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Padma Shanthi Jagadabhi

Methods to Enhance Hydrolysis During One and Two-stage Anaerobic Digestion of Energy Crops and Crop Residues

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ABSTRACT

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The objective of this thesis was to evaluate methods to enhance hydrolysis (measured as specific SCOD production, g SCOD g^{-1} VS) during one and two-stage anaerobic digestion (AD) of energy crops and crop residues. Addition of macro (NH_4Cl), micro nutrients (Fe, Ni, Co and Mo) and leachate replacement during mono-digestion of grass silage in one-stage leach bed reactors (LBRs) enhanced hydrolysis by 18 % (0.56 g SCOD g^{-1} VS), 7 % (0.45 g SCOD g^{-1} VS) and 34 % (0.51 g SCOD g^{-1} VS) respectively compared to respective controls. On the other hand, creating micro-aerobic conditions (@ 1 l min^{-1}, 2.5 l of air) did not improve hydrolysis but enhanced VFA production by 4 fold (from 2.2 g l^{-1} to 9 g l^{-1}). Application of rumen cultures improved hydrolysis by 10 % (0.33 g SCOD g^{-1} VS) more than control (0.30 g SCOD g^{-1} VS). Similarly, during two-stage AD in LBR-UASB reactor configuration leachate replacement enhanced hydrolysis in cucumber and grass silage (0.5 g SCOD g^{-1} VS) than in tomato and common reed (0.35 and 0.15 g SCOD g^{-1} VS respectively). During co-digestion of grass silage and cow manure at a ratio of 30:70 (VS) in CSTR, re-circulation of alkali treated solid fraction of digestate did not improve the anaerobic biodegradation rates or methane yields. Results from batch experiments showed that methane potential of grass silage varied from 0.28-0.39 m^3 CH_4 kg^{-1} VS_{added} in all the experiments. On the other hand, methane potentials of the studied crop residues were 0.32 m^3 CH_4 kg^{-1} VS_{added} for tomato and 0.26 m^3 CH_4 kg^{-1} VS_{added} for cucumber and common reed. Alkali pretreatment of solids, obtained from digestate (during co-digestion of grass silage and cow manure in one-stage CSTRs), at a low concentration of 20 g NaOH kg^{-1} VS resulted in higher methane yield (0.34 m^3 CH_4 kg^{-1} VS_{added}) than the other tested dosages (40 & 60 g NaOH kg^{-1} VS). Addition of macro nutrient (NH_4Cl) enhanced methane potential of grass silage by 17 % more than control. On the other hand, an increase in methane yield (15 %) was noticed only when micro nutrients were added at the highest tested concentration rather than at low and medium concentrations. Application of a mixed inoculum consisting of rumen culture and digestate from mesophilic biogas plant at a 50:50 (v/v) ratio enhanced methane yield of grass silage (0.5 m^3 CH_4 kg^{-1} VS_{added}) than pure inoculum (100 %) or other tested mixed inoculum combinations (0.2-0.29 m^3 CH_4 kg^{-1} VS_{added}). Therefore, the present study shows the feasibility of application above studied methods for enhancing hydrolysis and thus obtain improved methane yields during one and two-stage AD of energy crops and crop residues.

Keywords: Energy crops; leach bed reactors; leachate; methane; solubilization.

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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. I did the experimental work described in all the papers (I-V). Trace element analyses were carried out by one of the co-authors (A. Väisänen) in paper II. I wrote the first drafts of all papers which were completed in co-operation with my supervisor and co-authors.

I Jagadabhi, P.S., Lehtomäki, A. & Rintala, J. 2008. Co-digestion of grass silage and cow manure in a CSTR by re-circulation of alkali treated solids of the digestate. *Environmental Technology* 29: 1085-1093.

II Jagadabhi, P.S., Kaparaju, P., Väisänen A. & Rintala, J. Effect of macro and micro nutrients addition during mono-digestion of grass silage in one stage leach bed reactors. Submitted manuscript.

III Jagadabhi, P.S., Kaparaju, P. & Rintala, J. 2010. Effect of micro aeration and leachate replacement on COD solubilization and VFA production during mono-digestion of grass silage in one-stage leach bed reactors. *Bioresource Technology* 101: 2818-2824.

IV Jagadabhi, P.S., Kaparaju, P. & Rintala, J. Application of rumen cultures to enhance hydrolysis during anaerobic digestion of grass silage in one-stage leach bed reactors. Manuscript.

V Jagadabhi, P.S., Kaparaju, P. & Rintala, J. 2011. Two-stage anaerobic digestion of tomato, cucumber, common reed and grass silage in leach - bed reactors and upflow anaerobic sludge blanket reactors. *Bioresource Technology* 102: 4726-4733.

ABBREVIATIONS

AF	Anaerobic filter
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor system
EU	European union
FM	Fresh materials
GC	Gas chromatograph
Ha	Hectare
HRT	Hydraulic retention time
ICPMS	Inductively coupled plasma mass spectrometer
M_{toe}	Million tons of oil equivalent
LBR	Leach bed reactor
LCFA	Long chain fatty acids
OLR	Organic loading rate
RM	Residual materials
RMLE	Residual materials after extraction of leachate
SCOD	Soluble chemical oxygen demand
TCOD	Total soluble chemical oxygen demand
TS	Total solids
TKN	Total kjeldahl nitrogen
UASB	Up flow anaerobic sludge blanket reactor
VFA	Volatile fatty acids
VS	Volatile solids

1 INTRODUCTION

1.1 Anaerobic digestion of energy crops

Worldwide, renewable energy technologies are today considered as efficient options to reduce fossil fuel dependence and greenhouse gas emissions and to meet rising energy demands. Technical advancements in renewable energy technologies have also progressed in the last few decades, in terms of market introduction, lower costs of investment and increased reliability (Uyterlinde et al. 2007). Among the renewable energy technologies, biogas production through anaerobic digestion (AD) is one of the most environment-friendly technologies because of a vast potential for the availability of organic/agricultural materials, for the benefits of closed nutrient cycle (Döhler et al. 2006) and for reducing greenhouse gas emissions (Weiland 2010). In addition biogas is a cleaner, cheaper, sustainable and a versatile carrier of energy which can be replaced for fossil fuels in power and heat production and can also be used as a gaseous vehicle fuel (Eze & Agbo 2010, Weiland 2010). In Europe, biogas is mainly linked to agriculture (Holm- Nielsen & Al-saedi 2001) and crops which are specifically grown for the purpose of producing energy are known as energy crops.

It was estimated that the European Union (EU) has a biomass potential of 1500 million tons of oil equivalent (Mtoe) within agricultural sector which could be used for energy production via AD (Holm-Nielsen et al. 2009). The EU has set a target of achieving 20 % of total energy production from renewable energy sources by the year 2020 in comparison to 6.4 % in 2007, by adoption of Renewables Directive (European Parliament 2009/28/EC). Consequently, biogas production has been gaining significant attention as a renewable form of energy with high solid feedstocks such as energy crops and crop residues. Currently, the European energy production from biogas had reached 8 Mtoe in 2009 falling in line with the target of achieving 20 % renewable energy share from the gross energy consumption (EurObserv'er 2010). The main reason for this growth was stated to be a steep rise in agricultural biogas plants whose

production increasingly relied on crops (mainly maize). These plants produce 52 % of biogas energy and have exceeded the biogas production from landfills (36 %) and from waste water treatment plants (12 %) (EurObserv'er 2010). For example, Germany had about 4984 biogas plants operating with energy crops in 2009 with 1893 MW of electrical capacity and about 1093 biogas plants were planned to be initiated in 2010 (EurObserv'er 2010).

Apart from maize (silage), application of grass silage for biogas/methane production is at an early stage in Germany and Austria to be used either as transport fuel or for replacement of natural gas (Smyth et al. 2009). In addition to these energy crops, crop residues obtained after crop harvest can serve as the cheapest and most readily available organic and lignocellulosic sources of biomass for biogas production through AD. The annual yields of lignocellulosic biomass residues were estimated to be more than 220 billion tons worldwide (Ren et al. 2009). Furthermore, modern greenhouses are also estimated to produce about 40-60 tons ha^{-1} $year^{-1}$ of crop residues as a result of crop trimming and harvesting, which need to be disposed of properly (ODAF 2004). With proper harvesting practices, greenhouses can collect and utilize these crop residues for biogas production and in turn meet their own energy demands. In addition to lignocellulosic biomass from agriculture and greenhouses, aquatic grass species such as common reed, grown in temperate and tropical regions (Karunaratne et al. 2003), can also be harvested and used as a biomass resource for biogas production. Its extensive distribution and over-growth in water systems, especially in eutrophicated lakes, is considered to affect the quality of the water ecosystems, nutrient cycles and hydrological regimes (Huhta 2009). Therefore, its abundance and nutrient content can be exploited for biogas production. Furthermore, the effluents produced from the AD of the lignocellulosic biomass could be utilized as liquid fertilizers for crop production and recycle the nutrients at the greenhouses/agricultural fields.

1.2 Microbiology and nutritional requirements of AD process

1.2.1 Microbiology

AD of organic materials is a complex microbiological process and takes place in four steps such as hydrolysis, acidogenesis (fermentation of organic monomers to organic acids), acetogenesis (production of methanogenic substrates such as acetate and hydrogen (H_2/CO_2) and methanogenesis (production of methane) (Fig. 1). The AD process consists of various microbial consortia working in sequence with the products of one consortia serving as substrates to the next group. Thus, each group of microbial consortium is linked to the other consortia in a chain like fashion (Gerardi 2003). Hydrolysis is splitting of a compound (lysis) with water. The crop materials are particulate substrates containing

large, polymeric and insoluble compounds which further consist of small molecules joined together by specific chemical bonds.

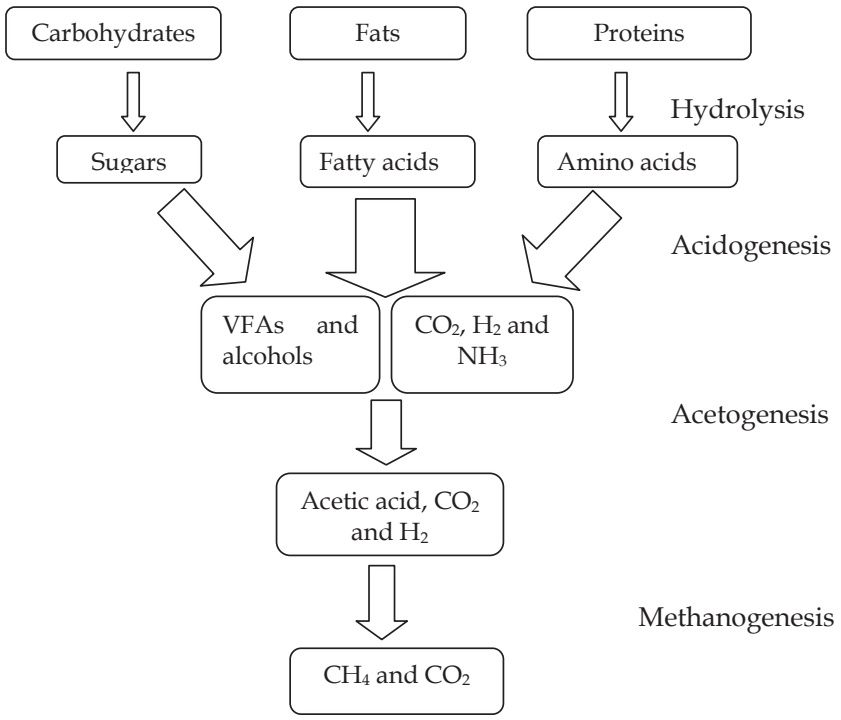

FIGURE 1 Basic schematic representation of AD of complex organic compounds to methane and carbon dioxide (Adapted from Gerardi 2003).

Hydrolytic bacterial consortia are capable of breaking down these specific chemical bonds by secreting specific hydrolytic enzymes such as cellulases, lipases, proteases, amylases etc. As a result, a complex polymeric substance such as cellulose is degraded into soluble monomers or oligomers i.e. sugars and aminoacids and long-chain fatty acids (LCFAs).

During acidogenesis, the solubilized monomers formed as a result of hydrolysis are degraded by diverse consortia of fermentative bacteria into CO_2, H_2, alcohols and volatile fatty acids (VFA). Sugars are converted to VFA while aminoacids are degraded to ammonium nitrogen (NH_4^+) thus increasing the ammonium nitrogen content of the digested end product. The LCFAs formed from lipid hydrolysis are further converted to acetate and propionate by ß-oxidation. For this degradation step to take place low hydrogen partial pressure is a favourable condition in the anaerobic reactor (Gerardi 2003). Methanogens consume hydrogen immediately as it is being produced unless in conditions of high concentrations of LCFAs and VFA, pH decreases inhibiting methanogenic activity. Hence, a rise in hydrogen partial pressure increases the degradation of

LCFAs to acetate which is otherwise a readily available intermediate for methanogens (Mackie et al. 1991). In the acetogenic step, lactate, alcohols and fatty acids longer than acetate are converted to acetate and H_2 by obligate H_2 producing acetogenic bacteria. This step is also dependent on presence of low hydrogen partial pressure in the reactor.

During methanogenesis, which is the final step, methane is formed from acetate in two ways; the first is aceticlastic methanogenesis. In this reaction, acetate is cleaved to methyl and carboxyl groups. The methyl group is directly converted to methane through several biochemical reactions while the carboxyl group is oxidized (Ferry 1992). Methanogens *Methanosarcina* and *Methanosaeta* are known to carry out this biochemical reaction (Hattori 2008). The second process consists of syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis.

In SAO, both methyl and carboxyl group of acetate are oxidized to CO_2 with the production of H_2 (Table 1, Fig. 2). This reaction is extremely unfavourable ($\triangle G^{\circ}$ =104 kJ/mol) but can occur when H_2 consuming methanogenesis eliminates hydrogen. Syntrophic acetate oxidation is carried out by syntrophic acetate-oxidizing bacteria and H_2 consuming methanogenesis is carried out by hydrogenotrophic methanogens. Hydrogenotrophic methanogens produce methane by reduction of C1 substrates such as methanol or methylamines and it is estimated that about 70 % of methane is formed from acetate by acetoclastic methanogens and 30 % from hydrogen and carbon dioxide by hydrogenotrophic methanogens. This is because acetate is the major end product of acidogenesis step in the anaerobic digester

TABLE 1 Biochemical reactions during methanogenesis (He et al. 2009).

Process	Reaction	$\triangle G^{\circ}$ (kJ/mol)
Acetoclastic	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31
Hydrogenotrophic	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-135.6
Methylotrophic	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-104.9
Homoacetogenesis	$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	-130.7
Syntrophic acetate oxidation	$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2$	104.6

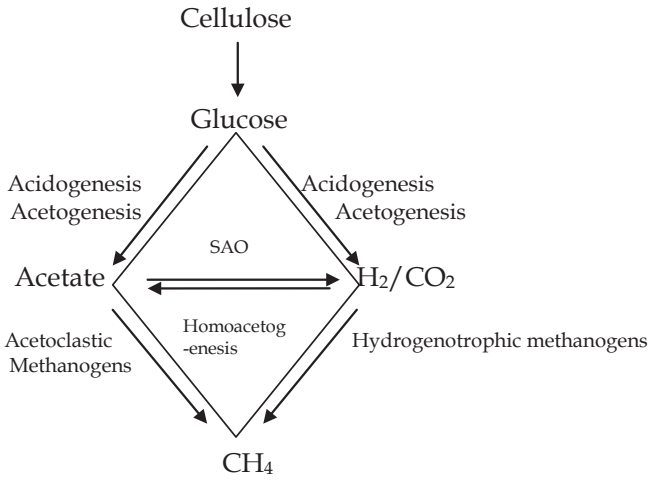

FIGURE 2 Metabolic reactions involved in anaerobic digestion of cellulose to CH_4. (Adapted from Lu et al. 2010).

Further, the decomposition of complex polymeric compounds into methane takes place as rapidly as the compounds can be converted to products that can be utilised by the methane forming bacteria. In the AD of easily degradable organic compounds (such as waste waters) acetate production is considered as the bottleneck or rate limiting while for the poorly degradable compounds (such as energy crops) hydrolysis is considered as the rate limiting step. When acetate levels accumulate in the anaerobic digesters, the conversion of acetate to methane lies in strengthening acetoclastic methanogenesis and reactions of syntrophic acetate oxidation from acetate to H_2/CO_2 and subsequent hydrogenotrophic methanogenesis (Lu et al. 2010). Lu et al. (2010) also reported that the pathway of syntrophic acetate oxidation was predominantly occurring in thermophilic anaerobic digesters fed with a wide range of substrates such as acetate, food waste, waste paper etc.

1.2.2 Nutritional requirements

The nutrients are essential for growth and synthesis of enzymes and cofactors responsible for biochemical and metabolic pathways. Nutrients are divided into two types, the macro nutrients and the micro nutrients. It is necessary that both macro and micro nutrients are present in the anaerobic digester to enable efficient uptake by the bacteria. It was reported that ideally these nutrients should be in excess in the digester as slightest deficiencies could inhibit the process (Mara & Horan 2003). Carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) are fundamental elements and are considered as macro nutrients required for growth and multiplication of microbes. The nitrogen content of a substrate plays a key role in the process for another reason that it will contribute to the neutral pH stability by releasing ammonium ions. Speece (1996) had suggested that the amount of N, P and S required in the anaerobic

digesters can be estimated by the empirical formula of the biomass ($C_5H_7O_2N$). Some researchers suggested an optimum C/N ratio as 30:1 (Gunnerson & Stuckey 1986). Among micro nutrients the elements such as iron (Fe), nickel (Ni), cobalt (Co), molybdenum (Mo), tungsten (W) and selinium (Se) are known to be the most essential elements for the growth and metabolism (Zandavoort et al. 2003) (Table 2). Several functions of anaerobic bacteria are dependent on the availability of micro nutrients as they form part of the active sites for several key enzymes (Oleszkiewicz & Sharma 1990) (Table 2). The microbial growth and the subsequent degradation of a substrate are highly dependent on the optimal availability of these nutrients within the digester. However, very few studies are reported in the literature for the optimum micro nutrient requirements of the anaerobic digesters. The reason could be that the concentrations have to be optimized specifically for each substrate subjected to AD depending on the scale of application, inherent micro nutrient concentrations of the substrate, inocula and the overall process conditions within the digester. Some of the reported concentrations in the literature for different substrates are given in Table 3.

TABLE 2 Micro nutrients in enzymes of microbial conversions.

Micro nutrient	Enzyme	Organism	Reference
Fe, Ni, Co	CO-dehyrodogenase	Methanogens/acetogens	Ferry (1999)
Co (B12)	Methyl transferase	Methanogens and acetogens	Beveridge & Doyle (1989)
Fe, Ni, Cu	Acetyl- CoA synthase	*Moorella thermoacetica*	Seravalli et al. (2003)
Ni	Methyl-CoM-reductase	Methanogens	Hausinger (1996)
Mo or W	Formiate dehydrogenase	Methylobacterium	Girio et al. (1992)
Mo, Fe	Nitrate reductase	*P.denitrificans*	Ferguson (1994)

TABLE 3 Reported optimum/useful/stimulatory/minimum micro nutrient concentrations in anaerobic digesters operating with waste waters, organic fraction of municipal solid wastes (OFMSW) and energy crops and crop residues.

Micro nutrient		Reference
Waste waters	Minimum/optimum/ stimulatory conc. (g m^{-3})	
Fe	1000 [α]	Takashima & Speece (1989)
	0.28-50.4	Takashima & Speece (1990)
Ni	0.2 [α]	Takashima & Speece (1989)
	0.012-5	Takashima & Speece (1990)
Co	0.1 [α]	Takashima & Speece (1989)
Mo	0.048	Takashima & Speece (1990)
OFMSW	Useful conc. (g m^{-3})	
Co	0.148-0.58	
Mo	0.044-53	Lo et al. (2010)
W	0.658-40	
	Stimulatory conc. (mg kg^{-1})	
Fe	0.39	
Ni	0.11-0.25	Kayhanian & Rich (1995)
Co	0.05-0.19	
Mo	0.16-0.3	
Se	0.062	
Energy crops/crop residues	Optimum/stimulatory conc. (mg kg^{-1})	
Ni	0.6 [π]	Pobeheim et al. (2010 & 2011)
	11 †	Hinken et al. (2008)
Co	0.02 [π]	Jarvis et al. (1997)
	0.05 †	Pobeheim et al. (2011)
	9	Hinken et al. (2008)
Fe	4000 [Ψ]	Raju et al. (1991)
	205 †	Hinken et al. (2008)

[α] Concentration expressed per day based on active reactor volume,
† Concentration expressed per kg of COD in the digester,
[Ψ] Concentration per kg volatile solids (VS),
[π] per kg of fresh materials,
The values for waste waters and OFMSW were taken from a review paper (Demirel & Scherer 2011).

1.3 Chemical composition and hydrolysis of energy crops

1.3.1 Chemical composition

Crop biomass is a heterogenous and chemically complex renewable energy resource. The efficiency of crop biomass conversion to a biofuel depends on the physical characteristics, chemical composition and type of fermentation technology used (Mckendry 2002, Sun & Cheng 2002). Crop biomass consists of mainly cellulose, hemicellulose and lignin (lignocellulose) (Taherzadeh & Karimi 2003) (Table 4). In addition to these primary polymers, crop biomass also contains non-structural carbohydrates (glucose, fructose, sucrose and fructans), proteins, pectins, extractives and lipids (Mc Donald, 1991). For example, the agricultural residues of wheat straw, rice straw and corn stover contain 32-47 % cellulose, 19-27 % hemicellulose and 5-24 % lignin (Ren et al. 2009). Cellulose is a linear polymer of glucose linked through α-1, 4 linkages and is arranged in microcrystalline structures that are difficult to be hydrolyzed under natural conditions. Hemicellulose is a heteropolysacharide composed of different hexoses, pentoses and gluconic acid. Hemicellulose is comparatively more soluble than cellulose whereas lignin is a highly irregular and insoluble polymer.

TABLE 4 Typical chemical composition of lignocellulosic materials (Betts et al. 1991).

Raw material	Cellulose %	Hemicellulose %	Lignin %
Grasses	10-30	25-40	25-50
Hardwoods	18-25	45-55	24-40
Softwoods	25-35	45-50	25-35

% of plant dry weight

Further, plants contain several nutrients of which nitrogen (N), phosphorus (P) and potassium (K) are considered as the primary elements responsible for important metabolic activities such as photosynthesis and respiration. The critical level of N in plants is reported to be around 3 %. In young plants, the N content would be close to 4 % or more and in leguminous plants (alfa alfa, soy beans, groundnut) it is 3-4.25 % (Plank 2010). High nitrogen levels (> 5 %) in forage crops like fescue are reported to be due to a low level of magnesium. Phosphorus levels in young plants can be as high as 0.5-1%. Tree crops have higher P requirements with the critical values ranging from 0.12-0.15 % while grasses have higher P requirements ranging from 0.2-0.25 %. Since P is a mobile element, its content decreases with ageing of the tissue and deficiencies occur in the plant. The K requirement of plant varies widely with crop and the critical value for K in leaves ranges from 0.75-1.25 %. For grasses, the K requirement

levels are high ranging from 1.2-2 % while in legumes the critical value is between 1.75-2 %. Young plants have high K concentration (3-5 %) and the deficiencies occur as the plant grows older (Plank 2010).

1.3.2 Hydrolysis

Polymer hydrolysis is complicated by enzyme and the substrate factors and bacteria cannot take up particulate organic material because a breakdown into soluble monomers is needed first (Gujer & Zender 1983). Hydrolysis of insoluble organic compounds is necessary to convert these materials into a size and form that can pass through the bacterial cells walls for use as energy and nutrient source (Kim et al. 2003). Crystalline cellulose which is a significant component of plant material is recalcitrant but completely hydrolysable through a combined action of endo and exoglucanases. Lignin does not contain any chains of repeating subunits thus making its enzymatic hydrolysis more difficult (Malherbe & Cloete 2002). The intricate associations between cellulose, hemicellulose and lignin prevent polymer hydrolysing enzymes access to its substrates (Cornu et al. 1994). It was previously reported that the accessibility of enzymes to wood and fibers is limited by factors such as adsorption to surface areas, low fiber porosity and low median pore size of fibers (Tomme et al. 1995, Grethlein 1985). Further, Reid (1995) suggested that the physical contact between enzyme and substrate is the rate-limiting step in lignin degradation.

Therefore, in order to increase the efficiency of degradation of lignocellulosic crop materials and obtain greater energy benefits it is inevitable to increase the accessibility of substrate surface to microorganisms by physical (particle size reduction, maceration) chemical (acid and alkaline hydrolysis), physico-chemical (size reduction and chemical treatments), thermal (heat treatments), thermo-chemical (in presence of heat and chemicals) and biological pretreatment (bacterial and fungal treatments) options. The pretreatment options have been studied extensively and a number of sophisticated techniques are in practice today in the industry (Ward et al. 2008).

Several other strategies to enhance the hydrolysis (rates as well as yields) of particulate organic substrates include optimization of inoculum to substrate ratio (I/S) (Banks et al. 2008), pH adjustment (Cysneiros et al. 2008), nutrient supplementation (Ren et al. 2008a, b, 2009), detoxification (de Vrije et al 2002), bioaugmentation (Jo et al. 2008) etc. However, hydrolysis of particulate substrates still remains a challenge and needs more alternatives to improve the overall degradation rates of these substrates and thus obtain higher methane yields.

1.4 Reactor configurations for AD of energy crops and crop residues

1.4.1 One-stage, continuously stirred tank reactor systems (CSTRs) for co-digestion of energy crops

The energy crops/crop residues can be anaerobically digested either in wet or dry processes and further in one-stage or two-stage reactor systems. Most of the farm-based anaerobic digesters are one-stage, wet digestion systems with < 12 % dry matter co-digesting liquid manures and energy crops such as maize and grass silage (Mata-Alvarez et al. 2000, EurObserv'er 2009, Demirel & Scherer 2009). Co-digestion concept has been studied and applied increasingly to also treat substrates such as municipal solid wastes, industrial wastes, food processing wastes, sludges etc in addition to animal manures and energy crops (Mata-Alvarez et al. 2000). During co-digestion, animal manures offer buffering capacity and a wide spectrum of nutrients while crop materials with high carbon content balance the carbon to nitrogen (C/N) ratio of the process thus reducing the risk of ammonia inhibition (Hashimoto 1983). The synergistic effects of co-digestion thus offer high methane yields.

On the contrary, AD of crops with high total solids (TS) of 20-50 % in one-stage reactor systems requires large quantities of water for homogenization thus increasing the volumes to be treated and the energy required for pumping, mixing and heating (Lehtomäki & Björnsson 2006). Scum formation of crop materials is another challenging factor during AD of energy crops in the conventional manure digesters/in wet-digestion systems such as continuously stirred tank reactor (CSTR) systems (Thamsiriroj & Murphy 2010). Therefore, AD of crops/crop residues in conventional liquid phase, one-stage digesters would be cost and energy intensive accompanied by the aforementioned challenges (Andersson & Björnsson 2002). Further, it is not possible to study/optimize/enhance either hydrolysis or methanogenesis independently in a one-stage CSTR system as both the steps would take place simultaneously in the reactor. The anaerobic degradation rates of a substrate in a one-stage reactor system (during co-digestion) could be enhanced by the application of pretreatment methods to the substrate. Pretreatment of the substrate increases the surface area available for enzymatic degradation and thus enables to obtain higher hydrolytic as well as methane yields.

1.4.2 One-stage, leach-bed reactor (LBR) systems for mono-digestion of energy crops

Application of mono-digestion systems or dry AD systems offer the benefits of lower need of water in the process, as no slurrying is required and also less waste water (effluent) is produced. This results in energy savings due to reduced heating and pumping requirements. In addition, the required fermentation volume is reduced. On the other hand, mixing and feeding of dry

systems requires more robust equipment than wet systems. The main advantage of mono-digestion of energy crops is the increased volumetric methane yields (Banks & Humphreys 1998). However, experience with mono-digestion plants showed that mono-digestion of energy crops is more sensitive to process imbalance than co-digestion with manure (Demirel & Scherer 2009, Pobeheim et al. 2010). Mono-digestion of energy crops has also been receiving attention because not all agricultural farmers are into animal husbandry and thus availability and accessibility (in terms of distance) of manure is a limitation. It is presumed that about 15 % of the biogas digesters in Germany are operated with crops alone (Demirel & Scherer 2009).

A biogasification technology that has recently started to receive attention is leach-bed reactor (LBR) technology which was mainly developed to treat the high-solid organic wastes and to recover biogas at high rates (Koppar & Pullammanapallil 2008, Dogan et al. 2008). LBR systems have been successfully used for treating various feedstocks like, organic fraction of municipal solid waste (Hegde & Pullammanappallil 2007), fruit and vegetable wastes (Martinez-Viturtia et al. 1995), food wastes (Han et al. 2002), animal manure (Demirer & Chen 2008) and energy crops (Banks et al. 2008, Lehtomäki et al. 2008). The leach-bed process is advantageous, as it does not require shredding of the substrate, does not require mixing of digester materials, does not require robust, high pressure equipment and they can operated stably at mesophilic and thermophilic conditions (Pullammanappallil 2005, Nizami et al. 2011). In an LBR system solids are subjected to hydrolysis by re-circulating the leachate collected at the bottom of the reactor continuously over the substrate bed. Re-circulation of leachate was shown to accelerate the hydrolysis of particulate matter as liquid re-circulation can increase the moisture content, promote mass transportation, redistribute the enzymes and microbes and minimize nutrient deficiency (Lü et al. 2008). Depending upon the feed characteristics and scale of operation, LBR systems can be operated either in a one-stage or two-stage system. The advantage of a two-stage system over one-stage system is that hydrolysis and methanogenesis can be optimized separately, with the former normally a rate limiting step if the substrate is rich in particulate matter e.g. lignocellulosic materials such as crops. Therefore, when crops are used as substrates in anaerobic process it becomes challenging to improve the rate and extent of hydrolysis in order to obtain maximum organic material solubilization for conversion into biogas.

1.4.3 Two-stage AD systems, LBRs coupled with high rate UASB reactors

The LBR process was originally devised to be operated as a two-stage reactor system (Chynoweth et al. 1992). In a two-stage reactor system, the solids can hydrolyzed in the first-stage reactor by re-circulation of liquid over a solid bed of crop materials. The liquid/leachate can then be pumped to the second-stage reactor for further degradation (methanogenesis) (Koppar & Pullammanapallil 2008). The first-stage reactor can be a dry batch reactor such as an LBR while the second-stage reactor can be a high rate reactor such as an upflow anaerobic

sludge blanket reactor (UASB) (Lehtomäki et al. 2008) or anaerobic filter (AF) (Cysneiros et al. 2008). In addition to reduced water consumption and waste water discharge, AD in LBR also enables increased volumetric methane yields when operated at high solids concentration (Lehtomäki et al. 2008).

Dry AD of lignocellulosic materials in reactor configuration consisting of LBR coupled to UASB or AF reactor is relatively more recent and has been successfully studied at laboratory-scale and pilot-scale with substrates like grass silage, maize silage and spent sugar beet pulp etc (Lehtomäki & Björnsson 2006, Lehtomäki et al. 2008, Cysneiros et al. 2008, Koppar & Pullammanapallil 2008). Methane yields of 0.2-0.4 m^3 kg^{-1} VS_{fed} were reported in two different studies when grass silage, sugar beet and willow were subjected to AD in a two-stage reactor system consisting of LBR-MF (methanogenic filter) and LBR-UASB reactors (Lehtomäki & Björnsson 2006, Lehtomäki et al. 2008). However, the effects of AD on the solubilization of nitrogen and conversion of the solubilized nitrogen to ammonium nitrogen are rarely reported. The digestate/effluent resulting from the two-stage reactor system would contain high quantities of plant accessible ammonium nitrogen which could serve as a liquid fertilizer for crop production.

1.5 Methods to enhance hydrolysis during AD of energy crops

1.5.1 Macro and micro nutrients addition

AD of energy crops (lignocellulosic substrates) have slow conversion efficiencies, long solid retention times at low loading rates specific to their complex chemical structure and composition. This is also due to the fact that hydrolysis is the rate limiting step in the AD of the lignocellulosics which is in turn due to the crystalline structures of cellulose with hemi-cellulose and lignin. Such complex structure offers higher recalcitrance for faster and efficient degradation (Kivaisi et al. 1990). Further, mono digestion of energy crops is a rather new practice in AD technology and the limitations involved in it have been less often studied. So far, the most reported limiting factor was that of insufficient availability of macro and micro nutrients thus resulting in an imbalance in the AD process and drastically affecting methane yields (Demirel et al. 2008). This is clearly due to the absence of manure which comes with the nutrients needed for the microbial consortia during the AD process.

Several authors have studied supplementation of macro and micro nutrients in one-stage or two-stage reactors for the improvement of methane yields from substrates like organic wastes, waste waters, manures etc., (Osuna et al. 2003, Zandavoort et al. 2006) while studies based on energy crops are limited (Jarvis et al. 1997, Gunaseelan 2006, Hinken et al. 2008, Lebuhn et al. 2008). For example, AD of bermuda grass was reported to be deficient in nitrogen and phosphorus (Ghosh et al. 1985). In the above study, the authors

reported about 96 % increase in methane yield (0.218 m^3 CH_4 kg^{-1} VS from a semi-continuously operated continuously stirred tank reactor systems (CSTRs) with external addition of nitrogen and phosphorus when compared to the methane yield obtained from the control reactors (0.112 m^3 CH_4 kg^{-1} VS). In another study, it was demonstrated that mesophilic AD of napier grass supplemented with nitrogen and phosphorus resulted in low methane yields and high VFAs (Wilkie et al. 1986). Daily addition of micro nutrients solution of nickel, cobalt, molybdenum and selenium and sulphate improved the methane production by 40 % (from 0.113 m^3 CH_4 kg^{-1} VS to 0.158 m^3 CH_4 kg^{-1} VS) and resulted in a significant decrease in VFA concentration.

Furthermore, studies on food processing/food wastes (Schanbacher et al. 2005), grass-clover silage (Jarvis et al., 1997), broiler and cattle manure (Güngör-Demirci & Demirer 2004) have shown that micro nutrients addition (Fe, Ni, Co, Mo, Se and W) had stimulated methane production. Most of these studies were carried out mainly to enhance methane yields (methanogenesis) and not much significance was given to study the influence of the nutrients addition on hydrolysis. Efforts to understand the nutritional needs and nutritional dynamics of microbial consortia during hydrolysis of crop biomass are still limited.

1.5.2 Leachate replacement and micro-aeration

When substrates such as MSW, food wastes, energy crops or landfill wastes are subjected to AD in LBRs or reactors similar to LBRs (called as percolating reactors) the leachates are saturated with high concentrations of SCOD (intermediary products of hydrolysis) and VFA as a result of re-circulation of leachate (Sanphoti et al. 2006, Zhang et al. 2007, Lehtomäki et al. 2008). In a one-stage LBR the high concentration of SCOD and VFA production cause accumulation of VFA and thus low pH conditions further resulting in inhibition of hydrolysis and methanogenic processes. Dilution of the inhibitory products by leachate replacement with tap water was shown to improve COD solubilization and methane yields from one-stage or two-stage AD in LBR systems with MSW (Hao et al. 2008) and maize silage (Cysneiros et al. 2008) respectively. Zhang et al. (2007) studied AD of vegetable and flower wastes in a two-stage reactor configuration and reported that hydrolysis of the substrates was enhanced with the increase in the dilution rate resulting in enhanced extracellular enzyme activities. During the first 2 days leachate produced from the hydrolytic reactor was re-circulated to avoid washing out of hydrolytic microorganisms and extracellular enzymes (Sanphoti et al. 2006). After 2 days of re-circulation, leachate replacement method was applied by introducing the effluent from the methanogenic reactor into the hydrolytic reactor. This enabled the dilution of the leachate and avoided the inhibition of hydrolytic products accumulation. As a result, the authors obtained 15-92 % of carbon and 9-80 % of nitrogen solubilization from the studied solid substrates. However, the method of leachate replacement for enhancing hydrolysis of specifically, the energy crops and crop residues during mono-digestion in LBRs was not reported.

Creating micro-aerobic conditions in the anaerobic reactor by introducing a controlled and limited amount of air (oxygen) was studied as a pre-treatment step or directly during AD process to improve hydrolysis and also methane yields from substrates such as organic sludge, primary sludge, municipal waste waters and municipal solid wastes (Hasegawa et al. 2000, Johansen & Bakke, 2006, Jenicek et al. 2008, Ngugyen et al. 2007 respectively). The increase in methane yields was attributed to the result of external enzymes secreted by aerobic bacteria (Hasegawa & Katsura 1999, Ngugyen et al. 2007). However, the method of micro-aeration for enhancing hydrolysis of crop biomass during mono-digestion in LBRs was never reported.

1.5.3 Application of rumen cultures

The process of AD also occurs in the natural anaerobic microbial ecosystems such as in the rumen of (ruminant) animals like in cow, sheep, deer, goat and kangaroos. The rumen is a complex anaerobic cellulolytic ecosystem with various bacteria, archea, protozoa and fungi. These microbial consortia collectively degrade plant polysaccharides to generate VFAs (acetic, propionic and butyric acids) as animals' energy source, biomass as the protein source for the animal and the biogas is eructated via the mouth (Barnes and Keller 2003). Therefore, the rumen uses the specific microbial community and the specific associated enzyme systems to anaerobically degrade the lignocellulosic materials that the animals consume. The rumen can be used as a model to develop an efficient AD system which could enhance the depolymerization and obtain higher degradation rates of the lignocellulosic substrates. Recently, potential applications of rumen microorganisms in artificial rumen reactors for the conversion of lignocellulosic materials were investigated (Dalhoff et al. 2003, Barnes & Keller 2003, 2004, Hu & Yu 2005).

For example, O'Sullivan et al. (2006) studied cellulose solubilization rates in batch reactors using rumen culture and MSW leachate as sources of inocula and microcrystalline cellulose as substrate. The authors obtained about 34-50 % higher cellulose solubilization rates when rumen culture was used as an inoculum source than with the MSW leachate. Furthermore, Hu & Yu (2005), studied AD of corn stover in batch and semi-continuous cultures and obtained a high VS conversion efficiency of 65-70 % after 10 days of incubation at 25-40˚C. In another such study by Yue et al. (2007), a degradation efficiency of 52.3 % was achieved during AD of aquatic plant such as *Canna indica L.* using rumen cultures in batch experiments. Therefore, application of rumen cultures as an inoculum source would be very interesting and useful in terms of obtaining higher degradation efficiencies and thus higher energy (biogas/methane) benefits.

2 OBJECTIVES

The broad objective of the study was to identify operational strategies for efficient AD of energy crops and crop residues in different reactor systems such as CSTRs and LBRs (with or without coupling UASB reactors) especially focusing on enhancement of hydrolysis. This was aimed to be achieved by the following specific objectives;

- To co-digest grass silage and cow manure in CSTRs and determine if the methane yield obtained from co-digestion can be improved by re-circulating solid fraction of the digestate fibres with and without alkali-treatment (I).
- To demonstrate the feasibility of mono-digestion of grass silage in LBRs with and without addition of macro and micro nutrients and the effects of nutrients on microbial hydrolysis (II).
- To enhance hydrolysis by micro-aeration and leachate replacement methods during mono-digestion grass silage in LBRs (III).
- To enhance hydrolysis of grass silage with the application of cellulolytic rumen cultures as an inoculum source during AD in LBRs (IV).
- To evaluate the two-stage AD of grass silage, common reed, tomato and cucumber, in an LBR-UASB reactor configuration along with an assessment of nitrogen solubilization into the leachates obtained (V).

3 MATERIALS AND METHODS

The materials and methods specific to each of the experiments are summarized in Table 5 and described in detail in the articles I-V.

TABLE 5 Objectives and the reactor experimental set-up(s) used in this study (papers I-V).

Objective	Type of reactor and no. of stages	Capacity of reactors (l)	No. of reactors	Temp. (°C)	Inoculum type	Paper
To improve the biodegradability of the solid fraction of the digestate by alkali treatment and obtain higher CH_4 yields by re-circulation of such treated fibres	CSTRs 1 stage	5	3	35 ± 1	Digested material from biogas plant	I
To study mono-digestion of grass silage with and without addition of micro and macro nutrients	LBRs 1 stage	1	4	35 ± 1	Digested material from biogas plant	II

TABLE 5 continued

Objective	Type of reactor and no. of stages	Capacity of reactors (l)	No. of reactors	Temp (°C)	Inoculum type	Paper
To enhance hydrolysis of grass silage in the anaerobic digesters by creating micro-aerobic conditions and leachate replacement	LBRs 1 stage	1	4	35 ± 1	Digested material from biogas plant	III
To enhance the hydrolysis of grass silage by application of rumen culture as an inoculum source (IV)	LBRs 1 stage	1	4	35 ± 1	Digested material from biogas plant + rumen culture	IV
To study two-stage AD of energy crops (grass silage and common reed) and crop residues (tomato and cucumber) for energy production (CH_4) and for assessing the nitrogen solubilization into leachates obtained	LBR+ UASB 2 stage	10 (LBRs) 1 (UASB) 0.5 (UASB)	4 3 1	20 37 37	Digested material from biogas plant + rumen culture	V

3.1 Origin of materials

Energy crops such as grass silage, aquatic grass species such as common reed, crop residues of tomato and cucumber were the lignocellulosic substrates used in the present study (I-V). Grass silage was used as substrate in all the one-stage experiments (I-IV) while in the two-stage AD experiments (V), crop materials of tomato, cucumber and common reed were used in addition to grass silage (Table 6). Grass silage was collected from silos of a local dairy farm (Kalmari farm, Laukaa) in Central Finland and was prepared at the farm from grass (seed mixture with 75 % timothy, *Phleum pratense* and 25 % of meadow fescue, *Festuca pratensis*) harvested at the early flowering stage, chopped with an agricultural precision chopper after 24 h pre-wilting and then ensiled after adding commercial silage additive (I-V). Cow manure (I) was also brought from the local dairy farm (Kalmari farm, Laukaa). Leaves and short stems of tomato (*Lycopersicon esculentum L.)* and cucumber (*Cucumis sativus*) crops (1-2 cm) were obtained from a green house in south-west Finland (Härkälän puutarha) while common reed (*Phragmites australis*) was collected from a lake (Kirrinsanta, Pori) (V). At the laboratory, grass silage (I-V) and common reed (V) were further cut to 2-4 cm size with kitchen scissors.

Three different inocula were used as inoculation material in the batch methane potential assays and the reactor experiments (I-V). Inoculum was digested material from a farm scale biogas plant treating cow manure, industrial confectionery waste and energy crops and was brought from the local farm (Kalmari farm, Laukaa) in Central Finland (I-V) (Table 7). Rumen fluid (IV and V) was obtained from a fistulated cow in MTT Agrifood research institute, Jokioinen, Finland. Rumen fluid was referred as 'whole rumen fluid' when it was unfiltered and as 'rumen culture' when filtered. For UASB reactor experiments (V) granular sludge was used as an inoculum and was obtained from a mesophilic UASB reactor treating waste waters from an oats processing industry, Jokioinen, Finland. In the co-digestion experiments (I), digestate from the laboratory CSTRs (operating with grass silage and cow manure for about 318 days at 35 °C) was also used as inoculum.

Micro nutrient (Fe, Co, Ni and Mo) concentrations (II) were modified based on the studies of Weiland et al. (1991), Jarvis et al. (1997) and Pobeheim et al. (2010) with substrates such as waste waters, grass silage and maize silage respectively (II). Further, concentration of NH_4Cl added as nitrogen source (macro nutrient) was based on studies of Zhang et al. (2003).

TABLE 6 Characteristics of the substrates used in the experiments I-V.

Substrate	TS (% ww)	VS (% ww)	pH	TKN (mg g^{-1} TS)	NH_4-N (mg g^{-1} TS)	Paper
Cow manure	6.5	5.1	6.9	32.0	0.002	I
Grass silage	38	35	4.5	19.3	2	I
Grass silage	27	26	4.6	9.8	2.8	II (set 1)
Grass silage	39	36	3.9	6.4	0.2	II (set 2)
Grass silage	37	34	4.5	7.28	0.25	III
Grass silage	42	39.5	4.09	6.4	0.24	IV
Grass silage	41	39	3.9	17	0.25	V
Common reed	44	41	4.3	8.9	0.05	V
Tomato	10	7.6	5.1	32.5	0.25	V
Cucumber	6.8	4.5	7.1	27.3	0.15	V

TABLE 7 Characteristics of the inocula used in the experiments I-V.

Type of inoculum	TS (% ww)	VS (% ww)	pH	TKN (mg g^{-1} TS)	NH_4-N (mg g^{-1} TS)	Paper
Inoculum (from the mesophilic farm digester treating cow manure, energy crops and industrial confectionery wastes)	5.8	4.5	7.9	42.2	1.6	I
	6.8	5.6	8.6	47	7.3	II (set 1)
	3.2	2.1	8.2	95	50	II (set 2)
	5.3	4.2	7.6	48	8.77	III
	6.0	5.0	7.74	50.7	13.2	IV
Rumen fluid*	3.3	2.3	6.55	47.5	0.23	IV
Rumen fluid*	3.5	2.6	6.8	NA	NA	V

NA- Not analysed, *- unfiltered

3.2 Experimental set-up

3.2.1 Co-digestion of grass silage and cow manure in CSTRs (I)

Co-digestion was studied in three identical continuously stirred (300 rpm), 5 l glass reactors (R1, R2 and R3) of liquid capacity of 4 l at 35 ± 1°C. The reactors were syringe fed semi-continuously (once a day 5 days a week). On day 0 of the run 4 l of inoculum (3 l of inoculum from farm and 1 l of inoculum from laboratory CSTRs operated with grass silage and straw) was added into the reactors. The substrate was fed at a loading rate of 2 kg VS m^{-3} d^{-1} (i.e., about 200 g of substrate/day) and the feed ratio was 70:30 (% of substrate VS) of cow manure and grass silage respectively. Before feeding, a volume equivalent to the feeding volume was removed with the syringe.

From day 75 onwards, suspended solids (referred to as 'solids' hereafter) from the digestate were re-circulated (ca 20 g of solids were fed based on the amount of solids obtained after centrifugation), to R2 and R3 while R1 was run as control. During the re-circulation period, the removed R2 and R3 digestates were collected every week (i.e. on the five feeding days of the week). The solids from R2 were treated with 20 g NaOH kg^{-1} VS (40 % NaOH solution) and the solids from R3 were untreated (no NaOH treatment) and were incubated in 250 ml bottles at 35 ± 1°C for about 65 hours. Reactor R2 was supplied with alkaline treated solids and R3 with untreated solids along with the original feed (grass silage and cow manure). Biogas produced from the reactors was collected in aluminium gas bags (I-V).

3.2.2 Enhancing hydrolysis during mono-digestion of grass silage in one-stage LBRs (II-IV)

LBRs were used to study mono-digestion of grass silage by employing methods to enhance microbial hydrolysis (II-IV). Effects of macro and micro nutrients addition (II), leachate replacement and micro-aerobic conditions (III) and application of rumen cultures (IV) on microbial hydrolysis were evaluated. Four LBRs were used and were referred to as L0, L1, L2 and L3 in all the studies (II-IV). The LBRs were connected to reservoirs and were referred as R1-R4 (II, set 1 and III). In LBR studies with micro nutrients (II, set 2) and for the application of rumen culture (IV) no reservoirs were used and the leachate was allowed to remain in contact with the grass silage in the LBRs.

The leachate collection system at the bottom of the LBRs included a 3 cm cylindrical acrylic column on top of which a steel mesh was placed (pore size about 2 mm) to support the biomass weight and several layers of nylon mesh (pore size <1 mm) were placed underneath to prevent clogging as well as solids entering into the leachate (II, set 1 and III). The leachate collection system was modified by shortening acrylic cylindrical column to 2 cm long on top of which a steel mesh (2 mm pore size) was placed to support the biomass weight. On

top of this steel mesh a layer of foam (1 cm thickness) and a layer of glass beads were placed to prevent microbial washout from the column. At the bottom of the cylindrical column two layers of nylon mesh (< 1 mm) were placed to further filter the solids from the leachate (II, set 2 and IV) (Fig. 3) .

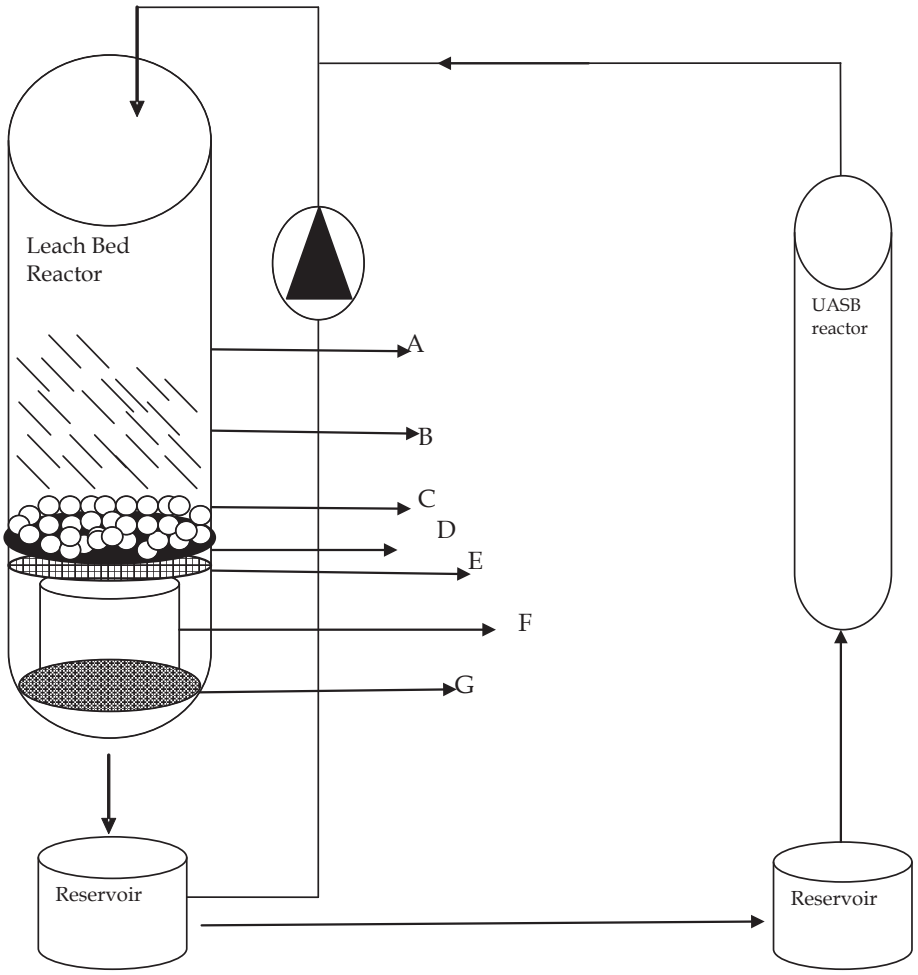

FIGURE 3 One-stage (LBR) and two-stage (LBR-UASB) reactor set-up(s) used in the experiments II-IV and V respectively. A- Acrylic column used as LBR, B- crop bed, C- Glass beads layer, D- Foam (1 cm thickness), E- Steel mesh (pore size- 2 mm), F-Inner acrylic column (Height 2-3 cm), G- Nylon mesh (pore size- 1 mm).

On day 0, LBR columns were loaded with 50 g VS of grass silage and 3 g VS of inoculum (II, set 1 and III). Grass silage and inocula were added in the LBRs at a $VS_{substrate}$ to $VS_{inoculum}$ ratio of 2 (IV). The reservoirs R0 and R1 were filled with 750 ml of distilled water and R1 was further added with 2.1 g l^{-1} of NH_4Cl as a nitrogen source (Zhang et al. 2003) (II, set 1). The control LBR (L2) was filled

with 470 ml of distilled water, while LBR with micro nutrients (L3) was filled with a micro nutrient solution of the same volume (470 ml) (II, set 2). Micro nutrient concentrations added in the LBR study (medium dosage level) are shown in Table 8. Leachate re-circulation was started immediately using peristaltic pumps at a flow rate of 750 ml d^{-1} (II, set 1 and III) and it was connected to timers to allow re-circulation every 15 minutes @470 ml d^{-1} (II, set 2) and @780 ml d^{-1} (IV).

TABLE 8 Micro nutrient dosages tested in the BMP assays (low, medium and high) and LBR experiments (medium) (set 2, II).

Micro nutrients concentration	**Dosage level**		
(mg l^{-1})	**Low**	**Medium**	**High**
Fe	50	75	375
Ni	0.1	1.7	9
Co	0.2	0.75	3.75
Mo	0.15	0.5	0.75

The effect of leachate replacement (day 22) with initial pH adjustment (days 1-7) was investigated (LBR L1) and was compared with the performance of LBR in which, leachate replacement (day 11) was done without pH adjustment (L3) (III). The pH in L1 was adjusted to 7.5 through NaOH addition (1M) and by using automatic pH regulator. On the other hand, the effect of micro-aeration was studied in L2. Air flow rates of 1 l min^{-1} (2.5 l of air) on day 11 and 4 l min^{-1} (24 l of air) on day 22 were employed in L2. The performance of LBRs L1, L2 and L3 was compared with control (L0), where no leachate replacement, pH adjustment or micro-aeration was carried out.

For application of rumen cultures (IV) also, the LBR L0 was studied as a control reactor with grass silage and inoculum from biogas plant while, LBR L1 was tested with grass silage and 100 % rumen inoculum. The LBR L2 was tested with grass silage and a mixture of inocula i.e., 25 % rumen fluid and 75 % cow manure while, LBR L3 was tested with NaOH pretreated grass silage and inoculum from biogas plant. In all the LBRs, 550 ml of water was added to make up the liquid (working) volume to about 780 ml. Leachate samples collected for chemical analyses were replaced by the same volume of distilled water (II-IV).

3.2.3 Two-stage AD of energy crops and crop residues in LBRs and UASB reactors (V)

AD of tomato, cucumber, common reed and grass silage was carried out in four parallel two-stage reactor set-ups. Each reactor set-up consisted of LBR and UASB reactor connected through their respective reservoirs. UASB reactors were started-up 10 days prior to the start-up of the LBRs. During the start-up, UASB reactors were inoculated with granular sludge and fed with glucose solution (1.5 g l^{-1}) at an organic loading rate (OLR) of 1.5 g chemical oxygen demand (COD) l^{-1} d^{-1}. Feed was diluted with basic anaerobic medium as described in Raposo et al. (2006).

Each LBR was provided with a leachate collection system at the bottom as described in section 3.2.2 for paper IV. Each LBR/UASB reactor was also provided with a gas collection/separation system. On day zero (start of the experiment), each LBR was loaded with the respective crop material to full capacity. Tomato and cucumber LBRs were filled with 10.5 kg of tomato leaves and 9.615 kg of cucumber leaves, respectively. The low dry matter content (Table 6) in these materials facilitated prompt leaching and thus no water addition was necessary. Within minutes, 3.85 l and 8.5 l of leachates were collected from these tomato and cucumber LBRs, respectively. However, only 2 l of the collected leachate was used as feed for their respective UASB reactors. The remaining leachate was stored separately at 4°C for future use. On the other hand, 2.3 kg of crop materials were loaded in grass silage and common reed LBRs, respectively, and 2 l of de-ionized water was added to initiate leaching in these two LBRs.

LBRs were sealed with PVC lids and anaerobic conditions were created by flushing the reactors with pure N_2 gas. Each LBR was provided with a reservoir (2 l glass bottle) for leachate collection. From days 1 to 6, leachate from each LBR was fed directly to their respective UASB reactor. Effluents from the UASB reactors were then returned to their respective LBRs. However, low biogas production and process failure in UASB reactors on day 7 prompted to operate the two-stage reactor system as two separate one-stage processes. From day 7 onwards, LBRs were operated as one-stage process with an internal re-circulation rate of 2 l d^{-1}. On the other hand, the effluent from UASB reactors were collected separately and fed to LBR. On day 10, leachates from all the LBRs were removed (stored at 4 °C) and 2 l of fresh de-ionized water was added to each LBR to facilitate further COD solubilization. In addition, the added water was allowed to stand over in the LBRs for 36 h before internal re-circulation was resumed at a higher rate of 4 l d^{-1}.

At the same time, UASB reactors were re-inoculated with 150-250 ml more sludge and operated as one-stage process with internal re-circulation (1 l for 1 l UASB and 0.5 l for 0.5 l UASB d^{-1}). Reactors were operated with an initial OLR of 1.5 g COD l^{-1} d^{-1} (day 7) and further increased to 3 g COD l^{-1} d^{-1} (days 13-29). To achieve the OLRs of 1.5 or 3 g COD l^{-1} d^{-1}, leachate volumes equivalent to 1.5 or 3 g COD l^{-1} were withdrawn from the respective LBRs and diluted with de-ionized water. From day 13 onwards, leachate volume equivalent to 3 g COD

l^{-1} was withdrawn from each LBR and an equivalent amount of effluent from UASB reactor was added to the respective LBR. This process continued for the rest of the experimental period. Thereby, no extra water was introduced into the system. Upon stable gas production, the OLR was increased from 3 to 5-7 g COD l^{-1} d^{-1} (days 29-89). Finally, process temperature was increased from room temperature (21°C) to 37 °C on day 19.

On day 31, LBR experiments were terminated and the residual materials were pressed manually and subsequently with weights (overnight) over a nylon mesh to extract the remaining leachate. About 2 l of leachate was extracted from the tomato and cucumber residual materials. The corresponding values for common reed and grass silage were 25-50 ml, respectively. The collected leachates were stored separately at 4 °C. The residual crop materials before (RM) and after extracting the remaining leachate through manual pressing and by overnight weight (RMLE) were also stored at 4 °C until further use. At the same time, the remaining untreated leachates and the effluents from UASB reactors (stored at 4 °C) were mixed and fed to UASB reactors (day 42 onwards). In total, the amount of leachate available for UASB reactors was 14 l for cucumber, 9 l for tomato, 3.1 l for grass silage and 3.4 l for common reed. However, only 6.6 l of cucumber and 5.5 l of tomato leachates were used for biomethanation in UASB reactors while for grass silage and common reed leachates available were completely fed.

3.3 Biological methane potential (BMP) assays (I-V)

The methane potential assays were carried out in duplicate/triplicate, in serum bottles (IV) or 1 l glass bottles (I, II, V) with liquid capacity of 40 ml and 750 ml respectively. The assays were carried out at 35 ± 1 °C. To each of the serum bottle or 1 l assays, 25 ml and 250 ml of inoculum was added respectively into the bottles and substrate was added to have a $VS_{substrate}$ / $VS_{inoculum}$ ratio of 1 (I, II, V). Distilled water was added to make the liquid volume 750 ml and $NaHCO_3$ (3 g l^{-1}) was added as buffer in 1 l assays (I, II). After preparing the assays the contents of the bottles were then flushed with nitrogen (98.8 %) for about 3 minutes before sealing with silicon stoppers. The bottles were manually shaken before analysing the methane content. Biogas from the assays was collected in aluminium gas bags. Assays with inoculum alone were used as controls to subtract its methane production from those of the substrates (I-V).

Effects of macro and micro nutrient additions on the AD of grass silage were analyzed with and without addition of NH_4Cl and micro nutrients (Fe, Ni, Co and Mo) (II, set 1). For assays with NH_4Cl, only one concentration was tested (II, set 1) while for assays with micro nutrients, three different dosages such as low, medium and high were tested (Table 8) (II, set 2).

Application of rumen culture (IV) was carried out in two sets (IV). The first set was to determine the effects of using a mixture of rumen culture and

digested cow manure as an inoculum at different ratios while, the second set was to determine the effect of whole rumen fluid (unfiltered/as obtained from the animal) and the solid fraction of the rumen fluid on biomethanation of grass silage. For the first set of assays, rumen culture was added at loading rates of 0, 25, 50, 75 and 100 % of the total inoculum amount (g VS basis). The corresponding ratio for digested cow manure was in reverse order i.e, 100, 75, 50, 25 and 0 %. The fresh substrates of tomato, cucumber, common reed and grass silage were assayed in parallel with two different inocula (V) *viz.*, inoculum from the farm-scale biogas plant and a mixture of inocula from with rumen culture (25 % v/v) and the digested material from the farm-scale biogas plant (75 % v/v) .

3.4 Residual methane potential assays

The residual methane potential assays were carried out to extract the left-over methane potential in the substrate (s) after AD in the reactor systems (I, II - set 1, V)/BMP assays (II, set 2). These assays were carried out in serum bottles of 120 ml capacity (I, II, set I, IV), 60 ml capacity (II, set 2) and in 1 l bottles (V). The working volumes were 40, 25 and 750 ml respectively. The effect of NaOH additions on methane potential of solid fraction of the digestate and digestate as such were carried out using digestate of R1 (I). The methane potential of the digestate layers (reactor materials stratified into the top, middle and bottom layers) were also studied at the end of the reactor experiments (I). NaOH was added (in 20, 30 and 40 and 60 g NaOH kg^{-1} VS, 40 % NaOH solution) and pH was adjusted to 7.2-7.5 with 5 M HCl in all the bottles immediately (I). In 1 l assays, inoculum and substrate were added to have a $VS_{substrate}$ /$VS_{inoculum}$ ratio 1 (I, V). $NaHCO_3$ was added as buffer @ 3 g l^{-1} (I, V). Finally, the prepared assays were flushed with nitrogen (98.8 %) for about 1 minute before sealing with silicon stoppers (1 l assays) butyl rubber stoppers and aluminium crimps (serum bottles). For serum bottle assays, nitrogen was flushed again through water lock system in order to ensure complete anaerobic conditions. For 1 l assays biogas was collected in aluminium gas bags.

Assays with inoculum alone were used as controls to subtract its methane potential from those of the substrate (I-V). After terminating the LBRs with NH_4Cl (II, set 1) residual solid materials and leachates were assayed separately and as a mixture (solid materials + leachate) with and without pH adjustment and inoculum. The residual materials from 'control' BMP assays (II, set 2, with micro nutrients) were assayed in 60 ml serum bottles to determine which micro nutrient (s) or micro nutrient combinations were stimulating higher methane potential in the BMP assays against control. On day 60 of BMP assays, one of the triplicates from 'control' assays (section 3.3) was opened and 25 ml of residual materials were transferred to each of the serum bottles. Four triplicate sets were prepared for four (Fe, Ni, Co and Mo) single micro nutrient additions and another four triplicate sets were prepared with combinations of the micro

nutrients and one triplicate was prepared to act as control (Table 8). The residual materials of tomato, cucumber, common reed and grass silage were assayed in parallel sets with two different inocula (V) as described in section 3.3.

3.5 Analyses and calculations

TS and VS were determined according to the Standard Methods (APHA 1998) (I-V). pH was measured with a Metrohm 774 pH meter (I, III, V) and Mettler Toledo S20 Seven easy pH meter (II and IV). COD, soluble (SCOD) and total (TCOD), were analyzed according to Finnish Standards (SFS 5504; Finnish standards association, 1988) (I-V). NH_4-N and total nitrogen (TKN) were determined by Tecator Application Note (Perstorp Analytical/Tecator AB) using a Kjeltec system 1002 distilling unit (I-V). Samples for SCOD and NH_4-N were filtered using GF 50 glass fibre filter papers (Schleicher & Schuell) (I-V). Soluble COD (SCOD) and NH_4-N from crop samples were analyzed after extraction according to SFS-EN 12457-4 (Finnish standards association 2002) (I-V). Methane and hydrogen contents in the biogas were analyzed using a gas chromatograph (Perkin Elmer Clarus 500 GC with thermal conductivity detector and Supelco Carboxen™ 1010 PLOT fused silica capillary column 30m×0.53mm, carrier gas argon, oven temperature 100°C, injection port 250 °C, detector 225 °C). A pressure lock syringe was used for sampling gas. VFAs were analyzed with gas chromatograph (PE Autosystem XL GC equipped with flame-ionization detector and PE FFAP column 30 m x 0.32 mm x 25 µm, carrier gas helium, injection port 225 °C, oven temperature 100-160 °C). Biogas volume was measured by water displacement method (I-V).

The total and soluble micro nutrient concentrations of Fe, Ni, Co and Mo were analyzed by using inductively coupled plasma optical emission spectrometry (ICP-OES). Leachate samples were acidified with HNO_3 (pH <2) and stored at -20 °C until further analyses whereas, crop samples (solids) of grass silage were oven dried at 105 °C for 24 hours and then kept in a muffle furnace (for 2 h) at 550 °C to obtain dry ash. The dry ash samples were then digested using the ultrasound-assisted digestion method. The sample solutions obtained from the digestion were analyzed for the metal concentrations according to Väisänen et al. (2008) using Perkin-Elmer (Norwalk, CT, USA), model Optima 4300 DV ICP-OES using the default parameters of the instrument (nebuliser flow 0.6 l m^{-1}, plasma power of 1400W and auxiliary gas flow of 15 l m^{-1}) (II).

In methane potential assays, specific methane yields from substrates/residual materials were calculated as cumulative methane produced per kg of added substrate VS (m^3 CH_4 kg^{-1} VS_{added}) (I-V). Specific SCOD (g COD g^{-1} VS_{added}), NH_4-N (mg NH_4-N g^{-1} VS_{added}), and TKN (mg TKN g^{-1} VS_{added}) production in LBRs was calculated by considering the total leachate produced

(including the leachate extracted by manual and overnight pressing with weights, V) and the sample volumes removed during the operation of LBRs (II-V). Methane yields from the two-stage system consisting of LBR-UASB reactors (V) were calculated as:

Methane yields (m^3 CH_4 kg^{-1} VS_{fed}) =

$$\frac{\text{Total methane produced (l)}}{\text{Total COD fed to UASB reactor (g COD)}} \times \frac{\text{Total COD leached from Leachbed reactor (g COD)}}{\text{Initial VS of substrate in Leachbed reactor (kg VS)}}$$

The data obtained in the BMP assays (II) was subjected to analysis of variance (ANOVA) using the SPSS program (SPSS 1999). The effects of the treatment were studied by comparing the means of treated groups. If the variances of groups differed, Welch's version of ANOVA was used. Further, if the ANOVA F-test was significant at $p \leq 0.05$, Tukey's test was used for multiple comparisons of the group means.

4 RESULTS

4.1 Co-digestion of grass silage and cow manure in CSTRs (I)

Co-digestion of grass silage and cow manure was studied in three lab-scale CSTRs (referred as R1, R2 and R3) for a period of 151 days to study if the biodegradability of the solid fraction of digestate can be improved by alkali treatment and if re-circulation of such solids enhances the overall methane yields from the process. The studied grass silage had ca 6, 7 and 4 fold TS, VS and nitrogen content than manure while its total methane potential was about 40 % higher than manure (I). On the other hand, the extractable NH_4-N was low compared to NH_4-N in manure.

The CSTRs were inoculated on day 0 and semi continuous feeding was started on day 6, when methane content reached about 50 % of gas phase in the reactors. Subsequently, all the three reactors were operated in parallel co-digesting with substrate VS constituting 30 % and 70 % of grass silage and cow manure respectively, with an OLR of 2 kg VS m^3 d^{-1} and HRT of 20 d. Methane yields in the three reactors increased from ca 165 to 180 l CH_4 kg^{-1} VS during the first eight weeks of operation while TS and VS removals were 20-26 % and 27-33 % respectively in the first four weeks and in the latter four weeks TS and VS removals ranged from 24-30 % and 31-38 % respectively. On day 57, the reactors were opened and the contents of all the three reactors were mixed and then redistributed into the three reactors to ensure presence of identical materials in parallel reactors. Feeding was started the next day. After about two weeks the methane production in the reactors reached to same level as before.

From day 75 onwards, solids separated from the reactor digestate were re-circulated as such (R3) and after alkaline treatment (R2) while one reactor without solids re-circulation (R1) was continued as control. During the study, the control reactor had the highest methane yield (R1, 182 ± 0.02 l CH_4 kg^{-1} VS) while reactor treating alkaline treated solids gave slightly more methane (R2, 161 ± 0.03 l CH_4 kg^{-1} VS) than reactor with untreated solids (R3, 143 ± 0.03 l CH_4

kg^{-1} VS) (Table 9, Fig. 4). Only slight changes in TS removal took place after re-circulation of solids; TS removal (per initial feed TS) decreased (about 3 %) in the reactor supplied with alkaline treated solids (R2) and increased slightly (2 %) in the reactor supplied with untreated solids (R3) while VS removal (in %) was same in both the reactors (R2 and R3) with re-circulation of solids (Table 9). In the reactor supplied with alkaline treated solids (R2) SCOD was higher (9.0 g l^{-1}) than in the reactor supplied with untreated solids (R3, 7.3) or control reactor (R1, 7.5) while NH_4-N concentrations were about the same in all reactors (0.75-0.80 g l^{-1}). TS and VS (%) of digestates of alkaline treated solids (R2) and untreated solids (R3) did not significantly change after solids re-circulation (Table 9).

The effect of alkali treatments and dosages on methane potential of solid fraction of digestate and digestate as such was studied in batch assays for 118 days (Fig. 5). Methane yield was about 12-20 % higher (340 ± 15 l CH_4 kg^{-1} VS) from solid fraction of digestate treated with 20g kg^{-1} VS of alkali than from 30, 40 and 60 g NaOH kg^{-1} VS additions and without NaOH addition; while alkaline treatments on digestate as such did not significantly affect the methane yield (Fig. 5) and did not show much difference from one another (data not shown). At the end of the reactor experiments, the characteristics of the contents of the reactors were studied as whole materials and as layers. Volume occupied by the top layers in both reactors supplied with solids (R2 and R3 respectively) was higher than the volume of the top layer in control reactor (R1) (26, 24 and 15 (%) respectively; Table 10). TS and VS % of top layers of reactor supplied with alkali treated solids (R2) and untreated solids (R3) were slightly higher than control reactor (R1) (Table 10).

Methane potentials of the top layers of the reactor supplied with alkali treated solids (R2) and untreated solids (R3) were about 17-20 % higher (64 ±4 and 67 ± 2 l CH_4 kg^{-1} VS) than control reactor (R1) (53 ± 4 l CH_4 kg^{-1} VS) (Fig. 6). Methane potential of the whole materials of reactor supplied with alkali treated solids (R2) was about 30 % higher (112 ± 4 l CH_4 kg^{-1} VS) than control reactor (R1) and reactor supplied with untreated solids (R3) (84 ± 5 and 75 ± 2 l CH_4 kg^{-1} VS) respectively (Fig. 6). SCOD in the top layer of reactor supplied with alkali treated solids (R2) was higher (14.7 g l^{-1}) and SCOD values of top layers of control reactor (R1) and the reactor supplied with untreated solids (R3) were identical (8.6 g l^{-1}). TCOD in top layer of reactor supplied with untreated solids (R3) was highest (97.3 g l^{-1}) and the values were identical in tops layers of control reactor (R1) and the reactor supplied with alkali treated solids (R2) (89.3 and 89.0 g l^{-1} respectively). TCOD and SCOD for whole materials were very similar for all the three reactors (Table 10).

TABLE 9 Substrate and digestate characteristics and methane yields from the reactors; R1 – control reactor, R2 – with re-circulation of alkali treated solids and R3 – with re-circulation of untreated solids.

	Day 0-24				Day 24-56				Day 75-151 (re-circulation of solids)			
Parameter	Sub	R1	R2	R3	Sub	R1	R2	R3	Sub	R1	R2	R3
TS (%)	4.9	3.6	3.9	3.7	4.6	3	3.5	3.8	4.6	3	3.7	3.5
VS (%)	4	2.7	2.9	2.9	3.8	2	2.7	3	4	2.4	2.6	2.7
SCOD ($g\ l^{-1}$)	24	13.6	14	14	17.6	11	11	12	17	7.5	9	7.2
TCOD ($g\ l^{-1}$)	72	38	37	37	68	36	36	36	46	29	29	26
NH_4-N ($g\ l^{-1}$)	1.1	1	0.9	0.9	1	0.9	0.9	0.9	0.8	0.8	0.8	0.75
TKN (gl^{-1})	2.4	2	2.2	2.1	2.2	1.8	2	2	1.9	2	2.1	1.9
TS removal (%)		26	20	24		30	26	24		28	23	25
VS removal (%)		33	27	28		38	33	31		38	33	31
Methane Conc. (%)		55	49	50		53	49	49		56	54	52
Specific CH_4 yield ($m^3\ CH_4\ kg^{-1}$ VS)		0.18 ± 0.03	0.17 ± 0.04	0.16 ± 0.04		0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.0		0.18 ± 0.02	0.16 ± 0.03	0.14 ± 0.03

(Calculated as averages based on weekly analyses), (Mean ± standard deviations) (I). Sub - substrate

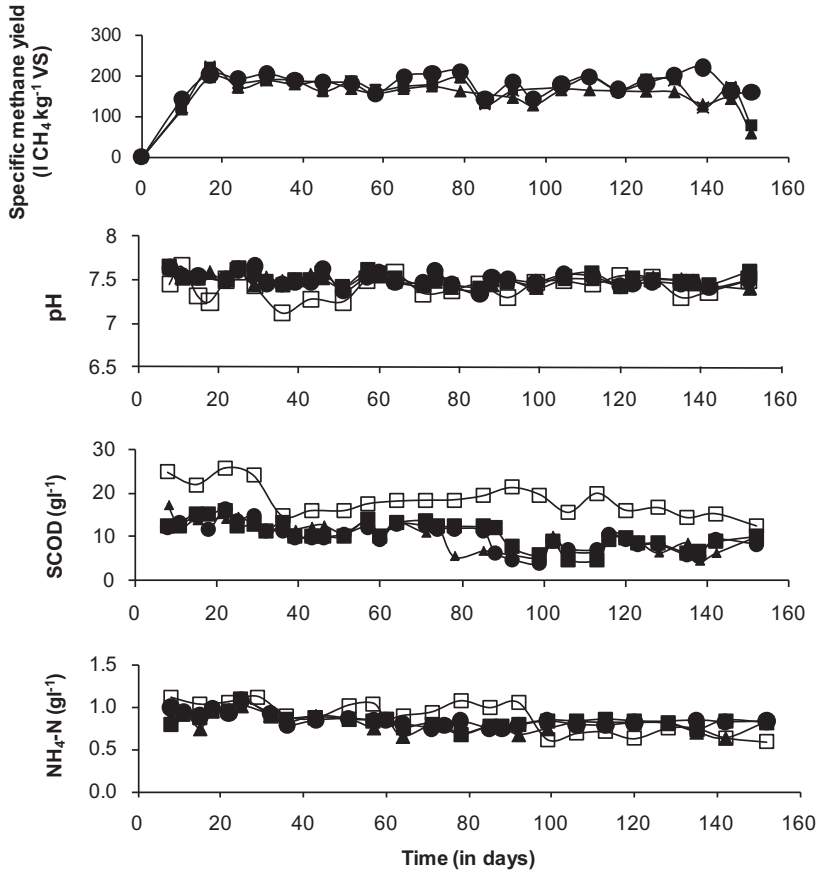

FIGURE 4 Specific methane yields, pH, SCOD and NH_4-N of the substrate and digestates in CSTRs; R1 - control reactor, R2 – with alkali treated solids and R3 - untreated solids, ● - control reactor R1, ■- reactor supplied with alkali treated solids R2, ▲ - reactor supplied with untreated solids, □ - substrate (feed).

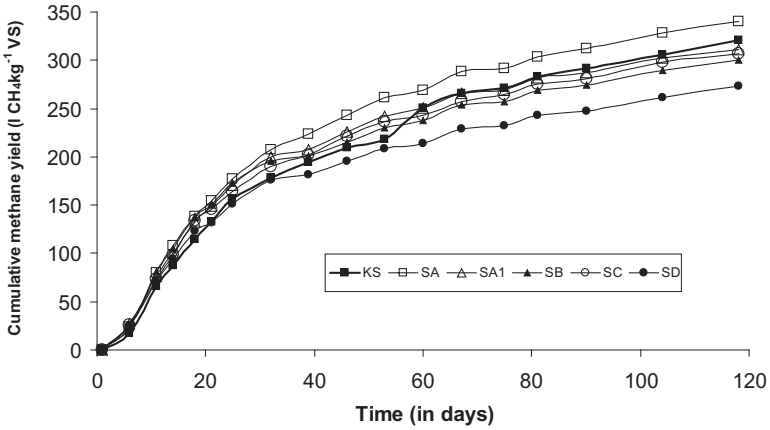

FIGURE 5 The effects of alkali treatments on (cumulative) methane yields of the solids fraction of digestate from the reactors; KS– Control (without NaOH addition) for solids, SA - solids with 20 g NaOH kg^{-1} VS, SA 1 – 20 g NaOH kg^{-1} VS without addition of water, SB - solids with 30 g NaOH kg^{-1} VS, SC solids with 40 g NaOH kg^{-1} VS, SD - solids with 60 g NaOH kg^{-1} VS.

TABLE 10 Characteristics of layers and whole materials in CSTRs co-digesting grass silage and cow manure; R1 - control reactor, R2– with alkali treated solids and R3 - untreated solids.

	Layer	Volume (%)	TS (%)	VS (%)	TCOD (g l^{-1})	SCOD (g l^{-1})	TKN (g l^{-1})	NH_4 (g l^{-1})	Methane (l CH_4 kg^{-1} VS)
R1	Top	15.6	8.2	6.5	89.3	8.6	3.8	0.8	53 ± 4
	Middle	60.1	4.7	3.6	60.2	7.4	2.6	0.8	79 ± 3
	Bottom	24.1	4.1	3.1	61.9	12.3	2.6	0.8	93 ± 7
	Whole	100	4.8	3.7	34	9.8	3.2	0.8	84 ± 5
R2	Top	24.9	8.6	6.9	89	14	3.8	0.9	64 ± 4
	Middle	50.0	4.2	3.2	62	11	2.3	0.9	81 ± 9
	Bottom	25.0	4.1	3.1	61	9.4	2.5	0.9	85 ± 10
	Whole	100	5.0	3.1	33	7.0	2.6	0.8	112 ± 4
R3	Top	26.3	8.6	6.9	97	8.6	4.0	0.9	67 ± 2
	Middle	48.4	4.2	3.3	69.5	8.9	2.4	0.9	66 ± 3
	Bottom	25.2	4.8	3.7	58.6	9.2	2.5	0.9	67 ± 3
	Whole	100	6.1	4.6	29.5	8.0	2.8	0.9	75 ± 2

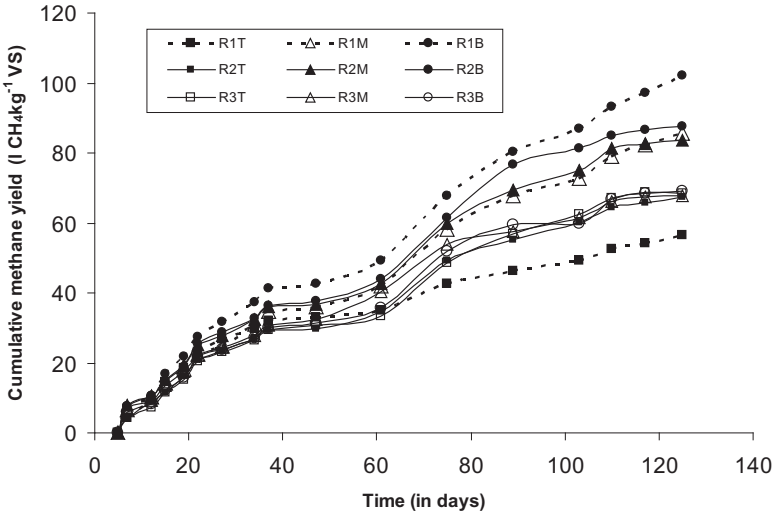

FIGURE 6 Cumulative methane yields from the (batch assays conducted for characterizing) stratification in the reactors at the end of operation of the CSTRs (R1 - control reactor, R2 – with alkali treated solids and R3 - untreated solids). T – top layers, M – middle layers, B – bottom layers.

4.2 Enhancing hydrolysis during mono-digestion of grass silage in one-stage LBRs (II-IV)

4.2.1 Chemical characteristics of substrate grass silage and inocula

Mono-digestion of grass silage was carried out in one-stage LBRs and methods to enhance microbial hydrolysis during AD were studied (II-IV). The chemical characteristics of the substrate grass silage, inoculum from biogas plant and rumen inoculum are shown in Table 6 and 7 (II-IV). Grass silage used for studying the effects of micro nutrients addition (II, set 2) showed lower pH and NH_4-N and higher TS & VS, than the grass silage used for studying effects of NH_4Cl addition (II, set 1). It can be seen that grass silage (II-IV) showed acidic pH and low NH_4-N concentration suggesting that these characteristics would lead to rapid acidification (due to low buffering capacity of grass silage) of the readily degradable compounds in the grass silage inhibiting further hydrolysis/acidification through decreased pH. High solids content in the grass silage also indicated the complexity involved in the degradation of this substrate (II-IV except set 2, II).

4.2.2 Batch experiments

Methane potential assays were performed for fresh materials (before AD in LBRs) as well as for residual materials from LBRs (materials obtained after AD in LBRs) with or without application of methods for enhancing hydrolysis of the substrate (II-IV).

Effect of macro and micro nutrient additions on methane yield of grass silage was tested in batch methane potential assays. Methane yield from grass silage was found to be enhanced with the addition of NH_4Cl (II, set 1) and showed 17 % higher methane yield (0.36 ± 0.02 m^3 CH_4 kg^{-1} VS) than control assays (0.30 ± 0.04 m^3 CH_4 kg^{-1} VS) (Table 11). It was observed that these differences were found to be statistically insignificant ($p > 0.05$). On the other hand, addition of micro nutrients (II, set 2) at high dosage level also enhanced the methane potential of grass silage by 15 % (0.33 ± 0.02 m^3 CH_4 kg^{-1} VS) than control (0.28 ± 0.004 m^3 CH_4 kg^{-1} VS) (II). These differences in yields were found to be statistically significant ($p < 0.05$). In order to verify the high methane potential obtained from the assays with high dosage of micro nutrients, two of the 'control' BMP assays were opened on day 60 and were added with 'high' dosage of micro nutrient solution (10 ml). These replicates showed a small (5 %) increase in methane yield over a period of 24 days (II). Methane potential assays of grass silage showed 0.4 ± 0.04 m^3 kg^{-1} VS_{added} after 75 d of incubation (III).

TABLE 11 Specific methane yields of grass silage from BMP assays tested with NH_4Cl (set 1) and micro nutrients (Fe, Ni, Co and Mo) addition (set 2) against respective controls (II).

Substrate	Methane yield (m^3 CH_4 kg^{-1} VS)
Set I	
Inoculum	0.10 ± 0.008
Grass silage (Control)	0.30 ± 0.040
Grass silage + NH_4Cl	0.36 ± 0.020
Set II	
Inoculum	0.12 ± 0.004
Grass silage (Control)	0.28 ± 0.018
Grass silage + low dosage of micro nutrients	0.29 ± 0.073
Grass silage + medium dosage of micro nutrients	0.30 ± 0.054
Grass silage + high dosage of micro nutrients	0.33 ± 0.020

Batch experiments performed to test the application of rumen culture at different loading rates (0, 25, 50, 75, and 100 %) (IV) showed that methane potential of grass silage was enhanced at a 50:50 combination of inoculum from biogas plant and rumen culture by 36-42 % (0.50 ± 0.013 m^3 CH_4 kg^{-1} VS) when compared to the yields obtained with 100 % of either of the inocula applied alone (Table 12). Application of either whole rumen fluid (unfiltered) or solids fraction of rumen fluid as a source of inoculum (IV) did not enhance the methane potential of grass silage and resulted in a methane yield of only 0.06 ± 0.01 m^3 CH_4 kg^{-1} VS and 0.004 ± 0.00 m^3 CH_4 kg^{-1} VS respectively (Table 12). These two inocula, i.e. solids fraction of rumen fluid and whole rumen fluid showed a methane potential of 0.03 ± 0.01 m^3 CH_4 kg^{-1} VS and 0.38 ± 0.01 m^3 CH_4 kg^{-1} VS respectively when they were digested alone (without the substrate). (Table 12) (IV).

The results of residual methane potential assays carried out at the end of LBR experiments for macro (II, set 1) and micro nutrients addition (II, set 2) are shown in Table 13. For set 1 (II) experiments, adjusting the pH of the reactor materials resulted in higher methane potential from control LBR (0.31 ± 0.05 m^3 CH_4 kg^{-1} VS) than LBR with NH_4Cl (0.25 ± 0.16 m^3 CH_4 kg^{-1} VS). The results of residual methane potential of reactor material in set 2 (II) with single and multiple micro nutrient addition are presented in Table 13. Results showed that there was no significant difference ($p > 0.05$) in residual methane potentials among the tested single micro nutrients and their combinations (Table 13).

TABLE 12 Specific methane yields obtained from grass silage with rumen culture and inoculum from biogas plant (set 1), solids fraction of rumen fluid and whole rumen fluid as inocula (set 2) (IV).

Set 1	CH_4 yield (m^3 CH_4 kg^{-1} VS)
Inoculum from biogas plant	0.05 ± 0.003
Rumen culture	0.44 ± 0.008
Grass + 100 % inoculum from biogas plant	0.32 ± 0.033
Grass + 100 % rumen culture	0.29 ± 0.020
Grass + 75 % inoculum from biogas plant + 25% rumen culture	0.20 ± 0.001
Grass + 50 % inoculum from biogas plant + 50 % rumen culture	0.50 ± 0.013
Grass + 25 % inoculum from biogas plant + 75 % rumen culture	0.33 ± 0.016
Set 2	
Whole rumen fluid (unfiltered)	0.38 ± 0.01
Grass silage + whole rumen fluid	0.06 ± 0.01
Solids fraction of whole rumen fluid	0.03 ± 0.01
Grass silage + solids fraction of whole rumen fluid	0.004 ± 0.00

TABLE 13 Specific methane yields obtained from residual methane potential assays of digested grass silage in LBRs tested for NH_4Cl addition (set 1, II) and in control BMP assays (set 2, II).

Set 1	(m^3 CH_4 $kg^{-1}VS$)	
Assays with inoculum addition	Control LBR	LBR withNH_4Cl
pH adjusted reactor material	0.31 ± 0.05	0.25 ± 0.16
pH adjusted leachate	0.33 ± 0.10	0.36 ± 0.14
Assays without inoculum addition		
Mixture of reactor material and leachate with pH adjustment	0.03 ± 0.03	0.03 ± 0.01
Mixture of reactor material and leachate without pH adjustment	0.03 ± 0	0.02 ± 0.01

Set 2 (from control BMP assays)	
Single micro nutrient addition	(m^3 CH_4 $kg^{-1}VS$)
Control	0.18 ± 0.00
Cobalt (Co)	0.21 ± 0.01
Iron (Fe)	0.21 ± 0.01
Nickel (Ni)	0.20 ± 0.00
Molybdenum (Mo)	0.20 ± 0.01
Multiple micro nutrient addition	
Fe + Ni + Co	0.20 ± 0.01
Ni + Co + Mo	0.20 ± 0.00
Fe + Ni + Mo	0.19 ± 0.02
Fe + Co + Mo	0.19 ± 0.02

4.2.3 Enhancing hydrolysis by addition of macro and micro nutrients in LBRs (II)

The effect of macro nutrient (NH_4Cl as nitrogen source) and micro nutrients addition on microbial hydrolysis (in terms of COD solubilization) of grass silage was investigated in LBRs for a period of 57 and 86 days respectively. The pH and SCOD trends in LBRs with NH_4Cl and micro nutrients are presented in Fig. 7 and Fig. 8 respectively. In all the LBRs, pH dropped on day 2 (3.9-4.9) and remained in the range of 4-5 till the end of the experiments. After day 45, pH in control LBR started to drop (pH ≤ 4.5) compared to the LBR with NH_4Cl (4.7-5.0). SCOD production was rapid during the first three days in all the LBRs and then stabilized thereafter. SCOD in the LBR with NH_4Cl was consistently higher than its control LBR till day 45 after which both the LBRs did not show much difference in SCOD production. On the other hand, in LBRs with micro nutrients, SCOD production did not appear to differ from its control either

before or after leachate replacement (on day 42). Specific SCOD production in the LBR with NH_4Cl addition was found to be enhanced by 18 % (0.56 g SCOD g^{-1} VS, Table 14) than in it's the control LBR (0.46 g SCOD g^{-1} VS). Specific SCOD production obtained in LBR with micro nutrients was found to be improved only by 7 % (0.46 g SCOD g^{-1} VS) than the control LBR (0.42 g SCOD g^{-1} VS) (Table 14).

The results of micro nutrient dynamics in the leachates (liquid phase) with initial and final concentrations in the grass silage (solid phase) are given in Fig. 9 and Table 14, respectively. Eighty percent of the externally added Fe and Co concentrations were found to be bioaccumulated (immobilised) in the solid phase (grass silage) when compared to Ni and Mo (72 and 53 % respectively). The remaining concentrations of the externally added micro nutrients (20-46 %) were found in the liquid phase (leachates) as shown in Fig. 9.

The results further showed that solids destruction (as TS and VS %) in LBR with NH_4Cl addition was improved (Table 14) by 7 % than the control while LBR with micro nutrients did not show any difference than its control. VFA production in LBR with NH_4Cl started quickly from day 1 and peaked to a maximum value of 8 g l^{-1} by day 23 while the maximum value in control LBR was 4.8 g l^{-1}. Acetic and butyric acids accounted for 80 % of the TVFA concentration in both LBRs. Gas production in these two LBRs was noticed mainly during the first 20 days and gas composition mainly contained 10-47 % of CO_2 and ≤ 15 % of H_2 and CH_4. However, gas production declined after 20 days. The LBR with micro nutrients addition showed a maximum VFA concentration of 9.4 g l^{-1} (day 13). The corresponding maximum value in control LBR was 7 g l^{-1} (day 23). However, leachate replacement on day 42 resulted in an increase in VFA production to maximum concentration of 8.8 g l^{-1} by day 43. Thereafter, VFA concentration remained unchanged.

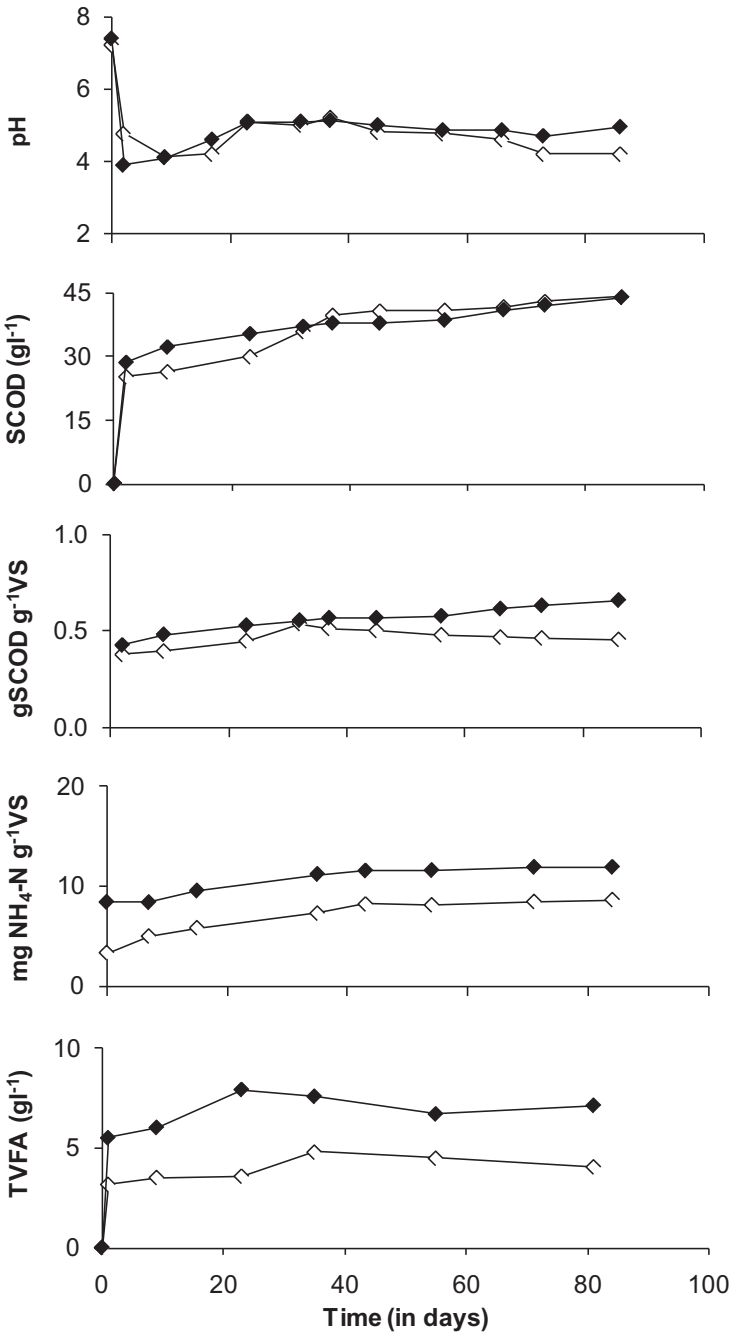

FIGURE 7 Process performance of LBRs during mono-digestion of grass silage with NH_4Cl addition (set 1, II). ◇ - Control LBR, ◆ - LBR with NH_4Cl

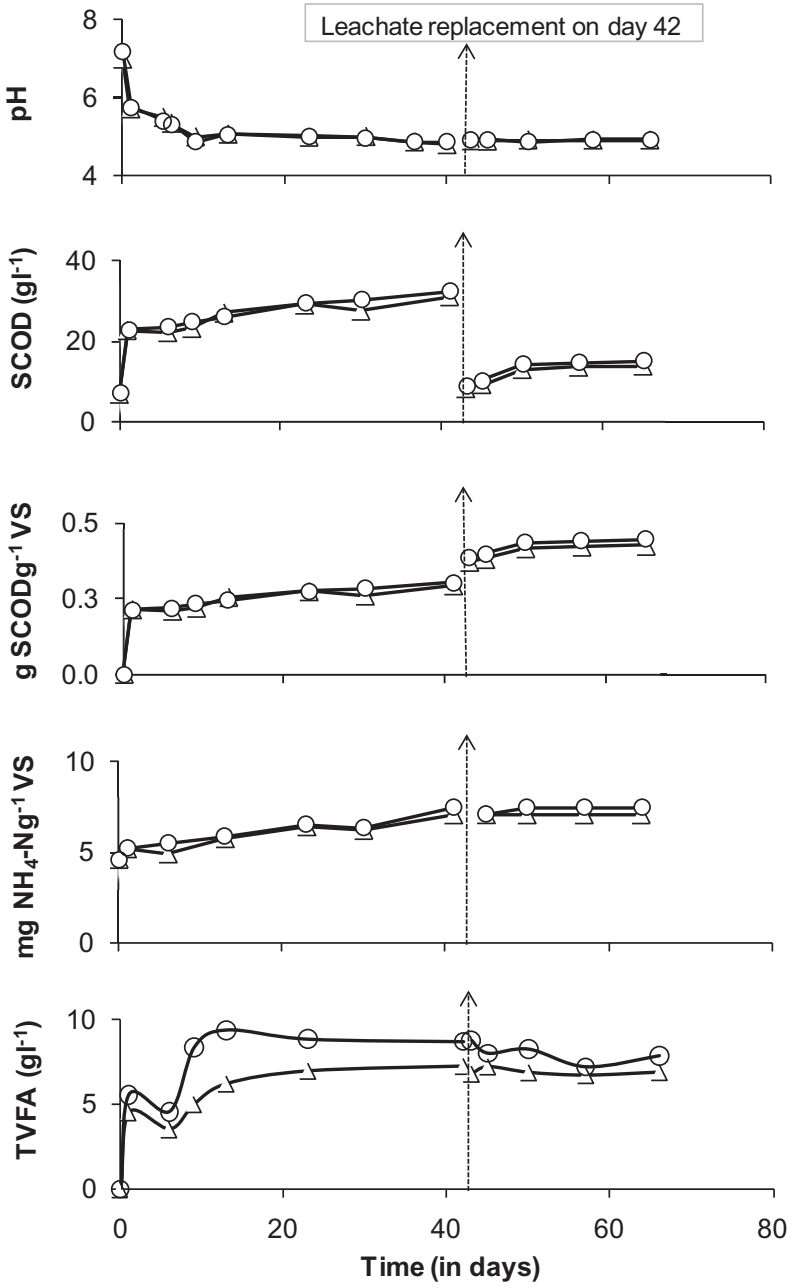

FIGURE 8 Process performance of LBRs during mono-digestion of grass silage with micro nutrients addition (set 2, II). Δ- Control LBR, ○- LBR with micro nutrients.

TABLE 14 Specific SCOD production, solids destruction, gas production and micro nutrient composition of grass silage in LBRs tested with NH_4Cl addition (set 1) and LBRs with micro nutrients (Fe, Ni, Co and Mo) addition (set 2) (II).

	Set 1		Set 2	
	L0 (Control)	L1 (NH_4Cl)	L2 (Control)	L3 (micro nutrients)
Specific SCOD production (g SCOD g^{-1} VS)	0.46	0.56	0.42	0.45
TS removal (%)	38.4	41.5	60.8	60.8
VS removal (%)	38.3	41.2	59.8	60.1
NH_4-N (mg g^{-1} VS)	8.6	11.8	7.1	7.5
Hydrogen (ml H_2 g^{-1} VS)	<1	<1	26	26
CO_2 (ml CO_2 g^{-1} VS)	<1	<1	40	31

Nutrient composition of LBRs	L2 Start	L2 End	L3 End
Fe (mg kg^{-1} TS)	1500 ± 120	2340 ± 110	7300 ± 300
Ni (mg kg^{-1} TS)	5.1 ± 1	9.1 ± 1	207 ± 6
Co (mg kg^{-1} TS)	1.8 ± 0.2	6.7 ± 0.7	94 ± 3
Mo (mg kg^{-1} TS)	18.8 ± 2.4	46 ± 3	212 ± 6

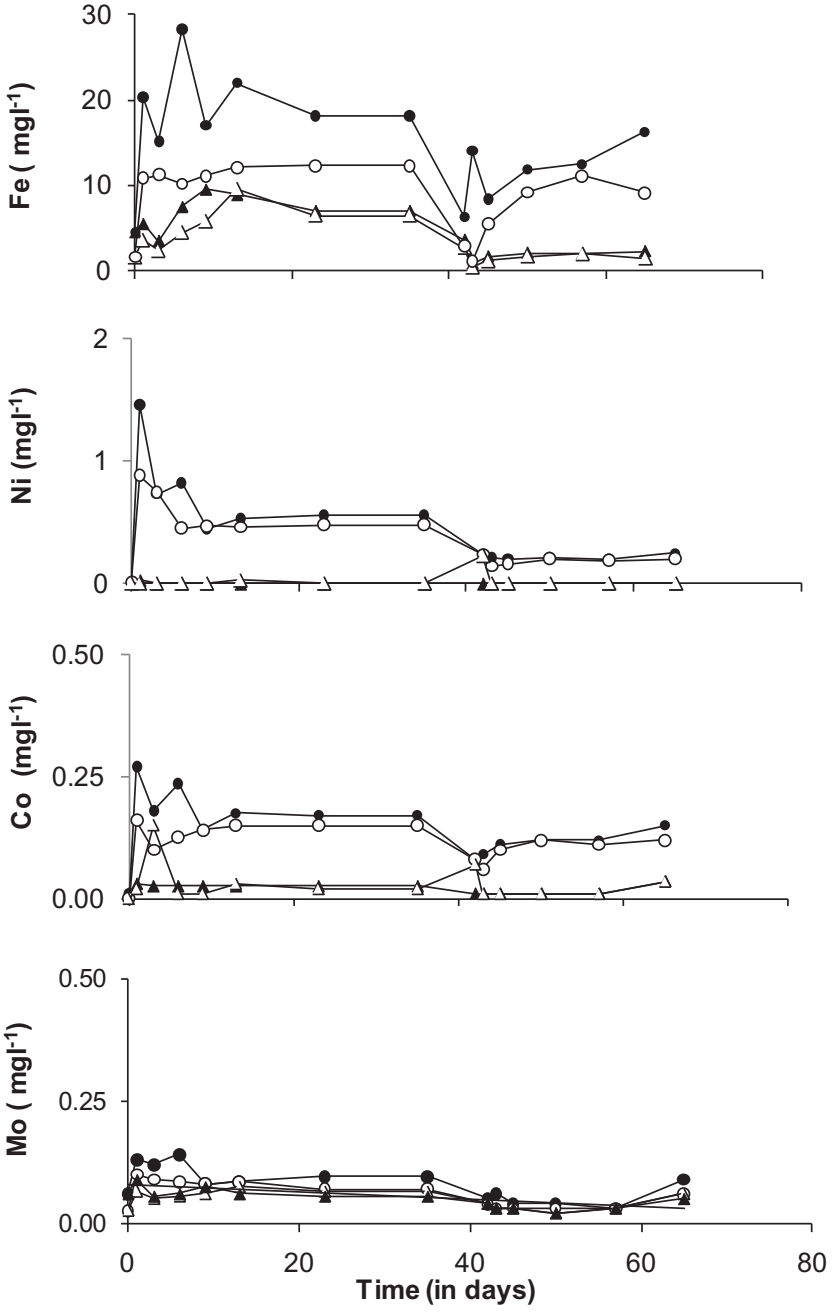

FIGURE 9 Soluble and total micro nutrients concentrations in leachates of control LBR and in LBR with micro nutrients addition. ▲, ●-Total nutrient concentrations Δ, ○- Soluble nutrient concentrations (II).

4.2.4 Enhancing hydrolysis by leachate replacement and micro-aerobic conditions in LBRs (III)

Anaerobic digestion of grass silage was carried out in one-stage LBRs for a period of 57 days to enhance the hydrolysis by adopting methods like leachate replacement and creating micro-aerobic conditions in the reactors. Hydrolysis was measured in terms of COD solubilization and acidification of grass silage in the LBRs. The effect of leachate replacement with initial pH adjustment (L1) and without pH adjustment (L3) was compared with the performance of control (L0), where no leachate replacement or pH adjustment was carried out. In addition, the effect of micro-aeration (L2) on COD solubilization and VFA production was also evaluated. The performance of the four LBRs is presented in Table 15 and Fig. 10, 11 and 12.

SCOD production was found to be enhanced by 2.7 and 1.3 times more in the LBR subjected to leachate replacement without pH adjustment (L3) compared to the control (L0) or LBR subjected to leachate replacement with initial pH adjustment (L1), respectively. Inducing micro-aerobic conditions at flow rate of 1 l min^{-1} (2.5 l of air) in L2 also resulted in a small but insignificant increase in cumulative SCOD in the leachate. Increasing the air flow rate to 4 l min^{-1} (24 l of air) however resulted in a decrease in SCOD extraction. The specific SCOD production (the amount of SCOD leached from the substrate) was also found to be enhanced by resulting in the highest COD solubilization (0.51 SCOD g^{-1} VS_{added}) in the LBR subjected to leachate replacement without pH adjustment (L3) than the control (L0) (0.34 g SCOD g^{-1} VS_{added}) or LBR subjected to leachate replacement with initial pH adjustment (L1) (0.33 g SCOD g^{-1} VS_{added}) or LBR subjected to micro-aeration (L2) (0.32 g SCOD g^{-1} VS_{added}) (Table 15).

TABLE 15 Composition of grass silage before and after AD in LBRs. L0 – control, L1 – LBR subjected to leachate replacement with initial pH adjustment, L2 – LBR subjected to micro-aeration, L3 - LBR subjected to leachate replacement with no pH adjustment.

	L0	L1	L2	L3
Total initial weight (g VS)	53	53	53	53
Initial inoculum weight (g VS)	3	3	3	3
Initial substrate weight (g VS)	50	50	50	50
I/S ratio	0.06	0.06	0.06	0.06
Final TS (%)	24.2	9.4	20.5	13
Final VS (%)	23.7	9	19.9	12.5
VS destroyed (g)	15	37	21	31
VS destroyed (%)	31	74	42	63
TS destroyed (%)	29	68	39	59
Degradation rate (gVS d^{-1})	0.27	0.64	0.37	0.55
Final SCOD of grass silage (mg g^{-1} VS)	159	34	150	78
Total COD solubilization (%) [a]	32	85	35	66

[a]Calculated using initial - final SCOD (mg g^{-1} VS)/initial VS of substrate

The variation in cumulative VFA and specific VFA production is shown in Fig. 10 and Table 16. The cumulative VFA and specific VFA production were found to be enhanced in the LBR with leachate replacement without initial pH adjustment (L3) showing an increase in VFA from 5.5 g l^{-1} on day 13 to a maximum of 12 g l^{-1} on day 35. On the other hand, cumulative VFA production in the LBR subjected to micro-aeration was also found to be enhanced by 4-fold with an increase in VFA production from 2.2 g l^{-1} on day 7 to 9.3 g l^{-1} by day 18. However, intensive aeration (day 22) resulted in a decrease in VFA production (6-7.5 g l^{-1}). Specific VFA production also followed similar trend as that of VFA production with values ranging between 0.1-0.33 g COD g^{-1} VS_{added}.

The evolution of individual VFAs is shown in Fig. 12. Acetic acid was the major organic acid and accounted for 80 % of TVFA in all LBRs. Acetic acid production started immediately and the concentration in the leachate increased sharply to reach 2-4 g l^{-1} within the first 7 days of operation (Fig. 12). Highest acetate concentration of 7 g l^{-1} was noticed on day 33 in LBR subjected to leachate replacement without pH adjustment (L3). *n*-Butyric acid was the second major acid and varied between 0.01 and 2.3 g l^{-1} with the highest concentration also noticed in L3. Despite acetic and *n*-butyric acids, propionic acid was also noticed, which reached to peak concentrations of 0.04-1 g l^{-1} in the first 10 days (Fig. 12). *Iso*-butyric, *iso*-valeric, *n*-valeric and caproic acids were also produced in all LBRs but only from day 7 onwards and their concentrations were <0.6 g l^{-1} (Fig. 16). The VFA equivalent COD (VFA-COD, g COD l^{-1}) also followed the same trend as that of SCOD (Fig. 11).

Solids destruction and degradation rates were observed to be enhanced in the LBRs subjected to leachate replacement with/without a pH adjustment (68 % in L1 and 59 % in L3) followed by LBR subjected to micro-aeration (39 %) and control (30 %) (Table 15).

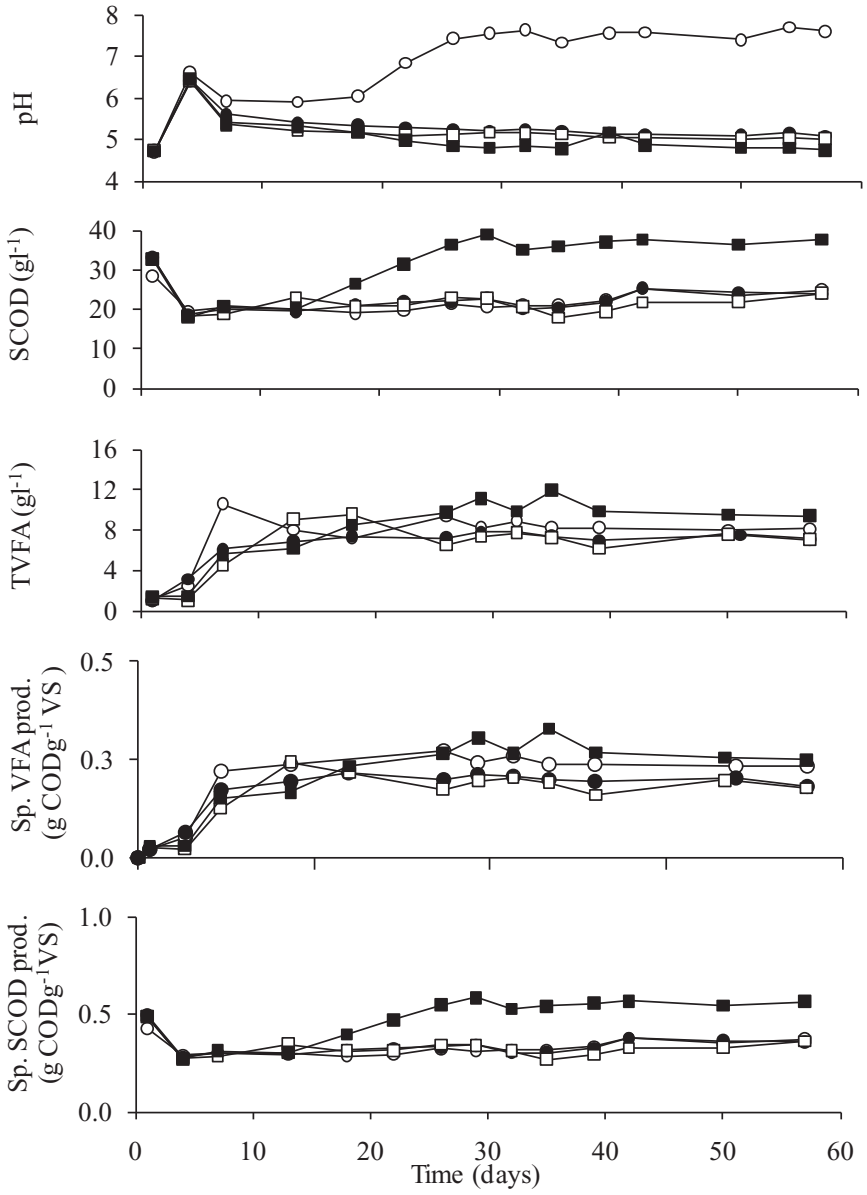

FIGURE 10 Variations of pH, SCOD, TVFA and specific SCOD and VFA production during mono-digestion of grass silage in LBRs: • L0, ○ L1, □ L2 and ■ L3.

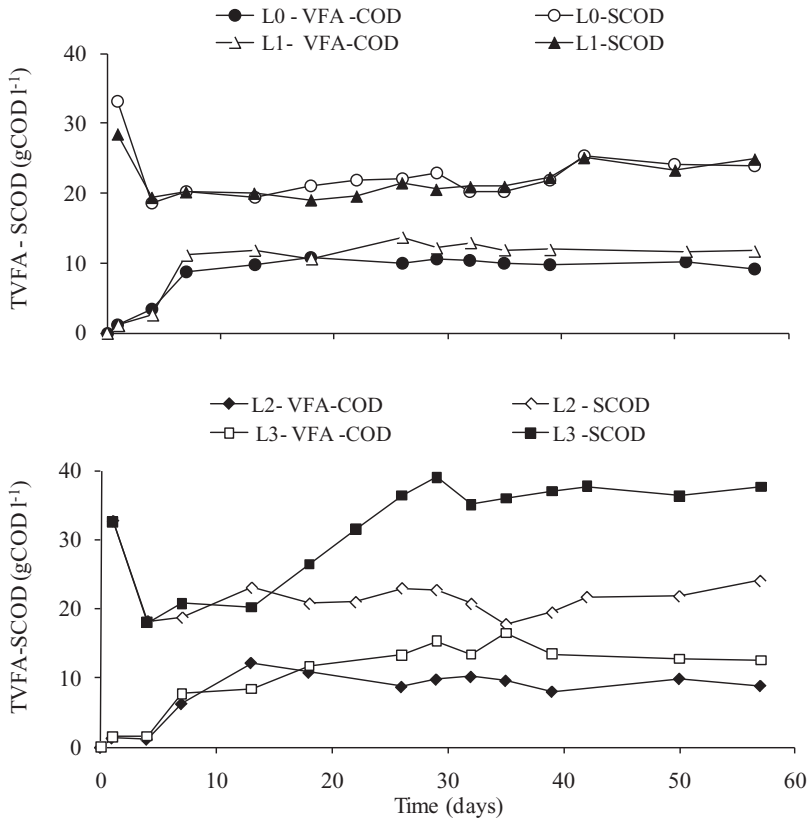

FIGURE 11 SCOD production and total VFA production (expressed as g COD l^{-1}) during mono-digestion of grass silage in LBRs (III).

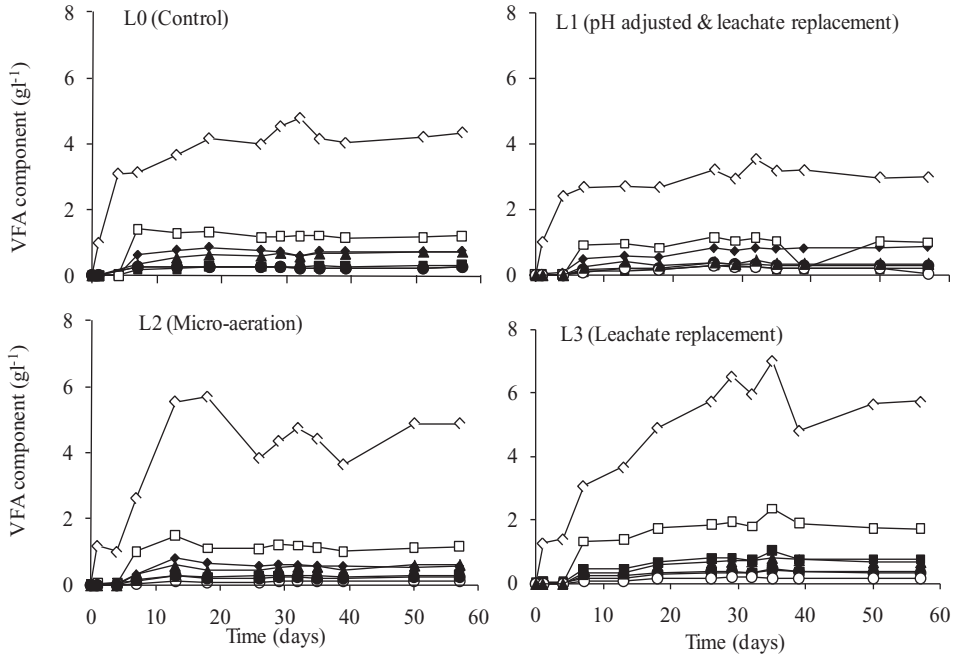

FIGURE 12 Individual VFA production during mono-digestion of grass silage in LBRs: ◊ acetic acid, ◆ propionic acid, ■ isobutyric acid, □butyric acid, • isovaleric acid, ○ valeric acid, ▲ caproic acid (III).

TABLE 16 Mean acetic acid, total volatile fatty acids (TVFA), VFA equivalent COD (VFA-COD, g COD l^{-1}) and specific SCOD production in the leachate (leachate volume per reactor- 750 ml) during mono-digestion of grass silage in LBRs (III).

	Acetic acid (g l^{-1})	TVFA (g l^{-1})	VFA-COD (g COD l^{-1})	SCOD (g l^{-1})	Specific SCOD production (g SCOD g^{-1} VS)
LBR, L0- control (Days 1-57)	3.75	6.3	8.6	22.4	0.34
LBR, L1- pH adj					
Before leachate replacement (Day 22)	2.2	5.8	7.4	21	0.31
After leachate replacement (Days 22–57)	3.1	8.4	12.2	22	0.33
LBR, L2					
Before micro-aeration (Day 11)	1.6	2.2	2.9	23	0.35
After micro-aeration (Days 11-22)	4	9.3	11.5	21	0.32
After intensive aeration (Days 22-57)	4.2	7.0	9.32	21	0.32
LBR, L3					
Before leachate replacement (Day 11)	2	2.8	3.6	24	0.36
After leachate replacement (Days 11-57)	5.6	9.8	13	34	0.51

4.2.5 Enhancing hydrolysis by application of rumen cultures in LBRs (IV)

Cellulolytic rumen cultures were employed as an inoculum source to study if hydrolysis of lignocellulosic substrate, grass silage would be enhanced in the LBRs operated for a period of 64 days. In addition, NaOH pre-treated grass silage was also used to study if alkali pretreated grass silage results in better hydrolysis and degradation and thus gives higher methane yields. The results showed that, hydrolysis (in terms of specific SCOD production) was improved by 10 % (0.33 g SCOD g^{-1} VS) in the LBRs either with 100 % (L1) or 25 % (L2) of rumen culture as inoculum (Table 17, Fig. 13) than the control LBR (0.30 g SCOD g^{-1} VS) which was operating with 100 % of inoculum from a (farm) biogas plant. On the other hand, LBR with NaOH pretreated grass silage did not show any improvement and resulted in lowest specific SCOD production (0.17 g SCOD g^{-1} VS) among all the LBRs. Specific NH_4-N production was found to be highest in the LBR with 100 % rumen culture as inoculum (L1, 26.4 mg NH_4-N g^{-1} VS) (Table 17, Fig. 13). Total nitrogen in the LBRs varied 1-1.5 g l^{-1} throughout the experimental period (Fig. 14). The results for solids destruction showed that highest rate of degradation in the LBR with inoculum from biogas plant (72 and 74 % of TS and VS removals, Table 16) following LBR L1, with rumen culture (100 %) as inoculum and LBR L3, with NaOH pretreated grass silage.

The results of VFA production were found to be peak high in the LBR, with rumen culture as inoculum (100%) (L1) showing upto 7.8 g l^{-1} by day 12, after which VFA production started to decline due to gas production (Fig. 14). The lowest VFA production was found to be in the LBR, with NaOH pretreated grass silage (L3) (3.6 g l^{-1}, Fig. 14). During the first 20 days methane composition was < 1% corresponding to the low pH conditions and increased only after day 20 when pH conditions rose to <6.5. High methane yields were obtained in the LBR with rumen culture (100 %) as inoculum (L1), (0.22 m^3 CH_4 kg^{-1} VS) followed by control LBR (100 % inoculum from biogas plant, L0)> LBR with 25 % rumen culture and 75 % inoculum from biogas plant (L2) > LBR with NaOH treated grass silage + 100 % inoculum from biogas plant (L3) (Table 17, Fig. 15).

TABLE 17 Process performance of LBRs (L0-L3) during AD of grass silage with the application of rumen culture as an inoculum source in combination with inoculum from biogas plant (IV).

LBRs	L0 (Gr + 100 % inoculum from biogas plant)	L1 (Gr + 100 % rumen culture)	L2 (Gr + 25 % rumen culture + 75 % inoculum from biogas plant)	L3 (NaOH treated Gr + 100 % inoculum from biogas plant)
Parameter				
Specific SCOD production *[a] (g SCOD g^{-1} VS)	0.30	0.33	0.33	0.17
Specific methane yield [a] (m^3 CH_4 kg^{-1} VS)	0.17 ± 0.07	0.22 ± 0.09	0.17 ± 0.07	0.06 ± 0.02
TS removal [a] (%)	72	66	52	61
VS removal [a] (%)	74	67	54	60
NH_4-N *[a] (mg g^{-1} VS)	19.2	26.4	18.7	15

Gr – grass silage, * - average values; [a] – g VS added in the LBRs was different i.e., L0 -19.2, L1 – 9.0, L2 – 16.5 and L3 – 19 gVS

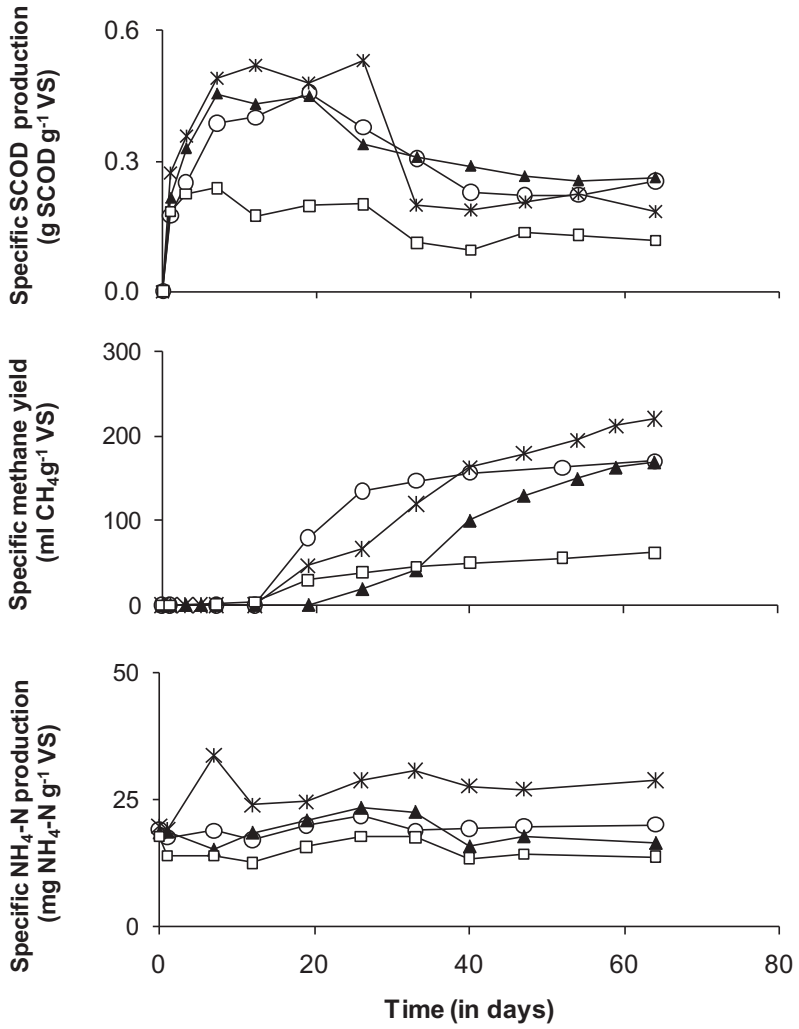

FIGURE 13 Specific SCOD, methane yields and NH_4-N production in the LBRs during AD of grass silage with the application of rumen culture as an inoculum in combination with inoculum from biogas plant (IV); ○ - LBR with grass silage +100 % inoculum from the biogas plant (L0), ✳ - grass silage + 100 % rumen culture (L1), ▲ - grass silage + 25 % rumen culture + 75 % inoculum from biogas plant (L2), and □ - NaOH pretreated grass silage + inoculum from biogas plant (L3) (IV).

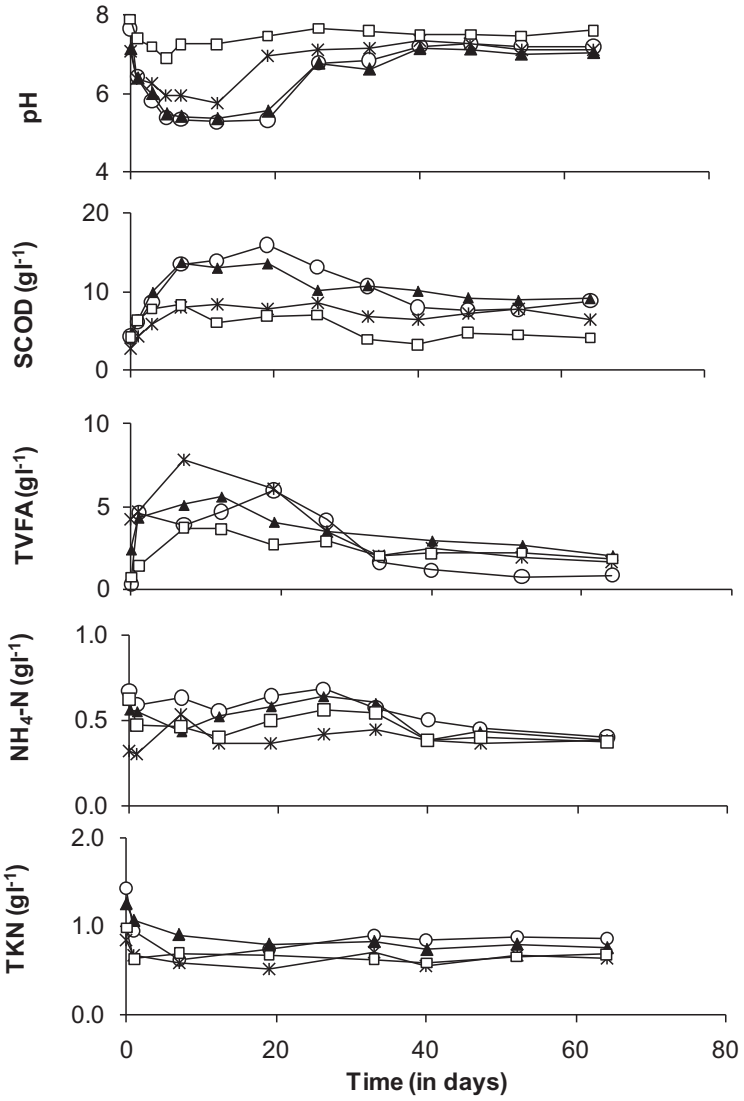

FIGURE 14 Process performance of LBRs during AD of grass silage with the application of rumen culture as an inoculum in combination with inoculum from biogas plant (IV); ○ - LBR with grass silage +100 % inoculum from the biogas plant (L0), ✳ - grass silage + 100 % rumen culture (L1), ▲ - grass silage + 25 % rumen culture + 75 % inoculum from biogas plant (L2) and □ - NaOH pretreated grass silage + inoculum from biogas plant (L3).

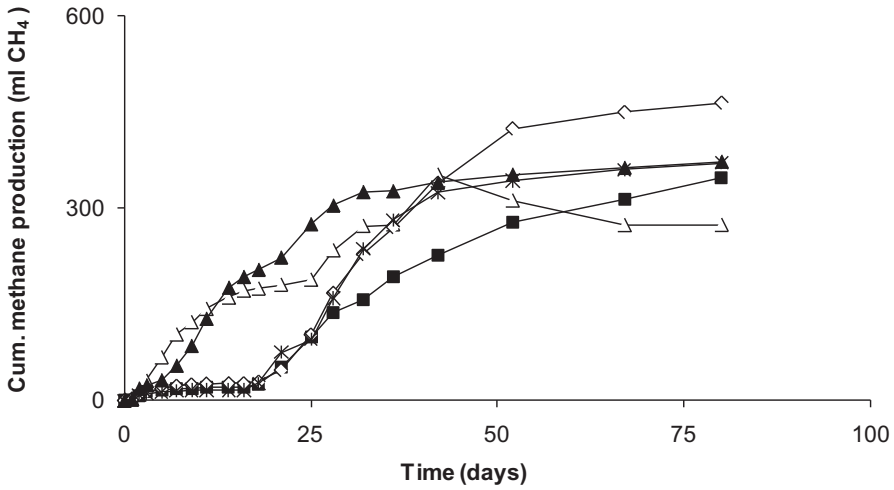

FIGURE 15 Cumulative methane production in methane potential assays of grass silage with rumen culture and inoculum from biogas plant ▲- Gr + 100 % Inoculum from biogas plant, ∗ - Gr + 100 % rumen culture, △- Gr + 75 % inoculum from biogas plant + 25 % rumen culture, ◇ - Gr + 50 % inoculum from biogas plant + 50 % rumen culture, ■ - Gr + 25 % inoculum from biogas plant + 75 % rumen culture.

4.3 Two-stage AD of energy crops and crop residues in LBRs and UASB reactors (V)

4.3.1 Chemical characteristics of the substrates and inocula

The chemical characteristics of the substrate grass silage, tomato, cucumber, common reed, inoculum from biogas plant and rumen inoculum are given in Table 18. Common reed and grass silage had higher solids content than tomato and cucumber. The VS contents of common reed and grass silage were 41 % and 39 %, respectively. The corresponding values for tomato and cucumber were 7.6 % and 4.5 %, respectively. On a dry weight basis, tomato and cucumber had higher TKN compared to common reed and grass silage. All substrates except cucumber had low initial pH of 3.9-5.08 and <1 % NH_4-N content. This low initial pH and NH_4-N suggests that the hydrolysis of the studied materials would be promoted as the optimum pH for the hydrolysis of lignocellulosic materials is 4-6.

TABLE 18 Chemical characteristics of the crop materials before and after AD in LBRs.

	Tomato			Cucumber			Common reed			Grass silage		
	Fresh material	RM	RMLE	Fresh material	RM	RMLE	Fresh material	RM	RMLE	Fresh material	RM	RMLE
pH	5.1	5.5	5.5	7.1	6.4	6.4	4.3	6.6	6.6	3.9	5.1	5.2
TS (%)	10	9.9	22	6.8	5.4	19	44.3	23.8	26	41	16.1	20
VS (%)	7.6	7.5	18	4.5	3.7	13	41	21.2	24	39	15.7	19
NH_4-N (g kg^{-1} TS)	0.25	0.62	8.6	0.15	0.47	6.1	0.05	0.12	0.34	0.25	0.39	0.67
N_{tot} (g kg^{-1} TS)	32.5	36.6	40	27.3	32	42	8.9	10.3	12	17	21.6	18
Cellulose (% TS)	12.5	NA	8.5	9.1	NA	4.2	34	NA	33	32	NA	25.6
Hemicellulose (% TS)	7.9	NA	4.1	4.9	NA	1.3	32	NA	31	24	NA	19.3
Lignin (% TS)	1.4	NA	1.2	1.1	NA	0.47	5.2	NA	5.0	3.6	NA	2.8

RM: Residual materials obtained at the end of LBR experiment.
RMLE: Residual materials obtained after leachate extraction by overnight pressing with weights.
NA- Not Analyzed

4.3.2 Batch experiments for fresh substrates and residual materials from LBRs

Batch experiments were carried out to estimate the methane potentials of the fresh and residual materials of tomato, cucumber, common reed and grass silage with digested material from farm-scale biogas plant as inoculum and with a combination of rumen inoculum and digested material from the biogas plant. When digested material from a farm-scale biogas plant was used as inoculum, methane yields of 0.26-0.36 m^3 kg^{-1} VS_{added} were obtained from the fresh substrates whereas mixed results were obtained with the residual materials. Methane yields obtained from the residual materials of cucumber and tomato were 0.29-0.32 m^3 kg^{-1} VS_{added}. On the other hand, the lower methane yields obtained from the residual materials of grass silage and common reed (0.10-0.32 m^3 kg^{-1} VS_{added}) than their fresh materials. Use of mixed inoculum (75 % digested material from farm-scale biogas plant and 25 % rumen fluid) resulted in methane yields of 0.22-0.36 m^3 kg^{-1} VS_{added} for the fresh crop materials (Table 19).

TABLE 19 Methane potentials from fresh and residual crop materials obtained after anaerobic digestion in LBRs (RMLE).

Materials	Methane yields (m^3 CH_4 $kg^{-1}VS_{added}$)			
	Inoculum from biogas plant		Inoculum mixture: biogas plant (75 %) & rumen fluid (25 %)	
Inoculum	0.04 ± 0.00		0.06 ± 0.03	
	Fresh substrates	RMLE*	Fresh substrates	RMLE*
Cucumber	0.26 ± 0.006	0.29 ± 0.001	0.24 ± 0.05	0.23 ± 0.016
Common reed	0.26 ± 0.008	0.10 ± 0.016	0.22 ± 0.013	0.17 ± 0.092
Tomato	0.32 ± 0.001	0.32 ± 0.009	0.32 ± 0.029	0.26 ± 0.143
Grass silage	0.36 ± 0.060	0.26 ± 0.100	0.36 ± 0.200	0.31 ± 0.054

*RMLE: Residual materials obtained after leachate extraction by overnight pressing with weights.

4.3.3 LBR experiments

Two-stage AD of tomato, cucumber, common reed and grass silage was performed in LBRs and UASB reactors to understand and optimize the hydrolysis and methanogenesis phases separately. The process performance of

the LBRs is presented in Fig. 16 and Fig. 17. During the first 7 days, LBRs were operated in conjunction with UASB reactors. In all LBRs, pH values of the leachate fluctuated throughout the experiment. The initial pH values of grass silage (3.9-6.2), tomato (5.1-6.9) and common reed (4-6.6) were low to near neutral compared to that of cucumber (6.3-7.8). SCOD production started immediately in all LBRs and maximum SCOD concentration of 14-38 g l^{-1} was noticed on day 10. These SCOD levels however decreased when the leachate was replaced with water on day 10, but increased thereafter to reach 15-47 g l^{-1} in the end. The maximum SCOD values obtained at the end of the experiment were 47 g l^{-1} for grass silage, 27 g l^{-1} for tomato, 18 g l^{-1} for cucumber and 15 g l^{-1} for common reed (Fig. 16).

The specific SCOD production (g SCOD g^{-1} VS_{added}) in the LBRs is presented in Fig. 17. The solubilization effect i.e. the amount of SCOD leached from the substrate was low during the first 10 days (0.11-0.32 g COD g^{-1} VS_{added}). However, leachate replacement (day 10) improved further hydrolysis and COD solubilization. The increase in SCOD solubilization upon leachate replacement was 65 % for tomato, 34 % for grass silage and cucumber and 23 % for common reed. At the end of the experiment the specific SCOD solubilization was 0.5 g COD g^{-1} VS_{added} for grass silage and cucumber, respectively. The corresponding values for tomato and common reed were 0.35 and 0.15 g COD g^{-1} VS_{added}, respectively (Fig. 17).

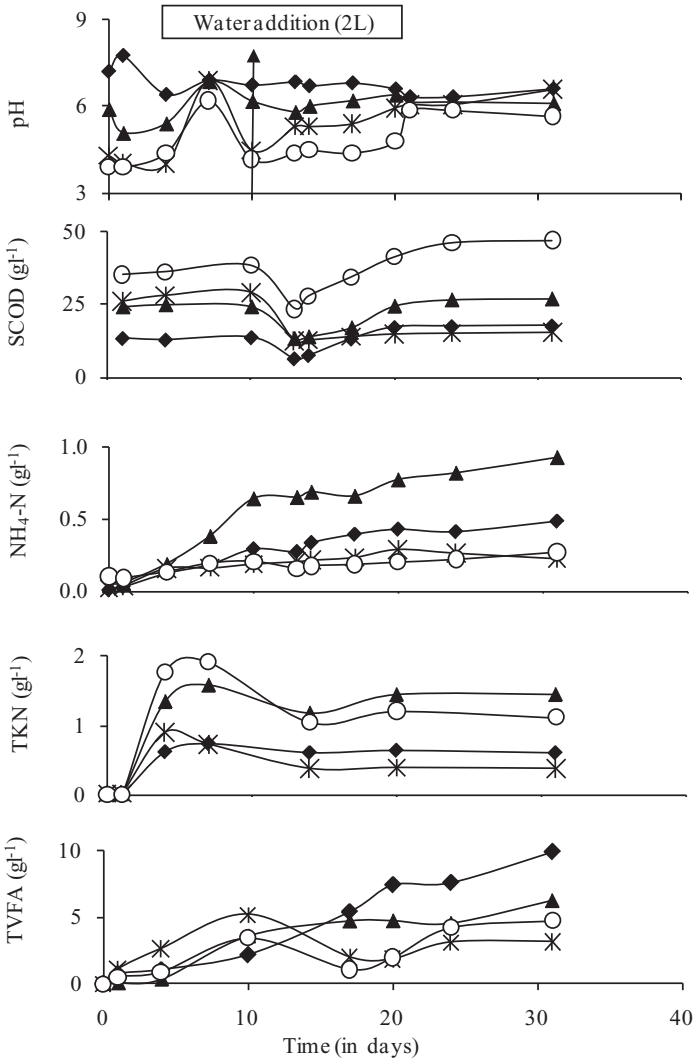

FIGURE 16 pH, COD, NH_4-N, TKN and TVFA concentrations in the leachates during mono-digestion of tomato, cucumber, common reed and grass silage in LBRs: (◆) Cucumber, (∗) Common reed, (▲) Tomato and (○) Grass silage in LBR.

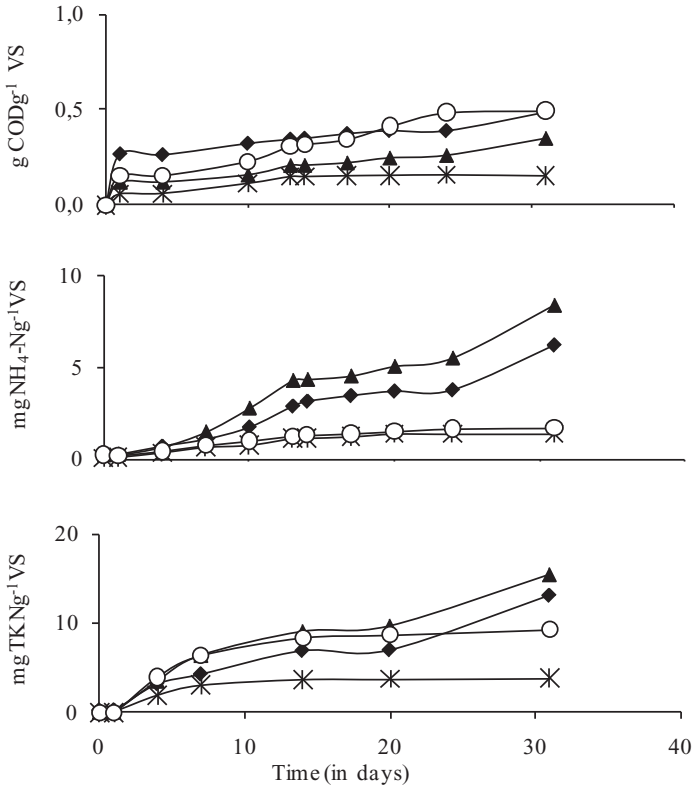

FIGURE 17 Specific COD, NH_4-N and TKN production in the leachates during the mono-digestion of tomato, cucumber, common reed and grass silage in LBRs: (◆) Cucumber, (∗) Common reed, (▲) Tomato and (○) Grass silage in LBRs.

TKN and NH_4-N concentration/production in the leachate is shown in Fig. 16 and Table 20 respectively. In all LBRs, TKN values of the leachate increased sharply from the initial value of 0.01-0.05 g l^{-1} to 0.75-1.92 g l^{-1} on day 4. Thereafter, TKN concentration decreased steadily to reach the lowest values on day 14 and remained more or less unchanged till the end of the experiment. Among the substrates, highest TKN concentrations were noticed in the leachates of tomato and grass silage compared with cucumber and common reed (Table 20). The NH_4-N concentration in the leachates increased more sharply for tomato from a very low level of 0.05-0.65 g l^{-1} (day 13) to a maximum concentration of 0.93 g l^{-1} at the end of the study (day 31). A similar trend was also noticed in cucumber LBR but with a maximum NH_4-N concentration of 0.48 g l^{-1}. On the other hand, the NH_4-N concentration in the leachates of grass silage and common reed remained more or less unchanged throughout the experiment. The specific TKN and NH_4-N extraction results are presented in Fig. 17. Results showed that leachate replacement improved the TKN extraction in tomato and cucumber but not for grass silage and common

reed. The specific TKN extraction values for tomato and cucumber were 13-15.5 mg g^{-1} VS_{added}. The corresponding values for grass silage and common reed were 9.3 and 3.9 mg g^{-1} VS_{added}, respectively. Nevertheless, NH_4-N extraction followed the same trend as that of TKN extraction (Fig. 17).

TABLE 20 TKN and NH_4-N extracted into the leachates during the anaerobic digestion of crop materials in LBR.

	Substrate (mg g^{-1} TS)	Leachate (mg g^{-1} TS)	TKN extracted into leachate (%)
TKN			
Cucumber	41	13	31
Common reed	9.6	3.8	38
Tomato	42	15.4	39
Grass silage	18.3	9.2	50
NH_4-N			
Cucumber	0.22	6.2	47
Common reed	0.05	1.37	36
Tomato	0.32	8.4	54
Grass silage	0.27	1.7	18

The solids destruction and degradation of the studied substrates in the LBR is presented in Table 21. The overall VS destruction for tomato and cucumber at the end of the LBR experiment was 48 % and 54 %, respectively. The corresponding values for common reed and grass silage were 8 % and 31 %, respectively. VFA production profile is present in Fig. 16. In all LBRs, VFA production started immediately from an initial value of 0.33-2.63 g l^{-1} on day 1 to reach a maximum value of 2-5 g l^{-1} on day 10. Highest VFA concentrations were noticed in the leachate of common reed (5.2 g l^{-1}) followed by tomato (3.6 g l^{-1}), grass silage (3.5 g l^{-1}) and cucumber (2.2 g l^{-1}). In the end, highest VFA concentrations were noticed in cucumber LBR (9.9 g l^{-1}) followed by tomato (6.3 g l^{-1}), grass silage (4.7 g l^{-1}) and common reed LBRs (3 g l^{-1}).

Process performance of the UASB reactors is presented in Fig 18. For the first 7 days, UASB reactors were coupled with the LBRs and operated at room temperature (21 °C). Use of low pH leachate (4-6.4) and high SCOD for biomethanation resulted in low methane production (<50 ml CH_4 d^{-1}) and unstable process (pH <4) in all UASB reactors. Thus, UASB reactors were decoupled from LBRs and operated as one-stage process (day 7). This led to an improved process performance and methane production in all UASB reactors. However, a shift in process temperature from 21 °C to 37 °C (day 19) further improved the methane production in all UASB reactors. This was evident through an increased pH (from <6 to 7.2-7.8), decreased TVFA concentration (from 5 to 0.14-2 g l^{-1}) and high COD removal efficiency (80 %).

TABLE 21 Initial and final weights along with the total solids (TS) and volatile solids (VS) removals during the AD of crop materials in LBR experiments.

Crop material	Amount of mass (kg, w/w)			Total solids (kg)		Volatile solids (kg)		TS removal (%)	VS removal (%)
	Fresh material	RM	RMLE	Fresh material	RM	Fresh material	RM		
Cucumber	9.62	5.4	3.00	0.65	0.29	0.43	0.20	55.5	54.0
Common reed	2.30	3.9	3.85	0.02	0.93	0.86	0.82	8.90	7.70
Tomato	10.5	5.5	3.50	1.05	0.54	0.79	0.41	48.0	47.0
Grass silage	2.30	3.9	3.75	0.94	0.62	0.89	0.61	33.4	31.6

RM: Residual materials obtained at the end of LBR experiment.

RMLE: Residual materials obtained after leachate extraction by overnight pressing with weights

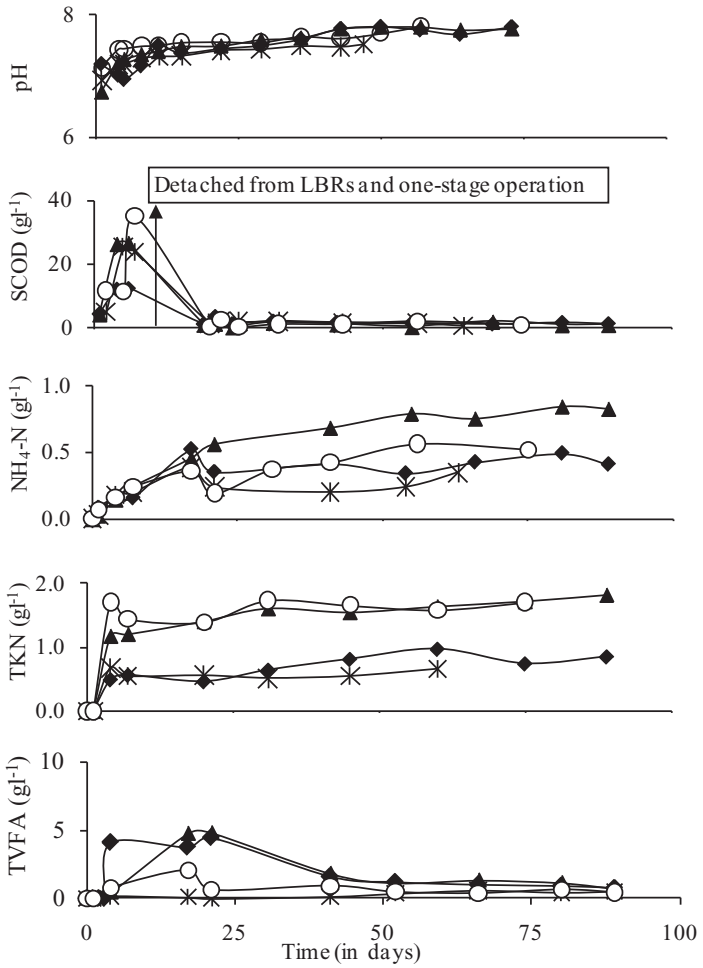

FIGURE 18 pH, NH_4-N, TKN and TVFA concentrations in the UASB effluents during the mono-digestion of tomato, cucumber, common reed and grass silage in LBRs: (◆) Cucumber, (∗) Common reed, (▲) Tomato and (○) Grass silage in UASB

The NH_4-N and TKN concentrations in the effluents are shown in Fig. 18. Tomato effluent had comparatively higher NH_4-N content (0.5-0.8 g l^{-1}) than cucumber, common reed and grass silage (0.3-0.05 g l^{-1}). However, TKN levels in all reactors appeared to be more or less stable and remained at 1-2 g l^{-1} throughout the experiment. AD of tomato and cucumber in the two-stage system consisting of LBR-UASB reactors resulted in methane yields of 0.09 and 0.13 m^3 CH_4 kg^{-1} VS_{fed}, respectively. These yields were 28 % and 50 % of those obtained in the BMP assays. On the other hand, methane yields of 0.14 and 0.034 m^3 CH_4 kg^{-1} VS_{fed} were obtained for grass silage and common reed, respectively.

5 DISCUSSION

5.1 Overview of significant findings of the study

The results of the present study showed the feasibility of application of methods like leachate replacement, micro-aeration, addition of macro and micro nutrients, and rumen cultures for enhancing hydrolysis during one and two-stage mono-digestion of energy crops and crop-residues. These methods have enhanced hydrolysis of energy crops and crop residues in LBRs by 7-34 % (in terms of specific SCOD solubilization g SCOD g^{-1} VS) and VS destruction up to 50 % in comparison to the control LBRs in which these methods were not applied. The significance of nutrients (macro and micro) addition on hydrolysis during the AD of lignocellulosic substrates was also shown in the present study. Furthermore, enhancing anaerobic degradation rates of fibres of digestates from CSTRs by alkali treatment and re-circulation of these fibres did not prove to be useful causing stratification and process inhibition. This finding is important in terms of knowledge concerning AD process technology (of energy crops) and would contribute to future research methods for enhancing degradation rates of the fibres and thus obtain additional methane benefits. In addition, two-stage AD of crop residues of tomato and cucumber and crop materials of common reed showed the feasibility of mono-digestion of these substrates in LBRs. The leachates of tomato and cucumber showed 47-54 % of (initial) total nitrogen mineralisation to NH_4-N and could be further investigated for their suitability in application as liquid fertilizers for crop production. The results are further discussed in detail in the following sections.

5.2 Co-digestion of grass silage and cow manure in CSTRs (I)

The present results show that re-circulation of solid fraction of digestate to the biogas process, both in treated and in untreated form were not effective and did not improve methane yields. Solids re-circulation in both the reactors basically

resulted in accumulation and scum formation which further lead to operational problems in the studied laboratory-scale system. The higher content by volume and TS % of materials at the end of the experiment are clear indicators for this (Table 10). However, the feasibility to co-digest cow manure and grass silage in CSTRs and the possibility of using about 30 % of VS of grass silage as an energy crop for methane (Table 9 and Fig. 4) has been reinforced as demonstrated in previous studies (Kaparaju et al. 2002, Lehtomäki et al. 2007). In the present study, a methane yield of 182 l CH_4 kg^{-1} VS was obtained from control reactor (R1) during days 75-151 and in the comparative CSTR study (Lehtomäki et al. 2007) a methane yield of 268 l CH_4 kg^{-1} VS was obtained during co-digestion of cow manure and grass silage by gradually raising the crop content from 0 to 40 %. On the other hand, methane potential obtained in the present batch assays was higher (376 l CH_4 kg^{-1} VS) than that reported by Lehtomäki et al. (2007) (306 l CH_4 kg^{-1} VS). Apparently the lower methane yield in the present CSTR study indicates that the process was not sufficiently adapted to degrade the substrate due to short operation periods than in previous study (Lehtomäki et al. 2007) and/or that the substrate mixture contained less readily biodegradable material than that in previous study. The lower methane yield could also be attributed to the differences in the mixture of crops used, harvest time of the crop, the maturity of the crop at the time of harvest and at the time of preparation of the silage. If any one of these variations were prevalent, then there will be variation in the substrate composition and subsequently variation in the microbial composition and dynamics in the reactors that might have affected overall methane yield.

Alkali treated solids when re-circulated to reactor (R2) yielded about 12 % higher methane than the reactor supplied with untreated solids (R3). This indicates some degree of destruction of lignocellulosic structures due to alkali treatment enabling solids digestion through hydrolysis (Fan et al. 1982, Hobson & Wheatley 1993, Angelidaki & Ahring 2000, Kaparaju & Rintala 2003). Further, organic content of the materials (VS %) in the reactor supplied with chemically treated solids (R2) was lower than in the control reactor (R1) (Table 9) and slightly higher than in the reactor supplied with untreated solids (R3). This indicates that solubilization of COD was somewhat better in the reactor supplied with alkali treated solids (R2) than the reactor supplied with untreated solids and the control reactor. One possible explanation for the higher SCOD in R2 but low methane yield could be non-conversion of solubilized COD to methane as found by (Haug et al. 1983). The authors reported that addition of NaOH enhanced COD solubilization, but solubilized COD conversion was not improved and thus did not favour digester performance. This situation indicates that in the present study also digestion performance was improved in the reactor supplied with alkali treated solids (R2) but over all conversion to methane was inhibited because of non-conversion of solubilized COD to methane.

Further, while studying thermo-chemical pre-treatment of microbial biomass and influence of NaOH addition on solubilization and anaerobic

biodegradability it was reported that (Kostyukovsky & Marounek 1995) addition of hydroxide caused an increase in pH causing COD solubilization. It further led to the formation of refractory compounds which are colored molecules, formed through reactions commonly named as 'Maillard' reaction. These colored compounds have been reported to be complex and very difficult to degrade even by rumen micro-organisms (Mc Millan 1994). Similar situation was also evidenced in our batch study, when characterising layers of the reactor materials at the end of the experiment. The top and middle layers of the reactor (R2) supplied with alkali treated solids showed higher SCOD values (Table 10) and the reason for lower methane yield could be because of inhibitory/refractory compounds forming as a result of increasing COD solubilization.

The lower methane yield from the reactor supplied with untreated solids (R3) could be only attributed to the recalcitrant lignocellulosic structures, visibly coarse and dry nature of the fibres (alkali treated fibres appeared soft and moist). However, it was not clear as to why the reactor supplied with untreated solids showed SCOD values comparable to the control reactor and still yielded less methane than the control reactor. On the other hand, multiple reasons could be attributed to low methane yield from the reactor supplied with alkali treated solids (R2) as alkali treatment depends on factors such as lignin content (Millet et al. 1976) and the type of lignocellulosic materials subjected to treatment (Kim et al. 2003). It was reported that cattle manure slurry in a quiescent state stratifies into 3 layers, namely the floating 'scum', the bottom sludge and a watery middle layer (Ong et al. 2000). The layer formation that took place in the present study is in complete agreement with the study of Ong et al. (2000). Top, middle and bottom regions of reactor supplied with untreated solids (R3) resulted in similar methane potentials indicating equal mass distribution but the methane potentials of the three layers were lower than the methane potentials (from the layers of) control reactor (R1) and the reactor supplied with chemically treated solids (R2). Accumulation and scum formation took place more intensively in the reactor supplied with untreated solids (R3) than in the reactor supplied with alkali treated solids which could also have inhibited degradation process in the reactor (R3).

Based on the batch study conducted to determine the effect of grading alkali treatments from 20-60 g NaOH kg^{-1} VS on solid and liquid fraction of digestate, 20 g NaOH kg^{-1} VS can be considered as the optimum amount for enhancing methane yield (12 % higher than control) for solid fraction of digestate as higher doses of alkali (30, 40 and 60 g NaOH kg^{-1} VS) did not enhance methane production. This is in agreement with the finding of (Kostyokovsky & Marounek 1995, Hartmann & Ahring 2005). It was reported that as NaOH concentration increased biogas production was still lower and most of the available COD was soluble and that hydrolysis was not the limiting step but the limitation was caused by the solubilized molecules (Kostyukovsky & Marounek 1995). The authors have also conducted biodegradability tests and found that highest biodegradability rates were obtained when sodium concentration was between 4-5 g NaOH l^{-1} and that biodegradability rate

progressively decreased with increasing NaOH concentrations. Batch assays with alkali treatments on digestate as such did not result in enhanced methane yields for the simple reason that the organic content (VS %) of the solid fibre samples extracted from the digestate for the experiment was more than double (10.3 %) the organic content of the digestate sample (4.4 %). The results obtained in this study suggest and necessitate the need to explore other methods for pre-treatment of solid fibres such as physical treatment methods like maceration, wet oxidation (Angelidaki & Ahring 2000, Kaparaju & Rintala 2003) and then study re-circulating to the reactor in order to exploit the residual methane potential of the digestate.

5.3 Enhancing hydrolysis during mono-digestion of grass silage in one-stage, LBRs (II-IV)

5.3.1 Enhancing hydrolysis by addition of macro and micro nutrients in LBRs (II)

Two LBRs were studied with the addition of macro nutrients (set 1) and two LBRs for micro nutrients (set 2) and the results showed that specific SCOD production in the LBR with NH_4Cl addition was about 18 % higher (0.56 g SCOD g^{-1} VS, Table 14, Fig. 7) than that obtained in control LBR (0.46 g SCOD g^{-1} VS) (Table 14, Fig. 8). This increase in specific SCOD production could be attributed to the increased microbial growth and activity due to the presence of additional nitrogen source, i.e. NH_4Cl (Chandrasekharan et al. 1991). Such enhanced microbial activity in the LBR resulted in an increased enzyme production (hydrolases) thus promoting higher polymer hydrolysis. Nitrogen is one of the macronutrients required for the anaerobic bacterial cell growth (Demirel & Scherer 2008) and specifically when degrading lignocellulosic substrates such as grass silage, the demand for nitrogen grows higher than usual since these substrates have lower nitrogen contents. Nitrogen supplementation during the AD process is practiced when the crops digested are either deficient of nitrogen or if the crops are highly acidic mainly to buffer the process and thus enhance the gas production rates. For example, Sterling et al. (2001), Demirel & Scherer (2008), Demirel et al. (2008) studied the effects of external addition of nitrogen in the form of NH_4HCO_3 or NH_4Cl during the AD of dairy cattle manure, sugar beet silage, fodder beet silage in different types of reactor systems and reported improved VS degradation rates and up to 30 % of increase in biogas production rates.

On the other hand, only 7 % increase in specific SCOD production was obtained in LBR with micro nutrients addition. It is difficult to attribute whether this small increase in specific SCOD production was either due to micro nutrients addition or leachate replacement (day 42). Because, the specific SCOD production before leachate replacement was similar in both LBRs (0.3 g

SCOD g^{-1} VS) but increased to 0.42 g SCOD g^{-1} VS in control LBR and to 0.46 g SCOD g^{-1} VS in LBR with micro nutrients after leachate replacement. Similar improvement in specific SCOD production after leachate replacement was reported earlier by Xu et al. (2011) and also in papers III and V. Higher specific SCOD production obtained in control LBR in set 1 experiments than in control LBR of set 2 could be attributed to the difference in the chemical nature of grass silage used and also the solid liquid ratio applied in both sets of experiments (V).

Methane potential assays with NH_4Cl addition showed increased higher methane yield (0.36 ± 0.02 m^3 CH_4 kg^{-1} VS) than control assays (0.30 ± 0.04 m^3 CH_4 kg^{-1} VS) (Table 11) this could be due to the availability of NH_4Cl as a nitrogen source during microbial hydrolysis and as a buffering source during microbial methanogenesis (Demirel and Scherer 2008) (II). However, since these results were found to be statistically insignificant ($p > 0.05$) further research is needed to optimize the amounts of addition of NH_4Cl and determine the subsequent effects on methane yields. On the other hand, higher methane yield from addition of micro nutrients at high dosage level (0.33 ± 0.02 m^3 CH_4 kg^{-1} VS) than control (0.28 ± 0.004 m^3 CH_4 kg^{-1} VS) indicates that VFA conversion was stimulated only at this dosage level in comparison to low and medium dosage assays (Table 11). Since these results were found to be statistically significant ($p < 0.05$) they indicate that the addition of micro nutrients stimulated methane production. Two replicates of control assays supplemented with micro nutrients solution (on day 60) showed a small (5 %) increase in methane yield over a period of 24 days. This is because, by day 60, easily degradable fraction of the substrate in these two assays was already converted to methane and what was left in the assays was the more recalcitrant fraction. Therefore, hydrolysis of this recalcitrant fraction would have been limited in these assays.

In a previous study by Jarvis et al. (1997) methane potential of digestates obtained from CSTRs fed with grass-clover silage were determined during week 4 and week 6 and found that higher methane production (about 80 % higher than control) was obtained when low concentration of trace/micro nutrients mixture (Fe - 109 mg l^{-1}, Ni - 0.7 mg l^{-1}, Co - 0.2 mg l^{-1} and Mo - 0.5 mg l^{-1}) was added in comparison to the higher concentration of trace nutrient mixture (Fe - 132 mg l^{-1}, Ni - 1.7 mg l^{-1}, Co - 0.8 mg l^{-1} and Mo - 1.6 mg l^{-1}). This is clearly attributed to the differences in chemical nature of the substrates (grass silage), micro nutrients requirements and uptake, differences in the process and digestates etc. Furthermore, several authors reported 35-60 % increase in the biogas/methane yields from anaerobic digesters operating with crops and crop residues when supplemented with micro nutrients such as Fe, Ni, Co, Mo, Se and W (Jarvis et al. 1997, Hinken et al. 2008, Lebuhn et al. 2008, Pobeheim et al. 2011). The concentrations of single or multiple micro nutrient additions in these studies were process-specific and also substrate specific. The increase in methane yield in the present study is still lower than those reported in literature for different crop substrates and thus, further research is necessary to determine the optimum concentration of these micro nutrients for obtaining higher

methane yields specifically, from the AD of grass silage. Nevertheless, the present results of methane potential assays (batch experiments) have by far shown that grass silage used in the present study can tolerate/withstand the tested high concentrations of the micro nutrients (II, Table. 11).

5.3.2 Enhancing hydrolysis by leachate replacement and micro-aerobic conditions in LBRs (III)

The results from the present study showed that COD solubilization and VFA production during mono-digestion of grass silage in one-stage LBRs could be improved through leachate replacement with or without pH adjustment. On the other hand, micro-aeration was shown to specifically improve the conversion of the produced leachate SCOD into VFA. The COD solubilization efficiency obtained in the present study through leachate replacement with/without a pH adjustment was 66-85 % of the substrate COD compared to 32 % obtained in the control. In a similar study, Lehtomäki et al. (2008) reported COD solubilization values of 80 % for grass silage in one-stage LBR without leachate replacement but with pH adjusted continuously to 7. However, COD solubilization efficiency of 92-95 % was obtained in the above study when one-stage LBR was coupled with a UASB reactor (without pH adjusted). Both these results thus suggest that the high concentrations of hydrolysis/acidogenesis products i.e. SCOD and VFA in the leachate can cause process inhibition in one-stage LBR system (Bhattacharya et al. 2008). Removal of the produced SCOD and/or VFA from the leachate through methanogenesis or dilution with water could improve further COD solubilization and acidification.

The highest specific SCOD production, solids destruction and COD solubilization efficiency noticed in LBR subjected to leachate replacement without initial pH adjustment (L3) was apparently due to the dilution of the leachate with tap water. Leachate replacement with fresh water may have diluted the inhibitory products of hydrolysis/acidogenesis and thus enabled further hydrolysis and COD solubilization (Bhatacharya et al. 2008). These results are in agreement with Hao et al. (2008) where leachate replacement with tap water showed to improve COD solubilization and methane yields through dilution of the hydrolysis/acidogenesis products in one-stage. In addition, leachate replacement during the early stages of digestion (day 11) in L3 may also have improved the COD solubilization. Sanphoti et al. (2006) also made a similar observation in simulated landfill experiments where supplemental water addition and re-circulation in the early acid phase of the reactor (day 203) helped to dilute the inhibitory substances and negated the need for buffer addition to overcome acid phase and resulted in higher COD removal (85 %) than without supplemental water addition and re-circulation (81 %).

The lower specific SCOD production in LBR subjected leachate replacement (on day 22) with initial pH adjustment (L1) compared to L3 (day 11), was probably due to the difference in the timing of leachate replacement and/or pH adjustment to 7 during the initial stages of digestion. For instance, leachate in LBR subjected to leachate replacement on day 11 (L3) (pH before

replacement, 5.4) showed a clear increase in leachate SCOD by day 13 indicating additional SCOD solubilization. This immediate increase in SCOD after leachate replacement on day 22 was not noticed in LBR subjected to leachate replacement on day 22 with initial pH adjustment (L1). In fact, SCOD in the leachate increased gradually to reach maximum (20.3 g l^{-1}) on day 42. On the other hand, adjusting the pH to neutral during the initial 7 days of digestion might not have aided in improving the hydrolysis and/or COD solubilization as the optimum pH for hydrolysis is 6.0-6.5 (Arntz et al. 1985) and hydrolysis is inhibited if pH <5 (Veeken et al. 2000). Moreover, COD solubilization is dependent on the pH and the initial COD and VFA concentrations (Sanphoti et al. 2006). Cysneiros et al. (2007) also reported that adjusting the pH to neutral has only partly improved hydrolysis of maize in one-stage LBR. The mean pH, SCOD and VFA concentration in L1 during the initial 7 days of operation was 6.2, 21.5 g l^{-1} and 4.7 g l^{-1} respectively. Further, the low specific SCOD yields in LBR with leachate replacement and initial pH adjustment (L1) are also attributed to the compositional changes in the substrate due to the use of NaOH, for adjusting the pH. Previous studies have shown that addition of strong bases such as NaOH or $Ca(OH)_2$ can increase the solubilization of organic matter through depolymerization reactions of lignin-cellulose complex (Bhatacharya et al. 2008).

The 4-fold increase in VFA levels but not in leachate SCOD upon inducing micro-aerobic conditions in LBR subjected to micro-aeration (L2) indicates that controlled and limited amount of oxygen at a flow rate of 1 l min^{-1} might have stimulated the degradation of slowly biodegradable compounds, through production of exoenzymes by aerobic microorganisms, which were otherwise resistant to degradation under fully anaerobic conditions (Hasegawa & Katsura 1999). Hasegawa et al. (2000) also reported similar acidification and accumulation of VFAs when micro-aerobic conditions were created during thermophilic pretreatment of organic sludge prior to AD. On the other hand, the decrease in COD solubilization and/or VFA production upon increasing the air flow rate to 4 l min^{-1} in the present study was probably due to oxidation of some of the VFAs (Jenicek et al. 2008). These results thus confirm that the presence of a controlled/limited amount of oxygen in the anaerobic digester does not inhibit the process as most of the oxygen is promptly consumed for organic matter oxidation.

The high leachate SCOD observed on day 1 in all LBRs was a result of simple wash-out of the soluble organics as well as fine organic particulate by the water added to the system (Demirer & Chen 2008). Based on the pH values of the leachate collected, it was observed that acidification started right after the start-up. However, the rapid VFA production and fluctuating pH values noticed during the initial 10 days of operation in all LBRs was attributed to the acidifying nature of the feedstock and/or buffering capacity of the system. The increase in pH during initial 4 days operation might have been due to buffering capacity of the system. Guerrero et al. (1999) also reported that pH during acidogenesis may vary as the system tends to buffer itself towards a pH value in the range of 5-6.5, if no control is carried out. On the other hand, the decrease

in pH from 6.4-6.6 to 5-5.6 after day 4 indicates the successful acidification (Bhattacharya et al. 2008). A similar observation was reported during AD of lignocellulosic feedstocks (Cysneiros et al. 2008, Guerrero et al. 1999, Lehtomäki et al. 2008).

The high TVFA and specific VFA productions noticed in all LBRs indicate imbalance between the VFA production and consumption, and build-up of VFA resulting in reduction or inhibition of hydrolysis. VFA levels in the present study reached 9 g l^{-1} (pH 5.6) by day 7 indicating the onset of inhibition of hydrolysis. Banks et al. (2005) also reported similar inhibition of cellulolytic activity at VFA concentrations of ≥2 g l^{-1} independent of pH system. Further, the concentration of some of the individual VFAs in the present study also exceeded the threshold levels reported to cause process inhibition (Hill and Holmberg 1988). For instance, acetic acid levels were 1-3.7 g l^{-1} (Table 16, Fig. 12) in all LBRs and exceeded the threshold level of 0.8 g l^{-1} reported to cause process inhibition (Hill & Holmberg 1988). Similarly, the concentrations of *iso*-butyric and *iso*-valeric acids varied between 0.01 and 0.3 g l^{-1} and also exceeded the levels of 0.005 g l^{-1} and 0.015 g l^{-1}, respectively, reported to cause inhibition (Wu et al. 1995). Butyric and propionic acids levels on the other hand, were low and did not seem to have exceeded the threshold levels of 2-3.2 g l^{-1} (Hanaki et al. 1994) indicating some extent of carbohydrate digestion but low protein digestion.

The temporal difference in the evolution of the higher molecular weight VFAs was probably due to the difference in the substrate utilized. It should be noted that acetic, propionic, *iso*-butyric and *n*-butyric acids are yielded directly from the fermentation of carbohydrates, proteins and lipids, while the higher molecular weight VFAs, such as *n*-valeric and *iso*-valeric acids are mainly associated with the fermentation of proteins (McInerney 1988). The latter two VFAs are usually formed through reductive deamination of single amino acids or by an oxidation-reduction between pairs of amino acids via the Stickland reaction (Parawira et al. 2004). The main soluble organic materials in grass silage consisted of carbohydrates and protein. Therefore it might be expected that propionate, *iso*-butyrate, *n*-butyrate, *iso*-valeric, and *n*-valerate could be produced and accumulated in the fermentation process. However, lower concentrations of these individual acids compared with acetic acid in the present study indicate that the former VFAs were easily biodegraded to form acetic acid (Gerardi 2003). Cysneiros et al. (2008) also reported that acetic and butyric acids accounted the most of the VFA produced during AD of maize-silage in one-stage LBR. The rapid accumulation of acetate during the initial 7 days of operation in the present study indicates that carbohydrates were more readily degraded than nitrogenous materials and lipids (Eastman and Ferguson 1981). The accumulation of acetate coincided with the pH drop and hydrogen production (data not shown) indicating acidification (Wu & Lin 2004). On the other hand, accumulation of propionate resulted in a corresponding decrease in acetic and butyric acid concentrations in the LBR.

The present study thus suggests that periodic dilution of the leachate with fresh water in practice could improve the extraction and solubilization of the organic material contained in lignocellulosic feedstocks into leachate. However, use of large amount of fresh water on a periodic basis in full-scale applications could result in high operational costs and accumulation of high strength leachates which need a secondary treatment. Alternatively, the produced leachate could be continuously or semi-continuously (every 10 d) fed to high rate anaerobic reactors such as anaerobic filters or UASB reactors to convert the produced VFA into methane and prevent VFA build-up and inhibition of hydrolysis in the LBR. Coupling of high rate anaerobic reactor as configuration during mono-digestion of grass silage (Lehtomäki et al. 2008) and maize-silage (Cysneiros et al. 2008) in LBRs has already been demonstrated successfully with higher biogas yields and lower reactor volumes.

LBRs when coupled with UASB reactors/AF will not require the continuous feeding of crops/grass silage to the system and thereby avoid problems associated with pumping, handling, mixing and clogging. In addition, a continuous supply of anaerobic seed for overcoming its continuous wash-out from the system could be overcome by recycle of the leachate back to LBR as an active acidifying culture is vital for successful application of this concept. This practice and its potential improvements needs to be further investigated.

5.3.3 Enhancing hydrolysis by application of rumen cultures in LBRs (IV)

The results obtained in this study showed the feasibility of application of cellulolytic rumen cultures for the enhancement of hydrolysis during AD of lignocellulosic substrates in LBRs. Maximum SCOD production was obtained in the first 20 days in all LBRs (7-16 g l^{-1}) except in LBR with NaOH pretreated grass silage (L3). pH rising above 6 after day 20 also confirmed that maximum hydrolysis was obtained during the first 20 days after which methanogenesis step was subsequently initiated. This result is in agreement with a previous study conducted by Chanakya et al. (1992). The authors studied AD of fresh and dry biomass feedstocks in solid phase biogas fermentors and observed that dry biomass feedstocks need 20-30 days of solids retention time to cross hydrolysis/acidogenic phase. Specific SCOD production was enhanced by 10 % in the LBRs in which rumen culture was applied as an inoculum source either alone (100 %) or in combination with inoculum from biogas plant (i.e. L1- 100 % rumen culture, L2- 25 % rumen culture + 75 % inoculum from biogas plant) (L1 and L2 respectively) than control LBR (L0) (Fig. 13). Such an increase in SCOD solubilization was previously reported by O'Sullivan et al. (2006) during AD of microcrystalline cellulose tested with rumen inoculum against MSW leachates as inoculum sources. The authors reported that SCOD solubilization was enhanced by 34-60 % in the reactor in which rumen culture was used as an inoculum source. The lower SCOD solubilization obtained in the present study could be due to the fact that the substrate grass silage was lignocellulosic in nature which offers more complex cell-wall structure than purified cellulose.

Further, specific NH_4-N production was about 27-43 % higher in the LBR with 100 % rumen culture (L1) than the LBRs with inoculum from biogas plant indicating higher nitrogen solubilization into the leachate. This also confirms the higher SCOD solubilization obtained in the LBR with 100 % rumen inoculum (L1).

It has to be also noted that, although the I/S ratio was same in all the LBRs the VS contents in the LBRs were different (L1 - 9, L2 - 16.5, L0 - 19.2 and L3 - 19 g VS) as seen in Table 17 (foot notes). Higher SCOD solubilization in the LBRs with rumen culture (L1 - 100 % and L2 - 25 %) than in LBRs with 100 % inoculum from biogas plant (L0 and L3) and this could be a result of lower substrate concentration in these LBRs. However, lower substrate concentration did not enable higher VS degradation efficiency from the LBRs with rumen culture as inoculum. Previosly, Hu & Yu (2005) studied AD of corn stover at two different temperatures (35 °C and 40 °C) using rumen cultures at different substrate loads (10-30 g VS l^{-1} d^{-1}). The authors reported that as loading rate increased the VS degradation efficiency also increased from 5 % to 13 %. This does not seem to be true in the present study if comparison is made between LBRs with rumen culture and the LBRs with inoculum from biogas plant. However, among the LBRs with 100 % rumen culture (L1) and 25 % rumen culture (L2), the former LBR showed higher VS degradation as a result of lower VS content (9 g VS) than the latter LBR (16.5 g VS).

On the other hand, the differences in degradation efficiencies between the LBRs with rumen culture (L1 and L2) can also be attributed to the differences in pH conditions within these LBRs (Fig. 14). Rumen microorganisms are reported to be highly sensitive to pH fluctuations within the reactors (Hu et al. 2004). The pH conditions in the LBR with 100 % rumen culture (L1) during the first 20 days, were higher (5.9-7) than the pH conditions in the LBR with 25 % rumen culture (L2) (5.4-5.5) which could be the reason for higher degradation in the former LBR. Because, for rumen microorganisms pH conditions of 6-7.5 were reported to be appropriate for obtaining higher cellulose degradation (Hu et al. 2004, Yue et al. 2007). Higher VFA production in the LBR with rumen fluid as inoculum (100 %) confirms the higher SCOD solubilization and also the higher methane yield obtained from this LBR while the opposite was true for the LBR with NaOH pretreated grass silage (L3). The lowest SCOD production in the LBR with NaOH pretreated grass silage (L3) could be clearly due to the unfavourable pH conditions in the reactor lowering the extent of hydrolysis of grass silage (Fig. 14). Because, unlike rumen microorganisms the ideal pH conditions for hydrolytic bacterial consortia in anaerobic digesters are between pH 5.5-6.5 (Kim et al. 2003). On the other hand, pH conditions in the LBR with 100 % inoculum from biogas plant (L0) had optimal pH conditions for better hydrolysis and thus resulted in higher specific SCOD production than LBR with NaOH pretreated grass silage (L3). Hence, L0 also showed higher VFA production and methane yields accompanied by higher VS degradation.

Higher methane yields from the methane potential assays with 50:50 (% volume) combination of rumen culture and inoculum from biogas plant could

be due to enhanced hydrolysis and methanogenesis and also possibly due to a balance achieved in these two phases. On the other hand, lower methane yields obtained from assays with >50 % of rumen culture as inoculum could be due to an imbalance in the process i.e. higher hydrolytic/cellulolytic activity and lesser methanogenic activity available for conversion of the formed VFAs to methane. Rumen culture alone showed about 89 % higher methane yield than the inoculum from the biogas plant indicating the high microbial activity of the rumen bacterial population. Methane yields from grass silage were not improved when solid fraction of the whole rumen fluid was used as a source of inoculum. This could be simply due to the microbial washout during filtration of whole rumen fluid and the microbial population adhered to the solids may not have been in enough to carry out the degradation process and thus resulted in lower methane yields.

5.4 Two-stage AD of energy crops and crop residues in LBRs and UASB reactors (V)

The results obtained in this study showed the feasibility of two-stage AD of tomato, cucumber, common reed and grass silage in LBRs with upto 50 % of hydrolytic degradation and with considerable methane yields from UASB reactors. The solubilization effect i.e. the amount of SCOD leached from the substrate(s) was low during the first 10 days (0.11-0.32 g COD g^{-1} VS_{added}). However, leachate replacement (day 10) improved further hydrolysis and COD solubilization in all the LBRs. A sharp increase (65 %) in COD solubilization in tomato LBR upon leachate replacement (day 20) suggests that dilution of the saturated leachate with fresh water had enabled further COD solubilization. Similar result was also obtained in a previous study (III). In that study (III), leachate replacement on day 11 resulted in about 66-85 % of COD solubilization compared to 32 % obtained in the control reactor. This confirms that, during the first 10 days of the LBR operation, rapid hydrolysis resulting in saturation of organic substances takes place and leachate replacement will prevent accumulation of these organic substances and thus enable further hydrolysis and COD solubilization.

The SCOD solubilization upon leachate replacement for grass silage and and common reed (34 and 23 % respectively) were lower indicating the dry nature of these substrates and that they require much higher amount of water for obtaining higher solubilization rates. The higher specific SCOD solubilization noticed for cucumber compared to tomato or common reed was attributed due to the difference in the chemical composition of these materials (Table 18, Fig. 16). For instance, cucumber had lower cellulose content (9.1 % TS) than tomato (12.5 %) and common reed (34 % TS) (Table 18). Moreover, crushing of the cucumber plant material, carried out at the production center in order to facilitate packing and transport, might have also facilitated hydrolysis

and thus COD solubilization through particle size reduction, enhanced surface area and reduction in cellulose crystallinity (Taherzadeh & Karimi 2008). On the other hand, the relatively high specific SCOD solubilization noticed for grass silage was due to the fact that ensiled grass contained high amount of readily available carbohydrates and low pH ideal for hydrolysis. In general, ensiling of energy crops such as grass or maize will result in an increase in the water soluble carbohydrate content from the degradation of carbohydrates by the lactic acid bacteria. The produced lactic acid will eventually inhibit further degradation of the carbohydrates by other bacteria and thereby preserve the nutritional value of the crops (Lehtomäki et al. 2008). In addition, the low pH (3.9), as a result of ensiling and particle size reduction (2-5 cm) prior to loading into the LBR, may have also promoted hydrolysis and COD solubilization in grass silage. A similar specific SCOD production (0.5 g SCOD g^{-1} VS_{added}) was also obtained for grass silage in the previous study (III). In the previous study (III), a 30 % increase in the solubilization of grass silage was observed in the LBR subjected to leachate replacement (day 11). On the other hand, the increase in COD solubilization with grass silage upon leachate replacement in the present study was 34 % (day 10). The lowest COD solubilization obtained for common reed was mainly attributed to the recalcitrant chemical nature of the substrate which was indicated by its highest lignin content (5.2 % TS) than the other crop materials studied (Table 18). The maximum increase in the COD solubilization that could be obtained with common reed was only 23 % and that too after leachate replacement (days 10-32). Chemical analyses further revealed that common reed contained high amounts of crude fibre, crude fat, starch and sugar (data not shown) than the other three studied materials. Apparently, the high TS and lignin content could be due to late harvesting of common reed (August).

The VS destruction rates obtained in the present study for common reed and grass silage were lower (8 % and 31 %) than those reported in the literature. For example Lehtomäki et al. (2008) reported VS removals of 34 % and 55 % for grass silage for one-stage (LBR) and two-stage (LBR-UASB) processes, respectively. The low VS removals for grass silage in the present study could be attributed to the differences in the substrate characteristics, harvest time, pretreatment methods and/or reactor operation. For instance, the solid to liquid ratio used in LBR and LBR-UASB reactor experiments of Lehtomäki et al. (2008) was 0.26 compared to 0.5 used in the present study. Therefore, lower solubilization efficiency noticed for grass silage and common reed in the present study could be mainly due to high solids and low moisture content in these LBRs. On the other hand, extraction of the leachate from the residual materials (RMLE) resulted in an increase in solids content for tomato and cucumber but not for common reed and grass silage (Table 21). This is probably due to high amount of matrix polysaccharides (hemicelluloses and pectin) in non-grass plants such as cucumber and tomato than in common reed and grass silage (Carpita 1996). It is presumed that the hydration gelling properties of the cell wall is influenced by matrix polysaccharides especially pectin, which acts as

a hydrophilic filler to prevent aggregation and collapse of the cellulose network (Jarvis 1992) and modulate the porosity of the cell wall to macromolecules (Baron-Epel et al. 1988).

The steady increase in VFA and SCOD levels along with a decrease in pH in all LBRs during the first 10 days indicate the rapid hydrolysis or solubilization of organic material. However, this trend changed upon leachate replacement as the produced VFAs and SCOD were diluted with the addition of fresh water. Nevertheless, the increase in VFA levels noticed for cucumber and tomato but not for grass silage and common reed upon leachate replacement indicates the resumption of hydrolysis and COD solubilization. In the end, highest VFA concentrations were noticed in cucumber LBR (9.9 g l^{-1}) followed by tomato (6.3 g l^{-1}), grass silage (4.7 g l^{-1}) and common reed LBRs (3.0 g l^{-1}). The probable reason for this variation could be attributed to the difference in the chemical composition of the studied substrates. Cucumber and tomato appeared to be easier materials to degrade than grass silage and common reed. The easily degradable fraction of the substrate was most probably exhausted around day 10 in the case of cucumber and tomato. A similar result in the build-up of VFAs and lactate during the first 10 days was reported during the AD of sugar beet tops and grass silage (Cirne et al. 2007 & III). Both these results indicate that rapid hydrolysis and acidification process generally occur during the first 10 days of the substrate degradation.

The high initial TKN solubilization noticed in the leachates of tomato and cucumber compared to common reed and grass silage could be attributed to various factors influencing protein degradation such as pH, temperature, dry matter content, inhibitory compounds (Slottner et al. 2006), hydraulic retention time (HRT) (Gallert & Winter 1997, Jokela & Rintala 2003) and water addition during AD (Jokela and Rintala 2003). For instance, some forage species with high dry matter content showed less proteolysis than species with low dry matter content (Muck et al. 1996). In the present study, the dry matter content in common reed (44 %) and grass silage (41 %) was higher than that of cucumber (6.8 %) and tomato (10 %). The 31-50 % of initial TKN solubilization obtained in the present study for the four crop materials was in agreement with the nitrogen solubilization of 30-50 % of TKN reported by Jokela & Rintala (2003) for putrescibles. The higher initial TKN solubilization noticed in the leachate of grass silage than in the other three plant materials (Table 20) was probably due to high protein content in grass silage. Lehtomäki et al. (2008) reported that during AD of grass silage in an LBR, proteins were the most rapidly hydrolysable components after 1 day of incubation.

The higher NH_4-N solubilization in tomato and cucumber (54 and 47 %, respectively) could be due to better proteolysis occurring at a higher pH (6-7) than that noticed for grass silage and common reed (3.9-5). In fact, lowering of pH is the key principle in ensiling technology to preserve the nutritive value of the crop for a longer period. Therefore, low pH (5.5) conditions in grass silage and common reed could have inhibited the growth of protein degrading bacteria (Slottner et al. 2006) and thus proteolysis. Higher NH_4-N solubilization obtained for tomato and cucumber in the present study are in agreement to

TKN to NH_4-N conversion efficiency of 50 % reported for biowaste (Gallert & Winter 1997) and 50-70 % reported for poultry and slaughterhouse wastes (Salminen 2001). These results are in accord to previous studies reported in the literature. Lehtomäki & Björnsson (2006) reported about 30 and 87 % of NH_4-N conversion in the liquid digestates during AD of grass silage and sugar beet, respectively and stated that higher NH_4-N in liquid fraction was beneficial when considering the use of liquid fraction as fertilizer since it will be easy for storage purposes and also for spreading onto the fields. The NH_4-N and TKN concentrations in the UASB effluents showed that tomato effluent had comparatively higher NH_4-N content (0.5-0.8 g l^{-1}) than cucumber, common reed and grass silage (0.3-0.05 g l^{-1}). These results in practice suggest that the NH_4-N rich effluents of tomato and cucumber could be used as liquid fertilizers for growing the same or different crops in the greenhouses or on agricultural lands. As NH_4-N is the preferred form of nitrogen for crop uptake (Demuynck 1984), use of the digestate, obtained after a two-stage process, as liquid fertilizer will not only reduce the need of inorganic fertilizers but also recycle the nutrients and thus close the energy and nutrient cycles within agricultural systems and promote sustainability. On the other hand, effluents of grass silage and common reed probably need further treatment prior to their use as liquid fertilizers.

AD of tomato and cucumber in the two-stage system consisting of LBR-UASB reactors resulted in methane yields of 0.09 and 0.13 m^3 CH_4 kg^{-1} VS_{fed}, respectively. These yields were 28 % and 50 % of those obtained in the BMP assays and are in agreement to the obtained SCOD solubilization rates for these two crop materials (Fig. 17). However, the methane yields obtained in the present study were lower than those reported for fruit and vegetable wastes (0.34-0.38 m^3 CH_4 kg^{-1} VS_{added}) in a two-stage system consisting of LBR-UASB/AF (Martinez-Viturtia & Mata-Alvarez 1989). On the other hand, methane yields of 0.14 and 0.034 m^3 CH_4 kg^{-1} VS_{fed} were obtained for grass silage and common reed, respectively. These yields were 38 % and 13 % of the yields obtained in the BMP assays and also reflect the SCOD solubilization rates of these two materials. However, methane yields obtained for grass silage in the present study were lower than the yields of 0.19 m^3 CH_4 kg^{-1} VS_{added} reported in a two-stage system consisting of LBR-UASB reactors (Lehtomäki et al. 2008). The low methane yields obtained from the studied crop materials in the present study could be attributed to various factors such as inefficient substrate VS solubilization in the LBR, differences in substrate composition, cellulose/lignin content, inefficiency of the UASB reactor inoculum etc. The methane yields obtained in the present study from the two-stage system (LBR-UASB reactors) were further compared with the methane yields of one-stage reactor systems reported in the literature. For example, methane yields of 0.13-0.26 m^3 CH_4 kg^{-1} VS_{added} were reported during co-digestion of cow manure and energy crops and crop residues such as sugar beet tops, grass silage and straw (up to 40 % feedstock VS) in one-stage CSTR systems (Lehtomäki et al. 2007) or 0.2-0.4 m^3 CH_4 kg^{-1} VS_{added} for vegetable wastes in BMP assays (Gunaseelan 2004). When

compared to the above two studies, the methane yields obtained for tomato and common reed in the present study were low (0. 09 and 0.03 m^3 CH_4 kg^{-1} VS_{added}) indicating an inefficient AD of tomato and common reed in the two-stage system. On the other hand, cucumber and grass silage showed comparable methane yields (0.13-0.14 m^3 CH_4 kg^{-1} VS_{added}) indicating a possibility for successful application of a two-stage system rather than one-stage system for these crop materials.

5.5 Future perspective

The present study mainly offers a preliminary insight into the mono and co-digestion of energy crops and crop residues with considerable success in enhancing the substrate hydrolysis at laboratory scale, by the application of methods like leachate replacement, micro-aeration, nutrient addition/supplementation and cellulolytic rumen cultures. In this study, mono-digestion of energy crops was studied only in laboratory scale LBRs and scaling up the research to a pilot plant would give more valuable data for practical application of LBR technology and for using these crops as substrates. The methods studied above for enhancing hydrolysis can be further investigated for application to a wider range of crop materials (than those studied in this work) with different chemical composition to investigate how these methods affect the degradation rates of substrates with different chemical composition and in different process conditions. The methods of micro-aeration and leachate replacement could be specifically studied to determine the optimum process conditions (duration of micro-aeration, timing and quantity of leachate replacement) in two stage reactor systems with different substrates and reactor technologies for enhancing hydrolysis of lignocellulosic energy crops and crop residues and thus achieving higher methane yields and energy-benefits. It is obvious that, rumen cultures would not be available at a scale that are required by full scale biogas plants and therefore, studies could be carried out to develop "artificial rumen ecosystems" (i.e. by growing rumen cultures *in vitro*) and these artificial cultures could be tested for their degradation efficiency against the rumen cultures obtained from live animals. The concentrations of macro and micro nutrients applied for enhancing hydrolysis of grass silage in this study would not obviously be suitable to apply for any other substrate. Therefore, further work could be carried out with substrates which are most commonly used in farm-based biogas plants (for eg. maize silage, other grass varieties etc) and determine the optimum nutrient concentrations needed for these substrates. The main results of this study are further compared with literature in Table 22.

TABLE 22 Summary of the (main) results obtained in the present study (I-V) for enhancement of hydrolysis and in the literature studies.

Method of enhancing hydrolysis	SCOD solubiliz-ation (%)	VS destruct-ion (%)	CH_4 yield (m^3 CH_4 kg^{-1} VS)	NH_4-N (% of initial TKN) in leachate	TVFA (g l^{-1})	This Study/Ref. study	Substrate used This study/Ref. study	Reactor system used This study/Ref. study
Macro nutrient addition	56	41	<0.01	NA	6.8	This study	Grass silage	LBRs
			0.48*		11	1	Sugar beet silage (without leaves)	One stage, continuous digester
			0.5*			2	Sugar beet (without tops and leaves)	Same as above
Micro nutrient addition	45	60	<0.01	NA	7.7	This study	Grass silage	LBRs
			0.3-0.4			3	Model substrate mimicking maize	Batch experiments
			0.3-0.45			4	Maize silage	One-stage, flow-through reactors
NaOH pre-treatment of substrate/ digestate	17	60	0.06(IV) /0.18 (I)	NA	2.3	This study	Grass silage	LBRs (IV)/CSTRs (I)
	80		0.32			5		
	81					6	Pulp and paper sludge	Completely mixed bioreactors
							Corn stover	NA
Leachate replacement	51 (III), 50 (V)	63	<0.01 (III)/ 0.14 (V)	54 (V)	9.8 (III)	This study	Grass, Tomato, Cucumber, Reed	LBR, LBR-UASB
	85					7	MSWVegetable and flower wastes	Simulated landfill reactors
	15-92					8		HR-UAF†

TABLE 22 continued

Method of enhancing hydrolysis	SCOD solubilization (%)	VS destruction (%)	CH_4 yield (m^3 CH_4 kg^{-1} VS)	NH_4-N (% of initial TKN) in leachate	TVFA (g l^{-1})	This Study/Ref. study	Substrate used	Reactor system used
						This study/Ref. study		This study/Ref. study
Leachate replacement & pH adj.	31	74	<0.01	NA	8.4	This study	Grass silage	LBRs
	59	63	0.22			9	Food waste	LBR-UASB
Micro-aeration	33	42	<0.01	NA	9.3	This study	Grass silage	LBRs
	50-60		0.3			10	Primary sludge	Batch reactors
	40					11	Organic sludge	Continuous -flow reactors
Rumen cultures	33	67	0.26	~80		This study	Grass silage	LBRs
	24	75	0.43			12	Waste activated sludge	Three stage continuous digesters
		52				13	Canna	Batch experiments
		71				14	Corn stover	Artificial rumen reactors
		75				15	Cellulose	Rumen enhanced anaerobic sequencing batch reactor

*- Calculated based on the reported yields, NA-Not applicable to this study, † -Hydrolytic reactor-Upflow anaerobic filter (HR-UAF), 1 Demirel & Scherer (2008), 2- Demirel & Scherer, (2009), 3- Pobeheim et al. (2010), 4- Lebuhn et al. (2008), 5- Yunqin et al. (2008), 6- Chen et al. (2009), 7- Sanphoti et al. (2006), 8- Zhang et al. (2007), 9- Xu et al. (2011), 10-Johanssen & Bakke (2006), 11- Hasagawa et al. (2000), 12- Park et al. (2005), 13- Yue et al. (2007), 14- Hu & Yu (2005), 15- Barnes & Keller, (2003).

6 CONCLUSIONS

The results obtained in the present study show that LBRs can be successfully applied for the one and two-stage AD of energy crops and crop residues in co-digestion or mono-digestion for renewable energy production as methane or biogas. Co-digestion of grass silage and cow manure reinforced the feasibility of co-digestion of energy crops with substrate VS containing up to 30 % (VS) of crop and 70 % (VS) of manure at an OLR 2 kg VS m^3 d^{-1} HRT 20 days. The concept of solids re-circulation with and without alkali treatment was proved not useful, resulted in stratification of reactor materials and finally process inhibition.

Mono-digestion of grass silage in LBRs with application of methods such as leachate replacement, micro-aeration, addition of macro and micro nutrients and application of cellulolytic rumen cultures were proved to have potential for enhancing hydrolysis during the AD of energy crops and crop residues. Particularly, the method of leachate replacement without pH adjustment proved to be the most useful by enhancing COD solubilization by 34 % (0.51 g SCOD g^{-1} VS) than other methods. Micro-aeration at low flow rates (1 l min^{-1}) improved acidification (VFA production) by 4-fold without any significant increase in COD solubilization. However, the amount of oxygen supplied and the time of micro-aeration introduced in to the process needs to be further optimized. Application of cellulolytic rumen cultures positively influenced the hydrolysis of the grass silage and improved the COD solubilization by 10 % than control LBR. The LBR with rumen cultures as the sole inoculum source also resulted in 23 % higher methane yield than its control indicating the potential for using rumen cultures as sources of inocula for enhancing both hydrolytic as well as methanogenic rates. Addition of macro (NH_4Cl) and micro nutrients (Fe, Ni, Co and Mo) enhanced specific soluble COD by 18 % and 7 % respectively and VFA production 40 % and 22 % respectively. About 50-80 % of the externally added micro nutrients showed accumulation in the grass silage and about 20-50 % were bioavailable in the leachates (in terms of soluble metal concentration analyzed). This study was a preliminary investigation and further research is needed to optimize the macro and micro nutrients concentrations to better understand the effects during the hydrolytic and methanogenic phases

ideally, in a two-stage reactor configuration. Further, the feasibility of using aquatic grass species such as common reed and crop residues of tomato and cucumber for methane production in a two stage reactor configuration (LBR-UASB) was proved with considerable degradation results and methane yields. LBR promoted the hydrolysis and acidogenesis and resulted in a maximum of 50 % COD solubilization efficiency and 54 % ammonification of the initial TKN solubilized from the studied substrates. On the other hand, UASB reactors facilitated efficient methanogenesis with about 80 % of COD removal efficiency.

Results from batch methane potential assays showed that methane potential of grass silage varied from 0.28-0.39 m^3 CH_4 kg^{-1} VS_{added} in all the experiments and were dependent on the harvest time and crop maturity at the time of the study. On the other hand, methane potentials of the studied crop residues were 0.32 m^3 CH_4 kg^{-1} VS_{added} for tomato and 0.26 m^3 CH_4 kg^{-1} VS_{added} for cucumber and common reed. Alkali pretreatment of solids, obtained from digestate (during co-digestion of grass silage and cow manure in one-stage CSTRs), at a low concentration of 20 g NaOH kg^{-1} VS resulted in higher methane yield (0.34 m^3 CH_4 kg^{-1} VS_{added}) than the other tested dosages (30, 40 & 60 g NaOH kg^{-1} VS). Addition of macro nutrient (NH_4Cl) enhanced methane potential of grass silage by 17 % than control. On the other hand, an increase in methane yields (15 %) was obtained when micro nutrients were added at the highest dosage than at low and medium dosages. Application of a mixed inoculum consisting of rumen culture and digestate from mesophilic biogas plant at 50:50 (v/v) ratio enhanced methane yield of grass silage (0.5 m^3 CH_4 kg^{-1} VS_{added}) than use of the pure inoculum (100 %) or other tested mixed inoculum combinations (0.2-0.29 m^3 CH_4 kg^{-1} VS_{added}). Therefore, the present study shows the feasibility of application above studied methods for enhancing hydrolysis and thus obtain improved methane yields during one and two-stage anaerobic digestion of energy crops and crop residues.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Hydrolyysin tehostaminen energiakasvien ja kasvitähteiden yksi- ja kaksivaiheisessa anaerobiprosessissa

Uusiutuvien energialähteiden käytöllä voidaan vähentää riippuvuutta fossiilisista energialähteistä ja samalla vähentää kasvihuonekaasupäästöjä. Biokaasun tuotanto anaerobiprosessilla on yksi ympäristöystävällisimmistä tavoista tuottaa uusiutuvaa energiaa, sillä raaka-ainepotentiaali on laaja ja prosessi mahdollistaa suljetun ravinnekierron. Lisäksi biokaasu on puhdas, kestävä ja monipuolinen energianlähde, jolla voidaan korvata fossiilisia polttoaineita lämmön- ja sähköntuotannossa, sekä liikennepolttoaineena. Biokaasuprosessissa muodostuva ravinnepitoinen käsittelyjäännös voidaan hyödyntää kasvintuotannossa, jolloin voidaan sulkea ravinnekiertoja ja korvata keinolannoitteiden käyttöä. Euroopassa biokaasua tuotetaan erityisesti maataloussektorilla ja raaka-aineina käytetään kasvi- ja eläinperäisiä tähteitä tai jätteitä sekä energiakasveja eli kasveja, joita viljellään energiantuotantoa varten. Kasvibiokaasuprosesseissa käytetään raaka-aineina lähinnä energiamaissia ja säilönurmea.

Kasvimateriaalin anaerobinen hajoaminen on usein hidasta, mikä johtuu sen monimutkaisesta kemiallisesta rakenteesta ja erityisesti lignoselluloosan suuresta pitoisuudesta. Kasvimateriaalissa selluloosa on tiukasti kiinni hemiselluloosassa ja ligniinissä. Hitaan hajoamisen vuoksi kasvimateriaalin käsittelyaika biokaasureaktorissa on usein pitkä ja kuormitukset suhteellisen alhaisia. Lisäksi osa kasvimateriaalista ei hajoa anaerobisessa prosessissa. Hydrolyysi onkin tyypillisesti rajoittava vaihe kasvimateriaalin anaerobisessa hajoamisessa. Kasvimateriaalin energiahyödyntämistä biokaasuteknologialla voidaan mahdollisesti tehostaa hydrolyysivaiheen prosessiteknologian ja prosessiolosuhteiden optimoinnilla.

Tässä työssä tutkittiin mahdollisuuksia tehostaa hydrolyysiä energiakasvien ja kasvitähteiden yksi- ja kaksivaiheisessa anaerobikäsittelyssä. Pääasiallisena kasvimateriaalina tutkimuksessa oli säilönurmi, ja lisäksi käytettiin tomaatin ja kurkun kasvihuoneviljelyn biomassoja sekä järviruokoa. Bioprosesseina tutkittiin yksivaiheista täyssekoitusreaktoria ja suotopetireaktoria sekä kaksivaiheista prosessia, jonka ensimmäinen vaihe oli suotopetireaktori ja toinen vaihe lietepatjareaktori. Suotopetireaktorissa kierrätetään reaktorin läpi suotautunutta vettä jatkuvasti reaktorin yläosaan, mikä tehostaa substraatin ja mikrobien kontaktia ja säätelee prosessin kosteutta. Suotopetireaktorin on esitetty soveltuvan erityisesti kuivien materiaalien, kuten kasvien, käsittelyyn. Suotopetireaktorissa syötteen partikkelikokoa ei tarvitse yleensä pienentää, siinä ei tarvita sekoitusta ja veden määrä on vähäinen. Hydrolyysin tehostamiseksi tässä työssä tutkittiin myös hivenainelisäysten, mikrobiympin, kasvimateriaalin kemiallisen käsittelyn (alkali) ja mikroilmastuksen sekä suotopetireaktorin kiertoveden korvaamisen vaikutusta.

Vaikutus hydrolyysiin määritettiin yleensä analysoimalla tutkittujen prosessien näytteistä liukoinen kemiallinen hapenkulutus (COD).

Työn tulosten perusteella säilönurmen yksivaiheisessa suotopetireaktorissa hydrolyysiä voidaan tehostaa korvaamalla kiertovesi ajoittain puhtaalla vedellä. Parhaimmillaan orgaanista ainesta hydrolysoitui 34 % enemmän kuin kontrollireaktorisssa, jossa kiertovettä ei korvattu. Tämä osoittaa, että hydrolyysi estyy, kun kiertoveteen kertyy liuennutta ainesta. Orgaanisen aineksen kertyminen saadaan estettyä myös kaksivaiheisessa prosessissa, kuten tässä työssä osoitettiin säilönurmen käsittelyssä. Kaksivaiheisen prosessin toisen vaiheen lietepatjareaktorissa orgaaninen aines hajosi tehokkaasti metaaniksi. Toisin kuin kiertoveden korvaaminen, kasvimateriaalin mikroilmastus ei tehostanut hydrolyysiä suotopetireaktorissa, mutta se hajotti liuennutta orgaanista ainesta lyhytketjuisiksi yhdisteiksi ja siten edisti anaerobista hajoamista.

Myös selluloosaa hajottavan pötsinesteen käyttö mikrobisiirroksena tehosti hydrolyysiä säilönurmen yksivaiheisessa suotopetireaktorissa. Orgaanista ainesta hydrolysoitui noin 10 % enemmän kuin mikrobisiirroksella, joka oli naudan lantaa käsittelevästä biokaasureaktorista. Myös metaanintuotto lisääntyi merkittävästi eli 23 %. Tulosten perusteella selluloosaa hajottava pötsineste on potentiaalinen mikrobien lähde tehokkaaseen kasvimateriaalin hydrolyysiin ja metaanintuottoon. Käytännön sovelluksessa mikrobipopulaatio tulee rikastaa melko pienistä lähtöpitoisuuksista.

Kasvibiokaasureaktoreiden toiminnan on esitetty vaativan hivenaine-lisäyksiä erityisesti maissia käsittelevissä reaktoreissa. Tässä tutkimuksessa makroravinteen (ammoniumkloridi) ja hivenravinneseoksen (rauta, nikkeli, koboltti, molybdeeni) havaittiin lisäävän vastaavasti 18 ja 7 % orgaanisen aineen hydrolyysiä säilönurmesta yksivaiheisessa suotopetireaktorissa kontrolliin verrattuna. Täten kasvibiokaasuprosesseissa on aiheellista kiinnittää huomiota ravinteiden vaikutukseen myös hydrolyysiin eikä vain metaanintuottoon, kuten yleensä tehdään.

Kaksivaiheisessa suotopeti-lietepatjareaktori -prosessissa kasvihuone-tomaatin ja -kurkun tähteiden ja järviruo'on orgaanisesta aineesta hydrolysoitui noin 50 %, ja liuennut orgaaninen aines saatiin konvertoitua metaaniksi lietepatjareaktorissa. Kasvihuonemassojen typestä mineralisoitui noin puolet ammoniumtypeksi. Prosessiyhdistelmässä voidaankin tuottaa energian lisäksi liukoista ravinneliuosta, jota mahdollisesti voidaan käyttää lannoitteena kasvintuotannossa.

Kasvibiomassan alkalikäsittely ei reaktorikoejoissa tehostanut hydrolyysiä eikä metaanintuottoa. Kasveja ja lantaa yhteiskäsittelevän täyssekoitteisen biokaasureaktorin käsittelyjäännöksen kiintoainejakeen kierrättäminen biokaasuprosessiin ei lisännyt metaanintuottoa. Kiintoainejakeen alkalikäsittelykään ennen kierrätystä ei lisännyt metaanintuottoa. Kierrätys aiheutti biokaasuprosessissa vaahdon muodostumista, kiintoaineen kertymistä ja merkkejä prosessin inhiboitumisesta. Alkalikäsittely ei myöskään tehostanut hydrolyysiä säilönurmen yksivaiheisessa suotopetireaktorissa.

Tämän työn tulosten perusteella kasvimateriaalin hydrolyysiä voidaan siis tehostaa eri tavoilla. Parhaimmillaan koeajoissa saatiin hydrolysoitua jopa 34 % enemmän orgaanista ainesta kuin kontrolliprosessissa. Tehostunut hydrolyysi mahdollistaa useimmiten myös suuremman metaanintuoton niin yksi- kuin kaksivaiheisissakin prosesseissa. Tässä työssä useita menetelmiä tutkittiin vasta ensimmäistä kertaa kasvimateriaalien hydrolyysin tehostamiseksi. Menetelmien käytännön soveltaminen edellyttääkin tapauskohtaista tutkimusta ensin laboratoriossa ja optimoitujen menetelmien demonstrointia pilot-mittakaavassa ja täyden mittakaavan laitoksissa.

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ORIGINAL PAPERS

I

CO-DIGESTION OF GRASS SILAGE AND COW MANURE IN A CSTR BY RE-CIRCULATION OF ALKALI TREATED SOLIDS OF THE DIGESTATE

by

Padma Shanthi Jagadabhi, Annimari Lehtomäki & Jukka Rintala 2008

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CO-DIGESTION OF GRASS SILAGE AND COW MANURE IN A CSTR BY RE-CIRCULATION OF ALKALI TREATED SOLIDS OF THE DIGESTATE

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ABSTRACT

Three laboratory, continuously stirred tank reactors (CSTRs) co-digesting grass silage and cow manure (forming 30% and 70% of substrate volatile solids (VS), respectively) were operated to evaluate the effects of re-circulating an alkali-treated and untreated solid fraction of the digestate back to the reactors. The CSTRs were operated at an organic loading rate (OLR) of 2 kg VS m^{-3} day^{-1} and hydraulic retention time (HRT) of 20 days with a semi-continuous mode of feeding. The feasibility of co-digestion with substrate VS containing 30% VS of crop was reinforced, resulting in average specific methane yield of about 180–185 l CH_4 kg^{-1} VS. Re-circulation of the solid fraction of digestate back to the reactors in both alkali-treated and untreated forms decreased the methane yield by 11% and 21%, respectively, and resulted in operational problems such as scum formation and accumulation of the reactor materials. Batch studies were conducted to evaluate (i) the methane potentials of the solid fraction of digestate, and whole digestate with alkali treatments ranging from 20–60 g NaOH kg^{-1} VS of substrate, and (ii) methane potentials of the accumulated reactor materials as top, middle and bottom layers. The solid fraction of digestate treated with 20 g NaOH kg^{-1} VS showed higher specific methane yield (340 l CH_4 kg^{-1} VS) than the higher range of alkali treatments. The bottom layers of the control reactor and the reactor fed with alkali-treated solids gave a higher specific methane yield (93 and 85 l CH_4 kg^{-1} VS, respectively), and all three layers of untreated solids gave similar methane potentials.

Keywords: Energy crops, biogas, solids, alkali treatment, stratification

INTRODUCTION

Co-digestion of solid wastes often results in a higher methane yield (measured in cubic metres of methane per kilogram of volatile solids (VS)) than monodigestion. This is apparently due to the synergistic effects of the co-substrates providing the missing nutrients and balancing the substrate composition [1, 2]. The co-digestion concept has been studied and applied increasingly to treat substrates such as municipal solid wastes, sludges, cow manure and energy crops [1]. The recent interest in producing renewable electricity through anaerobic digestion has rapidly stimulated the use of co-digestion of crops (such as maize silage) in farm-scale manure digesters, for example in Germany and Austria [3, 4]. Co-digestion of crops with manure results in higher methane production than manure alone. Co-digestion of 40% of sugar beet tops with dairy manure in batch and continuously fed laboratory reactors yielded about 1.5 times higher methane yield than from dairy manure alone [2]. Laboratory continuously stirred tank reactors (CSTRs) operated with 30% of feedstock VS consisting of crops (grass, sugar beet tops, straw) resulted in 16–65 % higher methane yield than from dairy manure alone, while 40% of feedstock VS with crops decreased the yields [5]. However, studies pertaining to co-digestion of crops with cow manure in typical CSTRs are still limited.

About 40–50% of the total solids (TS) of cow manure is present in the form of particulate matter [6]. It appears that the biodegradability of particulate material in the feedstock and in the digestate actually determines the overall methane potential of the substrate [7, 8]. In a biogas system with a typical hydraulic retention time (HRT) of 15–30 days not all of the fibres are degraded, with an average methane yield of 0.20–0.25 m^3 kg^{-1} VS against theoretical yield of 0.40–0.45 m^3 kg^{-1} VS, and thus about 25% of the methane potential remains untapped in the fibres [9]. It was also reported [10] that digester effluents still contain 6–33% of methane potential even after the production of substantial amounts of methane. This residual methane potential is recovered to a certain extent in covered post-storage tanks, commonly used on farms for storing (for several months) the digestate under ambient temperature for subsequent land spreading. For example, it was reported that digested cow manure from a farm digester had a methane potential of about 0.20–0.26 m^3 kg^{-1} VS during long-term incubation (345 days at 35–55°C) [11]. The authors also reported that the methane potential of the liquid fraction (particle size <0.25 mm) of the digestate could be recovered by long-term

storage in a post-storage tank (5–10°C) whereas recovery of methane from the >2 mm fraction of the digestate was more difficult; thus, this fraction could form the residual energy-rich fraction of the digestate. Therefore, increasing the biodegradability of such a recalcitrant fraction of the digestate with various physical or chemical treatment methods may be a viable option to recover its residual methane potential.

A number of methods treating the manure fibres chemically, physically and biologically, as a pre-treatment or post-treatment option before or after a primary digestion phase, have been studied [6, 12]. An increase in biogas yield of about 25% was obtained from fibres of manure when the feed was pre-treated by maceration and subjected to anaerobic digestion [9]. Further, an increase of about 13% and 23% in methane potential was obtained (determined in batch assays) with alkali-treated fresh cattle manure over untreated manure at a dosage of 20 g and 40 g NaOH kg^{-1} VS, respectively [6].

The main objective of this study was to evaluate the effects on methane production and process performance of re-circulating the solid fraction (referred as 'solids' hereafter) of the digestate back to the reactors, with and without alkali treatment. Three CSTRs co-digesting grass silage and cow manure (forming 30% and 70% of substrate VS, respectively) were operated at an organic loading rate (OLR) of 2 kg VS m^{-3} day^{-1} and HRT of 20 days. Alkali treatment of the solids was performed with the aim of destroying the ligno-cellulosic structures in the fibres and thereby enabling further substrate degradation. The re-circulation of untreated solids was studied to determine the impact of re-circulating the solids as such (that is, without any alkali treatment) to the reactor.

MATERIALS AND METHODS

Origin of Materials

The substrates used were grass silage and cow manure from a dairy farm (Kalmari farm, Laukaa) in Central Finland (Table 1). Two kinds of innocula were used; one was from the farm's mesophilic digester co-digesting cow manure and industrial confectionery by-products, and the other was from the laboratory CSTRs operated at 35°C with straw, grass silage and cow manure for about 318 days [5]; both inocula were stored at 4°C for 2–3 months before the experiment. Grass silage was prepared at the farm from grass (seed mixture comprising 75% timothy, *Phleum pratense* and 25% meadow fescue, *Festuca pratensis*) harvested at the early flowering stage, chopped with an agricultural precision chopper after 24 hours pre-wilting and then ensiled after adding a commercial silage additive. At the laboratory it was further cut to 2–3 cm size with scissors. A portion of the grass silage sample was immediately flushed with nitrogen, sealed tightly in a plastic bag and stored at –20°C until used in the batch experiments. The remaining portion of the silage was packed separately into portions sufficient to feed the reactors for 2–3 days and was immediately stored at –20°C.

Table 1. Characteristics and specific methane yields of substrate and inoculum (from farm digester).

	Grass	Cow manure	Inoculum
TS (%)	38	6.5	5.8
VS (%)	35	5.1	4.5
TKN (mg l^{-1} TS)	18.4	30.7	41.3
NH_4-N (mg l^{-1} TS)	0.2	18.5	14.9
SCOD (mg l^{-1} TS)	55.2	492	224
Specific methane Yield (l CH_4 kg^{-1} VS)	376 ± 32	309 ± 24	70 ± 14

Reactor Experiments

Co-digestion was studied in three identical continuously (300 rpm) stirred 5 l glass CSTR reactors (referred to as R1, R2 and R3) with a liquid capacity of 4 l at 35±1°C. The reactors' lids were provided with three tubular openings: one was tightly sealed, one was used to feed and to withdraw the effluent and the third was used as an outlet for the collection of biogas. The reactors were syringe-fed once a day, five days a week. Before feeding, a volume equivalent to the feeding volume was removed with the syringe.

On day 0 of the run 4 l of inoculum (3 l of inoculum from the farm and 1 l of inoculum from the laboratory CSTRs operated with grass silage and straw) was added into the reactors. The substrate was initially fed at an OLR of 2 kg VS m^{-3} day^{-1} (that is, 200 g of substrate per day) and the feed ratio was 70:30 (percentage of substrate VS) of cow manure and grass silage, respectively. On day 57, the reactors were opened and the contents of all the three reactors were poured into a single container, mixed and then redistributed into the three reactors to ensure that the materials in each reactor were identical before initiating solids re-circulation. One week before initiating the re-circulation of solids to the reactors, the digestates removed from R2 and R3 on all five feeding days of the week were collected, centrifuged (g = 905.58, 3000 rpm for 15 minutes) and supernatant was removed in order to obtain only the solids.

Solids thus obtained were introduced into 250 ml bottles with and without alkali treatments, and then were flushed with nitrogen (98.8%) for about three minutes and subjected to incubation at 35±1°C for about 65 hours (during the weekend). Alkali treatment of the solids obtained from R2 was performed by adding 20 g NaOH kg^{-1} VS (40% NaOH solution). After incubation, these solids were stored at 4°C during the following week for re-circulation along with the original feed. Re-circulation of solids was initiated on day 75. The process of collecting the digestates during the feeding days of the week, obtaining solids from the digestates and

incubation of solids was continued every week from day 75 until the end of the run (day 151). Thus, from day 75 onwards, feeding of R2 and R3 included 20 g of alkali-treated (R2) and untreated (R3) solids (8–10% VS) in addition to the original feed (about 200 g), while feeding of R1 was continued as previously, acting as a control process. Owing to the re-circulation of the solids, from day 75 onwards, the OLR in R2 and R3 ranged up to 2.5 kg VS m^{-3} day^{-1} while HRT was slightly less than 20 days.

Batch Treatments and Methane Potential Assays

Three different methane potential assays were carried out in this study. All the assays were performed at 35±1°C.

The methane potential of the substrates was assayed in triplicate one-litre glass bottles with a liquid capacity of 750 ml. 250 ml of inoculum (from farm; Table 1) was added into the bottles and substrate was added to obtain a VS inoculum/VS substrate ratio of one. Distilled water was added to obtain a liquid volume of 750 ml and $NaHCO_3$ (3 g l^{-1}) was added as a buffer. The contents of the bottles were then flushed with nitrogen (98.8%) for about three minutes before sealing with butyl rubber stoppers. Inoculum alone was assayed to subtract its methane production from those of the substrates. The bottles were manually shaken before analysing the methane content.

The effect of NaOH additions (ranging from 20–40 g NaOH kg^{-1} VS) on the methane potential of solids of the digestate and the whole of digestate was studied in triplicate 120 ml serum bottles. The working volume of these assays was about 42 ml. To obtain the solids, digestate of R1 was collected over five days in a week during days 88–104, stored at 4°C and subsequently centrifuged (g = 905.58, 3000 rpm for 15 minutes) before the start of the experiment. Assays of digestate solids were prepared with a VS inoculum/VS substrate ratio of one, and 7 g of solids were added into the assays while, for assays of the whole digestate, 40 g of digestate sample was directly added into triplicate 120 ml serum bottles. NaOH was then added (in 20, 30, 40 and 60 g NaOH kg^{-1} VS, 40% NaOH solution) in all bottles and pH was immediately adjusted to 7.2–7.5 with 5 M HCl. The gas phase was then flushed with nitrogen (98.8%) for about two to three minutes. The assays were immediately sealed with butyl rubber stoppers and then aluminium crimped. Nitrogen (98.8%) was flushed again for two minutes through a water lock system in order to ensure anaerobic conditions, and the assays were incubated for about 65 hours. Subsequently, the assays with solids were opened first and 25 ml of inoculum (from farm) was added. 9.7 ml of distilled water was added to the assays with solids, and 2 ml was added to the assays with the whole digestate, resulting in a liquid volume of 42 ml. The gas phase was then flushed with nitrogen (98.8%) for about two to three minutes. The assays were immediately sealed with butyl rubber stoppers and then aluminium crimped. Nitrogen (98.8%) was flushed again for two minutes through a water lock system in order to ensure anaerobic conditions. One solid sample was assayed with 20 g NaOH kg^{-1} VS without addition of water to determine the effect of adding water. The assays were immediately sealed with butyl rubber stoppers and then aluminium crimped. The assays were then kept on a shaker (Heidolph Instruments, Unimax 2010) at 300 rpm throughout the experimental period.

The methane potentials of the layers (reactor materials stratified into top, middle and bottom layers) and the 'whole materials' were studied at the end of the reactor experiments (day 151). The contents of the reactors were sampled as 'whole materials' and as three layers. Whole materials were sampled from all the three reactors after continuously mixing the contents of the reactors for five minutes. Materials for the layers were sampled after the reactors were left to settle for about 26 hours. Samples were taken from top, middle and bottom layers. Methane potential assays were carried out in triplicate 120 ml serum bottles. 40 ml of the materials (whole materials and materials forming top, middle and bottom layers) were directly added into the assays. Nitrogen was flushed (98.8%) into the assays for about two to three minutes, and they were immediately closed with butyl rubber stoppers and sealed with aluminium crimps. Nitrogen was flushed again for about two minutes through a water lock system in order to ensure complete anaerobic conditions.

ANALYSES AND CALCULATIONS

The methane content of the gas produced was analysed using a gas chromatograph (PE autosystem XL, Perkin Elmer Alumina column 30 m × 0.53 mm) with flame ionisation detectors (operating conditions: oven 100°C, injection port 250°C, detector 225°C). Argon gas was used as carrier gas. A pressure lock syringe was used for sampling the gas. The volume of gas produced was measured by the water displacement method. TS and VS were determined according to the APHA standard methods [13]. pH was measured with a Metrohm 774 pH metre. Chemical oxygen demand (COD) and ammonium nitrogen (NH_4-N) from the grass sample were analysed after extraction according to SFS-EN 12457-4 [14]. Samples for soluble COD and NH_4-N were filtered using GF50 glass fibre filter papers (Schleicher & Schhuell) before analyses. Soluble COD and total COD were measured according to the SFS 5504 (Finnish standards Association. 1988). Ammonium nitrogen (NH_4-N) and total nitrogen (TKN) were analysed by Tecator Application Note [15] (Perstorp Analytical/Tecator AB. 1995) with Kjeltec system 1002 distilling unit.

Specific methane yields of the batch assays were calculated as cumulative methane (in millilitres) per gram VS added and were expressed in litres of methane per kilogram of VS after subtracting the methane potential of the inoculum. Specific methane yields of the reactor experiments were calculated based on amounts of VS added weekly and weekly methane production. Specific methane yields, OLR, HRT, TS

and VS removals were calculated on the basis of the daily feed additions, and the amounts of re-circulated solids were not taken into account.

RESULTS

The studied grass silage had about a six-, seven- and four-fold higher TS, VS and nitrogen content respectively, than manure, while its total methane potential was about 40% higher than that of manure (Table 1). The extractable NH_4-N in grass silage was low in comparison with manure.

The CSTRs were inoculated on day 0 and semi-continuous feeding was started on day six, when methane content reached about 50% of the gas phase in the reactors. Subsequently, all three reactors were operated in parallel, co-digesting with substrate VS comprising 30% and 70% of grass silage and cow manure, respectively, with an OLR of 2 kg VS m^3 day^{-1} and HRT of 20 days. Specific methane yields in the three reactors increased from about 165 to 180 l $CH_4 kg^{-1}$ VS during the first eight weeks of operation, while TS and VS removals were 20–26% and 27–33%, respectively, in the first four weeks, and 24–30% and 31–38% in the latter four weeks. On day 57, the reactors were opened and the contents of all the three reactors were mixed and then redistributed into the three reactors to ensure that the materials in each reactor were identical. Feeding was started the next day. After about two weeks the methane production in the reactors reached the same level as before.

Subsequently from day 75 recirculation of solids to reactors R2 and R3 was initiated. During the re-circulation period, the control reactor (R1) gave a higher specific methane yield (182 ± 0.02 l CH_4 kg^{-1} VS) than reactors fed with alkali-treated (R2; 161 ± 0.03 l CH_4 kg^{-1} VS) and untreated solids (R3; 143 ± 0.03 l CH_4 kg^{-1} VS), respectively (Figure 1). Further re-circulation of solids in reactors R2 and R3 resulted in operational problems such as scum formation and accumulation. Only slight changes in TS removal were observed after the recirculation of solids: TS removal decreased (by 3%) in R2 and increased slightly (2%) in R3, while VS removal (as a percentage) was the same in both R2 and R3 (Table 2). TS and VS (%) of digestates from reactors R2 and R3 did not remarkably change after re-circulation of solids (Table 2). In R2, SCOD solubilisation was higher (9.0 g l^{-1}) than in R3 (7.3 g l^{-1}) and the control reactor (R1, 7.5 g l^{-1}) while NH_4-N concentrations were about the same in all three reactors (0.75–0.80 g l^{-1}) (Figure 1).

The effect of alkali treatments and dosages on methane potential of solids of digestate and whole digestate was studied in batch assays for 118 days (Table 3). Specific methane yield was about 12–20% higher (340 ± 15 l CH_4 kg^{-1} VS) from solids of digestate treated with 20 g kg^{-1} VS of alkali than from 30, 40 and 60 g NaOH kg^{-1} VS additions and without NaOH addition. Alkali treatments on whole digestate did not affect methane yields and all the dosages resulted in similar methane potentials (Table 3 and Figure 2).

At the end of the reactor experiments, the characteristics of the contents of the reactors were studied as whole materials and as layers. The volume occupied by the top layers in reactors R2 and R3 was higher than the volume of the top layer in the control reactor, R1 (26%, 24% and 15%, respectively; Table 4). TS and VS percentages of the top layers of reactors R2 and R3 were slightly higher than for R1 (Table 4). Specific methane yields of the top layers of reactors R2 and R3 were about 17–20% higher (64 ± 4 and 67 ± 2 l CH_4 kg^{-1} VS) than the control reactor, R1 (53 ± 4 l CH_4 kg^{-1} VS) (Table 4 and Figure 2). Specific methane yield of the whole materials of reactor R2 was about 30% higher (112 ± 4 l CH_4 kg^{-1} VS) than the control reactor R1 and R3 (84 ± 5 and 75 ± 2 l CH_4 kg^{-1} VS), respectively (Table 4 and Figure 2).

Table 2. Substrate and digestate characteristics and methane production in reactors co-digesting grass silage and cow manure; R1, control reactor; R2, with re-circulation of alkali-treated solids (from day 75 onwards); and R3, with re-circulation of untreated solids (day 75 onwards) (calculated as averages based on weekly analyses; for specific methane yields mean ± standard deviations are shown).

	Day 0–24				Day 24–56				Day 75–151			
Parameter	Substrate	R1	R2	R3	Substrate	R1	R2	R3	Substrate	R1	R2	R3
TS (%)	4.9	3.6	3.9	3.7	4.6	3	3.5	3.8	4.6	3	3.7	3.5
VS (%)	4	2.7	2.9	2.9	3.8	2	2.7	3	4	2.4	2.6	2.7
SCOD (g l^{-1})	24	13.6	14	14	17.6	11	11	12	17	7.5	9	7.2
TCOD (g l^{-1})	72	38	37	37	68	36	36	36	46	29	29	26
NH_4-N (g l^{-1})	1.1	1	0.9	0.9	1	0.9	0.9	0.9	0.8	0.8	0.8	0.75
TKN (g l^{-1})	2.4	2	2.2	2.1	2.2	1.8	2	2	1.9	2	2.1	1.9
TS removal (%)		26	20	24		30	26	24		28	23	25
VS removal (%)		33	27	28		38	33	31		38	33	31
Methane Content (%)		55	49	50		53	49	49		56	54	52
Specific Methane Yield		0.179	0.171	0.165		0.188	0.181	0.180		0.182	0.161	0.143
(m^3 CH_4 kg^{-1} VS)		±0.03	±0.04	±0.04		±0.01	±0.01	± 0.0		±0.02	±0.03	±0.03

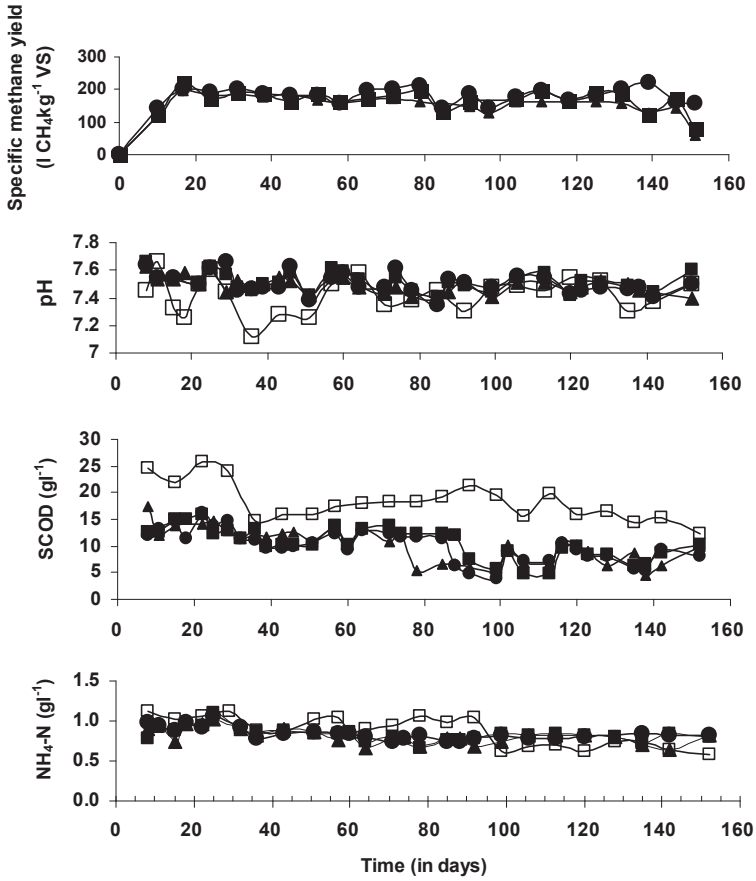

Figure 1. Specific methane yields, pH, SCOD and NH_4-N of the substrate and digestates of R1, R2 and R3; ●, control reactor R1; ■, reactor fed with alkali-treated solids (from day 75 onwards) R2; ▲, reactor fed with untreated solids (from day 75 onwards); and □, substrate (feed).

Table 3. Effect of NaOH treatment on specific methane yields from solid and whole digestate (mean ± standard deviations).

NaOH (g kg^{-1} VS)	Solid fraction (l CH_4 kg^{-1} VS)	Whole digestate (l CH_4 kg^{-1} VS)
0	301 ± 43	100 ± 6
20	340 ± 15	93 ± 7
20[a]	310 ± 50	–
30	300 ± 21	99 ± 4
40	306 ± 3	96 ± 4
60	272 ± 22	99 ± 10

[a]Solids only without the addition of water.

DISCUSSION

These results show that re-circulation of the solids to the biogas process, both in treated and in untreated form, was not effective and did not improve methane yields. Re-circulation of solids in both the reactors resulted in the accumulation of materials and scum formation which in turn led to operational problems in the laboratory reactors. The reactors showed higher content by volume and TS percentage of the materials by the end of the experiment (Table 4). However, the feasibility of co-digesting cow manure and grass silage in CSTRs and the possibility of using about 30% of VS of grass silage as an energy crop for methane (Tables 2 and 3) has been reinforced, as demonstrated in previous studies [5, 16]. The specific methane yield obtained from the CSTRs in the present study was

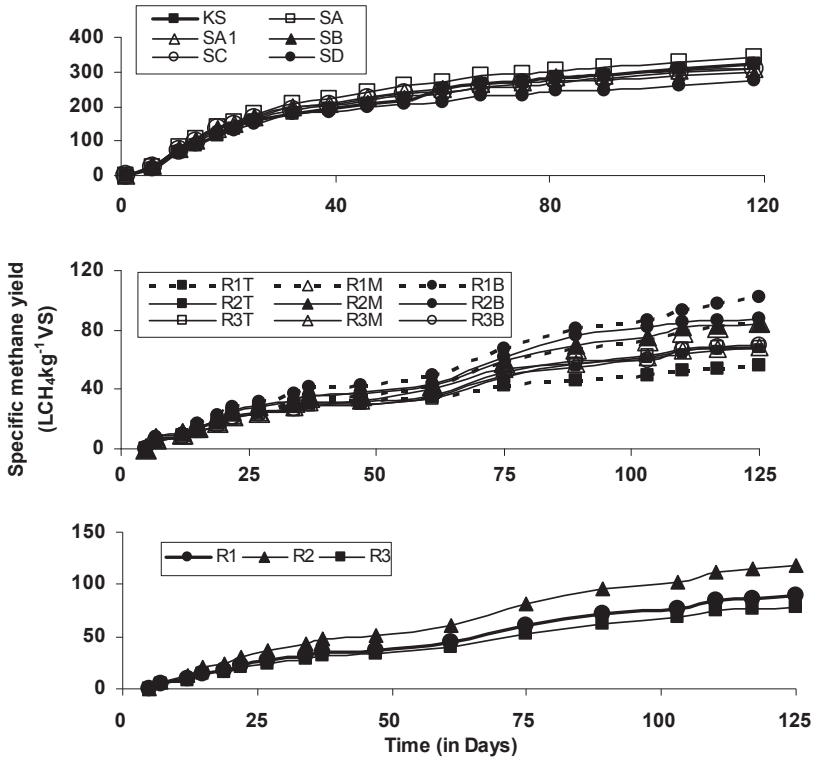

Figure 2. Specific methane yields of batch assays to determine the effects of alkali treatments on the solid fraction of digestate; KS, control (without NaOH addition) for solids, SA, solids with 20 g NaOH kg^{-1} VS; SA1, 20 g NaOH kg^{-1} VS without addition of water; SB, solids with 30 g NaOH kg^{-1} VS; SC, solids with 40g NaOH kg^{-1} VS; SD, solids with 60g NaOH kg^{-1} VS. Specific methane yields in the batch assays with stratified materials in CSTRs as T (top layers), M (middle layers), B (bottom layers) conducted at the end of operation of the CSTRs (R1, R2, R3). Specific methane yields from batch assays with whole materials of the reactors conducted at the end of the operation of CSTRs.

Table 4. Characteristics of layers and whole materials in reactors co-digesting grass silage and cow manure; R1, control reactor; R2, with re-circulation of alkali treated solids (from day 75 onwards); and R3, with re-circulation of untreated solids (day 75 onwards).

	Layer	Volume (%)	TS (%)	VS (%)	TKN (g l^{-1})	NH_4 (g l^{-1})	Specific methane yield (l CH_4 kg^{-1} VS)
R1	Top	15.6	8.2	6.5	3.8	0.8	53 ± 4
	Middle	60.1	4.7	3.6	2.6	0.8	79 ± 3
	Bottom	24.1	4.1	3.1	2.6	0.8	93 ± 7
	Whole	100	4.8	3.7	3.2	0.8	84 ± 5
R2	Top	24.9	8.6	6.9	3.8	0.9	64 ± 4
	Middle	50	4.2	3.2	2.3	0.9	81 ± 9
	Bottom	25	4.1	3.1	2.5	0.9	85 ± 10
	Whole	100	5.0	3.1	2.6	0.8	112 ± 4
R3	Top	26.3	8.6	6.9	4.0	0.9	67 ± 2
	Middle	48.4	4.2	3.3	2.4	0.9	66 ± 3
	Bottom	25.2	4.8	3.7	2.5	0.9	67 ± 3
	Whole	100	6.1	4.6	2.8	0.9	75 ± 2

lower than obtained in a previous study for the same substrate [5]; in the present study, 182 l CH_4 kg^{-1} VS was obtained (from R1 during days 75–151) and in an earlier study [5], a value of 268 l CH_4 kg^{-1} VS was obtained on co-digestion of cow manure and grass silage in CSTRs, conducted by gradually raising the crop content from 0 to 40%. However, methane potential in the present batch assays was higher (376 l CH_4 kg^{-1} VS) than obtained in the previous study [5] (306 l CH_4 kg^{-1} VS). The lower specific methane yield in the present CSTR study indicates that the process was not sufficiently adapted to degrade the substrate, probably due to shorter period of operation than in the previous study, or because no stepwise increase in the ratio of crop was used, unlike the previous study [5].

Alkali-treated solids, when re-circulated to R2, yielded about 12% higher methane than the reactor fed with untreated solids (R3); this could indicate some degree of destruction of ligno-cellulosic structures due to the alkali treatment enabling digestion of solids through hydrolysis [6, 11, 17, 18]. Further, SCOD solubilisation in digestate of R2 was higher than in R1 (Table 2), which indicates that the solubilisation of SCOD was somewhat better in R2 than in R1, and COD of R3 was less than R2 and R1, indicating lesser solubilisation probably due to accumulation of the reactor materials. One possible explanation for the higher SCOD solubilisation but low methane yield in R2 could be non-conversion of solubilised COD to methane [19]. Previously it was reported [19, 20] that the addition of NaOH enhanced COD solubilisation, but did not improve solubilised COD conversion to methane due to inhibition of methanisation by refractory molecules formed as a result of solubilisation by the alkali [19]. Similarly, in the batch studies conducted at the end of the experiment to characterise layers of the reactor materials, the high COD solubilisation (data not shown) and low methane yield from the top and middle layers of R2 (Table 4) could be mainly because of inhibitory/refractory compounds formed as a result of increased COD solubilisation. It appears that multiple reasons can be attributed to low methane yield with alkaline-treated materials, because the effects of alkali treatment depend on factors such as lignin content [21], incubation temperature and dosage of alkali [22] and also the type of ligno-cellulosic materials subjected to treatment [23].

The lower specific methane yield from R3 compared with R2 could be attributed to the presence of visibly coarse and dry fibres (alkali-treated fibres appeared soft and moist) and recalcitrant ligno-cellulosic structures. However, it was not clear why COD solubilisation values of R3 were comparable with R1 and yet yielded less methane than the control reactor (R1) (Table 2).

It was further reported that in the quiescent state cattle manure slurry stratifies into three layers, namely the floating 'scum', the bottom sludge and a watery middle layer [8]. The formation of layers that took place in the present study is in complete agreement with the previous study [8]. Top, middle and bottom layers of R3 resulted in similar specific methane yields, indicating equal mass distribution. However, the methane yields of layers of R3 were lower than those obtained from the layers of R1 and R2. Higher methane from assays of R2 can clearly be attributed to re-circulation of alkali-treated solids. Accumulation and scum formation took place more intensively in R3 than in R2, which could also have inhibited degradation processes in R3.

In the present batch study 20 g NaOH kg^{-1} VS was found to be the optimum amount for enhancing the methane yield of the solids of digestate, as higher doses (30, 40 and 60 g NaOH kg^{-1} VS) did not improve the methane yield (Table 3). It has been shown previously that increasing alkaline additions do not result in higher COD solubilisation rates or higher methane production rates, while an optimum dosage can be found. For example, in a study [20] conducted to determine the influence of alkaline pre-treatment on spent microbial biomass (of an industrial plant), it was reported that with NaOH concentrations of less than 4 g l^{-1} biodegradability rates remained below 17%. Biodegradability rates increased up to 50% for alkali concentrations ranging from 4–10 g l^{-1} and decreased for alkali concentrations higher than 10 g l^{-1}. It was also speculated that biogas production was low with low NaOH concentration because organic matter was still 'particulate' and the rate-limiting step in this condition was hydrolysis, while at higher NaOH concentrations biogas production was still low but due to the solubilised molecules. In another such study [24] conducted to determine the effect of pre-treatment of waste-activated sludge with NaOH concentrations ranging from 0–21 g l^{-1} on COD solubilisation and methane production, it was reported that up to 43.5% COD solubilisation was observed at 7 g l^{-1} of NaOH concentration. This concentration was considered as optimum since NaOH concentrations higher than 7 g l^{-1} resulted in lower COD solubilisation rates. Methane production obtained with 7 g l^{-1} of NaOH concentration was about 11% higher than without NaOH treatment. In contrast, it was reported [6] that about 10% higher methane potential (determined in batch assays) was obtained when fresh cattle manure was treated with 40 g NaOH kg^{-1} VS than when treated with 20 g NaOH kg^{-1} VS. Higher alkali concentrations in the present study did not favour such an increase in specific methane yield, which could be because of the additional recalcitrance offered by the ligno-cellulosic nature of the solids.

The batch assays with alkali treatments on whole digestate did not result in a higher methane yield than the assays with alkali treatments on solids; this could be because whole digestates are diluted due to the presence of water, and the organic content (VS %) of the whole digestates was less than half (4.4%) of the organic content of the solids (10.3%). Higher organic content (VS) indicates more carbon content, and thus more cellular material for microbial degradation, and thus a higher yield of methane.

The results obtained in this study suggest the need to explore other methods for the treatment of solid fibres in digestates, including physical treatment methods such as maceration and wet oxidation [6, 11, 25]. The residual methane

potential of the digestate can be exploited to the maximum once an efficient treatment option has been identified. On the other hand, it may turn out that the exploited methane does not cover the costs or resource requirements of the treatments.

CONCLUSIONS

This study reinforced the feasibility of co-digestion of crop and manure (forming 30 and 70% of substrate VS, respectively) at an OLR of 2 kg VS m^3 day^{-1} and HRT of 20 days. It seems that the concept of re-circulation of solids back to reactors, with and without alkali treatment, appears unjustified and even non-desirable, as it did not enhance specific methane yields (compared with the reactor without re-circulation of solids). Further re-circulation resulted in accumulation and stratification of reactor materials and operational problems. Nevertheless, alkaline treatment might have some potential for increasing methane production, as in the reactor runs higher methane yields were obtained from alkaline treated solids than from solids without alkaline treatment, and in the batch assays 20 g NaOH kg^{-1} VS was found optimum for enhancing the specific methane yield of the solids of digestate.

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II

EFFECT OF MACRO AND MICRO NUTRIENTS ADDITION DURING MONO-DIGESTION OF GRASS SILAGE IN ONE-STAGE LEACH BED REACTORS.

by

Padma Shanthi Jagadabhi, Prasad Kaparaju, Ari Väisänen & Jukka Rintala

Submitted Manuscript

III

EFFECT OF MICRO-AERATION AND LEACHATE REPLACEMENT ON COD SOLUBILIZATION AND VFA PRODUCTION DURING MONO-DIGESTION OF GRASS-SILAGE IN ONE-STAGE LEACH-BED REACTORS

by

Padma Shanthi Jagadabhi, Prasad Kaparaju & Jukka Rintala 2010

Bioresource Technology 101: 2818-2824

IV

APPLICATION OF RUMEN CULTURES FOR ENHANCING HYDROLYSIS OF GRASS SILAGE DURING ANAEROBIC DIGESTION IN LEACH-BED REACTORS

by

Padma Shanthi Jagadabhi, Prasad Kaparaju and Jukka Rintala

Manuscript

V

TWO-STAGE ANAEROBIC DIGESTION OF TOMATO, CUCUMBER, COMMON REED AND GRASS SILAGE IN LEACH-BED REACTORS AND UPFLOW ANAEROBIC SLUDGE BLANKET REACTORS

by

Padma Shanthi Jagadabhi, Prasad Kaparaju & Jukka Rintala 2011

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Two-stage anaerobic digestion of tomato, cucumber, common reed and grass silage in leach-bed reactors and upflow anaerobic sludge blanket reactors

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ABSTRACT

Anaerobic digestion of tomato, cucumber, common reed and grass silage was studied in four separate two-stage reactor configuration consisting of leach bed reactor (LBR) and upflow anaerobic sludge blanket reactor (UASB). LBR studies showed that COD solubilization for cucumber and grass silage was higher (50%) than tomato (35%) and common reed (15%). Results also showed that 31–39% of initial TKN present in tomato and cucumber was solubilized in the leachates and 47–54% of the solubilized TKN was converted to NH_4-N. The corresponding values for common reed and grass silage were 38–50% and 18–36%, respectively. Biomethanation of the leachates in UASB reactors resulted in methane yields of 0.03–0.14 m^3 CH_4 $kg^{-1}VS_{fed}$ for the studied crop materials. Thus, high COD solubilization, high nitrogen mineralization and solubilization rates were feasible during anaerobic digestion of lignocellulosic materials in a two-stage LBR–UASB reactor system.

1. Introduction

Anaerobic digestion (AD) of lignocellulosic biomass (agricultural crops, crop residues and aquatic plants) to produce biogas is a renewable and sustainable alternative to reduce fossil fuel consumption as well as CO_2 emissions (Cavinato et al., 2010). Among the agricultural crops, maize and grass–clover mixture are of the most commonly used feedstocks for biogas production, especially in central Europe, due to their high biomass yields per hectare (20–30 tons dry matter ha^{-1}). These crops are generally used either as fresh or as silage materials. The advantage of silage application is due to its ability to conserve the crop quality and thereby facilitate the possibility of year-round use of crops independent of crop season. In Finland, grass mixture consisting of timothy clover (75%) and meadow fescue (25%) is widely cultivated and ensiled for its year-round availability to serve as a fodder crop for the livestock. In addition to energy crops, crop residues obtained after crop harvest are the cheapest and most readily available organic and lignocellulosic sources of biomass for AD. The annual yields of lignocellulosic biomass residues were estimated to be more than 220 billion tons worldwide (Ren et al., 2009). Apart from the conventional agricultural sector, modern greenhouses, producing vegetables for commercial purpose, also produce about 40–60 tons ha^{-1} $year^{-1}$ of crop residues as a result of crop trimming and harvesting, which need to be disposed of properly (ODAF, 2004). In 2009, 70% of the total vegetable production from greenhouses in Finland comprised of tomato (350 tons ha^{-1} $year^{-1}$) and cucumber (120 tons ha^{-1} $year^{-1}$) crops (estimated from data of Matilda Agricultural Statistics, Finland, 2009). With proper harvesting practices, greenhouses can collect and utilize these crop residues for biogas production and in turn meet their own energy demands. In addition to lignocellulosic biomass from agriculture and greenhouses, aquatic grass species such as common reed, grown in temperate and tropical regions (Karunaratne et al., 2003), can also be harvested and used as biomass resource for biogas production. In Finland, common reed is grown in water bodies spread over 30 000 ha in southern Finland itself (Huhta, 2009). Extensive distribution and over-growth of common reed in water systems, especially in eutrophicated lakes, is considered to affect the quality of the water ecosystems, nutrient cycles and hydrological regimes (Huhta, 2009). Therefore, common reed with high biomass yield and nutrient content along with its abundance can be exploited for biogas production. Furthermore, the effluents produced from the AD of the lignocellulosic biomass can be utilized as liquid fertilizers for crop production and recycle the nutrients at the greenhouses/agricultural fields.

AD of crops with high total solids (TS) of 10–50% in one-stage reactor systems requires large quantities of water for homogenization thus increasing the volumes to be treated and the energy required for pumping, mixing and heating (Lehtomäki and Björnsson, 2006). Therefore, it is energetically and economically wasteful to treat crops/crop residues in conventional one-stage digesters (Andersson and Björnsson, 2002). Moreover, AD process cannot be optimized in one-stage reactor as the hydrolysis of complex polymeric substances is the rate-limiting step during the AD of

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lignocellulosic feedstocks (Lynd et al., 2002). In order to optimize AD of lignocellulosic feedstock, a two-stage process consisting of two separate reactors for hydrolysis and methanogenesis is recommended (Koppar and Pullammanapallil, 2008). In a two-stage reactor system, the solids are hydrolyzed in the first-stage reactor by re-circulation of liquid over a solid bed of crop materials. The liquid/leachate is then pumped to the second-stage reactor for further degradation (methanogenesis). The first-stage reactors can be a dry batch reactor such as leach bed reactor (LBR) while the second-stage reactor can be a high rate reactor such as an upflow anaerobic sludge blanket reactor (UASB) (Lehtomäki et al., 2008) or anaerobic filter (AF) (Cysneiros et al., 2008). In addition to reduced water consumption and waste water discharge, AD in LBR also enables increased volumetric methane yields when operated at high solids concentration (Lehtomäki et al., 2008).

AD of lignocellulosic materials in reactor configuration consisting of LBR coupled to UASB or AF reactor is relatively new and has been successfully studied at laboratory-scale and pilot-scale with substrates like grass silage, maize silage and spent sugar beet pulp (Lehtomäki and Björnsson, 2006; Lehtomäki et al., 2008; Cysneiros et al., 2008; Koppar and Pullammanappallil, 2008; Jagadabhi et al., 2010). Methane yields of 0.2–0.4 $m^3\ kg^{-1}$ volatile solids $(VS)_{fed}$ were reported in two different studies when grass silage, sugar beet and willow were subjected to AD in a two-stage reactor system consisting of LBR-MF (methanogenic filter) and LBR–UASB reactors (Lehtomäki and Björnsson, 2006; Lehtomäki et al., 2008). However, the effects of AD on the solubilization of nitrogen and conversion of the solubilized nitrogen to ammonium nitrogen are rarely reported. The digestate/effluent resulting from the two-stage reactor system would contain high quantities of plant accessible ammonium nitrogen which could serve as a very useful liquid fertilizer for crop production.

The aim of the present study was to investigate the suitability of tomato and cucumber crop residues generated in greenhouses along with common reed and grass silage for biogas production in a two-stage reactor system consisting of LBR and UASB reactor. Furthermore, nitrogen mineralization during the AD of lignocellulose was investigated with an aim to use the obtained digestate/effluents as liquid fertilizers. In addition, biological methane potential (BMP) assays were also performed to investigate the effect of different inocula and their mixing ratios on hydrolysis and methane yields of the studied substrates.

2. Methods

2.1. Origin of materials

Four different crop materials were used as substrates. Fresh leaves and stems (1–2 cm) of tomato (*Lycopersicon esculentum* L.) and cucumber (*Cucumis sativus* L.) were obtained from a greenhouse in South-West Finland (Härkälän puutarha). Common reed (*Phragmites australis*) was collected from a lake (Kirrinsanta, Pori) in South-West Finland. Grass silage was collected from a dairy farm (Kalmari Farm, Laukaa) located in Central Finland (Jagadabhi et al., 2010). Grass silage consisted of 75% timothy clover (*Phleum pratense* L.) and 25% of meadow fescue (*Festuca pratensis*). Grab samples of the prepared grass silage, stored at ambient conditions for about 6 months on the farm, were collected and used for the study. Upon arrival at the laboratory, crop materials were stored at −20 °C until further use. Frozen materials were thawed prior to experimental use. Common reed and grass silage were cut to a particle size of 2–5 cm prior to experimental use. The chemical composition of the crop materials is presented in Table 1.

Three different inocula were used in the study. For the batch experiments, digested material from a farm-scale biogas plant (Kalmari farm, Laukaa, Finland) treating cow manure, industrial confectionery waste and energy crops along with rumen fluid from a fistulized cow (MTT Agrifood research institute, Jokioinen, Finland) were used. For reactor experiments, granular sludge from a mesophilic UASB reactor treating wastewaters from an oats processing industry (Jokioinen, Finland) was used.

2.2. Batch experiments

BMPs for the studied substrates before (fresh substrates) and after AD from LBR experiments (residual materials) were determined in two parallel sets with two different inocula. In the first set, digested material from a farm-scale biogas plant was used as inoculum. For the second set, a mixture of rumen fluid (25% v/v) and the digested material from farm-scale biogas plant (75% v/v) was used.

BMPs were carried out in duplicate 1 L glass bottles with a liquid volume of 750 mL. To each assay, 250 mL of inoculum and substrate, at a $VS_{inoculum}$ to $VS_{substrate}$ ratio of 1, were added. Distilled water was added to make up the liquid volume of 750 mL. $NaHCO_3$ (3 g L^{-1}) was added as buffer in all the assays. Prepared assays were flushed with pure N_2 (98.8%) for about 3 min and incubated statically at 35 ± 1 °C. Assays with inoculum alone were used as controls. Methane production of the control assays was subtracted from those of the sample assays.

2.3. Reactor experiments

AD of tomato, cucumber, common reed and grass silage was carried out in four parallel two-stage reactor set-ups. Each reactor set-up consisted of LBR and UASB reactor connected through their respective reservoirs. Four glass UASB reactors, 3 of 500 mL capacity and 1 of 1 L capacity, were used. UASB reactors were started-up 10 days prior to the start-up of the LBRs (data not shown). During the start-up, UASB reactors were inoculated with granular sludge and fed with glucose solution (1.5 gL^{-1}) at an organic loading rate (OLR) of 1.5 g chemical oxygen demand (COD) $L^{-1}\ day^{-1}$. Feed was diluted with basic anaerobic medium as described in Raposo et al. (2006).

LBR experiments were conducted with four identical PVC columns of 10 L capacity. Each LBR was provided with a leachate collection system at the bottom. The leachate collection system consisted of a 0.5 cm thick perforated PVC plate (pore size < 1 mm), which was placed on an acrylic cylinder support system to withstand the biomass weight. On the top of the PVC plate, a layer of polyurethane foam (about 1 cm), three layers of nylon mesh (1 mm), a stainless steel mesh (2 mm pore size) and some glass beads were placed in order to facilitate proper leaching. Each LBR/UASB reactor was also provided with a gas collection/separation system. The biogas produced was collected using aluminum gas bags. LBRs were operated at room temperature (21 °C) throughout the experiment.

On day 0 (start of the experiment), each LBR was loaded with the respective crop material to full capacity. Tomato and cucumber LBRs were filled with 10.5 kg of tomato leaves and 9.615 kg of cucumber leaves, respectively. The low dry matter content (Table 1) in these materials facilitated prompt leaching and thus no water addition was necessary. Within minutes, 3.85 L and 8.5 L of leachates were collected from these tomato and cucumber LBRs, respectively. However, only 2 L of the collected leachate was used as feed for their respective UASB reactors. The remaining leachate was stored separately at 4 °C for future use. On the other hand, 2.3 kg of crop materials were loaded in grass silage and common reed LBRs, respectively, and 2 L of de-ionized water was added to initiate leaching in these two LBRs.

Table 1
Chemical characteristics of the crop materials before and after anaerobic digestion in LBRs.

	Tomato			Cucumber			Common reed			Grass silage		
	Fresh material	RM	RMLE	Fresh material	RM	RMLE	Fresh material	RM	RMLE	Fresh material	RM	RMLE
pH	5.1	5.5	5.5	7.1	6.4	6.4	4.3	6.6	6.6	3.9	5.1	5.2
TS (%)	10	9.9	22	6.8	5.4	19	44.3	23.8	26	41	16.1	20
VS (%)	7.6	7.5	18	4.5	3.7	13	41	21.2	24	39	15.7	19
NH_4-N (g kg^{-1} TS)	0.25	0.62	8.6	0.15	0.47	6.1	0.05	0.12	0.34	0.25	0.39	0.67
N_{tot} (g kg^{-1} TS)	32.5	36.6	40	27.3	32	42	8.9	10.3	12	17	21.6	18
Cellulose (% TS)	12.5	NA	8.5	9.1	NA	4.2	34	NA	33	32	NA	25.6
Hemicellulose (% TS)	7.9	NA	4.1	4.9	NA	1.3	32	NA	31	24	NA	19.3
Lignin (% TS)	1.4	NA	1.2	1.1	NA	0.47	5.2	NA	5.0	3.6	NA	2.8

RM: residual materials obtained at the end of LBR experiment.
RMLE: residual materials obtained after leachate extraction by overnight pressing with weights.
NA: not analyzed.

LBRs were sealed with PVC lids and anaerobic conditions were created by flushing the reactors with pure N_2 gas. Each LBR was provided with a reservoir (2 L glass bottle) for leachate collection. From days 1 to 6, leachate from each LBR was fed directly to their respective UASB reactor. Effluents from the UASB reactors were then returned to their respective LBRs. However, low biogas production and process failure in UASB reactors on day 7 prompted to operate the two-stage reactor system as two separate one-stage processes. From day 7 onwards, LBRs were operated as one-stage process with an internal re-circulation rate of 2 L day^{-1}. On the other hand, the effluent from UASB reactors were collected separately and fed to LBR. On day 10, leachates from all the LBRs were removed (stored at 4 °C) and 2 L of fresh de-ionized water was added to each LBR to facilitate further COD solubilization. In addition, the added water was allowed to stand over in the LBRs for 36 h before internal re-circulation was resumed at a higher rate of 4 L day^{-1}.

At the same time, UASB reactors were re-inoculated with 150–250 mL more sludge and operated as one-stage process with internal re-circulation (1 L for 1 L UASB and 0.5 L for 0.5 L UASB day^{-1}). Reactors were operated with an initial OLR of 1.5 gCOD L^{-1} day^{-1} (day 7) and further increased to 3 gCOD L^{-1} day^{-1} (days 13–29). To achieve the OLRs of 1.5 or 3 gCOD L^{-1} day^{-1}, leachate volumes equivalent to 1.5 or 3 gCOD L^{-1} were withdrawn from the respective LBRs and diluted with de-ionized water. From day 13 onwards, leachate volume equivalent to 3 gCOD L^{-1} was withdrawn from each LBR and an equivalent amount of effluent from UASB reactor was added to the respective LBR. This process continued for the rest of the experimental period. Thereby, no extra water was introduced into the system. Upon stable gas production, the OLR was increased from 3 to 5–7 gCOD L^{-1} day^{-1} (days 29–89). Finally, process temperature was increased from room temperature (21 °C) to 37 °C on day 19.

On day 31, LBR experiments were terminated and the residual materials were pressed manually and subsequently with weights (overnight) over a nylon mesh to extract the remaining leachate. About 2 L of leachate was extracted from the tomato and cucumber residual materials. The corresponding values for common reed and grass silage were 25–50 mL, respectively. The collected leachates were stored separately at 4 °C. The residual crop materials before (RM) and after extracting the remaining leachate through manual pressing and by overnight weight (RMLE) were also stored at 4 °C until further use. Residual methane potentials of RMLE were determined as described in Section 2.2. At the same time, the remaining untreated leachates and the effluents from UASB reactors (stored at 4 °C) were mixed and fed to UASB reactors (day 42 onwards). In total, the amount of leachate available for UASB reactors was 14 L for cucumber, 9 L for tomato, 3.1 L for grass silage and 3.4 L for common reed. However, only 6.6 L of cucumber and 5.5 L of tomato leachates were used for biomethanation in UASB reactors while for grass silage and common reed leachates available were completely fed.

2.4. Analyses and calculations

For chemical analyses, a sample volume of 25–50 mL leachate from LBR and 50 mL of effluent from UASB reactor was collected. This removed sample volume was not replaced with fresh water. TS and VS were determined according to the Standard Methods (APHA, 1998). pH was measured with a Metrohm 774 pH meter. COD, soluble (SCOD) and total (TCOD), were analyzed according to Finnish Standards (SFS 5504; SFS Standards, 1988). NH_4-N and total nitrogen (TKN) were determined using a Kjeltec system 1002 distilling unit (Tecator AB) as described elsewhere (Lehtomäki et al., 2008). Samples for SCOD and NH_4-N were filtered using GF 50 glass fiber filter papers (Schleicher & Schuell). Methane and hydrogen contents in the biogas were analyzed using a gas chromatograph (Perkin Elmer Clarus 500 GC with thermal conductivity detector and Supelco Carboxen™ 1010 PLOT fused silica capillary column 30 m × 0.53 mm) as described by Lehtomäki et al. (2008). Volatile fatty acids (VFAs) were analyzed with gas chromatograph (PE Autosystem XL GC equipped with flame-ionization detector and PE FFAP column 30 m × 0.32 mm) as described by Lehtomäki et al. (2008). Biogas volume was measured by water displacement method.

In batch assays, specific methane yields were calculated as cumulative methane produced per kg of added substrate VS (m^3 CH_4 kg^{-1} VS_{added}). Specific SCOD (gCOD g^{-1} VS_{added}), NH_4-N (mg NH_4-N g^{-1} VS_{added}), and TKN (mg TKN g^{-1} VS_{added}) production in each LBR was calculated by considering the total leachate produced (including the leachate extracted by manual and overnight pressing with weights) and the sample volumes removed during the operation of LBRs. Methane yields from the two-stage system consisting of LBR + UASB reactors were calculated as :

$$\text{Methane yields } (m^3\ CH_4\ kg^{-1}\ VS_{fed}) = \frac{\text{Total methane produced (L)}}{\text{Total COD fed to UASB reactor (gCOD)}} \times \frac{\text{Total COD leached from Leachbed reactor (gCOD)}}{\text{Initials VS of substrate in Leachbed reactor (kgVS)}}$$

3. Results and discussion

3.1. Substrate characteristics

The characteristics of the studied substrates are shown in Table 1. Common reed and grass silage had higher solids content than tomato and cucumber. The VS contents of common reed

and grass silage were 39% and 41%, respectively. The corresponding values for tomato and cucumber were 7.6% and 4.5%, respectively. On a dry weight basis, tomato and cucumber had higher TKN compared to common reed and grass silage. All substrates except cucumber had low initial pH of 3.9–5.08 and <1% NH_4-N content. This low initial pH and NH_4-N suggests that the hydrolysis of the studied materials would be promoted as the optimum pH for the hydrolysis of lignocellulosic materials is 4–6 (Veeken et al., 2000).

3.2. Batch experiments

The effect of inoculum type and their mixture ratio on the methane potential of the fresh and residual crop materials (after AD in LBRs and pressing for leachate extraction) was evaluated in batch assays at 35 °C. Results are presented in Table 2. When digested material from a farm-scale biogas plant was used as inoculum, methane yields of 0.26–0.36 $m^3\ kg^{-1}\ VS_{added}$ were obtained from the fresh substrates, whereas mixed results were obtained with the residual materials. For instance, methane yields obtained from the residual materials of cucumber and tomato were 0.29–0.32 $m^3\ kg^{-1}\ VS_{added}$. The similar or slightly higher methane yields obtained for residual materials compared to fresh materials of cucumber and tomato was due to the fact that hydrolysis of the particulate matter was improved in the residual materials after AD in LBR. On the other hand, the lower methane yields obtained from the residual materials of grass silage and common reed (0.10–0.32 $m^3\ kg^{-1}\ VS_{added}$) than their fresh materials was clearly due to low degradability of the final VS (Table 3).

Use of mixed inoculum (75% digested material from farm-scale biogas plant and 25% rumen fluid) resulted in methane yields of 0.22–0.36 $m^3\ kg^{-1}\ VS_{added}$ for the fresh crop materials. These methane yields were similar (tomato and grass silage) or slightly lower (cucumber and common reed) than the yields obtained when inoculum from farm-scale biogas plant was used (Table 2). The difference in response to mixed inoculum among the studied crop materials was however attributed to the difference in the chemical composition of the materials. Substrates with high amount of matrix polysaccharides (hemicelluloses and pectin) and lignin content were shown to result in low methane yields as hemicellulose and pectin digestion are influenced by type and amount of lignin content (Dehority et al., 1962; Benner et al., 1984). Moreover, lignin in higher plants has shown to severely affect the degradation rates of the associated polysaccharides by forming a barrier between plant hemicelluloses/pectin and the rumen bacteria (Dehority et al., 1962). On the other hand, use of mixed inoculum improved the methane yields from residual materials of grass silage (by 16%) and common reed (by 40%) but not from cucumber and tomato. The probable reason for this difference could be that high rates of biodegradation and solubilization of lignin and hemicellulose occurred during the AD of grass silage and common reed in the LBR. The removal of hemicelluloses and lignin may have improved the accessibility of cellulose by bacteria, especially rumen bacteria, leading to a more efficient cellulose hydrolysis. Several studies have shown that during AD, lignin is removed more efficiently due to solubilization than degradation (Kivaisi et al., 1990).

Table 2
Methane potentials from fresh and residual crop materials obtained after anaerobic digestion in LBRs (RMLE).

Materials	Methane yields ($m^3\ CH_4\ kg^{-1}\ VS_{added}$)			
	Inoculum from biogas plant		Inoculum mixture: biogas plant (75%) and rumen fluid (25%)	
Inoculum	0.04 ± 0.00		0.06 ± 0.03	
	Fresh substrates	RMLE[a]	Fresh substrates	Residual material[a]
Cucumber	0.26 ± 0.006	0.29 ± 0.001	0.24 ± 0.05	0.23 ± 0.016
Common reed	0.26 ± 0.008	0.10 ± 0.016	0.22 ± 0.013	0.17 ± 0.092
Tomato	0.32 ± 0.001	0.32 ± 0.009	0.32 ± 0.029	0.26 ± 0.143
Grass silage	0.36 ± 0.06	0.26 ± 0.1	0.36 ± 0.2	0.31 ± 0.054

[a] RMLE: residual materials obtained after leachate extraction by overnight pressing with weights.

3.3. Reactor experiments

3.3.1. LBR experiments

LBRs were operated for 31 days with an aim to improve the hydrolysis and COD solubilization of lignocellulosic materials. After the initial loading, leachate production started immediately in all LBRs. However, the amount of leachate produced and the leachate production rate varied depending upon the substrate characteristics.

3.3.2. pH and SCOD production

The process performance of the LBRs is presented in Fig. 1. During the first 7 days, LBRs were operated in conjunction with UASB reactors. In all LBRs, pH values of the leachate fluctuated throughout the experiment (Fig. 1). The initial pH values of grass silage (3.9–6.2), tomato (5.1–6.9) and common reed (4–6.6) were low to near neutral compared to that of cucumber (6.3–7.8) indicating a better buffering capacity in the latter LBR. SCOD production started immediately in all LBRs and maximum SCOD concentration of 14–38 $g\ L^{-1}$ was noticed on day 10 (Fig. 1). These SCOD levels however decreased when the leachate was replaced with water on day 10, but increased thereafter to reach 15–47 $g\ L^{-1}$ in the end. The maximum SCOD values obtained at the end of the experiment were 47 $g\ L^{-1}$ for grass silage, 27 $g\ L^{-1}$ for tomato, 18 $g\ L^{-1}$ for cucumber and 15 $g\ L^{-1}$ for common reed.

Generally, under anaerobic conditions, tomato with an initial pH of 5.1 should show better hydrolysis and higher COD solubilization rate than cucumber (pH 7.1) as the optimal pH for hydrolysis is 4–6 (Veeken et al., 2000). However, low pH in tomato LBR did not seem to promote further hydrolysis and COD solubilization. Nevertheless, a sharp increase (65%) in COD solubilization in tomato LBR upon leachate replacement (day 20) suggests that dilution of the saturated leachate with fresh water had enabled further

Table 3
Initial and final weights along with the total solids (TS) and volatile solids (VS) removals during the anaerobic digestion of crop materials in LBR experiments.

Crop material	Amount of mass (kg, w/w)			Total solids (kg)		Volatile solids (kg)		TS removal (%)	VS removal (%)
	Fresh material	RM	RMLE	Fresh material	RM	Fresh material	RMLE		
Cucumber	9.62	5.4	3.0	0.65	0.29	0.43	0.20	55.5	54
Common reed	2.3	3.9	3.85	0.02	0.93	0.86	0.82	8.9	7.7
Tomato	10.5	5.5	3.5	1.05	0.54	0.79	0.41	48	47
Grass silage	2.3	3.9	3.75	0.94	0.62	0.89	0.61	33.4	31.6

RM: residual materials obtained at the end of LBR experiment.
RMLE: residual materials obtained after leachate extraction by overnight pressing with weights.

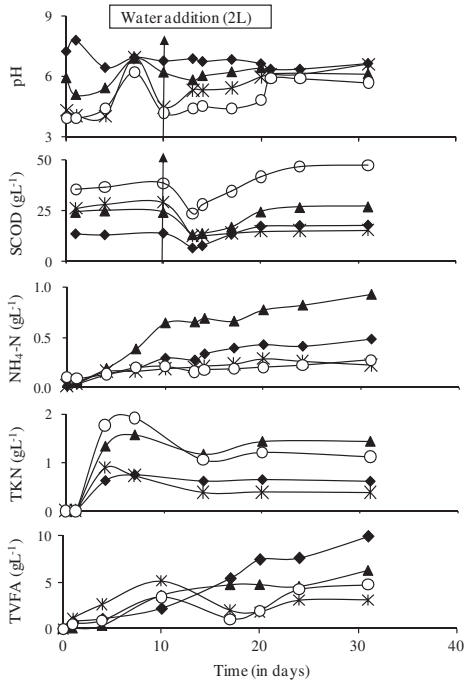

Fig. 1. pH, COD, NH_4-N, TKN and TVFA concentrations in the leachates during the anaerobic digestion of (♦) cucumber, (✳) common reed, (▲) tomato and (○) grass silage in LBR.

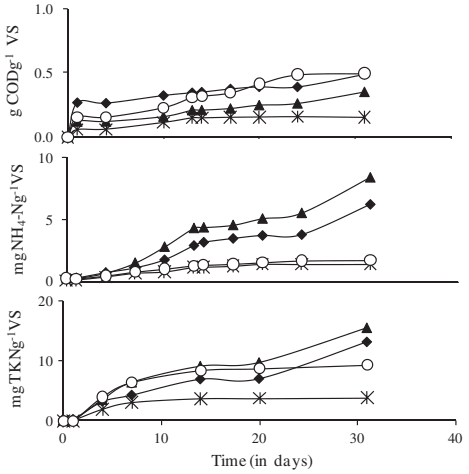

Fig. 2. Specific COD, NH_4-N and TKN production in the leachates during the anaerobic digestion of (♦) cucumber, (✳) common reed, (▲) tomato and (○) grass silage in LBR.

COD solubilization. Previously, Jagadabhi et al. (2010) reported the importance of leachate replacement with fresh (distilled) water on COD solubilization of grass silage in LBRs. In that study, leachate replacement on day 11 resulted in about 66–85% of COD solubilization compared to 32% obtained in the control reactor. This confirms that, during the first 10 days of the LBR operation, rapid hydrolysis resulting in saturation of organic substances takes place and leachate replacement will prevent accumulation of these organic substances and thus enable further hydrolysis and COD solubilization.

3.3.3. Specific SCOD production

The specific SCOD production (gSCOD g^{-1} VS_{added}) in the LBRs is presented in Fig. 2. The solubilization effect, i.e. the amount of SCOD leached from the substrate was low during the first 10 days (0.11–0.32 gCOD g^{-1} VS_{added}). However, leachate replacement (day 10) improved further hydrolysis and COD solubilization. The increase in SCOD solubilization upon leachate replacement was 65% for tomato, 34% for grass silage and cucumber and 23% for common reed. At the end of the experiment the specific SCOD solubilization was 0.5 gCOD g^{-1} VS_{added} for grass silage and cucumber, respectively. The corresponding values for tomato and common reed were 0.35 and 0.15 gCOD g^{-1} VS_{added}, respectively. The higher specific SCOD solubilization noticed for cucumber compared to tomato or common reed was attributed due to the difference in the chemical composition of these materials. For instance, cucumber had lower cellulose content (9.1% TS) than tomato (12.5%) and common reed (34% TS) (Table 1). Moreover, crushing of the cucumber plant material, carried out at the production center in order to facilitate packing and transport, might have also facilitated hydrolysis and thus COD solubilization through particle size reduction, enhanced surface area and reduction in cellulose crystallinity (Taherzadeh and Karimi 2008). On the other hand, the relatively high specific SCOD solubilization noticed for grass silage was due to the fact that ensiled grass contained high amount of readily available carbohydrates and low pH ideal for hydrolysis. In general, ensiling of energy crops such as grass or maize will result in an increase in the water soluble carbohydrate content from the degradation of carbohydrates by the lactic acid bacteria. The produced lactic acid will eventually inhibit further degradation of the carbohydrates by other bacteria and thereby preserve the nutritional value of the crops (Lehtomäki et al., 2008). In addition, the low pH (3.9), as a result of ensiling and particle size reduction (2–5 cm) prior to loading into the LBR, may have also promoted hydrolysis and COD solubilization in grass silage. A similar specific SCOD production of 0.5 gSCOD g^{-1} VS_{added} was reported for grass silage by Jagadabhi et al. (2010). In the above study, the authors also reported a 30% increase in the solubilization of grass silage in LBR subjected to leachate replacement (day 11). On the other hand, the increase in COD solubilization with grass silage upon leachate replacement in the present study was 34% (day 10). The lowest COD solubilization obtained for common reed was mainly attributed to the recalcitrant chemical nature of the substrate which was indicated by its highest lignin content (5.2% TS) than the other crop materials studied (Table 1). The maximum increase in the COD solubilization that could be obtained with common reed was only 23% and that too after leachate replacement (days 10–32). Chemical analyses further revealed that common reed contained high amounts of crude fiber, crude fat, starch and sugar (data not shown) than the other three studied materials. Apparently, the high TS and lignin content could be due to late harvesting of common reed (August).

3.3.4. Solids destruction

The solids destruction and degradation of the studied substrates in the LBR is presented in Table 3. The overall VS destruction for to-

mato and cucumber at the end of the LBR experiment was 48% and 54%, respectively. The corresponding values for common reed and grass silage were 8% and 31%, respectively. Lehtomäki et al. (2008) reported VS removals of 34% and 55% for grass silage for one-stage (LBR) and two-stage (LBR–UASB) processes, respectively. The low VS removals in the present study could be attributed to the differences in the substrate characteristics, harvest time, pretreatment methods and/or reactor operation. For instance, the solid to liquid ratio used in LBR and LBR + UASB reactor experiments of Lehtomäki et al. (2008) was 0.26 compared to 0.5 used in the present study. Therefore, lower solubilization efficiency noticed for grass silage and common reed in the present study could be mainly due to high solids and low moisture content in these LBRs. On the other hand, extraction of the leachate from the residual materials (RMLE) resulted in an increase in solids content for tomato and cucumber but not for common reed and grass silage (Table 1). This is probably due to high amount of matrix polysaccharides (hemicelluloses and pectin) in non-grass plants such as cucumber and tomato than in common reed and grass silage (Carpita, 1996). It is presumed that the hydration gelling properties of the cell wall is influenced by matrix polysaccharides especially pectin, which acts as a hydrophilic filler to prevent aggregation and collapse of the cellulose network (Jarvis, 1992) and modulate the porosity of the cell wall to macromolecules (Baron-Epel et al., 1988).

3.3.5. VFA production

VFA production profile is presented in Fig. 1. In all LBRs, VFA production started immediately from an initial value of 0.33–2.63 g L^{-1} on day 1 to reach a maximum value of 2–5 g L^{-1} on day 10. Highest VFA concentrations were noticed in the leachate of common reed (5.2 g L^{-1}) followed by tomato (3.6 g L^{-1}), grass silage (3.5 g L^{-1}) and cucumber (2.2 g L^{-1}). The steady increase in VFA and SCOD levels along with a decrease in pH in all LBRs during the first 10 days indicate the rapid hydrolysis or solubilization of organic material. However, this trend changed upon leachate replacement as the produced VFAs and SCOD were diluted with the addition of fresh water. Nevertheless, the increase in VFA levels noticed for cucumber and tomato but not for grass silage and common reed upon leachate replacement indicates the resumption of hydrolysis and COD solubilization. In the end, highest VFA concentrations were noticed in cucumber LBR (9.9 g L^{-1}) followed by tomato (6.3 g L^{-1}), grass silage (4.7 g L^{-1}) and common reed LBRs (3 g L^{-1}). The probable reason for this variation could be attributed to the difference in the chemical composition of the studied substrates. Cucumber and tomato appeared to be easier materials to degrade than grass silage and common reed. The easily degradable fraction of the substrate was most probably exhausted around day 10 in the case of cucumber and tomato. A similar result in the build-up of VFAs and lactate during the first 10 days was reported during the AD of sugar beet tops and grass silage (Cirne et al., 2007; Jagadabhi et al., 2010). Both these results indicate that rapid hydrolysis and acidification process generally occur during the first 10 days of the substrate degradation.

3.3.6. TKN and NH_4-N production

TKN and NH_4-N concentration in the leachate is shown in Fig. 1. In all LBRs, TKN values of the leachate increased sharply from the initial value of 0.01–0.05 g L^{-1} to 0.75–1.92 g L^{-1} on day 4. Thereafter, TKN concentration decreased steadily to reach the lowest values on day 14 and remained more or less unchanged till the end of the experiment. Among the substrates, highest TKN concentrations were noticed in the leachates of tomato and grass silage compared with cucumber and common reed. The high initial TKN solubilization noticed in the leachates of tomato and cucumber compared to common reed and grass silage could be attributed to various factors influencing protein degradation such as pH, temperature, dry matter content, inhibitory compounds (Slottner and Bertilsson, 2006), hydraulic retention time (HRT) (Gallert and Winter, 1997; Jokela and Rintala, 2003) and water addition during AD (Jokela and Rintala, 2003). For instance, some forage species with high dry matter content showed less proteolysis than species with low dry matter content (Muck and Shinners, 2001). In the present study, the dry matter content in common reed (44%) and grass silage (41%) was higher than that of cucumber (6.8%) and tomato (10%). The initial TKN solubilization (31–50%) obtained in the present study for the four crop materials was in agreement with the nitrogen solubilization of 30–50% of TKN reported by Jokela and Rintala (2003) for putrescibles. The higher initial TKN solubilization noticed in the leachate of grass silage than in the other three plant materials (Table 4) was probably due to high protein content in grass silage. Lehtomäki et al. (2008) reported that during AD of grass silage in an LBR, proteins were the most rapidly hydrolysable components after 1 day of incubation.

The NH_4-N concentration in the leachates increased more sharply for tomato from a very low level of 0.05–0.65 g L^{-1} (day 13) to a maximum concentration of 0.93 g L^{-1} at the end of the study (day 31). A similar trend was also noticed in cucumber LBR but with a maximum NH_4-N concentration of 0.48 g L^{-1}. On the other hand, the NH_4-N concentration in the leachates of grass silage and common reed remained more or less unchanged throughout the experiment. The higher NH_4-N solubilization in tomato and cucumber (54% and 47%, respectively) could be due to better proteolysis occurring at a higher pH (6–7) than that noticed for grass silage and common reed (3.9–5). In fact, lowering of pH is the key principle in ensiling technology to preserve the nutritive value of the crop for a longer period. Therefore, low pH (5.5) conditions in grass silage and common reed could have inhibited the growth of protein degrading bacteria (Slottner and Bertilsson, 2006) and thus proteolysis. Higher NH_4-N solubilization obtained for tomato and cucumber in the present study are in agreement to TKN to NH_4-N conversion efficiency of 50% reported for biowaste (Gallert and Winter, 1997) and 50–70% reported for poultry and slaughterhouse wastes (Salminen, 2001). These results are in accord to previous studies reported in the literature. Lehtomäki and Björnsson (2006) reported about 30% and 87% of NH_4-N conversion in the liquid digestates during AD of grass silage and sugar beet, respectively and stated that higher NH_4-N in liquid fraction was beneficial when considering the use of liquid fraction as fertilizer since it will be easy for storage purposes and also for spreading onto the fields.

The specific TKN and NH_4-N extraction results are presented in Fig. 2. The specific TKN and NH_4-N extraction from the studied substrates showed somewhat different trend to that of COD solubilization trend. Results showed that leachate replacement improved the TKN extraction in tomato and cucumber but not for grass silage

Table 4
TKN and NH_4-N extracted into the leachates during the anaerobic digestion of crop materials in LBR.

	Substrate (mg g^{-1}TS)	Leachate (mg g^{-1}TS)	TKN extracted into leachate (%)
TKN			
Cucumber	41	13	31
Common reed	9.6	3.8	38
Tomato	42	15.4	39
Grass silage	18.3	9.2	50
NH_4-N			
Cucumber	0.22	6.2	47
Common reed	0.05	1.37	36
Tomato	0.32	8.4	54
Grass silage	0.27	1.7	18

and common reed. The specific TKN extraction values for tomato and cucumber were 13–15.5 mg g^{-1} VS_{added}. The corresponding values for grass silage and common reed were 9.3 and 3.9 mg g^{-1} VS_{added}, respectively. Nevertheless, NH_4-N extraction followed the same trend as that of TKN extraction (Fig. 2). Leachate replacement improved the NH_4-N extraction for cucumber and tomato (from 1.7 to 3.1 g L^{-1} and from 2.8 to 4.3 g L^{-1}, respectively) but not for common reed and grass silage. Among the four substrates, cucumber had the highest NH_4-N extraction (8.4 mg g^{-1} VS_{added}) followed by tomato (6.2 mg g^{-1} VS_{added}). On the contrary, NH_4-N extraction in the leachates of common reed and grass silage remained low (1.7 mg g^{-1} VS_{added}) throughout the experiment. Further, Table 4 shows the amount of the initial TKN extracted into the leachate and the conversion of the extracted TKN into NH_4-N. Grass silage had the highest TKN solubilization (50% of initial TKN was solubilized into the leachate) followed by tomato, common reed and cucumber. On the other hand, tomato showed the highest conversion of the TKN into NH_4-N (54%) followed by cucumber, common reed and grass silage.

3.3.7. UASB reactor experiments

Process performance of the UASB reactors is presented in Fig 3. For the first 7 days, UASB reactors were coupled with the LBRs and operated at room temperature (21 °C). Use of low pH leachate (4–6.4) and high SCOD for biomethanation resulted in low methane production (<50 mL CH_4 day^{-1}) and unstable process (pH < 4) in all UASB reactors. Thus, UASB reactors were decoupled from LBRs and operated as one-stage process (day 7). This led to an improved process performance and methane production in all UASB reactors. However, a shift in process temperature from 21 °C to 37 °C (day 19) further improved the methane production in all UASB reactors. This was evident through an increased pH (from < 6 to 7.2–7.8, decreased TVFA concentration (from 5 to 0.14–2 g L^{-1}) and high COD removal efficiency (80%).

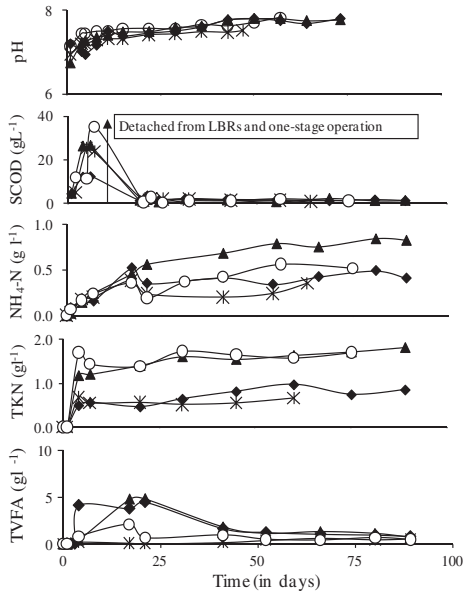

Fig. 3. pH, NH_4-N, TKN and TVFA concentrations in the effluents during the anaerobic digestion of (♦) cucumber, (✳) common reed, (▲) tomato and (○) grass silage in UASB.

The NH_4-N and TKN concentrations in the effluents are shown in Fig. 3. Tomato effluent had comparatively higher NH_4-N content (0.5–0.8 g L^{-1}) than cucumber, common reed and grass silage (0.3–0.05 g L^{-1}). However, TKN levels in all reactors appeared to be more or less stable and remained at 1–2 g L^{-1} throughout the experiment. These results in practice suggest that the NH_4-N rich effluents of tomato and cucumber could be used as liquid fertilizers for growing the same or different crops in the greenhouses or on agricultural lands. As NH_4-N is the preferred form of nitrogen for crop uptake (Demuynck, 1984), use of the digestate, obtained after a two-stage process, as liquid fertilizer will not only reduce the need of inorganic fertilizers but also recycle the nutrients and thus close the energy and nutrient cycles within agricultural systems and promote sustainability. On the other hand, effluents of grass silage and common reed probably need further treatment prior to their use as liquid fertilizers.

3.3.8. Methane yields from the two-stage AD of crops in LBR–UASB reactors

AD of tomato and cucumber in the two-stage system consisting of LBR–UASB reactors resulted in methane yields of 0.09 and 0.13 m^3 CH_4 kg^{-1} VS_{fed}, respectively. These yields were 28% and 50% of those obtained in the BMP assays and are in agreement to the obtained SCOD solubilization rates for these two crop materials (Fig. 2). However, the methane yields obtained in the present study were lower than those reported for fruit and vegetable wastes (0.34–0.38 m^3 CH_4 kg^{-1} VS_{added}) in a two-stage system consisting of LBR–UASB/AF (Martinez-Viturtia and Mata-Alvarez, 1989). On the other hand, methane yields of 0.14 and 0.034 m^3 CH_4 kg^{-1} VS_{fed} were obtained for grass silage and common reed, respectively. These yields were 38% and 13% of the yields obtained in the BMP assays and also reflect the SCOD solubilization rates of these two materials. However, methane yields obtained for grass silage in the present study were lower than the yields of 0.19 m^3 CH_4 kg^{-1} VS_{added} reported in a two-stage system consisting of LBR–UASB reactors (Lehtomäki et al., 2008). The low methane yields obtained from the studied crop materials in the present study could be attributed to various factors such as inefficient substrate VS solubilization in the LBR, differences in substrate composition, cellulose/lignin content, and inefficiency of the UASB reactor inoculum.

The methane yields obtained in the present study from the two-stage system (LBR–UASB reactors) were further compared with the methane yields of one-stage reactor systems reported in the literature. For example, methane yields of 0.13–0.26 m^3 CH_4 kg^{-1} VS_{added} were reported during co-digestion of cow manure and energy crops and crop residues such as sugar beet tops, grass silage and straw (up to 40% feedstock VS) in one-stage CSTR systems (Lehtomäki et al., 2007) or 0.2–0.4 m^3 CH_4 kg^{-1} VS_{added} for vegetable wastes in BMP assays (Gunaseelan, 2004). When compared to the above two studies, the methane yields obtained for tomato and common reed in the present study were low (0. 09 and 0.03 m^3 CH_4 kg^{-1} VS_{added}) indicating an inefficient AD of tomato and common reed in the two-stage system. On the other hand, cucumber and grass silage showed comparable methane yields (0.13–0.14 m^3 CH_4 kg^{-1} VS_{added}) indicating a possibility for successful application of a two-stage system rather than one-stage system for these crop materials.

4. Conclusions

The present study showed that AD of common reed, grass silage, cucumber and tomato can be successfully performed in a two-

stage reactor system consisting of LBR and UASB reactor. LBR promoted the hydrolysis and acidogenesis and resulted in a maximum of 50% COD solubilization efficiency and 54% ammonification of the initial TKN extracted from the studied substrates. On the other hand, UASB reactors facilitated efficient methanogenesis with about 80% of COD removal efficiency. The NH_4-N rich effluents from UASB reactors could be used as liquid fertilizers in crop production.

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