix ***A 4***].

Enzyme dosage	Enzyme state	Change of the methane yield (%)	
		Single-step process	Two-step process with enzymatic pretreatment
Without enzyme		0.0 - Reference	-4.1
1.3 g / kg VS	Inactivated	-1.8	-0.1
	Active	+0.1	+2.0
13 g / kg VS	Inactivated	+0.0	+0.6
	Active	+0.6	+0.2

Methane production patterns for all variants in both single-step and two-step processes were quite homogenous (**Figure 28**). Apparently enzymatic pretreatment did not affect methane production kinetics.

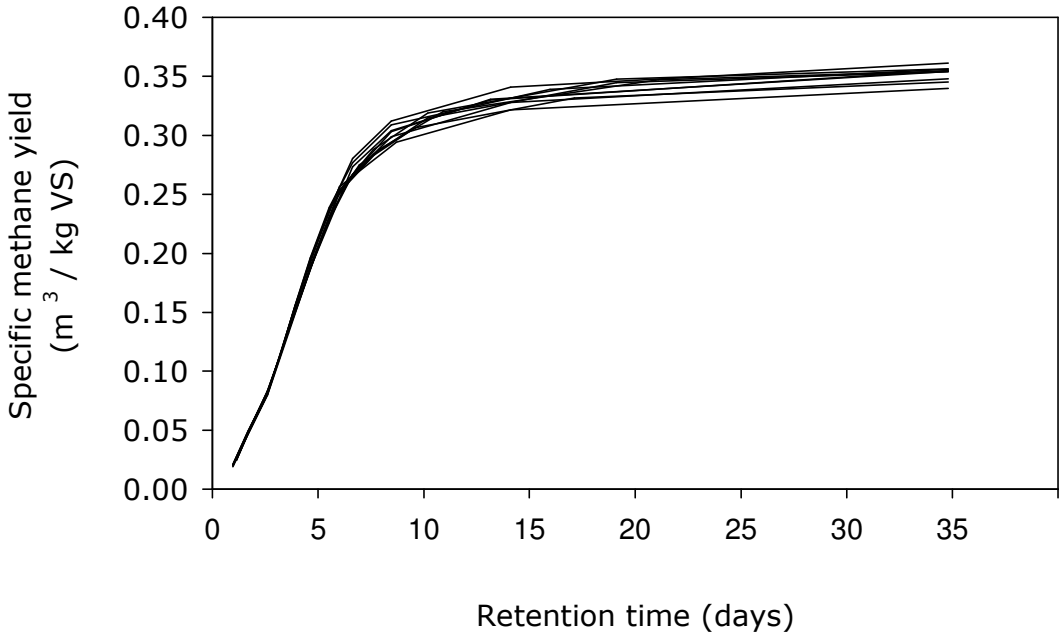


Figure 28. Methane production of ensiled maize straw [Digestion temperature: 37°C. Average values. n=3].

Batch digestion of effluent from a biogas plant

Batch digestion at mesophilic temperature

Methane yields without enzyme addition expressed in relation to fresh weight and to volatile solids of substrate after 87 days are shown in **Table 21**. The final methane yield decreased from the first reactor to the second reactor to an extent of 46% relative to substrate FW and of 31% relative to substrate VS. The organic mass (VS) content of the sample from the second reactor was 23% lower than in the first reactor.

Table 21. Relationship between volatile solids content of reactor samples and methane production without enzyme addition [* Percent decrease between values of first and second reactor].

	Reactor sample content			**Methane yield after 87 days**	
	TS (% FW)	**VS (% TS)**	**VS (% FW)**	**Related to FW of reactor sample (m^3/kg FW)**	**Related to VS of reactor sample (m^3/kg VS)**
First reactor	10.4	78	8.1	0.0137	0.170
Second reactor	8.5	73	6.2	0.0073	0.118
Decrease* (%)	18	6	23	46	31

The decrease of methane yield relative to substrate VS might be related to increased organic matter recalcitrance. According to Van Soest analysis, lignin content in the second reactor was 18% higher than in the first reactor. Lignin is a fraction of organic matter that is considered not to be degradable in anaerobic environment (Tong et al. 1990).

The course of methane production for all variants of digested effluent (both with and without enzyme added) from first and second reactor in HBT process are shown in **Figure 29**. According to the curves, substrate degradation occurred at a very constant rate. Methane production of substrate from the first digester occurred at a rapid rate between day 0 and day 8. Following this start-up phase, methane production velocity diminished continuously. The pattern of methane production from the second digester was more uniform. To the end of the digestion period, substrate degradation velocity tended to be linear. As substrate was degraded along the digestion process, methane production rate diminished, while the methane production curve evolved from a parabolic form towards a more linear form. An almost linear increase of the methane yield at the end of the digestion period proved that substrate degradation was not fully completed within the relatively long digestion period of 87 days. Apparently, tremendously high digestion durations would be required for reaching complete substrate degradation.

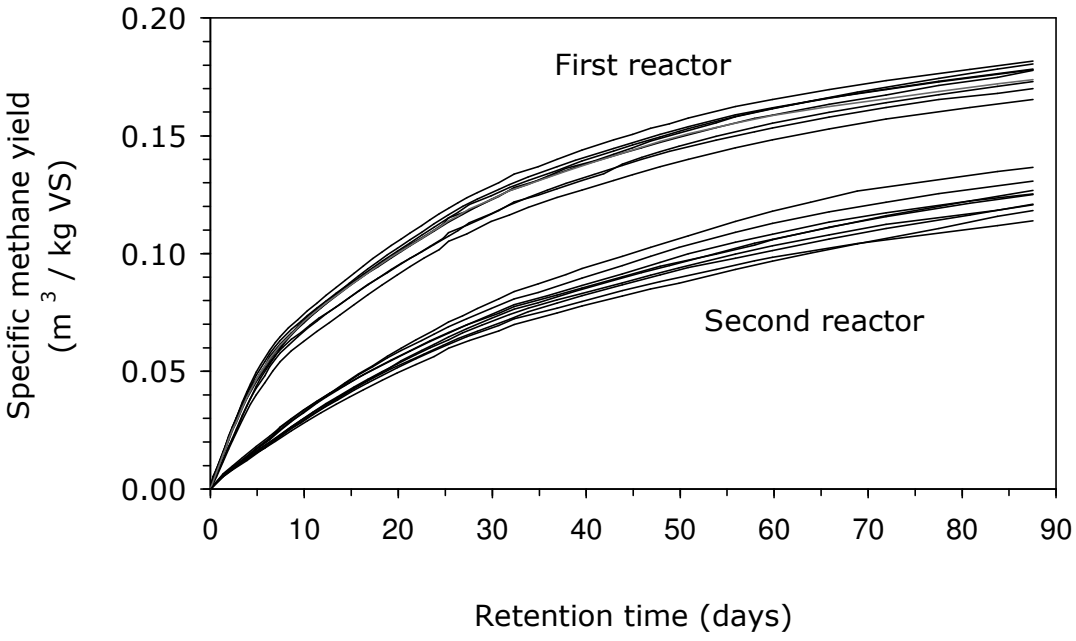


Figure 29. Methane production of reactor samples digested at 37°C [Average values. n=3. Enzyme products: MethaPlus L100 or Novozym 342/Pulpzyme HC (50/50 w/w). Enzyme dosages: 0.2 (low) or 2 g/kg FW (high). Dosage divided into 20 additions performed every ~3 days from the start of the digestion period].

The standard deviation of the final methane yields ranged between 0.6 und 8.2%. **Table 22** shows the increase in methane yields of reactor samples after 87 days of digestion through enzyme addition. The first reactor with Novozym 342/Pulpzyme HC enzyme mixture at high concentration (i.e. 2 g/kg substrate FW) produced 15.7% more methane than the enzyme-free variant. According to a Student t-test this increase was significant (at $p<0.05$). However, there was no significant difference compared with the inactivated enzyme variant, whose methane yield was 10.7% higher than the enzyme-free variant.

At high enzyme dosage, the increase in methane yields in relation to the enzyme-free variant may be due to the biodegradation of enzyme proteins as well as organic components of enzyme products. Such effects become more visible as enzyme dosage is high, and concomitantly the amounts of methane released from the fibre-rich, predigested substrate is low due to its limited biodegradability.

Table 22. Effect of enzyme additives on methane yields of reactors samples at mesophilic temperature [Final values after 87 days of digestion at 37°C. Average values. n=3. Numerical methane yield values cf. Appendix ***A 4***. * Significant difference to a tolerance degree of 5% ($p<0.05$) relative to the variant without enzyme addition, but no significant difference relative to the corresponding variant with inactivated enzyme].

Reactor	Enzyme state	Change in methane yield (%)			
		Addition of MethaPlus L100		Addition of Novozym 342 - Pulpzyme HC mixture	
		Low dosage 0.2 g/kg FW	High dosage 2 g/kg FW	Low dosage 0.2 g/kg FW	High dosage 2 g/kg FW
First reactor	Active	+1.8	-2.7	+5.3	+6.2
	Inactivated	+4.6	+6.9	+4.7	+4.9
Second reactor	Active	+2.9	+7.3	+2.1	+15.7 *
	Inactivated	-3.6	+6.0	+2.4	+10.7

Batch digestion at psychrophilic temperature

The course of methane production for all variants of digested effluent from the first and second reactors in the HBT process at psychrophilic temperature are shown in **Figure 30**.

Considering methane production rate, one could distinguish between three periods. Between day 0 and 60, methane production occurred at a constant rate. Between day 60 and 100, methane production rate diminished. Between day 100 and 180, methane production rate increased again. The average room temperature was 13, 14 and 18°C in the periods 0-60, 60-100, and 100-180 days, respectively. The increase in methane production rate for the last period was probably related to an increase in room temperature.

Cooler temperatures at the beginning are due to the fact that the experiment began during the winter period. Temperatures later increased at the end of the winter, as the spring season began. Although differences in average temperatures for the three periods previously mentioned was not higher than 5°C, this temperature gap had a tremendous influence on methane production kinetics. This statement is very interesting for evaluating methane production patterns of unheated digesters. Average winter temperatures of 13°C are probably not sufficient to maintain an efficient digestion process. Following that statement, methane production from unheated digesters in Germany would probably be drastically reduced during the winter season.

The addition of Novozym 342/Pulpzyme HC mixture to samples from the second reactor increased the methane yield by 21.8% (**Table 23**). A Student t-test revealed a highly significant ($p<0.01$) difference of this enzyme mixture compared to enzyme-free variants. However, this effect was probably related to the biodegradation of enzyme products and their conversion into biogas, since no control variant was run with the same concentration of inactivated enzyme. No significant difference ($p<0.05$) was found between the variant with the enzyme product MethaPlus L100 and the enzyme-free variant.

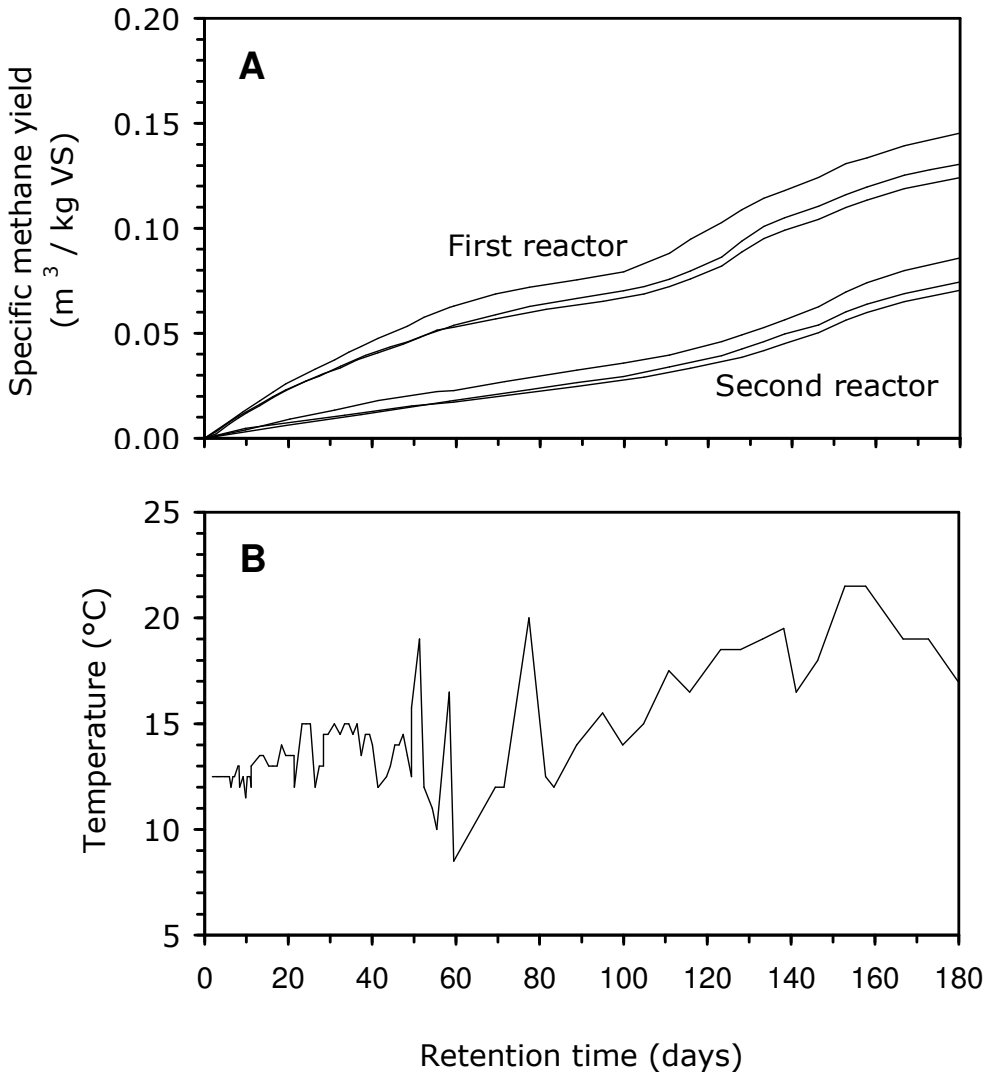


Figure 30. Methane production (A) and temperatures (B) of reactor samples digested at room temperature [Average values. n=3. Enzyme products: MethaPlus L100 or Novozym 342/Pulpzyme HC (50/50 w/w). Enzyme dosage: 2 g/kg FW. Dosage divided into 20 additions performed every ~3 days from the start of the digestion period].

Table 23. Effect of enzyme additives on methane yields of reactors samples at psychrophilic temperature [Final values after 180 days of digestion. n=3. Enzyme dosage: 2 g/kg FW. Numerical methane yield values cf. Appendix ***A 4***. ** Significant difference to a tolerance degree of 1% ($p<0.01$) relative to the variant without enzyme addition].

Reactor	Addition of MethaPlus L100	Addition of Novozym 342 - Pulpzyme HC mixture
First reactor	+5.2	+17.1
Second reactor	+5.6	+21.8 **

Comparison between psychrophylic and mesophilic temperatures

Although digestion duration in the psychrophilic trial was much higher than in the mesophilic trial (180 days instead of 87 days), final values of methane yields were still lower in the psychrophilic trial (**Table 24**). Methane yields at psychrophilic temperature were 27% and 41% lower than at mesophilic temperature regarding the first and second reactor, respectively. The more important decrease of the methane yield for the second reactor shows that anaerobic digestion of hardly degradable compounds should be more affected by the decrease in temperature. Following this hypothesis, the influence of temperature on anaerobic digestion under stable conditions would depend on substrate's degradability.

Table 24. Final methane yields of reactor samples at psychrophilic and mesophilic temperatures without enzyme addition [Final values of enzyme-free samples. n=3. Mesophilic digestion: temperature of 37°C, digestion time of 87 days. Psychrophilic digestion: room temperature, digestion time of 180 days].

Reactor	Final value of the methane yield (m^3/kg VS)	
	Mesophilic digestion	Psychrophilic digestion
First reactor	0.170	0.124
Second reactor	0.118	0.070

Considering absolute change instead of relative change in methane yields could be more adequate while comparing enzyme addition in different digestion conditions. As shown in **Table 25**, the absolute increase in methane yield at psychrophilic temperature (room temperature) was not much higher than at mesophilic temperature. Only the higher enzyme dosage is presented because it had the highest effect on methane yields.

Table 25. Comparison of the effects of enzyme additives on methane yields of reactor samples at psychrophylic and mesophilic temperatures [Final values after completion of the digestion period. n=3. High enzyme dosage: 2 g/kg FW. Mesophilic digestion: temperature of 37°C, digestion time of 87 days. Psychrophilic digestion: room temperature, digestion time of 180 days].

Reactor	Absolute change in methane yield (m^3/kg VS)			
	Addition of MethaPlus L100		Addition of Novozym 342 - Pulpzyme HC mixture	
	Mesophilic digestion	Psychrophylic digestion	Mesophilic digestion	Psychrophylic digestion
First reactor	-0.005	+0.006	+0.011	+0.021
Second reactor	+0.009	+0.004	+0.019	+0.015

5.3 Semi-continuous acidogenic digestion

pH values

pH values were measured daily on every digester (**Figure 31**). Following high lime addition to the beginning of the trial, pH rose up to 6.2 at day 5. After reaching this peak, pH continuously decreased until an equilibrium state was reached around day 30. pH values for both 4-digester groups (with and without enzyme addition) are shown along the whole trial period, differences between these two groups occur only after enzyme addition started, i.e. from day 51, marked as **(b)**. Between day 55 and day 58, digesters with enzyme addition were ~0.3 pH units lower than digesters without enzyme addition. However, after reduction of enzyme addition on day 66 **(c)**, this effect progressively disappeared, and both groups converged again to the same pH levels. The following hypotheses can be drawn:

1. Enzyme addition (enzyme product MethaPlus L100) increased the release of organic acids, thus enhancing the acidity in the fermentation medium.
2. This effect occurred only at high enzyme dosage (i.e. 10 g/kg VS).

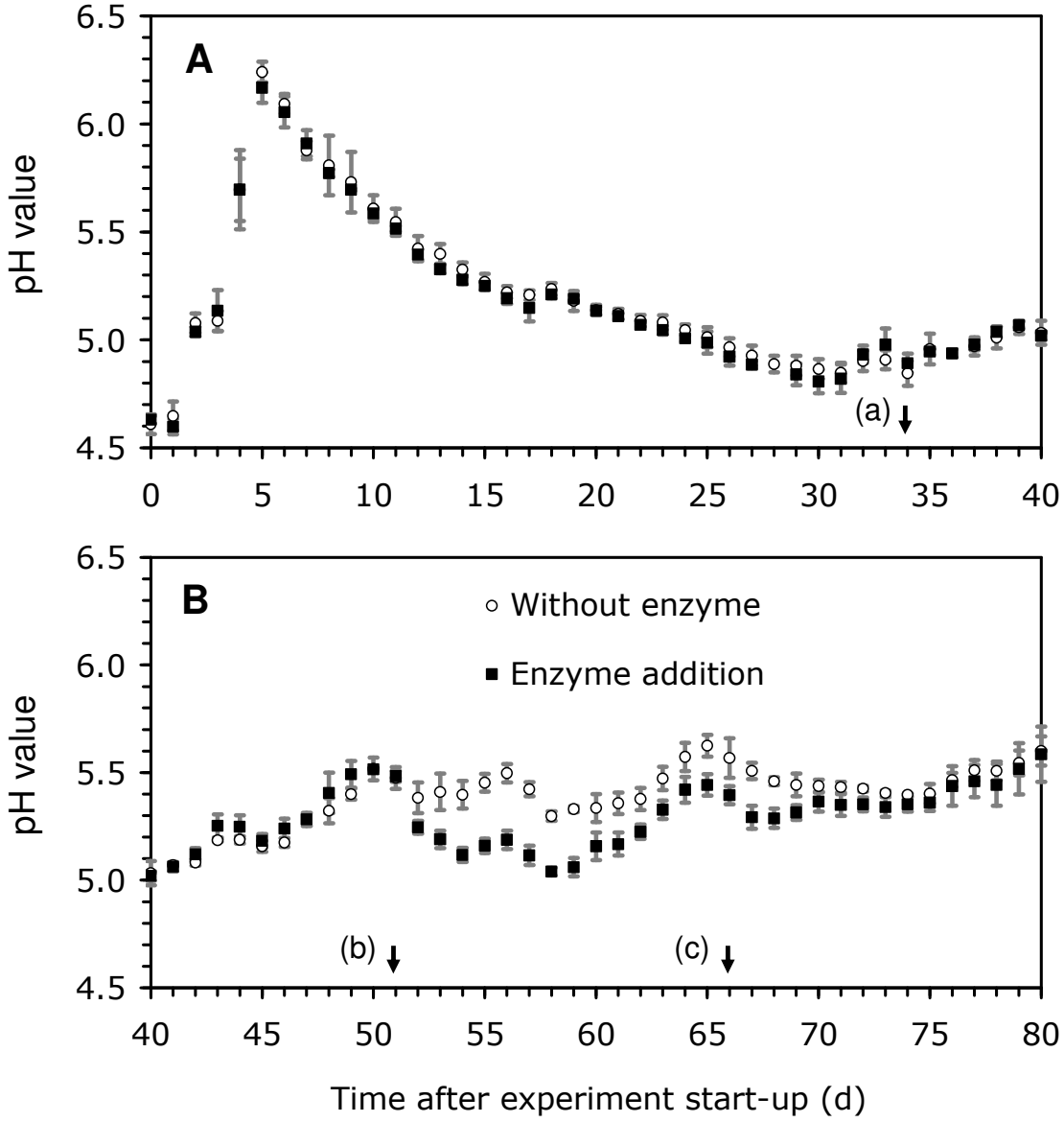


Figure 31. pH of digesters during acidogenic fermentation with and without enzyme addition [Average values. n=4. Vertical arrows stand for SD. (a) HRT reduced from 10 to 5 days. (b) Enzyme additive MethaPlus L100 10 g/kg VS. (c) Enzyme additive 1 g/kg VS].

Liquid reaction products

Volatile Fatty Acids (VFA)

VFA analysis was performed only in the second half of the trial. VFAs produced during the acidogenic process were mainly acetate and n-butyrate, which were in the range 2500-3000 ppm and 2000-2500 ppm, respectively (**Table 26**). Caproate concentration ranged between 600 and 700 mg/L.

Table 26. VFA concentrations of digester samples [Average values. n=4. VFA and total ± SD as mg/L. Values were not converted to acetic acid equivalents].

VFA	Enzyme addition	Time after experiment start-up (d)					
		46	53	60	67	74	80
Acetate	No	3005 ±199	2418 ±149	2551 ±71	2533 ±154	2533 ±264	2689 ±188
	Yes	2930 ±237	2670 ±117	2946 ±63	2946 ±94	2798 ±140	2618 ±267
Propionate	No	298 ±10	308 ±15	289 ±5	272 ±5	283 ±14	294 ±29
	Yes	308 ±20	305 ±10	268 ±22	259 ±9	296 ±11	304 ±18
Iso-Butyrate	No	0	53 ±5	60 ±7	76 ±7	66 ±14	47 ±4
	Yes	0	67 ±8	57 ±6	74 ±9	64 ±7	53 ±6
n-Butyrate	No	2230 ±165	2239 ±148	2141 ±232	2177 ±169	2325 ±285	2338 ±63
	Yes	2181 ±270	2540 ±84	2697 ±173	2426 ±35	2587 ±57	2310 ±93
Iso-Valerate	No	39 ±5	80 ±12	88 ±13	100 ±8	89 ±18	69 ±9
	Yes	44 ±8	101 ±4	88 ±5	97 ±9	88 ±10	77 ±10
n-Valerate	No	32 ±21	37 ±5	36 ±1	34 ±3	37 ±7	40 ±4
	Yes	37 ±7	43 ±13	35 ±4	34 ±3	36 ±2	39 ±2
Caproate	No	714 ±48	559 ±26	524 ±34	495 ±29	528 ±79	556 ±25
	Yes	680 ±111	554 ±48	656 ±54	567 ±170	626 ±12	607 ±57
Total	No	6317 ±358	5694 ±110	5688 ±313	5686 ±147	5943 ±626	6032 ±197
	Yes	6178 ±493	6279 ±191	6747 ±145	6255 ±239	6503 ±142	6008 ±375

Table 27 shows the relative change of VFA concentrations due to enzyme addition. Enzyme addition at a high level of 10 g/kg substrate VS significantly increased VFA concentrations in the digesters. Acetate and butyrate concentrations significantly increased through enzyme addition between day 53 and day 67. Other VFAs also increased, but the type of VFA affected changed depending on the time elapsed after the first enzyme addition. Significant increases of iso-butyrate and iso-valerate concentrations occurred on day 53, but this effect disappeared from the next sampling date on day 60, where caproate concentration increased. Subsequently, on day 67, significant increases of VFA concentrations concerned solely acetate and butyrate. On days 74 and 80, increases of VFA concentration dropped below the significance level of 5%. Enzyme addition had been reduced to 1 g/kg substrate VS on day 64. The lower level of enzyme addition was seemingly not sufficient to yield significant increases of VFA production.

According to **Table 26**, total VFA concentration increased of ~500 mg/L or more in the period between day 53 and day 74 (although the increase was not significant at day 74).

Table 27. Change in VFA concentrations in the variant with enzyme addition versus the enzyme-free variant [Percent values. Average values. n=4. Enzyme addition from day 51. Significant difference to a tolerance degree of * 5% ($p<0.05$), ** 1% ($p<0.01$), *** 0.1% ($p<0.001$) relative to the variant without enzyme addition. n=4].

VFA	Time after experiment start-up (d)					
	46	**53**	**60**	**67**	**74**	**80**
Acetate	-2	+10 *	+15 ***	+10 *	+7	-3
Propionate	+3	-1	-7	-5	+5	+3
Iso-Butyrate	0	+27 *	-6	-2	-4	+13
n-Butyrate	-2	+13 *	+26 **	+11 *	+11	-1
Iso-Valerate	+13	+25 *	0	-3	-2	+12
n-Valerate	+18	+17	-3	0	-3	-2
Caproate	-5	-1	+25 **	+15	+18	+9
Total	-2	+10 **	+19 ***	+10 **	+9	0

Lactic acid

Lactic acid concentration was measured enzymatically on digesters samples of day 53. No significant effect was found at $p<0.05$.

Table 28. Lactic acid concentrations of digesters samples [Average values. n=4.. Values ± SD as mg/L].

Day	Without enzyme	With enzyme
53	36 ±8	29 ±11

Alcohols

Ethanol, n-propanol and n-butanol concentrations were measured through gas chromatography. Diols and iso-alcohols could not be determined. As shown previously (**Table 26**), the total amount of VFA was ~6000 mg/L. Alcohol concentrations were one order of magnitude lower than VFA concentrations. Enzyme addition was directly followed by a decrease of ethanol and propanol concentrations on day 53, but this tendency was not maintained on later measurements.

Table 29. Alcohol concentrations of digester samples [Average values. n=4. Values ± SD as mg/L. Significant difference to a tolerance degree of * 5% ($p<0.05$), ** 1% ($p<0.01$) relative to the variant without enzyme addition].

Day	Ethanol			n-propanol			n-butanol		
	Without enzyme	With enzyme	Change (%)	Without enzyme	With enzyme	Change (%)	Without enzyme	With enzyme	Change (%)
46	88 ±7	91 ±20	+4	115 ±16	124 ±21	+8	7 ±1	9 ±1	+19
53	114 ±5	89 ±6	-22**	178 ±11	153 ±15	-14*	22 ±3	21 ±4	-7
60	120 ±26	139 ±33	+16	140 ±38	174 ±46	+24	18 ±5	18 ±6	-2
67	120 ±21	106 ±9	-11	117 ±23	125 ±10	+7	21 ±2	21 ±2	+1
74	128 ±43	128 ±23	+1	127 ±51	140 ±30	+11	24 ±8	27 ±8	+13
81	134 ±17	107 ±17	-21	104 ±11	87 ±16	-16	33 ±9	27 ±9	-18

COD of filtrated effluent

Digester samples were passed through filter paper (Whatman 602 H 1/2) and COD was determined on the filtrates (i.e. dissolved COD in the liquid phase). COD values were in the range 13,000-16,000 mg O_2/L (**Table 30**). Increases in COD and VFA values through enzyme addition followed similar trends. However, due to higher discrepancy between the replicates, significant differences in the COD of the variant with enzyme addition relative to the enzyme-free variant appeared only at day 60 and day 67 of the experiment. Surprisingly, consequently to the reduction of enzyme dosage, COD concentration of the variant with enzyme addition became 18% lower at day 80 compared to the enzyme-free variant. However, no conclusions can be drawn from this phenomenon because according to a Student t-test this difference was not significant.

Table 30. COD in the liquid phase of filtrated digester samples [Average values. n=4. Values ± SD in mg O_2/L. Significant difference to a tolerance degree of * 5% ($p<0.05$), ** 1% ($p<0.01$) towards the variant without enzyme addition].

	Time after experiment start-up (d)					
	46	53	60	67	74	80
Without enzyme	15197 ±1565	13118 ±2243	14141 ±1228	12925 ±640	12303 ±2559	14834 ±3441
With enzyme	15389 ±3426	14278 ±2654	15950 ±816	15083 ± 607	14151 ±498	12195 ± 931
Change of COD (%)	+1	+9	+13 *	+17 **	+15	-18

Gas production

Carbon dioxide content of biogas

According to **Figure 32**, no clear difference in the CO_2 content appears between enzyme-free variant and the variant with enzyme addition. Reducing the HRT from 10 to 5 days on day 34 also had no effect on carbon dioxide contents. Considering the whole trial period, carbon dioxide contents seemed to fluctuate randomly around an average of 50% (v/v).

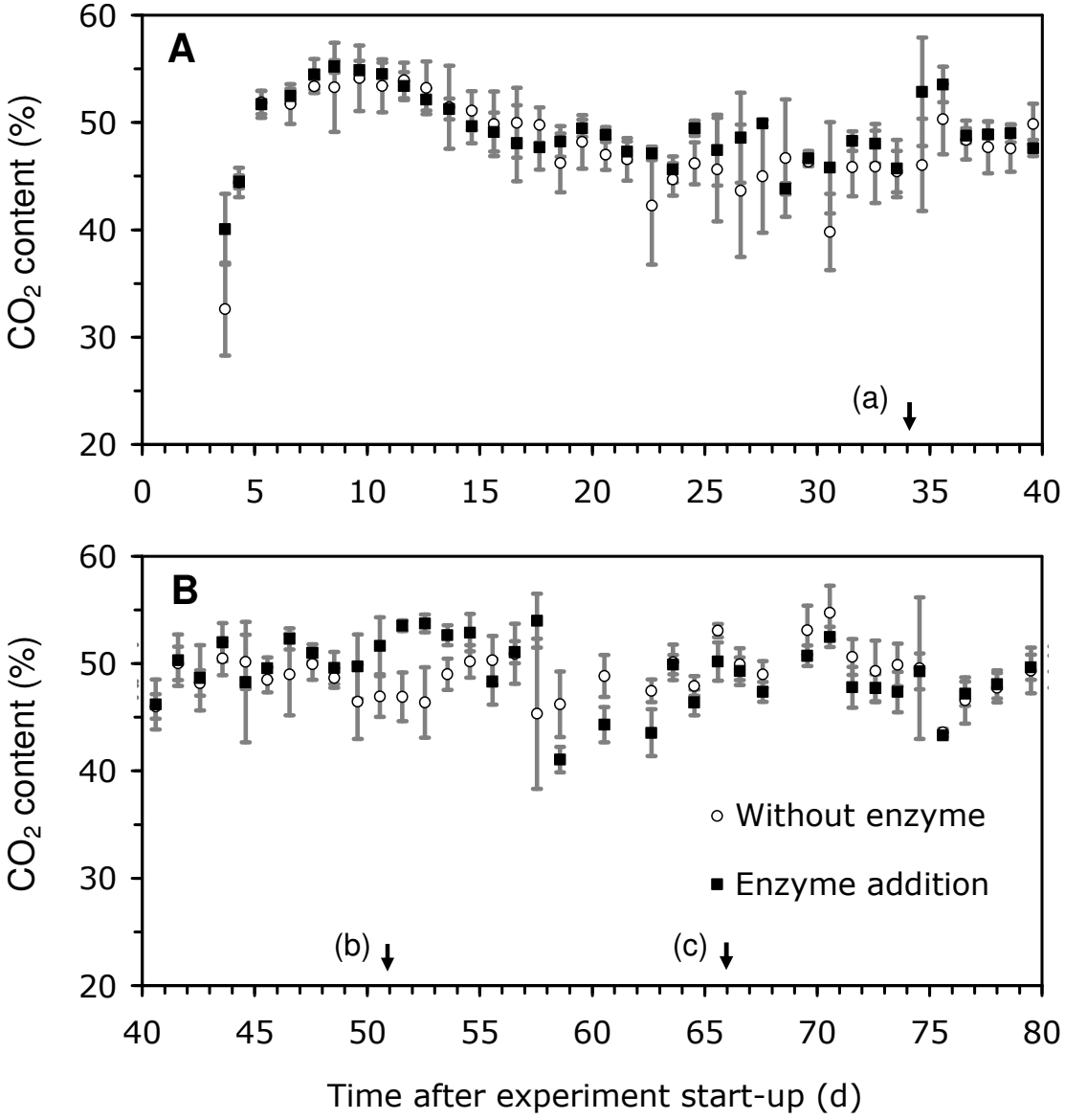


Figure 32. Carbon dioxide content of gas during acidogenic fermentation [Average values. n=4. Vertical arrows stand for SD. (a) HRT reduced from 10 to 5 days. (b) Enzyme additive MethaPlus L100 10 g/kg VS. (c) Enzyme additive 1 g/kg VS].

Specific gas yield

Gas yield was very unstable and varied between 0.1 and 0.3 m^3/kg VS. This issue might originate from the sampling of inhomogenous maize silage, which was taken from a full-scale bunker silo at the University research farm of Meiereihof. As shown in **Figure 33**, the specific gas yields of the enzyme-added variant increased directly after enzyme addition on day 51, noted as **(b)**, from the following day. Subsequently gas yields of the enzyme-added variant remained continuously higher than the enzyme-free variant, even after enzyme dosage had been decreased.

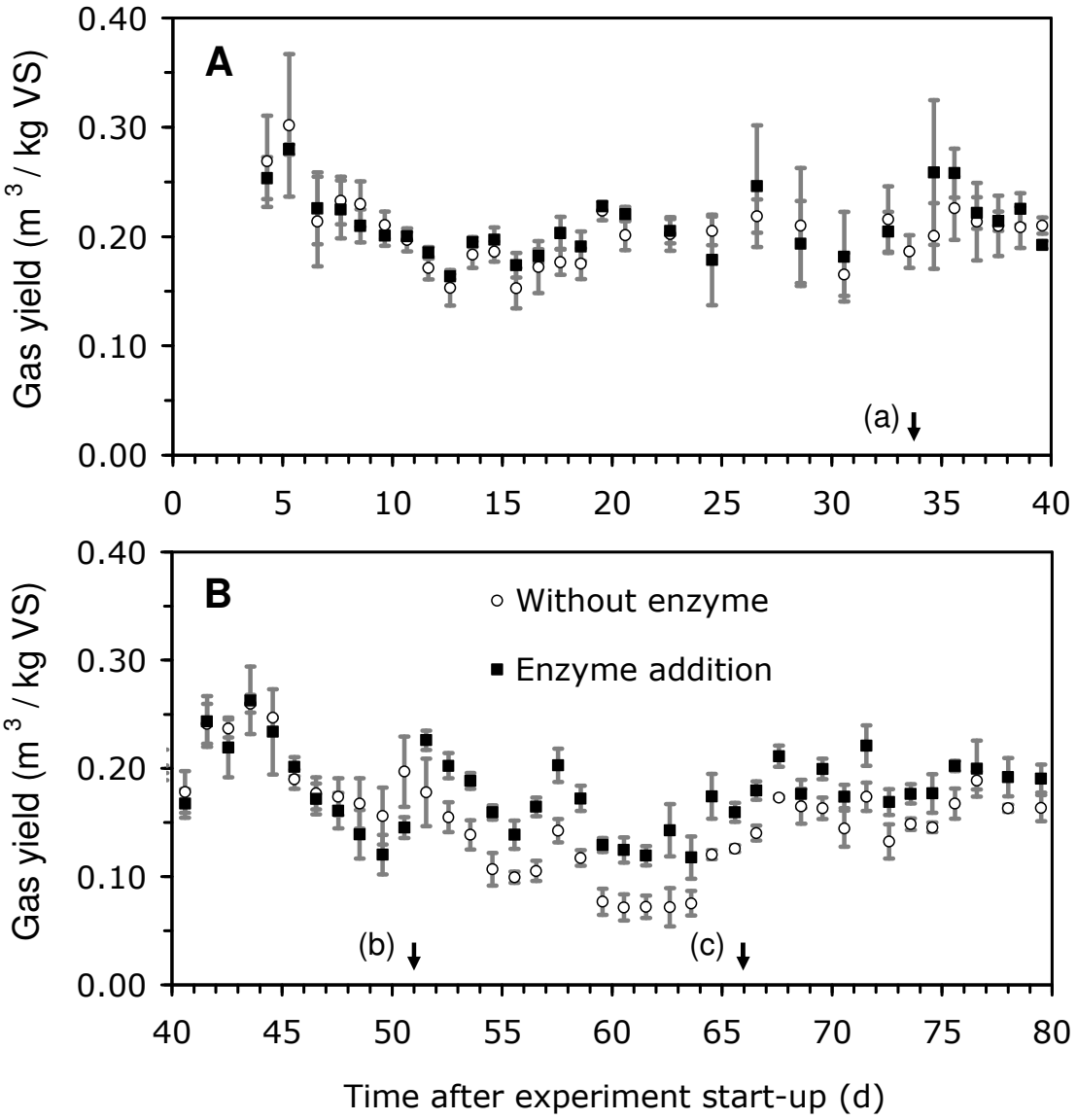


Figure 33. Specific gas yield during acidogenic fermentation with and without enzyme addition [Average values. n=4. Vertical arrows stand for SD. (a) HRT reduced from 10 to 5 days. (b) Enzyme additive MethaPlus L100 10 g/kg VS. (c) Enzyme additive 1 g/kg VS].

A more precise representation of enzyme effect appears in **Table 31**. Before enzyme addition, gas yields of the variant without enzyme addition were generally higher than for the variant in which enzymes were later dosed. The reasons for this discrepancy are unknown.

Given the lack of accuracy of the measurements, it is important to search for steady and prolongated effects. Even a very significant change ($p<0.01$) occurring on day 40, may not have any practical meaning other than an artifact if the trend would not be continued on the following days. This was the case as values of the enzyme-added variant remained higher than the enzyme-free variant for every day after the first day of enzyme addition.

Table 31. Specific gas yield during acidogenic fermentation [Average values ± SD. n=4. Significant difference to a tolerance degree of * 5% ($p<0.05$), ** 1% ($p<0.01$), *** 0.1% ($p<0.001$) relative to the variant without enzyme addition].

Day	Specific gas yield (m^3/kg VS)		Change (%)	Day	Specific gas yield (m^3/kg VS)		Change (%)
	Without enzyme	With enzyme			Without enzyme	With enzyme	
40	0.210 ±0.007	0.192 ±0.003	-9 **	**60**	0.077 ±0.012	0.129 ±0.006	+41 ***
41	0.178 ±0.019	0.167 ±0.013	-6	**61**	0.071 ±0.012	0.125 ±0.012	+43 **
42	0.241 ±0.018	0.243 ±0.023	+1	**62**	0.072 ±0.010	0.119 ±0.009	+40 ***
43	0.237 ±0.008	0.219 ±0.028	-8	**63**	0.072 ±0.018	0.143 ±0.024	+50 **
44	0.260 ±0.008	0.263 ±0.031	+1	**64**	0.075 ±0.011	0.118 ±0.020	+36 **
45	0.247 ±0.001	0.234 ±0.040	-6	**65**	0.120 ±0.004	0.174 ±0.021	+31 **
46	0.190 ±0.009	0.202 ±0.009	+6	**66**	0.126 ±0.003	0.159 ±0.009	+21 ***
47	0.177 ±0.015	0.172 ±0.014	-3	**67**	0.140 ±0.007	0.179 ±0.008	+22 ***
48	0.174 ±0.017	0.161 ±0.016	-8	**68**	0.173 ±0.001	0.211 ±0.010	+18 ***
49	0.167 ±0.023	0.139 ±0.022	-20	**69**	0.165 ±0.016	0.176 ±0.013	+7
50	0.156 ±0.026	0.120 ±0.018	-30	**70**	0.163 ±0.010	0.200 ±0.010	+18 **
51	0.197 ±0.033	0.145 ±0.010	-36 *	**71**	0.144 ±0.017	0.174 ±0.011	+17 *
52	0.178 ±0.031	0.226 ±0.009	+21 *	**72**	0.174 ±0.013	0.221 ±0.019	+21 **
53	0.155 ±0.014	0.202 ±0.012	+24 **	**73**	0.132 ±0.016	0.169 ±0.012	+22 *
54	0.139 ±0.014	0.188 ±0.007	+26 ***	**74**	0.148 ±0.005	0.177 ±0.009	+16 **
55	0.107 ±0.015	0.159 ±0.007	+33 ***	**75**	0.146 ±0.005	0.177 ±0.018	+18 *
56	0.099 ±0.013	0.139 ±0.013	+28 **	**76**	0.167 ±0.014	0.202 ±0.005	+17 **
57	0.105 ±0.009	0.164 ±0.009	+36 ***	**77**	0.189 ±0.008	0.200 ±0.026	+6
58	0.142 ±0.015	0.203 ±0.015	+30 ***	**78**	0.163 ±0.003	0.192 ±0.018	+15 *
59	0.117 ±0.012	0.172 ±0.012	+32 ***	**79**	0.163 ±0.012	0.191 ±0.013	+14 *

Specific biohydrogen yield

The specific biohydrogen yield follows the same trend as the specific gas yield, due to the relatively constant CO_2 content of biogas.

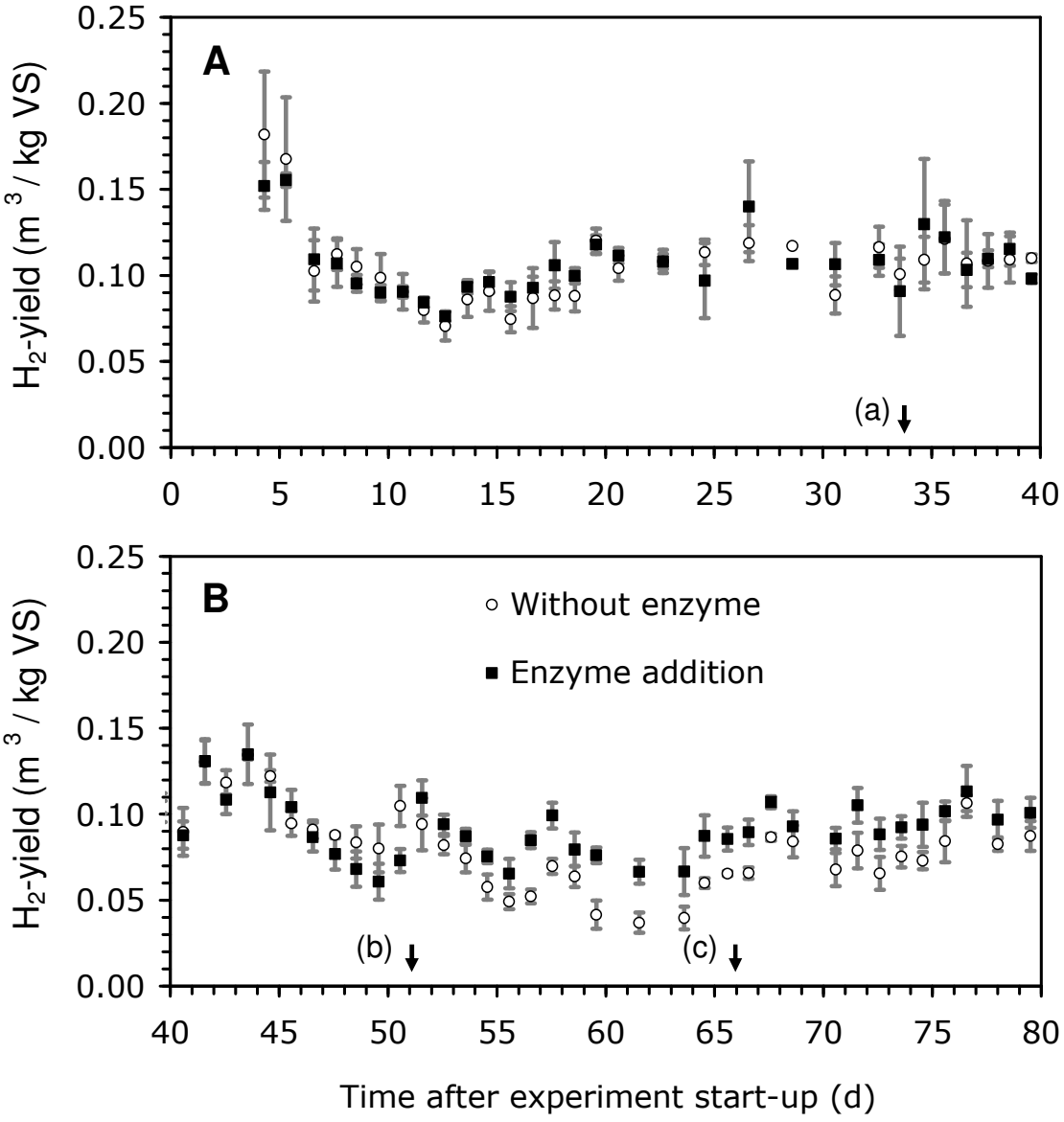


Figure 34. Specific biohydrogen yield of gas during acidogenic fermentation with and without enzyme addition [n=4. Vertical arrows stand for SD. (a) HRT reduced from 10 to 5 days. (b) Enzyme additive MethaPlus L100 10 g/kg VS. (c) Enzyme additive 1 g/kg VS].

Table 32. Specific biohydrogen yield during acidogenic fermentation [Average values ± SD. n=4. Significant difference to a tolerance degree of * 5% ($p<0.05$), ** 1% ($p<0.01$), *** 0.1% ($p<0.001$) relative to the variant without enzyme addition. n.v.: no measurement value available].

Day	Specific hydrogen yield (m^3/kg VS)		Change (%)	Day	Specific hydrogen yield (m^3/kg VS)		Change (%)
	Without enzyme	With enzyme			Without enzyme	With enzyme	
40	0.110 ±0.002	0.098 ±0.003	-12 **	**60**	0.042 ±0.008	0.076 ±0.005	+45 ***
41	0.090 ±0.014	0.088 ±0.008	-2	**61**	*n.v.*	*n.v.*	*n.v.*
42	0.130 ±0.013	0.131 ±0.013	0	**62**	0.037 ±0.006	0.067 ±0.007	+45 ***
43	0.118 ±0.007	0.109 ±0.008	-9	**63**	*n.v.*	*n.v.*	*n.v.*
44	0.135 ±0.003	0.135 ±0.017	0	**64**	0.040 ±0.007	0.067 ±0.014	+41 *
45	0.122 ±0.003	0.113 ±0.022	-8	**65**	0.060 ±0.003	0.087 ±0.012	+31 **
46	0.095 ±0.007	0.104 ±0.010	+9	**66**	0.066 ±0.001	0.086 ±0.007	+23 **
47	0.091 ±0.005	0.087 ±0.008	-5	**67**	0.066 ±0.003	0.089 ±0.007	+26 **
48	0.088 ±0.001	0.077 ±0.009	-14	**68**	0.087 ±0.002	0.107 ±0.003	+19 ***
49	0.084 ±0.009	0.068 ±0.010	-23	**69**	0.084 ±0.009	0.093 ±0.009	+9
50	0.080 ±0.014	0.061 ±0.010	-32	**70**	*n.v.*	*n.v.*	*n.v.*
51	0.105 ±0.012	0.073 ±0.007	-43 *	**71**	0.068 ±0.010	0.086 ±0.006	+21 *
52	0.094 ±0.015	0.109 ±0.010	+14	**72**	0.079 ±0.010	0.105 ±0.010	+25 *
53	0.082 ±0.005	0.094 ±0.006	+13 *	**73**	0.066 ±0.010	0.088 ±0.009	+26 *
54	0.074 ±0.008	0.087 ±0.004	+15 *	**74**	0.075 ±0.006	0.092 ±0.007	+18 **
55	0.058 ±0.007	0.075 ±0.004	+24 **	**75**	0.073 ±0.005	0.094 ±0.013	+22 *
56	0.049 ±0.004	0.065 ±0.009	+25 *	**76**	0.084 ±0.012	0.102 ±0.006	+17
57	0.052 ±0.004	0.085 ±0.005	+39 ***	**77**	0.106 ±0.005	0.113 ±0.015	+6
58	0.070 ±0.004	0.099 ±0.007	+30 ***	**78**	0.083 ±0.004	0.097 ±0.011	+15
59	0.064 ±0.006	0.079 ±0.010	+20 *	**79**	0.087 ±0.009	0.101 ±0.009	+13

6 Discussion

6.1 Analysis of literature findings

Challenges for enzyme additives in the biogas process

Enzymes added into the biogas process face several challenges:

1. **Inactivation by proteases**: being constituted of proteins, enzymes face degradation by proteases released by the bacteria present in the digestion medium (Marquardt and Brufau 1997; Morgavi et al. 2001; Schimpf et al. 2012a).
2. **Thermal inactivation**: while thermal inactivation is less problematic for rapid industrial enzymatic processes, it can become an issue when enzymes are expected to remain active over a longer period. Some fungal enzymes may already be significantly affected by thermal inactivation at 30°C (Schimpf et al. 2012a).
3. **Non-productive binding**: enzymes can be severely inactivated by irreversible non-productive binding on substrate fibers, especially on lignin (Berlin et al. 2006; Berlin et al. 2005; Eriksson et al. 2002; Zhao et al. 2012).
4. **Neutral pH**: many fungal enzymes are most active at acidic pH levels and their activity decreases at neutral to slightly alkaline pH levels, which prevail in methanogenic reactors (Adney et al. 1991; Akao et al. 1992; Dashtban et al. 2009; Durand et al. 1984; Schimpf et al. 2011b; Schimpf et al. 2012a).
5. **Washout**: in continuous digestion, a fraction of both fermenting substrate and enzyme is regularly removed from the reactors while new substrate is being fed (Koch et al. 2010).

Strategies to increase effects of enzyme additives in laboratory assays

Different strategies have been implemented by research groups in order to maximize enzyme efficiency. Accounts of these strategies can be found in ***3.3 Enzyme additives in anaerobic digestion processes***. These strategies are summarized as follows:

1. **Digestion at low temperature** (≤ 35°C) may reduce thermal inactivation of enzyme additives.
2. **Manure-free digestion** of energy crops can reduce the amount of recalcitrant fibers and lignin (lignocellulose) in the medium, since these recalcitrant fibers hamper the efficiency of enzyme additives.
3. **Separate pretreatment steps** can be implemented to optimize conditions for enzymatic hydrolysis of the substrate:
    a. Water may be added to the substrate and the preferred pH may be set by means of chemicals (bacteria-free pretreatment).
    b. The pretreatment step may be performed along with acidogenic fermentation that performs at pH values closer to enzyme requirements.

While performing acidogenic fermentation of wheat straw, Quéméneur et al. (2012) noticed that direct enzyme addition into an acidogenic reactor was more efficient for increasing hydrogen production than performing a separate enzymatic hydrolysis step. Indeed, a pretreatment comprising both a high dilution with water and the addition of chemicals to increase the performance of enzyme additives may not be economical in practice.

6.2 Analysis of results

Hydrolysis trials

The efficiency of enzymatic hydrolysis of native substrate (fresh maize straw) was very low regardless of reaction conditions. Applying an enzyme dosage of 3% of substrate VS to fresh maize straw, the maximal yield of reducing sugars was ~35%, compared with ~23% without enzyme addition. Sugar release without enzyme addition corresponded to the amount of soluble sugars originating from plant sap in fresh maize straw, as analyzed by Chen et al. (2007). The findings confirm that enzymatic hydrolysis is inefficient towards native lignocellulosic biomass, the maximal sugar release being ~20% (Zhang and Lynd 2004).

An enzyme product from acidophilic fungi was active only at low pH. Its activity was greatly reduced in enzymatic hydrolysis trials run at neutral pH. According to Adney et al. (1991), most fungal enzymes may not be appropriate for direct addition into biogas reactors that operate at neutral pH, because they are most active at low pH. Nevertheless, enzymatic hydrolysis with an enzyme product designed to function at slightly alkaline pH yielded ~35% of reducing sugars relative to substrate VS at pH 7.5, under the hydrolysis conditions mentioned previously. Comparatively, enzyme products from acidogenic fungi in an optimized mixture yielded ~34% reducing sugars when tested under similar conditions at their optimal pH of 4.5.

Unfortunately, there was no sufficient information available to determine which linkages were cleaved by enzyme action. Pectin may be more readily available to enzymatic degradation than cellulose and xylan. Interestingly, an enzyme product that has applications in the brewing industry for its high pectinase activity yielded high levels of reducing sugars. Other substrate fractions, such as mixed-linkage glucans (a major hemicellulose component of grasses) and sugars from glycoproteins, may also be degraded to an unknown extent.

In industrial processes, a pretreatment of native plant biomass is required for enzyme additives to perform efficiently. In the production of bioethanol from lignocellulosic biomass, intensive physicochemical pretreatments take place upstream from enzymatic hydrolysis. These pretreatments involve pressure, temperature, acidic and alkaline reagents, and oxidants (Chundawat et al. 2012; Taherzadeh and Karimi 2008).

A question arises, that can not be answered by enzymatic hydrolysis assays alone: may bacteria take over the role of a physicochemical pretreatment to render plant substrate amenable to enzymatic degradation? Consequently, may synergy effects between added enzyme and bacteria take place?

Batch digestion trials

Standard batch digestion

Under standard conditions of batch anaerobic digestion (BMP assays), i.e. batch process, inoculum with optimized efficiency (with high bacterial activity against all substrate fractions and strong pH buffer capacity), reactor maintained at 37°C for 35 days, no significant effect of enzyme addition on the biogas process could be observed.

Batch digestion following enzymatic hydrolysis step

Adding an enzymatic hydrolysis step upstream of standard batch anaerobic digestion (BMP assay) did not bring any effect on methane production, presumably due to the low efficiency of enzymatic hydrolysis on native plant biomass. The enzymatic hydrolysis step was performed with enzyme addition at a high dose of 13 g/kg substrate VS on fresh maize straw at pH 4.5 and 45°C for 24 hours and a substrate:water ratio of 1:6.

Batch digestion under modified conditions

Standard batch digestion trials, also designated as BMP (Biochemical Methane Potential) assays are designed to measure the maximal methane production, which can be achieved under optimal digestion conditions. These optimal conditions are obtained by controlling both substrate and inoculum properties. The digestion medium of BMP assays usually consists of a high share of inoculum with the following properties:

1. Containing high amounts of residual lignocellulose (residues from previous anaerobic digestion of fibrous substrates applied to trigger bacterial adaptation of the inoculum to fiber biodegradation);
2. Being a strong chemical buffer, which maintains a slightly alkaline pH range required for optimal bacterial development in the biogas process (i.e. pH in the range 7.5-8.3 at the beginning and at the end of the digestion period);
3. Displaying a high bacterial activity against lignocellulose (allowing rapid bacterial biodegradation of lignocellulose in the assay).

In addition to the optimization of inoculum characteristics for lignocellulose degradation, substrate is finely ground to ensure high conversion rates as well as sample homogeneity. These experimental conditions are optimal to ensure extended substrate degradation. However, they may not be favorable for enzyme additives to yield an increase of methane production, because effects are more likely to occur when the anaerobic digestion process is ineffective and both methane production rate and extent remain at low levels.

In order to reveal potential effects of enzyme additives, changes have to be made to both substrate and inoculum characteristics:

1. Reduction of inoculum activity: two different approaches were followed to obtain weak inoculums with reduced bacterial activity:

- **Batch digestion with weak inoculum:** a weak inoculum was taken from a methanogenic fixed bed reactor which had been left in dormancy at room temperature for several months. Enzyme additives applied at 11 g/kg VS generated a temporary increase of the methane production rate of grass silage was noticed, occurring between day 10 and day 25 of the experiment. However, methane production curves of variants with and without enzyme addition converged at the end of the 35-day digestion period and final methane yields were similar.
- **Batch digestion with diluted inoculum:** an experiment was performed with 10-fold diluted standard inoculum ("manure inoculum"). Minerals were supplemented to diluted inoculum in order to ensure stable reaction conditions. Minerals supplementation consisted of pH-buffering substances, micronutrients and trace metals. At a dosage of 7 g/kg VS of enzyme additive, a ~40% increase of final methane yields of grass silage after a 60-day digestion period was noticed.

2. Reduction of substrate degradability: concurrently, fiber-rich grass silage was used as a substrate, and was not finely chopped: a coarse fiber length of 0.5-1 cm was kept on purpose to reduce substrate degradability.

Weaker inoculums seemed to favor enzyme effects in BMP assays. Possible reasons for improved enzyme efficiency are listed as follows:

1. **Low share of lignocellulose (fibers) in the inoculum:** may increase the efficiency of enzyme additives. Non-productive binding on recalcitrant lignocellulosic material contained in the inoculum may inhibit enzyme activity (Berlin et al. 2006; Berlin et al. 2005; Eriksson et al. 2002; Zhao et al. 2012). This inhibition effect may be mitigated when the share of lignocellulose contained in the inoculum is lower, e.g. when the inoculum is more dilute.
2. **Lower pH values of digestion medium:** weak or diluted inoculum may contain a lower share of pH-buffering substances. In batch digestion, pH generally decreases a few days after the beginning of the digestion process due to a high release of VFA, before eventually recovering (Mukengele et al. 2005). Within this digestion period at lower pH the medium may become more favorable to enzyme additives from acidophilic fungi.
3. **Lower bacterial activity:** as the fiber-degrading activity of enzyme additives gets in the same range as bacterial activity, synergy effects between enzyme and bacteria become visible. On the opposite, if bacterial activity is much higher than enzyme activity from the additives, their effect on the substrate become negligible compared with bacterial degradation, and would not be visible anymore.
4. **Lower load of bacterial proteases:** would be linked to lower bacterial activity and increase the lifespan of enzyme additives in the digestion medium, as protease degradation of enzyme additives occur at a lower rate (Morgavi et al. 2001).

Further trials would be necessary to confirm these hypotheses, which may provide a direction to future investigations on the effect of enzyme additives in anaerobic digestion processes.

Batch digestion of effluent from a biogas plant

Enzyme additives applied at a very high enzyme dosage of 2 g/kg FW had no significant effect on methane production of effluent from an agricultural biogas plant compared with inactivated enzymes. At this high dosage, even methane generation caused by the biodegradation of inactivated enzyme was not negligible. The anaerobic degradation of enzyme proteins and other organic components produced significant amounts of methane.

The substrate fed into the sampled biogas plant contained a high share of manure (~49% FW), resulting in a high share of recalcitrant fibres in reactor samples, that may lead to the deactivation of enzyme additives via non-productive binding. Even enzyme products specifically designed to function at neutral to alkaline pH did not bring any significant increase of methane production compared with the variant with inactivated enzyme. Other adverse factors that may counter the effect of enzyme additives in reactor samples may be: high pH, high bacterial activity, and high share of bacterial proteases in the medium.

Semi-continuous acidogenic digestion

Laboratory horizontal digesters were operated semi-continuously with maize silage, water and quicklime in an acidogenic fermentation process. Operating conditions were a hybrid between dark fermentation, which is optimized for H_2 production, and hydrolysis or acidification step, which is optimized for the production of organic acids (Bartacek et al. 2007; Fang and Liu 2002; Kyazze et al. 2006; Lay 2000; Logan et al. 2002; Puchajda and Oleszkiewicz 2006; Traverso et al. 2000; Van Ginkel et al. 2001; Veeken et al. 2000; Yu and Fang 2002; Zoetemeyer et al. 1982a; Zoetemeyer et al. 1982b). Contrary to methane fermentation, the process did not generate biogas consisting in CH_4 and CO_2. Instead, bacterial acidogenic fermentation produced organic acids, alcohols and ketones together with a gas mixture of H_2 and CO_2.

The low pH range of the process (5.0 – 5.5) was expected to be beneficial to maximize the efficiency of enzyme additives from acidophilic fungi. Extensive, continuous stirring would have been preferable to achieve a higher removal rate of diluted H_2, which inhibits bacterial fermentation (Logan et al. 2002). Nevertheless, the acidogenic process generated as much as ~0.100 m^3/kg VS of gas containing ~50% H_2 and ~6 g/L VFA in the liquid phase (i.e. ~1.5 g VFA/g VS of substrate). However, VS contents of the substrate (maize silage) were not corrected for VFA losses resulting from the determination method, and clogging of digester outlets may have further affected the validity of the mass balance.

An enzyme product commonly used in agricultural biogas plants and originating from the acidophilic fungi *Trichoderma reesei* was added at a dosage of 10 g/kg VS. The enzyme additive increased VFA production by ~10% as well as both H_2 and CO_2 production by ~20% compared with the enzyme-free variant. However, reducing the enzyme load to 1 g/kg VS greatly diminished enzyme effects.

The results of the semi-continuous acidogenic digestion experiment are in line with the findings of other research groups (***3.3 Enzyme additives in anaerobic digestion processes***) that demonstrate the efficiency of enzyme additives in acidogenic fermentation processes. However, enzyme dosages used were still very high. Furthermore, the effects of enzyme additives recorded in this trial may be related to the degradation of non-fibrous fractions of maize silage, such as starch.

6.3 Analysis of methods

Scope of the methodological analysis

Methodological issues have been detailed previously in section ***4 Materials and methods***. The methodological analysis developed in this section focuses on investigating experimental approaches and critical areas of improvement with regards to the evaluation of enzyme additive and anaerobic digestion efficiencies.

Evaluation of the efficiency of enzyme additives

Comparison of enzyme dosages

In enzymatic hydrolysis trials carried out in this thesis, the effects of enzyme products on the hydrolysis of maize straw were compared by adding the same amount of each enzyme product in each variant. However, caution should be taken in comparing enzyme efficiencies because certain enzyme products could be more dilute than others. A common method, which could have been applied to evaluate the enzyme concentration of commercial enzyme products is to measure their protein concentration, since the protein fraction usually consists of enzymes. However, protein determination may also yield different results depending on the method applied. Hence, it is important to select a standard protocol for protein analysis before comparing enzyme products (Nieves et al. 1998).

Evaluation of enzyme efficiency from an economical point of view

When comparing similar amounts of pure enzyme, certain enzymes could be more active than others. Therefore, rather than considering enzyme concentration alone, the enzyme production cost required to obtain the desired effect can also considered as a valuable parameter (Schober 2008).

Challenges of enzyme stability assessment

According to Suárez Quiñones et al. (2012b), research into enzyme application still lacks a systematic approach. More emphasis should be placed on enzyme-feedstock interactions. It may be of interest to study enzyme behavior in the digestion medium. Binner et al. (2011) claimed that added enzyme may be already almost completely inactivated after 1 h of residence in a biogas reactor. Their measurements relied on the analysis of the supernatant produced by centrifugation of a sample of digester contents. He et al. (2006) followed a different protocol to measure enzyme activities in acidogenic fermentation reactors and discarded the initial supernatant before extracting enzymes from the solid residue with phosphate buffer. According to Marquardt and Brufau (1997), due to the challenge of measuring the residual activity of enzyme acting on solid polymers in a digestion medium, there is no reliable method to measure enzyme stability in fermentation processes. Moreover, a significant share of active enzymes may be bound to fibers in non-productive, irreversible binding (Berlin et al. 2006; Berlin et al. 2005; Eriksson et al. 2002; Zhao et al. 2012). These facts may lead to the conclusion that researchers might still be unable to know for how long enzyme additives remain active in the biogas process.

Challenges of batch digestion trials

Measurement accuracy

According to enzyme suppliers, an increase in methane yields as low as 5% might lead enzyme additives to be economically profitable in biogas plants (Gerhardt et al. 2007). Unfortunately, the accurracy of BMP assays is in the same range as the detection level required to validate such a tiny increase of methane yields at the laboratory.

Experimental approaches exist to demonstrate effects that are below the accurracy range of an assay. These approaches include: drastically increasing the number of replicates (so that replicates can be considered as a statistical population distributed around the true value), repeating the experiments several times, randomizing the assays, and following double-blind protocols (i.e. where the experimenter is unaware that he is testing a placebo instead of the enzyme additive). No instance of these approaches being followed by biogas researchers have been found in the literature. Testing enzyme additives under chosen conditions known to yield high increases in methane yields is a much easier approach that may turn to be more valuable at first to gain better understanding of enzyme effects.

Experimental protocols

There is a lack of commonly known and widely accepted standards for batch digestion trials (also named BMP assays). International norms are not widely accepted because the preparation of a complex mineral medium is not convenient for low-cost routine analyses. It is easier for scientists to collect and apply directly undiluted inoculums without addition of minerals, a procedure that proves to be satisfactory in most cases. There is a need to promote common rules which would be accepted as good practices by the scientific community (Angelidaki et al. 2009). The German directive VDI 4630 (2006) is a first step towards a simplification of the protocols for BMP assays. However, this German standard may be too detailed and still lacks international recognition.

Lemmer (2005) suggested that the increased use of standard substrates of defined characteristics could build a basis for comparison of BMP assays. In the experiments described in this thesis, hay was used as a standard substrate (data not shown). Laboratory-grade crystalline cellulose may be a good candidate for use as a standard substrate by research groups on international level (Raposo et al. 2011).

Researchers often face methodological issues of which they are not aware. In this work, air drying of substrate occurred during weighing at the beginning of one experiment. This bias could be eliminated by portioning fresh maize straw into numerous subsamples that were distributed into impermeable plastic bags, a procedure that drastically reduced the duration of air contact with the fresh material. Schimpf and Valbuena (2009) noticed that the amount of water added into the digestion medium could have a tremendous influence on methane production rate. In line with this observation, Abbassi-Guendouz et al. (2012) noticed that higher dry matter contents (i.e. lower water contents) reduce the efficiency of anaerobic digestion. Listing the most common methodological issues related to the performance of BMP assays may be beneficial to the scientific community.

Assay conditions

BMP assays were originally designed to determine the maximal methane yields that can be achieved from a given substrate under optimized fermentation conditions, but such an approach may not be appropriate to reveal the performance of additives. Additives usually show the highest effects when digestion conditions are not optimal, as is often the case in full-scale biogas plants for which they have been designed (Demmig et al. 2010; Koch et al. 2010).

Optimizing batch digestion trials for the evaluation of enzyme additives

Suggestions can be made to optimize batch digestion trials for enzyme additives:

1. Designing the experimental protocol:

    - **Increase measurement accuracy** by testing and optimizing both the experimental protocol and the equipment for sample processing and digestion.
    - **Run a sufficient number of replicates,** at least 4-5 per variant to reach statistical significance of the effects of enzyme additives.
    - **Run a standard substrate,** e.g. cristalline cellulose, to evaluate the validity, accuracy and repeatability of the assay methodology.
    - **Run negative controls** with thermally inactivated enzyme added strictly under the same conditions as active enzymes.
    - **Mesure proteins concentration in the enzyme product** as an evaluation of true enzyme concentration, since the protein fraction in commercial enzyme products generally consists of enzyme.
    - **Measure pH values** in the course of the digestion process, because pH may change along with VFA release and VFA consumption and affect enzyme activity.
    - **Measure lignin and fiber contents** of both substrate and inoculum.
    - **Test a wide range of enzyme products** at medium to high dosages in order to discover enzyme characteristics and activity spectrum that yield highest effects.

2. Setting digestion conditions:

    - **Apply substrate with a coarse particle size** to reduce the rate of degradation.
    - **Generate suboptimal digestion conditions for the bacteria,** possible options are the development of weakened inoculums from reactors left in dormancy over a long period, or the dilution of inoculum in a watery medium supplemented with mineral buffer and trace metals.
    - **Generate favorable medium conditions for enzyme additives**: the weak inoculums should contain a low share of particulate matter, especially fiber (lignocellulose) and lignin, a characteristic that can be achieved via inoculum selection and inoculum processing via sedimentation, filtration or dilution.

6.4 Efficiency of enzyme additives in the biogas process

Features of lignocellulose degradation in the biogas process

On the basis of the knowledge gathered in the literature review, a schematic description of interactions occurring in anaerobic digestion is drawn in **Figure 35**. While this representation does not account for the complexity of microbiological processes, it might help in understanding issues related to the efficiency of enzyme additives in anaerobic digestion processes. The main challenge for enzyme additives is to compete with a highly efficient system for lignocellulose degradation: hydrolytic bacteria. Tightly bound to the substrate via exopolymers (glyocalyx), hydrolytic bacteria possess cell-bound enzyme complexes (cellulosomes) and can release free enzymes as well. They avoid end-product inhibition of their own enzymes by absorbing and digesting oligosaccharides inside their cells. They use the energy yield from substrate digestion to multiply and to produce more enzymes. A synthrophic community of acetogenic and methanogenic bacteria consumes organic acids and alcohols released as degradation products by hydrolytic bacteria and turn them into biogas, avoiding self-inhibition of the bacteria from these substances.

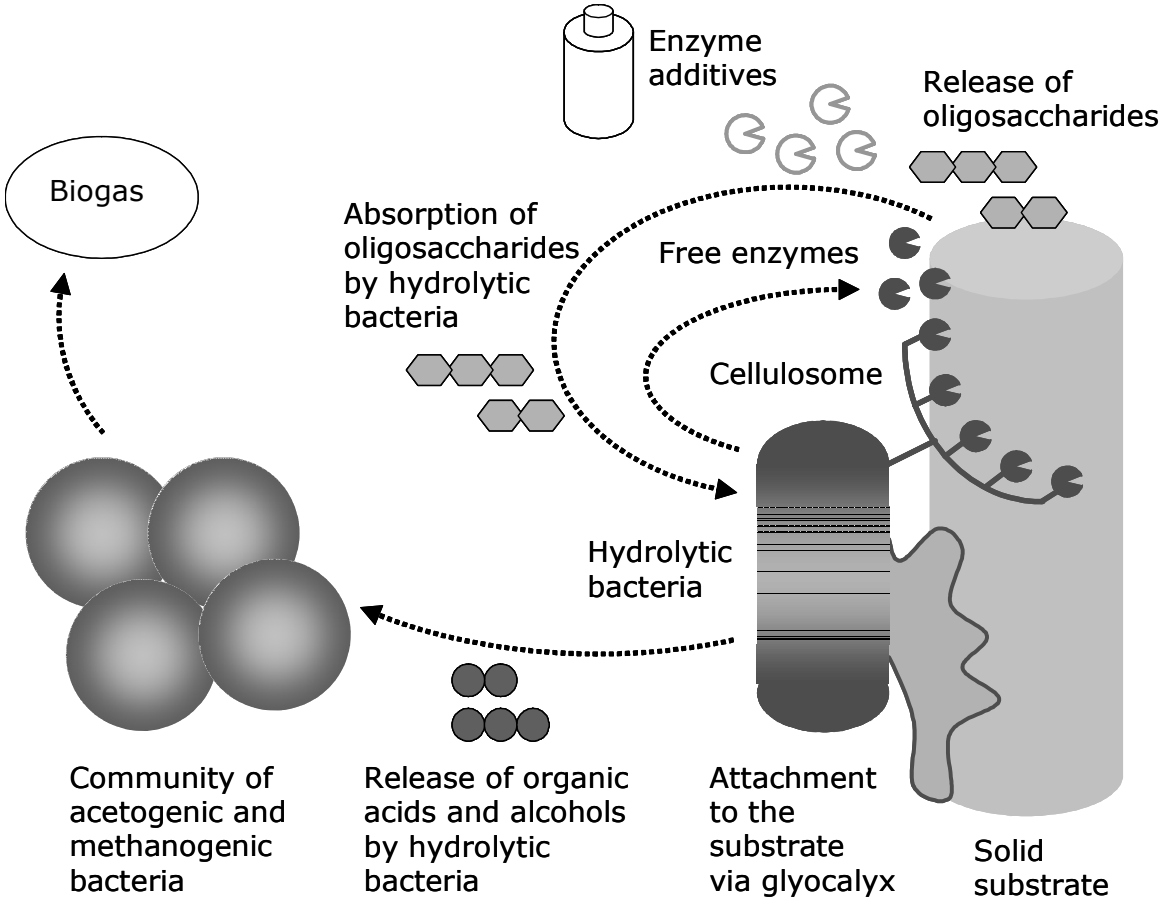


Figure 35. Mechanisms of lignocellulosic substrate degradation in the biogas process.

Conditions favoring maximal efficiency of enzyme additives

Based on hypotheses developed in this thesis, assumptions about the best conditions to reach the highest effects of enzyme additives in anaerobic digestion processes, can be summarized as follows:

1. **High share of lignocellulosic substrate:** this substrate should have a coarse particle size, and contain much cellulose and hemicellulose, but few lignin in order to avoid enzyme inhibition. Energy crops may be appropriate substrates to fulfill these requirements.
2. **Operating conditions favorable to enzyme additives:** low temperature (for higher enzyme stability), low pH (for optimal action of enzyme from acidophilic fungi), low amount of recalcitrant lignin and fiber in the medium (to prevent non-productive binding of enzymes).
3. **Suboptimal digestion conditions:** poor activity of hydrolytic bacteria and bacterial proteins, which can be favored by coarse fiber length of substrate, high OLR, low HRT, and in the case of batch processes a low activity of the inoculum.

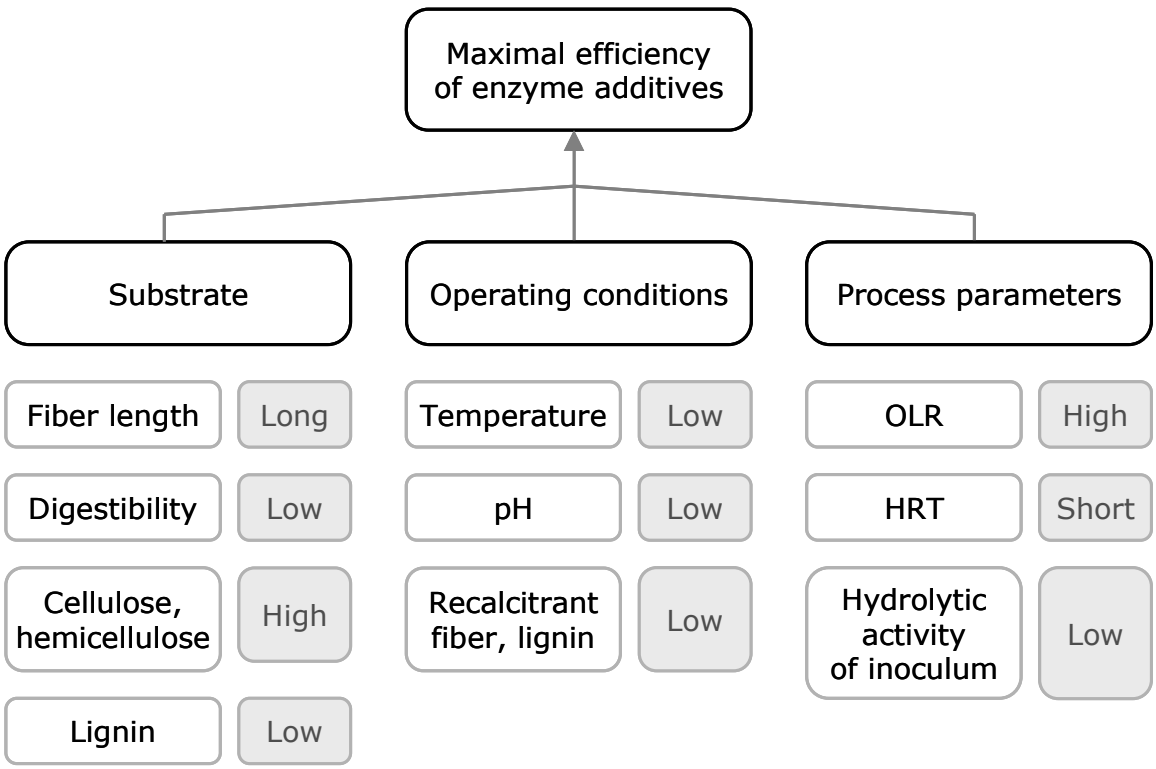


Figure 36. Requirements for maximal efficiency of enzyme additives in anaerobic digestion processes.

6.5 Recommendations for the practical use of enzyme additives

Maximal effects of enzyme additives in agricultural biogas plants may occur under following conditions:

1. **High OLR** (e.g. >4 kg VS/(m^3 × d));
2. **Low HRT** (e.g. <40 d);
3. **Maximal share of energy crops and minimal share of manure** in the substrate mix, because manure contains a high share of recalcitrant fibers and lignin;
4. **Low pH,** which is more favorable to enzyme additives from acidophilic fungi.

The experiments carried out in this thesis revealed enzyme effects on the anaerobic digestion of energy crops at an enzyme dosage of ~10 g/kg VS. This dosage corresponds to ~2-3 kg of enzyme product per ton of ensiled material. At such high dosages, the profitability of prolongated enzyme addition over a long period can be questioned (Parawira 2012). Nevertheless, temporary enzyme addition may be a technical solution to tackle problems related to floating layers that can occur in digesters where a high share of energy crops is applied (Demmig et al. 2010). Advantages become evident if enzyme addition can be performed in replacement to other measures, such as resorting to a drastic reduction of the substrate charge, or emptying digesters.

Enzyme effects that occur at a low HRT tend to disappear as the overall digestion period increases (Koch et al. 2010). Hence enzyme effects occurring at a short HRT in the digester where enzyme addition is performed may be annihilated when downstream digesters are considered because the overall HRT becomes higher. Hence, in 2-step (acidogenic-methanogenic) and 2-stage (methanogenic-methanogenic) biogas plants, comprising a cascade of 2 reactors or more, which are common in Germany, enzymes may not yield a significant increase of the methane yield, but only a reduction in the viscosity of fermenting substrate contained in the first digester.

Further batch and semi-continuous digestion trials are required to validate the efficiency of enzyme additives. Pilot-scale and full-scale trials may also be useful but in such trials experimental conditions can not be easily controlled, an issue that makes the interpretation of the results more difficult (Koch et al. 2010). Future improvements of enzyme products, especially designing efficient fibre-degrading enzymes with an activity optimum at neutral pH for direct addition into biogas reactors, as well as the discovery of cheaper enzyme production techniques, may improve the profitability of enzyme additives.

7 Conclusion

This thesis investigated mechanisms governing the efficiency of commercial fiber-degrading enzyme additives on the anaerobic digestion of energy crops.

Enzymatic hydrolysis trials revealed the poor efficiency of enzyme additives towards native (untreated) plant biomass. According to scientific literature, enzyme efficiency may be favored by low contents in recalcitrant fiber and lignin (to limit non-productive binding of enzyme), low pH (for most common enzymes from acidophilic fungi) and low temperatures (to enhance enzyme lifespan by reducing thermal inactivation).

Anaerobic microbial degradation processes outperform microbe-free enzymatic hydrolysis of lignocellulose. Mechanisms that make bacteria more efficient than enzymes alone include tight bacterial attachment to solid substrate, bacteria producing cell-bound enzyme complexes (cellulosomes), while also releasing free enzymes, and absorbing oligosaccharides that would otherwise inhibit enzyme action, as well as bacteria growing and multiplying during substrate degradation. Hence, experiments should be performed under suboptimal digestion conditions to observe effects of enzyme additives (weak inoculum, coarse particle size of substrate, high loading rate).

For enzyme addition to be effective in practice, biogas reactors should be heavily loaded (high OLR, low HRT) with a substrate mix containing a high share of energy crops. The dosage of enzyme product required for effects to appear (~10 g/kg VS) may be too high to envision prolongated enzyme addition, but temporary applications might resolve issues related to floating layers and viscosity. The semi-continuous experiment confirmed that enzyme additives increase the release of digestion products in acidogenic fermentation processes that can be performed either for the production of organic acids (acidification step or hydrolysis step, sometimes applied in practice upstream of biogas reactors) or of hydrogen (dark fermentation). Further research is required to confirm these hypotheses.

Scientists working in the fields of plant physiology, polymer science and animal nutrition gathered important knowledge about plant structure (Carpita 1996; Ding and Himmel 2009; Ebringerová 2006; Gilbert et al. 2008; Harris and Smith 2006; Knox 2008; Wilson 1993). Unfortunately, only few attempts have been made to correlate plant features at molecular, cellular, tissue and plant levels with the efficiency of enzymatic hydrolysis and bioethanol conversion, or of biogas production. Focusing research on plant structure is critical to foster the development of more efficient bioenergy processes.

8 Summary

The mechanisms governing the efficiency of commercial fiber-degrading enzyme additives at improving the anaerobic digestion of energy crops were investigated.

Enzymatic hydrolysis of fresh maize straw was performed for 24 h with commercial cellulases, xylanases and pectinases at an enzyme dosage of ~30 g/kg of substrate VS and a substrate:water ratio of 1:6. A maximal yield of ~35% reducing sugars, compared with ~23% without enzyme addition, was achieved. Enzyme products from acidophilic fungi were active only at low pH (<7.0).

Standard batch digestion trials (BMP-assays) were performed using the Hohenheim Biogas Test (HBT) on maize straw, maize corn, and rye silage with different inocula. These BMP-assays showed no significant effect of enzyme additives (including commercial cellulase, xylanase, pectinase, laccase) on the methane production rate. However, batch digestion trials performed under suboptimal conditions with inoculum of weak bacterial activity from a digester that had been left in dormancy for several months, revealed a temporary increase of the methane production rate from grass silage between day 10 and day 25 of the experiment. In another batch digestion trial, a standard inoculum was diluted 10-fold to reduce its bacterial activity, and a 40% increase of the final methane yield was reached after 60 days of digestion of grass silage. Alternately, in batch digestion trials performed directly on samples of reactor content from an agricultural biogas plant, enzyme additives at a very high dosage of 2 g/kg substrate FW had no significant effect on the methane yield. Even a mixture of cellulase and xylanase specifically designed to function under slightly alkaline pH conditions was ineffective.

In semi-continuous acidogenic fermentation performed in laboratory digesters with maize silage and water added for dilution at OLR 4 kg VS/(m^3 × d), HRT 5 days, with the medium kept in the pH-range 5-5.5 through quicklime addition, enzyme additive at a dosage of 10 g/kg substrate VS significantly increased VFA release (+10%) as well as gas production, including H_2 production (+20%).

Combining the results of the assays with scientific knowledge gathered from the literature, assumptions were drawn about the optimal conditions to foster the performance of enzyme additives in anaerobic digestion processes. In agricultural biogas plants effects of enzyme additives may be most expected in reactors that are heavily loaded (high OLR >4 kg VS/(m^3 × d) and short HRT) with a substrate mix comprising a maximal share of energy crops and a minimal share of manure. Nevertheless, the experimental results of this thesis show that a very high enzyme dosage was required to observe effects of enzyme additives on anaerobic digestion. Hence, the profitability of enzyme addition into the biogas process should be examined carefully.

9 Zusammenfassung

In der Promotionsarbeit wurde die Wirkungsweise von Enzymzusätzen auf die anaerobe Vergärung von Energiepflanzen systematisch untersucht.

Die enzymatische Hydrolyse von frischem Mais-Stroh wurde für 24 h mit handelsüblichen Zellulasen, Xylanasen und Pektinasen durchgeführt. Unter Anwendung einer Dosierung von 30 g/kg Substrat-oTS und einem Substrat:Wasser Verhältnis von 1:6 wurde ein maximaler Ertrag an reduzierendem Zucker von ~35% erreicht, im Vergleich zu ~23% ohne Enzymzugabe. Enzymprodukte aus azidophilen Pilzen blieben nur bei geringen pH-Werten aktiv (pH<7).

Standard-Batchgärversuche mit dem Hohenheimer Biogasertragstest (HBT) und Mais-Stroh, Maiskorn und Roggensilage unter Anwendung verschiedener Inokula zeigten keine signifikante Wirkung der Enzymzusätze (handelsübliche Zellulase, Xylanase, Pektinase und Laccase) auf die Methanbildung. Hingegen zeigten Batchgärversuche, die unter suboptimalen Bedingungen mit einem mikrobiologisch wenig aktiven Inokulum durchgeführt wurden, eine temporäre Steigerung der Methanbildung aus Grassilage zwischen Tag 10 und Tag 25 des Versuches. Das verwendete Inokulum stammte aus einem Fermenter, der für Monate nicht gefüttert wurde. In weiteren Gärversuche wurde die bakterielle Aktivität durch 10-facher Verdünnung eines Standard-Inokulums künstlich reduziert. Dabei brachte die Enzymzugabe nach einer Gärdauer von 60 Tagen eine 40-prozentige Steigerung des Endmethanertrages von Grassilage. Hingegen wurde in Batchgärversuche mit Biogasanlagengärrest als Substrat keine signifikante Steigerung der Methanbildung beobachtet, auch nicht bei höherer Enzymdosierung und bei Verwendung von speziell angepasster Zellulase und Xylanase (wirksam bei leicht alkalischem pH).

Die azidogene Vergärung von Maissilage wurde semi-kontinuierlich unter Zugabe von Wasser und Branntkalk bei einer Raumbelastung von 4 kg oTS/($m^3 \times d$), einer hydraulischen Verweilzeit von 5 Tagen und einem pH-Wert von 5-5,5 durchgeführt. Dabei brachten Enzymzusätze mit einer Dosierung von 10 g/kg oTS eine signifikante Erhöhung der Fettsäureproduktion (+10%), sowie der Gas- und Wasserstoffproduktion (+20%).

Aus einer Literaturanalyse und den Versuchsergebnissen wurden Schlussfolgerungen zu optimalen Bedingungen für die maximale Wirkung von Enzymzusätzen in anaeroben Gärprozessen gezogen. In landwirtschaftlichen Biogasanlagen dürfte die stärkste Wirkung von Enzymzusätzen bei hoher Belastung (hohe Raumbelastung und geringe Verweilzeit) zu erwarten sein. Dabei muss der Substrat-Mix einen möglichst hohen Anteil an Energiepflanzen und einen möglichst geringen Anteil an Flüssigmist enthalten. Die Versuchsergebnisse der Promotionsarbeit deuten jedoch darauf hin, dass eine Wirkung der Enzymzusätze erst bei sehr hoher Dosierung auftrat, so dass die Wirtschaftlichkeit eines Enzymeinsatzes im Biogasverfahren kritisch betrachtet werden muss.

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11 Appendix

A 1 Reducing sugar yields in enzymatic hydrolysis assays

Table A1. Enzymatic hydrolysis of maize straw at low pH [Hydrolysis of 1.8 g maize straw + 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer for 24 hours at 50°C. n=3].

Reducing sugars (% substrate VS)

pH	Without enzyme	Celluclast 1.5 L	Novozym 188	Ultraflo Max	Viscozyme L
3.5	21.9	29.2	25.2	29.9	24.5
4	22.7	31.5	24.6	32.3	26.7
4.5	22.9	30.5	25.4	32.8	26.4
5	23.2	30.7	24.9	32.8	26.8
5.5	23.1	30.9	24.5	32.2	27.0

Standard deviation

pH	Without enzyme	Celluclast 1.5 L	Novozym 188	Ultraflo Max	Viscozyme L
3.5	0.6	0.8	1.1	0.5	0.4
4	0.3	1.2	1.2	1.6	1.2
4.5	0.3	1.0	1.0	0.0	1.1
5	1.1	0.5	0.3	0.4	1.2
5.5	0.9	0.0	0.7	0.5	0.5

Table A2. Enzymatic hydrolysis of maize straw at high pH [Hydrolysis of 1.8 g maize straw + 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer (pH 6-6.5) or phosphate buffer (pH 7-8) for 24 hours at 50°C. Addition of sodium azide at 1 g/L in each sample for bacterial inhibition. n=3].

Reducing sugars (% substrate VS)

pH	Without enzyme	Novozym 342	Ultraflo Max
6	24.07	28.39	32.37
6.5	24.85	30.30	34.10
7	25.04	30.17	26.19
7.5	25.22	35.14	25.97
8	24.76	33.10	27.66

Standard deviation

pH	Without enzyme	Novozym 342	Ultraflo Max
6	0.64	0.95	0.81
6.5	0.73	0.90	0.86
7	0.86	0.58	0.34
7.5	1.14	2.83	0.46
8	0.77	1.32	0.28

Table A3. Enzymatic hydrolysis of maize straw at different temperatures [Hydrolysis of 1.8 g maize straw + 10 µL enzyme in 10 mL of 0.1 M citrate, 24 hours. n=3].

Reducing sugars (% substrate VS)

Temperature	Without enzyme	Celluclast 1.5 L	MethaPlus L100	Novozym 188	Ultraflo Max	Viscozyme L
20	20.74	25.19	24.55	23.77	26.58	23.12
25	21.17	27.14	25.93	22.64	27.27	22.08
30	21.48	27.49	27.10	21.65	27.70	22.77
35	23.08	29.04	28.22	25.28	31.46	26.23
40	21.30	31.20	26.88	23.42	31.16	26.06
45	24.40	30.17	29.21	25.41	32.63	27.01
50	22.60	30.77	29.17	24.20	33.36	27.18
55	24.01	30.55	29.39	25.50	33.75	25.05
60	23.16	29.43	27.14	24.52	29.73	25.58

Standard deviation

Temperature	Without enzyme	Celluclast 1.5 L	MethaPlus L100	Novozym 188	Ultraflo Max	Viscozyme L
20	0.15	0.00	0.69	0.59	0.15	0.72
25	0.45	1.40	0.46	0.15	0.45	0.13
30	1.01	0.52	1.13	0.91	1.47	0.86
35	0.54	0.20	0.27	0.37	0.46	0.34
40	1.53	1.63	1.12	3.70	1.28	1.13
45	0.78	1.67	0.94	0.96	0.91	1.91
50	0.47	1.24	0.15	0.76	0.90	0.46
55	0.59	1.39	1.17	1.98	1.06	0.55
60	0.46	0.92	0.79	0.27	0.45	0.13

Table A4. Enzymatic hydrolysis of maize straw with enzyme mixtures [Hydrolysis of 1.8 g maize straw + 10 µL enzyme, 10 mL of 0.1 M citrate, 24 h, 50°C. n=3].

Reducing sugars (% substrate VS)

% enzyme A / % enzyme B (w/w)	Wirhout enzyme	Celluclast 1.5 L - Ultraflo Max	Celluclast 1.5 L- Viscozyme L	Celluclast 1.5 L- Novozym 188	MethaPlus L100- Ultraflo Max	MethaPlus L100- Viscozyme L	MethaPlus L100- Novozym 188	Ultraflo Max- Viscozyme L	Ultraflo Max- Novozym 188
100/0	21.9	28.2	28.2	28.2	28.0	29.1	29.1	31.3	31.3
75/25	21.9	28.1	31.0	29.3	29.1	29.3	29.1	31.9	33.2
50/50	21.9	28.9	30.0	29.2	31.1	29.4	29.0	31.2	33.9
25/75	21.9	29.3	29.0	28.7	29.2	28.6	25.8	31.5	33.8
0/100	21.9	30.7	24.4	24.6	28.0	25.0	24.3	26.1	26.7

Standard deviation

% enzyme A / % enzyme B (w/w)	Without enzyme	Celluclast 1.5 L - Ultraflo Max	Celluclast 1.5 L- Viscozyme L	Celluclast 1.5 L- Novozym 188	MethaPlus L100- Ultraflo Max	MethaPlus L100- Viscozyme L	MethaPlus L100- Novozym 188	Ultraflo Max- Viscozyme L	Ultraflo Max- Novozym 188
100/0	0.6	0.5	0.5	0.5	0.4	0.5	0.5	0.5	0.5
75/25	0.6	0.5	0.3	0.6	0.6	0.4	0.6	0.6	0.8
50/50	0.6	0.6	0.7	0.4	0.7	0.7	0.6	0.7	0.6
25/75	0.6	0.3	0.9	0.3	0.5	0.1	0.3	0.5	0.6
0/100	0.6	0.7	0.5	0.6	0.3	0.8	0.7	0.7	0.7

Table A5. Enzymatic hydrolysis of maize straw at different durations [Hydrolysis of 1.8 g maize straw + 10 µL enzyme in 10 mL of 0.1 M citrate. n=3].

Duration (days)	0	0.02	0.04	0.17	0.33	0.50	0.67	1	2	3	4	5
Reducing sugars (% substrate VS)	22.0	23.7	26.5	30.0	30.5	31.4	31.8	33.0	35.6	36.4	36.9	37.3
Standard deviation	0.7	0.3	0.6	0.6	0.3	0.2	0.4	0.1	0.3	0.1	0.2	0.3

Table A6. Enzymatic hydrolysis of maize straw at different dosages [Hydrolysis of 1.8 g maize straw in 10 mL of 0.1 M citrate, 24 hours. n=3].

Enzyme dosage (% substrate VS)	**Reducing sugars (% substrate VS)**	**Standard deviation**
Without enzyme	22.0	0.4
0.05%	26.5	0.1
0.10%	26.6	0.1
0.20%	27.8	0.4
0.50%	30.4	0.5
1%	31.9	0.6

Table A7. Enzymatic hydrolysis of maize straw at different substrate amounts [Hydrolysis of maize straw + 10 µL enzyme in 10 mL of 0.1 M citrate, 24 hours. n=3].

	Without enzyme		**With enzyme**	
Substrate amount (g FW)	**Reducing sugars (% substrate VS)**	**Standard deviation**	**Reducing sugars (% substrate VS)**	**Standard deviation**
1.8	23.6	0.3	32.8	0.6
3.6	21.6	0.1	27.9	0.3
7.2	17.1	0.1	20.7	0.1
9	15.9	0.1	18.2	0.0

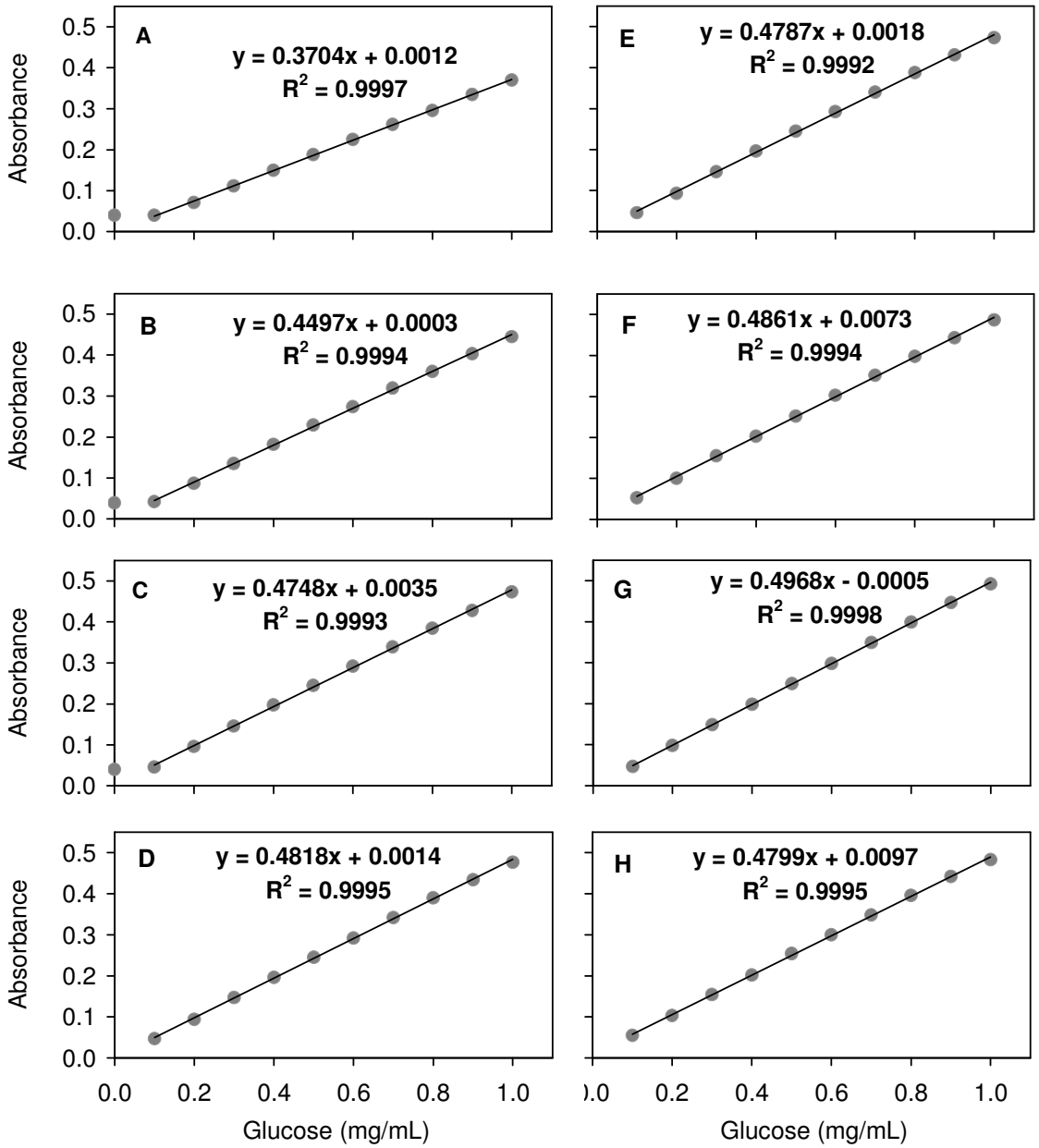

Figure A1. Glucose calibration curves for reducing sugars determination [Glucose concentration measured in citrate buffer 0.1 M (pH 5) with different DNS reagent solutions on 16.01.2008 (A), 14.02.2008 (B), 31.03.2008 (C). Effect of different mediums on the calibration curves measured on 13.03.2009: glucose dilutions carried out in redistilled water (D), citrate buffer pH 7 (E), phosphate buffer pH 7 (F), citrate buffer pH 7 + 1 g/L sodium azide (G), phosphate buffer pH 7 + 1 g/L sodium azide (H)].

A 2 Reducing sugars against glucose determination

In order to verify the validity of the determination method for reducing sugars, a comparable assays were run with colorimetric enzyme-based glucose determination assay. The kit, including all reagents, was purchased from Roche-Biopharm (D-Glucose Test kit, Roche-Biopharm, Darmstadt, Germany).

The kit was based on a two-step enzymatic reaction for glucose measurement. First, glucose was phosphorylated to glucose-6-phosphate (G-6-P) through hexokinase enzyme, while adenosine-5'-triphosphate (ATP) was converted to adenosine-5'-diphosphate (ADP).

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP} \qquad \textbf{(11)}$$

In a second step, G-6-P was oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate through glucose-6-phosphate dehydrogenase (G6P-DH) while nicotinamide-adenine dinucleotide phosphate (NADPH) was formed.

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+ \qquad \textbf{(12)}$$

Before analysis, samples were passed through filter paper (Whatman 602 H 1/2) and diluted 1:50 in 20 mL volumetric flasks. The assay was run according to the manufacturer's specifications. Absorbances were read at 340 nm with a photometer (UV Mini 1240 UV-VIS-Spectrophotometer, Shimadzu, Kyoto, Japan).

This method is known for its high specificity: the enzyme should react with glucose only and with no other product. However, the method is sensitive to interferences from other enzymes and from colorants (Urginovits 1980).

The enzymatic hydrolysis experiment with variation of pH values was repeated with the glucose enzyme-based kit. Glucose contents partially confirmed the trends of sugar production, though results differed due to the fact that enzyme-based kit recorded only glucose content, while the dinitrosalicylic reagent method recorded all end chains of sugars having reducing properties, treating indifferently sugar monomers and polymers, regardless of molecule size. Therefore, reducing sugars determination provide a better picture of the overall degree of hydrolysis, but might be less relevant for the evaluation of hydrolytic processes where sugar monomers are the end products required.

Glucose content was approximately half of total reducing sugars content. The rankings of enzyme products efficiencies with regard to glucose release and reducing sugars release were similar. At a low pH, glucose release was markedly lower, while reducing sugars

release decreased to a lesser extent in relative values. As an exception, glucose and reducing sugars release from Novozym 188 did not decrease at lower pH.

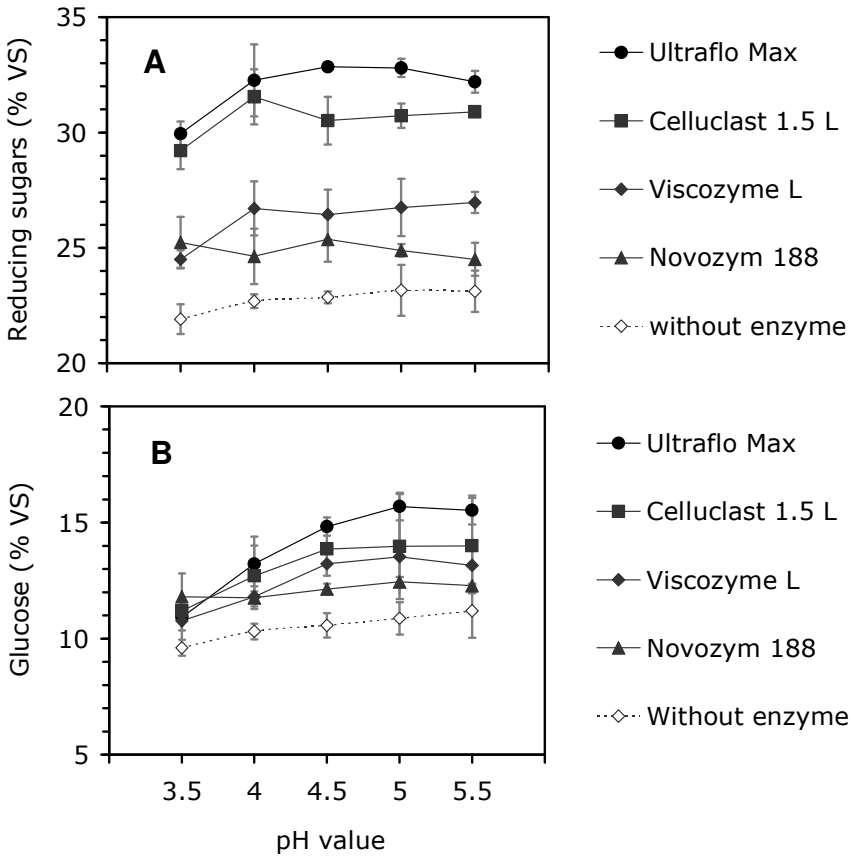

Figure A2. Reducing sugars (A) and glucose (B) release from maize straw at different pH values [Hydrolysis of 1.8 g maize straw with 10 µL enzyme solution in 10 mL of 0.1 M citrate buffer solution at 50°C for 24 hours. n=3. Vertical arrows stand for SD. Numerical values of glucose assay cf. **Table A8**].

Table A8. Enzymatic hydrolysis of maize straw with glucose kit [Hydrolysis of 1.8 g maize straw + 10 µL enzyme, 10 mL of 0.1 M citrate, 24 h, 50°C. n=3].

Glucose (% substrate VS)

pH	Ultraflo Max	Viscozyme L	Without enzyme	Celluclast 1.5 L	Novozym 188
3.5	10.9	10.8	9.6	11.2	11.8
4	13.2	11.8	10.3	12.7	11.8
4.5	14.8	13.2	10.6	13.9	12.1
5	15.7	13.5	10.9	14.0	12.4
5.5	15.5	13.2	11.2	14.0	12.3

Standard deviation

pH	Ultraflo Max	Viscozyme L	Without enzyme	Celluclast 1.5 L	Novozym 188
3.5	0.6	1.0	0.3	0.1	1.0
4	1.2	0.3	0.3	1.3	0.5
4.5	0.4	0.5	0.5	1.1	0.2
5	0.6	1.6	0.7	2.3	0.2
5.5	0.6	1.2	1.2	2.1	0.1

A 3 Experiment to optimize the hydrolysis step

A preliminary experiment was run to optimize the enzymatic hydrolysis step before coupling it to a subsequent anaerobic digestion step. Two different enzyme concentrations were applied, namely 14 and 140 g/kg substrate VS. Diluted enzyme solutions were prepared for both concentration levels with 100 and 1000 µL enzyme added to 10 mL distilled water, respectively. Following variants were tested:

1. Only water (control variant);
2. Novozym 188;
3. Genencor Laminex BG;
4. Mixture Genencor Laminex BG / Novozym 188 (80%/20% w/w, respectively);
5. Mixture MethaPlus L100 / Novozym 188 (80%/20% w/w, respectively).

Both Genencor Laminex BG and MethaPlus L100 products had cellulase and xylanase activities and contained enzymes from the fungi *Trichoderma reesei*. Novozym 188 had β-glucosidase activity originating from the fungi *Aspergillus niger*.

1.8 g ensiled maize straw were put inside glass tubes of the HBT process together with 10 mL water and 0.5 mL of diluted enzyme. Enzyme loads corresponded to 13 and 130 g/kg substrate VS for low and high dosage, respectively. Enzymatic hydrolysis was performed at 55°C during 24 hours. This trial was performed without pH control; pH value was 3.61 before hydrolysis and ranged between 3.51 and 3.58 after hydrolysis. Organic acids contained in ensiled maize straw probably had a high buffering effect that maintained pH in a narrow range.

The release of soluble nonosaccharides following enzymatic hydrolysis was determined with HPLC (**Figure A3**). The maximal glucose release was 4.8 and 8.3% of substrate VS at low and high enzyme dosage, respectively. The release of monosaccharides (i.e. xylose, galactose, mannose, fructose or arabinose) was almost insignificant. Novozym 188 had a low effect on monosaccharide release when applied alone, however mixing this enzyme together with Genencor Laminex BG yielded the highest monosaccharide release.

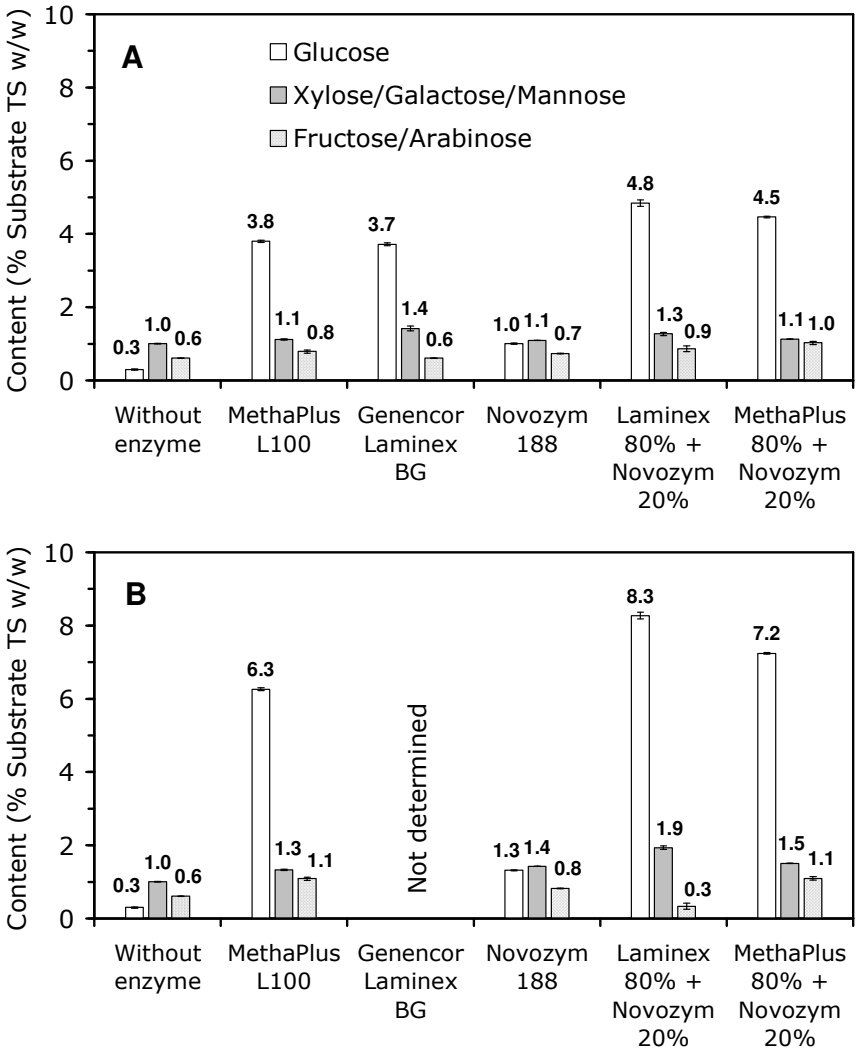

Figure A3. Concentration of soluble monosaccharides after enzymatic hydrolysis of ensiled maize straw in the screening experiment [Hydrolysis of 1.8 g maize straw in 10 mL of water at pH 3.6 and 55°C for 24 hours. n=2. Vertical arrows stand for SD. (A) 13 g/kg substrate VS; (B) 130 g/kg substrate VS].

A 4 Final methane yields in batch digestion trials

Standard batch digestion of energy crops

Table A9. Final methane yield of maize straw and maize corn [Batch digestion in HBT process at 37°C for 35 days. IN = thermally inactivated 95°C - 15 min. n=3. Enzyme additives were also tested on manure inoculum alone under the same conditions].

		Specific methane yield (m^3/kg VS)	Standard deviation	Relative Standard deviation (%)	Change of the methane yield (%)
Maize corn	Without enzyme	0.353	0.006	1.7	0.0
	+ MethaPlus 0.13 g/kg VS	0.359	0.001	0.2	1.6
	+ GoldFerm 0.13 g/kg VS	0.355	0.002	0.7	0.4
	+ Genencor 0.13 g/kg VS	0.356	0.003	0.7	0.8
	+ Novozyme 0.13 g/kg VS	0.355	0.008	2.3	0.4
	+ MethaPlus 1.3 g/kg VS	0.359	0.009	2.5	1.7
	+ GoldFerm 1.3 g/kg VS	0.369	0.002	0.7	4.5
	+ Genencor 1.3 g/kg VS	0.353	0.007	1.9	0.1
	+ Novozyme 1.3 g/kg VS	0.356	0.004	1.1	0.9
	+ GoldFerm 1.3 g/kg VS - IN	0.366	0.008	2.3	3.5
	+ Genencor 1.3 g/kg VS - IN	0.362	0.007	1.9	2.4
	+ Novozyme 1.3 g/kg VS - IN	0.365	0.001	0.3	3.3
Maize straw	Without Enzyme	0.314	0.007	2.2	0.0
	+ MethaPlus 0.13 g/kg VS	0.313	0.004	1.1	-0.2
	+ GoldFerm 0.13 g/kg VS	0.310	0.005	1.6	-1.2
	+ Genencor 0.13 g/kg VS	0.317	0.002	0.6	1.0
	+ Novozyme 0.13 g/kg VS	0.313	0.006	1.9	-0.4
	+ MethaPlus 1.3 g/kg VS	0.309	0.007	2.1	-1.7
	+ GoldFerm 1.3 g/kg VS	0.330	0.005	1.6	5.0
	+ Genencor 1.3 g/kg VS	0.317	0.003	1.1	1.0
	+ Novozyme 1.3 g/kg VS	0.315	0.001	0.5	0.5
	+ MethaPlus 1.3 g/kg VS - IN	0.322	0.011	3.4	2.5
	+ GoldFerm 1.3 g/kg VS _ IN	0.315	0.011	3.6	0.2
	+ Genencor 1.3 g/kg VS - IN	0.321	0.006	1.9	2.3
	+ Novozymes 1.3 g/kg VS - IN	0.309	0.004	1.4	-1.7
Manure inoculum	Without Enzyme	0.015	0.001	3.8	0.0
	+ MethaPlus 0.13 g/kg VS	0.014	0.000	1.9	-0.5
	+ GoldFerm 0.13 g/kg VS	0.015	0.001	3.8	0.0
	+ Genencor 0.13 g/kg VS	0.015	0.000	0.4	2.6
	+ Novozymes 0.13 g/kg VS	0.015	0.000	1.6	1.6
	+ MethaPlus 1.3 g/kg VS	0.015	0.000	2.4	2.6
	+ GoldFerm 1.3 g/kg VS	0.016	0.001	5.9	6.8
	+ Genencor 1.3 g/kg VS	0.014	0.001	8.5	-4.3
	+ Novozyme 1.3 g/kg VS	0.013	0.001	6.0	-8.1
	+ MethaPlus 1.3 g/kg VS - IN	0.015	0.000	3.0	4.7
	+ GoldFerm 1.3 g/kg VS - IN	0.015	0.000	1.8	5.1
	+ Genencor 1.3 g/kg VS - IN	0.015	0.000	2.4	1.7
	+ Novozyme 1.3 g/kg VS - IN	0.015	0.001	3.6	1.3

Table A10. Final methane yield of coarse rye silage [Batch digestion in stirred glass digesters at 37°C for 35 days. Addition of each enzyme component in the mixture at a dosage of 0.17 g/kg VS].

	Number of replicates used	Methane content of biogas (% v/v)	Specific methane yield (m^3/kg VS)	Standard deviation	Relative standard deviation (%)
Hay (standard substrate)	3	54	0.297	0.001	0.2
Rye silage	3	53	0.299	0.007	2.4
Rye silage + Pectinase	2	51	0.291	0.008	2.6
Rye silage + Pectinase + Laccase	2	52	0.296	0.010	3.4
Rye silage + Cellulase + Pectinase + Laccase	2	54	0.302	0.008	2.5

Table A11. Comparison between final methane yields in the HBT process and in stirred glass digesters [Batch digestion in stirred glass digesters or HBT process at 37°C for 35 days. Addition of each component in the mixture at a dosage of 0.17 g/kg VS of enzyme product in stirred glass digesters and of 0.13 g/kg VS in the HBT process, respectively].

Specific methane yields (m^3 / kg VS)

	Stirred glass digesters with standard inoculum	HBT process with standard inoculum	HBT process with sewage sludge
Hay (standard substrate)	0.297	0.308	0.306
Rye silage	0.299	0.341	0.332
Rye silage + Pectinase	0.291	0.333	0.344
Rye silage + Pectinase + Laccase	0.296	0.334	0.339
Rye silage + Cellulase + Pectinase + Laccase	0.302	0.335	0.334

Relative standard deviation (%)

	Stirred glass digesters with standard inoculum	HBT process with standard inoculum	HBT process with sewage sludge
Hay (standard substrate)	0.2	0.6	1.2
Rye silage	2.4	2.4	3.4
Rye silage + Pectinase	2.6	3.1	0.6
Rye silage + Pectinase + Laccase	3.4	2.1	2.4
Rye silage + Cellulase + Pectinase + Laccase	2.5	2.0	3.1

Batch digestion of grass silage with weak inoculum

Table A12. Final methane yield of grass silage with weak inoculum [Batch digestion in HBT process at 37°C for 35 days. n=2. Application of the enzyme product MethaPlus L 100 at a dosage of 11 g/kg VS of substrate].

	Specific methane yield (m^3/kg VS)	Standard deviation
Grass silage without enzyme	0.187	0.005
Grass silage + MethaPlus L100	0.194	0.018

Batch digestion of grass silage with diluted inoculum

Table A13. Final methane yield of grass silage with diluted inoculum [Batch digestion in HBT process at 37°C for 60 days].

Inoculum dilution rate	Enzyme addition	Number of replicates used	Specific methane yield (m^3/kg VS)	Standard deviation
3-fold	without enzyme	3	0.245	12.2
	0.7 g/kg VS MethaPlus L100	3	0.269	5.9
	7 g/kg VS MethaPlus L100	3	0.265	3.6
5-fold	without enzyme	3	0.271	5.5
	0.7 g/kg VS MethaPlus L100	2	0.263	8.3
	7 g/kg VS MethaPlus L100	3	0.262	1.6
10-fold	without enzyme	2	0.209	9.3
	0.7 g/kg VS MethaPlus L100	3	0.280	4.4
	7 g/kg VS MethaPlus L100	2	0.290	3.3

Batch digestion of maize straw after separate enzymatic pretreatment

Table A14. Final methane yields of maize straw after an enzymatic pretreatment step [Enzymatic pretreatment step: hydrolysis of 1.8 g maize straw + enzyme in 10 mL water for 24 hours at 45°C after setting the pH to 4.5. Enzyme mixture: Genencor Laminex BG 80% + Novozym 188 20% (w/w). Anaerobic digestion: Batch digestion in HBT process at 37°C for 35 days].

			Specific methane yield (m^3/kg VS)	Relative standard deviation (%)	Change of the methane yield (%)
Direct enzyme addition at the start of anaerobic digestion	without enzyme		0.354	3.9	0.0
	Inactivated enzyme	1.3 g/kg VS	0.348	2.4	-1.8
		13 g/kg VS	0.354	2.2	0.0
	Active enzyme	1.3 g/kg VS	0.354	2.4	0.1
		13 g/kg VS	0.356	1.2	0.6
Enzymatic hydrolyis 24h at pH 4.5 and substrate:water ratio of 1:6	without enzyme		0.340	2.6	-4.1
	Inactivated enzyme	1.3 g/kg VS	0.354	2.1	-0.1
		13 g/kg VS	0.356	0.4	0.6
	Active enzyme	1.3 g/kg VS	0.361	1.8	2.0
		13 g/kg VS	0.355	1.4	0.2

Batch digestion of reactor samples from a biogas plant

Table A15. Final methane yields of effluent from a biogas plant at mesophilic temperature [Batch digestion in HBT process at 37°C for 87 days. n=3. Enzyme product at low (0.2 g/kg FW) and high dosage (2 g/kg FW of reactor sample). Dose added in 20 equal portions every 3 days from the start of the experiment].

Effluent from first reactor			Specific methane yield (m^3/kg VS)	Relative standard deviation (%)	Increase of methane yield (%)
Without enzyme (only water)			0.170	2.8	0.0
Active enzyme	Low dosage	MethaPlus L100	0.173	4.5	1.8
		Pulpzym HC + Novozym 342	0.179	5.5	5.3
	High dosage	MethaPlus L100	0.165	5.8	-2.7
		Pulpzym HC + Novozym 342	0.180	7.9	6.2
Thermally inactivated enzyme	Low dosage	MethaPlus L100	0.178	0.9	4.6
		Pulpzym HC + Novozym 342	0.178	8.0	4.7
	High dosage	MethaPlus L100	0.182	5.3	6.9
		Pulpzym HC + Novozym 342	0.178	2.5	4.9

Effluent from second reactor			Specific methane yield (m^3/kg VS)	Relative standard deviation (%)	Increase of methane yield (%)
Without enzyme (only water)			0.118	5.4	0.0
Active enzyme	Low dosage	MethaPlus L100	0.122	5.4	2.9
		Pulpzym HC + Novozym 342	0.121	6.2	2.1
	High dosage	MethaPlus L100	0.127	2.1	7.3
		Pulpzym HC + Novozym 342	0.137	0.4	15.7
Thermally inactivated enzyme	Low dosage	MethaPlus L100	0.114	8.2	-3.6
		Pulpzym HC + Novozym 342	0.121	0.6	2.4
	High dosage	MethaPlus L100	0.125	3.1	6.0
		Pulpzym HC + Novozym 342	0.131	7.9	10.7

Table A16. Final methane yields of effluent from a biogas plant at psychrophilic temperature [Batch digestion in HBT process at room temperature for 180 days. n=3. Enzyme product at high dosage: 2 g/kg FW of reactor sample. Dose added in 20 equal portions every 3 days from the start of the experiment].

		Specific methane yield (m^3/kg VS)	Relative standard deviation (%)	Increase of methane yield (%)
Effluent from first reactor	Without enzyme (only water)	0.124	11.0	0.0
	MethaPlus L100	0.130	5.5	5.2
	Pulpzym HC + Novozym 342	0.145	3.9	17.1
Effluent from second reactor	Without enzyme (only water)	0.070	4.4	0.0
	MethaPlus L100	0.074	6.9	5.6
	Pulpzym HC + Novozym 342	0.086	4.4	21.8

A 5 Complementary experiment with the enzyme product Goldferm-Mais

A batch anaerobic digestion experiment showing significant effects of an enzyme additive on maize straw and maize corncob in the HBT process was replicated to verify the validity of the results. The experimental protocol was optimized to improve the reliability of the assay. Substrate grinding using a laboratory mixer-grinder was thought to be insufficient to reduce particle size to a level ensuring homogenous samples with low amounts of substrate applied in the HBT process. Therefore, the laboratory mill used for substrate grinding was replaced with a laboratory mortar (KM 100, Retsch, Haan, Germany). It was also thought that a drying effect on substrate in open air during weighing of the feed, at the beginning of the experiment, which lasts for several hours, could influence the results. Therefore, caution was taken in using several subsamples and tightly closing samples bags between each weighing step. Inactivated enzyme variants were prepared through heating of diluted enzyme product in water at 121°C for 30 min using an autoclave.

In previous trials Goldferm-Mais seemed to yield higher increases of the methane production of maize straw and maize corncob at 1 g/kg TS. In order to verify the results, complementary experiments were run on maize straw using Goldferm-Mais, under similar operating conditions, at concentrations of 0.1 g/kg TS, 1 g/kg TS and 10 g/kg substrate TS. Standard deviations of the cumulated methane yields of maize straw after 35 days ranged between 1.1 and 3.6%. Although enzyme concentration(10 g/kg substrate-TS) was 10-fold higher than in the first trials, no significant difference to the variant without enzyme addition was observed, according to student t-test at $p<0.05$. Therefore, previous results showing a significant effect of the enzyme additive Goldferm –Mais were not confirmed.

Additional research would be useful to characterize and assess the effects of Goldferm Mais, as this product clearly differs from classical purified enzymes. Analyses run by Suzanne Herr, scientific assistant, under the direction of Prof. Dr. Lutz Fischer, head of the Biotechnology department of the Institute of Food science and Biotechnology of the University of Hohenheim, revealed a high content of living microorganisms, mostly anaerobic yeasts and bacteria, and very low enzymatic activity. Therefore, this product should be considered as a microbial additive rather than as an enzyme product. Testing, characterizing and developing such microbial additives could be the topic of another work, but does not match the scope of this thesis. Microorganisms adaptation, rather than enzymatic activity, would be the key to efficient use of such products. Therefore, investigations with Goldferm Mais were abandoned.

Table A17. Final methane yield of maize straw tested with Goldferm-Mais [Batch digestion in HBT process at 37°C for 35 days. n=3].

	Specific methane yield (m^3/kg VS)	Standard deviation	Relative standard deviation (%)
Maize straw without additive	0.314	0.007	2.1
Maize straw + Goldferm-Mais 0.1 g/kg VS thermally inactivated	0.322	0.010	3.3
Maize straw + Goldferm-Mais 1.1 g/kg VS thermally inactivated	0.314	0.002	0.7
Maize straw + Goldferm-Mais 11 g/kg VS thermally inactivated	0.314	0.001	0.4
Maize straw + Goldferm-Mais 0.1 g/kg VS	0.317	0.012	3.6
Maize straw + Goldferm-Mais 1.1 g/kg VS	0.318	0.013	4.2
Maize straw + Goldferm-Mais 11 g/kg VS	0.324	0.004	1.1

Table A18. Effect of Goldferm Mais on final methane yields of maize straw [Final values after 35 days of digestion at 37°C. Average values. n=3].

Enzyme State	Enzyme concentration	Change of the methane yield of maize straw (%)
	None	0.0 – Reference
Inactivated	0.1 g/kg VS	+2.3
	1.1 g/kg VS	+0.1
	11 g/kg VS	-0.1
Active	0.1 g/kg VS	+0.9
	1.1 g/kg VS	+1.2
	11 g/kg VS	+3.2

A 6 List of publications related to the thesis

Brulé M., Vogtherr J., Lemmer A., Oechsner H., Jungbluth T (2011). Effect of enzyme addition on the methane yields of effluents from a full-scale biogas plant. Landtechnik 66(1):50-52. ISSN 0023-8082.

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Stefana J., Diguta C., Israel-Roming F., Stelian P., Vintiloiu A., Brule M., Mukengele M. (2007). Hydrolysis of lignocellulosic biomass using hydrolytic enzymes and chemical pretreatments, Biotehnologii, Seria F, 2007. Vol. 12:47-52. ISSN 1224-7774.

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Brulé M., Oechsner H. Fischer L., Lemmer A., Jungbluth T. (2007). Effect of enzymatic substrate preparation on the biogas yield of energy crops. Landtechnik 62(6):414-415. ISSN 0023-8082.

Diguta C., Jurcoane S., Israel-Roming F., Brulé M., Mukengele M., Lemmer A., Oechsner H. (2007). Studies concerning enzymatic hydrolysis of energy crops, Romanian biotechnological letters 12(2):3203-3207. ISBN 1224-5984.