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Outi Pakarinen

Methane and Hydrogen Production from Crop Biomass through Anaerobic Digestion

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ABSTRACT

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Yhteenveto: Metaanin ja vedyn tuottaminen energiakasveista anaerobiprosessissa
Diss.

The feasibility of methane and hydrogen production from energy crops through anaerobic digestion was evaluated in this thesis. The effects of environmental conditions, e.g. pH and temperature, as well as inoculum source on H_2 yield were studied in batch assays. In addition, the effects of pre-treatments on methane and hydrogen yield as well as the feasibility of two-stage H_2 + CH_4 production was evaluated. Moreover, the effect of storage on methane yield of grasses was evaluated. Monodigestion of grass silage for methane production was studied, as well as shifting the methanogenic process to hydrogenic. Hydrogen production from grass silage and maize was shown to be possible with heat-treated inoculum in batch assays, with highest H_2 yields of 16.0 and 9.9 ml gVS_{added}^{-1} from untreated grass silage and maize, respectively. Pre-treatments (NaOH, HCl and water-extraction) showed some potential in increasing H_2 yields, while methane yields were not affected. Two-stage H_2 + CH_4 producing process was shown to improve CH_4 yields when compared to traditional one-stage CH_4 process. Methane yield from grass silage monodigestion in continuously stirred tank reactor (CSTR) with organic loading rate (OLR) of 2 kgVS $(m^3d)^{-1}$ and hydraulic retention time (HRT) of 30 days was at most 218 l $kgVS_{fed}^{-1}$. Methanogenic process was shifted to hydrogenic by increasing the OLR to 10 kgVS $(m^3d)^{-1}$ and shortening the HRT to 6 days. Highest H_2 yield from grass silage was 42 l $kgVS_{fed}^{-1}$ with a maximum H_2 content of 24 %. Energy crops can be successfully stored even for prolonged periods without decrease in methane yield. However, under sub-optimal storage conditions loss in volatile solids (VS) content and methane yield can occur. According to present results energy crops such as grass silage and maize can be converted to hydrogen or methane in AD process. Hydrogen energy yields are typically only 2-5 % of the methane energy yields, but the overall energy yield of the process can be increased by two-stage H_2 + CH_4 producing process. In addition, the ongoing methanogenic process can be shifted towards hydrogen production by increasing the OLR and shortening the HRT. However, the process needs further research to optimize especially the H_2 production.

Keywords: Energy crop; grass silage; hydrogen; methane; pre-treatment; storage; two-stage anaerobic process.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V.

I planned the experimental work with my supervisor and co-authors and did the main part of the experimental work. I wrote the first draft of all the papers and finalized them with my co-authors and supervisor.

I Pakarinen O., Lehtomäki A., Rissanen S. & Rintala J. 2008. Storing energy crops for methane production: effects of solids content and biological additive. *Bioresource Technology* 99: 7074–7082.

II Pakarinen O., Lehtomäki A. & Rintala J. 2008. Batch dark fermentative hydrogen production from grass silage: the effect of inoculum, pH, temperature and VS-ratio. *International Journal of Hydrogen Energy* 33: 594–601.

III Pakarinen O., Tähti H. & Rintala J. 2009. One-stage H_2 and CH_4 and two-stage H_2 + CH_4 production from grass silage and from solid and liquid fractions of NaOH pre-treated grass silage. *Biomass & Bioenergy* 33: 1419–1427.

IV Pakarinen O., Kaparaju P. & Rintala J. Hydrogen and methane yields of untreated, water-extracted and acid (HCl) treated maize in one and two-stage batch assays. *International Journal of Hydrogen Energy*. In press.

V Pakarinen O., Kaparaju P. & Rintala J. 2011. The effect of organic loading rate and retention time on hydrogen production from a methanogenic CSTR. *Bioresource Technology* 102: 8952–8957.

ABBREVIATIONS

AA	acetic acid
ACF	upflow anaerobic contact filter
AD	anaerobic digestion
BA	butyric acid
BESA	2-bromoethanesulfonic acid
CA	caproic acid
CFU	colony forming unit
ch	carbohydrate
COD	chemical oxygen demand
CSTR	continuously stirred tank reactor
D	dilution rate
DP	degree of polymerization
FID	flame ionizatiion detector
FM	fresh matter
GC	gas chromatograph
ha	hectare
HMF	hydroxymethyl furfural
HRT	hydraulic retention time
HSW	household solid waste
IBA	iso-butyric acid
IVA	iso-valeric acid
LAB	lactic acid bacteria
OLR	organic loading rate
oww	original wet weight
PA	propionic acid
PEMFC	proton-exchange membrane fuel cell
pH_2	partial pressure of hydrogen
rpm	revolutions per minute
SC	semi-continuous
SCOD	soluble chemical oxygen demand
t	metric ton
TCD	thermal conductivity detector
TS	total solids
TVFA	total volatile fatty acids
TVS	total volatile solids
UASB	upflow anaerobic sludge blanket
VA	valeric acid
VFA	volatile fatty acid
VS	volatile solids
ww	wet weight
WWTP	wastewater treatment plant

1 INTRODUCTION

1.1 Background

Methane (CH_4) and hydrogen (H_2) are valuable gaseous compounds which can be used as fuels or chemicals. Nowadays, hydrogen is mainly utilized as a reductant in oil refining, and ammonia and methanol production (Muradov & Veziroğlu 2008), while methane is almost entirely used for heat and power production and increasingly also as a vehicle fuel (Thomas & Dave 2003). For fuel cells hydrogen is often the preferred fuel, while methane or even biogas (containing CH_4 and CO_2) can be used in e.g. solid oxide fuel cells (Murray et al. 1999, Xuan et al. 2009). When methane is used as a traffic fuel instead of gasoline CO_2 emissions can be reduced about 25 % (Wang & Huang 1999), while the main advantage of H_2 as a fuel is the absence of CO_2, CO and hydrocarbon emissions (Marbán & Valdés-Solís 2007, Balat 2008) as the major oxidation product is water vapour (small amount of NO_x).

Hydrogen has a high energy content on mass basis (lower heating value of 120 MJ kg^{-1}) as compared to methane (50 MJ kg^{-1}) and gasoline (44 MJ kg^{-1}), while the energy content on volume basis (10.8 MJ $(Nm^3)^{-1}$), is, however, less than one third that of CH_4 (35.9 MJ $(Nm^3)^{-1}$, Balat 2008). The sustainability of methane and hydrogen depends on the production process and the original energy source (fossil or renewable) (Ball & Wietschel 2009). Currently, H_2 is almost entirely produced from fossil energy sources, mainly by steam-reforming of natural gas (Mueller-Langer et al. 2007) and the methane used derives almost entirely from fossil natural gas.

Biomass is available in various forms such as organic waste, animal manure, energy crops and crop residues for renewable energy production (Hoogwijk et al. 2003). Biomass can be converted into energy using thermochemical and biotechnological processes of which anaerobic digestion (AD) is a competitive concept for methane production in both energy efficiency and environmental impact comparison studies (Fredriksson et al. 2006). AD can use various crop materials and wastes as substrates while nutrients can be

recirculated for further cultivation (Fredriksson et al. 2006). Moreover, carbohydrate rich crop biomass could be especially suitable for H_2 production through AD (Chong et al. 2009). Energy crops are considered as the major resource among the biomass for renewable energy production (Hoogwijk et al. 2003) and crops with high biomass yields and efficient conversion into energy are considered most sustainable.

Grasses are classified among potential crops for biogas production in northern conditions due to their potential high CH_4 yield per hectare and suitability in current agricultural cultivation, harvest and storage practices (e.g. Lehtomäki et al. 2008a, Prochnow et al. 2009). Moreover, maize is increasingly used as feedstock for CH_4 production especially in Germany and Austria due to its high biomass yield (Amon et al. 2007) and maize and grass silage are the most applied co-substrates in agricultural biogas plants in Germany (Weiland 2006). In addition, grass and maize based biogas production has been found feasible in terms of energy and CO_2 balance (Gerin et al. 2008). In this summary the main focus is on AD process as a means for converting crop biomass for renewable energy, especially for hydrogen production.

1.2 Anaerobic digestion pathways

1.2.1 Hydrolysis

Anaerobic digestion of solid substrates is typically divided to four main steps (Fig. 1). In hydrolysis, organic polymers are degraded by enzymes to soluble compounds, which are further degraded to e.g. volatile fatty acids (VFA), H_2 and CO_2 during acidogenesis. VFAs are oxidized in acetogenesis to acetate, H_2 and CO_2 (very low partial pressure of H_2 is needed) which are further converted to methane in methanogenesis (Madigan et al. 2009).

In the hydrolysis (Fig. 1) organic polymers are degraded into soluble monomers, e.g. cellulose is hydrolyzed to glucose units in enzymatic reactions (Malherbe & Cloete 2002). Efficient hydrolysis of cellulose involves at least three groups of enzymes, namely endoglucanases, exoglucanases and β-glucosidase. More enzymes are required for complete degradation of hemicellulose because of its greater complexity compared to cellulose. Of these, xylanase is the best studied. Anaerobic bacterial species, especially *Clostridium* spp. (e.g. *C. cellobioparum*, *C. acetobutylicum*) contain complexed cellulase systems which enables high hydrolysis efficiency (Ljungdahl & Eriksson 1985, Malherbe & Cloete 2002). Other anaerobic bacteria with cellulolytic activity are e.g. *Acetivibrio cellulolyticus*, *Ruminococcus albus* and *Eubacterium cellusolvens* (Ljungdahl & Eriksson 1985). The enzymatic hydrolysis of lignin is limited due to its irregular shape (Malherbe & Cloete 2002) and lignin is hardly degraded in anaerobic conditions (Jimenez et al. 1990). Hydrolysis rate coefficients (constants) for solid materials are normally in the order of 0.1-0.3 day^{-1}

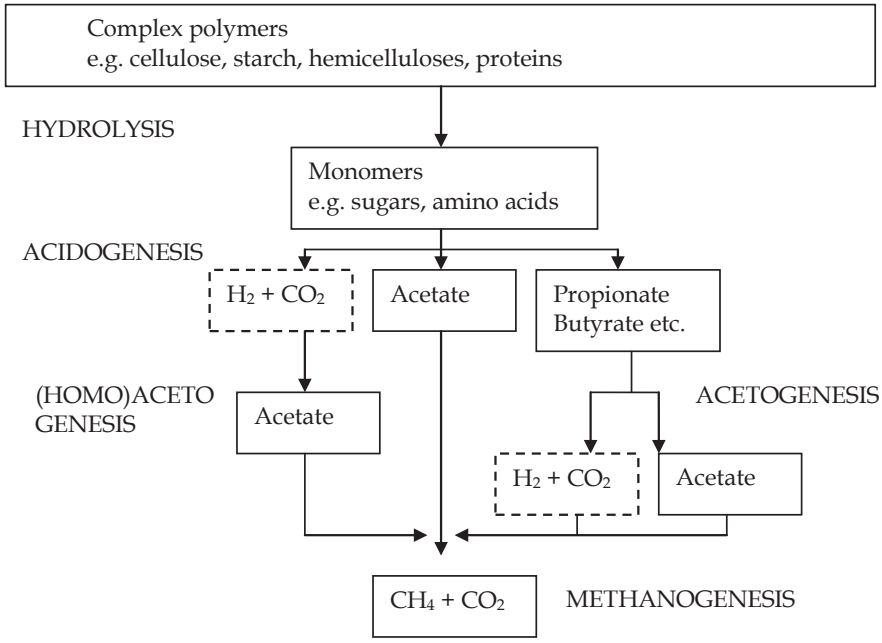

FIGURE 1 CH_4 and H_2 production from polymeric substrates. Dark fermentative hydrogen production shown with dashed line (partially adapted from Madigan et al. 2009).

(Weiland 2010, Angelidaki et al. 2011), while kinetic coefficients of the first-order rate of hydrolysis for energy crops and crop residues are reported to be 0.009-0.094 day^{-1} (Lehtomäki et al. 2005a).

1.2.2 Acidogenesis

The soluble compounds formed in the hydrolysis (e.g. glucose, xylose) are further oxidized to e.g. VFAs (acetic, propionic, butyric etc.), H_2 and CO_2 in the acidogenesis step (Fig. 1) (also referred to as fermentation) by fermentative bacteria. In fermentation, some of the molecules of the substrate are reduced, whereas others are oxidized, usually to CO_2. In many fermentation reactions redox balance is maintained by the production of molecular hydrogen (H_2) as protons (H^+) of the water serve as electron acceptor. Production of hydrogen is related to the activity of an iron-sulfur protein called ferredoxin, an electron carrier of low redox potential. The transfer of electrons from ferredoxin to H^+ is catalyzed by the enzyme hydrogenase (Madigan et al. 2009). Thus, hydrogen production is dependent on the presence of a hydrogen-producing enzyme containing complex metallo-clusters as active sites (Bartacek et al. 2007). The energetics of hydrogen production are somewhat unfavourable, so that most fermentative organisms only produce a relatively small amount of H_2 along

with other fermentation products (Madigan et al. 2009) as fermentations have been optimized by evolution to produce cell biomass and not hydrogen (Hallenbeck 2005). Hydrogen production in acidogenesis, i.e. dark fermentative hydrogen production, is covered in more detail in Section 1.2.

1.2.3 Acetogenesis

In acetogenesis VFAs (e.g. propionic and butyric acids) are oxidized by acetogenic bacteria to acetic acid and H_2, which are used as substrates in methanogenesis (Fig. 1). Interspecies hydrogen transfer (e.g. to methanogens) makes otherwise energetically unfavourable reaction possible. Most acetogenic bacteria that produce acetate are gram-positive *Bacteria*, and many are species of the spore-forming *Clostridium* (e.g. *C. aceticum*) or the non-spore-forming *Acetobacterium* (e.g. *A. woodii*, Madigan et al. 2009). Acetogenic bacteria typically grow more slowly when compared to acidogenic bacteria. Most acetogenic bacteria can grow heterotrophically by fermenting sugars (Madigan et al. 2009). Homoacetogens consume CO_2 and H_2 producing acetate according to the following equation (1).

$$4\ H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O \quad (1)$$

1.2.4 Methanogenesis

Methanogens are obligate anaerobic *Archaea* (Madigan et al. 2009), divided into five phylogenetic orders, namely *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales* and *Methanopyrales* showing diverse cell morphology and optimal growth conditions (Angelidaki et al. 2011). Three classes of methanogenic substrates are known, i.e. CO_2 type substrates (CO_2, CO, formate), methyl substrates (methanol, methylamine, dimethylamine, trimethylamine) and acetotrophic substrates (acetate) (Madigan et al. 2009). In anaerobic digesters treating wastewater biosolids, 70 % of the methane derives from acetate and 30 % from hydrogen. Acetotrophic methanogens degrade acetic acid according to Equation (2).

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3 \quad (2)$$

Hydrogenotrophic methanogens produce methane autotrophically from carbon dioxide (carbon source and electron acceptor) and hydrogen (electron donor). Methanogenesis from H_2 and CO_2 is presented in Equation (3).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2\ H_2O \quad (3)$$

Anaerobic reactions will ultimately lead to production of CH_4 and CO_2, thus, in traditional AD process the biogas is mainly composed of methane (50-70 %) and carbon dioxide (Madigan et al. 2009).

1.3 Hydrogen production in acidogenesis

1.3.1 Theoretical hydrogen yield and hydrogen producing micro-organisms

Theoretical maximum of 12 moles of H_2 from one mole of glucose is not thermodynamically possible reaction. Instead, the highest H_2 production (4 moles) can be achieved, when one mole of glucose ($C_6H_{12}O_6$) is degraded to 2 moles of acetate (Equation 4), resulting in COD reduction of 33 % in the form of H_2 (Bartacek et al. 2007). When glucose is degraded to butyrate, in theory 2 moles of H_2 per one mole of glucose (Equation 5) can be produced (Hallenbeck 2005). Theoretical maximum from one mole of xylose ($C_5H_{10}O_5$) is 3.33 moles H_2 with acetate as the sole end product (Cui et al. 2010), while theoretical maximum from one mole of sucrose ($C_{12}H_{22}O_{11}$) is 8 moles of H_2 (Logan et al. 2002).

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2 \text{ acetate} + 2CO_2 + 4H_2 \quad (4)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow \text{butyrate} + 2CO_2 + 2H_2 \quad (5)$$

Among a large number of microbial species, strict anaerobes such as clostridia (e.g. *C. pasteurianum, C. butyricum, C. beijerinkii*), are efficient producers of hydrogen, with theoretical H_2 yield of 4 moles per mole of glucose. Practical yields from these fermentations are near 2 or slightly above (Nath & Das 2004, Hallenbeck 2005, Mohan 2009), as some of the substrate is used as energy and carbon source for bacteria (Kapdan & Kargi 2006), other degradation products than acetic acid can be produced, and H_2 consuming reactions can occur (Li & Fang 2007). Hydrogen is produced during the exponential growth phase of clostridia (Bartacek et al. 2007) and in addition to acetate, fermentation can yield ethanol, butyrate, butanol and acetone (Hawkes et al. 2002, Nath & Das 2004, Hallenbeck 2005). Clostridia form spores under unfavourable conditions, such as lack of nutrients or heat-treatment and are highly sensitive to oxygen (Bartacek et al. 2007).

In addition, facultative anaerobic bacteria, such as enteric bacteria, can produce at most 2 moles of H_2 per mole of glucose (Nath & Das 2004, Hallenbeck 2005, Kapdan & Kargi 2006), while in practise about one half of this theoretical H_2 yield is observed (Hallenbeck 2005). *Enterobacter* sp. can tolerate oxygen (Bartacek et al. 2007) and carry out a mixed-acid fermentation producing lactate, ethanol, acetate, formate, H_2, CO_2, succinate and butanediol. In mixed cultures, mesophilic *Clostridium* sp. and thermophilic *Thermoanaerobacterium* sp. are the species most often indicated (Bartacek et al. 2007). Moreover, extreme-thermophilic (70 °C) hydrogen (+ethanol) producers (from glucose) have been found; e.g. *Thermoanaerobacter*, *Thermoanaerobacterium* and *Caldanaerobacter* (Zhao et al. 2009).

In addition to hexose or pentose utilizing H_2-producers, some bacteria, e.g. *C. cellulolyticum*, *C. acetobutylicum X9, C. cellobioparum* and *C. thermocellum*

are capable of H_2 production from cellulose (Levin et al. 2009, Madigan et al. 2009, Ren et al. 2009), with e.g. acetic acid, lactic acid, succinic acid and ethanol as fermentation products (Madigan et al. 2009). Moreover, species such as *C. butyricum*, *C. acetobutylicum*, *C. cellobioparum*, *C. pasteurianum* and *C. perfringens* are capable of fermentation of starch and pectin in addition to sugars, with fermentation products of acetone, butanol, ethanol, isopropanol, butyric acid, acetic acid, propionic acid and lactic acid (Madigan et al. 2009). H_2 yields from cellulose are typically between 1-2 mol (mol hexose)$^{-1}$ (Levin et al. 2009). Recently H_2 yield of 10.1 mmol (g cellulose)$^{-1}$ has been reported (Wang et al. 2011a), while 2.32 mol (mol glucose)$^{-1}$ has been obtained from starch in CSTR (Akutsu et al. 2008). Factors affecting cellulose degradation are among others initial and final pH, substrate type and concentration as well as inhibition by degradation products (Ren et al. 2009).

1.3.2 Inoculum for hydrogen production

In a typical AD process hydrogen is produced, but is not detected, as it is immediately consumed by hydrogen consuming micro-organisms, e.g. methanogens, homoacetogens and sulphate-reducing bacteria (Madigan et al. 2009). It has been shown, that hydrogen production instead of methane is possible, by adjusting the process parameters and by inactivating H_2 consuming micro-organisms. In batch hydrogen production this is typically achieved by heat-treating the inoculum, as hydrogen producers are spore-forming, and can thus survive under severe conditions (heat, acidic or alkaline pH), while hydrogen consumers, e.g. methanogens can not (O-Thong et al. 2009). However, heat-treatment is energy intensive (Wang & Zhao 2009) and H_2 consuming micro-organisms can be introduced with the substrate in continuous processes. It has also been shown, that bacterial diversity can be diminished after heat-treatment (Baghchehsaraee et al. 2008) and heat-treatment is not always necessary for hydrogen production (e.g. Antonopoulou et al. 2008, Pan et al. 2008, Ohnishi et al. 2010). Other methods for preparation of hydrogen producing inoculum include organic shock load, acid, base and chemical inhibitors, e.g. 2-bromoethanesulfonic acid (BESA) and acetylene (Bartacek et al. 2007, Hawkes et al. 2007, O-Thong et al. 2009). During shock load VFAs, H_2, CO_2 and formate accumulate, which can lead to inhibition of methanogens. Load-shock has been found as an effective method for preparing H_2 producing inoculum with H_2 yield of 1.96 mol (mol hexose)$^{-1}$ (O-Thong et al. 2009) and with higher species diversity as compared to heat-treated inoculum. In addition, some researchers prefer to use the indigenous microflora of the substrate without any inoculum addition (Antonopoulou et al. 2008, Wang & Zhao 2009, Antonopoulou et al. 2011). In practical applications, hydrogen producing system should be easily obtained and thus shifting the ongoing (typically methanogenic) process to hydrogen production could be an interesting opportunity. Typically, as compared to methanogenic process, shorter HRT, higher OLR and lower pH are favoured in hydrogenic process (Hawkes et al. 2002).

In addition to mixed culture, pure cultures can be used for H_2 production. However, the use of pure cultures is expensive and technically difficult requiring aseptic conditions. Besides, pure cultures have limited metabolic capabilities in degrading polymeric carbohydrates such as starch and cellulose (Argun & Kargi 2009) and they can easily be contaminated by H_2 consuming micro-organisms (Bartacek et al. 2007). Thus, mixed cultures could be more suitable to complex substrates, such as energy crops (Hallenbeck 2009, Hallenbeck & Ghosh 2009).

1.3.3 The effect of pH and substrate concentration on hydrogen production

Optimal pH (Table 1) for hydrogen production differs from one study to another, but a pH level between 5 and 7 is usually favoured (Fang & Liu 2002, Khanal et al. 2004, Kapdan & Kargi 2006, Bartacek et al. 2007, Guo et al. 2010). Simplest and most economic method for methanogen inhibition could be biokinetic control, mainly utilization of low pH (Valdez-Vazquez & Poggi-Varaldo 2009) as the optimum pH for growth of *Clostridium* sp. is in the range 4.5-5.5, whereas optimum pH for methanogens is around 7 (Bartacek et al. 2007). Low pH (5.5) has been found as an effective method for continuous H_2 production from household solid waste as otherwise methane was produced even with short HRT of 2-6 days at pH controlled to 7 (Liu et al. 2008a).

In acidogenesis VFAs are formed in addition to hydrogen as metabolic products. Fermentative bacteria are incapable of further breaking down the acids and acid accumulation causes a rapid drop of pH and subsequent inhibition of bacterial hydrogen production (Nath & Das 2004). When hydrogen production is prevented, more reduced end-products e.g. ethanol (Equation 6), butanol and lactic acid (7), will be formed. These degradation products contain additional H atoms that are not liberated as gas (Nath & Das 2004, Akutsu et al. 2009a, Madigan et al. 2009). Hydrogen consumption can occur e.g. when formic acid (8) is produced. Thus, drop in pH can result in shift in metabolic pathways as well as changes in microbial communities (Guo et al. 2010).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (6)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3C(OH)HCOOH \quad (7)$$

$$H_2 + HCO_3^- \rightarrow HCOO^- + H_2O \quad (8)$$

However, hydrogen and ethanol can be formed simultaneously according to Equation (9) (Akutsu et al. 2009b).

$$C_6H_{12}O_6 + H_2O \rightarrow 2H_2 + 2CO_2 + C_2H_5OH + CH_3COOH \quad (9)$$

TABLE 1 Several factors (such as temperature and pH) have an effect on H_2 yield in dark fermentation. The main factors are listed below as well as some of the relevant references.

Factor	Effect	Mechanism	References
Temperature	Higher H_2 yields can be obtained in higher temperatures.	Conversion of acetate to H_2 can become favorable. H_2 consuming reactions, e.g. lactate and propionate production and homoacetogenesis can be prevented. Growth is typically faster at higher temperatures.	Nath & Das (2004) Akutsu et al. (2009a) Luo et al. (2010a)
pH_2	Higher H_2 yields can be obtained if pH_2 is kept low.	Increasing pH_2 inhibits H_2 production.	Nath & Das (2004) Liu et al. (2006) Nguyen et al. (2010)
Loading/ substrate concentration	Optimal substrate concentration is dependent on e.g. substrate and inoculum.	After optimal substrate concentration bacterial metabolism can shift towards alcohol production, which results in decreased H_2 yield. High load can be used to inhibit methanogens.	Fan et al. (2006a) Wang et al. (2006) Zhang et al. (2007) Fan et al. (2008) Akutsu et al. (2009a) García-Peña et al. (2009) O-Thong et al. (2009)
pH	Optimal pH differs from one study to another, typically pH between 5-7 is favoured.	Optimum pH for clostridia is around 5, in addition, low pH can be used to inhibit methanogens.	Fang & Liu (2002) Khanal et al. (2004) Kapdan & Kargi (2006) Bartacek et al. (2007) Liu et al. (2008a) Valdez-Vazquez & Poggi-Varaldo (2009) Guo et al. (2010)
HRT	Typically short HRT favors H_2 production	Acidogenic H_2 producers grow faster compared to H_2 consuming methanogens, which can be washed away from the reactor.	Das & Veziroğlu (2001) Hawkes et al. (2002) Davila-Vazquez et al. (2008) Valdez-Vazquez & Poggi-Varaldo (2009)

Substrate concentration affects the metabolites produced and thus the H_2 yield (Table 1). In one study, the optimum glucose concentration was found to be 10 g l^{-1}, as higher (20 and 30 g l^{-1}) concentrations resulted in decreased H_2 yield and more reduced end products such as ethanol (García-Peña et al. 2009). Metabolic shift from acid to solvent production occurred with sucrose concentration of 30 gCOD l^{-1}, whereas optimum concentration for H_2 production was found to be 20 gCOD l^{-1} (Wang et al. 2006). A maximum H_2 yield from starch was obtained at a substrate concentration of 20 g l^{-1}, as at

higher concentrations the amount of formic and lactic acids increased (Akutsu et al. 2009a). Optimal substrate concentration for H_2 production from beer lees (Fan et al. 2006a), bio-pretreated corn stalk (Fan et al. 2008) and HCl-treated cornstalk (Zhang et al. 2007) was 20, 15 and 15 g l^{-1}, respectively, whereas with higher substrate concentrations H_2 yields decreased.

1.3.4 Effect of temperature on hydrogen production

Thermophilic process (Table 1) has the potential to achieve a greater hydrogen yield and higher hydrogen production rate than mesophilic process (Hallenbeck 2005, Valdez-Vazquez et al. 2005, Bartacek et al. 2007). The conversion of acetate to hydrogen (Equation 10) is thermodynamically unfavourable at moderate temperature (ΔG^0 = +104.6 kJ $(mol)^{-1}$) and is strongly determined by the H_2 partial pressure (Nath & Das 2004).

$$CH_3COOH + 2\ H_2O \rightarrow 4H_2 + CO_2 \quad (10)$$

Moreover, higher temperatures might inhibit H_2 consumers and suppress lactate forming bacteria (Davila-Vazquez et al. 2008, Chong et al. 2009). Propionic acid production from glucose consumes 2 moles of H_2 per one mole of glucose degraded (Li & Fang 2007) and it is known that propionic acid bacteria can ferment e.g. lactic acid and carbohydrates (Madigan et al. 2009). Hydrogen production from starch was found more stable in thermophilic conditions, as in mesophilic conditions hydrogen was consumed by homoacetogens (Akutsu et al. 2009a). Moreover, hydrogen yield from cassava stillage improved from 14 to 70 ml gVS^{-1} when temperature was increased from 37 to 60 °C. This was caused by decrease in propionate concentration and inhibition of homoacetogenesis in thermophilic conditions (Luo et al. 2010a).

1.3.5 The effect of HRT on hydrogen production

Short HRT of 0.5-12 h (i.e. high dilution rate) can be used to wash out methanogens in continuous processes with liquid substrates (Table 1), e.g. with sucrose or glucose containing wastewaters or hydrolysates (Davila-Vazquez et al. 2008, Valdez-Vazquez & Poggi-Varaldo 2009). This is based on the fact, that methanogens grow slower compared to acidogens (Hawkes et al. 2002) and the specific growth rates of methanogens are much lower than those of H_2-producing bacteria (0.0167 and 0.083 h^{-1}, respectively; Valdez-Vazquez and Poggi-Varaldo 2009). Microbial populations, which have larger growth rate than the dilution rate ($\mu_{max} > D$, inverse of HRT) can stay in the reactor. Fermentative bacteria have doubling time between 0.16 and 2 h (Das & Veziroğlu 2001), whereas the doubling time of methanogens is typically longer. However, with solid substrates, like energy crops, the hydrolysis is typically rate-limiting (Vavilin et al. 1996) and longer HRT is needed to allow hydrolysis.

1.3.6 Partial pressure of hydrogen

Several factors have been shown to affect H_2 yield and rate of production in dark fermentation. Increasing partial pressure of hydrogen (pH_2; Table 1) is known to be the major factor inhibiting H_2 production (Hawkes et al. 2002, Nath & Das 2004). In batch assays without sparging the head-space volume can be critical. The optimal headspace to liquid ratio for hydrogen production in a study with batch assays was found to be 80:40 (Nguyen et al. 2010). Increase in pH_2 can be prevented by constant flushing with some inert gas, e.g. N_2 and thus also interspecies hydrogen transfer can be prevented (Hallenbeck 2005, Massanet-Nicolau et al. 2010). In addition to removal of H_2, removal of CO_2 can lead to increased H_2 yield (Nath & Das 2004) due to inhibition of homoacetogenesis. Intermittent sparging with N_2 increased H_2 yield from 1.82 to 3.24 mol (mol glucose)$^{-1}$ by *Thermotoga neapolitana* (Nguyen et al. 2010). Upgraded biogas (CO_2 and H_2S removed) was used to sparge a laboratory reactor treating HSW and resulted in doubling the hydrogen production (Liu et al. 2006). However, sparging may not be practical, as the diluted gas stream is more expensive to purify (Hallenbeck 2005).

1.3.7 The effect of nutrients on hydrogen production

Hydrogen production requires nutrients for bacterial metabolism and growth. Effects of nitrogen and phosphate have been studied to some extent, as well as the effects of micronutrients. However, the results obtained are sometimes controversial and very dependent on e.g. the substrate and inoculum used (Li & Fang 2007). Micronutrients found essential for hydrogen production are e.g. magnesium, sodium, zinc and iron (sucrose as a substrate, Lin & Lay 2005), as iron is needed in hydrogenase-enzyme (Kapdan & Kargi 2006). Moderate addition of Fe^{2+} (113.7 mg l^{-1}) was shown to improve H_2 yield from HCl-treated beer lees (Cui et al. 2009), whereas the highest H_2 yield of 311 ml (g glucose)$^{-1}$ by mixed culture was obtained with Fe^{2+} concentration of 300-350 mg l^{-1} (Wang & Wan 2008a). Moreover, addition of Ni^{2+} at concentration of 0.1 mg l^{-1} resulted in highest H_2 yield of 296 ml (g glucose)$^{-1}$ by mixed culture (Wang & Wan 2008b). However, micronutrients can inhibit hydrogen production if applied in too high concentration. It has been shown, that acclimated microbial consortia remained active with Na^+ concentration of up to 6 g l^{-1} while without acclimation decrease in specific hydrogen production activity was observed with Na^+ concentration of 0.27 to 21 g l^{-1} (sucrose as a substrate, heat-treated inoculum, Kim et al. 2009).

1.3.8 Hydrogen production from model substrates

Most studies on H_2 production have used soluble model substrates like sucrose or glucose (e.g. Lin et al. 2007, García-Peña et al. 2009). Rather high H_2 yield of 3.6 molH_2 (mol glucose)$^{-1}$ was obtained with heat-treated methanogenic inoculum and the produced biogas (H_2 content 43 %) was used to fuel proton-

exchange-membrane fuel cell (PEMFC, García-Peña et al. 2009). In addition, continuous hydrogen production from sucrose (3.71 molH_2 (mol sucrose)$^{-1}$) by immobilized culture for over 300 days has been successfully demonstrated. The produced biogas (around 40 % H_2) was purified via a CO_2 absorber and silica-gel desiccator (H_2 purity > 99 %) for PEMFC (Lin et al. 2007). However, to reduce the cost of H_2 production, a lower-cost and more sustainable substrate (biomass or organic waste) should be used as a feedstock (Lin et al. 2007).

1.4 Two-stage hydrogen and methane production

In dark fermentative hydrogen production the energy yield in the form of H_2 is rather low, and lot of degradation by-products, e.g. VFAs and alcohols are present in the digestate. Two-stage AD producing both H_2 and CH_4 has been suggested as a feasible technology to improve the overall energy conversion efficiency (Hallenbeck 2009, Hallenbeck & Ghosh 2009). The growth rates and pH optima are different for acidogens and methanogens (Liu et al. 2004) and thus, in a two-stage AD system, faster growing acidogens are developed in the first-stage hydrogenic reactor and are involved in the production of VFAs and H_2. On the other hand, slow growing acetogens and methanogens are developed in the second-stage methanogenic reactor, in which the produced VFAs are further converted to CH_4 and CO_2 (Fig. 2). In addition, the optimal temperature for hydrolysis/acidogenesis can differ from optimal temperature for methanogenesis (Ward et al. 2008). Two-phase processes thus allow the selection and enrichment of different micro-organisms in each phase and can increase the stability of the process (Demirer & Chen 2005).

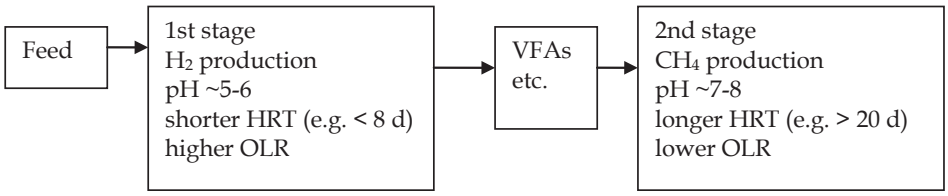

FIGURE 2 Two-stage hydrogen and methane production with some suggestions as optimal conditions.

The application of two-stage AD process for sequential H_2 and CH_4 production has been proposed as a promising technology for better process performance and higher energy yields as compared to the traditional one-stage CH_4 production process (e.g. Cooney et al. 2007, Antonopoulou et al. 2008). Two-stage H_2 + CH_4 system has been shown to improve CH_4 yield when compared to traditional one-stage methane process, as e.g. 21 % more CH_4 was obtained in a two-stage system from household solid waste (Liu et al. 2006) and 22 % more

from lipid-extracted microalgae (Yang et al. 2011). Besides improving methane yield, H_2 stage has been shown to enable higher OLR and shorter HRT in the subsequent methanogenic stage (Ueno et al. 2007a) and better effluent quality with less propionate and other VFAs (Wang et al. 2011b) when compared to one-stage system. Continuous laboratory experiments for two-stage H_2 and CH_4 production have been carried out, by using e.g. cheese whey (Venetsaneas et al. 2009), olive pulp (Koutrouli et al. 2009), sweet sorghum extract (Antanopoulou et al. 2008) food waste (Chu et al. 2008, Wang & Zhao 2009, Lee et al. 2010), potato waste (Zhu et al. 2008), and household solid waste (Liu et al. 2006) as substrates. Even pilot-scale H_2 + CH_4 production has been applied, with garbage and office paper as a substrate (Ueno et al. 2007b).

In the traditional two-stage system the first stage is not optimized for H_2 production (Zhu et al. 2008). Extraction of H_2 formed during acidogenic first stage should in theory result in reduced CH_4 yields as H_2 is now longer available for methanogens (Mohan 2009). It has been previously suggested, that H_2 and CO_2 containing gas from the first stage could be fed into the methanogenic stage (Jarvis et al. 1995). However, in practise CH_4 yields from the second stage have in many cases increased when compared to methane yields from one-stage systems, most probably due to improved hydrolysis and acidogenesis in the first stage.

In theory one mole of glucose is degraded to 3 moles of CH_4 and 3 moles of CO_2 (Angelidaki et al. 2011) in traditional AD, ignoring biomass synthesis (DiStefano & Palomar 2010; Table 2). In two-stage H_2 + CH_4 process one mole of glucose could be degraded in the first stage to 4 moles of H_2, 2 moles of CO_2 and 2 moles of acetate. These two moles of acetate could be degraded in the second, methanogenic process to 2 moles of CH_4 and 2 moles of CO_2. Thus, the overall equation (11) in two-stage system would be (Cheng et al. 2010, DiStefano & Palomar 2010):

$$C_6H_{12}O_6 \rightarrow 4H_2 + 2CH_4 + 4CO_2 \quad (11)$$

According to these reactions, the total energy yield in two-stage H_2 + CH_4 system could in theory be increased by 6.7 % (from 2.41 to 2.57 MJ (mol glucose)$^{-1}$) and the share of H_2 could be at most 38 % of the total energy yield (Table 2).

TABLE 2 Theoretical H_2, CH_4 and energy (MJ and kWh) yield from one mole of glucose (M = 180 g mol^{-1}) assuming that glucose is degraded to acetate, H_2 and CO_2 in the first stage.

Process	Unit	H_2	CH_4
H_2 production in the first stage	mol	4	0
	L	89.6	0
	MJ	0.97	0
	kWh	0.27	0
CH_4 production in the second stage	mol	0	2
	L	0	44.8
	MJ	0	1.61
	kWh	0	0.45
	total (MJ/kWh)	2.57/0.71	
CH_4 production in one-stage process	mol	0	3
	L	0	67.2
	MJ	0	2.41
	kWh	0	0.67

1.5 Crop biomass for methane and hydrogen production

1.5.1 Composition of crops

The chemical composition of crops determines among other factors the rate of hydrogen or methane production and the ultimate hydrogen or methane yield. Herbaceous plants are mainly composed of cellulose, hemicelluloses and lignin, with smaller amounts of other structural polymers, e.g. waxes and proteins (McKendry 2002). Cellulose is the most abundant organic material on the earth (Cowling & Kirk 1976). It is a linear polymer of glucose linked through β-1,4-linkages and contains both amorphous and crystalline regions, the crystalline regions considered to be more difficult to degrade (Walker & Wilson 1991, Malherbe & Cloete 2002). The degree of polymerization (DP), i.e. the number of glucose units, range from 500 to 25 000 (Malherbe & Cloete 2002). The disaccharide obtained from partial hydrolysis of cellulose is called cellobiose (McMurry 1998). Hemicellulose is a heteropolysaccharide composed of different hexoses (glucose, mannose), pentoses (e.g. xylose) and glucoronic acid (Malherbe & Cloete 2002, McKendry 2002). It is more soluble than cellulose and is frequently branched with DP of 100 to 200. Xylan is the most common hemicellulose component in grasses (Malherbe & Cloete 2002, McKendry 2002). Starch is branched glucose polymer linked through α-1,4-linkages and the disaccharide from partial hydrolysis of starch is called maltose (McMurry et al. 1998). Lignin is a highly irregular and insoluble, high molecular-weight polymer consisting of phenylpropane subunits, namely *p*-hydroxyphenyl (H-

type), guaiacyl (G-type) and syringyl (S-type) units (Malherbe & Cloete 2002, McKendry 2002). Typically cellulose accounts 40-50 % of plant material by weight, while hemicelluloses account 20-40 % (McKendry 2002). When plant matures the lignin content typically increase (McDonald et al. 1991).

Several factors, e.g. growth conditions, fertilization, harvesting and storage can affect on composition of crops. Grass silage has been shown to be composed of carbohydrates (45 % of TS), lignin (17 % of TS), proteins (10 % of TS) and extractives (8.4 % of TS) with higher heat content of 18.3 MJ $kgVS^{-1}$ (Lehtomäki et al. 2007), while in another study the cellulose, hemicelluloses and lignin contents were found as 32, 24 and 3.6 % of grass silage TS (Jagadabhi et al. 2011). The composition of maize varies as well, but according to one study maize was mainly composed of cellulose (44 % of TS), hemicelluloses (15 % of TS), starch (29 % of TS) and lignin (7 % of TS) (Oleskowicz-Popiel et al. 2011). The energy content of biomass is similar for all plant species, laying in the range 17-21 MJ $kgVS^{-1}$ (4.7-5.8 kWh $kgVS^{-1}$) (McKendry 2002).

1.5.2 Storage of energy crops

Energy crops need to be stored so that methane (or hydrogen) can be produced throughout the year and/or when the demand and/or price for energy are highest. Ensiling is a biological storage process during which lactic acid bacteria (LAB) use the sugars in the crop to produce lactic acid (lactic acid fermentation) and lower the pH to a level inhibitory to other bacteria (McDonald et al. 1991). This traditional way of storing fodder crops could also be suitable for energy crops (Egg et al. 1993, Oleskowicz-Popiel et al. 2011). Crops contain high amounts of non-structural carbohydrates which are easily degradable and thus can be lost during processing and suboptimal storage conditions. During storage, it is important to minimize energy losses, and ensiling has been shown to conserve over 90 % of the energy content of crops (Egg et al. 1993). However, prolonged storage typically increases organic matter losses (Herrmann et al. 2011).

Different kind of additives, such as acids or biological ones can be used to promote the ensiling process. Addition of acid lowers the pH and thus inhibits the growth of detrimental microorganisms; however, acids may cause corrosion of equipment and health problems. Enzymes enhance the hydrolysis of crop material and subsequently increase the content of sugars convertible by LAB. Bacterial inoculants can be used to increase the amount of LAB, and in combination with the addition of enzymes and LAB, enzymes degrade the plant cell wall and release carbohydrates for lactic acid fermentation (McDonald et al. 1991).

Ensiling is affected by several factors such as the chemical characteristics of crop in question and the solids content (i.e. moisture content), which can be controlled by the stage of maturity of the crop, by pre-wilting (Egg et al. 1993) and by using an absorbent during ensiling (Singh et al. 1996). The solids content of the crop to be ensiled affects the total bacterial count and the rate of fermentation, which is usually more restricted the higher the solids content.

This is reflected in higher pH, higher soluble carbohydrate values, lower levels of lactic, acetic and butyric acids, and inhibition of the deamination of amino acids with high solids content (McDonald et al. 1991). With low solids content pH critical for well preserved silage is lower compared to that in high solids contents. For grasses with dry matter content of 20 % the critical pH has been found to be 4.0. Unless the soluble carbohydrate levels are very high, the ensiling of crops with a low solids content will encourage a clostridial fermentation, resulting in energy losses and a silage of low nutritional value (Egg et al. 1993, McDonald et al. 1991). During the storage of low solids crops baling might be impossible due to leachate formation. It has been assumed that leachate would not be formed, if crops are dried to a TS content of 29 % or above and that overall losses of solids would be minimised around a TS content of 25-30 % (McDonald et al. 1991). In contrast, if the pre-wilting period is too long, respiration will cause energy losses and the sugar content of the crop may fall. Moreover, high solids crops are also susceptible to mould (Buxton & O´Kiely 2003). When the crops are used for energy production, ensiling conditions do not necessarily have to be as strictly controlled as with fodder crops. Field drying can lower transportation costs since much less water would be transported with the biomass; however, the savings in transportation must be balanced with the dry matter losses that occur during field drying (Egg et al. 1993).

1.5.3 Pre-treatments of energy crops

Lignocellulosic biomass, such as energy crops, is mainly composed of cellulose, hemicelluloses and lignin, which are tightly linked to each other in a complex structure and due to its heterogeneity and crystallinity, microbial hydrolysis is rather slow (Taherzadeh & Karimi 2008). Therefore, pre-treatment of lignocellulosic biomass might be needed in order to improve the rate of hydrolysis and to increase the carbohydrate availability and hence H_2 and CH_4 production rates and yields (Fan et al. 2006a,b, Hendriks & Zeeman 2009). The purpose is to break the lignin seal, disrupt the crystalline structure of cellulose and increase the surface area of cellulose (Walker & Wilson 1991, Mosier et al. 2005). Different physical, chemical or biological pretreatments have shown to enhance the degradation of the lignocellulose for biofuel production (e.g. reviews by Taherzadeh & Karimi 2008, Hendriks & Zeeman 2009).

Pre-treatments can be performed either in ambient or elevated temperature and pressure. For instance hydrothermal treatment (Oleskowicz-Popiel et al. 2011) and steam-explosion are stated as an effective method to pre-treat lignocellulosic biomass (Kaparaju et al. 2009), however, the applied high temperature and pressure can result in formation of inhibitory products (Datar et al. 2007). For example acetic acid, terpenes, alcohols and aromatic compounds can be produced from hemicellulose degradation, furfural, 5-HMF and levulinic acid from sugar degradation and aromatic or polyaromatic compounds from lignin degradation (Ren et al. 2009). Moreover, thermochemical treatments combining either acids or alkalis and high

temperature have been applied for lignocellulose pre-treatment (e.g. Taherzadeh & Karimi 2008, Cao et al. 2009, Chen et al. 2009). The disadvantages associated with chemical treatments are the cost of reagent, requirement of heat/and pressure and the capital cost of the pre-treatment system. Moreover, chemical pre-treatment can result in loss of fermentable carbohydrates and production of inhibitory compounds (Weimer et al. 2009).

Pre-treatments can also be carried out in ambient temperatures and pressures. Among the methods of pre-treatment, water extraction alone can enhance the hydrolysis of lignocellulosic biomass. For example, free sugars can be extracted from sweet sorghum stalks using water at 30 °C (Antonopoulou et al. 2008). Alkali pre-treatment processes typically utilize lower temperatures and pressures compared to other pretreatment technologies (Mosier et al. 2005). Alkalis such as NaOH and $Ca(OH)_2$ have been shown to improve hydrolysis by breaking the bonds between hemicelluloses and lignin as well as swell the fibres and increase the pore size even when applied at room temperature (Pavlostathis & Gossett 1985, Gunaseelan 1995, Neves et al. 2006). Besides alkalis, acids can also be used in pre-treatment methods for lignocellulosic biomass. Acids, such as HCl, can be used to hydrolyse cellulose to glucose (Goldstein et al. 1984) and to improve solubility of hemicelluloses and thus enhance anaerobic degradation even at ambient or moderate temperatures (Hendriks & Zeeman 2009).

1.5.4 Methane production from crop biomass

There are several methods of renewable energy production from crop biomass, one being the AD process. Energy crops can be co-digested for instance in farm-scale digesters with animal manure for methane production. Moreover, monodigestion of energy crops in different one- or two-stage reactor configurations has also been studied (e.g. Lehtomäki et al. 2008b, Koch et al. 2009) and applied in full-scale plants (Resch et al. 2008) despite the possible drawbacks associated with the nutrient deficiency and lack of buffer capacity (Koch et al. 2009). Typical methane yields from crop material vary between 250 and 400 m^3 tVS^{-1} (Amon et al. 2007, Seppälä et al. 2009) and the methane content in the biogas is around 50 % (Lübken et al. 2010).

Pre-treatments have been applied to improve hydrolysis and methane yield from energy crops (Taherzadeh & Karimi 2008, Hendriks & Zeeman 2009). Alkalis such as NaOH and $Ca(OH)_2$ have been shown to improve hydrolysis and CH_4 production from biomass (Pavlostathis & Gossett 1985, Gunaseelan 1995, Neves et al. 2006). However, no clear conclusion on the effect of pre-treatments on methane yield can be given, as the effect of pre-treatment can differ e.g. between crop species (Table 3). When evaluating the effect of a pre-treatment (or storage), the methane (or hydrogen) yield should be expressed based on the original VS of the substrate, thus taking into account the possible losses during the treatment.

TABLE 3 The effect of (thermo)-chemical pre-treatments on methane potential of some selected crop-based substrates.

Substrate	Pre-treatment	CH_4 yield (ml gVS^{-1})	Reference
Hay	untreated	320	Fernandes et al. (2009)
	calcium hydroxide 85 °C	280	
	ammonium 120 °C	300	
	maleic acid 150 °C	230	
Straw	untreated	250	
	ammonium 120 °C	320	
Bracken	untreated	70	
	calcium hydroxide 85 °C	170	
Rice straw	untreated	250	Zhao et al. (2010)
	acetic+propionic acid+autoclaving	280	
Grass	untreated	230	Lehtomäki et al. (2004)
	alkaline	270	
Sugar beet tops	untreated	310	
	alkaline	340	

1.5.5 Hydrogen production from crop biomass

H_2 production from biomass through different methods has been reviewed in the literature recently (Kalinci et al. 2009, Balat & Kırtay 2010). Of late, the interest towards dark fermentative H_2 production has increased as the rates of H_2 production are rather high, variety of feedstock can be used as substrate and the process is not dependent on light energy (Nath & Das 2004, Chong et al. 2009). Crop biomass containing carbohydrates is considered as an ideal feedstock for dark fermentative H_2 production (Kapdan & Kargi 2006, Akutsu et al. 2009b, Chong et al. 2009) and dark fermentative H_2 production from agricultural feedstock has been reviewed recently (Chong et al. 2009, Guo et al. 2010). Hydrogen yield from energy crops, crop residues or plant materials show wide variation (Table 4), which are largely explained by differences in experimental conditions (pH, inoculum source, temperature, loading etc.) and characteristics of the substrate. Some of the research focus on H_2 production from pre-treated energy crops, namely the sugar rich extract, typically extracted either after water extraction (Antonopoulou et al. 2011) or steam-explosion (Datar et al. 2007, Kongjan et al. 2010). Both mixed and pure cultures have been used for H_2 production from energy crops and crop residues. Most of the earlier studies are carried out in batch experiments, while some continuous H_2 production studies have been reported more recently. H_2 contents in semi-continuous or continuos experiments have been around 58, 45, 52-57 and 30-40 % for cassava stillage (Luo et al. 2010b), potato waste (Zhu et al. 2008), sugar beet (Hussy et al. 2005) and sweet sorghum extract (Antonopoulou et al. 2008, 2011).

TABLE 4 Hydrogen yields from some crop based and agro industrial substrates.

Substrate	System	T (°C)	H_2 yield	Reference
Bean curd waste	Batch	35	14-21 ml gVS^{-1}	Noike & Mizuno (2000)
Beer lees	Batch	36		Fan et al. (2006)
-untreated			7.6 ml $gTVS^{-1}$	
-NaOH-treated[a]			11.5 ml $gTVS^{-1}$	
-HCl-treated[a]			68.6 ml $gTVS^{-1}$	
Beer lees	Batch	35		Cui et al. (2009)
-untreated			3 ml (g dry beer lees)$^{-1}$	
-HCl-treated [a]			53 ml (g dry beer lees)$^{-1}$	
Cabbage	Batch	37	26-62 ml gVS^{-1}	Okamoto et al. (2000)
Carrot	Batch	37	45-71 ml gVS^{-1}	Okamoto et al. (2000)
Cassava stillage	Batch	60	65 ml gVS^{-1}	Luo et al. (2010b)
	SC		52 ml gVS^{-1}	
Cheese whey	CSTR	35	0.61-0.78 mol (mol glucose consumed)$^{-1}$	Venetsaneas et al. (2009)
Corn stalk	Batch	36		Fan et al. (2008)
-untreated			20 ml gVS^{-1}	
-lactic acid treated			133 ml gVS^{-1}	
-bio-pretreated			176 ml gVS^{-1}	
Corn stalk	Batch	36		Zhang et al. (2007)
-untreated			3 ml $gTVS^{-1}$	
-NaOH-treated[a]			57 ml $gTVS^{-1}$	
-HCl-treated[a]			150 ml $gTVS^{-1}$	
Fodder maize	Batch	35	62 ml gTS_{added}^{-1}	Kyazze et al. (2008)
Fruit peel waste	ACF	nr	459 ml $gVS_{destroyed}^{-1}$[b]	Vijayaraghavan et al. (2007)
Olive pulp	CSTR	35	0.19 mmol gTS^{-1}	Koutrouli et al. (2009)
Olive pulp	Batch	55	1.6 mmol gTS^{-1}	Gavala et al. (2005)
	CSTR		0.32 mmol gTS^{-1}	
Pineapple waste	Batch	37	5.92 mmol $gCOD^{-1}$	Wang et al. (2006)
Poplar leaves	Batch	35		Cui et al. (2010)
-untreated			15 ml gTS–1	
-HCl-treated			33.5 ml gTS–1	
-enzyme-treated			44.9 ml gTS–1	
Potato waste	CSTR	35	30 ml gTS–1	Zhu et al. (2008)
Rice (boiled)	Batch	37	19-96 ml gVS–1	Okamoto et al. (2000)
Rice bran	Batch	35	31-61 ml gVS–1	Noike & Mizuno (2000)
Rice slurryd	Batch	37	346 ml (g ch)–1	Fang et al. (2006)
		55	210 ml (g ch)–1	
Ryegrass (wilted)	Batch	35	76 ml gTSadded–1	Kyazze et al. (2008)
Ryegrass (fresh)	Batch	35	22 ml gTSadded–1	Kyazze et al. (2008)
Spent grains	Batch	40	13 ml gTVS–1	Chou et al. (2008)
Sugarcane	Batch	35	170 ml (=7.5 mmol) gVS–1	Hafner (2007)
Sugar beet pulp+extract	CSTR	32	0.9 mol (mol hexose converted)–1	Hussy et al. (2005)

TABLE 4 Continues.

Substrate	System	T (°C)	H_2 yield	Reference
Sugar beet pulp	Batch	35		Ozkan et al. (2011)
-untreated			90 ml $gCOD^{-1}$	
-alkaline-treated			116 ml $gCOD^{-1}$ = 50 ml $(g\ pulp)^{-1}$	
Sweet sorghum extract	CSTR	35	10.4 ml $(g\ sweet\ sorghum)^{-1}$ = 0.86 mol $(mol\ glucose\ consumed)^{-1}$	Antonopoulou et al. (2008)
Sweet sorghum extract	CSTR	35	8.8 ml $(g\ sweet\ sorghum)^{-1}$ = 0.74 mol $(mol\ glucose\ consumed)^{-1}$	Antonopoulou et al. (2011)
Water hyacinth (NaOH+enzymatic hydrolysis)	Batch	35	52 ml $gTVS^{-1}$	Cheng et al. (2010)
Wheat bran	Batch	35	10-43 ml gVS^{-1}	Noike & Mizuno (2000)
Wheat straw	Batch	36		Fan et al. (2006b)
-untreated			0.5 ml $gTVS^{-1}$	
-HCl-treated[c]			68.1 ml $gTVS^{-1}$	
Wheatfeed	Batch	35	56 ml gTS^{-1}	Hawkes et al. (2008)
	CSTR		56 ml gTS^{-1}	
Wheat straw hydrolysate	CSTR	70	178 ml $(g\ sugars)^{-1}$	Kongjan et al. (2010)

[a]boiled for 30 minutes
[b]calculated from the data given
[c]microwave heating
[d]steaming 100 °C for 30 min
nr = not reported

Pre-treatments can be applied to improve hydrolysis and thus H_2 yield (Taherzadeh & Karimi 2008). HCl treatment has been shown to improve H_2 production from corn stalk (Table 4), as H_2 yield of 150 ml $gTVS^{-1}$ was obtained from pre-treated corn stalk compared to H_2 yield of 3 ml $gTVS^{-1}$ from untreated material (Zhang et al. 2007). In another study 20 ml gVS^{-1} was obtained from untreated corn stalk (Table 4) while the bio-pretreatment (microbial additive, not specified) increased H_2 yield to 176 ml gVS^{-1} (Fan et al. 2008). Furthermore, hydrogen yields from HCl-treated beer lees (0.2 % HCl, boiling 30 min) and wheat straw wastes were roughly nine and 136-times greater compared to yield from these untreated substrates (Fan et al. 2006a,b; Table 4). With higher HCl concentrations H_2 yield decreased, apparently due to inhibition caused by the Cl^- anion (Fan et al. 2006a). Hydrolysate of steam-exploded corn stover was used for H_2 production with yield of 2.84-3 mol $(mol\ sugar)^{-1}$ (Datar et al. 2007). However, after steam-explosion 50-85 % of the carbohydrates remained in the solid fraction and the applied microbial consortia was not able to produce H_2 from this solid fraction, apparently due to lack of cellulolytic micro-organisms

(Datar et al. 2007). In another study corn stalk pre-treated by steam-explosion produced at most 63.7 ml H_2 g^{-1} corn stalk (Lu et al. 2009). It has to be noticed, that comparison of the different studies is difficult due to different experimental conditions and different units and temperatures used to express the H_2 yield.

In addition to mixed cultures, pure cultures have been used for H_2 production from crop biomass. H_2 yield by *Caldicellulosiruptor saccharolyticus* at 70 °C was 50 ml gTS^{-1} from wheat straw, 30 ml gTS^{-1} from sweet sorghum and 16 ml gTS^{-1} from maize leaves (Ivanova et al. 2009). Corn stalk pre-treated by mild acid pre-treatment (sulfuric acid, 170 °C, 30 min) and enzymatic hydrolysis was used for H_2 production by *Caldicellulosiruptor saccharolyticus*. Severe inhibition was observed when sugar concentration was 7.5 g l^{-1} or more, possibly caused by HMF and furfural formed during the pre-treatment (Panagiotopoulos et al. 2009). H_2 yield of 2.6 mol H_2 (mol glucose consumed)$^{-1}$ was obtained with *Ruminococcus albus* from the sorghum water extract (mainly sucrose), corresponding to 60 l H_2 kg^{-1} wet sorghum biomass (Ntaikou et al. 2008). *Clostridium thermocellum* produced 1.67 mol H_2 (mol glucose)$^{-1}$ from pre-treated corn stover, containing 59 % cellulose and 25 % lignin (Lalaurette et al. 2009). Hydrolysate from corn stover pre-treated with dilute acid hydrolysis was used for H_2 production by *Thermoanaerobacterium thermosaccharolyticum* W16 with hydrogen yield of 2.24 mol (mol sugar)$^{-1}$ (Cao et al. 2009). Steam-exploded corn straw was used for H_2 production by *C. butyricum* AS1 .209. H_2 yield was at most 68 ml g^{-1}, whereas H_2 yield of 9 ml g^{-1} corn straw was obtained without pre-treatment (Li & Chen 2007).

2 OBJECTIVES

The main objective of this thesis was to evaluate the feasibility of hydrogen and methane production from energy crops through the anaerobic digestion process. The subobjectives were:

- To evaluate the effect of storage in field conditions and in the laboratory on the CH_4 yield of a mixture of grasses and ryegrass (I).
- To determine the H_2 production potential of grass silage and the effects of the source and heat-treatment of the inoculum, as well as the effects of initial pH, temperature and the VS ratio on H_2 yield in batch processes (II).
- To evaluate the effects of pre-treatments on H_2 and CH_4 yield from grass silage (III) and maize (IV) in batch assays.
- To assess the feasibility of two-stage H_2 + CH_4 production from grass silage (III) and maize (IV) in batch assays.
- To evaluate the possibility of shifting the ongoing methanogenic process to hydrogen production and to determine the feasibility of grass silage monodigestion for methane production in CSTR (V).

3 MATERIALS AND METHODS

3.1 Main experiments of the thesis

Main experiments, substrates used and the target energy carrier (H_2 and/or CH_4) in this thesis are summarized in Table 5.

TABLE 5 Main experiments in this thesis showing the objectives, substrates used, energy carrier (H_2 and/or CH_4) produced, mode and size of the system as well as the temperature used.

Substrate	Objective	Energy carrier	System (volume)	Temp. (°C)	Paper
Grass mixture and ryegrass	The effects of storage on VS loss and CH_4 yield – The effect of initial TS content – The effect of biological ensiling additive	CH_4	Batch (1000 ml)	35	I
Grass silage and glucose	To determine the H_2 production potential from grass silage – The effects of inoculum source, initial pH, temperature and VS ratio	H_2	Batch (118 ml)	35, 55 and 70	II
Grass silage	Two-stage H_2+CH_4 production from grass silage – The effect of NaOH-pretreatment	H_2 and CH_4	Batch (1000 ml)	55 (H_2) 35 (CH_4)	III
Maize	Two-stage H_2+CH_4 production from maize – The effects of water-extraction and HCl-treatment	H_2 and CH_4	Batch (118 ml)	55 (H_2) 35 (CH_4)	IV
Grass silage	The possibility of shifting CH_4 producing process to H_2 production – Monodigestion of grass silage for CH_4 production	H_2 and CH_4	CSTR (300-1500 ml)	35	V

Detailed description of the materials and methods are in the following chapters and in the original articles (I-V).

3.2 Substrates and inocula

The crop materials used in (I) were a grassmixture of timothy (*Phleum pratense*, 63 % of seed mixture), red clover (*Trifolium pratense*, 17 %) and meadow fescue (*Festuca arundinacea*, 20 %) (henceforth referred to as grass) and ryegrass (*Lolium multiflorum*). Grass silage (a mixture of timothy, *Phleum pratense* and meadow fescue, *Festuca pratensis*) was used as a substrate in II, III and V. Besides, analytical grade D(+)-glucose (EC NO 200-075-1, Sigma, Steinheim, Germany) was used as a control substrate (II). Dried maize (variety Cerruti) was used as a substrate in experiments described in paper IV. All crop materials were obtained from a farm in Laukaa, Central-Finland (Table 6).

TABLE 6 Characteristics of the substrates used in the experiments.

Substrate	TS (% ww)	VS (% ww)	VS/TS (%)	pH	SCOD (mg gTS^{-1})	Paper
Grass	15.6	13.9	89	6.1	158	I
Ryegrass	13.3	11.7	88	6.4	217	I
Grass-field	14.6	13.4	92	6.1	71	I
Ryegrass-field (pre-wilted)	44.4	39.6	89	6.2	242	I
Grass silage	25.9	24.0	93	4.3	229	II
Grass silage	27.2	23.4	86	4.1	190	III
Maize (dried)	91.8	89.0	97	6.3	211	IV
Grass silage	43.7	40.8	93	4.0	239-373	V

In the laboratory storage experiments (I) fresh crop material was first chopped with a garden chopper to ca. 5 cm particle size. In II, III and V grass silage was stored at –20 °C until used. Prior to use, it was thawed overnight at room temperature and chopped into particles of ~ 1 cm with scissors or a household blender. In IV fresh maize (whole crop, including stem, leaves and corn) was chopped with garden chopper to a particle size of 1-2 cm and dried at 60 °C for 24 h. Dried maize was stored at 20 °C for six weeks and was cut to a particle size of ca. 0.5-1 cm prior to start of the experiments.

The feed for CSTRs (V) was prepared daily by diluting 7.4 g grass silage wet weight (ww) (corresponding to 3.3 gTS and 3.1 gVS) with 43 g of water (days 1-27) or nutrient solution (day 28 onwards). Thus, during the whole run, feed TS and VS was maintained at 6.6 and 6.2 %, respectively. Nutrient solution contained (mg $(kg\ feed)^{-1}$) 1183 NH_4Cl, 1056 K_2HPO_4, 422 $MgSO_4$, 42 $CaCl_2*2H_2O$, 8.45 $FeCl_2*4H_2O$, 0.21 H_2BO_3, 0.21 $ZnCl_2$, 0.21 $NiCl_2*6H_2O$, 0.16 $CuCl_2*2H_2O$, 2.11 $MnCl_2*4H_2O$, 0.21 $(NH_4)_6Mo_7O_{24}*4H_2O$, 0.38 $AlCl_3*6H_2O$ and 8.45 $CoCl_2*6H_2O$.

The inoculum was obtained from a mesophilic farm biogas reactor treating cow manure and confectionery by-products (Laukaa, Finland). Besides, in II, inoculum from a mesophilic digester at a municipal WWTP (Jyväskylä, Finland) was used, after increasing the TS concentration by centrifuging. In hydrogen production batch assays (III, IV) inoculum was heat-treated by boiling for 30 minutes to inactivate H_2-consuming micro-organisms and to enrich spore-forming H_2 producers. In II, inoculum was used with and without heat-treatment, whereas in V, no heat-treatment was applied (Table 7).

TABLE 7 Characteristics of the inocula used in the experiments.

Source	HT	TS (%ww)	VS (%ww)	pH	SCOD (g l^{-1})	Paper
Farm	no	5.6	4.3	7.9	10.6	I
Farm	no	6.3	4.8	8.1	7.9	II
	yes	6.3	4.9	9.6	10.6	
WWTP[a]	no	6.1	3.0	7.8	1.8	
	yes	7.2	3.5	9.0	8.2	
Farm	no	5.6	4.3	7.9	12.0	III
	yes	7.8	6.0	9.2	15.9	
Farm	no	6.0	4.8	7.6	3.7	IV
	yes	6.4	5.1	9.2	4.4	
Farm	no	4.1	3.0	7.8	3.0	V

[a]solid fraction after centrifuging
HT = heat-treatment

3.3 Experimental set-up

3.3.1 Storage experiments (I)

Storage experiments were performed both in laboratory and in field scale. In laboratory storage experiments part of the chopped material was spread on top of a plastic net and dried in a thin layer for 24 and 48 h at 20 °C, while part of the material was used fresh (drying 0 h). Biological ensiling additive (Josilac, manufacturer Josera Erbacher GmbH & Co) containing both LAB (*Lactobacillus plantarum* and *Pediococcus acidlactiti*, total amount 1.5×10^{11} CFU (g Josilac)$^{-1}$) and enzymes (cellulase, pectinase and xylanase) was added (6.8 g (t_{ww})$^{-1}$) to part of the fresh and dried crop materials while part of the materials did not receive additive. The crop materials (range 154-500 g ww) were packed in polyethylene bags and placed in a 5 l plastic silo equipped with water locks to enable the release of gas from the silos. Silos were flushed for about 3 minutes with N_2 to remove O_2 and maintained at 20 °C. After storage (3 and 6 months) the silos were weighed and samples taken for analysis. Experiments were performed in duplicate.

In field storage experiments crops were baled in plastic-covered round bales immediately (only grass) after cutting or after 24 h pre-wilting in the field.

Additive (same as in laboratory trials) was added to part of the pre-wilted crops during baling with round baler (19 g $(t_{ww})^{-1}$ for grass and 24 g $(t_{ww})^{-1}$ for ryegrass). Bales were weighed with pallet truck scales (Tamtron, Finland) at the beginning of the storage trials and after eleven months of storage. Bales were stored outside in ambient conditions, the temperature ranging during the year from ca. –30 °C to 30 °C. From the bales samples were taken manually using an auger, and after sampling the plastic cover was repaired with tape. Samples were taken from different bales at different times.

3.3.2 Pre-treatments (III, IV)

For alkaline pretreatment experiment (III) grass silage (184 g ww, corresponding to 50 gTS and 46.5 gVS, particle size ca. 1-2 cm) was placed into 1 l glass bottles and distilled water (866 g) was added to obtain a TS concentration of 5 %. Solid NaOH (2 g) was added to obtain a dose of 4 % NaOH gTS^{-1}. For water-extraction and acid treatment experiment (IV) maize (32.7 g ww, corresponding to 30 gTS and 28.1 gVS), was placed into two 1 l glass bottles and distilled water (297 g) was added to obtain a TS concentration of 10 %. To have water extracted material one bottle was incubated as such. For acid treated material, 0.6 ml HCl (37 %) was added to obtain a dose of 2 % HCl gTS^{-1}. Prepared bottles were mixed in an orbital shaker for 24 h at 20 °C (III, IV). After treatment the materials were sieved by gentle manual pressure through a metallic sieve (bore size approximately 1 mm) into solid and liquid fractions. For batch assays, the solid and liquid fractions were used separately (III) or combined in the ratios that were actually generated during the treatments (IV).

3.3.3 Methane and hydrogen potential batch assays (I-IV)

Methane potentials were determined in batch assays in duplicate or triplicate in either 1 l glass bottles (I, III) or 118 ml serum bottles (IV) incubated statically at 35 °C. 250 ml (I, III) or 20 (IV) g of inoculum was added in each bottle and requisite amount of substrate to give substrate to inoculum VS-ratio of 1 (except for stored crops in laboratory conditions when ratio was 0.5 (I) and for NaOH treated solid and liquid fractions the ratios were 0.8 and 0.2 (III)). Bottles were filled to a liquid volume of 750 ml (I, III) or 60 ml (IV) with distilled water and $NaHCO_3$ (3 g l^{-1}) was added as buffer (I, III, IV). Finally, bottles were flushed with N_2 to remove O_2 from the headspace and closed with silicon rubber caps (I, III) or butyl rubber stoppers (IV). The produced gas was collected in aluminium gas bags (I, III). The CH_4 assays were performed at 35 °C for 70-80 (I), 56 (III) or 77 (IV) days.

Hydrogen potentials were determined in batch assays in duplicate or triplicate in either 1 l glass bottles (III) or 118 ml serum bottles (II, IV), incubated statically at 35 (II), 55 (II-IV) or 70 °C (II). First, 28-41 g (II), 100 g (III) or 12 g (IV) inoculum was added and subsequently substrate to obtain the desired substrate to inoculum VS ratio (1-2 in II, 2, 1.6 and 0.2 for grass silage, NaOH treated solid and liquid fractions, respectively, in III and 2 in IV). In the control substrate

assays glucose (5 g l^{-1}) was added (II). Bottles were filled to total volume of 60 ml (II), 650 ml (III) or 78 ml (IV) with distilled water. When necessary, pH was adjusted to 4 (II), 5 (II) or 6 (II, III, IV) with 5 M HCl and 5 M NaOH. Finally, bottles were flushed with N_2 to remove O_2 from the headspace and closed with silicon rubber caps (III) or butyl rubber stoppers (II, IV). Assays were terminated after H_2 production ceased, which was after 11-31 days of incubation (II). The control assays, with inoculum and water only, were incubated under the same conditions. All bottles were mixed manually before each gas analysis.

In the H_2 assays (III, IV) bottles were first incubated for two (IV) and/or 14 days (III, IV), and then sampled (200 g in III and 38 g in IV) after which 250 (III) or 20 g (IV) of methanogenic inoculum was added and the contents of the bottles were flushed and closed as in the CH_4 assays and then incubated for 57 (III), 75 (IV, after 2 d H_2 stage) or 63 days (IV, after 14 d H_2 stage) at 35 °C.

3.3.4 CSTR reactors (V)

CH_4 production from grass silage was studied in 2 parallel semi-continuously fed CSTRs (M1 and M2) each with a total volume of 2 l at 35 °C. During the start-up, reactors were filled with 1500 ml of inoculum (working volume) and flushed with N_2 for 5 minutes to ensure anaerobic conditions. Semi-continuous feeding was initiated 14 days after the start-up and considered as day 1 of the experimental period. Reactors were fed manually once on every weekday (Monday through Friday) with a plastic syringe. Digestate was removed just prior to the each feeding. The amount removed was about 5-10 % less than the daily feed volume in order to maintain the constant working volume. The reactors were mixed continuously using a magnetic stirrer (300 rpm).

$NaHCO_3$ was added as buffer at a total dosage of 9 g $reactor^{-1}$ during days 23-30 and at 0.5 g d^{-1} (9.8 g $(kg\ feed)^{-1}$) during days 41-105. From day 106 onwards, $NaHCO_3$ was added only in M1 at a dosage of 2 g d^{-1} (corresponds to 38 g $(kg\ feed)^{-1}$).

On day 78, both reactors were opened and the reactor contents were mixed and distributed equally between M1 and M2. From day 79 onwards, M1 was continued as methanogenic reactor and operated at the same OLR and HRT as earlier (OLR 2 kgVS $(m^3d)^{-1}$, HRT 30 days, liquid volume 1.5 l). On the other hand, hydrogen production was induced in M2 by reducing the working volume from 1500 to 300 ml and feeding at the same feed amount. Thus, the OLR in M2 was increased to 10 kgVS $(m^3d)^{-1}$ and HRT was decreased to 6 days. OLR and HRT were calculated for five feeding days per week.

3.4 Analyses and calculations

TS and VS were analysed according to Standard Methods (APHA 1998) and pH was measured with a Metrohm 774 pH-meter (Metrohm, Switzerland, I-IV) or

with a Radiometer Copenhagen PHM82 (V). COD was analysed according to SFS 5504 (Finnish Standards Association 1988). Soluble COD (SCOD) from the crops was analysed after the leaching test, which was modified from SFS-EN 12457-4 (Finnish Standards Association 2002). Particle size used in the leaching test varied between 10 and 25 mm (as against ≤10 mm in the standard), amount of crop was 50 gTS (as against 90 ± 5 gTS in the standard) and filtration was done through a glass fibre filter paper (GF50, Schleicher & Schuell, Dassel, Germany, as against 0.45 µm membrane filter in the standard).

VFA (I, III-V) content were measured with a GC equipped with a FID (Perkin Elmer Autosystem XL GC, PE FFAP column 30 m × 0.32 mm × 25 µm, carrier gas helium, oven 100 to 160 °C (20 °C min^{-1}), detector and injector 225 °C). Individual VFAs were expressed as mg l^{-1} or converted to COD according to conversion factors (g COD (g acid)$^{-1}$ 1.066 for AA, 1.512 for PA, 1.816 for BA and IBA, 2.037 for VA and IVA and 2.204 for CA, respectively).

Gas samples were taken through stoppers from the gas phase with a pressure-locked glass syringe (Supelco, Pressure-Lok® Series A-2 Syringe, Bellefonte, USA). In reactor experiments (V), gas sample was taken before daily removal and feeding. CH_4 (I, IV: methane assays) content were measured with a GC equipped with a FID (Perkin Elmer Arnel Clarus 500 GC, Perkin Elmer Alumina column 30 m × 0.53 mm, carrier gas argon, oven 100 °C, detector 225 °C and injector 250 °C). Gas composition (H_2, CH_4 and CO_2) was analysed with a Perkin Elmer Arnel Clarus 500 gas chromatograph (II-IV H_2 assays and V)) equipped with a thermal conductivity detector (TCD) and Supelco Carboxen™ 1010 PLOT fused silica capillary column (30 m × 0.53 mm). Argon (15 ml min^{-1}) was used as the carrier gas and the temperature of the oven, detector and injector were 200, 230 and 225 °C, respectively. The amount of biogas formed in methane potential assays (I, III) and in reactor experiments (V) was measured using the water displacement method, while in other assays (II-IV) manometric method was used.

In calculating the CH_4 and H_2 potentials and yields (I-IV) the gas production from inoculum was subtracted from those of the samples. In the article I CH_4 potentials were calculated as m^3 $kgVS_{added}^{-1}$. CH_4 yields were calculated as m^3 t_{ww}^{-1} (based on the mass of wet material added) and m^3 t_{oww}^{-1} (based on the original wet weight of the material taking into account losses during storage). Gas (H_2 and CH_4) yield (II-IV) is given as ml gVS_{added}^{-1}, except from glucose (II), when unit ml or ml (g glucose added)$^{-1}$ (II) is used. In some assays H_2 peaked twice (II, III) but, when calculating the actual H_2 yield, only the higher peak was taken into account. In the alkaline pre-treatment experiments (III) the gas yield (unit ml $gVS_{original}^{-1}$) was calculated by relating the gas produced from treated fractions to the initial VS of grass silage (untreated). To be able to compare the pre-treated to untreated grass silage, the gas yield of solid and liquid fractions were counted (defined as combined gas yield) (III). With pre-treated maize, amounts of solid and liquid that would have been generated during the pretreatments were used in batch assays. The gas yields of the pre-treated maize were thus directly related to the VS of

untreated maize (IV). In the two-stage processes CH_4 yield was related to the amount of VS added at the beginning of the first stage (III, IV). In CSTR experiments (V) gas yield is given as 1 $kgVS_{fed}^{-1}$. Results were converted to standard conditions (T = 273K, p = 1 bar) in IV and V.

When calculating the energy yields of produced hydrogen and/or methane, lower heating values of 10.8 and 35.9 MJ $(Nm^3)^{-1}$ (corresponding to 3 and 10 kWh $(Nm^3)^{-1}$) were used, respectively.

In IV the data were subjected to analysis of variance (ANOVA) (SPSS 1999). Dunnett´s t-test was used to compare all other treatments against control if the F-test was significant at $P \leq 0.05$. Before performing ANOVA, data were subjected to Welch's test to evaluate the homogeneity of variance.

4 RESULTS

4.1 The effect of storage on methane yield of grasses

The effect of storage on VS losses and methane yield of grass and ryegrass was studied in laboratory and field scale (I). The effects of storage for 2 and 6 months on VS loss and CH_4 yield of grass and ryegrass stored at different solids contents (i.e. after initial drying for 0, 24 and 48 h) and with and without biological additive (Josilac) were studied in laboratory conditions (Tables 8-10). Drying increased the initial TS from 13-16 (nondried, defined as low solids) to 19-20 (24 h dried, defined as medium solids) and to 27-30 % (48 h, defined as high solids). After two months pH had fallen from initial 6.1-6.5 below 5.6 in all the experimental conditions except with high solids grass, which had higher initial pH (6.5), only additive addition enabling a lower pH (5.5). Further storage to 6 months increased pH by over two units at most and a pH below 6.2 was maintained only with low solids grass and high solids ryegrass. Additive enabled lower pH at all solid contents compared to crops without additive, more clearly with ryegrass. Storage decreased the VS/TS ratio at all solids contents with both crops (from 89 to 83 % with grass and from 88 to 78 % with ryegrass), more clearly with crops stored for 6 months. Storage of grass at all solids contents resulted in a loss of VS of about 20 % at 2 months and about 28-35 % at 6 months, while with ryegrass loss of VS at 6 months was lower at high solids (VS loss 20-27 %) compared to low solid contents (VS loss 52 %; Tables 8, 9). Storage increased SCOD values and increasing solids content resulted in a lower VFA concentration and a lower proportion of VFA from SCOD. Acetic acid was the main VFA with grass and ryegrass stored for six months, along with smaller amounts of propionic, isobutyric, butyric, isovaleric, valeric and caproic acids (Table 10). Storage increased CH_4 potential (m^3 $kgVS_{added}^{-1}$) by at most 42 and 25 % with grass and ryegrass, respectively, although, with no clear trends in relation to solids content or storage time. In some cases CH_4 potential decreased during storage. However, storage mainly decreased CH_4 yield (m^3 t_{oww}^{-1}, taking the VS losses into account) which was best preserved with high

solids ryegrass where the percentages of original CH_4 yield were 98 and 91 after 2 and 6 months, respectively (Tables 8, 9).

The effect of storage on VS loss and CH_4 yield (storage losses taken into account) of grass and ryegrass stored with and without additive was studied in field conditions for 11 months (Tables 11, 12). Grass was stored immediately after harvesting and after 24 h pre-wilting, which increased the TS from 14.6 to 18.2 %. After 3 months storage, pH was 5.0-5.2 and 4.5-4.9 with grass and ryegrass, respectively, and remained around 5 even after 11 months of storage, except in the bale with pre-wilted grass stored for 6 months, in which pH had increased to 8.8. The measured SCOD values, TS and VS concentrations varied during the follow-up period without showing clear trends or permanent changes in their ranges, which is probably an effect of the ambient conditions but is also due to variation between individual bales. This was observed especially with ryegass with lower TS concentration after one month of storage compared to fresh crop and crop stored for longer periods. Storage with nondried and pre-wilted grass slightly increased the CH_4 potential, and storage with ryegrass decreased it. Loss of mass (ww) in grass bales during 11 months of storage was between 18 and 29 % (data not shown), but with ryegrass no mass loss occurred. After 11 months of storage the best preserved CH_4 yield (m^3 t_{oww}^{-1}, VS loss included) was found with nondried grass, 96 % of the original yield (Tables 11, 12).

TABLE 8 Effect of drying and storage on chemical characteristics and CH_4 yield ($m^3\ t_{oww}^{-1}$, storage losses taken into account) of grass in laboratory trials. Standard deviation of methane potential is given after ±.

Pre-wilting time (h)		Storage time (months)	pH	SCOD (mg gTS^{-1})	TS (%)	VS (%)	VS/TS (%)	VS loss (%)	CH_4 ($m^3\ kgVS_{added}^{-1}$)	CH_4 ($m^3\ t_{ww}^{-1}$)	CH_4 ($m^3\ t_{oww}^{-1}$)
0	Without	0	6.1	160	15.6	13.9	89.1	nr	0.36	49.8	49.8
	additive	2	5.0	310	13.0	11.3	86.9	20.0	0.42 ± 0.01	47.8	47.0
		6	6.0	350	12.1	10.3	85.1	28.1	0.43 ± 0.05	44.0	42.7
	With	0	6.1	160	15.6	13.9	89.1	nr	0.36[a]	49.8	49.8
	additive	2	5.0	270	13.3	11.7	88.0	17.8	0.42 ± 0.02	49.7	48.6
		6	5.5	330	12.1	10.4	86.0	28.3	0.43 ± 0.03	45.0	43.1
24	Without	0	6.2	130	19.8	17.6	89.0	nr	0.36[a]	63.3	49.8
	additive	2	5.4	320	16.4	14.3	87.2	21.1	0.51 ± 0.01	72.9	55.9
		6	8.2	280	14.6	12.2	83.4	34.1	0.39 ± 0.01	47.6	35.7
	With	0	6.2	130	19.8	17.6	89.0	nr	0.36[a]	63.3	49.8
	additive	2	5.6	260	16.3	14.1	86.5	21.8	0.48 ± 0.06	68.2	52.5
		6	7.7	360	13.9	11.9	85.6	35.3	0.39 ± 0.05	46.8	35.4
48	Without	0	6.5	130	26.7	23.6	88.4	nr	0.36[a]	84.8	49.8
	additive	2	6.5	170	22.8	19.8	86.8	19.1	0.42 ± 0.01	83.8	47.6
		6	8.8	190	21.1	17.7	83.9	29.2	0.32 ± 0.04	56.6	31.5
	With	0	6.5	130	26.7	23.6	88.4	nr	0.36[a]	84.8	49.8
	additive	2	5.5	170	22.5	19.6	87.1	19.8	0.41 ± 0.01	80.7	45.9
		6	8.6	340	19.9	16.9	84.9	31.5	0.41 ± 0.00	69.6	39.1

[a]initial CH_4 production potential was tested only with the low solids sample, without additive.
nr = not relevant

TABLE 9 Effect of drying and storage on chemical characteristics and CH_4 yield ($m^3\ t_{oww}^{-1}$, storage losses taken into account) of ryegrass in laboratory trials. Standard deviation of methane potential is given after ±.

Pre-wilting time (h)		Storage time (months)	pH	SCOD (mg gTS^{-1})	TS (%)	VS (%)	VS/TS (%)	VS loss (%)	CH_4 ($m^3\ kgVS_{added}^{-1}$)	CH_4 ($m^3\ t_{ww}^{-1}$)	CH_4 ($m^3\ t_{oww}^{-1}$)
0	Without additive	0	6.4	220	13.3	11.7	88.0	nr	0.41 ± 0.02	47.6	47.6
		2	4.8	370	9.9	8.4	84.8	29.6	0.47 ± 0.04	39.9	39.1
		6	6.7	350	7.6	5.9	77.6	51.8	0.45 ± 0.04	26.7	25.6
	With additive	0	6.4	220	13.3	11.7	88.0	nr	0.41[a]	47.6	47.6
		2	4.3	350	10.1	8.7	86.1	26.9	0.44 ± 0.01	38.5	37.9
		6	7.0	340	7.6	6.0	78.9	50.8	0.48 ± 0.01	28.8	27.6
24	Without additive	0	6.3	200	18.8	16.6	88.3	nr	0.41[a]	67.5	47.6
		2	4.5	190	16.1	13.9	86.3	18.0	0.41 ± 0.02	56.3	38.9
		6	7.5	330	10.8	8.5	78.7	51.3	0.40 ± 0.01	33.8	22.7
	With additive	0	6.3	200	18.8	16.6	88.3	nr	0.41[a]	67.5	47.6
		2	4.7	240	14.9	12.7	85.2	25.1	0.49 ± 0.01	62.8	43.4
		6	5.9	320	12.4	10.2	82.2	51.3	0.43 ± 0.07	43.7	29.7
48	Without additive	0	6.5	230	30.4	26.5	87.2	nr	0.41[a]	107.9	47.6
		2	4.4	170	27.2	23.4	86.0	14.2	0.46 ± 0.01	108.3	46.5
		6	6.2	320	24.4	20.4	83.6	27.0	0.51 ± 0.00	103.9	43.5
	With additive	0	6.5	230	30.4	26.5	87.2	nr	0.41[a]	107.9	47.6
		2	4.2	160	28.3	24.4	86.2	10.4	0.39 ± 0.01	94.7	40.7
		6	4.9	300	26.1	22.2	85.1	20.0	0.43 ± 0.02	94.5	39.8

[a]initial CH_4 production potential was tested only with the low solids sample, without additive.
nr = not relevant

TABLE 10 VFAs (mgCOD gTS^{-1}) of grass and ryegrass stored for 6 months.

Crop	Drying time (h) (+ A)	AA	PA	IBA	BA	IVA	VA	CA	Total VFA	VFA/ SCOD (%)
Grass	Fresh	0.2	nd	nd	nd	nd	nd	nd	0.2	0.1
	0	71.1	21.7	16.4	50.3	16.1	7.6	9.7	192.9	55.1
	24	43.6	14.7	9.9	16.3	10.3	2.8	2.4	100.0	35.3
	48	18.0	8.8	3.6	3.4	5.1	0.7	0.0	39.7	20.5
	0 + A	71.8	23.8	8.8	30.0	13.0	2.9	1.2	151.5	46.2
	24 + A	-	-	-	-	-	-	-	-	-
	48 + A	5.1	1.5	0.5	1.1	0.6	0.0	0.0	8.9	2.6
Ryegrass	Fresh	0.1	nd	nd	nd	nd	nd	nd	0.1	0.05
	0	42.9	8.0	7.5	17.0	8.4	5.7	13.9	103.4	29.8
	24	27.9	8.1	3.7	4.2	5.7	1.6	6.6	57.8	17.3
	48	25.1	6.2	0.7	2.2	1.3	0.0	0.0	35.5	11.0
	0 + A	73.6	26.0	8.7	30.0	12.9	2.8	0.0	154.0	45.6
	24 + A	31.6	12.9	7.8	19.0	11.4	5.9	21.2	109.7	34.7
	48 + A	10.2	3.0	1.1	1.2	0.9	0.0	0.0	16.4	5.5

A = additive
nd = not detected
- = no sample

TABLE 11 Effects of pre-wilting, storage time and biological additive on chemical characteristics and CH_4 potential of grass under field conditions. Standard deviation of methane potential is given after ±.

Treatment	Storage time (months)	pH	SCOD (mg gTS^{-1})	TS (%)	VS (%)	VS/TS (%)	CH_4 (m^3 $kgVS_{added}^{-1}$)	CH_4 (m^3 t_{ww}^{-1})	CH_4 (m^3 t_{oww}^{-1})
Nondried	0	6.1	70	14.6	13.4	91.8	0.47 ± 0.02	62.6	62.6
	1	4.7	210	17.1	15.7	91.8	0.14 ± 0.03	21.5	nd
	3	5.0	270	17.1	15.7	91.8	0.49 ± 0.05	76.3	nd
	6	4.8	170	17.3	16.0	92.5	0.47 ± 0.01	74.8	nd
	11	5.4	160	16.4	15.0	91.5	0.49 ± 0.02	73.4	60.0
Pre-wilted	0	6.0	100	18.2	16.8	92.3	0.41 ± 0.00	68.4	54.5
	1	5.1	250	20.0	18.3	91.5	0.48 ± 0.01	87.2	nd
	3	5.2	280	17.4	15.7	90.2	0.42 ± 0.02	66.1	nd
	6	8.8	70	17.9	15.9	88.8	0.26 ± 0.01	40.8	nd
	11	5.3	310	17.7	16.2	91.5	0.48 ± 0.02	78.2	46.0
Pre-wilted+ additive	0	6.2	180	17.0	15.7	92.4	0.50 ± 0.04	78.8	67.3
	1	5.4	320	17.0	15.2	89.4	0.38 ± 0.01	58.0	nd
	3	5.1	180	20.3	18.5	91.1	0.37 ± 0.04	57.9	nd
	6	5.4	150	17.9	16.5	92.2	0.37 ± 0.00	61.1	nd
	11	5.0	170	21.5	20.0	93.0	0.46 ± 0.01	92.0	55.7

nd = not determined

TABLE 12 Effects of pre-wilting, storage time and biological additive on chemical characteristics and CH_4 potential of ryegrass under field conditions. Standard deviation of methane potential is given after ±.

Treatment	Storage time (months)	pH	SCOD (mg gTS^{-1})	TS (%)	VS (%)	VS/TS (%)	CH_4 (m^3 $kgVS_{added}^{-1}$)	CH_4 (m^3 t_{ww}^{-1})	CH_4 (m^3 t_{oww}^{-1})
Pre-wilted	0	6.2	240	44.4	39.6	89.2	0.48 ± 0.09	188.7	188.7
	1	7.5	140	30.3	26.0	85.8	0.33 ± 0.04	85.1	nd
	3	4.9	200	44.4	39.9	89.9	0.44 ± 0.01	177.7	nd
	11	4.5	260	37.4	32.9	88.0	0.39 ± 0.02	127.8	127.8
Pre-wilted+ additive	0	5.8	280	42.2	37.8	89.6	0.48 ± 0.09	180.0	188.7
	1	7.1	180	27.6	24.4	88.4	0.32 ± 0.08	79.2	nd
	3	4.5	140	26.6	22.6	85.0	0.39 ± 0.02	88.4	nd
	11	4.3	350	33.3	29.0	87.1	0.37 ± 0.01	106.2	111.8

nd = not determined

4.2 Hydrogen production from grass silage and maize

4.2.1 The effect of inoculum on hydrogen production from grass silage

The effect of heat-treatment of the inocula and initial pH adjustment (to pH 6) on H_2 production from grass silage (VS ratio of 1) and from glucose was studied in batch assays (35 °C) using two different inocula, namely inoculum from farm and inoculum from WWTP (II; Table 13).

H_2 was produced from grass silage and glucose with heat-treated and/or pH-adjusted inoculum of farm origin, while inoculum from WWTP produced H_2 from glucose only (Table 13). With inoculum of farm origin the highest H_2 yield from grass silage (11.5 ml gVS_{added}^{-1}, 31 % of the biogas) and glucose (45.3 ml (g glucose added)$^{-1}$) occurred when the inoculum was both heat-treated and pH-adjusted. Without heat-treatment both inocula produced mainly CH_4 from grass silage and glucose, while pH-adjusted inoculum from farm also produced some H_2 (3.6 ml gVS_{added}^{-1}) from grass silage and glucose (8.4 ml (g glucose added)$^{-1}$). The heat-treatment inhibited CH_4 production from both substrates with both inocula (Fig. 3). Typically, in all the assays H_2 production started within 24 hours and ceased within 5 days of incubation (Fig. 3). The final pH in all the assays with grass silage was around 6 (5.8-6.6), except with heat-treated inoculum from WWTP (final pH of 6.9-7.1) (Table 13).

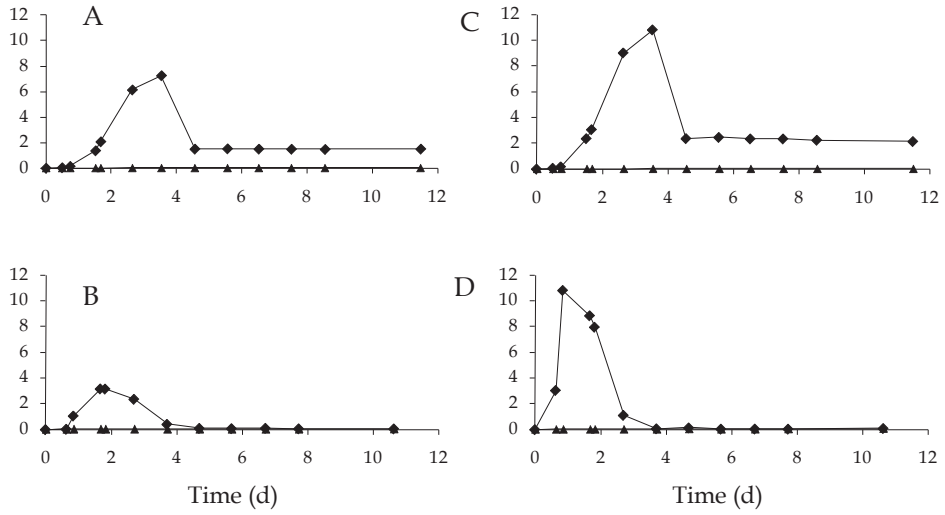

FIGURE 3 Mean H_2 (♦) and CH_4 (▲) production from grass silage (above, unit ml gVS^{-1}) using heat-treated inoculum of farm origin and from glucose (below, unit ml g^{-1}) using heat-treated inoculum from WWTP without (A & B) and with (C & D) initial pH adjustment to 6.

TABLE 13 H_2 and CH_4 yield (±standard deviation) from inocula and substrates (inoculum subtracted), SCOD at the end of the assays and pH at the beginning and at the end of the assays (VS ratio of 1, assays done in duplicate).

Inoculum	HT	pH (initial/final)			SCOD (g l^{-1})			H_2 (ml gVS^{-1})			CH_4 (ml gVS^{-1})		
		No substrate	Grass	Glucose	No substrate	Grass	Glucose	No substrate	Grass	Glucose[a]	No substrate	Grass	Glucose[a]
Farm	no	8.6/7.6	6.5/6.6	8.4/7.9	4.3	9.5	3.6	0.3 ± 0.3	2.1 ± 1.3	0.0 ± 0	27.0 ± 0.1	6.3 ± 1.4	301.1 ± 7.6
Farm	no	6/6.7	6/6.2	6/4.3	2.5	7.0	4.7	0.4 ± 0.1	3.6 ± 0.7	8.4 ± 6.9	9.5 ± 1.0	13.8 ± 0.5	0.0 ± 0.0
Farm	yes	9.5/8.5	6.2/6.1	9.5/6.6	8.2	12.2	4.4	0.3 ± 0	7.2 ± 2.7	0.5 ± 0.7	0.3 ± 0.3	0.0 ± 0	0.0 ± 0
Farm	yes	6/6.4	6/5.9	6/5.8	4.2	12.8	5.3	0.5 ± 0.4	11.5 ± 0	45.3 ± 25.5	1.4 ± 0.1	0.0 ± 0	0.0 ± 0
WWTP	no	7.9/7.3	5.9/7.1	7.9/6.9	0.3	1.4	0.2	0.0 ± 0	0.0 ± 0	0.0 ± 0	9.7 ± 0.7	49.0 ± 1.2	87.6 ± 5.1
WWTP	no	6/6.5	6/6.9	6/6.4	0.2	1.6	0.3	0.0 ± 0	0.0 ± 0	6.6 ± 0.3	8.1 ± 0	48.2 ± 1.4	90.2 ± 1.7
WWTP	yes	9.1/7.3	6.1/5.9	9.1/6.0	3.2	6.2	4.5	0.2 ± 0	0.1 ± 0.2	9.7 ± 1.2	0.4 ± 0.3	0.0 ± 0	0.0 ± 0
WWTP	yes	6/6.7	6/5.8	6/5.7	2.0	7.4	4.5	0.0 ± 0	0.2 ± 0.1	35.9 ± 3.2	0.7 ± 0	0.0 ± 0.0	0.0 ± 0

[a]unit ml (g glucose)$^{-1}$

4.2.2 The effects of VS ratio, temperature and initial pH on hydrogen yield from grass silage

The effects of different VS ratios and temperature (VS ratio of 1) on H_2 yield was studied in batch assays using heat-treated inoculum of farm biogas plant (II). H_2 yield increased from 3.2 to 6.2 ml gVS_{added}^{-1} when the VS ratio was increased from 1 to 2 (Table 14, Fig. 4). H_2 production increased from 3.2 to 7.2 and 16.0 ml gVS^{-1} with corresponding H_2 percentages of 6, 15 and 35 %, when temperature was increased from 35 to 55 and 70 °C, respectively. Time taken to reach maximum H_2 yield was longer at 70 °C (around 25 days) compared to 55 °C (10 days) and 35 °C (3-4 days). At 55 °C H_2 was consumed after the initial peak on day 1, and a second H_2 production phase was detected after day 3 (Fig. 4). SCOD at the end of the assays increased when the higher VS ratio was used. In contrast pH was not affected (range 5.1-5.2). The final pH was lower at 55 °C compared to 35 °C and 70 °C and SCOD was lowest at 70 °C (9.5 g l^{-1}), second lowest at 35 °C (10.5 g l^{-1}) and highest at 55 °C (11.8 g l^{-1}) (Table 14).

The effect of initial pH (4, 5 and 6) on H_2 yield from grass silage was studied in batch assays at 35 °C using a VS ratio of 2. H_2 yield was highest at initial pH of 5 (4.0 ml gVS_{added}^{-1}), while at pH 6 H_2 yield was 0.9 ml gVS_{added}^{-1}. At pH 4 no H_2 was produced. The final pH (4.9) and SCOD of the grass silage at initial pH 5 and 6 did not differ, while at pH 4 final pH and SCOD were lower (Table 14).

TABLE 14 Effect of temperature, VS ratio and initial pH on final pH, SCOD and H_2 yield (±standard deviation) from heat-treated inoculum and grass silage (H_2 production from inoculum subtracted).

T	VS	pH (initial/final)		SCOD (g l^{-1})		H_2 (ml gVS_{added}^{-1})	
(°C)	ratio	No substrate	Grass	No substrate	Grass	No substrate	Grass
35	1:1	6/6.1	6/5.2	5.0	10.5	0.4 ± 0.2	3.2 ± 1.2
35	1.5:1	nr	6/5.1	nr	14.5	nr	4.3 ± 1.3
35	2:1	nr	6/5.2	nr	16.5	nr	6.2 ± 0.7
55	1:1	6/5.8	6/5.1	5.5	11.8	1.4 ± 0.5	7.2 ± 0.2
70	1:1	6/5.9	6/5.4	5.0	9.5	4.0 ± 0.8	16.0 ± 1.7
35	2:1	6/6.2	6/4.9	5.0	14.7	bd	0.9 ± 0.3
35	2:1	5/5.8	5/4.9	3.7	14.2	bd	4.0 ± 3.2
35	2:1	4/4.5	4/4.4	2.3	8.6	bd	bd

bd = below detection limit
nr = not relevant

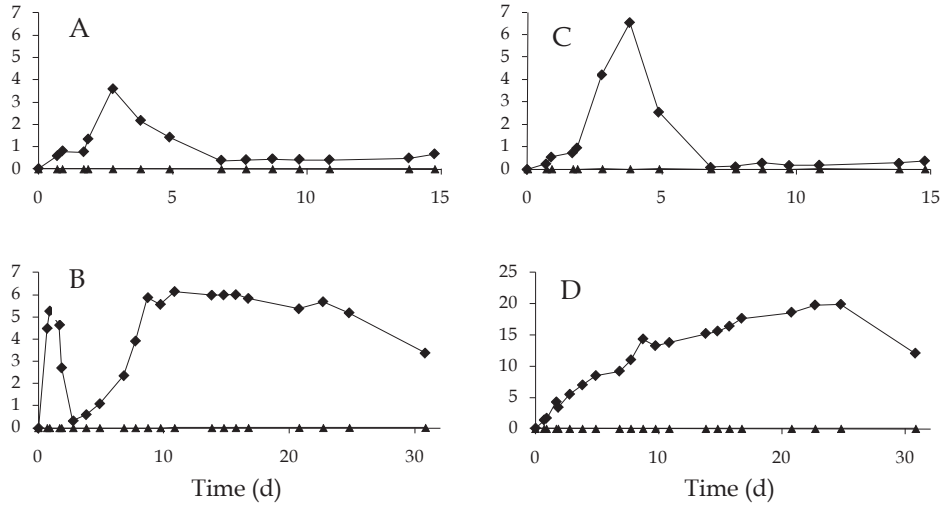

FIGURE 4 Mean H_2 (♦) and CH_4 (▲) production (unit ml gVS_{added}^{-1}) from grass silage using VS ratio of 1 (A) and 2 (C) (temperature 35 °C) and at temperature of 55 °C (B) and of 70 °C (D) (VS ratio of 1).

4.2.3 The effects of pre-treatments on hydrogen production from grass silage and maize

Grass silage (III) and maize (IV) were subjected to water extraction (maize), alkaline (NaOH, grass silage) and acid (HCl, maize) pre-treatments (3.2.2) to study their effects on H_2 and CH_4 yields. After pre-treatments crops were separated into solid and liquid fractions. NaOH treatment increased the pH of grass silage from 4.1 to 6.4, whereas water-extraction and HCl-treatment decreased the pH of maize from 6.3 to 5.5 and 4.5, respectively. 18 % of the grass silage VS was solubilised into liquid fraction after NaOH-treatment, whereas 9 % of the maize VS was solubilised into liquid fraction after water-extraction and HCl-treatment (Table 15).

TABLE 15 Characteristics of the pre-treated fractions of grass silage (III) and maize (IV).

Crop	Fraction	ww (g)	TS (g)	VS (g)	pH	SCOD (g l^{-1})	Paper
Grass silage	untreated	184	50	46.5	4.1	190[a]	III
	NaOH-treated solid	302	41	39	6.4	na	
	NaOH-treated liquid	750	9	5	6.4	10.1	
Maize	untreated	32.7	30	28.1	6.29	211[a]	IV
	water-extracted solid	228	30.1	28.9	5.46	na	
	water-extracted liquid	102	2.7	2.3	5.46	21.1	
	HCl-treated solid	229	27.6	26.2	4.53	na	
	HCl-treated liquid	102	2.8	2.3	4.53	18.1	

[a]unit mg gTS^{-1}
na = not available

H_2 production from grass silage and NaOH treated solid and liquid fractions (separately) were studied in batch assays at 55 °C with heat-treated inoculum from farm (III; Fig. 5, Table 16). H_2 production peaked within 1-2 days with all the studied substrates but H_2 was consumed within 2-3 days (Fig. 5). Secondary H_2 production was observed with silage and solid fraction as substrates although the replicates showed some variation especially with silage (Fig. 5). The average H_2 potential of grass silage was 5.6 ml gVS_{added}^{-1}. The corresponding values for solid and liquid fractions were 3.4 and 31.1 ml gVS_{added}^{-1}, respectively (Table 16).

The calculated combined H_2 yield from NaOH treated grass silage was slightly higher (6.5 ml g $VS_{original}^{-1}$) than that of untreated grass silage (5.6 ml gVS^{-1}) with liquid fraction accounting for 56 % of the total H_2 potential (Table 16). No CH_4 production was noticed in the H_2 assays except for the assays with liquid fraction, where CH_4 production was noticed after 4 days of incubation. The CH_4 potential obtained from the liquid fraction was 135 ml gVS_{added}^{-1} (Table 16).

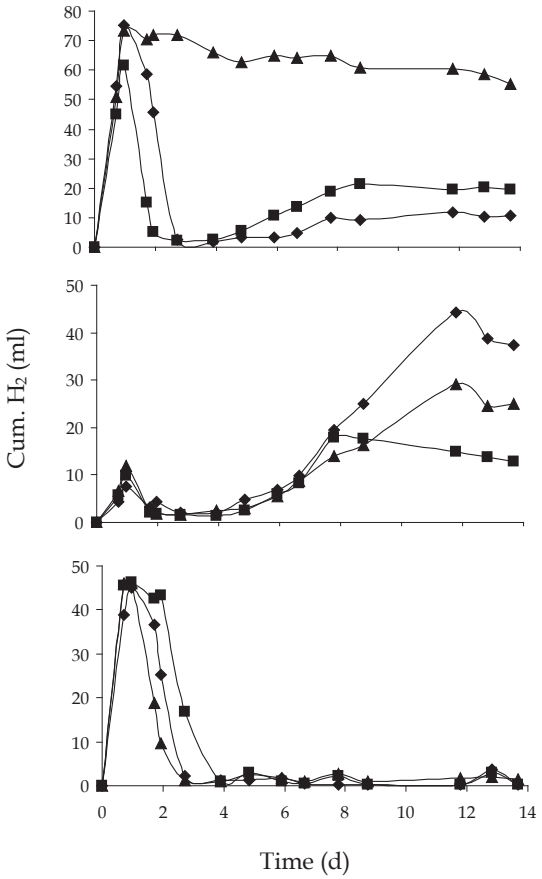

FIGURE 5 H_2 production from grass silage (up), NaOH-treated solid (middle) and liquid fractions (down). Data from the triplicate assays (♦, ■, ▲).

At the end of the H_2 assays, pH was 4.8-5.0 in all assays except with the liquid fraction where it was 6.3. The final SCOD values were as high as 7.3 g l^{-1} with grass silage after the H_2 stage. In the H_2 assays, the final total VFA-CODs with substrates were 1810-4790 mg l^{-1}, contributing from 48 to 70 % of the final SCOD (Table 16). Acetate was the main VFA (Table 16). In the H_2 assays the amount of SCOD increased by 39 and 49 % with grass silage and solid fraction as substrates. In contrast, with liquid fraction the amount of SCOD decreased by 43 % during H_2 assay (Table 16).

TABLE 16 H_2 and CH_4 yields of untreated and pre-treated grass silage in one- and two-stage batch assays as well as pH, SCOD and main metabolic products after H_2-stage. Moreover, the changes in SCOD, AA and PA concentrations during H_2 assays are shown. Standard deviation in parenthesis when applicable.

		Untreated	NaOH-treated solid	NaOH-treated liquid
H_2-stage	pH	5.02	4.77	6.29
	SCOD (g l^{-1})	7.3 (0.23)	3.6 (0.1)	3.8
	SCOD change (%)	+39	+49	–43
	H_2 (ml gVS_{added}^{-1})	5.64 (0.63)	3.38 (1.05)	31.14 (0.47)
	H_2 (ml $gVS_{original}^{-1}$)	na	2.85	3.61
	H_2 (ml $gVS_{original}^{-1}$) comb	na	6.46	
	TVFA (mg l^{-1})	3569	2028	1463
	TVFA/SCOD (%)	65	70	48
	AA(mg l^{-1}), % of TVFA	2197 (179), 62	1520 (90), 75	1027, 70
	AA change (%)	+179	+3556	+36
	PA (mg l^{-1}), % of TVFA	284 (28), 8	136 (58), 7	299, 20
	PA change (%)	+137	+193	+250
	BA (mg l^{-1}), % of TVFA	906 (30), 25	325 (39), 16	48, 3
+ CH_4 stage	pH	7.48	7.43	7.52
	SCOD (g l^{-1})	3.0 (0.1)	2.9 (0.1)	3.4 (0.2)
	CH_4 (ml gVS_{added}^{-1})	467 (18)	490 (32)	520
	CH_4 (ml $gVS_{original}^{-1}$)	na	413	60
	(ml $gVS_{original}^{-1}$) comb	na	473	
One-stage CH_4	pH	7.36	7.40	7.47
	SCOD (g l^{-1})	3.0 (0.23)	2.4 (0.1)	2.4 (0.14)
	CH_4 (ml gVS_{added}^{-1})	431 (3)	299 (30)	703 (10)
	CH_4 (ml $gVS_{original}^{-1}$)	na	252	82
	(ml $gVS_{original}^{-1}$) comb	na	334	

na = not applicable

H_2 production from untreated, water-extracted and HCl-treated maize (IV) was studied in batch assays for 2 and 14 d (Fig. 6, Table 17). For batch assays, the fractions after pre-treatments were combined in actual ratios that were formed during the pre-treatments. The produced biogas was composed of H_2 and CO_2, and was free of CH_4. After 2 d of incubation, average H_2 yields of 5.6 and 1.9 ml gVS_{added}^{-1} were obtained from untreated and water-extracted maize, respectively ($P < 0.05$). In contrast, no H_2 was produced from HCl-treated maize. After 14 d of incubation, the highest average H_2 yield of 20.5 ml gVS_{added}^{-1} was obtained from HCl-treated maize, followed by water-extracted (18.0 ml gVS_{added}^{-1}) and untreated maize (9.9 ml gVS_{added}^{-1}) (Table 17). However, no statistically significant difference in H_2 yields was noticed between the treatments ($P > 0.05$).

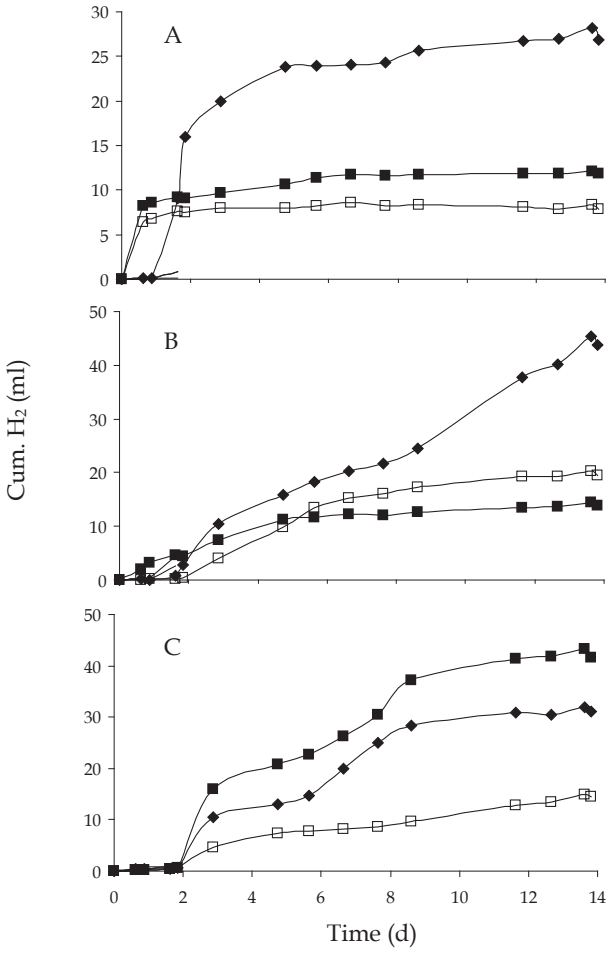

FIGURE 6 Cumulative H_2 production from untreated (A), water-extracted (B) and HCl-treated (C) maize in 14 d H_2 assays (replicates shown). Note the different scale in y-axis. Results from 2 d assays shown with lines only.

Table 17 presents the pH levels in H_2 assays at the end of 2 d and 14 d of incubation. The pH decreased due to fermentation from 6 to 4.9 (2 d assay) and to 4.3 (14 d) with untreated maize as a substrate. The dominant metabolic products were acetic acid and butyric acid (Table 17). The concentration of VFA varied with incubation period and pre-treatment method. In 2 d H_2 assays, the predominant VFA was acetic acid in the untreated (77 % of total VFA) and HCl-treated maize (78% of total VFA), while more or less equal concentration of acetic and butyric acids were present in the water-extracted maize (43 % of total VFA). Average acetic acid concentrations were approximately the same in the HCl-treated (288 mg l^{-1}) and water-extracted maize (275 mg l^{-1}) after 2 d H_2

TABLE 17 H_2, CH_4 and energy yields of untreated and pre-treated maize in one- and two-stage batch assays as well as pH, SCOD and main metabolic products after H_2-stage. Standard deviation in parenthesis when applicable. Conversion factors of 3 and 10 kWh per 1 Nm^3 have been used for H_2 and CH_4, respectively.

		Untreated	Water-extracted	HCl-treated
2 d	pH	4.85	5.28	5.19
H_2-stage	SCOD (g l^{-1})	4.83 (0.76)	4.50 (0.35)	5.00 (0.30)
	H_2 (ml gVS_{added}^{-1})	5.6 (0.7)	1.9 (0.8)	0.0
	energy (kWh tVS^{-1})	16.8	5.7	0
	TVFA (mg l^{-1})	122	634	368
	TVFA/SCOD (%)	3	21	9
	AA(mg l^{-1}), % of TVFA	94 (29), 77	275 (8), 43	288 (35), 78
	PA (mg l) $^{-1}$, % of TVFA	15 (0), 12	64 (2), 10	21 (1), 6
	BA (mg l^{-1}), % of TVFA	5 (4), 4	274 (390), 43	8 (8), 2
+ CH_4 stage	CH_4 (ml gVS_{added}^{-1})	342 (8)	358 (37)	397 (5)
	energy (kWh tVS^{-1})	3420	3580	3970
	energy total (kWh tVS^{-1})	3437	3586	3970
14 d	pH	4.30	5.15	5.02
H_2-stage	SCOD (g l^{-1})	3.70 (0.35)	3.70 (0.35)	4.60 (0.20)
	H_2 (ml gVS_{added}^{-1})	9.9 (8.0)	18.0 (12.6)	20.5 (11.1)
	energy (kWh tVS^{-1})	29.7	54.0	61.5
	TVFA (mg l^{-1})	454	1196	1007
	TVFA/SCOD (%)	18	47	34
	AA(mg l^{-1}), % of TVFA	201 (124), 44	562 (504), 47	346 (138), 34
	PA (mg l^{-1}), % of TVFA	17 (3), 4	63 (18), 5	26 (3), 3
	BA (mg l^{-1}), % of TVFA	226 (165), 50	544 (324), 45	580 (261), 58
+ CH_4 stage	CH_4 (ml gVS_{added}^{-1})	311 (38)	357 (47)	368 (41)
	energy (kWh tVS^{-1})	3110	3570	3680
	energy total (kWh tVS^{-1})	3140	3624	3742
One-stage CH_4	CH_4 (ml gVS_{added}^{-1})	321 (23)	328 (7)	312 (15)
	energy (kWh tVS^{-1})	3210	3280	3120

assays (Table 17). In 14 d H_2 assays, the share of butyric acid increased in all assays, being highest in the HCl-treated maize assays (58 % of total VFA). The concentration of butyric acid (580 mg l^{-1}) in the HCl-treated maize was 1.6 times higher than that of acetic acid. On the other hand, the concentration of propionic acid remained unchanged with increase in incubation time in the respective assays. However, the highest propionic acid concentration was observed in the water-extracted maize (63 mg l^{-1}) compared to that of HCl-treated maize (21-26 mg l^{-1}) or untreated maize (15-17 mg l^{-1}).

4.3 Methane production from grass silage and maize

4.3.1 Methane yield in batch assays, the effect of pre-treatments

CH_4 production from grass silage and NaOH treated solid and liquid fractions (separately) were studied in batch assays at 35 °C (III). Highest CH_4 yield of 703 ml gVS_{added}^{-1} was obtained from the liquid fraction followed by 430 ml gVS_{added}^{-1} from grass silage and 299 ml gVS_{added}^{-1} from the solid fraction (Table 16). The calculated combined CH_4 yield of the NaOH pre-treated grass silage was 334 ml $gVS_{original}^{-1}$ and was lower than that of the untreated grass silage (430 ml gVS_{added}^{-1}) (Table 16).

Methane production from untreated, water-extracted and HCl-treated maize (IV) was studied in batch assays at 35 °C (Table 17). In one-stage CH_4 assays, CH_4 yield of 321 ml gVS_{added}^{-1} was obtained from untreated maize. However, no significant difference in CH_4 yield was obtained with the studied pre-treatments ($P > 0.05$). The average CH_4 yields were 328 and 312 ml gVS_{added}^{-1} for water-extracted and HCl-treated maize, respectively.

4.3.2 Methane production after hydrogen stage

Methane production from grass silage and NaOH treated solid and liquid fractions were studied in batch assays after H_2 stage (III). On comparison to one-stage process, two-stage process (H_2 and CH_4 assays) resulted in increased CH_4 yields by 8 % for grass silage and 64 % for solid fraction. The increase in CH_4 yields for grass silage was from 431 to 467 ml $gVS_{original}^{-1}$ and for solids fraction from 252 to 412 ml $gVS_{original}^{-1}$. On the other hand, CH_4 yield of the liquid fraction in two-stage process was lower (60 ml $gVS_{original}^{-1}$) compared with one-stage process (80 ml $gVS_{original}^{-1}$) (Table 16). CH_4 production of solid fraction was faster in two-stage process compared to one-stage process. The opposite was true with liquid fraction (Fig. 7). At the end of all the CH_4 assays, pH varied between 7.3 and 7.5 (Table 16). SCOD values between 2.4 and 3.5 g l^{-1} were detected after the one- and two-stage CH_4 assays (Table 16). After CH_4 assays no VFAs were detected.

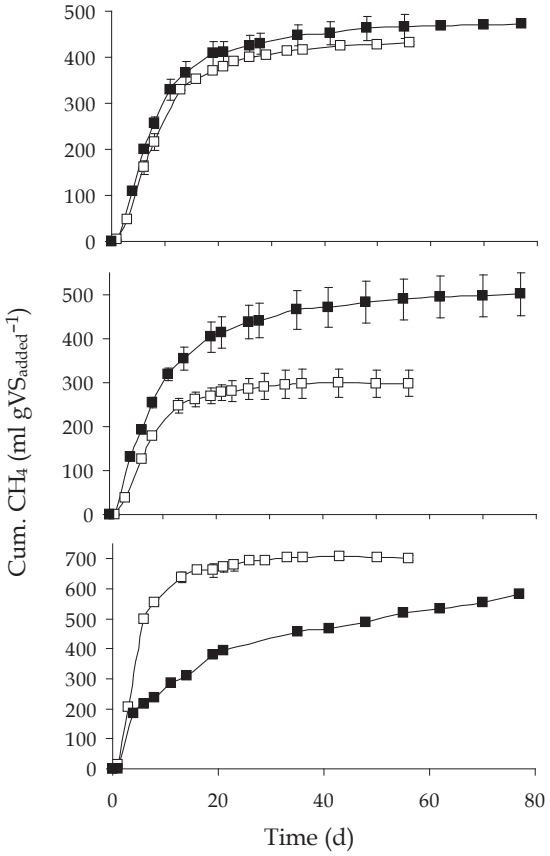

FIGURE 7 Average cumulative CH_4 yield (ml VS_{added}^{-1}) from grass silage (up), NaOH-treated solid (middle) and liquid fractions (down) without (□) and with (■) H_2 stage.

Methane production from untreated, water-extracted and HCl-treated maize (IV) was studied in batch assays after H_2 stage (2 and 14 days). The highest CH_4 yield of 397 ml gVS_{added}^{-1} was obtained with HCl-treated maize. The average increase in CH_4 yields were 24 % and 27 % compared to the CH_4 yields obtained from untreated (321 ml gVS_{added}^{-1}) and HCl-treated (312 ml gVS_{added}^{-1}) maize in one-stage processes. This difference in yield was statistically significant when compared to that obtained with untreated and HCl-treated maize in one-stage assays ($P < 0.05$). On comparison to 2 d H_2 stage, 14 d incubation period resulted in either decreased CH_4 yields (untreated and HCl-treated maize) or did not further improve (water-extracted) the CH_4 yields (Table 17). Two-stage process with 14 d H_2 stage showed higher initial CH_4 production rates compared with the two-stage process with 2 d H_2 stage or one-stage CH_4 process (Fig. 8).

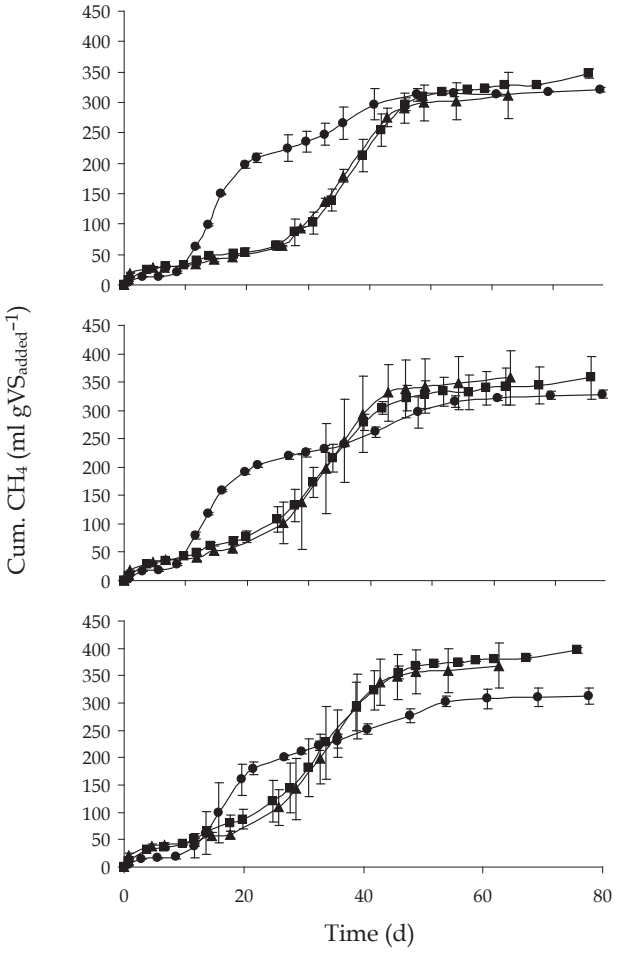

FIGURE 8 Cumulative CH_4 production (ml gVS_{added}^{-1}, inoculum subtracted) from untreated (A), water-extracted (B) and HCl-treated maize (C) in one-stage CH_4 (●) assays and in CH_4 assays after two (■) and 14 days (▲) H_2 stage.

4.3.3 Methane production from grass silage in CSTRs

Methane production from grass silage (V) was studied in 2 parallel CSTRs at 35 ^oC with an OLR of 2 kgVS $(m^3d)^{-1}$ and HRT of 30 days (Fig. 9, 10, Table 18). After the initial start-up of feeding, specific methane yield rose to around 200 l $kgVS_{fed}^{-1}$ by day 15. Thereafter, methane production dropped sharply with a corresponding decrease in pH (Fig. 9) and increase in total volatile fatty acid (TVFA) concentration (Fig. 10). In order to raise the pH, buffer ($NaHCO_3$) addition was initiated on day 23 and was substituted with nutrient solution

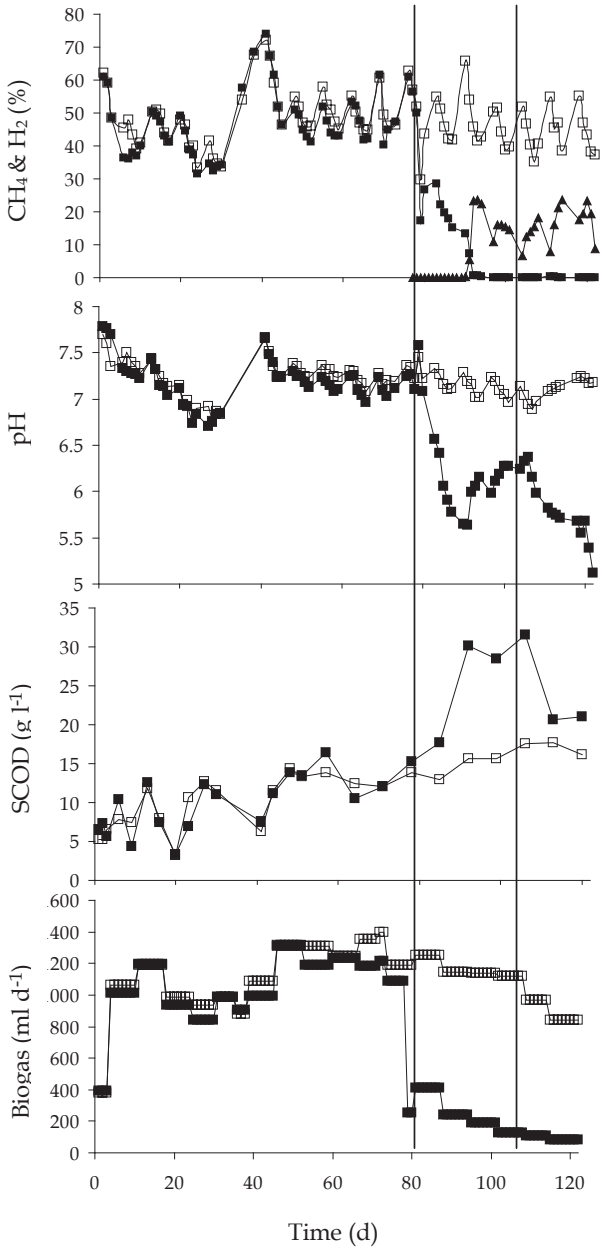

FIGURE 9 CH_4 and H_2 (▲) contents, pH, SCOD and daily biogas volume of M1 (open) and M2 (closed). Lines represent the shift (d. 79) and change in buffer dosage (d. 106). Note that liquid volume of M_2 was reduced from 1.5 l to 0.3 l on day 79.

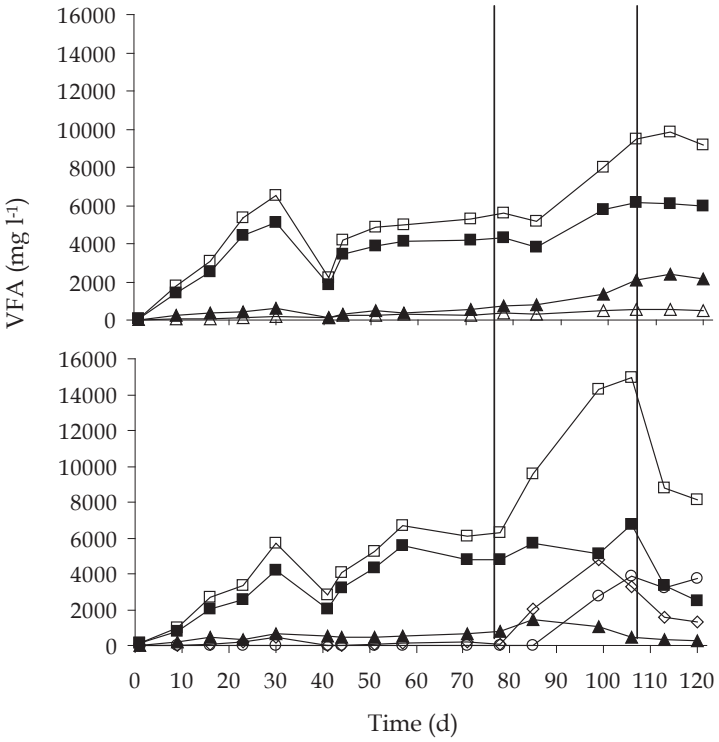

FIGURE 10 TVFA (□), acetic (■), propionic (▲), butyric (◊), iso-valeric (Δ) and caproic acid (○) concentrations in M1 (up) and M2 (below). Lines represent the shift (d. 79) and change in buffer dosage (d. 106).

TABLE 18 Average specific and volumetric methane yields of methanogenic CSTRs. Results of M2 shown between days 41-78 only. Standard deviation in parenthesis.

Days	CH_4 l $kgVS_{fed}^{-1}$	CH_4 m^3 $(m^3d)^{-1}$
41-78	218 (25)	0.44 (0.05)
	198 (18) (M2)	0.40 (0.04) (M2)
79-105	185 (20)	0.37 (0.04)
106-122	140 (18)	0.28 (0.04)

from day 28 onwards. However, TVFA concentration continued to increase further to reach 5.7-6.5 g l^{-1} by day 31. The concentration of acetic acid and propionic acid, the main components of TVFAs, were 4.2-5.1 and 0.6-0.7 g l^{-1}, respectively (Fig. 10).

Despite the addition of buffer and nutrients, both reactors did not recover and thus reactors were kept unfed between days 31 and 40 (Fig. 9, 10). During this unfed period, TVFA concentration decreased to 2.2-2.9 g l^{-1} and pH increased to 7.7. Feeding was resumed on day 41 with an OLR of 2 kgVS $(m^3d)^{-1}$ and HRT of 30 days. The mean methane production during days 41-78 was 198-218 l CH_4 $kgVS_{fed}^{-1}$ with an average CH_4 content of 50-54 % (Table 18). During the same period, SCOD concentration was between 12 and 15 g l^{-1}. However, daily buffer addition (9.8 g (kg feed)$^{-1}$) was needed in order to keep the pH close to 7. Nevertheless, TVFA concentration increased from 2.2 to 5.7 g l^{-1} in M1 and from 2.9 to 6.3 g l^{-1} in M2. Besides acetic acid, propionic acid accumulation was also noticed in both M1 and M2. The increase in propionic acid concentration was from 0.1 to 0.7 g l^{-1} in M1 and from 0.5 to 0.8 g l^{-1} in M2. VS reduction during this period was between 49 and 57 % in both reactors.

Mixing the M1 and M2 reactor contents (day 78) and continuing M1 as methanogenic reactor with an OLR of 2 kgVS $(m^3d)^{-1}$ and HRT of 30 days resulted in a mean specific methane yield of 140 l $kgVS_{fed}^{-1}$ and volumetric methane yield of 0.28 m^3 $(m^3d)^{-1}$ (Table 18). This yield was lower than the methane yield of 218 l $kgVS_{fed}^{-1}$ obtained prior to mixing of the reactor contents. The SCOD concentration during the days 78-105 increased from 13.8 to 17.6 g l^{-1} with a corresponding decrease in pH (Fig. 9). Due to this decrease in pH, more buffer was added (38.2 g (kg feed)$^{-1}$) from day 106 onwards. However, TVFA concentration increased from 5.5 g l^{-1} to 9.9 g l^{-1}. The main components of TVFA were acetic (6.1 g l^{-1}), propionic (2.4 g l^{-1}) and iso-valeric acids (0.5 g l^{-1}). Iso-butyric (0.3 g l^{-1}), butyric (0.3 g l^{-1}) and valeric acids (0.1 g l^{-1}) were also present but at a lower concentrations (Fig. 10).

4.4 Shifting methanogenic process to hydrogenic process

Methanogenic CSTR (V) treating grass silage (M2) was shifted to H_2 production by increasing the OLR from 2 to 10 kgVS $(m^3d)^{-1}$ and by decreasing the HRT from 30 to 6 days (day 79; Fig. 9, 10). Immediately after the changes in operation strategy, CH_4 concentration dropped rapidly from ca. 50 % to below detection limit. H_2 production was first detected 12 days (on day 90) after the shift in operational strategy. Thereafter, H_2 concentration increased steadily during the next 30 days of operation and fluctuated between 10 and 24 % depending upon the feeding cycle, with lowest H_2 concentration of < 10 % noticed after the non-fed weekends (Fig. 9). This shift in operational strategy through increased OLR and decreased HRT resulted in a sharp drop in pH from 7 to 5.6 (day 90) and then increased slowly to reach 6 (since day 92). On the other hand, SCOD and TVFA concentrations increased from 15.3 to 31.6 g l^{-1} and from 6.3 to 15 g l^{-1}, respectively (day 106). Among the TVFA components, the increase in acetic acid concentration was small (from 4.8 to 6.8 g l^{-1}) compared to caproic acid, which increased from negligible to 3.9 g l^{-1} (day 106). Butyric acid concentration reached its highest concentration of 4.8 g l^{-1} on day 99. On day 106, buffer

addition was stopped (29 days of addition) which resulted in a decrease in SCOD concentration to around 20 g l^{-1} with a simultaneous drop in pH (Fig. 9). However, H_2 content remained at the same level as noticed during the buffer addition period. In addition, the concentration of TVFA especially that of acetic acid decreased while the concentration of caproic acid remained more or less constant (Fig. 10). TVFA-COD accounted 68-80 % of the SCOD during the shifting period (days 79-120). VS reduction was at the end of reactor experiment 18 %.

During the 40 days of operation at a higher OLR of 10 kgVS $(m^3d)^{-1}$, the highest daily H_2 yield was 42 l $kgVS_{fed}^{-1}$. Nevertheless, the mean daily biogas production showed a decreasing trend, being at highest around 400 ml d^{-1} (Fig. 9) and thus, the average specific and volumetric H_2 yields obtained were 9 l $kgVS_{fed}^{-1}$ and 0.06 m^3 $(m^3d)^{-1}$, respectively.

5 DISCUSSION

5.1 Main findings of the thesis

The results of the present study showed that both hydrogen and methane can be harnessed from energy crops by anaerobic digestion process. Highest hydrogen yields from batch assays were 16 and 9.9 ml gVS_{added}^{-1} for untreated grass silage and maize, respectively, under the studied experimental conditions. The methane yields from untreated crops were in the range 320-480 ml gVS_{added}^{-1}. The effects of temperature and pH on hydrogen yield in batch assays were shown. Inducing hydrogen production in a methanogenic CSTR treating grass silage was shown to be possible by increasing the OLR and shortening the HRT with highest hydrogen yield of 42 l $kgVS_{fed}^{-1}$. Pre-treatments (alkaline, acid and water-extraction) showed some potential for elevating H_2 yields, whereas the methane yields were not affected. Two-stage H_2 + CH_4 process resulted in increased methane yields when compared to traditional one-stage CH_4 process, mainly due to improved hydrolysis and acidogenesis in the first step. Monodigestion of grass silage for methane production in CSTR resulted in methane yield of 198-218 l $kgVS_{fed}^{-1}$. However, long term monodigestion was not feasible with the applied OLR and HRT due to accumulation of VFAs. Ensiling was proven to be a proper method for storage of crops intended for biomethane production. These results thus provide knowledge on how crop biomass could be converted into H_2 and/or CH_4, although further research is needed especially for optimizing the hydrogen production step. These results are discussed in detail in the following sections.

5.2 Effect of storage on methane yield of grasses

The present results show that the CH_4 yield (VS losses during storage taken into account) of energy crops can be maintained by appropriate ensiling conditions for even after 11 months in ambient conditions, while, in contrast, in suboptimal

storage conditions over 50 % of the CH_4 yield can be lost. Several factors, such as crop species, pre-wilting, harvest time, additives and storage time can affect the ensiling process, and thus the final effect on CH_4 yield can be complex. Ensiling has been found as an appropriate method for storing crops for biogas and ethanol production in other studies as well (Mähnert et al. 2005, Vervaeren et al. 2010, Herrmann et al. 2011, Oleskowicz-Popiel et al. 2011).

This and other studies (Table 19) suggest that the CH_4 potential (m^3 $kgVS_{added}^{-1}$, storage losses not included) of energy crops can in some cases be increased during storage, which thus acts as a pre-treatment step. The increase in CH_4 potential is due to degradation of structural polysaccharides into more easily degradable intermediates (Egg et al. 1993, Kung et al. 2003). However, the methane potential can even decrease during the storage in some cases. In this study, storage time had no clear impact on CH_4 potential, which was also the situation in a previous study with grass (Lehtomäki et al. 2005b). It has to be noted, that the increase in methane potential does not necessarily result in increase in energy yield per hectare due to possible storage losses.

TABLE 19 Examples of selected studies with crop-based materials showing the improvement in methane potential (per VS added) after storage.

Substrate	CH_4 potential (ml gVS_{added}^{-1})		Reference
	before storage	after storage	
Grass	360	480	This study, Paper I
Ryegrass	410	490	
Grass	230	310	(Lehtomäki et al. 2005b)
Sugar beet tops	310	370	
Whole crop maize	380	540	(Neureiter et al. 2005)
Maize	330	380	(Herrmann et al. 2011)
Sorghum	320	350	
Forage rye	290	350	
Triticale	340	370	
Maize	380	420	(Vervaeren et al. 2010)

According to this study VS loss during storage seems to be a major factor in determining the preservation of CH_4 yield: the smaller the VS loss the better was the CH_4 yield preserved. With ryegrass the smallest VS loss was obtained with high solids ryegrass (48 h dried, TS 30.4 %) while with grass the effect of initial TS on VS loss was less evident VS losses being even slightly higher for high solids than for low solids grass. High VS losses were characterised by a higher/increased final pH, while low VS losses were obtained in conditions in which final pH was low, as in the case of high solids ryegrass and low solids grass, with additive addition further lowering pH. The chemical characteristics of the crop species in question also have an effect on ensiling properties; for example the buffering capacity of legumes (such as clover) is usually higher than of grasses (McDonald et al. 1991), which may partly explain the relatively high pH of grass in some of the study samples.

Thus, during suboptimal storage conditions large proportion of CH_4 yield can be lost, as shown by the fact that after 6 months of storage, losses in CH_4 yield were at most 37 and 52 % with grass and ryegrass, respectively, in laboratory studies, and 17 and 41 %, respectively, in field studies after 11 months. These losses in CH_4 yield were probably caused by secondary fermentation (e.g. Sebastian et al. 1996), which led to a rise in pH and loss of VS and, in some cases also, to loss of CH_4 potential. It has been stated that if there are insufficient amount of water soluble carbohydrates present in the silage, or the solids content is too low, secondary fermentation by clostridia can occur. Clostridia ferment sugars and lactate mainly into butyrate, while minor amounts of formate, acetate, propionate, ethanol and butanol can also be produced. In secondary fermentation CO_2 is released, resulting in increased pH and, with rising pH, conditions may become favourable for the proteolytic clostridia, which break down proteins and amino acids into amines, amides, and ammonia, thus causing a further increase in pH (Egg et al. 1993, Woolford 1984). Clostridial fermentation during storage is undesirable, because the butyrate fermentation pathway results in considerable loss of gross energy through the loss of molecular H_2 (Egg et al. 1993). In the present study higher initial solids content resulted in a decreased VFA concentration at six months with stored grass and ryegrass. This is partly in accordance with previous results, hence lower concentrations of propionic and n-butyric acids and higher concentrations of lactic- and acetic acids were found with wilted comfrey silage compared to unwilted crop (Wilkinson 2003). In this study, which included crops with lower solids, other VFAs, such as propionic and butyric acid, were also present. This is, as discussed above, an indication of secondary fermentation. With low solids crops small amounts of valeric and caproic acids were also present. These acids are thought to be formed from acetic and propionic or acetic and n-butyric acid by removal of molecular H_2 and it is known that some clostridia and a rumen bacterium are capable of catalysing these reactions (Zauner & Küntzel 1986). Unfortunately, lactic acid was not analysed in this study. Usually VS losses during ensiling are lower (McDonald et al. 1991, Egg et al. 1993) as obtained in this study. In earlier studies between 1.6 and 15.7 % of TS was lost when storing elephantgrass and energycane (Woodard et al. 1991), 2.9 % when storing maize silage for 90 days (Filya 2004) and TS losses of switchgrass during six months of storage (round bales, no plastic) averaged 13 % (Sanderson et al. 1997).

In the present study the role of biological additive in improving or preserving CH_4 yield was not noteworthy, as was also the situation with grass and sugar beet tops (Lehtomäki et al. 2005b) and whole crop maize (Neureiter et al. 2005). Additives have led to lower pHs in previous studies as well; e.g., bacterial inoculant resulted in lower pH in lupin silages (Fraser et al. 2005), whole crop barley silage (Zahiroddini et al. 2004) and forage pea and field bean silages (Fraser et al. 2001). According to this study addition of biological additive resulted in reduced VS loss with ryegrass at six months of storage, whereas with grass the effect of additive on VS loss was less clear.

In this study mass losses on the field scale were negligible with ryegrass, probably due to the high initial TS content (44.4 %), while mass losses of grass varied between 18 and 29 %, the smallest mass loss observed with grass stored without pre-wilting. Mass losses for crops stored in laboratory studies have generally been less than 2.5 % (Weinberg et al. 2002, Neureiter et al. 2005). In the present study mass losses varied between 2.9 and 4.6 % for grass and between 4.0 and 5.1 % for ryegrass after 3 months of storage. Mass losses in laboratory studies are usually lower than in field studies as in the field leachate losses increase mass loss.

5.3 Hydrogen production from grass silage and maize in batch process

5.3.1 Hydrogen production from grass silage and maize

The present results show that it is possible to produce H_2 from grass silage (II, III) and maize (IV) in a batch process and that the yields are highly dependent on several factors (II-IV). The highest H_2 yield from grass silage (16 ml gVS_{added}^{-1}) was obtained at 70 °C using heat-treated inoculum from a farm digester (II), while the highest average H_2 yield from untreated maize (IV) was 9.9 ml gVS_{added}^{-1} under the studied experimental conditions (heat-treated inoculum, 55 °C, substrate to inoculum VS-ratio of 2). The H_2 yields were comparable or slightly lower to yields obtained with other energy crops or crop residues. Previously, H_2 yields of 3, 16, 20 and 62 ml gTS^{-1} have been reported from untreated corn stalk (Zhang et al. 2007), maize leaves (Ivanova et al. 2009), corn stalk (Fan et al. 2008) and fodder maize (Kyazze et al. 2008), respectively, while H_2 yield of 22-76 ml gVS^{-1} has been obtained from ryegrass (Kyazze et al. 2008). Reasons for this variation in H_2 yields even with similar substrates in the literature are e.g. differences in operational conditions (e.g. pH, temperature, loading and headspace volume), inoculum and chemical characteristics of the substrate in question.

5.3.2 The effect of inoculum on hydrogen production from grass silage

Heat-treated inoculum from the farm biogas digester treating cow manure was more efficient in producing H_2 from grass silage compared to the digested sludge from WWTP (II), and was thus selected for further use (III, IV). Inoculum derived from cow manure presumably contains rumen micro-organisms which are capable of degrading lignocellulosic substrates such as grass silage. Unlike dairy farm sludge, sewage sludge apparently contains low amounts of cellulose utilizing micro-organisms while the dominant micro-organisms in sewage sludge are capable of degrading, e.g., glucose (Chen 1983). Under the studied experimental conditions (VS-ratio 1, temperature 35 °C), heat-treatment of the inoculum seemed to be necessary for H_2 production from

grass silage in batch assays (II), as without heat-treatment, methane was produced. Thus, heat-treatment (boiling 30 minutes) was chosen as a suitable pre-treatment method for H_2 production batch assays (III, IV). In addition, initial pH adjustment to 6 as compared to situation without pH adjustment was shown to improve H_2 yield from grass silage (II), and was thus chosen as a suitable pH in further experiments (III, IV).

In the present batch assays (II-IV), inoculum was heat-treated at the normal boiling temperature of water, which has recently been shown to decrease the species diversity and H_2 yield compared to lower (e.g. 65 °C) treatment temperatures (Baghchehsaraee et al. 2008). It could be possible to produce H_2 from grass silage and maize without heat-treating the inoculum, as H_2 production from different substrates without heat-treating the inocula has been shown to be possible (Antonopoulou et al. 2008, Pan et al. 2008, O-Thong et al. 2009, Ohnishi et al. 2010). In addition, the results obtained in the present study (V) further confirm, that heat-treatment is not necessary for obtaining H_2 production in continuous process.

5.3.3 The effect of temperature on hydrogen production

Both the present (II) and previous studies (Table 20; Pan et al. 2008) indicate that thermophilic conditions can favour H_2 production compared to mesophilic conditions. H_2 yield from grass silage increased with increasing temperature and highest H_2 yield was obtained at 70 °C. Higher H_2 yields at higher temperatures might be expected given the fact that higher temperatures favour H_2 formation but not H_2-consuming reactions (Lepistö 1999, Shin et al. 2004, Luo et al. 2010a), such as methane and propionate formation. Moreover, the higher H_2 yield in thermophilic conditions is also explained by the optimal temperature of the hydrogenase enzyme, which is reported to lie between 50 and 70 °C (Koesnandar et al. 1991). In addition, acidogenic H_2 producers are reported to be mostly thermophilic heat-resistant bacteria (Fang et al. 2002, Liu & Fang 2002).

TABLE 20 Examples of studies with different substrates showing improved hydrogen yields at elevated temperatures.

Substrate	Temperature (°C)	H_2 yield	Reference
Grass silage	35	3 ml gVS^{-1}	This study, Paper II
	55	7 ml gVS^{-1}	
	70	16 ml gVS^{-1}	
Food waste	35	12 ml (g hexose)$^{-1a}$	(Shin et al. 2004)
	55	112 ml (g hexose)$^{-1a}$	
Organic waste	35	165 ml $gVS_{removed}^{-1}$	(Valdez-Vazquez et al. 2005)
	55	360 ml $gVS_{removed}^{-1}$	
Starch-rich	35	47 ml (g starch)$^{-1}$	(Zhang et al. 2003)
wastewater	55	78 ml (g starch)$^{-1}$	

a = calculated from the data given

In both the present and earlier studies the time taken to reach the maximum H_2 yield was longer at thermophilic conditions compared to mesophilic conditions. This was probably caused by the low initial amounts of thermophilic micro-organisms in the inoculum, which was of mesophilic origin (Shin et al. 2004). Heat-treated inoculum from farm digester was thus shown to be suitable for hydrogen production from grass silage, even with elevated temperatures of 55 and 70 °C despite the mesophilic origin of the inoculum. Temperature of 55 °C was chosen for further H_2 production experiments (III, IV), as it was thought to be more easily applicable in practice than the higher temperature of 70 °C.

5.3.4 The effect of substrate concentration and pH on hydrogen production

Substrate concentration apparently has a significant effect on microbial metabolic pathways and on H_2 production. According to results obtained in the present study (II) a higher substrate concentration (i.e. higher substrate to inoculum VS ratio) in batches might be preferable for dark fermentative H_2 production from grass silage. However, when a certain substrate threshold is exceeded and the partial pressure of H_2 is increased, bacteria may shift their metabolism from H_2 and VFA to alcohol production (Okamoto et al. 2000, Fan et al. 2004, Fan et al. 2006a). Too high a substrate concentration would result in the accumulation of VFA and a fall in pH, which would inhibit H_2 producers. The optimal TS concentration for H_2 production seems to be very dependent, e.g., on the substrate used and, apparently, on the substrate to micro-organism ratio. Increased H_2 yield with increasing substrate concentration has been reported, e.g., for lean meat (2.5 ml gVS^{-1} at TS 4 %, 7.1 ml gVS^{-1} at TS 12 %; Okamoto et al. 2000), food waste (46 ml gVS^{-1} at VS 0.3 %, 92 ml gVS^{-1} at VS 0.6 %; Shin et al. 2004) and wheat straw waste (13.8 and 68.1ml gVS^{-1} at substrate concentrations of 0.5 and 2.5 %, respectively; Fan et al. 2006b). In contrast, with carbohydrate-rich substrates a TS concentration of 2-5 % was found preferable for H_2 production, while at higher TS concentrations (tested up to 15 %) H_2 yield per gram VS decreased (Okamoto et al. 2000). Similarly, H_2 yield from wheat straw waste (Fan et al. 2006b) and HSW (Liu et al. 2008b) fell with higher substrate concentrations from 68 ml gVS^{-1} to 16 ml gVS^{-1} at substrate concentrations of 2.5 and 3.5 %, respectively (wheat straw) and from 84 to 24 ml gVS_{added}^{-1} at substrate concentrations of 1 and 2 g l^{-1} (HSW). Despite the initial results (II) of the increased H_2 yield with increasing substrate concentration, the VS-ratio of 2 might have been too high in further experiments (III, IV) and resulted in rapid drop in pH and suboptimal conditions for H_2 production.

The optimal pH for H_2 production from grass silage seems to lie between 5 and 6 (II). At the optimal pH acetate and butyrate producers are assumed to overcome propionate producers, thereby increasing the H_2 yield (Fan et al. 2006a). Optimal pH for H_2 production by *Clostridium butyricum* was reported to be in the range 5.5-6.0, as at a lower pH both cell growth and H_2 production were inhibited and at a higher pH cell growth was more efficient than H_2 production (Chen et al. 2005). With initial pH 4 no H_2 was produced (II). The

pH 4 has also been found inhibitory in other studies using substrates such as starch (Liu & Shen 2004) and wheat straw wastes (Fan et al. 2006b). Thus, based on these results from II, pH was initially adjusted to 6 in H_2 batch assays (III and IV).

According to present results (II-IV) optimal conditions for H_2 production are very case-dependent. High substrate concentration (i.e. VS ratio) applied resulted in enhanced acidification and drop in pH, which in some cases dropped close or even below 5 (II-IV). This makes the interpretation of the results challenging, as H_2 production might have ceased due to low pH and potential H_2 yields are thus underestimated. For example with untreated maize (IV) pH dropped to 4.85, which was below the optimum pH of 5.5 required for H_2 production (Fang & Liu 2002). Therefore, with proper control of pH, H_2 yield from grass silage and maize could probably be improved, as has been shown e.g. with glucose (Karadag & Puhakka 2010).

5.3.5 The effect of pre-treatments

The pre-treatments applied in the present study showed some potential in improving hydrogen yields, although pre-treatment conditions needs further optimization. The slightly enhanced combined H_2 yield (from 5.6 ml VS^{-1} to 6.5 ml $VS_{original}^{-1}$) noticed with NaOH treated grass silage in the present study (III) could be due to enhanced hydrolysis. Water-extraction and HCl-treatment increased the average H_2 yield from maize (IV), even though the result was not statistically significant, most probably due to suboptimal conditions, as discussed previously. Thus, the effect of pre-treatments on H_2 yield in the present study can be unreliable, as the low final pH in solid fraction of NaOH-treated grass silage (III) and untreated maize assays (IV) may have led to low H_2 yield of these respective substrates. Previously, pre-treatments have been applied with the purpose of elevating H_2 production. For example 68 mlH_2 gVS^{-1} was obtained with acid-treated straw compared to 0.5 mlH_2 gVS^{-1} with untreated straw (Fan et al. 2006b) and 3, 57 and 147 ml H_2 $gTVS^{-1}$ were obtained from untreated, NaOH and HCl treated cornstalk (Zhang et al. 2007), respectively. During the HCl pre-treatment, hydrolysis can be promoted by partial removal of hemicellulose or lignin and an increase in the amount of soluble sugars (Zhang et al. 2007). The increase in H_2 yield following water extraction was apparently due to the enhanced hydrolysis and acidogenesis. This was evident from the decrease in pH from 6.3 to 5.5 after water extraction. Previously, water incubation in slightly acidic (initial pH 5) conditions has shown to improve hydrolysis of grass (Lehtomäki et al. 2004) and free sugars from sweet sorghum stalks was extracted by using water at 30 °C (Ntaikou et al. 2008).

5.3.6 Pattern of hydrogen production and consumption

The present results show, that H_2 production from grass silage and maize typically occur fast after initiation of the batch assays (II-IV). The phenomenon

of two peaks in hydrogen production at 55 °C (II, III) could be due to the fact, that due to rapid acidification pH dropped and led to inhibition of H_2-producers. Then, a population that tolerated lower pH was enriched and second peak of hydrogen was produced (Pan et al. 2008). Similar observation has been previously reported in the case of acidified sludge (Ting & Lee 2007) and food waste (Pan et al. 2008, Han & Shin 2004). It was noticed, that H_2 production from HCl-treated maize started after longer lag phase (2 days) compared to untreated and water-extracted maize (IV), indicating that the process was inhibited. A similar observation was also reported by Cui et al. (2010). In the above study, H_2 production from HCl-treated poplar leaves was initially inhibited but the final H_2 yield was however higher than that obtained from untreated poplar leaves (Cui et al. 2010). With the aim of H_2 production from solid crop materials, longer incubation time might be needed, as was also suggested by previous researchers (Ntaikou et al. 2008). In the above mentioned study, H_2 production from sorghum continued for 12 d and the authors suggested that the limiting factor in H_2 production from cellulosic and hemicellulosic materials was the hydrolysis phase (Ntaikou et al. 2008). However, use of adapted microbial consortia could probably improve the H_2 production rates and yields as indicated by a study in which repeated batch cultivation were performed on household solid waste (Liu et al. 2008b). In the above study, H_2 yield of 84 ml gVS_{added}^{-1} was obtained in 15 d with first generation, whereas 170 ml gVS_{added}^{-1} was obtained after 4 d of incubation with fifth generation culture.

In the present study with grass silage as a substrate (II, III) H_2 was in most cases consumed after the exponential H_2 production phase, apparently by homoacetogenic and propionate-producing bacteria, as only a negligible amount of CH_4 was produced (Hussy et al. 2003, Oh et al. 2003, Park et al. 2005, Hawkes et al. 2007). High concentration of acetic acid (62-79 % of VFA) at the end of the H_2 assays (III) could indicate the homoacetogenesis, and that the homoacetogens survived the heat-treatment, as has been shown previously (Oh et al. 2003) and/or were introduced with non-sterilized substrate. In addition, also propionate fermentation consumes H_2 (Hussy et al. 2003) and produces propionate, acetate and valeriate (Lee et al. 2004) and might have occurred in the present study (III). However, it should be noted that VFA analysis at the end of the experiment can not be used as a reliable indicator of H_2 production and consumption pathways, as H_2 is constantly produced and consumed during the assay with mixed culture. Therefore, continuous monitoring of VFAs during the experimental run would give more reliable data of hydrogen production and consumption.

The fact that in the H_2 assays with NaOH-treated liquid fraction (III) CH_4 production started after H_2 production phase, despite the use of heat-treated inoculum could indicate that the applied heat-treatment (boiling 30 minutes) of the inoculum was not capable of destroying all methanogenic activity. In fact methanogens have been shown to survive even 10 h at 105 °C (Ueki et al. 1997). The reason that CH_4 production was observed only in assays with liquid fraction could be due to its higher final pH (6.3) compared to solid fraction and

grass silage, in which the final pHs were 4.7-5.0, thus too low for methanogens. Methane production has been observed from heat-treated inoculum (100 °C 1 h) with HSW as a substrate, methane production being inhibited only at pH of 5.5 or less (Liu et al. 2006).

One possible explanation for the rather low H_2 yield of grass silage (II, III) in the present study could be the composition of the substrate. During ensiling, water-soluble carbohydrates of the crop material are converted to fermentation products (e.g. lactic and acetic acid) and some H_2 can be lost during the ensiling process, losses being higher during suboptimal conditions (McDonald et al. 1991). These fermentation products (lactic and acetic acids) are stated to be hardly degraded into H_2 during acidogenesis, thus the ensiling has been suggested as a suboptimal storage method for crops intended for dark fermentative hydrogen production (Martínez-Pérez et al. 2007). However, the impact of ensiling on H_2 yield from energy crops is not clear, as actually, lactic acid has been shown to be degraded into H_2 in some studies (Matsumoto & Nishimura 2007, Baghchehsaraee et al. 2009).

5.3.7 Effects of methodology on hydrogen yield in batch assays

In batch-type assays the increasing partial pressure of H_2 is known to inhibite H_2 production (Hawkes et al. 2002). The headspace to liquid volume ratio in the present study has apparently been too low (58:60 (II), 350:650 (III), 40:78 (IV)) for efficient H_2 production, and inhibition due to increased partial pressure of H_2 probably occurred (II-IV). To be able to obtain more reliable results, larger headspace volume should be used (Oh et al. 2009) as the optimal headspace to liquid volume ratio for hydrogen production was found to be 80:40 (Nguyen et al. 2010). The inhibiting effect of pH_2 can be avoided by the constant removal of H_2 from the system. Logan et al. (2002) obtained a 43 % higher H_2 yield when using a respirometric (continuous gas release) method compared to the traditional Owen (an intermittent pressure release) method. In addition, H_2-consuming homoacetogenesis can be inhibited by CO_2 removal, and, higher H_2 yields were achieved when CO_2 was removed from the culture liquid by bubbling with argon gas (H_2 yield increased from 0.52 to 1.09 mol mol^{-1} glucose; Tanisho et al. 1998) or by chemical absorption into KOH (H_2 yield increased from 1.4 to 2.0 mol (mol glucose)$^{-1}$; Park et al. 2005). Thus, H_2 production from grass silage and maize in batch assays could be improved e.g. through gas sparging of a reactor content, which has previously shown to improve H_2 yield from grass silage by 75 % (from 13 to 23 ml gVS_{added}^{-1}; Tähti et al. 2008).

It seems that traditional batch assays may not be the best way to evaluate the H_2 production potential of different substrates as this method tends to underestimate the H_2 yield achievable in continuous reactors (Oh & Logan 2005). In the present study, some variation in H_2 yields between replicate bottles was observed; a phenomenon, which has also been reported by previous researchers (Kalogo & Bagley 2008, Liu et al. 2008b). The reason for variation in H_2 yields might be due the presence of relatively low number of H_2-producing

bacteria in the heat-treated inoculum (Baghchehsaraee et al. 2008), the heterogeneity of the substrate and/or inoculum used and the several pathways for H_2 production/consumption. Besides, the lack of continuous gas composition measurements may also have influenced the H_2 yields found here (II-IV). Thus, better methods would apparently improve the reliability of the results obtained from the type of batch assays generally used to study H_2 potential. Moreover, H_2 yield from grass silage and maize could probably be increased by use of well adapted inoculum, which has been accomplished in a previous study with HSW (Liu et al. 2008b).

5.4 Shifting methanogenic process to hydrogenic

According to present results (V), shifting methanogenic reactor to hydrogenic is possible by increasing the OLR and shortening the HRT. High OLR resulted in the build-up of VFAs and decrease in pH, which inhibited the methane production and hydrogen consumption by the hydrogenotrophic methanogens. The low pH (5.5-6.5) in the present study has apparently favoured acidogens instead of methanogens. The optimal pH for methanogens is in the quite narrow range close to 7, whereas the acidogenic H_2 producing bacteria can grow at lower pH of < 6 (Valdez-Vazquez & Poggi-Varaldo 2009). Thus, increase in OLR as an operational strategy was shown to be a proper method for inducing H_2 production from an already operating mesophilic methanogenic system. The fact that CH_4 production ceased and H_2 accumulated in the reactor indicates the shift in microbial community (Liu et al. 2002, Demirel & Scherer 2008) and the inhibition of methanogens throughout the experimental run. It has previously been reported that hydrogen production without treating the inoculum has been feasible e.g. from garbage waste (Ohnishi et al. 2010) and household solid waste (Liu et al. 2008a) and load-shock method was found as a simple method for enriching H_2 producers (O-Thong et al. 2009). Moreover, low pH (5.5) has shown to be an effective method for continuous H_2 production from household solid waste (Liu et al. 2008a) as methane production was noticed even with short HRT of 2-6 days at pH controlled to 7.

Short HRT of 0.5-12 h (i.e. high dilution rate) can be used to wash out methanogens in continuous processes with liquid substrates, e.g., with sucrose or glucose containing wastewaters (Davila-Vazquez et al. 2008, Valdez-Vazquez & Poggi-Varaldo 2009). However, with solid substrates, like grass silage, the hydrolysis is the typically rate-limiting (Vavilin et al. 1996) and longer HRTs are needed to allow hydrolysis. It is thus concluded that the high OLR was the main operational strategy that could affect the shift in the anaerobic digestion process from methane to hydrogen production rather than the short HRT of 6 days. One possible way to increase H_2 yield from grass silage could be through pre-treatment of the substrate and use of hydrolysate for H_2 production, as in that case very short HRT could be used.

The highest specific H_2 yield of 42 l $kgVS_{fed}^{-1}$ obtained in the present study is comparable to H_2 yield obtained in batch assays (at most 44 l $kgVS_{added}^{-1}$, data not shown). However, the low mean specific (9 l $kgVS_{fed}^{-1}$) and volumetric H_2 yields (0.06 m^3 $(m^3d)^{-1}$) obtained in the present study might be due to high concentrations of VFAs. Previous studies have shown that high VFA levels would inhibit hydrogen production (Wang et al. 2008, Chong et al. 2009, Valdez-Vazquez & Poggi-Varaldo 2009). For instance, H_2 yield in the present study decreased with a corresponding increase in the concentration of caproic acid. A similar observation in the increased caproic acid production in continuous reactor processes under mesophilic condition was reported (Jung et al. 2010). This is attributed to the fact that at pH 4-5, caproic acid production is thermodynamically favoured by the consumption of 1 mol of butyric and acetic acids along with 2 mol of H_2 (Jung et al. 2010). Based on the present results, it seems advantageous to adjust the pH close to 6 as higher H_2 yields were obtained in the period with constant buffer addition.

The high TVFAs (from acetic to caproic acids) conversion to SCOD, accounted for up to 80 % of the measured SCOD, indicates high acidification efficiency. The remaining degradation products have most probably been lactic acid and alcohols (not measured). Lactic acid was probably present in the substrate, as in a typical ensiling process, water soluble carbohydrates of the crop are mainly degraded to lactic acid (McDonald et al. 1991). Moreover, lactic acid can be produced during acidogenesis and it does not result in H_2 production (Nath & Das 2004).

Further research would be needed to find optimal conditions for both hydrogen and VFAs production, as high concentrations of VFAs can inhibit both hydrogen production (Wang et al. 2008, Chong et al. 2009) and hydrolysis (Vavilin et al. 2008). In addition, hydrogen production from sewage biosolids in continuous mode was shown to be improved by nitrogen sparging (Massanet-Nicolau et al. 2010), a method, that could be tested with crops as well. Moreover, research of prevailing microbial population could give valuable data of hydrogen production and consumption processes. This data could be combined to data of pH (Yasin et al. 2011) and metabolic products (Karadag & Puhakka 2010) to be able to control the process towards hydrogen production.

5.5 Methane production from grass silage and maize

5.5.1 Methane yield in batch assays, the effect of pre-treatments

According to present results methane yield from untreated grass silage (431 ml gVS_{added}^{-1}, III) and maize (321 ml gVS_{added}^{-1}, IV) in batch assays are in the same range as previously reported from similar crop materials as methane yield from different grasses and different maize varieties varied between 253-394 ml gVS^{-1} (Seppälä et al. 2009) and 268-365 ml gVS^{-1} (Amon et al. 2007), respectively. The pre-treatments applied in the present study (NaOH-treatment for grass silage

(III) and water-extraction and HCl-treatment for maize (IV)) did not show any positive effect on methane yield. Actually, the alkaline treatment decreased the combined CH_4 yield (334 ml $gVS_{original}^{-1}$) compared to untreated grass silage (431 ml gVS_{added}^{-1}) which was especially due to low CH_4 potential of the solid fraction under the current experimental conditions (III). Alkaline treatment is expected to break the lignocellulosic structure, swell the fibres and increase the pore size, thus improving hydrolysis (Pavlostathis & Gossett 1985, Gunaseelan 1995, Neves et al. 2006). Some studies have previously shown that alkalis such as NaOH can increase methane yield of e.g. wheat straw (Pavlostathis & Gossett 1985). Definitely the dose of alkali (or acid) and conditions of the treatment (e.g. temperature) play also a major role in the hydrolysis, and they should be optimised to obtain stimulating effects on CH_4 yield. In their study with wheat straw (Pavlostathis & Gossett 1985), the best methane yield (280 ml $gCOD_{added}^{-1}$ as compared to 120 ml $gCOD_{added}^{-1}$ from untreated) was obtained when alkali concentration of 50 g NaOH 100 gTS^{-1} was applied, which was more than 10 times higher than in the present study (4 g $100gTS^{-1}$). In the study of Neves et al. (2006) NaOH concentration of 30 g $100gTS^{-1}$ was used, which increased the methane yield from barley waste from 25 to 222 ml $gVS_{initial}^{-1}$, while methane yield from Parthenium increased from 152 to 203 and 236 ml gVS_{added}^{-1} after HCl (32 g $100gTS^{-1}$) and NaOH (12 g $100gTS^{-1}$) treatments at room temperature, respectively (Gunaseelan 1995).

5.5.2 Methane production after hydrogen stage

Acidogenic H_2 production can not be considered a complete treatment process due to high amount of undegraded by-products such as VFA (Ueno et al. 2004). VFAs can be degraded to methane in traditional AD thus improving the overall energy efficiency (Kovács et al. 2004, Kraemer & Bagley 2005). The present results (III, IV) show that application of two-stage anaerobic digestion with a thermophilic H_2 production as first stage and mesophilic CH_4 production as second stage can improve CH_4 yields compared with one-stage mesophilic CH_4 process. The increase in methane yields in two-stage process compared with one-stage process were 8, 64, 7, 9 and 27 % with grass silage, NaOH-treated solid fraction of grass silage (III), untreated maize, water-extracted maize and HCl-treated maize (IV), respectively. The higher methane yields in a two-stage compared with one-stage process was attributed to the fact the thermophilic H_2 production stage apparently enhanced hydrolysis of the solid substrates and resulted in increased solubilisation and VFA production. This was evident from increase in SCOD and VFA concentrations after the H_2 stage (III, IV).

In two-stage assays with maize (IV), methane yields after 14 d H_2-stage were similar (water-extracted) or slightly lower (untreated and HCl-treated maize) on comparison to 2 d H_2 stage. This was evident from the decrease in the SCOD levels with increase in duration of H_2 stage from 2 to 14 d, although H_2 yields and the amount of VFA typically increased with longer H_2-stage (14 d). A similar observation of higher methane yield after lower hydrogen and VFA yield was observed with pre-treated (NaOH+enzymatic hydrolysis) water

hyacinth as a substrate (Cheng et al. 2010). The authors concluded that the mixed methanogenic culture was able to hydrolyse the substrate and further utilize the hydrolysis by-products (Cheng et al. 2010). However, in the present study, the two-stage process with 14 d H_2 stage showed higher initial CH_4 production rates compared with the two-stage process with 2 d H_2 stage or one-stage CH_4 process (IV) due to the higher amount of VFA.

Overall, these results were in agreement with previous studies (Liu et al. 2006, Cooney et al. 2007, Ting & Lee 2007, Ueno et al. 2007a) which showed that hydrolysis and acidogenesis in the first stage can be enhanced by low pH and high temperature leading to an elevated digestion efficiency (Zhu et al. 2008) and CH_4 yields in the second stage (Liu et al. 2006, DiStefano & Palomar 2010). However, it seems essential to find the optimal conditions for the first stage, as in a recent study (Siddiqui et al. 2011) with food waste and sewage sludge the two-stage H_2 + CH_4 process actually led to decrease in energy production when compared to one-stage CH_4 process. This was mainly attributed to high VS degradation in the first stage (47 %; Siddiqui et al. 2011).

5.5.3 Methane production from grass silage in CSTRs

The results from the present study showed that the long-term monodigestion of grass silage at an OLR of 2 kgVS $(m^3d)^{-1}$ and HRT of 30 days is not feasible and would result in low methane yields due to accumulation of VFA. The mean methane yields of 200-220 l CH_4 $kgVS_{fed}^{-1}$ obtained (Table 21) were slightly lower than the methane yields of 260 l CH_4 $kgVS_{fed}^{-1}$ reported during the monodigestion of grass silage at an OLR of 3.5 $kgVS^{-1}$ and HRT of 50 days in a loop reactor (Koch et al. 2009). On the other hand, the volumetric CH_4 yield of 0.40-0.44 m^3 $(m^3d)^{-1}$ and VS reductions of 49-57 % obtained in the present study were in the same range (0.4 m^3 $(m^3d)^{-1}$ and 41-52 %, respectively) as those reported by Lehtomäki et al. (2007) during the co-digestion of grass silage with cow manure in CSTR.

TABLE 21 Methane yields from grass monodigestion in different kind of reactors.

Substrate	Reactor	OLR kgVS $(m^3d)^{-1}$	HRT days	CH_4 yield l $kgVS^{-1}$	Reference
Grass silage	CSTR	2	30	200-220	This study
Grass silage	Loop reactor	3.5	50	260	(Koch et al. 2009)
Grass silage	LBR + UASB	na	55	200	(Lehtomäki et al. 2008b)
Grass	CSTR	1.4	153[a]	300	(Mähnert et al. 2005)
Grass-clover silage	CSTR	up to 7	20	250-300	(Jarvis et al. 1997)

na = not available
[a]calculated from the data given
LBR = leach bed reactor

The reason for the low methane production efficiency in the present study was most probably due to the build-up of VFAs, which is attributed to the inhibition of acetate consumption by acetate utilizing methanogens and VFA degradation by acetogens. This was evident by the high concentrations of VFAs especially acetic acid (6.1 g l^{-1}) and propionic acid (2.4 g l^{-1}). This high concentration of propionic acid in the present study might have been a reason for the process failure. Previous studies have shown that propionic acid at a concentration of 0.9 g l^{-1} has resulted in decreased methanogenic growth rates and thus methane yields (Wang et al. 2009a). Propionate accumulation has been shown to inhibit propionate degradation while acetate accumulation can inhbite both acetate and propionate degradation (Kus & Wiesmann 1995). Besides direct VFA accumulation, other factors such as lack of trace nutrients or accumulation of inhibitory levels of Na^+ through $NaHCO_3$ additions may have also resulted in the process failure. For instance, the amount of nickel (Ni) supplied through nutrient solution addition in the present study (0.05 mg (kg feed)$^{-1}$) might have been too low. Previous study with maize model substrate (cellulose and starch) showed that a 12-fold higher addition of Ni (0.6 mg kg FM^{-1}) was required for stable biogas production (Pobeheim et al. 2011). Ni has been reported to be an essential trace nutrient to achieve high acetate to methane conversion rate (Kida et al. 2001). On the other hand, no selenium (Se) or tungsten (W) was added in the present study, both of which have previously been shown to be advantageous for biogas process (Lebuhn et al. 2008, Plugge et al. 2009). However, the impact of nutrient addition in the present study was not clear, as the recommended concentrations for trace elements show high variation (Demirel & Scherer 2011) and no control without nutrient addition was operated. Furthermore, addition of $NaHCO_3$ for buffering the process had apparently resulted in accumulation of Na^+ in the reactor. The calculated Na^+ concentration was 2.7 g (kg feed)$^{-1}$, which was apparently reached in the reactor around day 73 of the experiment and was further increased to 10.4 g kg^{-1} at day 106. The role of $NaHCO_3$ on decreasing process performance is however not clear as the concentration of sodium that causes 50 % reduction in cumulative methane yield has been reported to show wide range, from 5.6 to 53 g l^{-1} (Chen et al. 2008), depending on e.g. adaptation of the system.

According to present and previous studies (e.g Lebuhn et al. 2008; Table 21), step-wise increase in OLR or generally lower OLR and/or longer HRT might be feasible in energy crop monodigestion. Higher methane yields have been obtained typically when the reactors were operated either with lower OLR and/or longer HRTs, as applied in the present study. In addition to OLR and HRT, feeding regime and mixing can affect the process. In the present study the substrate was fed only once per day, which is the method typically applied in laboratory studies. However, it has been shown that feeding of the silage should be done several times (12-24) per day due to the high lactic acid concentration and the low substrate pH, which can affect the process stability and gas yield (Krieg 2005). In mono-digestion of grass, special attention has to be given to proper mixing (e.g. Koch et al. 2009) as grass tends to float more

easily when compared e.g. to maize (Thamsiriroj & Murphy 2010), a phenomenon, which was observed in the present study as well.

5.6 Energy aspect of hydrogen and methane from energy crops

According to present results H_2 production from energy crops, such as grass silage and maize is possible even without pre-treatment of the substrate. However, the potential for energy production per hectare in the form of H_2 from untreated grass silage and maize through dark fermentation under these circumstances remain low when compared to CH_4 production, which is in accordance with other studies (Zhu et al. 2008, Wang et al. 2009b, DiStefano & Palomar 2010). According to the present results from batch assays, annual energy production in the form of hydrogen from one hectare of grass and maize would only be around 74-163 m^3, thus corresponding to around 0.5 MWh ha^{-1} (Table 22). However, when calculated from the highest hydrogen yield in the CSTR (V), hydrogen and energy yields of 314-431 m^3 ha^{-1} and 0.9-1.3 MWh ha^{-1} could be obtained, which is in the same range, as estimated from ryegrass cultivated in UK (392-501 m^3H_2 ha^{-1}, 18-23 tTS ha^{-1} a^{-1}, Kyazze et al. 2007). Under optimal experimental conditions hydrogen yields from these energy crops could be even further improved. According to present study up to 41 and 51 MWh ha^{-1} of energy could be obtained if grass silage or maize were directly converted to CH_4 by a traditional anaerobic digestion process (Table 22). This yield is in the same range as previously calculated for grasses (12-54 MWh ha^{-1}) (Lehtomäki et al. 2008a, Seppälä et al. 2009), while the highest energy yield from maize has been calculated to be even 90 MWh $ha^{-1}a^{-1}$ (Seppälä et al. submitted).

TABLE 22 H_2, CH_4 and energy yields of grass silage and maize. Conversion factors of 3 and 10 kWh per 1 Nm^3 have been used for H_2 and CH_4, respectively.

Crop	Yield tTS ha^{-1}	H_2			CH_4		
		m^3 tTS^{-1}	m^3 ha^{-1}	MWh ha^{-1}	m^3 tTS^{-1}	m^3 ha^{-1}	MWh ha^{-1}
Grass silage	8-11 [a]	14.8[c]	119-163	0.36-0.49	371[d]	2966-4079	30-41
		39.2[f]	314-431	0.94-1.29			
Maize	8-17 [b]	9.3 [e]	74-158	0.22-0.47	301[e]	2406-5112	24-51

[a]Lehtomäki et al. (2008a) [b]Seppälä et al. submitted, [c]Paper II, [d]Paper III, [e]Paper IV, [f]Paper V

Theoretical hydrogen yield from crops can be calculated, if the chemical composition is known. Carbohydrate content of grass silage was 45 % of TS (Lehtomäki et al. 2007), while in another study the content of cellulose and hemicellulose accounted 56 % of TS (Jagadabhi et al. 2011). One ton TS would thus contribute to around 450-560 kg of carbohydrates. If all the carbohydrates are assumed to be glucose (M=180 g mol^{-1}) and theoretical conversion of 4

moles of H_2 per one mole of glucose could be achieved, this could give 224-279 m^3H_2 tTS^{-1}, thus corresponding to 672-836 kWh tTS^{-1}. When converted to methane in traditional AD (Table 2), this could give 168-209 m^3CH_4 tTS^{-1}, thus corresponding to 1680-2090 kWh tTS^{-1}.

Hydrogen production alone clearly is not beneficial due to lower energy yield as compared to traditional methane production. However, it was shown in this study (III, IV), that the acidogenic H_2 stage could be used as a pre-treatment method to enhance the methane yield in the second stage. Non-sterile fermentation was actually mentioned as one of low-cost pre-treatment of lignocellulose already in 1981 (Datta 1981). Besides improving methane yield, H_2 stage has been shown to enable higher OLR and shorter HRT in the subsequent methanogenic stage (Ueno et al. 2007a) and better effluent quality with less propionate (Wang et al. 2011b) when compared to one-stage system. The results from the present study (III) suggests that the highest (calculated) CH_4 yield from grass silage (495 ml $gVS_{original}^{-1}$) could be obtained, if grass silage is first pre-treated with NaOH and the solid fraction obtained after solids-liquid separation is incubated in two-stage process consisting of a thermophilic H_2 production as the first stage and mesophilic CH_4 production as the second stage. On the other hand, the liquid fraction could be used for one-stage CH_4 production directly. In the present study (IV), the highest CH_4 yield (397 ml gVS_{added}^{-1}) from maize was obtained when maize was first subjected to HCl-treatment and then digested in a two-stage process consisting (2 d H_2 stage). However, it should be noted that the results were obtained from batch experiments and cannot be directly extrapolated to large-scale continuous processes. In practice, the increases in CH_4 yields have to be balanced with the costs for the pre-treatment, additional equipment and higher investment and operational costs of two-stage processes. Nevertheless, this short first H_2 stage could probably be embedded in a current pre-treatment and/or the mixing tank in agricultural biogas reactors.

Thus, in the future it might be possible to produce both H_2 and CH_4 from energy crops (Fig. 11).

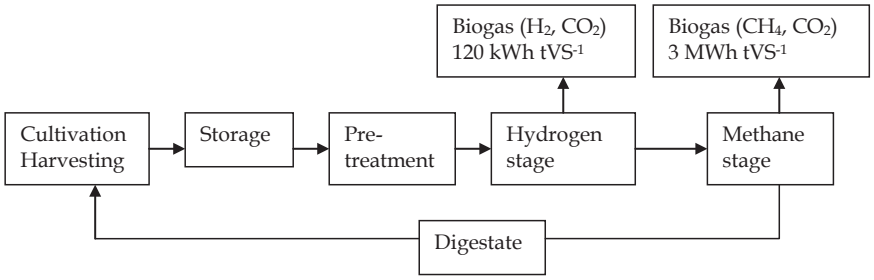

FIGURE 11 The possible biohydrogen and –methane from energy crops-chain. According to present study at least around 120 kWh tVS^{-1} could be produced in the form of H_2 without negative impact on following methanogenic step producing around 3 MWh tVS^{-1}.

The process could be preceded by one or several pre-treatments (e.g. acid and/or enzymatic hydrolysis) followed by H_2 producing acidogenic reactor (short HRT, high OLR, pH around 5-6) and subsequently the traditional methanogenic stage (longer HRT, lower OLR, neutral pH). In this system both H_2 and CH_4 could be produced, and, moreover, the actual energy yield could be improved when compared to traditional one-stage CH_4 system. In this kind of two-stage system at least around 40 m^3H_2 tVS^{-1} (=120 kWh, highest H_2 yield in CSTR obtained in this study) and around 300 m^3CH_4 tVS^{-1} (=3000 kWh) could be produced. The total energy production would thus be 3120 kWh tVS^{-1}, and the share of H_2 would be around 4 % of the energy and about 12 % of the volume of H_2 and CH_4 mixture. However, more research would be needed especially for optimizing the hydrogenic first stage to improve and stabilize both H_2 and VFA yields. Produced hydrogen and methane could be upgraded either as separate or mixed gas streams for further use. Hydrogen, up to at least 17 % of the volume, could be injected into natural gas grid to improve the properties of methane (Haeseldonckxa & D'haeseleer 2007) and hydrogen and methane mixtures have shown good combustion characteristics even with low H_2 percentages (Karim et al. 1996).

6 CONCLUSIONS

This thesis shows that anaerobic digestion process can be used to convert energy crops for both hydrogen and/or methane. The two-stage H_2 + CH_4 process showed potential in improving methane yield from both grass silage and maize. With the aim of hydrogen production the ongoing methanogenic process could be shifted on hydrogen production by controlling the operational parameters (OLR and HRT).

H_2 production from grass silage and maize through dark fermentation was shown to be possible. The preferred inoculum was obtained from a farm scale digester, while digested sewage sludge did not produce H_2 from grass silage. The highest H_2 yield from grass silage was achieved at a temperature of 70 °C. Highest hydrogen yields from batch assays were 16 and 9.9 ml gVS_{added}^{-1} for untreated grass silage and maize, respectively, under the studied experimental conditions. The optimal initial pH for H_2 production from grass silage according to this study was between 5 and 6, while at pH 4 no H_2 was produced. A VS ratio of 2 was shown to increase H_2 production compared to lower VS ratios under the experimental conditions. Pre-treatments (alkaline, acid and water-extraction) showed some potential for elevating H_2 yields. Hydrogen producing CSTR can be obtained by increasing the OLR and shortening the HRT of methane producing CSTR. This leads to increase in TVFAs and decrease in pH, which inhibits hydrogen consuming methanogens. At most 42 l H_2 $kgVS^{-1}$ was obtained from grass silage with OLR of 10 kgVS $(m^3d)^{-1}$ and HRT of 6 days.

In a two-stage H_2 and CH_4 process, CH_4 production from grass silage, NaOH treated solid fraction of grass silage, untreated, water-extracted and HCl-treated maize was found to increase by 8, 64, 7, 9 and 27 %. In addition, the initial CH_4 production was faster when compared to CH_4 production in one-stage CH_4 assays. Pre-treatments applied in the present study did not improve CH_4 yield from grass silage and maize in one-stage batch assays. According to the present study at most about 218 l CH_4 $kgVS^{-1}$ can be obtained from grass silage monodigestion in CSTR with OLR of 2 kgVS $(m^3d)^{-1}$ and HRT of 30 days. However, initial OLR of 2 kgVS $(m^3d)^{-1}$ was shown to be too high and HRT of

30 days too short for stable methane production from grass silage monodigestion in CSTR, thus stepwise increase in OLR and/or longer HRT could be suggested.

Ensiling of energy crops used for CH_4 production was shown to be a feasible method for preserving the CH_4 yield even 11 months in boreal field conditions. Under appropriate storage conditions 87 and 91 % in laboratory conditions and 96 and 68 % in field conditions, of CH_4 yield of grass and ryegrass, respectively, were recovered. According to this study VS loss during storage seems to be a major factor in determining the preservation of CH_4 yield.

H_2 yield from grass silage and maize were moderate and the energy value is not comparable to CH_4 energy yield. However, H_2 production stage could act as a pre-treatment step, thus improving hydrolysis and acidogenesis for the subsequent methanogenic step. In the future, it could be possible to produce both H_2 and CH_4 from energy crops in a two-stage concept.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Metaanin ja vedyn tuottaminen energiakasveista anaerobiprosessissa

Siirtymällä uusiutuvaan energiaan voidaan vähentää fossiilisten energialähteiden käytöstä aiheutuvia ympäristövaikutuksia. Metaania ja vetyä voidaan käyttää lämmön ja sähkön tuotannossa sekä liikennepolttoaineena. Molempia voidaan tuottaa anaerobiprosessissa esimerkiksi kasvibiomassasta.

Tässä väitöstyössä tutkittiin metaanin ja vedyn tuottamista kasveista anaerobiprosessissa. Työssä tutkittiin varastointitavan ja -keston vaikutusta kasvien orgaanisen aineen ja metaanintuottopotentiaalin säilymiseen. Lisäksi tutkittiin pimeäfermentatiivista vedyn tuotantoa säilönurmesta ja maissista, sekä eri olosuhdetekijöiden vaikutusta vetysaantoon. Myös kaksivaiheisen vety + metaani -prosessin toimintaa arvioitiin panoskokeissa. Lisäksi selvitettiin pelkän kasvimateriaalin (säilönurmi) soveltuvuutta jatkuvatoimiseen metaanintuottoon sekä metanogeenisen prosessin muuntamista vetyä tuottavaksi.

Kasvien varastointitutkimuksessa merkittävimmäksi tekijäksi metaanisaannon säilymisessä osoittautui orgaanisen aineen häviöt varastoinnin aikana. Monissa tapauksissa varastointi jopa paransi kasvien metaanipotentiaalia (m^3CH_4 $tVS_{lisätty}^{-1}$) (lisättyä orgaanista ainetta kohti). Tämä johtui luultavasti siitä, että varastoinnin aikana kasvimassa hajoaa mm. orgaanisiksi hapoiksi, jotka ovat nopeasti hyödynnettävissä biokaasuprosessissa. Enimmillään 96 ja 68 % heinäseoksen ja raiheinän metaanisaannosta (varastointihäviöt huomioiden) säilyi kenttäolosuhteissa 11 kuukauden varastoinnin aikana.

Tässä tutkimuksessa havaittiin, että säilönurmesta ja maissista voidaan tuottaa vetyä anaerobisesti. Parhaiten vedyntuotantoon soveltui maatilakohtaisesta biokaasureaktorista peräisin oleva ymppi (ts. mikrobisiirros), joka lämpökäsiteltiin keittämällä vetyä kuluttavien metanogeenien inhiboimiseksi. Olosuhteiden, kuten lämpötilan, pH:n ja substraatti-ymppi -suhteen vaikutusta vedyntuotantoprosessiin tutkittiin. Esikäsittelyjen avulla voitiin hieman kasvattaa vetysaantoa, ja suurimmat vetysaannot säilönurmesta ja maissista panosprosesseissa olivat 16 ja 22 m^3H_2 $tVS_{lisätty}^{-1}$.

Tutkitut esikäsittelyt eivät vaikuttaneet säilönurmen ja maissin metaanisaantoihin panosprosesseissa. Sen sijaan vetyvaihe lisäsi metaanisaantoa kaksivaiheisessa panosprosessissa verrattuna yksivaiheiseen metaaniprosessiin. Vedyntuotantovaihe toimi esikäsittelynä edistäen kasvimassan hydrolyysiä ja happokäymistä, joiden seurauksena metaanintuotanto tehostui. Jatkuvatoimisessa reaktorikokeessa säilönurmen hajotus metaaniksi ei toiminut valituilla prosessiparametreilla (kuormitus 2 kgVS $(m^3d)^{-1}$ ja viipymä 30 d), sillä orgaanisten happojen kertymisen vuoksi metaanintuotanto inhiboitui. Jos kasvimassaa käsitellään ilman lantaa tai muuta substraattia tämänkaltaisessa jatkuvatoimisessa reaktorissa, voisi operoiminen pidemmällä viipymällä, alhaisemmalla kuormituksella ja/tai kuormituksen asteittainen nostaminen edesauttaa prosessin toimivuutta.

Kun säilönurmesta metaania tuottavan reaktorin kuormitusta nostettiin (2 → 10 kgVS $(m^3d)^{-1}$) ja viipymää lyhennettiin (30 → 6 d), metaanintuotto inhiboitui ja reaktori alkoi tuottaa vetyä. Korkein vetysaanto tässä reaktorissa oli 42 m^3H_2 $tVS_{lisätty}^{-1}$. Vedyntuotanto ei kuitenkaan ollut stabiilia ja lisätutkimuksia tarvitaan erityisesti optimaalisten prosessiolosuhteiden löytämiseksi vedyntuoton maksimoimiseksi.

Hehtaarikohtainen energiasaanto kasveista vetynä oli pieni verrattuna energiasaantoon metaanina, ollen enimmillään 1.3 MWh ha^{-1}, kun taas metaanienergiasaanto oli enimmillään 51 MWh ha^{-1}. Silti vedyntuotanto kasveista voisi olla kannattavaa, sillä kaksivaiheinen vety + metaani -prosessi voi lisätä metaanisaantoa verrattuna yksivaiheiseen metaanin tuotantoon. Tulevaisuudessa voisi olla mahdollista tuottaa kasvimassasta sekä vetyä että metaania kaksivaiheisella anaerobiprosessilla.

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ORIGINAL PAPERS

I

STORING ENERGY CROPS FOR METHANE PRODUCTION: EFFECTS OF SOLIDS CONTENT AND BIOLOGICAL ADDITIVE

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Storing energy crops for methane production: Effects of solids content and biological additive

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Abstract

The effect of storage on chemical characteristics and CH_4 yield (taking into account loss of VS during storage) of a mixture of grasses and ryegrass, ensiled as such (low solids content) and after drying (medium and high solids) with and without biological additive, were studied in field and laboratory trials. Up to 87% and 98% of CH_4 yield was preserved with low solids grass (initial TS 15.6%) and high solids ryegrass (initial TS 30.4%), respectively, after storage for 6 months, while under suboptimal conditions at most 37% and 52% of CH_4 yield were lost. Loss in CH_4 yield was mainly due to VS loss, presumably caused by secondary fermentation as also suggested by increasing pH during storage. Biological additive did not assist in preserving the CH_4 yield.

Keywords: Anaerobic digestion; Biogas; Energy crop; Grass; Storage

1. Introduction

Renewable energy can be produced from crops through different conversion processes. CH_4 production through anaerobic digestion appears to be a competitive concept in both energy efficiency and environmental impact comparison studies (e.g. Fredriksson et al., 2006). Anaerobic digestion appears as a widely applicable technology, as it can use various crops and wastes as substrates and nutrients can be recirculated. The valuable gaseous end-product, CH_4, is a flexible energy carrier which can be used for heat, power and traffic biofuel production (e.g. Plöchl and Heiermann, 2006).

It has been proposed that 1545 million tons of agricultural biomass, half in the form of energy crops, could be used for CH_4 production each year in the European Union (Amon et al., 2001). In Europe, especially in Austria and Germany, the biogas production is tightly linked to agricultural sector (Plöchl and Heiermann, 2006). For example, up to 4000 m^3 of CH_4 could be obtained from 1 ha of grass cultivated in Finland (Lehtomäki et al., in press) and up to 9000 N m^3 from maize cultivated in Austria (Amon et al., 2007). Grasses are classified among potential crops for biogas production in northern conditions due to their potential high CH_4 yield per hectare and suitability in current agricultural cultivation, harvest and storage practices (e.g. Lehtomäki et al., in press).

Energy crops can rather easily be stored so that energy can be produced throughout the year and/or when the demand and/or price for energy are highest. Crops contain high amounts of non-structural carbohydrates which are easily degradable and thus can be lost during processing and suboptimal storage conditions. Ensiling is a traditional way of storing fodder crops and may also suit energy crops used for CH_4 production (Egg et al., 1993). Ensiling is a biological process during which LAB break down the sugars in the crop (lactic acid fermentation) and lower the pH to a level inhibitory to other bacteria (McDonald et al.,

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Nomenclature

CFU	colony-forming unit	oww	original wet weight
FID	flame ionization detector	SCOD	soluble chemical oxygen demand
GC	gas chromatograph	TCOD	total chemical oxygen demand
h	hour	TS	total solids
LAB	lactic acid bacteria	VFA	volatile fatty acid
NH_4-N	ammonium nitrogen	VS	volatile solids
N_{tot}	total nitrogen	WSC	water soluble carbohydrates

1991). During storage, it is important to minimize energy losses, and ensiling has been shown to conserve over 90% of the energy content of crops (Egg et al., 1993). Both fresh and ensiled grass species have been found suitable for biogas production in previous studies (Mähnert et al., 2002, 2005).

Ensiling is affected by several factors such as the solids content (i.e. moisture content) and chemical characteristics of the crop in question. Previous studies, performed mostly with fodder crops, have shown that the solids content of silage can be controlled by the stage of maturity of the crop, by pre-wilting (Egg et al., 1993) and by using an absorbent during ensiling (Singh et al., 1996). The solids content of the crop to be ensiled affects the total bacterial count and the rate of fermentation, which is usually more restricted the higher the solids content, as reflected in higher pH, higher soluble carbohydrate values, lower levels of lactic, acetic and butyric acids, and inhibition of the deamination of amino acids (McDonald et al., 1991). With low solids content pH critical for well preserved silage is lower compared to that in high solids contents. For grasses with dry matter content of 20% the critical pH has been found to be 4.0. Unless the soluble carbohydrate levels are very high, the ensiling of crops with a low solids content will encourage a clostridial fermentation, resulting in energy losses and a silage of low nutritional value (Egg et al., 1993; McDonald et al., 1991). During the storage of low solids crops baling might be impossible due to leachate formation. It has been assumed that leachate would not be formed, if crops are dried to a TS content of 29% or above and that overall losses of solids would be minimized around a TS content of 25–30% (McDonald et al., 1991). In contrast, if the pre-wilting period is too long, respiration will cause energy losses and the sugar content of the crop may fall. Moreover, high solids crops are also susceptible to mould (Buxton and O'Kiely, 2003). When the crops are used for energy production, ensiling conditions do not necessarily have to be as strictly controlled as with fodder crops. Field drying can lower transportation costs since much less water would be transported with the biomass; however, the savings in transportation must be balanced with the dry matter losses that occur during field drying (Egg et al., 1993). To our knowledge the effect of initial solids (TS) content on ensiling and CH_4 production has not been reported earlier.

Different kind of additives can be used to promote the ensiling process. Addition of acid lowers the pH; however, acids may cause corrosion of equipment and health problems. Enzymes enhance the hydrolysis of crop material and subsequently increase the content of sugars convertible by LAB. Bacterial inoculants can be used to increase the amount of LAB, and in combination with the addition of enzymes and LAB, enzymes degrade the plant cell wall and release carbohydrates for lactic acid fermentation (McDonald et al., 1991). Some authors suggest the use of these inoculants in the storage of grasses (e.g. Lehtomäki et al., submitted for publication).

The objective of this study was to evaluate the effect of storage (2–11 months) with and without biological additive containing both enzymes and LAB in boreal field conditions and in the laboratory on the CH_4 yield and chemical characteristics of a mixture of grasses (timothy, red clover and meadow fescue) and ryegrass, considered suitable for CH_4 production. Also the effect of drying, i.e., initial solids content was studied in the laboratory.

2. Methods

2.1. Substrates

The substrates used were (1) a mixture of timothy (*Phleum pratense*, 63% of seed mixture), red clover (*Trifolium pratense*, 17%) and meadow fescue (*Festuca arundinacea*, 20%), henceforth grass, and (2) ryegrass (*Lolium multiflorum*, 17% of seed mixture, 83% oat, *Avena sativa*, harvest was mainly composed of ryegrass as oat was only used as a companion crop and was harvested at the first harvest in June) harvested (Laukaa, Finland) in June (grass) and in August 2005 (ryegrass) for field and in September for the laboratory trials.

2.2. Laboratory trials

Crop material was first chopped with a garden chopper to ca. 5 cm particle size. Part of the chopped material was spread on top of a plastic net and dried in a thin layer for 24 and 48 h at 20 °C, while part of the material was used fresh.

Biological ensiling additive (Josilac, manufacturer Josera Erbacher GmbH & Co) containing both LAB (*Lactoba-*

cillus plantarum and *Pediococcus acidlactiti*, total amount $1.5 * 10^{11}$ CFU/g Josilac) and enzymes (cellulase, pectinase and xylanase) was added (6.8 g/t_{ww}) to part of the fresh and dried crop materials while part of the materials did not receive additive. The crop materials (range 154–500 g (ww)) were packed in polyethylene bags and placed in a 5 l plastic silo equipped with water locks to enable the release of gas from the silos. Silos were flushed for about 3 min with N_2 to remove O_2 and placed in 20 °C. After storage the silos were weighed and samples taken for analysis. Experiments were performed in duplicate.

2.3. Field trials

Crops were baled in plastic-covered round bales immediately (only grass) or after 24 h pre-wilting in the field. Additive (same as in laboratory trials) was added evenly to part of the pre-wilted crops from a container (connected to tractor and round baler) by continuously pumping through small nozzles (19 g/t_{ww} for grass and 24 g/t_{ww} for ryegrass). Bales were weighed with pallet truck scales (Tamtron, Finland) at the beginning of the storage trials and after 11 months of storage. Bales were stored outside in ambient conditions, the temperature ranging during the year from −30 °C to 30 °C. After each studied storage time, one sample (ca. 10 l) was taken manually using an auger, and after sampling the plastic cover was repaired with tape.

2.4. CH_4 assays

For the CH_4 assays inoculum (average values from different assays; pH 7.9, TS 5.6%, VS 4.3%, TCOD 43.5 g/l, SCOD 10.6 g/l, N_{tot} 2.5 g/l, NH_4-N 1.3 g/l) was obtained from farm digester treating cow manure and industrial confectionary by-products (Laukaa, Finland). Assays were performed in triplicate 1 l glass bottles. Two hundred and fifty millilitre of inoculum was added in each bottle and requisite amount of crop to give substrate to inoculum VS-ratio of 1:1 (except for stored crops in laboratory conditions when ratio was 1:2). Bottles were filled to a liquid volume of 750 ml with distilled water and 3 g/l $NaHCO_3$ was added as buffer. Assays with inoculum only were incubated as controls. Finally, bottles were flushed with N_2 to remove O_2 from the headspace and closed with silicon rubber caps. The produced gas was collected in aluminium gas bags. CH_4 potentials were calculated as m^3CH_4/kgVS added with CH_4 of inoculum subtracted. CH_4 yields were calculated as m^3CH_4/t_{ww} and m^3CH_4/t_{oww}, in which VS and mass losses during storage were taken into account.

2.5. Analysis

TS and VS were analysed according to Standard Methods (APHA, 1998) and pH was measured with a Metrohm 774 pH-meter. COD was analysed according to SFS 5504 (Finnish Standard Association, 1988) and SCOD from the fresh and stored crops was analysed according to the modified SFS-EN 12457-4 (Finnish Standard Association, 2002). VFA and CH_4 content were measured with GCs equipped with a FID (VFA: Perkin–Elmer Autosystem XL GC, PE FFAP column 30 m * 0.32 mm * 25 μm, carrier gas helium, oven 100–160 °C (20 °C/min), detector and injector 225 °C; CH_4: Perkin–Elmer Arnel Clarus 500 GC, Perkin–Elmer Alumina column 30 m * 0.53 mm, carrier gas argon, oven 100 °C, detector 225 °C and injector

Table 1
Effect of drying and storage on chemical characteristics and CH_4 yield of grass in laboratory trials

Pre-wilting time (h)		Storage time (months)	pH	SCOD (mg/gTS)	TS (%)	VS (%)	VS/TS (%)	VS loss (%)	CH_4 (m^3/kgVS)	CH_4 (m^3/t_{ww})	CH_4 (m^3/t_{oww})
0	Without	0	6.14	158	15.6	13.9	89.1	0	0.36	49.8	49.8
	additive	2	4.99	309	13.0	11.3	86.9	20.0	0.42 ± 0.01	47.8	47.0
		6	5.98	350	12.1	10.3	85.1	28.1	0.43 ± 0.05	44.0	42.7
	With	0	6.14	158	15.6	13.9	89.1	0	0.36[a]	49.8	49.8
	additive	2	4.95	274	13.3	11.7	88.0	17.8	0.42 ± 0.02	49.7	48.6
		6	5.49	328	12.1	10.4	86.0	28.3	0.43 ± 0.03	45.0	43.1
24	Without	0	6.2	131	19.8	17.6	89.0	0	0.36[a]	63.3	49.8
	additive	2	5.35	320	16.4	14.3	87.2	21.1	0.51 ± 0.01	72.9	55.9
		6	8.20	283	14.6	12.2	83.4	34.1	0.39 ± 0.01	47.6	35.7
	With	0	6.2	131	19.8	17.6	89.0	0	0.36[a]	63.3	49.8
	additive	2	5.62	261	16.3	14.1	86.5	21.8	0.48 ± 0.06	68.2	52.5
		6	7.71	356	13.9	11.9	85.6	35.3	0.39 ± 0.05	46.8	35.4
48	Without	0	6.47	131	26.7	23.6	88.4	0	0.36[a]	84.8	49.8
	additive	2	6.5	169	22.8	19.8	86.8	19.1	0.42 ± 0.01	83.8	47.6
		6	8.76	194	21.1	17.7	83.9	29.2	0.32 ± 0.04	56.6	31.5
	With	0	6.47	131	26.7	23.6	88.4	0	0.36[a]	84.8	49.8
	additive	2	5.47	171	22.5	19.6	87.1	19.8	0.41 ± 0.01	80.7	45.9
		6	8.58	344	19.9	16.9	84.9	31.5	0.41 ± 0.00	69.6	39.1

[a] Initial CH_4 production potential was tested only with the low solids sample, without additive.

250 °C). The amount of biogas was measured using the water displacement method.

3. Results

The effects of initial drying on the chemical characteristics of grass and ryegrass were studied in laboratory conditions (Tables 1 and 2). Drying increased the initial TS of the two crops from 13.3–15.6 (nondried, defined as low solids) to 18.8–19.8 (24 h dried, defined as medium solids) and to 26.7–30.4% (48 h, defined as high solids). Drying increased the pH from 6.14 up to 6.47 (48 h drying) and from 6.36 to 6.5 for grass and ryegrass, respectively.

The effects of storage for 2 and 6 months on the chemical characteristics and CH_4 yield of grass and ryegrass stored at different solids contents and with and without biological additive were studied in laboratory conditions (Tables 1–3). After 2 months pH had fallen below 5.6 in all the experimental conditions except with high solids grass, which had higher initial pH (6.47), only additive addition enabling a lower pH (5.47). Further storage to 6 months increased pH by over two units at most and a pH below 6.2 was maintained only with low solids grass and high solids ryegrass. Additive enabled lower pH at all solid contents compared to crops without additive, more clearly with ryegrass. Storage decreased the VS/TS ratio at all solids contents with

Table 2
Effect of drying and storage on chemical characteristics and CH_4 yield of ryegrass in laboratory trials

Pre-wilting time (h)		Storage time (months)	pH	SCOD (mg/gTS)	TS (%)	VS (%)	VS/TS (%)	VS loss (%)	CH_4 (m^3/kgVS)	CH_4 (m^3/t_{ww})	CH_4 (m^3/t_{oww})
0	Without additive	0	6.36	217	13.3	11.7	88.0	0	0.41 ± 0.02	47.6	47.6
		2	4.82	374	9.9	8.4	84.8	29.6	0.47 ± 0.04	39.9	39.1
		6	6.73	347	7.6	5.9	77.6	51.8	0.45 ± 0.04	26.7	25.6
	With additive	0	6.36	217	13.3	11.7	88.0	0	0.41[a]	47.6	47.6
		2	4.3	354	10.1	8.7	86.1	26.9	0.44 ± 0.01	38.5	37.9
		6	7.04	338	7.6	6.0	78.9	50.8	0.48 ± 0.01	28.8	27.6
24	Without additive	0	6.28	196	18.8	16.6	88.3	0	0.41[a]	67.5	47.6
		2	4.54	185	16.1	13.9	86.3	18.0	0.41 ± 0.02	56.3	38.9
		6	7.46	334	10.8	8.5	78.7	51.3	0.40 ± 0.01	33.8	22.7
	With additive	0	6.28	196	18.8	16.6	88.3	0	0.41[a]	67.5	47.6
		2	4.65	237	14.9	12.7	85.2	25.1	0.49 ± 0.01	62.8	43.4
		6	5.92	316	12.4	10.2	82.2	51.3	0.43 ± 0.07	43.7	29.7
48	Without additive	0	6.5	234	30.4	26.5	87.2	0	0.41[a]	107.9	47.6
		2	4.39	172	27.2	23.4	86.0	14.2	0.46 ± 0.01	108.3	46.5
		6	6.16	324	24.4	20.4	83.6	27.0	0.51 ± 0.00	103.9	43.5
	With additive	0	6.5	234	30.4	26.5	87.2	0	0.41[a]	107.9	47.6
		2	4.19	158	28.3	24.4	86.2	10.4	0.39 ± 0.01	94.7	40.7
		6	4.90	300	26.1	22.2	85.1	20.0	0.43 ± 0.02	94.5	39.8

[a] Initial CH_4 production potential was tested only with the low solids sample, without additive.

Table 3
VFAs (mgCOD/gTS) of grass and ryegrass stored for 6 months

Crop	Drying time (h) (+A)	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Caproic acid	Total VFA	VFA/SCOD (%)
Grass	Fresh	0.2	nd	nd	nd	nd	nd	nd	0.2	0.1
	0	71.1	21.7	16.4	50.3	16.1	7.6	9.7	192.9	55.1
	24	43.6	14.7	9.9	16.3	10.3	2.8	2.4	100.0	35.3
	48	18.0	8.8	3.6	3.4	5.1	0.7	0.0	39.7	20.5
	0 + A	71.8	23.8	8.8	30.0	13.0	2.9	1.2	151.5	46.2
	24 + A	–	–	–	–	–	–	–	–	–
	48 + A	5.1	1.5	0.5	1.1	0.6	0.0	0.0	8.9	2.6
Ryegrass	Fresh	0.1	nd	nd	nd	nd	nd	nd	0.1	0.05
	0	42.9	8.0	7.5	17.0	8.4	5.7	13.9	103.4	29.8
	24	27.9	8.1	3.7	4.2	5.7	1.6	6.6	57.8	17.3
	48	25.1	6.2	0.7	2.2	1.3	0.0	0.0	35.5	11.0
	0 + A	73.6	26.0	8.7	30.0	12.9	2.8	0.0	154.0	45.6
	24 + A	31.6	12.9	7.8	19.0	11.4	5.9	21.2	109.7	34.7
	48 + A	10.2	3.0	1.1	1.2	0.9	0.0	0.0	16.4	5.5

A = Additive.
– = No sample.
nd = Not detected.

both crops (from 89.1% to 83.4% with grass and from 88.0% to 77.6% with ryegrass), more clearly with crops stored for 6 months. Storage of grass at all solids contents resulted in a loss of VS of about 20% at 2 months and about 28–35% at 6 months, while with ryegrass loss of VS at 6 months was lower at high solids (VS loss 20–27%) compared to low solid contents (VS loss 52%; Tables 1 and 2). Storage increased SCOD values and increasing solids content resulted in a lower VFA concentration and a lower proportion of VFA from SCOD. Acetic acid was the main VFA with grass and ryegrass stored for 6 months, along with smaller amounts of propionic, isobutyric, butyric, isovaleric, valeric and caproic acids (Table 3). Storage increased CH_4 potential (m^3/kgVS) by at most 42% and 25% with grass and ryegrass, respectively, although, with no clear trends in relation to solids content or storage time. In some cases CH_4 potential decreased during storage. Storage mainly decreased CH_4 yield (m^3/t_{oww}) which was best preserved with high solids ryegrass where the percentages of original CH_4 yield were 98 and 91 after 2 and 6 months, respectively (Tables 1 and 2).

The effect of storage on chemical characteristics and CH_4 yield of grass and ryegrass stored with and without additive was studied in field conditions (Tables 4 and 5). Grass was stored immediately after harvesting and after 24 h pre-wilting, which increased the TS from 14.6% to 18.2%. After 3 months storage, pH was 5.0–5.2 and 4.5–4.9 with grass and ryegrass, respectively, and remaining around 5 even after 11 months of storage, except in the bale with pre-wilted grass stored for 6 months, in which pH had increased to 8.8. The measured SCOD values, TS and VS concentrations varied during the follow-up period without showing clear trends or permanent changes in their ranges, which is probably an effect of the ambient conditions but is also due to variation between individual bales. This was observed especially with ryegass with lower TS concentration after 1 month of storage compared to fresh crop and crop stored for longer periods. Storage with nondried and pre-wilted grass slightly increased the CH_4 potential, and storage with ryegrass decreased it. Loss of mass (ww) in grass bales during 11 months of storage was between 18% and 29% (data not shown), but with ryegrass no mass

Table 4
Effects of pre-wilting, storage time and biological additive on chemical characteristics and CH_4 potential of grass under field conditions

Treatment	Storage time (months)[a]	pH	SCOD (mg/gTS)	TS (%)	VS (%)	VS/TS (%)	CH_4 (m^3/kgVS)	CH_4 (m^3/t_{ww})	CH_4 (m^3/t_{oww})
Nondried	0 (June)	6.08	71	14.6	13.4	91.8	0.47 ± 0.02	62.6	62.6
	1 (July)	4.72	213	17.1	15.7	91.8	0.14 ± 0.03	21.5	nd
	3 (September)	5.02	265	17.1	15.7	91.8	0.49 ± 0.05	76.3	nd
	6 (December)	4.79	165	17.3	16.0	92.5	0.47 ± 0.01	74.8	nd
	11 (May)	5.38	162	16.4	15.0	91.5	0.49 ± 0.02	73.4	60.0
Pre-wilted	0 (June)	6.02	100	18.2	16.8	92.3	0.41 ± 0.00	68.4	54.5
	1 (July)	5.05	247	20.0	18.3	91.5	0.48 ± 0.01	87.2	nd
	3 (September)	5.22	280	17.4	15.7	90.2	0.42 ± 0.02	66.1	nd
	6 (December)	8.79	72	17.9	15.9	88.8	0.26 ± 0.01	40.8	nd
	11 (May)	5.27	307	17.7	16.2	91.5	0.48 ± 0.02	78.2	46.0
Pre-wilted + additive	0 (June)	6.22	177	17.0	15.7	92.4	0.50 ± 0.04	78.8	67.3
	1 (July)	5.44	316	17.0	15.2	89.4	0.38 ± 0.01	58.0	nd
	3 (September)	5.12	176	20.3	18.5	91.1	0.37 ± 0.04	57.9	nd
	6 (December)	5.44	148	17.9	16.5	92.2	0.37 ± 0.00	61.1	nd
	11 (May)	5.03	171	21.5	20.0	93.0	0.46 ± 0.01	92.0	55.7

nd = Not determined.
[a] Sampling month in parenthesis.

Table 5
Effects of storage time and biological additive on chemical characteristics and CH_4 potential of ryegrass under field conditions

Treatment	Storage time (months)[a]	pH	SCOD (mg/gTS)	TS (%)	VS (%)	VS/TS (%)	CH_4 (m^3/kgVS)	CH_4 (m^3/t_{ww})	CH_4 (m^3/t_{oww})
Pre-wilted	0 (August)	6.2	242	44.4	39.6	89.2	0.48 ± 0.09	188.7	188.7
	1 (September)	7.47	143	30.3	26.0	85.8	0.33 ± 0.04	85.1	nd
	3 (November)	4.88	200	44.4	39.9	89.9	0.44 ± 0.01	177.7	nd
	11 (July)	4.51	262	37.4	32.9	88.0	0.39 ± 0.02	127.8	127.8
Pre-wilted + additive	0 (August)	5.81	281	42.2	37.8	89.6	0.48 ± 0.09	180.0	188.7
	1 (September)	7.09	176	27.6	24.4	88.4	0.32 ± 0.08	79.2	nd
	3 (November)	4.49	142	26.6	22.6	85.0	0.39 ± 0.02	88.4	nd
	11 (July)	4.32	351	33.3	29.0	87.1	0.37 ± 0.01	106.2	111.8

nd = Not determined.
[a] Sampling month in parenthesis.

loss occurred. After 11 months of storage the best preserved CH_4 yield was found with nondried grass, 96% of the original yield (Tables 4 and 5).

4. Discussion

The present results show that the CH_4 yield of energy crops can be maintained by appropriate ensiling conditions for even after 11 months in ambient conditions, while, in contrast, in suboptimal storage conditions over 50% of the CH_4 yield can be lost. Several factors, such as crop species, pre-wilting, harvest time, additives and storage time can affect the ensiling process, and thus the final effect on CH_4 yield can be complex. Ensiling has been found as an appropriate method for storing grasses for biogas production earlier as well (Mähnert et al., 2002, 2005).

According to our study VS loss during storage seems to be a major factor in determining the preservation of CH_4 yield: the smaller the VS loss the better was the CH_4 yield preserved. With ryegrass the smallest VS loss was obtained with high solids ryegrass (48 h dried, TS 30.4%) while with grass the effect of initial TS on VS loss was less evident VS losses being even slightly higher for high solids than for low solids grass. One of the main reasons for pre-wilting silage is to increase the content of dry matter and thereby prevent the growth of clostridia, which is usually restricted at TS above 25–30%, which range is also thought to minimize dry matter losses (McDonald et al., 1991). High VS losses were characterised by a higher/increased final pH, while low VS losses were obtained in conditions in which final pH was low, as in the case of high solids ryegrass and low solids grass, with additive addition further lowering pH. Dawson et al. (1999) also observed higher pH with wilted grass silage, which might have been caused by proteolysis and changes in nitrogenous components occurring during wilting and thus leading to inhibition of acidification (McDonald et al., 1991). The chemical characteristics of the crop species in question also have an effect on ensiling properties; for example the buffering capacity of legumes (such as clover) is usually higher than of grasses (McDonald et al., 1991), which may partly explain the relatively high pH of grass in some of our study samples.

According to our results storage can enhance the CH_4 potential (m^3/kgVS) of crops, which can further help in maintaining a high CH_4 yield (m^3/t_{oww}) despite quite high VS losses in some cases. The present and previous studies (e.g. Lehtomäki et al., submitted for publication) suggest that the CH_4 potential (m^3/kgVS) of energy crops can in some cases be increased during storage, which thus acts as a pre-treatment step. Fifty-two percent higher CH_4 potential was obtained from sugar beet tops stored for 6 months compared to fresh crop (Lehtomäki et al., submitted for publication) and 15% higher CH_4 potential was estimated from ensiled elephantgrass and energycane compared to fresh crops (Woodard et al., 1991). The CH_4 potential of stored whole crop maize (0.48 m^3/kgVS) increased by 25% compared to fresh crop (0.38 m^3/kgVS) (Neureiter et al., 2005), and the biogas potentials of ensiled green pea shells (Madhukara et al., 1997), ensiled mango-peel (Madhukara et al., 1993) and ensiled pineapple peel (Rani and Nand, 2004) increased by 9%, 58% and 22% during ensiling, respectively. The observed CH_4 potential in our study (0.51 m^3/kgVS at maximum) was higher than the CH_4 potentials of fresh and ensiled perennial ryegrass, cocksfoot and meadow foxtail (0.31–0.36 m^3/kgVS) (Mähnert et al., 2005), but this can be explained by different chemical composition of the grass species and also by different duration of the batch assays (70–80 days in our study compared to 28 days in study of Mähnert et al., 2005). Storage with additives can further improve the maintenance or even enhancement of CH_4 potential; thus the highest CH_4 potential was obtained with grass and sugar beet tops stored for 6 months with formic acid (35% and 68% increase compared to fresh crops), while LAB inoculant also increased CH_4 potential by 4% and 42%, respectively (Lehtomäki et al., submitted for publication). Bacterial inoculant, amylase and *Clostridium tyrobutyricum* increased CH_4 potential of stored whole crop maize by 10%, 27% and 40% compared to fresh crop (Neureiter et al., 2005). In our study, storage time had no significant impact on CH_4 potential, which was also the situation in a previous study with grass, whereas with sugar beet tops CH_4 potential was usually higher after storage for 6 months compared to 3 months (Lehtomäki et al., submitted for publication). The increase in CH_4 potential during storage is assumed to be caused by degradation of structural polysaccharides of plant material into more easily degradable intermediates (Egg et al., 1993). Cellulose- and hemicellulose-degrading enzymes can enhance the hydrolysis and improve the digestibility of organic matter (Kung et al., 2003). In some situations silage might have promoted degradation of the inoculum and thus caused overestimation of the CH_4 potentials of the substrates.

During suboptimal storage conditions large proportion of CH_4 yield can be lost, as shown by the fact that after 6 months of storage losses were at most 37% and 52% with grass and ryegrass, respectively, in laboratory studies, and 17% and 41%, respectively, in field studies after 11 months. These losses in CH_4 yield were probably caused by secondary fermentation (e.g. Sebastian et al., 1996), which led to a rise in pH and loss of VS and, in some cases also, to loss of CH_4 potential. It has been stated that if there are insufficient WSC present in the silage, or the solids content is too low, secondary fermentation by *Clostridia* bacteria can occur. Clostridia ferment sugars and lactate mainly into butyrate, while minor amounts of formate, acetate, propionate, ethanol and butanol can also be produced. In secondary fermentation CO_2 is released, resulting in increased pH and, with rising pH, conditions may become favourable for the proteolytic clostridia, which break down proteins and amino acids into amines, amides, and ammonia, thus causing a further increase in pH (Egg et al., 1993; Woolford, 1984). Clostridial fermentation is undesirable, because the butyrate fermentation pathway results in con-

siderable loss of gross energy through the loss of molecular H_2 (Egg et al., 1993). In the present study a higher initial solids content resulted in a decreased VFA concentration at 6 months with stored grass and ryegrass, which is partly in accordance with previous results, hence lower concentrations of propionic and *n*-butyric acids and higher concentrations of lactic- and acetic-acids were found with wilted comfrey silage compared to unwilted crop (Wilkinson, 2003). In our study, which included crops with lower solids, other VFAs, such as propionic and butyric acid, were also present. This is, as discussed above, an indication of secondary fermentation. With low solids crops small amounts of valeric and caproic acids were also present. These acids are thought to be formed from acetic and propionic or acetic and *n*-butyric acid by removal of molecular H_2 and it is known that some clostridia and a rumen bacterium are capable of catalysing these reactions (Zauner and Küntzel, 1986). Unfortunately, lactic acid was not analysed in our study.

In our study the role of biological additive in improving or preserving CH_4 yield was not noteworthy, as was also the situation with grass and sugar beet tops (Lehtomäki et al., submitted for publication) and whole crop maize (Neureiter et al., 2005). In contrast, previous study suggested that formic acid could improve CH_4 yield of grass stored for 3 months by 22% compared to fresh crop (Lehtomäki et al., submitted for publication). According to our results additive increased acetic acid concentration in low and medium solids crops and decreased it in high solids crops. In an earlier study addition of urea reduced the concentration of fermentation acids (Hill and Leaver, 2002) and bacterial inoculant (*L. plantarum*) increased lactic acid and reduced the acetic acid concentration in lupin (Fraser et al., 2005), forage pea and field bean silages (Fraser et al., 2001). Additives have led to lower pHs in previous studies as well; e.g., bacterial inoculant resulted in lower pH in lupin silages (Fraser et al., 2005), whole crop barley silage (Zahiroddini et al., 2004) and forage pea and field bean silages (Fraser et al., 2001). LAB additive was found to be effective only for wilted alfalfa (TS 31%), resulting in a faster drop in pH compared with control (TS 22%) (Schmidt et al., 2001). According to our study addition of biological additive resulted in reduced VS loss with ryegrass at 6 months of storage, whereas with grass the effect of additive on VS loss was less clear. In earlier studies additives, e.g., urea (Hill and Leaver, 2002), bacterial inoculant and hydrolytic enzymes (Zahiroddini et al., 2004), have reduced dry matter losses during ensiling. However, organic matter losses have also been higher in enzyme treated than untreated maize silage (Colombatto et al., 2004). It should be noted that as in our study, as is usually done, the additive was dosed on the basis of the wet weight of the crop, drier crops received less additive per TS.

In our study mass losses on the field scale were negligible with ryegrass, probably due to the high initial TS content (44.4%), while mass losses of grass varied between 18% and 29%, the smallest mass loss observed with grass stored without pre-wilting. In earlier studies between 1.6% and 15.7% of TS was lost when storing elephantgrass and energycane (Woodard et al., 1991), 2.9% when storing maize silage for 90 days (Filya, 2004) and TS losses of switchgrass during 6 months of storage (round bales, no plastic) averaged 13% (Sanderson et al., 1997). Mass losses for crops stored in laboratory studies have generally been less than 5%. In the present study mass losses varied between 2.9% and 4.6% for grass and between 4.0% and 5.1% for ryegrass and after 3 months of storage 1.5–2.5% of mass of wheat silage, 1.5–1.7% of corn silage (Weinberg et al., 2002) and after 119 days 0.9–2.4% of whole crop maize silage were lost (Neureiter et al., 2005). Mass losses in laboratory studies are usually lower than in field studies as in the field leachate losses increase mass loss.

As the present results show, how energy crops for CH_4 production are stored is an important issue since with appropriate storage practices CH_4 yield can be rather well maintained, whereas with suboptimal storage conditions a large proportion of CH_4 yield can be lost. In this study the more detailed investigation on the initial TS content was performed in controlled, small-scale laboratory conditions in 20 °C, while storage in field conditions was performed in large round bales in varying temperature conditions. The results obtained from the laboratory may not be fully comparative with those obtained in the field; nevertheless the CH_4 recovery in the latter studies was promising. CH_4 yield in field conditions could be probably further enhanced by selection the correct type of storage; e.g., in large silos losses might be lower compared to plastic-covered round bales. The results obtained from the storage of fodder crops may not be fully comparable with those of energy crops, as VS losses of the energy crops can be balanced by their enhanced CH_4 potential (m^3CH_4/kgVS). Initial drying, i.e., solids content, seems to have an important effect on maintaining CH_4 yield, but if crops are used in a biogas process without storage, it should be borne in mind that the use of pre-wilted crops reduces transportation costs and that drier crops are also easier to handle (Egg et al., 1993; McDonald et al., 1991). However, in the field losses during drying might occur and these losses need to be balanced against the gains from lower transportation and handling costs.

5. Conclusions

Storage of energy crops to be used in biogas production is an important issue to handle as crops need to be available throughout the year. Ensiling of energy crops used for CH_4 production was shown to be a feasible method for preserving the CH_4 yield even 11 months in boreal field conditions. Under appropriate storage conditions 87% and 91% in laboratory conditions and 96% and 68% in field conditions, of CH_4 yield of grass and ryegrass, respectively, were recovered. Initial solids content can affect the ensiling process and thus on chemical characteristics and CH_4 potential of the crop. According to present study VS loss

during storage seems to be a major factor in determining the preservation of CH_4 yield. Prolonged drying, i.e., a higher initial TS content enabled better VS and CH_4 yield recovery with ryegrass, whereas with grass a lower initial TS content resulted in the best preserved CH_4 yield. Storage usually resulted in a lowered pH, which was necessary for the preservation of CH_4 yield. In the laboratory pH increased between 2 and 6 months due to secondary fermentation. Biological additive did not improve preservation of the CH_4 yield of the crops studied even though it usually resulted in lower pH.

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II

BATCH DARK FERMENTATIVE HYDROGEN PRODUCTION FROM GRASS SILAGE: THE EFFECT OF INOCULUM, PH, TEMPERATURE AND VS-RATIO

by

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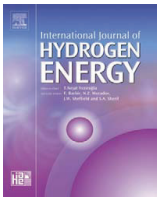

Batch dark fermentative hydrogen production from grass silage: The effect of inoculum, pH, temperature and VS ratio

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ABSTRACT

The potential for fermentative hydrogen (H_2) production from grass silage was evaluated in laboratory batch assays. First, two different inocula (from a dairy farm digester and digested sewage sludge) were studied with and without prior heat treatment and pH adjustment. Only the inoculum from the dairy farm digester produced H_2 from grass silage. Without heat treatment, methane (CH_4) was mainly produced, but heat treatment efficiently inhibited CH_4 production. pH adjustment to 6 further increased H_2 production. The effects of initial pH (4, 5 and 6), temperature (35, 55 and 70 °C) and the substrate to inoculum volatile solids (VS) ratio (henceforth VS ratio) (1:1; 1.5:1 and 2:1) on H_2 production from grass silage were evaluated with heat-treated dairy farm digester sludge as inoculum. Optimal pH was found to be between 5 and 6, while at pH 4 no H_2 was formed. The highest H_2 yield was achieved at 70 °C. H_2 production also increased when the VS ratio was increased. However, the overall energy value of H_2 compared to that of CH_4 production remained low.

1. Introduction

H_2 is expected to be a major energy carrier in the future, mainly due to its non-polluting nature and versatility as fuel. H_2 can be converted to heat and electricity or used as traffic fuel. In particular, it is assumed that the development of fuel cell technology will lead to the utilization of H_2 [1]. H_2 should be produced from renewable energy sources instead of non-renewable ones, thereby reducing both the negative impact of energy production on the environment and the use of non-renewable resources. A variety of electrochemical, thermochemical and biological processes can be used in the production of renewable H_2 [2].

Increasing interest has been shown in the development and cultivation of crops which have high biomass yields and which could efficiently be converted into energy. Anaerobic digestion is considered a promising technology also for crop conversion, as it uses most of the energy content of the crop while the residual material, containing nutrients and some carbon, can be recycled for further crop cultivation. Grasses have been proposed as potential candidates for energy production in northern climatic conditions due to their ease and long tradition of cultivation, harvesting and storage. Dry matter yields per hectare are also quite high (8–11 t/ha) and it has been estimated that annually 28–38 MW h of CH_4 energy can be produced through anaerobic digestion from 1 ha of grass [3].

CH_4-rich biogas is increasingly being produced from wastes, wastewaters, energy crops and agricultural residues, and used for electricity, heat and traffic fuel production [4]. Biogas (main components CH_4 and carbon dioxide (CO_2)) is the end

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product of the gas phase in the anaerobic conversion of organic material. This is a multistep process, ending with methanogenesis in which acetate and H_2 are converted to CH_4. In this digestion process H_2 is formed during the acidogenesis phase in combination with volatile fatty acid (VFA) production and, as a by-product, is usually converted to CH_4 by methanogens. H_2-producing bacteria species have been found, e.g., among the *Enterobacter*, *Bacillus* and *Clostridium*. It might be possible to produce H_2 instead of CH_4 by adjusting the process parameters, e.g. hydraulic retention time (HRT), pH and temperature and by inactivating H_2-consuming bacteria, e.g., by heat treatment [5]. However, as it seems that the H_2-producing pathway cannot convert all the organic material [6] to H_2, the process could be used to produce both H_2 and CH_4, depending on the relative need for the two energy sources in the future.

Several factors have been shown to affect H_2 yield and rate of production in dark fermentation. Increasing partial pressure of H_2 (pH_2) inhibits H_2 production [5]. In some studies thermophilic conditions have given a higher [7–10] H_2 yield than mesophilic conditions. Optimal pH differs from one study to another, but a pH level between 5 and 7 is usually favoured [11–13] for H_2 production. Most studies on H_2 production have used model substrates like sucrose or glucose [8,11,12,14–18], while studies on solid substrates such as food waste have been few [19–23]. Plant material, such as pea-shells, rice, potato and carrot, has also been tested as substrates for fermentative H_2 production [20,22,24], but in general studies on the use of potential energy crops are scarce.

The objective of this study was to evaluate the H_2 production potential of grass silage and the effects of the source and heat treatment of the inoculum, as well as the effects of pH adjustment, temperature and the volatile solids (VS) ratio on H_2 production in batch processes.

2. Materials and methods

2.1. Inocula and substrates

Two different inocula were used, namely, the digestate from a mesophilic farm biogas process (Laukaa, Finland) digesting cow manure and confectionery by-products, defined as inoculumF, and the mesophically digested sewage sludge from a municipal wastewater treatment plant (Jyväskylä, Finland), defined as inoculumS. The latter was centrifuged to increase the total solids (TS) concentration (Table 1). Both inocula were heat-treated by boiling for 30 min to inactivate H_2-consuming bacteria and to enrich spore-forming H_2 producers.

Grass silage (a mixture of timothy, *Phleum pratense* and meadow fescue, *Festuca pratensis*) was obtained from a farm (Laukaa, Finland, 1.11.2004) and stored at −20 °C until used. Prior to use, it was thawed overnight at room temperature and chopped into particles of ~1 cm with a household blender (Table 1). Glucose, used as a control substrate, was of analytical grade D(+)-glucose (EC NO 200-075-1, Sigma, Steinheim, Germany). Glucose was chosen as a control substrate to evaluate the H_2 production of the inocula with a commonly used model compound.

2.2. Batch experiments

The batch assays were performed in duplicate or triplicate in 118 mL glass bottles (Table 2). First, inoculum was added (1.0 or 1.5 g VS of inoculumF per bottle; 1.2 g VS of inoculumS per bottle). Subsequently, grass silage (1.0–2.0 g VS per bottle) was added to obtain the desired VS ratio. In the control substrate assays, glucose (5 g/L, corresponding to 300 mg/bottle, VS ratio of 0.2 with inoculumF and 0.25 with inoculumS, corresponding to theoretical H_2 and CH_4 yields of 149 and 112 mL, respectively) was added from 30 g/L stock solution. Bottles were filled to total volume of 60 mL with distilled water. When necessary, pH was adjusted to 4, 5 or 6 with 5 M HCl and 5 M NaOH and contents were flushed with nitrogen gas to remove oxygen (Table 2). The control assays, with inoculum only, were incubated under the same conditions. Bottles were sealed with butyl rubber stoppers (Bellco Glass Inc., NJ, USA) and aluminium crimps (Sigma-Aldrich, Wheaton aluminium cap, Steinheim, Germany), and incubated statically at a constant temperature. Gas samples were taken through stoppers from the gas phase with a pressure-locked glass syringe (Supelco, Pressure-Lok® Series A-2 Syringe, Bellefonte, USA). Gas composition was analysed twice per day during the first 48 h and daily after that. Assays were terminated after H_2 production ceased, which was after 11–31 days of incubation.

Gas (H_2 and CH_4) production from the inocula only and from grass silage is given as mL/g VS. Gas production of the

Table 1 – Characteristics of inocula and substrates (average values from all the experiments)

Inoculum	Heat treatment	TS (%)	VS (%)	TCOD (g/l)	SCOD (g/l)	pH
Farm	No	6.3	4.8	76	7.9	8.1
Farm	Yes	6.3	4.9	107	10.6	9.6
Sewage	No	6.1	3.0	58	1.8	7.8
Sewage	Yes	7.2	3.5	51	8.2	9.0
Grass silage	–	25.9	24.0	na	229[a]	4.3

na: not analysed.
[a] Unit mg/g TS.

Table 2 – Experimental set-up for different batch assays

Experiment	Inoculum	Substrate	VS-ratio	pH adjustment (target pH)	Temperature (°C)
The effects of inoculum source, heat treatment and initial pH adjustment to 6	InoculumF 1.5 g VS	Grass silage	1:1	No	35
				Yes (6)	35
		Glucose		No	35
				Yes (6)	35
	Heat-treated inoculumF 1.5 g VS	Grass silage	1:1	No	35
				Yes (6)	35
		Glucose		No	35
				Yes (6)	35
	InoculumS 1.2 g VS	Grass silage	1:1	No	35
				Yes (6)	35
		Glucose		No	35
				Yes (6)	35
	Heat-treated inoculumS 1.2 g VS	Grass silage	1:1	No	35
				Yes (6)	35
		Glucose		No	35
				Yes (6)	35
The effects of temperature, initial pH and VS-ratio[a]	Heat-treated inoculumF 1.0 g VS	Grass silage	1.5:1	Yes (6)	35
			2:1	Yes (6)	35
			2:1	Yes (5)	35
			2:1	Yes (4)	35
			1:1	Yes (6)	55
			1:1	Yes (6)	70

[a] Triplicates.

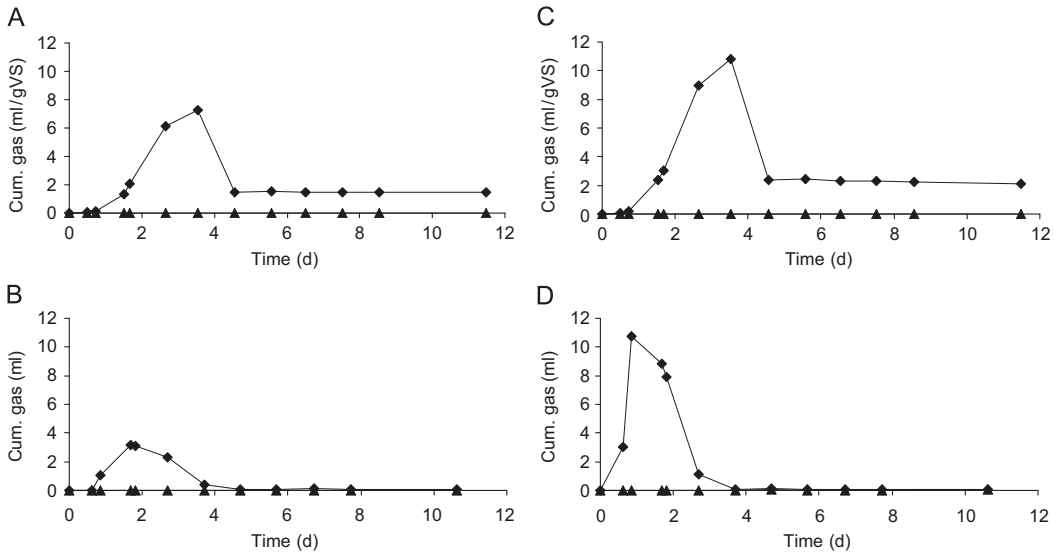

Fig. 1 – Mean H_2 (◆) and CH_4 (▲) production from grass silage (above) using heat-treated inoculumF and from glucose (below) using heat-treated inoculumS without (A and B) and with (C and D) initial pH adjustment to 6.

substrates is given minus the gas production of the inoculum. Gas production from glucose is given as mL (Fig. 1) or mL/g-glucose added (Table 3 and text). In some assays H_2 peaked twice (discussed more in discussion section) but, when calculating the actual H_2 yield, only the higher peak was taken into account.

Table 3 – H_2 and CH_4 production (± standard deviation) from inocula and substrates (inoculum subtracted), SCOD at the end of the assays and pH at the beginning and at the end of the assays (VS ratio of 1:1, assays done in duplicate)

Inoculum	Heat treatment	pH (initial/final)			SCOD (g/L)			H_2 (mL/g VS)			CH_4 (mL/g VS)		
		No substrate	Grass	Glucose	No substrate	Grass	Glucose	No substrate	Grass	Glucose[a]	No substrate	Grass	Glucose[a]
Farm	No	8.6/7.6	6.5/6.6	8.4/7.9	4.3	9.5	3.6	0.3 ± 0.3	2.1 ± 1.3	0.0 ± 0	27.0 ± 0.1	6.3 ± 1.4	301.1 ± 7.6
Farm	No	6/6.7	6/6.2	6/4.3	2.5	7.0	4.7	0.4 ± 0.1	3.6 ± 0.7	8.4 ± 6.9	9.5 ± 1.0	13.8 ± 0.5	0.0 ± 0.0
Farm	Yes	9.5/8.5	6.2/6.1	9.5/6.6	8.2	12.2	4.4	0.3 ± 0	7.2 ± 2.7	0.5 ± 0.7	0.3 ± 0.3	0.0 ± 0	0.0 ± 0
Farm	Yes	6/6.4	6/5.9	6/5.8	4.2	12.8	5.3	0.5 ± 0.4	11.5 ± 0	45.3 ± 25.5	1.4 ± 0.1	0.0 ± 0	0.0 ± 0
Sewage	No	7.9/7.3	5.9/7.1	7.9/6.9	0.3	1.4	0.2	0.0 ± 0	0.0 ± 0	0.0 ± 0	9.7 ± 0.7	49.0 ± 1.2	87.6 ± 5.1
Sewage	No	6/6.5	6/6.9	6/6.4	0.2	1.6	0.3	0.0 ± 0	0.0 ± 0	6.6 ± 0.3	8.1 ± 0	48.2 ± 1.4	90.2 ± 1.7
Sewage	Yes	9.1/7.3	6.1/5.9	9.1/6	3.2	6.2	4.5	0.2 ± 0	0.1 ± 0.2	9.7 ± 1.2	0.4 ± 0.3	0.0 ± 0	0.0 ± 0
Sewage	Yes	6/6.7	6/5.8	6/5.7	2.0	7.4	4.5	0.0 ± 0	0.2 ± 0.1	35.9 ± 3.2	0.7 ± 0	0.0 ± 0	0.0 ± 0

[a] Unit mL/g glucose.

2.3. Analysis

The TS and VS were analysed according to Standard Methods [25], and chemical oxygen demand (COD) according to SFS 5504 [26]. The pH was measured with a Metrohm 774 pH-meter (Metrohm, Switzerland). Soluble COD (SCOD) from the grass silage was analysed after the leaching test, which was modified from SFS-EN 12457-4 (particle size used in the leaching test varied between 10 and 25 mm (as against ⩽10 mm in the standard), amount of crop was 50 g TS (as against 90 ± 5 g TS in the standard) and filtration was done through a glass fibre filter paper (GF50, Schleicher & Schuell, Dassel, Germany, as against 0.45 μm membrane filter in the standard)) [27].

Gas composition (H_2, CH_4 and CO_2) was analysed with a Perkin Elmer Arnel Clarus 500 gas chromatograph equipped with a thermal conductivity detector (TCD) and Supelco Carboxen™ 1010 PLOT fused silica capillary column (30 m * 0.53 mm). Argon (15 mL/min) was used as the carrier gas and the temperatures of the oven, detector and injector were 200, 230 and 225 °C, respectively.

3. Results

3.1. The effects of source of inoculum, heat treatment and initial pH adjustment on H_2 production from grass silage and from glucose as control

The effect of heat treatment of the inocula and initial pH adjustment (to pH 6) on H_2 and CH_4 production from glucose and from grass silage (VS ratio of 1:1) was studied in batch assays (35 °C) using two different inocula (Table 3). Typically, in all the assays H_2 production started within 24 h and ceased after 5 days of incubation (Fig. 1). Each of the inocula alone produced negligible amounts (⩽0.5 mL/g VS) of H_2 in all conditions while, without heat treatment and pH adjustment, both inocula produced CH_4 (9.7–27.0 mL/g VS). pH adjustment alone decreased CH_4 production (8.1–9.5 mL/g VS), and heat treatment further decreased CH_4 production to negligible amounts (⩽0.4 mL/g VS). At the end of the assays the final SCOD values of the pH-adjusted inocula were lower than those of the unadjusted inocula (Table 3).

H_2 was produced from grass silage and glucose with heat-treated and/or pH-adjusted inoculumF, while inoculumS produced H_2 from glucose only. With inoculumF the highest H_2 production from grass silage (11.5 mL/g VS, 31% of the biogas) and glucose (45.3 mL/g-glucose added) occurred when the inoculum was both heat-treated and pH-adjusted. Without heat treatment both inocula produced mainly CH_4 from grass silage and glucose, while pH-adjusted inoculumF also produced some H_2 (3.6 mL/g VS) from grass silage and glucose (8.4 mL/g-glucose added). The heat treatment inhibited CH_4 production from both substrates with both inocula. The final pH in all the assays with grass silage was around 6 (5.8–6.6), except with heat-treated inoculumS (final pH of 6.9–7.1) (Table 3).

With glucose, pH decreased by as much as around 3 units with heat-treated inocula and 1.7 units with pH-adjusted inoculumF, apparently due to the acidification of glucose.

In these assays no CH_4 and small amounts of H_2 (<10 mL/g-glucose) were detected. pH increased during the assays with both pH-adjusted inocula alone (increase 0.4–0.7 units), and in all CH_4-producing assays with grass (increase 0.1–1.2 units), while with glucose pH increased only with pH-adjusted and CH_4-producing inoculumS (Table 3).

3.2. *The effects of VS ratio, temperature and initial pH adjustment on H_2 production from grass silage*

The effects of different VS ratios and temperature (VS ratio of 1:1) on H_2 production were studied in batch assays using heat-treated inoculumF. Inoculum as such and grass silage produced no CH_4 under any conditions. H_2 production increased from 3.2 to 6.2 mL/g VS (percentage of H_2 in biogas increased from 6% to 23%) when the VS ratio was increased from 1:1 to 2:1 (Table 4, Fig. 2). H_2 production increased from 3.2 to 7.2 and 16.0 mL/g VS with corresponding H_2 percentages of 6%, 15% and 35%, when temperature was increased from 35 to 55 and 70 °C, respectively. Time taken to reach maximum H_2 yield was longer at 70 °C (around 25 days) compared to 55 °C (10 days) and 35 °C (3–4 days). At 55 °C H_2 was consumed after the initial peak on day 1, and a second H_2 production phase was detected after day 3 (Fig. 2). SCOD at the end of the assays increased when the higher VS ratio was used. In contrast pH was not affected (range 5.1–5.2). The final pH was lower at 55 °C compared to 35 and 70 °C and SCOD was lowest at 70 °C (9.5 g/L), second lowest at 35 °C (10.5 g/L) and highest at 55 °C (11.8 g/L) (Table 4).

The effect of initial pH (4, 5 and 6) on H_2 production from grass silage was studied in batch assays at 35 °C using a VS ratio of 2:1. H_2 production was highest at pH 5 (4.0 mL/g VS),

Table 4 – Effect of temperature, VS ratio and initial pH on final pH, SCOD and H_2 production (± standard deviation) from heat-treated inoculumF and grass silage (H_2 production from inoculum subtracted)

T (°C)	VS ratio	pH (initial/final)		SCOD (g/L)		H_2 (mL/g VS)	
		No substrate	Grass	No substrate	Grass	No substrate	Grass
35	1:1	6/6.1	6/5.2	5.0	10.5	0.4 ± 0.2	3.2 ± 1.2
35	1.5:1	[a]	6/5.1	[a]	14.5	[a]	4.3 ± 1.3
35	2:1	[a]	6/5.2	[a]	16.5	[a]	6.2 ± 0.7
55	1:1	6/5.8	6/5.1	5.5	11.8	1.4 ± 0.5	7.2 ± 0.2
70	1:1	6/5.9	6/5.4	5.0	9.5	4.0 ± 0.8	16.0 ± 1.7
35	2:1	6/6.2	6/4.9	5.0	14.7	0.0 ± 0	0.9 ± 0.3
35	2:1	5/5.8	5/4.9	3.7	14.2	0.0 ± 0	4.0 ± 3.2
35	2:1	4/4.5	4/4.4	2.3	8.6	0.0 ± 0	0.0 ± 0

[a] As control upper values can be used.

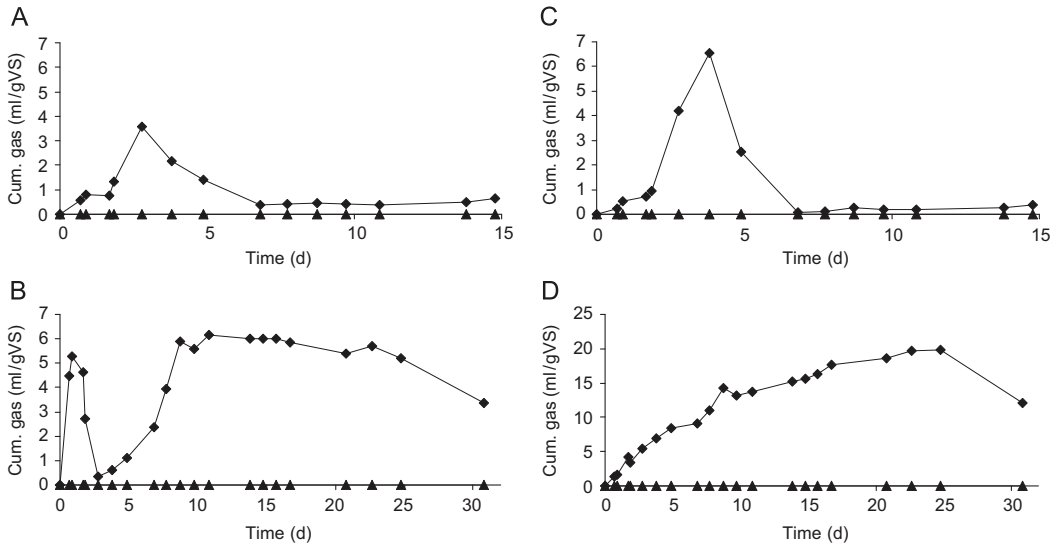

Fig. 2 – Mean H_2 (◆) and CH_4 (▲) production from grass silage using VS ratio of 1:1 (A) and 2:1 (C) (temperature 35 °C) and at temperature of 55 °C (B) and of 70 °C (D) (VS ratio of 1:1).

while at pH 6 H_2 production was 0.9 mL/g VS. At pH 4 no H_2 was produced. The final pH and SCOD of the grass silage at initial pH 5 and 6 did not differ, while at pH 4 final pH and SCOD were lower (Table 4).

4. Discussion

The present results show that it is possible to produce H_2 from grass silage in a batch process and that the yields are highly dependent on several factors. The highest amount was obtained at 70 °C using heat-treated inoculum from a dairy farm digester. The H_2 yield (16 mL/g VS added) was comparable to yields obtained with other solid substrates. For example, 14–21 mL H_2/g VS was produced from bean curd manufacturing waste, 31–61 from rice bran, 10–43 from wheat bran [28], 26–62 from cabbage and 19–96 from rice [20]. However, the potential for energy production from grass silage through dark fermentation under these circumstances would appear to remain low. According to the present results, annual energy production from 1 ha of grass would only be between 400 and 500 kW h. It is estimated that around 28–38 MW h of energy could be obtained if grass were directly converted to CH_4 by a traditional anaerobic digestion process [3]. Thus to increase H_2 yield from solid substrates such as grass silage, measures such as substrate pre-treatment appear to be essential. Pre-treatments have been applied with the purpose of elevating H_2 production [16,29–31]. For example 68 mL H_2/g VS was obtained with acid-treated straw compared to 0.5 mL H_2/g VS with untreated straw [31]. Moreover, it is possible that the H_2 yield in the present study was underestimated, as discussed below. Furthermore, H_2 production through anaerobic digestion cannot be considered a complete treatment process, since high amounts of by-products such as VFA remain undegraded [6]. One way to improve overall energy efficiency would be to produce CH_4 from the digestate produced by the dark fermentation process [17,32].

Both the present and previous studies [7–10] indicate that thermophilic conditions favour H_2 production compared to mesophilic conditions. This might be expected given the fact that higher temperatures favour H_2 formation but not H_2-consuming reactions [33]. The higher H_2 yield is also explained by the optimal temperature of the hydrogenase enzyme, which is reported to lie between 50 and 70 °C [34]. Acidogenic H_2 producers are reported to be mostly thermophilic heat-resistant bacteria [18,35]. A higher H_2 yield was obtained at 55 °C than at 35 °C with food waste [9] (112 (calculated by the authors) and 12 mL H_2/g-hexose at 55 and 35 °C, respectively) and with organic waste [10] (360 and 165 mL H_2/g VS removed at 55 and 35 °C, respectively). A higher H_2 yield was also reported at 55 °C (78 mL H_2/g-starch) than at 35 °C (47 mL H_2/g-starch) with starch-rich wastewater [8], due to H_2-consuming reactions (propionate and CH_4 formation) at 35 °C [9]. In both the present and earlier studies, lag time and the time taken to reach the maximum H_2 yield were longer in thermophilic conditions compared to mesophilic conditions. This was probably caused by the low initial amounts of thermophilic micro-organisms in the inoculum, which was of mesophilic origin [8].

Heat-treated inoculum from the farm biogas digester treating cow manure was more efficient in producing H_2 from grass silage compared to the digested sewage sludge. Inoculum derived from cow manure presumably contains rumen micro-organisms which are capable of degrading lignocellulosic substrates such as grass silage. Unlike dairy farm sludge, sewage sludge apparently contains low amounts of cellulose utilizing micro-organisms while the dominant micro-organisms in sewage sludge are capable of degrading, e.g., glucose [36]. H_2 production studies have mostly used heat-treated digested sludge from municipal wastewater treatment plants as inocula. To our knowledge, the use of heat-treated sludge from a farm-scale anaerobic digester treating cow manure has not been reported previously, while heat-treated composted cow dung has been proven capable of H_2 production from sucrose and wheat straw waste [31,37]. According to the present results, heat treatment of the inoculum seems to be necessary for H_2 production from grass silage. In contrast, H_2 production from glucose, using digested sludge and compost without heat treatment as inocula, has been shown to be possible [38], although other treatments such as pH control might also be necessary [30,39].

Substrate concentration apparently has a significant effect on microbial metabolic pathways and on H_2 production. According to our results a higher substrate concentration (i.e., higher substrate to inoculum VS ratio) in batches might be preferable for fermentative H_2 production from grass silage. However, when a certain substrate threshold is exceeded and the partial pressure of H_2 is increased, bacteria may shift their metabolism from H_2 and VFA to alcohol production [20,31,37]. Too high a substrate concentration would also result in the accumulation of VFA and a fall in pH, which would inhibit H_2 producers. The optimal TS concentration for H_2 production seems to be very dependent, e.g., on the substrate used and, apparently, on the substrate to micro-organism ratio. In practical applications with mixed cultures the latter is generally estimated using VS values, on the understanding that these are only a very rough estimation of the bacterial population. Increased H_2 yield with increasing substrate concentration has been reported, e.g., for lean meat (2.5 mL/g VS at TS 4%, 7.1 mL/g VS at TS 12%) [20], food waste (46 mL/g VS at VS 0.3%, 92 mL/g VS at VS 0.6%) [9] and wheat straw waste (13.8 and 68.1 mL/g VS at substrate concentrations of 0.5% and 2.5%, respectively) [31]. In contrast, with carbohydrate-rich substrates a TS concentration of 2–5% was found preferable for H_2 production, while at higher TS concentrations (tested up to 15%) H_2 yield per gram VS decreased [20]. With wheat straw waste H_2 yield fell with higher substrate concentrations (68.1 and 16.3 mL/g VS at substrate concentrations of 2.5% and 3.5%, respectively) [31]. In the present study, VS ratios up to 2 were studied. It might, however, be worth studying VS ratios higher than 2, as the highest H_2 yield was achieved at a VS ratio of 2.

The optimal pH for H_2 production from grass silage seems to lie between 5 and 6, while at pH 4 no H_2 was produced. However, some H_2 was produced from grass silage also at an initial pH of ca. 6.2–6.5, which was the initial pH in assays with inoculumF (pH 8.6 and 9.5) and grass silage (pH 4.3) without initial pH adjustment. In contrast, pH adjustment to 6 was necessary for H_2 production from glucose; thus the initial

pH in glucose assays without pH adjustment was too high (7.9–9.5). pH 4 has also been found inhibitory in other studies using substrates such as starch [40] and wheat straw wastes [31]. At the optimal pH, acetate and butyrate producers are assumed to overcome propionate producers, thereby increasing the H_2 yield [31]. Optimal pH for H_2 production by *Clostridium butyricum* was reported to be in the range of 5.5–6.0, as at a lower pH both cell growth and H_2 production were inhibited and at a higher pH cell growth was more efficient than H_2 production [41]. The initial pH adjustment used was apparently not sufficient to buffer the pH in assays with inocula alone as the pH tended to rise towards the original pH of the inocula. With glucose, pH decreased (by as much as 3 units), apparently due to effective acidification, and H_2 was produced when the final pH of $\leqslant$ 6.6 was reached. In some assays without heat treatment, methanogens were able to convert H_2 and the acids to CH_4 and thus increase the pH.

It seems that traditional batch assays may not be the best way to evaluate the H_2 production potential of different substrates as this method tends to underestimate the H_2 yield achievable in continuous reactors [42]. H_2 is consumed by homoacetogenic bacteria, which convert H_2 and CO_2 to acetate [43–45] and propionate-producing bacteria [43]. In some studies heat treatment has been incapable of destroying homoacetogenic bacteria [44]. H_2-consuming homoacetogenesis is assumed to be inhibited by CO_2 removal, thus increasing H_2 yield. Hence, higher H_2 yields were achieved when CO_2 was removed from the culture liquid by bubbling with argon gas (H_2 yield increased from 0.52 to 1.09 mol-H_2/mol-glucose) [46] or by chemical absorption into KOH (H_2 yield increased from 1.4 to 2.0 mol-H_2/mol-glucose) [45]. In batch-type assays the increasing partial pressure of H_2 may also inhibit H_2 production [5] and this may also have occurred in this study. The inhibiting effect of pH_2 can be avoided by the constant removal of H_2 from the system. Logan et al. [47] obtained a 43% higher H_2 yield when using a respirometric (continuous gas release) method compared to the traditional Owen (an intermittent pressure release) method. In the present study H_2 was in most cases consumed after the exponential H_2 production phase, apparently by homoacetogenic and propionate-producing bacteria, as only a negligible amount of CH_4 was produced.

The complex metabolism of H_2 production and consumption and the heterogeneity of the substrate are possible explanations for the slightly higher variation in H_2 yield between assays observed in this study compared with typical CH_4 production assays. The possible simultaneous production and consumption of H_2, as well as the lack of continuous gas composition measurements, may also have influenced the H_2 yields found here. Thus, better methods would apparently improve the reliability of the results obtained from the type of batch assays generally used to study H_2 potential.

5. Conclusions

- H_2 production from grass silage through dark fermentation was shown to be feasible.
- The preferred inoculum was obtained from a farm scale digester, while digested sewage sludge did not produce H_2 from grass silage. The inoculum needs to be heat-treated to inhibit H_2 consumers and enrich spore-forming H_2 producers.
- The highest H_2 yield was achieved at a temperature of 70 °C (under the experimental conditions at VS ratio 1 and pH 6). At higher temperatures a longer time was required to reach the maximum H_2 yield than at lower temperatures (35 and 55 °C).
- The optimal pH for H_2 production from grass silage according to this study was between 5 and 6, while at pH 4 no H_2 was produced.
- A VS ratio of 2 was shown to increase H_2 production compared to lower VS ratios under this experimental condition.
- H_2 yield from grass silage was moderate and its energy value is not comparable to its CH_4 yield. If grass silage or other lignocellulosic material is to be used for H_2 production, efficient pre-treatment technologies or two-stage systems (combined H_2 and CH_4 production) might be needed to increase overall energy efficiency.

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ONE-STAGE H_2 AND CH_4 AND TWO-STAGE H_2 + CH_4 PRODUCTION FROM GRASS SILAGE AND FROM SOLID AND LIQUID FRACTIONS OF NAOH PRE-TREATED GRASS SILAGE

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One-stage H_2 and CH_4 and two-stage H_2 + CH_4 production from grass silage and from solid and liquid fractions of NaOH pre-treated grass silage

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ABSTRACT

In the present study, mesophilic CH_4 production from grass silage in a one-stage process was compared with the combined thermophilic H_2 and mesophilic CH_4 production in a two-stage process. In addition, solid and liquid fractions separated from NaOH pre-treated grass silage were also used as substrates. Results showed that higher CH_4 yield was obtained from grass silage in a two-stage process (467 ml g^{-1} volatile solids $(VS)_{original}$) compared with a one-stage process (431 ml g^{-1} $VS_{original}$). Similarly, CH_4 yield from solid fraction increased from 252 to 413 ml g^{-1} $VS_{original}$ whereas CH_4 yield from liquid fraction decreased from 82 to 60 ml g^{-1} $VS_{original}$ in a two-stage compared to a one-stage process. NaOH pre-treatment increased combined H_2 yield by 15% (from 5.54 to 6.46 ml g^{-1} $VS_{original}$). In contrast, NaOH pre-treatment decreased the combined CH_4 yield by 23%. Compared to the energy value of CH_4 yield obtained, the energy value of H_2 yield remained low. According to this study, highest CH_4 yield (495 ml g^{-1} $VS_{original}$) could be obtained, if grass silage was first pre-treated with NaOH, and the separated solid fraction was digested in a two-stage (thermophilic H_2 and mesophilic CH_4) process while the liquid fraction could be treated directly in a one-stage CH_4 process.

1. Introduction

Hydrogen (H_2) and methane (CH_4) are both valuable fuels and can be used for heat and electricity production or used as traffic fuels – either separately or as a mixture of H_2 and CH_4 known as hythane [1]. H_2 is the preferred fuel for fuel cells, while CH_4 can also be used in solid oxide fuel cells (e.g. see Ref. [2]). On mass basis, H_2 has a lower heating value (LHV) of 33.33 kWh kg^{-1} which is over two times higher than LHV of CH_4 (13.90 kWh kg^{-1}). On volume basis, the energy content of H_2 (LHV of 2.995 kWh Nm^{-3} (N meaning here the normal conditions of temperature and pressure)) is, however, three times less than that of CH_4 (LHV of 9.968 kWh Nm^{-3}) (e.g. see Refs. [3,4]).

Anaerobic degradation of organic matter is a complex series of metabolic interactions among different anaerobic microorganisms and is classified into four main stages, namely, hydrolysis, acidogenesis, acetogenesis and methanogenesis. During hydrolysis, organic polymers, such as cellulose, are degraded and solubilized into monomers, e.g. glucose. Acidogenic bacteria then convert these solubilized monomers to, e.g. volatile fatty acids (VFA) and H_2. In the traditional anaerobic digestion process H_2 is usually not detected as H_2 is consumed during, e.g. homoacetogenesis

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and methanogenesis to produce CH_4 and CO_2 as final end products. However, the process can be moved towards H_2 production instead of CH_4 by controlling the operational parameters, e.g. pH and temperature (e.g. see Refs. [5,6]).

Owing to the advantageous properties of H_2 as a fuel, there is an increasing interest in H_2 production from organic substrates through anaerobic digestion, i.e. dark fermentative H_2 production (e.g. reviews of Refs. [5,6]). Dark fermentative H_2 production has been intensively studied with model organic compounds, e.g. glucose and sucrose, whereas less work has been done with solid substrates (e.g. see Refs. [7,8]) and real wastewaters. H_2 yields from solid substrates were shown to be dependent on the chemical nature of the substrate and operational conditions. Previous researchers reported H_2 yields of 36 ml g^{-1} total solids (TS, converted by the authors from 1.6 mmol g^{-1} TS) from olive pulp [9], 170 ml g^{-1} VS from sugarcane [10], and 360 Nml g^{-1} $VS_{removed}$ from organic fraction of municipal solid waste (OFMSW) [11].

However, the H_2 yields obtained through dark fermentation are typically only about 10–20% of the energy content of the substrate [12]. Digestates or effluents from dark fermentation can further be used to recover the residual energy content as they usually contain VFAs and other degradation products which are not degraded further to H_2 due to thermodynamic restrictions (e.g. see Ref. [13]). According to Hawkes et al. [14] there are three different possibilities for a second stage in digestate treatment. These are photofermentation of acetate and butyrate to H_2, microbial fuel cells converting acetate and butyrate to electricity, and an anaerobic digestion process converting VFAs to CH_4. A combined two-stage H_2 and CH_4 process has been proposed as a promising technology as it has shown enhanced hydrolysis and higher energy yields than a one-stage methanogenic process (e.g. see Refs. [14–16]). Previous studies have demonstrated that CH_4 yields from household solid waste [16], artificial organic solid waste [15] and wastewater sludge [17] can be increased in two-stage processes compared to one-stage processes. Moreover, two-stage H_2 and CH_4 production in a pilot scale (first stage 50 l, second stage 220 l) has also been demonstrated successfully. In the above study, H_2 and CH_4 yields of 0.29 and 0.24 m^3 kg^{-1} VS added were obtained from food waste at an organic loading rate of 12.5 kg VS m^{3-1} d^{-1} [18]. In a two-stage system, the growth of acidogenic and methanogenic bacteria is optimized separately. In the first stage, low pH (e.g. see Refs. [5,6]) and short hydraulic residence times (HRT) (1–2 days) are maintained thus favoring acidogenic, H_2-forming bacteria, while the conditions for slower growing methanogens with neutral pH and longer HRT (typically 10–20 days) are maintained in the second stage (e.g. see Refs. [12,16]). In addition, a thermophilic or hyperthermophilic first stage, which improves hydrolysis, is also an efficient method of pathogenic destruction (e.g. see Ref. [16]) and thermophilic conditions have so far shown to favour H_2 formation (e.g. see Ref. [11]) by depressing H_2 consuming reactions [19].

Energy crops, i.e. crops grown specifically for the purpose of energy production, are abundant producers of biomass. The produced biomass can be used for CH_4 and/or H_2 production. Crops are mainly composed of lignocellulose, that is, cellulose, hemicelluloses and lignin, which are tightly linked to each other [20]. For successful utilisation of lignocellulosic biomass for bioenergy production, pre-treatment such as thermo-chemical [21] or steam-explosion [22] might be essential. These pre-treatments have been shown to increase carbohydrate availability and thus CH_4 and H_2 yields, e.g. see Refs. [21,22]. In addition, alkaline treatments have also shown to increase CH_4 production of different lignocellulosic materials, e.g. see Refs. [23–26]. For example 9 and 15% more CH_4 was obtained from alkaline-treated grass and sugar beet tops, respectively, compared to untreated crops [26]. The increase in CH_4 yield was attributed mainly to the improved hydrolysis as alkalis are known to break the bonds between hemicelluloses and lignin as well as swell the fibres and increase the pore size, e.g. see Refs. [23–26]. However, Na and K ions present in the alkali can inhibit H_2 and CH_4 production [23,27] and alkaline treatment can cause the degradation of lignocellulose to refractory and/or inhibitory aromatic compounds, e.g. see Ref. [23].

The objective of this study was to evaluate the CH_4 (mesophilic) production from grass silage in one-stage process and to compare that with the combined H_2 (thermophilic) and CH_4 (mesophilic) production in two-stage process. In addition, solid and liquid fractions separated from NaOH pre-treated grass silage were also used as substrates. Finally, the total energy production from one- and two-stage processes was estimated. A potential energy crop, namely, grass silage, was chosen as a substrate, as it is rather abundant in agricultural sector and also showed potential in CH_4 and H_2 production in our previous studies [28,29].

2. Materials and methods

2.1. *Substrates*

Grass silage (mixture of timothy, *Phleum pratense*, and meadow fescue, *Festuca pratensis*, ensiled with bacterial inoculant AIV Bioprofit (Kemira Growhow Ltd.) and stored in a silo for 2 months) was obtained from a farm (Laukaa, Finland). In laboratory grass silage was stored at −20 °C until used. Before the analysis and experiments it was thawed overnight and cut to a particle size of ca. 1–2 cm with scissors. Inoculum was obtained from a mesophilic farm biogas reactor treating cow manure and confectionary by-products as substrate (from the same farm as the silage; Table 1). For the H_2 assays it was heat-treated by boiling for 30 min to inactivate methanogens and to enrich spore-forming H_2-producers (Table 1).

2.2. *Alkaline pre-treatment*

Grass silage (184 g wet weight (ww), corresponding to 50 g TS and 46.5 g VS, particle size ca. 1–2 cm) was placed into 1 l glass bottle and distilled water (866 g) was added to obtain a TS concentration of 5%, resulting in a final mass of 1050 g. TS concentration of 5% was chosen to enhance fractionation of grass silage into solid and liquid fractions. Solid NaOH (2 g) was added to obtain a dose of 4% NaOH g^{-1} TS. Bottles were closed and mixed in an orbital shaker for 24 h at 20 °C. After treatment the material was sieved by gentle manual pressure through a metallic sieve (bore size approximately 1 mm) into

Table 1 – Characteristics of substrates and inocula.

Substrate	pH	TS (%)	VS (%)	SCOD ($g l^{-1}$)	Acetic acid ($mg l^{-1}$)	Propionic acid ($mg l^{-1}$)	Isobutyric acid ($mg l^{-1}$)
Grass silage	4.1	27.2	23.4	190.0[a]	32.7[a]	5.4[a]	bd
Solid fraction[b]	6.4	13.6	13.0	62.4[a]	1.9[a]	2.2[a]	bd
Liquid fraction[b]	6.4	1.2	0.7	10.1	1566	258	12
Inoculum	7.9	5.6	4.3	12.0	7.4	bd	bd
Heat-treated inoculum	9.2	7.8	6.0	15.9	264	bd	bd

bd = below detection limit.
a Unit $mg g^{-1}$ TS.
b Solid and liquid fractions from NaOH treatment.

solid (302 g ww, 41 g TS, 39 g VS) and liquid (750 g ww, 9 g TS, 5 g VS) fractions (Table 1, Fig. 1).

2.3. Batch assays

Batch assays were used to determine (1) CH_4 (one-stage) and (2) H_2 followed by CH_4 (two-stage) production from grass silage as well as from solid and liquid fractions separated (in a way expressed above) from NaOH pre-treated grass silage. All the batch assays were performed in triplicate, using 1-l glass bottles.

In the one-stage CH_4 assays 250 g of inoculum and substrate (41.9, 68.8 and 171 g ww of grass silage, solid and liquid fractions, respectively) were added into the bottles. With grass silage, a substrate to inoculum VS:VS-ratio of 1:1 was used while the amounts of solid and liquid fractions were chosen to correspond to the volumes that would have been generated from the grass silage (41.9 g) in the alkaline treatment. Thus, the substrate to inoculum VS:VS-ratios were

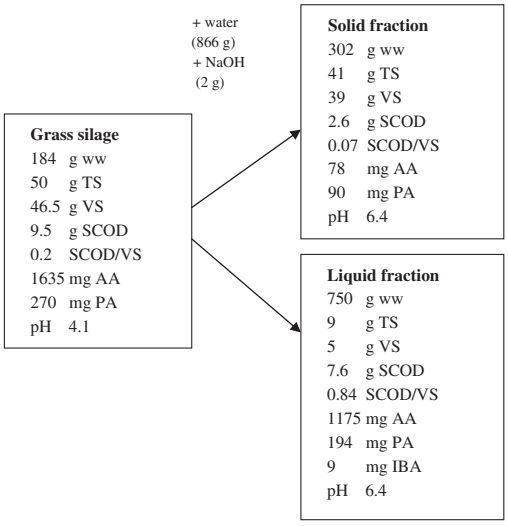

Fig. 1 – Mass balance and chemical composition of grass silage before and after NaOH pre-treatment and solid–liquid separation: TS, VS, SCOD, VFAs. AA = acetic acid, PA = propionic acid, IBA = isobutyric acid.

0.8 and 0.2 for the solid and liquid fractions, respectively. Then distilled water was added to produce a liquid volume of 750 ml, and $NaHCO_3$ (3 $g l^{-1}$) was added as buffer. Finally, the contents of the bottles were flushed with N_2 to remove O_2 from the headspace and sealed with butyl rubber stoppers. The CH_4 assays were incubated at 35 °C for 56 days.

In the H_2 assays 100 g of heat-treated inoculum and substrate (47.1, 77.3 and 192.0 g ww of grass silage, solid and liquid fractions, respectively) were added into the bottles. A substrate to inoculum VS:VS-ratio was 2:1 with grass silage and amounts of solid and liquid fractions were chosen as they were produced from pre-treating 47.1 g of grass silage. The substrate to inoculum VS:VS-ratios were 1.6 and 0.2 for the solid and liquid fractions, respectively. Distilled water was then added to produce a liquid volume of 650 ml and pH was adjusted to 6 with 5 M NaOH and 5 M HCl. The bottles were closed similarly as in the CH_4 assays. In the H_2 assays bottles were first incubated at 55 °C for 14 days, and then sampled (200 ml of the content for chemical analysis) after which 250 g of methanogenic inoculum (Table 1) was added to initiate the second-stage CH_4 assay. Subsequently, the contents of the bottles were flushed and closed as in the one-stage CH_4 assays and then incubated for 57 days at 35 °C.

In all assays control assays with inoculum (plus distilled water) only were carried out to determine the H_2 and CH_4 potentials of the inocula, which were subtracted from those of the substrates. In all CH_4 assays the biogas produced was collected in aluminium gas bags.

2.4. Analysis and calculations

TS, VS, pH, soluble chemical oxygen demand (SCOD), gas composition (H_2 and CH_4) and VFAs were analysed as described previously [28,29]. Gas volume in the CH_4 assays was measured using water displacement method.

The gas production potential (defined as H_2 or CH_4 potential) of the substrates is given as ml g^{-1} VS added minus the gas production of the inoculum. In the two-stage processes CH_4 potential was related to the amount of VS added at the beginning of the first stage, as VS loss in first stage was assumed to be minimal. In some H_2 assays, H_2 content in the gas phase peaked twice. In these cases only the higher value peak was used for calculating H_2 potential. The gas yields in assays incubated with NaOH pre-treated substrates were calculated by relating the amount of gas produced from treated fractions to the initial VS of grass silage (untreated)

and expressed as ml g^{-1} $VS_{original}$. In order to compare the pre-treated with untreated grass silage, the gas yield obtained from solid and liquid fractions was summed up (defined as combined gas yield). In H_2 assays with liquid fraction as substrate, data were presented from only one bottle as two of the replicate assays were observed to leak after 7 days of incubation.

3. Results

3.1. NaOH pre-treatment

Chemical composition of grass silage before and after solid–liquid separation of NaOH pre-treated grass silage (24 h, 20 °C) is presented in Fig. 1. Chemical analysis showed that grass silage had TS of 27% and VS of 23% with an SCOD/VS ratio of 0.2. After solid–liquid fractionation, about 84% of the original VS were retained in the solid fraction whereas most of the SCOD (80% of original SCOD) was transferred to liquid fraction (Fig. 1). Thus, a solid fraction with relatively high VS and low SCOD/VS (0.07) and a liquid fraction with low VS and high (0.84) SCOD/VS were obtained. Among the analysed VFAs, acetate was the main component in grass silage. Fractionation resulted in a liquid fraction retaining ca. 72% of the original acetate. pH of grass silage increased from 4.1 to 6.4 after NaOH pre-treatment and solid–liquid fractionation (Fig. 1, Table 1).

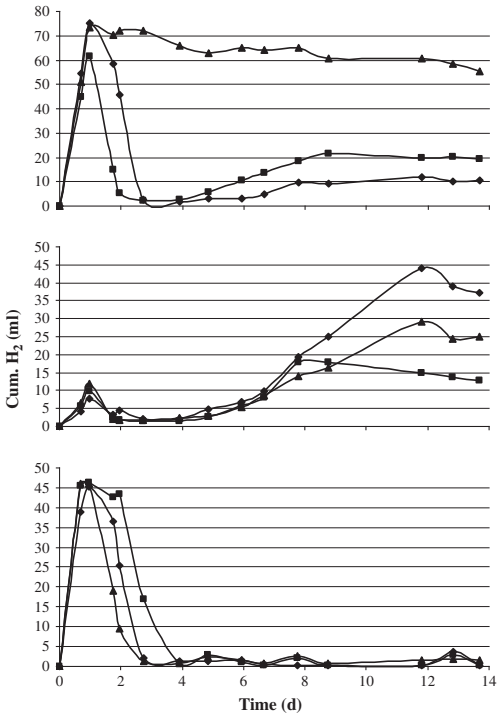

Fig. 2 – H_2 (◆, ■, ▲) production from grass silage (up), NaOH-treated solid (middle) and liquid fractions (down). Data from the triplicate assays.

3.2. One-stage H_2 or CH_4 versus two-stage H_2 and CH_4 production

In the H_2 assays, the H_2 production peaked within 1–2 days with all the studied substrates but H_2 was consumed within 2–3 days (Fig. 2). Secondary H_2 production was observed with silage and solid fraction as substrates although the replicates showed some variation especially with silage (Fig. 2). The mean H_2 potential (calculated as the maximum H_2 production during the experiment) of grass silage was 5.6 ml g^{-1} VS. The corresponding values for solid and liquid fractions were 3.4 and 31.1 ml g^{-1} VS, respectively.

Similarly, CH_4 production in the one-stage CH_4 assays also started immediately. Highest CH_4 potential of 703 ml g^{-1} VS was obtained from the liquid fraction followed by 430 ml g^{-1} VS from grass silage and 299 ml g^{-1} VS from the solid fraction (Table 2).

On comparison to one-stage process, two-stage process (H_2 and CH_4 assays) resulted in increased CH_4 yields by 8% for grass silage and 64% for solid fraction. The increase in CH_4 yields for grass silage was from 431 to 467 ml g^{-1} $VS_{original}$ and for solid fraction from 252 to 412 ml g^{-1} $VS_{original}$. On the other hand, CH_4 yield of the liquid fraction in two-stage process was lower (60 ml g^{-1} $VS_{original}$) compared with one-stage process (80 ml g^{-1} $VS_{original}$) (Table 2). CH_4 production of solid fraction was faster in two-stage process compared to one-stage process. The opposite was true with liquid fraction (Fig. 3).

At the end of the H_2 assays, pH was 4.8–5.0 in all assays except with the liquid fraction where it was 6.3. At the end of all the CH_4 assays, pH varied between 7.3 and 7.5 (Table 3). The final SCOD values were as high as 7.3 g l^{-1} with grass silage after the H_2 stage, whereas SCOD values between 2.4 and 3.5 g l^{-1} were detected after the one- and two-stage CH_4 assays (Table 3). In the H_2 assays, the final total VFA-CODs with substrates were 1810–4790 mg l^{-1}, contributing from 48 to 70% of the final SCOD (Table 4). Acetate was the main VFA (Table 4). After CH_4 assays no VFAs were detected. In the H_2 assays the amount of SCOD increased by 39 and 49% with grass silage and solid fraction as substrates. In contrast, with liquid fraction the amount of SCOD decreased by 43% during H_2 assay (Table 5).

3.3. Effect of NaOH pre-treatment on H_2 and CH_4 production

The calculated combined H_2 yield from NaOH-treated grass silage was higher (6.5 ml g^{-1} $VS_{original}$) than that of untreated grass silage (5.6 ml g^{-1} VS) with liquid fraction accounting for 56% of the total H_2 potential (Table 2). No CH_4 production was noticed in the H_2 assays except for the assays with liquid fraction, where CH_4 production was noticed after 4 days of incubation. The CH_4 potential obtained from the liquid fraction was 135 ml g^{-1} VS (Table 2). The calculated combined CH_4 yield of the NaOH pre-treated grass silage was 334 ml g^{-1} $VS_{original}$ and was lower than that of the untreated grass silage (430 ml g^{-1} VS) (Table 2).

Table 2 – H_2 and CH_4 production of the substrates in H_2 and CH_4 assays (standard deviation in parenthesis where applicable).

Substrate	H_2 assay		CH_4 assay (after H_2 stage)		CH_4 assay (without H_2 stage)	
	H_2 ml g^{-1} VS[a]	H_2 ml g^{-1} VS[b]	CH_4 ml g^{-1} VS[a]	CH_4 ml g^{-1} VS[b]	CH_4 ml g^{-1} VS[a]	CH_4 ml g^{-1} VS[b]
Grass silage	5.64 (0.63)	na	467 (18)	na	431 (3)	na
Solid fraction	3.38 (1.05)	2.85	490 (32)	413	299 (30)	252
Liquid fraction	31.14 (0.47)	3.61	520	60	703 (10)	82
Combined fractions	na	6.46	na	473	na	334

na = not applicable.
Liquid fraction started to produce CH_4 in H_2 assay on day 4, with cumulative CH_4 yield of 135 ml g^{-1} VS[a] and 16 ml g^{-1} VS[b] on day 14.
a Calculated per VS of the sample.
b Calculated per VS of the original grass silage (VS of the material before NaOH treatment or H_2 assay).

4. Discussion

The present results show that the application of two-stage anaerobic digestion with a thermophilic H_2 production as first stage and mesophilic CH_4 production as second stage can improve CH_4 yields compared with one-stage mesophilic CH_4 process. The increase in methane yields in two-stage process compared with one-stage process were 8 and 64% with grass silage (Scenario 2) and solid fraction, respectively (Fig. 4, Table 2). The higher methane yields in a two-stage compared with a one-stage process was attributed to the fact the thermophilic H_2 production stage apparently enhanced hydrolysis of the solid substrates and resulted in increased solubilisation and VFA production. This was evident from 1.4-fold and 2.8-fold increase in SCOD and acetic acid concentrations from grass silage after the first H_2 stage. The corresponding values for solid fraction were 1.5 and 36.5, respectively. These results are in accordance with previous studies [12,15–17], e.g. 21% more CH_4 was obtained from household solid waste from a two-stage H_2 and CH_4 process compared to a one-stage process [16]. With liquid fraction the effect of H_2 stage was the opposite, as SCOD concentration decreased by 43% during H_2 assay and CH_4 potential in second stage decreased to 193 ml g^{-1} VS compared to CH_4 potential of 700 ml g^{-1} VS in one-stage CH_4 assay. However, it should be noted that the CH_4 production of liquid fraction in two-stage process was still increasing and could have reached the CH_4 potential obtained in the one-stage process. Shorter H_2 stage for liquid fraction could have been better and short HRT (e.g. 2 days) have been used in H_2 assays in previous studies, e.g. see Ref. [16].

NaOH pre-treatment of grass silage and fractionation into solid and liquid fractions after treatment resulted in solid and liquid fractions of quite different chemical characteristics and gas production potentials. However, the alkaline treatment decreased the combined CH_4 yield (334 ml g^{-1} $VS_{original}$) compared to untreated grass silage (431 ml g^{-1} VS) which was especially due to low CH_4 potential of the solid fraction. Alkaline treatment is expected to break the lignocellulosic structure, swell the fibres and increase the pore size, thus improving hydrolysis, e.g. see Refs. [23–25], but, on the other hand, inhibiting aromatic degradation products can be formed, e.g. see Ref. [23], which can partially explain the low H_2 and CH_4 potentials of solid fraction in our study. Definitely the dose of alkali and conditions of the treatment (e.g. temperature) also play a major role in the hydrolysis, and they should be optimized to obtain stimulating effects on CH_4 potentials. However, it should also be noted that the effect of NaOH addition cannot be reliably interpreted as addition of water as such can also enhance hydrolysis. Moreover, control treatment without NaOH addition was not performed in this

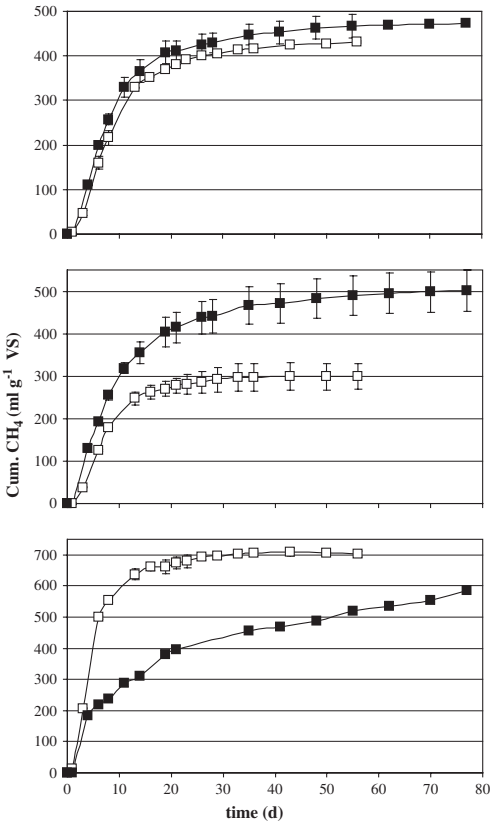

Fig. 3 – Cumulative CH_4 production (ml g^{-1} VS) from grass silage (up), NaOH-treated solid (middle) and liquid fractions (down) without (□) and with (■) H_2 stage.

Table 3 – pH and SCOD after the H_2 assays and after two-stage and one-stage CH_4 assays (standard deviation in parenthesis).

Substrate	H_2 assay		Two-stage and one-stage CH_4 assay	
	pH	SCOD ($g\,l^{-1}$)	pH	SCOD ($g\,l^{-1}$)
Inoculum	6.17 (0.27)	2.1 (0.38)	7.41 (0.01) 7.35 (0.06)	3.5 (0.23) 3.3 (0.15)
Grass silage	5.02 (0.05)	7.3 (0.23)	7.48 (0.02) 7.36 (0.02)	3.0 (0.1) 3.0 (0.23)
Solid fraction	4.77 (0.02)	3.6 (0.1)	7.43 (0.01) 7.40 (0.04)	2.9 (0.1) 2.4 (0.1)
Liquid fraction	6.29	3.8	7.52 (0.04) 7.47 (0.02)	3.4 (0.21) 2.4 (0.14)

experiment. Nevertheless, some studies have shown that alkalis such as NaOH can increase methane yields of, e.g. wheat straw [23], grass and sugar beet tops [26].

The results from the present study also showed that dark fermentative H_2 production from grass silage with heat-treated inoculum was possible. However, the H_2 yield (5.6 ml g^{-1} VS) was rather low compared to other studies with crop material, e.g. 170, 3.2, 57 and 147 ml $H_2\,g^{-1}$ VS were obtained from sugarcane [10], and untreated, NaOH- and HCl-treated cornstalk [7], respectively. One possible explanation for the rather low H_2 yield in the present study is the composition of the substrate. During ensiling, carbohydrates of the crop material are converted to fermentation products (e.g. lactic and acetic acid) which are not further degraded to H_2, and some H_2 can be lost during the ensiling process, e.g. see Refs. [30,31]. The slightly enhanced combined H_2 yield (from 5.6 to 6.5 ml g^{-1} $VS_{original}$) noticed with NaOH-treated grass silage in the present study was probably due to enhanced hydrolysis. However, high variation in H_2 production and consumption between the replicates with solid substrates was observed in the present study, similarly, as reported previously, e.g. see Ref. [32]. The possible reason for this variation could be the heterogeneity of the substrate and/or inoculum. In our study, both the liquid and solid fractions produced H_2. However, the H_2 production curves were not similar, as from the liquid fraction H_2 was produced within 2 experimental days, whereas H_2 production from grass silage and solid fraction peaked twice (Fig. 1), a phenomenon which has previously been reported in the case of acidified sludge [17] and food waste [33]. The observed H_2 consumption after the initial peaking (ca. day 3, Fig. 2) in the present study could be due to homoacetogenesis, where H_2 and CO_2 are converted to acetate, e.g. see Ref. [14]. This was evident from the high concentration of acetic acid (62–79% of VFA) at the end of the H_2 assays (Table 4). Thus, the probable homoacetogenesis in the assays suggests that homoacetogens survived the heat treatment and/or were introduced with non-sterilized substrate. In addition, also propionate fermentation consumes H_2 and produces propionate, acetate and valeriate, e.g. see Ref. [34] and might have occurred in the present study. However, it should be noted that VFA analysis at the end of the experiment cannot be used as a reliable indicator of H_2 production and consumption pathways, as H_2 is constantly produced and consumed during the assay with mixed culture. Therefore, continuous monitoring of VFAs during the experimental run would give more reliable data.

The fact that in the H_2 assays with liquid fraction CH_4 production started after H_2 production phase, despite the use of heat-treated inoculum could indicate that the applied heat treatment (boiling 30 min) to the inoculum was not capable of destroying all methanogenic activities. In fact methanogens have been shown to survive even 10 h at 105 °C [35]. The reason that CH_4 production was observed only in assays with liquid fraction could be due to its higher final pH (6.3) compared to solid fraction and grass silage, in which the final pHs were 4.7–5.0.

The results from the present study suggests that the highest (calculated) CH_4 yield from grass silage (495 ml g^{-1} $VS_{original}$) can be obtained, if grass silage is first pre-treated with NaOH and the solid fraction obtained after solid–liquid

Table 4 – VFA concentrations ($mg\,l^{-1}$) and proportion of individual VFAs of total VFAs at the end of the H_2 assays (standard deviation in parenthesis).

Substrate	Acetic acid		Propionic acid		Butyric acid		Isob. acid	Isoval. acid	Caproic acid	Total ($mg\,l^{-1}$)		% of SCOD
	$mg\,l^{-1}$	% VFA	$mg\,l^{-1}$	% VFA	$mg\,l^{-1}$	% VFA				VFA	COD	
Inoculum	398 (222)	79	32 (8)	6	32 (21)	7	15 (8)	25 (8)	0	502	608	29
Grass silage	2197 (179)	62	284 (28)	8	906 (30)	25	42 (2)	63 (2)	77 (9)	3569	4790	65
Solid fraction	1520 (90)	75	136 (58)	7	325 (39)	16	3 (5)	0	43 (9)	2028	2518	70
Liquid fraction	1027	70	299	20	48	3	40	58	0	1463	1811	48

Table 5 – Amount of SCOD, acetic and propionic acids at the beginning and at the end of H_2 assays, and their changes.

Substrate	SCOD (g)			AA (mg)			PA (mg)		
	Beginning	End	Change (%)	Beginning	End	Change (%)	Beginning	End	Change (%)
Inoculum	1.59	1.37	−14	26	259	880	–	21	–
Grass silage	2.43	3.38	39	419	1169	179	69	164	137
Solid fraction	0.66	0.98	49	20	729	3556	23	68	193
Liquid fraction	1.94	1.11	−43	301	409	36	50	174	250

Impact of the inoculum subtracted.

separation is incubated in two-stage process consisting of a thermophilic H_2 production as the first stage and mesophilic CH_4 production as the second stage. On the other hand, the liquid fraction could be used for one-stage CH_4 production directly. However, the increase in CH_4 yield (6–15%) has to be compared with the costs associated with the NaOH treatment and investment in the more complex two-stage process. Although H_2 production from grass silage was shown to be possible, the energy yields per hectare remained low compared to CH_4 production. According to our previous results only 0.5 MWh of energy was obtained from 1 ha of grass silage converted to H_2 [29], whereas 28–38 MWh ha^{-1} could be obtained if grass were converted to CH_4 [36]. Thus, if H_2 production is the main aim, then a more practical approach would be to produce CH_4 by a traditional anaerobic digestion process and then reform the produced CH_4 to H_2 [12]. In the future, if needed, also hythane could be produced from energy crops even though the process needs further optimization. Although alkaline treatment showed some improvement in H_2 yield in the present study, acid treatment could be also tested as previous studies involving acid treatments showed to be more efficient than alkaline treatments in improving

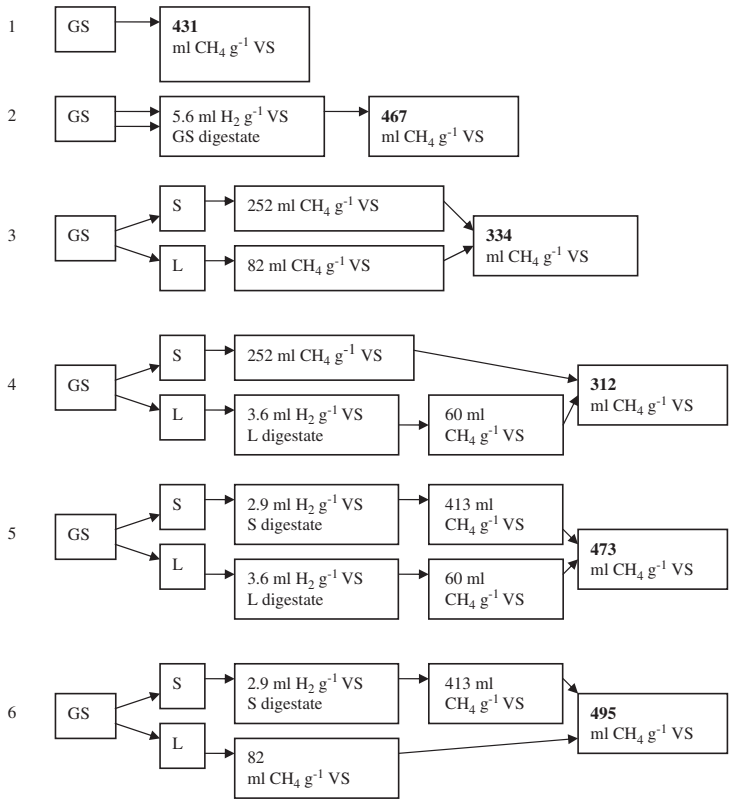

Fig. 4 – Six different H_2 and/or CH_4 energy scenarios from grass silage with and without NaOH pre-treatment. GS = grass silage, S = solid fraction from NaOH treatment, L = liquid fraction from NaOH treatment. Gas yields (ml g^{-1} VS) are calculated per original VS of untreated grass silage for easier comparison.

H_2 yields [7,17]. Alternatively, H_2 production from grass silage could be improved, e.g. through gas sparging of a reactor content [37].

5. Conclusions

- CH_4 yield from grass silage and solid fraction from NaOH treatment could be improved by 8 and 64% in a two-stage H_2 and CH_4 process compared to one-stage CH_4 production.
- NaOH pre-treatment of grass silage increased the combined H_2 yield by 15%, whereas the pre-treatment resulted in 23% decrease in the combined CH_4 yield.
- Both H_2 and CH_4 potentials (per VS of sample) of liquid fraction were higher than that noticed with solid fraction.
- Highest CH_4 yield (495 ml g^{-1} $VS_{original}$) could be obtained, if grass silage was first pre-treated with NaOH, and the separated solid fraction was digested in a two-stage (thermophilic H_2 and mesophilic CH_4) process while the liquid fraction could be treated in a one-stage CH_4 process directly.

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IV

HYDROGEN AND METHANE YIELDS OF UNTREATED, WATER-EXTRACTED AND ACID (HCL) TREATED MAIZE IN ONE AND TWO-STAGE BATCH ASSAYS

by

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Hydrogen and methane yields of untreated, water-extracted and acid (HCl) treated maize in one- and two-stage batch assays

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ABSTRACT

In the present study, two-stage H_2 and CH_4 production was compared with one-stage CH_4 production from maize subjected to water extraction and acid (HCl) treatment. In addition, the effect of duration (2 and 14 days) of the first-stage H_2 process on the H_2 yields and subsequent CH_4 yields from the second-stage was also investigated. Results showed that the average H_2 yields from untreated maize were 5.6 and 9.9 ml/g volatile solids added (VS_{added}) after 2 and 14 days, respectively. On the other hand, H_2 yields from water-extracted and HCl-treated maize were 18.0 and 20.5 ml/gVS_{added} (14 d), respectively. On comparison to one-stage CH_4 assays, the average increase in CH_4 yields from two-stage assays with 2 d H_2 stage were 7, 9 and 27% for untreated, water-extracted and HCl-treated maize, respectively.

1. Introduction

AD is a multi-step biological process with H_2 as a non-accumulating intermediate product. Recently, the interest in H_2 production through AD, also known as dark fermentative H_2 production, has increased [1–3]. This is due to the fact that the rates of H_2 production are rather high and a variety of feedstock can be used as a substrate. In traditional AD, H_2 is not detected as it is consumed immediately e.g. by hydrogenotrophic methanogens to produce CH_4 and CO_2. On the other hand, H_2 can be produced separately by engineering the process conditions. However, the main limitation of dark fermentative H_2 production is the rather low energy recovery. In order to completely utilize the organic acids produced during dark fermentation and improve the overall energy conversion efficiency, a two-stage AD concept consisting of hydrogenic process followed by methanogenic process has been suggested [1,4].

The application of a two-stage AD process for sequential H_2 and CH_4 production has been proposed as a promising technology for better process performance and higher energy yields as compared to the traditional one-stage CH_4

Abbreviations: AA, acetic acid; AD, anaerobic digestion; BA, butyric acid; CH_4, methane; CO_2, carbon dioxide; d, day; FID, flame ionization detector; H_2, hydrogen; HSW, household solid waste; kWh, kilowatt-hour; MWh, Megawatt-hour; PA, propionic acid; SCOD, soluble chemical oxygen demand; TCD, thermal conductivity detector; TS, total solids; TVS, total volatile solids; TVFA, total volatile fatty acids; VFA, volatile fatty acids; VS, volatile solids; ww, wet weight.

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production process [5,6]. In a two-stage AD system, relatively fast growing acidogens and H_2-producing microorganisms are developed in the first-stage hydrogenic reactor and are involved in the production of VFA and H_2. On the other hand, the slow growing acetogens and methanogens are developed in the second-stage methanogenic reactor, in which the produced VFA are further converted to CH_4 and CO_2 [7]. In a previous study, two-stage H_2 and CH_4 production in a batch process has shown to improve the CH_4 yield from grass silage by 8% compared to a one-stage CH_4 batch process [8]. Similarly, 21% more CH_4 was obtained from a two-stage than one-stage system loaded with household solid waste [9]. However, the duration of the H_2 process in the first-stage has shown to influence the H_2 yields e.g. with a pure culture of *Ruminococcus albus* [10] and, presumably, the CH_4 yields from the subsequent second-stage methanogenic reactor. For instance, a longer residence time may be needed for lignocellulosic materials in order to enhance hydrolysis and acidogenesis. However, the obtainable CH_4 yields in the subsequent CH_4 stage can be reduced if the duration of the H_2 stage is too long. Nevertheless, a short H_2 stage could increase the loading potential and thus reduce the operational costs.

Lignocellulosic biomass is an abundant and renewable feedstock that is increasingly used for biofuels, chemicals and power generation [11,12]. Moreover, H_2 production from biomass has been extensively reviewed in the literature [13,14]. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin, and is considered as an ideal feedstock for dark fermentative H_2 production due to its high carbohydrate content, the preferred substrate for H_2 production [2]. However, direct utilization of biomass by microorganism is very slow due to its heterogeneity and high degree of polymerization and crystallinity. Therefore, pre-treatment of lignocellulosic biomass is essential in order to increase the carbohydrate availability and hence H_2 and CH_4 yield [15–17]. Several pre-treatment methods viz., physical, chemical or biological have shown to enhance the biodegradation of the lignocellulosic biomass [18]. Among the different pre-treatments, water extraction alone can enhance the hydrolysis of lignocellulosic biomass. For instance, free sugars can be extracted from sweet sorghum stalks by using water at 30 °C [10]. Similarly, alkalis such as NaOH and $Ca(OH)_2$ have also shown to improve hydrolysis [18] as well as H_2 [8] and CH_4 [19] production from the biomass. In an earlier study, pre-treatment of grass silage with NaOH resulted in a 15% increase in H_2 yield compared to untreated material [8]. In addition to alkali, acids such as HCl, can be used to hydrolyze cellulose to glucose [20] and improve solubility of hemicelluloses and thus enhance anaerobic degradation at ambient or moderate temperatures [17]. HCl-treated corn stalk resulted in higher H_2 yields of 150 ml/gTVS compared to 3 ml/gTVS obtained with untreated material [21]. Furthermore, H_2 yields obtained from HCl-treated beer lees and wheat straw wastes were roughly 9 and 136-times more than those obtained from their respective untreated substrates [15,16].

Among the energy crops, maize is increasingly used as feedstock for CH_4 production, especially in Germany and Austria, due to its high biomass yield [12]. Maize has proven to be a potential energy crop for biomethanation even in southern Finland with a maximum energy yield of 90 MWh per hectare [22]. Research on the production of H_2 from maize and its derivatives is still at an early stage. By using a mixed microflora, H_2 yields of 62 ml/gTS from fodder maize without any pre-treatment has been reported in the literature [23]. To our knowledge, H_2 and CH_4 production from pre-treated maize in a two-stage sequential AD process by using a mixed anaerobic culture has not been studied so far. The objective of the present study was to evaluate the effects of water extraction and acid (HCl) treatments on H_2 and CH_4 production from maize in one-stage (H_2 or CH_4) and two-stage (H_2 and CH_4) batch assays. In addition, the effect of duration (2 d and 14 d) of the first-stage H_2 process on the CH_4 yields from the second-stage was also investigated. Finally, the gross energy production from one-stage (CH_4 or H_2) and two-stage processes (H_2 and CH_4) was estimated.

2. Materials and methods

2.1. Substrates

Maize (variety Cerruti) was obtained from a farm in Laukaa, Finland. At the laboratory, the whole maize crop (including stem, leaves and corn) was chopped with a garden chopper to a particle size of 1–2 cm and dried at 60 °C for 24 h. The dried maize was stored at room temperature (20 °C) for 6 weeks (Table 1). Prior to the start of the experiment, dried maize was cut to a particle size of 0.5–1 cm. Inoculum was obtained from a farm-scale biogas reactor treating cow manure and confectionary by-products (Table 1). For the H_2 assays, inoculum was heat-treated by boiling for 30 min in order to inactivate methanogens and to enrich spore-forming H_2-producers.

2.2. Water extraction and acid pre-treatment

Water extraction and acid pre-treatments were performed in 1 L glass bottles. To each assay, 32.7 g ww of maize sample (TS of 91.8% and VS of 86%, thus corresponding to 30 gTS and 28.1 gVS, respectively) and 297 ml of distilled water were added to obtain a TS concentration of 10%. In order to obtain water-extracted material, one of the two replicate bottles was

Table 1 – Characteristics of inoculum, maize and solid and liquid fractions from water extraction and HCl treatment.

Substrate	pH	TS (%)	VS (%)	SCOD (g/l)
Untreated maize	6.29	91.8	86.0	211[a]
Water extraction				
Solid fraction	5.46	13.2	12.7	[b]
Liquid fraction	5.46	2.6	2.2	21.1
HCl-treatment				
Solid fraction	4.53	12.0	11.4	[b]
Liquid fraction	4.53	2.7	2.3	18.1
Inoculum	7.60	6.0	4.8	3.7
Heat–treated inoculum	9.19	6.4	5.1	4.4

a unit mg/gTS.
b not applicable.

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incubated as such. For acid pre-treatment, 0.6 ml of HCl (37%) was added to obtain a concentration of 2% HCl/gTS. Prepared bottles were mixed in an orbital shaker for 24 h at 20 °C. Thereafter, the water-extracted (pH of 5.46) and HCl-treated materials (pH of 4.53, Table 1) were sieved through a metallic sieve (pore size approximately 1 mm) by gentle manual pressure into solid (TS of 13.2% and 12.0% with water-extracted and HCl-treated, respectively) and liquid fractions. For batch assays, the solid and liquid fractions were combined in the ratios that were actually generated during the pre-treatments.

2.3. Batch experiments

H_2 and CH_4 yield of the substrates were determined in batch assays by using 118 ml serum bottles. To each assay, inoculum (20 ml in CH_4 assays and 12 ml in H_2 assays) and subsequently substrate were added. The substrate to inoculum VS-ratio used was 1 for CH_4 assays and 2 for H_2 assays. Distilled water was added to obtain a working volume of 60 ml in CH_4 assays and 78 ml in H_2 assays. The pH was adjusted to 7 (CH_4 assays) and 6 (H_2 assays) with 5 M NaOH and 5 M HCl. $NaHCO_3$ (0.2 g) was added as a buffer in CH_4 assays only. The reactor contents were flushed with nitrogen gas and sealed with butyl rubber stoppers and aluminium crimps. Assays with inoculum only (with distilled water) were used as control. The prepared CH_4 assays were incubated statically in triplicate at 35 °C for 77 days. On the other hand, H_2 assays were first incubated at 55 °C either for 2 d or 14 d. Upon completion of H_2 experiments, the assays were opened and well homogenized samples were drawn for chemical analysis (38 ml). The assays were re-inoculated with 20 ml of methanogenic inoculum (Table 1). The prepared assays were flushed with nitrogen gas once again and continued incubation at 35 °C to obtain a total incubation time of 77 d (75 d and 63 d after 2 d and 14 d H_2 assays, respectively).

2.4. Analysis and calculations

TS, VS, pH, SCOD and VFA were analysed as previously described [24,25]. CH_4 content in the methanogenic assays was analysed with a GC (Perkin Elmer Arnel Clarus 500 GC) equipped with a FID (Perkin Elmer Alumina column 30 m*0.53 mm, carrier gas argon, oven 100 °C, detector 225 °C and injector 250 °C). Gas composition (H_2, CH_4 and CO_2) in hydrogenic assays was analysed with the same GC equipped with a TCD (Supelco Carboxen™ 1010 PLOT fused silica capillary column 30 m*0.53 mm, carrier gas argon, oven 200 °C, detector 230 °C and injector 225 °C). In H_2 assays, gas composition was analysed twice per day during the first 2 days and once per day thereafter. Over pressure was released through a water lock system after every measurement and gas composition was analysed before and after each pressure release.

In batch assays, gas yields of the substrates were calculated as the amount of gas produced in millilitres per added gVS (ml/gVS_{added}). Gas produced from the control assays was subtracted from the sample assays. With pre-treated maize, the amounts of solid and liquid fractions that were generated during the pre-treatment were used in the batch assays. The gas yields from the pre-treated maize were thus related to the VS of untreated maize. In the two-stage processes, CH_4 yields were calculated as the amount of VS added at the start of the H_2 stage. All results were converted to standard conditions ($T = 273$ K, $p = 1$ bar).

The data were subjected to analysis of variance (ANOVA) using the SPSS program [26]. Dunnett t-test was used to compare all other treatments against control if the F-test was significant at $P \leq 0.05$. Before performing ANOVA, data were subjected to Welch's test to evaluate the homogeneity of variance.

3. Results and discussion

3.1. H_2 production from maize

H_2 production from untreated, water-extracted and HCl-treated maize was studied in batch assays for 2 and 14 d (Table 2, Fig. 1). The produced biogas was composed of H_2 and CO_2, and was free of CH_4. After 2 d of incubation, average H_2 yields of 5.6 and 1.9 ml/gVS_{added} were obtained from untreated and water-extracted maize, respectively ($P < 0.05$). On the contrary, no H_2 was produced from HCl-treated maize, indicating that the process was inhibited. After 14 d of incubation, the highest average H_2 yield of 20.5 ml/gVS_{added} was obtained from HCl-treated maize, followed by water-extracted (18.0 ml/gVS_{added}) and untreated maize (9.9 ml/gVS_{added}) (Table 2). However, no significant difference in H_2 yields was noticed between the treatments ($P > 0.05$). On the other hand, the interpretation of the H_2 assays was indeed more challenging as some variation in H_2 yields between the replicate bottles was observed even during the 14 d of incubation (Fig. 1). Similar type variation has been reported also previously [8,27].

The present data shows that several days of incubation time e.g. from 6 to 14 d was needed for major H_2 production from the studied materials (Fig. 1). The observed lag phase in the present study was apparently due to the use of unadapted inoculum and/or inoculum containing low amounts of H_2 producers. Low or negligible H_2 production during the initial 2 d indicated that the H_2 yield was from the lag phase or initial growth rate of the population. Furthermore, initial inhibitory effect of HCl-treatment was evident. A similar observation was also reported earlier where H_2 production from HCl-treated poplar leaves was initially inhibited but the final H_2 yield was however higher than that obtained from untreated poplar leaves [28]. In addition, hydrolysis is considered as the rate limiting step during the degradation of lignocellulosic materials. For instance H_2 production from sorghum continued for 12 d and the authors suggested that hydrolysis was the limiting step in H_2 production from cellulosic and hemicellulosic materials [10].

The reason for the variation in H_2 yields might be due to the low adaptation of the inoculum, presence of relatively low number of H_2-producing bacterial species in the heat-treated inoculum [29], the heterogeneity of the substrate and/or inoculum used and the several pathways for H_2 production/consumption. In the present study, inoculum was heat treated at the normal boiling temperature of water, which has recently been shown to decrease the species diversity and H_2

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Table 2 – H_2, CH_4 and energy yields of untreated and pre-treated maize in one- and two-stage batch assays as well as pH, SCOD and main metabolic products after H_2-stage. Standard deviation in parenthesis when applicable. Conversion factors of 3 and 10 kWh per 1 Nm^3 have been used for H_2 and CH_4, respectively.

		Untreated	Water-extracted	HCl-treated
2 d H_2-stage	pH	4.85	5.28	5.19
	SCOD	4.83 (0.76)	4.50 (0.35)	5.00 (0.30)
	H_2 (ml/gVS_{added})	5.6 (0.7)	1.9 (0.8)	0.0
	Energy (kWh/tVS)	16.7 (2.0)	5.8 (2.5)	0
	TVFA (mg/l)	122	634	368
	TVFA/SCOD (%)	3	21	9
	AA (mg/l), % of TVFA	94 (29), 77	275 (8), 43	288 (35), 78
	PA (mg/l), % of TVFA	15 (0), 12	64 (2), 10	21 (1), 6
	BA (mg/l), % of TVFA	5 (4), 4	274 (390), 43	8 (8), 2
+ CH_4 stage	CH_4 (ml/gVS_{added})	342 (8)	358 (37)	397 (5)
	Energy (kWh/tVS)	3422 (81)	3577 (371)	3970 (50)
	Energy total (kWh/tVS)	3438 (63)	3582 (321)	3970(50)
14 d H_2-stage	pH	4.30	5.15	5.02
	SCOD	3.70 (0.35)	3.70 (0.35)	4.60 (0.20)
	H_2 (ml/gVS_{added})	9.9 (8.0)	18.0 (12.6)	20.5 (11.1)
	Energy (kWh/tVS)	29.7 (24.1)	53.8 (37.8)	61.4 (33.2)
	TVFA (mg/l)	454	1196	1007
	TVFA/SCOD (%)	18	47	34
	AA (mg/l), % of TVFA	201 (124), 44	562 (504), 47	346 (138), 34
	PA (mg/l), % of TVFA	17 (3), 4	63 (18), 5	26 (3), 3
	BA (mg/l), % of TVFA	226 (165), 50	544 (324), 45	580 (261), 58
+ CH_4 stage	CH_4 (ml/gVS_{added})	311 (38)	357 (47)	368 (41)
	Energy (kWh/tVS)	3110 (378)	3576 (471)	3684 (411)
	Energy total (kWh/tVS)	3140 (328)	3630 (366)	3746 (357)
One-stage CH_4	CH_4 (ml/gVS_{added})	321 (23)	328 (7)	312 (15)
	Energy (kWh/tVS)	3210 (230)	3280 (70)	3120 (151)

yield compared to lower (e.g. 65 °C) treatment temperatures [29]. Nevertheless, use of adapted microbial consortia could probably even greatly improve the H_2 production rates and yields as supposed by a study in which repeated batch cultivation were performed on household solid waste [27]. In the above study, H_2 yield of 84 ml/gVS_{added} was obtained in 15 d with first generation, whereas 170 ml/gVS_{added} was obtained after 4 d of incubation with fifth generation culture.

Water extraction and HCl treatment enhanced the average H_2 yields from maize from 14 d assays. However, the difference was statistically insignificant due to rather high variation between the replicates ($P > 0.05$). On comparison to untreated maize yields, the average increase in H_2 yields were 1.8 times for water extraction and 2 times for HCl-treatment. During the HCl pre-treatment, hydrolysis can be promoted by partial removal of hemicellulose or lignin and thus resulting in an increase in the amount of soluble sugars. Previously, an almost 50-fold increase in H_2 yield was obtained from HCl-treated corn stalk (150 ml/gTVS) compared to untreated stalks [21]. The increase in H_2 yield was mainly attributed to the increased amount of soluble sugars after HCl treatment [21]. The increase in H_2 yield following water extraction was apparently due to the enhanced hydrolysis and acidogenesis. This was evident from the decrease in pH from 6.3 to 5.5 after water extraction. Water incubation in slightly acidic (initial pH 5) conditions has shown to improve hydrolysis of grass [19]. In addition, liquid hot water pre-treatment is known to remove hemicelluloses and make cellulose more accessible [18]. Ntaikou et al. [10] also extracted free sugars from sweet sorghum stalks by using water at 30 °C. In the above study, H_2 yield of 2.5 mol H_2/mol glucose was obtained from the sorghum water extract, which mainly contained sucrose. This H_2 yield was similar to the yield obtained from glucose [10].

Table 2 presents the pH levels in H_2 assays at the end of 2 d and 14 d of incubation. The pH decreased due to fermentation from 6 to 4.9 (2 d assay) and to 4.3 (14 d) in assays with untreated maize as a substrate. This indicates that the pH, especially with untreated maize, was below the optimum pH of 5.5 required for H_2 production [30]. This low pH clearly demonstrates that the conditions were not optimal for H_2 production. Therefore, with proper control of pH, H_2 yield from maize could probably be improved. Moreover, the effect of pre-treatments on H_2 yield can be unreliable, as the low final pH in untreated maize assays may have led to low H_2 yield. In addition, substrate concentration might have been too high and may have also resulted in low pH, which in turn may have underestimated the obtainable H_2 yield in this study. Previous studies have shown that substrate concentration has a profound effect on H_2 yields. For instance, H_2 yield from HSW decreased from 84 to 24 ml/gVS_{added} when the substrate concentration was increased from 1 to 2 g/l [31]. In addition, the headspace volume in the present study has apparently been too low (40 ml as compared to liquid volume of 78 ml) for efficient H_2 production, and inhibition due to increased partial pressure of H_2 has probably occurred. To be able to obtain more reliable results, larger headspace volume should be used. Previously, H_2 yields of 16, 20 and 62 ml/gTS have been reported from untreated maize leaves [31], corn stalk [32] and fodder

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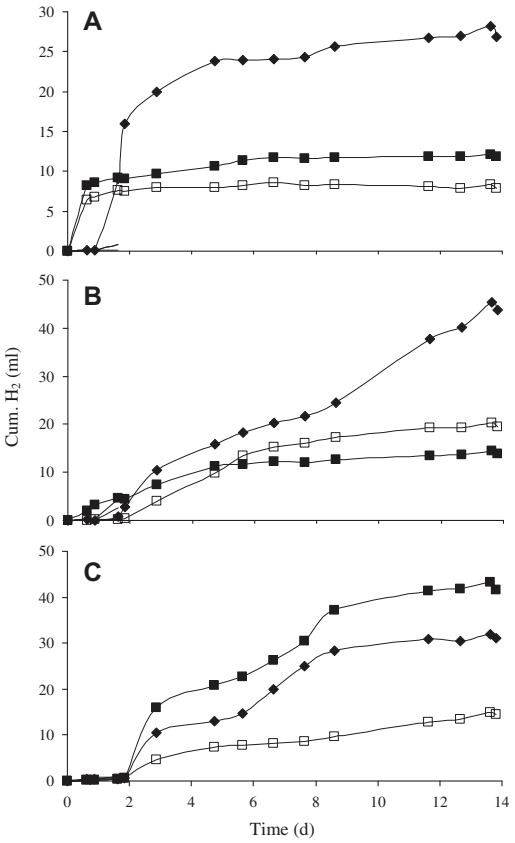

Fig. 1 – Cumulative H_2 production from untreated (A), water-extracted (B) and HCl-treated (C) maize in 14 d H_2 assays (replicates shown). Note the different scale in y-axis. Results from 2 d assays shown with lines only.

maize [23], respectively. Reasons for this variation in H_2 yields from maize in the literature are e.g. differences in operational conditions (e.g. pH, temperature, loading and headspace volume), inoculum and chemical composition of maize.

The concentrations of the main metabolic products are presented in Table 2. The dominant metabolic products were acetic acid and butyric acid. The concentration of VFA varied with incubation period and pre-treatment method. In 2 d H_2 assays, the predominant VFA was acetic acid in the untreated (77% of total VFA) and HCl-treated maize (78% of total VFA), while more or less equal concentration of acetic and butyric acids were present in the water-extracted maize (43% of total VFA). Average acetic acid concentrations in the HCl-treated maize (288 mg/l) and water-extracted maize (275 mg/l) were approximately the same (Table 2). In 14 d H_2 assays, the share of butyric acid increased in all assays, being highest in the HCl-treated maize assays (58% of total VFA). The concentration of butyric acid (580 mg/l) in the HCl-treated maize was 1.6 times higher than that of acetic acid. On the other hand, the concentration of propionic acid remained unchanged with increase in incubation time in the respective assays. However, the highest propionic acid concentration was observed in the water-extracted maize (63 mg/l) compared to that of HCl-treated maize (21–26 mg/l) or untreated maize (15–17 mg/l).

3.2. *CH_4 production from maize*

The results from one-stage and two-stage CH_4 production are presented in Table 2 and Fig. 2. Methane production started immediately in all assays. In one-stage CH_4 assays, CH_4 yield of 321 ml/gVS_{added} was obtained from untreated maize. This yield was in the same range as that reported from different maize hybrids [33]. However, no significant difference in CH_4 yield was obtained with the studied pre-treatments ($P > 0.05$). The average CH_4 yields were 328 and 312 ml/gVS_{added} for water-extracted and HCl-treated maize, respectively.

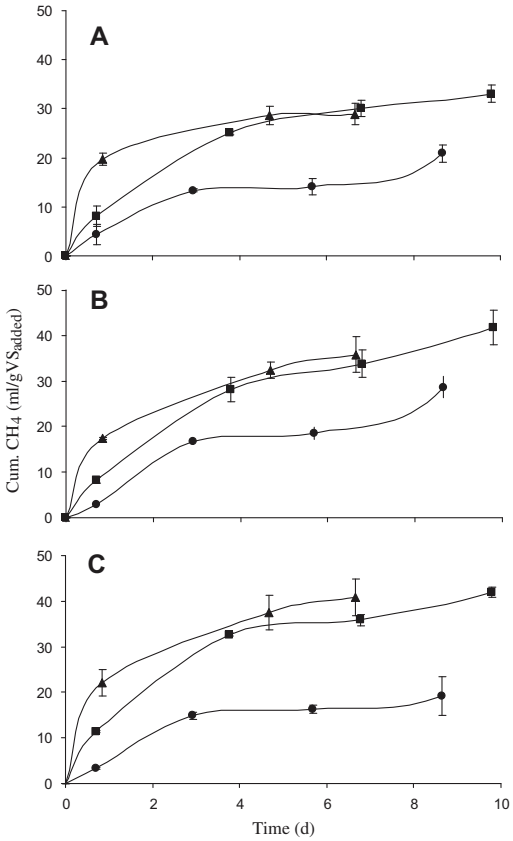

Fig. 2 – Initial cumulative CH_4 production (ml/gVS_{added}, inoculum subtracted) from untreated (A), water-extracted (B) and HCl-treated maize (C) in one-stage CH_4 (●) assays and in CH_4 assays after two (■) and 14 days (▲) H_2 stage. The trend line is representative of the whole CH_4 production batch test.

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Results from the two-stage process (Table 2) showed that the hydrogenic stage could improve the average CH_4 yields in the second stage. The highest CH_4 yield of 397 ml/gVS_{added} was obtained with HCl-treated maize. The average increase in CH_4 yields were 24% and 27% compared to the CH_4 yields obtained from untreated (321 ml/gVS_{added}) and HCl-treated (312 ml/gVS_{added}) maize in one-stage processes. This yield was statistically significant when compared to that obtained with untreated and HCl-treated maize in one-stage assays ($P < 0.05$). The increase in methane yields in the two-stage assays with the H_2 stage was probably due to the fact that hydrolysis and acidogenesis were improved thereby promoting the production of VFA which were subsequently converted into CH_4 in the methanogenic reactor. On comparison to 2 d H_2 stage, 14 d incubation period resulted in either decreased CH_4 yields (untreated and HCl-treated maize) or did not further improve (water-extracted) the CH_4 yields (Table 2). This was evident from the decrease in the SCOD levels with increase in duration of H_2 stage from 2 to 14 d, although H_2 yields and the amount of VFA typically increased with increase in retention time (14 d). However, due to the higher amount of VFA, the two-stage process with 14 d H_2 stage showed higher initial CH_4 production rates compared with the two-stage process with 2 d H_2 stage or one-stage CH_4 process (Fig. 2). However, the conversion of VFAs of first stage to CH_4 in the second stage accounted only 1–12% of the total CH_4 yield, thus suggesting, that hydrolysis and acidogenesis in the first stage were sub-optimal. The slightly higher methane production rates in two-stage than in one-stage assays during the initial 10 days indicate that the readily available VFA, produced during the hydrogenic stage of the two-stage assays, was converted rapidly into methane while hydrolysis was rate limiting in one-stage assays. This trend however reversed during the next 20–25 days. The reason for the low and steady methane production rates during days 10–40 in two-stage assays might be due to the time required for the further hydrolysis of the less degradable material and/or limitations in conversion of solubilized COD during several steps of anaerobic degradation. Nevertheless, the higher methane production rates in two-stage than in one-stage assays, especially in pre-treated assays at the end indicate that pretreatments improved the hydrolysis. Thus, these results were in agreement with previous studies [6,8,9] which showed that hydrolysis and acidogenesis in the first stage can be enhanced by low pH and high temperature leading to an elevated digestion efficiency [34] and CH_4 yields in the second stage. The gas yields obtained from the two-stage process with 14 d H_2 stage were 9.9–20.5 ml/gVS_{added} of H_2 and 311–368 ml/gVS_{added} of CH_4. The corresponding values from the two-stage process with 2 d H_2 stage were 0–5.6 ml/gVS_{added} of H_2 and 342–397 ml/gVS_{added} of CH_4 (Table 2).

In the present study, the highest CH_4 yields (397 ml/gVS_{added}) and calculated energy yields (3970 kWh/tVS_{added}) from maize were obtained when maize was first subjected to HCl-treatment and then digested in a two-stage process consisting of a short (2 d) thermophilic H_2 stage followed by a mesophilic CH_4 stage (Table 2). On the other hand, the highest energy yield from H_2 production alone was significantly lower (61 kWh/tVS_{added}) than that obtained from CH_4 production. However, it should be noted that the results in the present study were obtained from batch experiments and cannot be extrapolated to large-scale continuous process. In practice, the increase in CH_4 yields have to be balanced with the costs for chemical pre-treatment, additional equipment and higher investment and operational costs of two-stage processes. Nevertheless, this short first H_2 stage could probably be embedded in a current pre-treatment and/or the mixing tank in agricultural biogas reactors. However, more research would be needed especially for optimizing the hydrogenic first stage to improve both H_2 and VFA yields.

4. Conclusions

The study showed that two-stage process consisting of a short H_2 (2 d) stage could improve CH_4 yields by 7–27% than from one-stage CH_4 assays. In addition, initial methane production was faster when compared to CH_4 production in one-stage assays. The average H_2 yields from untreated, water-extracted and HCl-treated maize were 9.9, 18.0 and 20.5 ml/gVS_{added}, respectively. However, due to suboptimal conditions (pH decreased due to high substrate concentration) and low adaptation of the inoculum, the substrate conversion in H_2 assays was not maximal and resulted in low H_2 yields as well as variation between replicates. The pre-treatments (water extraction and HCl-treatment) applied in the present study resulted in no statistically significant differences in one-stage H_2 (14 d) or CH_4 yields. According to present study, highest CH_4 yield (397 ml/gVS_{added}) from maize could be obtained if maize was first pre-treated with HCl and then digested in two-stage process.

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V

THE EFFECT OF ORGANIC LOADING RATE AND RETENTION TIME ON HYDROGEN PRODUCTION FROM A METHANOGENIC CSTR

by

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The effect of organic loading rate and retention time on hydrogen production from a methanogenic CSTR

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ABSTRACT

The possibility of shifting a methanogenic process for hydrogen production by changing the process parameters viz., organic loading rate (OLR) and hydraulic retention time (HRT) was evaluated. At first, two parallel semi-continuously fed continuously stirred tank reactors (CSTR) were operated as methanogenic reactors (M1 and M2) for 78 days. Results showed that a methane yield of 198–218 L/kg volatile solids fed (VS_{fed}) was obtained when fed with grass silage at an OLR of 2 kgVS/m^3/d and HRT of 30 days. After 78 days of operation, hydrogen production was induced in M2 by increasing the OLR from 2 to 10 kgVS/m^3/d and shortening the HRT from 30 to 6 days. The highest H_2 yield of 42 L/kgVS_{fed} was obtained with a maximum H_2 content of 24%. The present results thus demonstrate that methanogenic process can be shifted towards hydrogen production by increasing the OLR and decreasing HRT.

1. Introduction

AD of solid substrates is a multi-step process, in which organic polymers are first degraded into soluble monomers during the hydrolysis step e.g., cellulose is degraded to glucose units. During the acidogenesis step, these soluble compounds are further degraded to e.g., VFA, H_2 and CO_2. Volatile fatty acids (e.g., propionic and butyric acids) are then degraded by acetogenic bacteria to acetic acid and H_2, which are used as substrates during the methanogenesis (Demirel and Scherer, 2008; Valdez-Vazquez and Poggi-Varaldo, 2009). In the traditional AD process, biogas is mainly composed of methane (50–70%) and carbon dioxide (30–50%) with some traces of H_2S and water vapor. Although H_2 is produced during the AD process, it is not detected in the biogas as it is consumed immediately by the hydrogen consuming bacteria e.g., methanogens, homoacetogens and sulphate-reducing bacteria (Valdez-Vazquez and Poggi-Varaldo, 2009).

In Europe, energy crops are commonly co-digested with animal manure for methane production. However, monodigestion of energy crops in different reactor configurations viz., one- or two-stage have been also studied (e.g., Koch et al., 2009; Lehtomäki et al., 2008) and applied in full-scale plants (Resch et al., 2008) despite the possible drawbacks associated with the nutrient deficiency and lack of buffer capacity (Koch et al., 2009). Besides methane production, hydrogen production from energy crops through dark fermentation has been shown to be possible (Pakarinen et al., 2008, 2009). However, research on hydrogen production from energy crops in continuous experiments is limited.

Previously, several studies have shown that H_2 production in addition to CH_4 production is possible from a wide variety of feedstocks by adjusting the process parameters and/or by inactivating H_2-consuming bacteria (Chong et al., 2009; Valdez-Vazquez and Poggi-Varaldo, 2009). Typically in batch hydrogen production, inoculum is generally heat-treated to inhibit H_2-consuming bacteria e.g., methanogens (Davila-Vazquez et al., 2008). However, heat-treatment is energy intensive and H_2 consuming bacteria can be introduced along with the substrate in a continuous process. Recently, it has been shown that heat-treatment is not necessary to facilitate hydrogen production (Ohnishi et al., 2010). On comparison to methanogenic process, process parameters viz., shorter HRT, higher OLR and lower pH (e.g., 5–6) are favored for hydrogen production. Thus, simplest and most economic method for methanogen inhibition could be biokinetic control, mainly through utilization of low pH (Valdez-Vazquez and Poggi-Varaldo, 2009). In practical applications, hydrogen producing system should be easily established and thus shifting the ongoing (typically methanogenic) process to hydrogen production could be an interesting opport unity.

The objective of this study was to evaluate the possibility of shifting the ongoing methanogenic process to hydrogen production

Abbreviations: AD, anaerobic digestion; CSTR, continuously stirred tank reactor; FM, fresh matter; HRT, hydraulic retention time; OLR, organic loading rate; SCOD, soluble chemical oxygen demand; TS, total solids; TVFA, total volatile fatty acids; VFA, volatile fatty acid; VS, volatile solids.

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by increasing the OLR and shortening the HRT. At first, two parallel mesophilic (35 °C) CSTRs were operated with an OLR of 2 kgVS/m^3/d and HRT of 30 days with grass silage as a substrate. After 79 days of operation, the OLR of one of the two reactors was increased to 10 kgVS/m^3/d and HRT was shortened to 6 days in order to inhibit H_2-consuming methanogens and facilitate hydrogen production.

2. Methods

2.1. Substrate and inoculum

Grass silage (mixture of timothy and meadow fescue) stored in a silo for about 5 months (Kalmari farm, Laukaa, Middle-Finland) was used as substrate. At the laboratory, grass silage was packed in small plastic bags and stored at −20 °C before further use. Before each feeding, the substrate was cut to a particle size of ca. 0.5 cm with a coffee bean grinder (Krups).

Inoculum was obtained from a mesophilic farm-scale reactor treating cow manure and confectionary by-products. The characteristics of substrate and inoculum are presented in Table 1.

2.2. Reactor experiments

CH_4 production from grass silage was studied in two parallel semi-continuously fed CSTRs (M1 and M2) with a total volume of 2 L at 35 °C. During the start-up, reactors were filled with 1500 mL of inoculum (working volume) and flushed with N_2 for 5 min to ensure anaerobic conditions. Semi-continuous feeding was initiated 14 days after the start-up and considered as day 1 of the experimental period. Reactors were fed manually once on every weekday (Monday through Friday) with a plastic syringe. Digestate was removed just prior to the each feeding. The amount removed was about 5–10% less than the daily feed volume in order to maintain the constant working volume. The reactors were mixed continuously using a magnetic stirrer (300 rpm).

During the whole run, feed TS and VS was maintained at 6.6% and 6.2%, respectively, by diluting 7.4 gFM of grass silage (3.3 gTS or 3.1 gVS) with 43 g of water (days 1–27) or nutrient solution (day 28 onwards). Nutrient solution contained (mg/kg feed) 1183 NH_4Cl, 1056 K_2HPO_4, 422 $MgSO_4$, 42 $CaCl_2{\cdot}2H_2O$, 8.45 $FeCl_2{\cdot}4H_2O$, 0.21 H_2BO_3, 0.21 $ZnCl_2$, 0.21 $NiCl_2{\cdot}6H_2O$, 0.16 $CuCl_2{\cdot}2H_2O$, 2.11 $MnCl_2{\cdot}4H_2O$, 0.21 $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.38 $AlCl_3{\cdot}6H_2O$ and 8.45 $CoCl_2{\cdot}6H_2O$. $NaHCO_3$ was added as buffer at a dosage of 9 g/reactor during days 23–30 and at 0.5 g/d (9.8 g/kg feed) during days 41–105. From day 106 onwards, $NaHCO_3$ was added only in M1 at a dosage of 2 g/d (38 g/kg feed).

On day 78, both reactors were opened and the reactor contents were mixed and distributed equally between M1 and M2. From day 79 onwards, M1 was continued as methanogenic reactor and operated at the same OLR and HRT as earlier (liquid volume 1500 mL). On the other hand, hydrogen production was induced in M2 by reducing the working volume from 1500 to 300 mL with constant amount of feed. Thus, the OLR in M2 was increased from 2 to 10 kgVS/m^3/d and HRT was decreased from 30 to 6 days.

Table 1
Characteristics of the grass silage and inoculum.

	pH	TS (%/FM)	VS (%/FM)	SCOD (g/l)	Acetic acid (mg/l)
Grass silage	4.0	45.4	42.5	239–373[a]	3.8–5.9[a]
Inoculum	7.8	4.1	3.0	7.2	9

[a] Unit mg/g TS.

2.3. Analysis and calculations

Analyses were done as described previously (Pakarinen et al., 2008, 2009). Gas composition was sampled and measured before daily feeding. OLR and HRT were calculated for five feeding days per week. H_2 and CH_4 yields were converted to standard temperature and pressure. When calculating the energy yields (in kWh) of hydrogen and methane yields, conversion factors of 3 and 10 kWh/Nm^3 were used for hydrogen and methane, respectively.

3. Results and discussion

3.1. CH_4 production in CSTRs M1 and M2 at 35 °C

Methane production from grass silage was studied in two parallel CSTRs at 35 °C with an OLR of 2 kgVS/m^3/d and HRT of 30 days (Figs. 1 and 2 and Table 2). After the initial start-up of feeding, specific methane yield rose to around 200 L/kgVS_{fed} by day 15. Thereafter, methane production dropped sharply with a corresponding decrease in pH (Fig. 1) and increase in TVFA concentration (Fig. 2). In order to raise the pH, buffer ($NaHCO_3$) addition was initiated on day 23 and was substituted with nutrient solution from day 28 onwards. However, TVFA concentration continued to increase further to reach 5.7–6.5 g/l by day 31. The concentration of acetic acid and propionic acid, the main components of TVFA, were 4.2–5.1 and 0.6–0.7 g/l, respectively (Fig. 2).

Despite the addition of buffer and nutrients, both reactors did not recover and thus reactors were kept unfed between days 31 and 40 (Figs. 1 and 2). During this unfed period, TVFAs concentration decreased to 2.2–2.9 g/l and pH increased to 7.7. Feeding was resumed on day 41 with an OLR of 2 kgVS/m^3/d and HRT of 30 days. The mean methane production during days 41–78 was 198–218 L CH_4/kgVS_{fed} with an average CH_4 content of 50–54% (Table 2 and Fig. 1). Thus, the highest average methane yield corresponded to energy yield of 2180 kWh/tVS_{fed}. These methane yields were slightly lower than the methane yields of 260 L/kgVS_{fed} reported during the monodigestion of grass silage at an OLR of 3.5 kgVS/m^3/d and HRT of 50 days in a loop reactor (Koch et al., 2009). However, the methane yields obtained in the present study were comparable to those obtained during the monodigestion of grass silage (197 L/kgVS_{fed}) in a two-stage systems consisting of leach bed and UASB reactor system with a total HRT of 55 days (Lehtomäki et al., 2008). On the other hand, the volumetric CH_4 yield of 0.40–0.44 m^3/m^3/d and VS reductions of 49–57% (days 41–78) obtained in the present study were in the same range (0.4 m^3/m^3/d and 41–52%, respectively) as those reported by Lehtomäki et al. (2007) during the co-digestion of grass silage with cow manure in CSTR.

Between the days 41–78, SCOD concentration was between 12 and 15 g/l. However, daily buffer addition (9.8 g/kg feed) was needed in order to keep the pH close to 7. Nevertheless, TVFA concentration increased from 2.2 to 5.7 g/l in M1 and from 2.9 to 6.3 g/l in M2. Besides acetic acid, propionic acid accumulation was also noticed in both M1 and M2. The increase in propionic acid concentration was from 0.1 to 0.7 g/l in M1 and from 0.5 to 0.8 g/l in M2. Mixing the M1 and M2 reactor contents (day 78) and continuing M1 as methanogenic reactor with an OLR of 2 kgVS/m^3/d and HRT of 30 days resulted in a mean specific methane yield of 140 L/kgVS_{fed} and volumetric methane yield of 0.28 m^3/m^3/d (Table 2). This yield was lower than the methane yields of 218 L/kgVS_{fed} obtained prior to mixing of the reactors contents. The SCOD concentration during the days 78–105 increased from 13.8 to 17.6 g/l with a corresponding decrease in pH (Fig. 1). Due to this decrease in pH, more buffer was added (38.2 g/kg feed) from day 106 onwards. However, TVFA concentration increased from 5.5 to

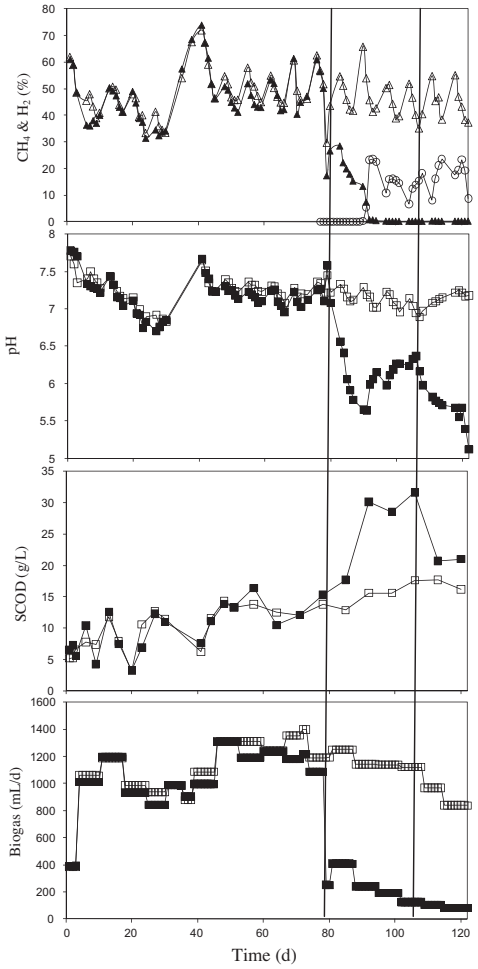

Fig. 1. CH_4 and H_2 (● in M2) contents in the biogas, pH, SCOD and daily biogas volume of M1 (open) and M2 (black). Lines show the time of the shift of M2 (day 79) and the time of change in buffer addition (day 106). Note that the working volume of M2 was reduced from 1500 to 300 mL on day 79.

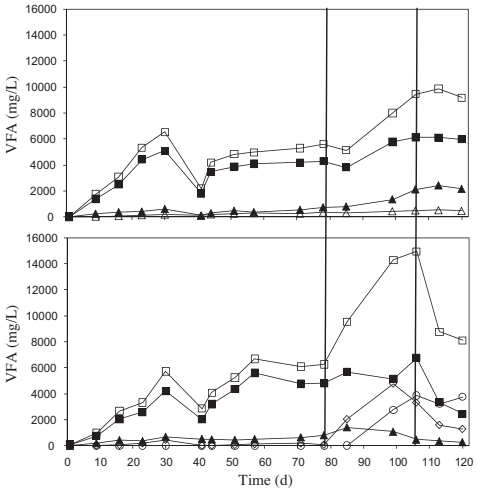

Fig. 2. Main VFAs in the M1 (up) and M2 (down). TVFA (□), acetic (■), propionic (▲), butyric (◇), iso-valeric (Δ) and caproic (○) acids. Lines show the time of the shift of M2 (day 79) and the time of change in buffer addition (day 106).

Table 2
Average specific and volumetric methane yields of methanogenic CSTR (M1). Values from M2 are shown between days 41 and 78 only. Standard deviation in parenthesis.

Days	CH_4 (L/kgVS_{fed})	CH_4 ($m^3/m^3/d$)
41–78	218 (25)	0.44 (0.05)
	M2:198 (18)	M2: 0.40 (0.04)
79–105	185 (20)	0.37 (0.04)
106–122	140 (18)	0.28 (0.04)

9.9 g/l. The main components of TVFA were acetic (6.1 g/l), propionic (2.4 g/l) and iso-valeric acids (0.5 g/l). Iso-butyric (0.3 g/l), butyric (0.3 g/l) and valeric acids (0.1 g/l) were also present but at a lower concentrations (Fig. 2).

The results from the present study showed that the long-term monodigestion of grass silage at an OLR of 2 kgVS/m^3/d and HRT of 30 days is not feasible and would result in low methane yields due to accumulation of VFA, which is attributed to the inhibition of acetate consumption by acetate utilizing methanogens and VFA degradation by acetogens. This was evident by the high concentrations of VFAs especially acetic acid (6.1 g/l) and propionic acid (2.4 g/l). This high concentration of propionic acid in the present study might have been a reason for the process failure. Previous studies have shown that propionic acid at a concentration of 0.9 g/l has resulted in decreased methanogenic growth rates and thus methane yields (Wang et al., 2009). Propionate accumulation has been shown to inhibit propionate degradation while acetate accumulation inhibits both acetate and propionate degradation (Kus and Wiesmann, 1995). Besides direct VFA accumulation, other factors such as lack of trace nutrients or accumulation of inhibitory levels of Na^+ through $NaHCO_3$ additions may have also resulted in the process failure. For instance, the amount of nickel (Ni) supplied through nutrient solution addition in the present study (0.05 mg/kg feed) might have been too low, as Ni has been reported to be an essential trace nutrient to achieve high acetate to methane conversion rate (Kida et al., 2001). On the other hand, no selenium (Se) or tungsten (W) was added in the present study, both of which have previously been shown to be advantageous for biogas process (Lebuhn et al., 2008; Plugge et al., 2009). However, the impact of nutrient addition in the present study was not clear, as the recommended concentrations for trace elements show high variation (Demirel and Scherer, 2011) and no control without nutrient addition was operated. Furthermore, addition of $NaHCO_3$ for buffering the process had apparently resulted in accumulation of Na^+ in the reactor. The calculated Na^+ concentration was 2.7 g/kg feed, which was apparently reached in the reactor around day 73 of the experiment and was further increased to 10.4 g/kg feed at day 106. The role of $NaHCO_3$ on decreasing process performance

is however not clear as IC_{50} for sodium inhibition has been reported to show wide range, from 5.6 to 53 g/l (Chen et al., 2008), depending on e.g., adaptation of the bacterial population.

According to present and previous studies (e.g., Lebuhn et al., 2008), step-wise increase in OLR or generally lower OLR and/or longer HRT might be feasible in energy crop monodigestion. Higher methane yields have been obtained typically when the reactors were operated either with lower OLR and/or longer HRTs, as applied in the present study. When OLR was increased step-wisely from 1 to 3.5 kgVS/m^3/d (HRT decreased from 440 to 50 days) during 300 days, methane yields of 260 L/kgVS were obtained without indication of reactor failure during monodigestion of grass silage in a loop reactor (Koch et al., 2009). Mähnert et al. (2005) on the other hand reported high methane yields of 300 L/kgVS during the monodigestion of a mixture of three fresh grass species at a low OLR of 1.4 kgVS/m^3/d and long HRT of 153 days (calculated from data given). Similarly, high methane yields of 455 L/kgVS have been reported from perennial ryegrass silage in pilot-scale two-stage CSTR with relatively low OLR of 0.5 kgVS/m^3/d and long HRT of 221 days (Thamsiriroj and Murphy, 2010). In addition to OLR and HRT, feeding regime and mixing can affect the process. In the present study the substrate was fed only once per day, which is the method typically applied in laboratory studies. However, it has been shown that feeding of the silage should be done several times (12–24) per day due to the high lactic acid concentration and the low substrate pH, which can affect the process stability and gas yield (Krieg, 2005). In mono-digestion of grass, special attention has to be given to proper mixing (e.g., Koch et al., 2009) as grass tends to float more easily when compared e.g. to maize (Thamsiriroj and Murphy, 2010), a phenomenon, which was observed in the present study as well.

3.2. Shifting methanogenic process to hydrogenic process

Methanogenic process in M2 was shifted to H_2 production by increasing the OLR from 2 to 10 kgVS/m^3/d and by decreasing the HRT from 30 to 6 days (day 79). Immediately after the changes in operation strategy, CH_4 concentration dropped rapidly from ca. 50% to below detection limit. H_2 production was first detected 12 days (on day 90) after the shift in operational strategy. Thereafter, H_2 concentration increased steadily during the next 30 days of operation and fluctuated between 10% and 24% depending upon the feeding cycle, with lowest H_2 concentration of <10% noticed after the non-fed weekends (Fig. 1). This shift in operational strategy through increased OLR and decreased HRT resulted in a sharp drop in pH from 7 to 5.6 (day 90) and then increased slowly to reach 6 (since day 92). On the other hand, SCOD and TVFA concentrations increased from 15.3 to 31.6 g/l and from 6.3 to 15 g/l, respectively (day 106). Among the TVFA components, the increase in acetic acid concentration was small (from 4.8 to 6.8 g/l) compared to caproic acid, which increased from negligible to 3.9 g/l (day 106). Butyric acid concentration reached its highest concentration of 4.8 g/l on day 99. On day 106, buffer addition was stopped (29 days of addition) which resulted in a decrease in SCOD concentration to around 20 g/l with a simultaneous drop in pH (Fig. 1). However, H_2 content remained at the same level as noticed during the buffer addition period. In addition, the concentration of TVFA especially that of acetic acid decreased while the concentration of caproic acid remained more or less constant (Fig. 2). VS reduction was at the end of reactor experiment 18%.

According to present results, shifting methanogenic reactor to hydrogenic is possible by increasing the OLR and shortening the HRT. High OLR resulted in the build-up of VFA and decrease in pH, which inhibited the methane production and hydrogen consumption by the hydrogenotrophic methanogens. The low pH (5.5–6.5) in the present study has apparently favoured acidogens instead of methanogens. The optimal pH for methanogens is in the quite narrow range close to 7, whereas the acidogenic H_2 producing bacteria can grow at lower pH of <6 (Valdez-Vazquez and Poggi-Varaldo, 2009). Thus, increase in OLR as an operational strategy was shown to be a proper method for inducing H_2 production from an already operating mesophilic methanogenic system. The fact that CH_4 production ceased and H_2 accumulated in the reactor indicates the shift in microbial community (Demirel and Scherer, 2008) and the inhibition of methanogens throughout the experimental run. It has previously been reported that hydrogen production without treating the inoculum has been feasible e.g. from garbage waste (Ohnishi et al., 2010) and household solid waste (Liu et al., 2008) and load–shock method was found as a simple method for enriching H_2 producers (O-Thong et al., 2009). Moreover, low pH (5.5) has shown to be an effective method for continuous H_2 production from household solid waste (Liu et al., 2008) as methane production was noticed even with short HRT of 2–6 days at pH controlled to 7.

Short HRT of 0.5–12 h (i.e. high dilution rate) can be used to wash out methanogens in continuous processes with liquid substrates, e.g., with sucrose or glucose containing wastewaters (Davila-Vazquez et al., 2008; Valdez-Vazquez and Poggi-Varaldo, 2009). However, with solid substrates, like grass silage, the hydrolysis is typically rate-limiting (Vavilin et al., 2008) and longer HRTs are needed to allow hydrolysis. HRT of 3 days was found optimal for hydrogen production from household waste in CSTR, while HRT less than 2 days limited hydrolysis and longer HRTs (up to tested 6 days) stimulated methanogenesis (Liu et al., 2008). High OLR can be used to inhibit methanogens through shock–load, while too high OLR can result in solvent production and thus reduce the hydrogen yield. It is thus concluded that in the present study the high OLR was the main operational strategy that could affect the shift in the anaerobic digestion process from methane to hydrogen production rather than the short HRT of 6 days. One possible way to increase H_2 yield from grass silage could be through pre-treatment of the substrate and use of hydrolysate for H_2 production, as in that case very short HRT could be used.

The highest daily H_2 yield of 42 L/kgVS_{fed}, which corresponds to an energy yield of 126 kWh/tVS_{fed}, is comparable to H_2 yield obtained in batch assays (at most 44 L/kgVS_{added}, data not shown). This yield is in the same range, as previously obtained in semi-continuously fed CSTR from household solid waste (Liu et al., 2006, Table 3), while the yield was slightly higher than obtained from sweet sorghum extract (Antonopoulou et al., 2008), olive pulp (Koutrouli et al., 2009) and garbage slurry (Ohnishi et al., 2010, Table 3). However, the yields from potato waste (Zhu et al., 2008) and cassava stillage (Wang et al., 2011) were higher than obtained in the present study (Table 3). The chemical composition of the substrate, together with operational parameters, affects the hydrogen yield. The effect of OLR and HRT on hydrogen yield in CSTR from solid substrates can be complex and research regarding this topic is limited. In the present study the OLR was lower and the HRT longer than in the above mentioned studies (Table 3), but it must be noted that we didn't try to optimize the process parameters.

In the present study, the mean daily biogas production showed a decreasing trend, being at highest around 400 mL/d (Fig. 1). Thus, the highest specific and volumetric H_2 yields obtained were 19 L/kgVS_{fed} and 0.19 m^3/m^3/d (average between days 90 and 94), respectively, while, during the 40 days of operation at a higher OLR of 10 kgVS/m^3/d, the average specific and volumetric H_2 yields obtained were 9 L/kgVS_{fed} and 0.06 m^3/m^3/d, respectively. This decrease in H_2 yield might be due to high concentrations of VFAs. Previous studies have been shown that high VFA levels would inhibit hydrogen production (Chong et al., 2009; Valdez-Vazquez and Poggi-Varaldo, 2009; Wang et al., 2008). For instance, H_2 yield in the present study decreased with a corresponding increase in the

Table 3
Examples of maximum H_2 yields, OLR and HRT of some selected organic residues and crop based materials in semi-continuously fed CSTRs.

Substrate	H_2 yield (L/kgVS$_{fed}$)	OLR (kgVS/m^3/d)	HRT	Reference
Sugar beet pulp	1.2[a]	16[b]	14 h	Hussy et al. (2005)
Household solid waste	43	37.5	2 d	Liu et al. (2006)
Sweet sorghum extract	10.4[c]	34[d,g]	12 h	Antonopoulou et al. (2008)
Olive pulp	4.3[e,g]	43[f,g]	30 h	Koutrouli et al. (2009)
Garbage slurry	20–30	nr	14–48 h	Ohnishi et al. (2010)
Potato waste	65	41[e]	6 h	Zhu et al. (2008)
Cassava stillage	74	nr	3 d	Wang et al. (2011)
Grass silage	42	10	6 d	This study

nr = Not reported.
[a] Unit mol/mol hexose converted, over 95% of the substrate was converted.
[b] Unit kg total sugars/m^3/d.
[c] Unit L/kg sweet sorghum, as compared to 17.9 L/kg grass silage in the present study.
[d] Unit kg glucose/m^3/d.
[e] Unit L/kg TS.
[f] Unit kgTS/m^3/d.
[g] Calculated from the data given.

concentration of caproic acid. A similar observation in the increased caproic acid production in continuous reactor processes under mesophilic condition was reported (Jung et al., 2010). This is attributed to the fact that at pH 4–5, caproic acid production is thermodynamically favoured by the consumption of 1 mol of butyric and acetic acids along with 2 mol of H_2 (Jung et al., 2010). Based on the present results, it seems advantageous to adjust the pH close to 6 as higher H_2 yields were obtained in the period with constant buffer addition.

TVFA–COD accounted 68–80% of the SCOD during the shifting period (days 79–120), thus indicating high degradation efficiency. The remaining degradation products have most probably been lactic acid and alcohols (not measured). Lactic acid was probably present in the substrate, as in a typical ensiling process, water soluble carbohydrates of the crop are mainly degraded to lactic acid. Moreover, lactic acid can be produced during anaerobic digestion process as well. In addition, lactic acid can be converted to propionic acid and the produced propionate may accumulate during the subsequent methanogenic process (Wang et al., 2009). Furthermore, lactic acid bacteria (also present in silage) can inhibit H_2-producers through the excretion of bacteriocins (Valdez-Vazquez and Poggi-Varaldo, 2009).

In the present study, the highest H_2 energy yield (19 m^3/tVS$_{fed}$, 57 kWh/tVS$_{fed}$) was less than 3% of the highest methane energy yield (218 m^3/tVS$_{fed}$, 2180 kWh/tVS$_{fed}$). In a similar study with potato waste, about 5% of the energy was obtained from hydrogen production (111 kWh/tTS) compared to methane production (2040 kWh/tTS) (Zhu et al., 2008). Thus, the energy value of hydrogen production remained rather low and hydrogen production alone clearly is not beneficial. However, in practical applications, H_2 producing acidogenic stage could be coupled to traditional methanogenic stage converting VFAs and other degradation products to CH_4, thus improving the overall energy efficiency. Moreover, the digestate of the hydrogenic process could be a good substrate for the subsequent methanogenic step as the propionic acid concentration was rather low and butyric and caproic acids can be rather easily converted to acetic acid by acetogenic bacteria (Ding and Wang, 2008; Wang et al., 2009). Two-stage H_2 + CH_4 system has been shown to improve CH_4 yield when compared to traditional one-stage methane process (Liu et al., 2006; Pakarinen et al., 2009), mainly by improving hydrolysis and acidogenesis. For instance, Liu et al. (2006) reported 21% more CH_4 in a two-stage system compared with one-stage system fed with household solid waste. Besides improving methane yield, H_2 stage has been shown to enable higher OLR and shorter HRT in the subsequent methanogenic stage (Ueno et al., 2007) and better effluent quality with less propionate (Wang et al., 2011) when compared to one-stage system. Further research would be needed to find optimal conditions for both hydrogen and VFAs production, as high concentrations of VFAs can inhibit both hydrogen production (Chong et al., 2009; Wang et al., 2008) and hydrolysis (Vavilin et al., 2008). In the future it could be possible to produce both hydrogen and methane from energy crops in a two-stage concept. Hydrogen could be upgraded and used separately, or, on the other hand, it could be, up to at least 17% of the volume, injected into natural gas grid to improve the properties of methane (Haeseldonckx and Ðhaeseleer, 2007).

4. Conclusions

Methanogenic process can be changed towards hydrogen production by increasing the OLR and shortening the HRT. This leads to increase in TVFAs and decrease in pH, which inhibits hydrogen consuming methanogens. At most 42 L H_2/kgVS$_{fed}$ was obtained with OLR of 10 kgVS/m^3/d and HRT of 6 days. According to present study at most about 218 L CH_4/kgVS$_{fed}$ can be obtained from grass silage monodigestion. However, initial OLR of 2 kgVS/m^3/d was shown to be too high and HRT of 30 days too short for stable methane production, thus stepwise increase in OLR and/or longer HRT could be suggested.

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