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2017

Document Version:
Publisher's PDF, also known as Version of record

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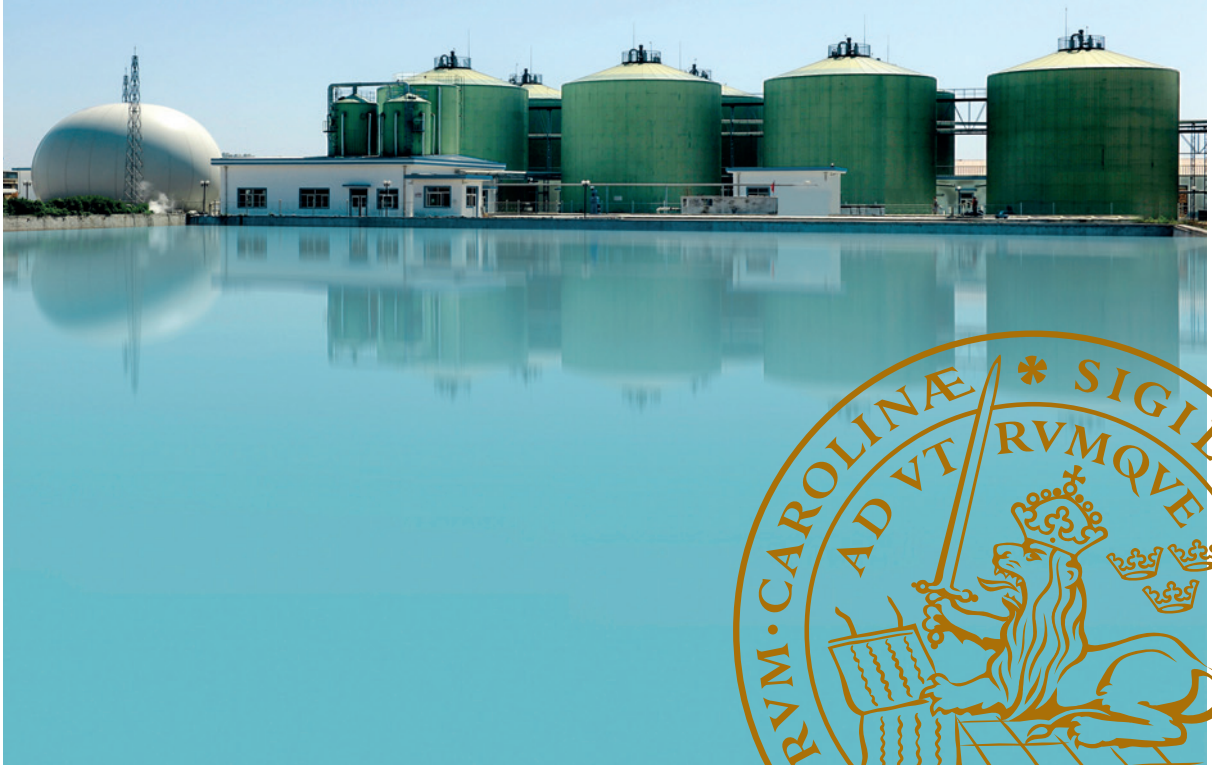

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LUND UNIVERSITY

DOCTORAL DISSERTATION
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To be defended at the Center for Chemistry and Chemical Engineering. Date 27th of September 2017 and time 09.00.

Faculty opponent
Associate Prof. Hinrich Uellendahl, Department of Chemistry and Bioscience, Ålborg University Copenhagen AAU-CPH, Denmark

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
	Date of issue 2017-09-01
Author(s)	Sponsoring organization
Chao Li	

Title and subtitle

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Abstract

Biogas or methane production through anaerobic digestion (AD) is gaining increasing attention worldwide due to concerns over global warming, energy security and the need for sustainable waste management. AD of lignocellulosic biomass is one facet that is highly appreciated since the conflict over biomass for food/feed or energy can be avoided. As a result the need for non-food based lignocellulosic biomass feedstock has emerged as (co-) feedstock of choice for the AD process. Despite these advantages, lignocellulosic biomasses are generally viewed as recalcitrant to hydrolysis, laden with insoluble lignin, poor in essential nutrients, and unmanageable in conventional wet/liquid AD processes i.e. may float in continuous stirred tank reactors.

This thesis presents the feasibility of using lignocellulosic biomasses (Miscanthus sp. and seagrass) as feedstock for biogas production via AD. Various operations (pre-treatment, co-digestion, harvest time management and inoculation optimization) were adopted and investigated as means to accumulate both experience and knowledge for evaluation and suitable utilization of the abundant lignocellulosic biomass. Furthermore, the use of solid-state anaerobic digestion (SS-AD) was investigated by means of biogas production from lignocellulosic biomass. Specifically, a sequential aerobic (pre-treatment) and SS-AD process together with liquor supplementation was studied as means to improve performance and recovery of inhibited/failed SS-AD processes.

Results of the experiment showed that cellulose degradability in different species of Miscathus decreased with longer cultivation period or age (later cut). This was inversely proportion to the methane yields at early harvest (2 months) ranged from 247 to 266 ml CH_4/g VS which were noted to decrease with as much as 35% when the late harvest was carried out. It was demonstrated that when using Miscanthus as feedstock, pre-treatment, especially steam explosion, could lead to approximately 50% increase in methane yield. In addition, aerobic pre-treatment of rice straw at 35°C for 2 days was shown to be a viable method to improve hydrolysis and decomposition of lignocellulosic structure and therefore resulted in highest biochemical methane potential (BMP) (355.3 ± 18.7 ml CH_4/gVS).

Co-digestion of Miscathus and manure, and sea grass and manure was demonstrated to ameliorate the nutrient content, especially the carbon to nitrogen ratio, in the AD process. The co-digestion of Miscanthus or seagrass with manure demonstrated to show a synergistic effect with 11 to 34% higher methane yields as compared to values obtained from the mono-digestion.

The SS-AD proved to be feasible in the treatment of rice straw as biomethane production reached over 70% of the biochemical methane potential especially after supplementation (of the inoculum) with recycled water. In fact, the addition of recycled water could improve the buffering capacity and mitigate the accumulation of toxic intermediates such as volatile fatty acids which could have led to an improved process performance and greater stability. In a related mesophilic and thermophilic SS-AD study, liquor (recycled water and sludge supernatant) was demonstrated to alleviate process inhibition or failure. The liquor supplementation minimized inhibition and boosts the SS-AD process performance as evident by the doubling of the methane yield under the mesophilic conditions.

Making the lignocellulosic biomass more accessible to anaerobic consortium (appropriate harvest time, pre-treatment and co-digestion), treatment of the inoculum in the SS-AD process and the sequential aerobic and batch SS-AD process were therefore demonstrated as viable means to improve methane production from lignocellulosic biomass.

Key words

Anaerobic digestion; Lignocellulosic biomass; Pretreatment; Co-digestion; Inoculation

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language English
ISSN and key title		ISBN 978-91-7422-540-2
Recipient's notes	Number of pages 138	Price
	Security classification	

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Cover: First phase of Minhe biogas plant

Photo by: Shandong Minhe Biological Sci-tech Co. Ltd

Division of Biotechnology
Faculty of Engineering
Lund University

ISBN 978-91-7422-540-2 (printed)
ISBN 978-91-7422-541-9 (pdf)
ISRN LUTKDH / TKBT - 17 / 1168 - SE

Printed in Sweden by Media-Tryck, Lund University
Lund 2017

“There is nothing more powerful than an idea whose time has come”. *-Victor Hugo*

“最強而有力的莫過於時機成熟的理念”- 維克多·雨果

Content

Acknowledgement

In 2008, just a few days after I graduated with my Master's degree from the School of Economics and Management at Lund University, I moved, alone, to Beijing to work as a country manager for Bioprocess Control AB. When my business reached a stable point in 2012, it marked the milestone at which many young entrepreneurs like myself begin to develop their plans for the future. Faced with the difficulties of explaining the technical side of my business to my customers, I was beginning to think it was time to return to Sweden to pursue a PhD.

As the co-founder of a small environmental engineering company in China, it would be hard to do without the decade that I put into learning the technology through a PhD and then a Postdoc. I found that like experimental methods, data analysis, assessment, process optimization, etc., could only be learned by doing them over and over again. It is not something you can learn in a 6-week class.

I have long wondered why most Swedish biogas projects are more successful than projects in my home country. The answer to this question is very complex and broad but two important factors are: i) differences in the fundamental understanding of the science, including the mechanisms of action, behind the hypotheses to be tested, and ii) varying degrees of appreciation of the importance of collaborating across disciplines and the ability to build networks and partnerships outside your normal environment. Accordingly, I wish to acknowledge Dr. **Jing Liu**, my main supervisor, for helping both me and all of his others students develop these types of competence. Jing also shared his spirit of trust, optimism, and integrity, which has been crucial for my personal career development and my enjoyment of research and entrepreneurial work.

To my co-supervisor, Dr. **Ivo Achu Nges**, thank you for giving me a deep understanding of the biogas process and for going the extra mile by spending your spare time and weekends with me in scientific discussion. Your days spent in discussion with me helped bring my research and writing to the next level.

I would like to thank the past and present members of the Environmental Group **Bing**, **Emma**, **Anders**, **Ying**, **Linda**, **Mingxing**, **Zhidan** and **Roxana**, and all my other past and present colleagues and friends at the Division of Biotechnology. My thanks also go to the administrative staff in the division: **Emma Poaches**, **Frans**

Peder and **Christina**. You have been a constant in my life and your contributions have been invaluable.

I would like to acknowledge and extend my heartfelt gratitude to **Mihaela**, **Sten**, **Alberto**, **Nicklas** and **Susanne** and to all my past and present colleagues and friends at Bioprocess Control for giving me a stimulating and cosmopolitan environment to work in. I also greatly appreciate the assistance of all my past and present colleagues at Nova Skantek Beijing: **Honglei**, **Qiang**, **Qi, Gangjin**, **Jingxi**, **Chuyan**, and **Guohuan**. Unfortunately, I could not spend as much time as I would have liked in our office at the CRCC plaza in Beijing, but you have always made me feel supported.

To **Wenjing**, you will be my post-doc supervisor in Tsinghua University soon and I cannot wait for the opportunity. Thank you for your enthusiastic discussion about waste management in Hamburg as a Humboldt research fellow two years ago.

I would also like to take this opportunity to thank Prof. **Yuansong Wei** (RCEES of Chinese Academy of Sciences) and Prof. **Xiangyang Lu** (Hunan Agricultural University), along with Prof. **Jiane Zuo** and Prof. **Kaijun Wang** of Tsinghua University, who welcomed me into their research group. And I want to thank to all the Ph.D. students, master students, and staff in the laboratory that took good care of me. Special thanks to Dr. **Dawei Yu** and Dr. **Junya Zhang**.

Konrad Koch (Technical University of Munich), thanks for your insightful scientific discussions and advice. **Yu Tao**, thank you for helping me work with characterization of microbial community structure, and thanks for all the discussions in the cafeteria at the Imperial Colleague London. **Fuqing Xu** at the Ohio State University, thanks for your kindness, for being always being available to help and for teaching me how to write a review paper. **Taili Dong**, thank you for teaching me how to operate the biggest biogas plant in China!

Delia Amalia Pocan-Aulisio, **Kyle Jaros** and all other friends of Hopkins-Nanjing Center, thanks for always being there and giving advice. The Sten K. Johnson Centre for Entrepreneurship is acknowledged, especially **Hans Landström** and **Marie Löwegren**.

This doctoral thesis work has been conducted in a very close collaboration with Nova Skantek as an industrial partner. We are greatly appreciative of financial support from the China-EU Innovation Funding for Small and Medium Size Enterprise from the China Ministry of Science & Technology (SQ2013ZOA000002, SQ2013ZOC500005).

Lastly, I would like to thank my wife, **Angela Gao**, for making everything happen and always supporting and accompanying me through these years. To my daughter **Isabelle (Yijie Li)**, you make me happy every day and encourage me to continue to strive for a better future.

Popular summary

About two hundred years ago, biomass was the primary source of materials used to produce energy until the industrial revolution set off a series of major changes. Since the beginning of the industrial revolution, the energy required for developed industries has increased drastically and fossil fuels became the primary source of energy especially in the forms of electricity and heat. However, these resources are not only limited in supply, but also have negative effects on the environment, due to the emission of green-house gases (GHGs) into the atmosphere. An urgent need exists for alternative energy sources and sustainable biomass utilization. Is there any abundant, possible biomass and a method that can be a highly promising solution for energy generation, waste management and reduce GHGs emissions? The answer is yes: lignocellulosic biomass and methane production via anaerobic digestion (AD).

Bioenergy is one of the most realistic alternatives to fossil fuels and can be produced by the anaerobic decomposition of biomass or wastewater. Gaseous fuel in the form of biogas or bio-methane generated via AD represents one secure, competitive and low carbon energy alternative method that is growing in importance, and has the potential to become a key contributor to the field of renewable energy. Many organic feedstocks have been employed as substrates for commercial biogas production. Lignocellulosic biomass, such as energy crops and agricultural residues, has gained much interest as a candidates for methane production. It is an inexpensive, renewable, abundant and provides a unique natural resource. Furthermore, lignocellulosic biomass generally does not compete with food and feed production.

Lignocellulosic biomass is composed of cellulose, hemicellulose, lignin, and other extraneous components: proteins, lipids, and inorganic substances. Since the digestible cellulose and hemicelluloses are covered by sheath of insoluble lignin, the bacterial hydrolysis/solublisation of lignocellulose is regarded as the rate-limiting step during AD. Lignocellulosic biomass is also poor in essential nutrients needed in the AD process. There is therefore a need to overcome these hurdles for an effective and efficient degradation process. To achieve this goal, various pre-treatment methods have been proposed and investigated. The outcome of different pre-treatments vary widely in terms of the physical, chemical and biological characteristics of the feedstock. In general, a suitable pre-treatment can increase

the biodegradability of feedstock which could improve the yield and rate of feedstock degradation. Biodegradability of lignocellulosic biomass, such as energy crops, can also be influenced by cultivation in terms of harvest timing and frequency paying cognizance to the fact that cellulose crystallinity and lignin content (resilience to hydrolysis) increases with harvest age or time.

To boost biogas production, many full-scale biogas plants have employed co-digestion strategy that biomass from various sources could be selected and used to feed biogas digesters including the addition of lignocellulosic biomass. One of the main reasons for co-digestion in biogas plants is to ensure that there is a sufficient supply of feedstock. This is always difficult to arrange from single source. Furthermore, co-digestion also augment and or balance the nutrient content in the biogas reactor thereby leading to higher methane yields and more stable processes.

For biogas production from lignocellulosic biomass, solid-state anaerobic digestion (SS-AD) has emerged as one of the cutting-edge technologies for its many desirable features. Operating under SS-AD conditions allows the increase of the organic loading, avoids the floating and stratification of fibrous materials, lowers reactor volumes, decreases the energy requirements for heating the digester and simplifies the final step for the digestate processing. However, a main drawback in the SS-AD process, apart from the hydrolysis, is the poor mass transfer between the substrate and the microbial consortium. As a result, special inoculation techniques (dilution with fresh water and recirculation) have been employed to improve process performance during SS-AD.

In all, the study presented in this thesis demonstrates that making the lignocellulosic biomass more digestible (appropriate harvest time, pre-treatment and co-digestion), inoculation optimization and inhibition recovery in the SS-AD process are viable ways to improve biogas production.

Abstract

Biogas or methane production through anaerobic digestion (AD) is gaining increasing attention worldwide due to concerns over global warming, energy security and the need for sustainable waste management. AD of lignocellulosic biomass is one facet that is highly appreciated since the conflict over biomass for food/feed or energy can be avoided. As a result the need for non-food based lignocellulosic biomass feedstock has emerged as (co-) feedstock of choice for the AD process. Despite these advantages, lignocellulosic biomasses are generally viewed as recalcitrant to hydrolysis, laden with insoluble lignin, poor in essential nutrients, and unmanageable in conventional wet/liquid AD processes i.e. may float in continuous stirred tank reactors.

This thesis presents the feasibility of using lignocellulosic biomasses (*Miscanthus* sp. and seagrass) as feedstock for biogas production via AD. Various operations (pre-treatment, co-digestion, harvest time management and inoculation optimization) were adopted and investigated as means to accumulate both experience and knowledge to evaluate and suitable utilize of the abundant lignocellulosic biomass. Furthermore, the use of solid-state anaerobic digestion (SS-AD) was investigated as a means of biogas production from lignocellulosic biomass. Specifically, a sequential aerobic (pre-treatment) and SS-AD process together with liquor supplementation was studied as means to improve performance and recovery of inhibited/failed SS-AD processes.

Results of the experiment showed that cellulose degradability in different species of *Miscanthus* decreased with longer cultivation period or age (later cut). This was inversely proportional to the methane yields i.e. the methane yields at early harvest (2 months) ranged from 247 to 266 ml CH_4/g VS which were noted to decrease with as much as 35% when the late harvest was carried out.

It was demonstrated that when using *Miscanthus* as feedstock, pre-treatment, especially steam explosion, could lead to approximately 50% increase in methane yield. In addition, aerobic pre-treatment of rice straw at 35°C for 2 days was shown to be a viable method to improve hydrolysis and decomposition of lignocellulosic structure and therefore resulted in the highest biochemical methane potential (BMP) (355.3 ± 18.7 ml CH_4/gVS).

Co-digestion of *Miscanthus* and manure, and sea grass and manure was demonstrated to ameliorate the nutrient content, especially the carbon to nitrogen ratio, in the AD process. The co-digestion of *Miscanthus* or seagrass with manure demonstrated to show a synergistic effect with 11 to 34% higher methane yields as compared to values obtained from the mono-digestion.

The SS-AD proved to be feasible in the treatment of rice straw, as biomethane production reached over 70% of the biochemical methane potential especially after supplementation (of the inoculum) with recycled water. In fact, the addition of recycled water could improve the buffering capacity and mitigate the accumulation of toxic intermediates such as volatile fatty acids which could have led to an improved process performance and greater stability. In a related mesophilic and thermophilic SS-AD study, liquor (recycled water and sludge supernatant) was demonstrated to alleviate process inhibition or failure. The liquor supplementation minimized inhibition and boosted the SS-AD process performance as evident by the doubling of the methane yield under the mesophilic conditions.

Making the lignocellulosic biomass more accessible to anaerobic consortium (appropriate harvest time, pre-treatment and co-digestion), special treatment of the inoculum in the SS-AD process and the sequential aerobic and batch SS-AD process were therefore demonstrated as viable means to improve methane production from lignocellulosic biomass.

List of papers

This thesis is based on the following papers, which are referred to their respective Roman numerals (I-VI) in the text. The papers are attached as appendices at the end of the thesis.

Paper I **Li C**[*], Liu G, Nges IA, Liu J. Enhanced biomethane production from *Miscanthus lutarioriparius* using steam explosion pretreatment. *Fuel,* 179 (2016), 267-273.

Paper II Nges IA, **Li C**, Wang B, Xiao L, Yi Z, Liu J[*]. Physio-chemical pretreatments for improved methane potential of *Miscanthus lutarioriparius*. *Fuel,* 166 (2016), 29-35.

Paper III Peng X, **Li C**[*], Liu J, Yi Z, Han Y[*]. Changes in composition, cellulose degradability and biochemical methane potential of Miscanthus species during the growing season. *Bioresource Technology,* 235 (2017), 389-395.

Paper IV **Li C**[*], Strömberg S, Liu G, Nges IA, Liu J. Assessment of regional biomass as co-substrate in the anaerobic digestion of chicken manure: Impact of co-digestion with chicken processing waste, seagrass and Miscanthus. *Biochemical Engineering Journal*, 118 (2017), 1-10.

Paper V Zhou Y, **Li C**[*], Nges IA, Liu J. The effects of pre-aeration and inoculation on solid-state anaerobic digestion of rice straw. *Bioresource Technology,* 224 (2017), 78-86.

Paper VI **Li C**[*], Zhou Y, Nges IA, Liu J, Lu W. Enhancement of the solid state anaerobic digestion of rice straw by liquor addition under mesophilic and thermophilic conditions.
Submitted to Waste Management

My contributions to the papers

Paper I I planed and executed the experimental work. I also performed the majority of statistical analysis and modelling, and wrote most part of the manuscript.

Paper II This work was planned by Ivo Achu Nges. I and Nges performed the experiments and was involved in manuscript preparation together with other co-authors.

Paper III I designed the study with co-authors and performed the experimental work for biochemical methane potential analysis. I also had a major role in data analysis and manuscript preparation

Paper IV I took the main responsibility for planning the study and made the major contribution to the experimental design. I performed the laboratory trials, coordinated manuscript preparation, and wrote a large part of the manuscript.

Paper V I had a major role in planning of the study and I performed the experiments and analysed major parts of the data together with Ying Zhou. Ying Zhou and I wrote the paper together.

Paper VI I designed the study together with the co-authors. Ying Zhou helped in experiment preparation and VFA testing. I performed the laboratory trials and made a large contribution to data analysis, and wrote the majority part of the manuscript.

Abbreviations

AD	Anaerobic Digestion
BMP	Biochemical Methane Potential
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
EC	Energy Crops
FTIR	Fourier Transform Infrared Spectroscopy
GHGs	Green-house gases
HRT	Hydraulic Retention Time
ISR	Inoculum to Substrate Ratio
LCFA	Long Chain Fatty Acids
MSW	Municipal Solid Waste
NIRS	Near Infrared Spectroscopy
OFMSW	Organic Fraction of Municipal Solid Waste
OLR	Organic Loading Rate
PA	Partial Alkalinity
RS	Rice Straw
SEM	Scanning Electron Microscopy
SS-AD	Solid State Anaerobic Digestion
SRT	Solid Retention Time
TA	Total Alkalinity
TS	Total Solids
VFA	Volatile Fatty Acids
VS	Volatile Solids

1.Introduction

The energy crisis, fluctuating prices of fossil fuel, and the severity of global warming caused by green-house gases (GHGs) emissions has promoted the continuous development of renewable energy technologies. When it comes to production of non-fossil fuel energy-carriers, there are only a limited numbers of potential chemical compounds. Biogas production through the anaerobic digestion (AD) processes provides exciting possibilities and solutions for effective decomposition of organic wastes with a net energy production (Weiland, 2010). Biogas or biomethane as a form of renewable energy is expected in the near future to play an increasingly important role in the energy systems of the world, especially in Sweden and China.

Sweden is among the trailblazers in biogas production especially with regards to waste-based biogas processes. Among the members of the EU, Sweden specializes in a long-term vision for a sustainable energy supply involving zero emissions of GHG and a transportation sector supplied by non-fossil fuels by 2030 (Ahlberg-Eliasson et al., 2017) . The Swedish Energy Agency reported that there are over 260 biogas plants in Sweden, which facilitates the utilization of sewage sludge, manure, agricultural crops and food waste (IGR, 2015). Concerning the biogas utilization, Sweden has demonstrated that upgraded biogas can be utilized as a vehicle fuel and distributed via the natural gas grid. Swedish technologies and experiences in utilizing biomass for bioenergy production and applications can potentially have a large impact on biomass utilization and renewable energy production in China. Renewable resource utilization is regarded as a vital component for China's sustained future development. As of 2015, the Chinese National Development and Reform Commission had issued a biogas transformation and upgrading program, investing 2 billion yuan to support 386 large-scale biogas projects (collectively with a daily biogas production of over 500 m^3), and 28 super large-scale biogas projects (with a total daily biogas production of over 10 000 m^3). For these full-scale biogas plants, sufficient feedstock supply has become the most important issue. Therefore, the utilization of energy crops and agricultural residues as a (co-) feedstocks for biogas production is being promoted as one of the main routes for new generation biogas production.

Despite of all the positive features and development thus far, the production and utilization of biomethane is still challenged in leading "cleantech" economies. The industry is still in an early development phase and suffering from low profit margins and slow return on investment. One of the challenges is related to the need for securing a reliable and economically viable feedstock supply to support the biogas production.

Lignocellulosic biomass, such as energy crops, wood, as well as agricultural residues, exists as the most abundant raw materials for biogas production. It has been earning much interest as a promising candidate feedstock for producing biogas. Unlike conventional lignocellulosic feedstock (i.e., maize, wheat and triticale), non-conventional lignocellulosic biomass such as *Miscanthus* and seagrass, may not directly compete with food or feed production (Sawatdeenarunat et al., 2015). Lignocellulosic biomasses such as *Miscanthus* may therefore play an important role as feedstock or co-feedstock especially in China. In addition, high biomass yields even under low requirement of energy, water, and nutrients conditions, and its ecological adaptability to varying environments, make *Miscanthus* an ideal energy crop for biogas production. Moreover, rice straw, which mainly contains non-edible plant material, and is one of the major agricultural wastes in China also falls under this category. These lignocellulosic materials contain a large amount of biodegradable biomass that can be a potential resource. Therefore, it is imperative to find environmentally friendly methods for efficient utilization.

Lignocellulosic biomass contains mainly cellulose and hemicelluloses glued together by a network of lignin, that physically shields the utilizable cellulose and hemicelluloses part from the degrading enzymes. Consequently, this make the hydrolysis stage of AD the rate-limiting step. An appropriate pre-treatment is needed prior to AD in order to open up the recalcitrant structure and reduce the crystallinity of cellulose (Taherzadeh & Karimi, 2008b). However, the pre-treatment should ideally produce low amounts of fermentation inhibitors, such as furfural and hydroxymethylfurfural, as these might inhibit the hydrolysis and further degradation. Therefore, the success/suitability of different pre-treatment methods should be assessed in terms of biogas or biomethane yield and rates together with the related cost and energy consumption of the pre-treatment process.

Apart from different pre-treatment methods, harvest time significantly influences the physiochemical characteristics and digestibility of plant material (Li et al., 2015a). In the tropical and subtropical areas, such as the south part of China, *Miscanthus* can be harvested at least twice yearly. It is commonly known that crop maturity negatively affects methane production and digestibility (Nizami et al., 2009). Double cutting may prevent the crops from senescing and the harvest of

juvenile plants prevents the increase in the degree of polymerization and crystallinity of cellulose.

Co-digestion is yet another method used to enhance methane production and efficient utilize lignocellulosic materials. Energy crops and rice straw are often rich in carbohydrates, but are low in nitrogen (Li et al., 2009). Thus, the co-digestion of carbohydrate-rich lignocellulosic biomass with nitrogen-rich manure waste can improve the methane yield due to the positive synergisms established by specific blend ratio which provides a favourable C/N ratio and better nutritional balance that is required in the digester feed (Aichinger et al., 2015). Co-digestion may also lead to antagonism, so the choosing the right blend is vital for an efficient and effective biogas process.

The mono-digestion of lignocellulosic biomass, such as energy crops or rice straw, is always not recommended as it can result in low methane yield and volatile fatty acids (VFAs) accumulation. Additionally, there is high risk of lack of micro and marco nutrients with mono-digestion of lignocellulosic biomass, in particular continuous operation over a long-time period. Even more, it may float in conventional wet/liquid AD process i.e. continuous stirred tank reactors. The solid-state anaerobic digestion (SS-AD) is alternative process configuration for high solids organic feedstock like energy crops and agricultural residues due to its smaller reactor volume, less water for dilution, low costs for digestate management and lower energy requirements for mixing (Karthikeyan & Visvanathan, 2013). However, SS-AD suffers from two major drawbacks: First, VFAs and ammonia accumulation are likely to occur due to initial high organic loading rate (OLR) for each batch operation run. Second, the digestion of solid wastes may require supplementation of water, nitrogen and micronutrients to both improve mass transfer and maintain adequate microbial activities; meantime, these supplementations may increase the capital cost of SS-AD.

This thesis includes five sections. Section 1 presents an introduction to the research field and the aims of the study. Section 2 offers the general background about anaerobic digestion. Section 3 presents feedstocks types and methane potential analysis. Section 4 presents the technologies for improving degradation of lignocellulosic biomass and Section 5 includes the conclusions of this study and future perspectives.

1.1 Objectives of the study

The study was aimed at elucidating the viability of lignocellulosic biomass as feedstock in the AD process and investigating the impact of various pre-treatments, co-digestion, impact of harvest time/frequency on biodegradability and the impact inoculum optimization as well as liquor supplementation in the SS-AD process. The lignocellulosic feedstock explored during these investigations were (i) *Miscanthus* (**Papers I-IV**), (ii) Seagrass (**Paper IV**) and (iii) rice straw (**Papers V-VI**).

The specific goals of this study can be summarized as follows: (a) how pre-treatments improve feedstock characteristics and their effects on the biochemical methane potential (BMP) and the SS-AD process (**Papers I, II and V**). (b) how harvest time and frequency impact the biodegradability of biomass (**Paper III**) (c) how co-substrate addition (co-digestion) ameliorate feedstock characteristics to improve methane production (BMP) (**Paper IV**). (d) how combine pre-aeration and SS-AD process was established and optimized to accelerate methane production (**paper V**). (e) how liquor addition in an inhibited or retarded SS-AD could influence process performance and stability in a SS-AD process fed with lignocellulosic biomass (**papers VI**). The overall PhD study can be summarized into a literature survey, experimental protocol, conclusions and perspectives as presented in Figure 1.

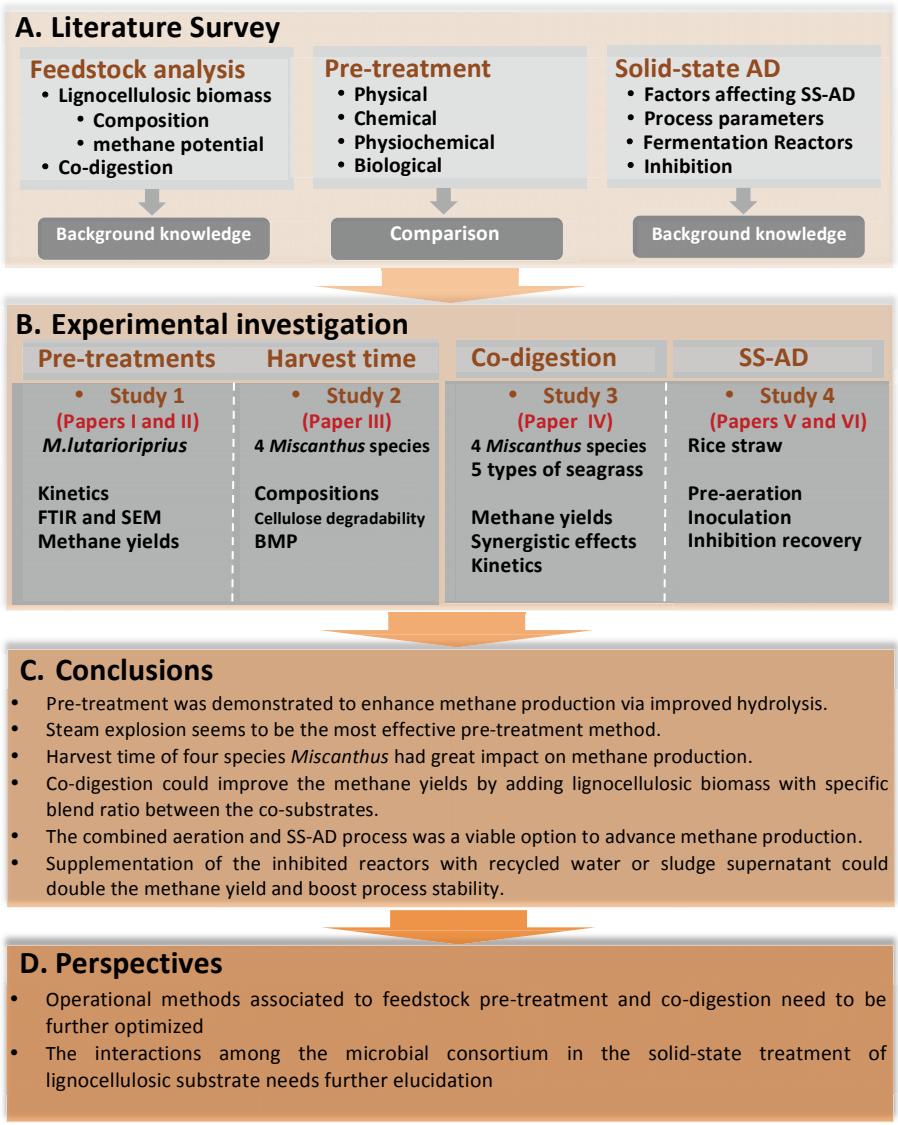

Figure 1. Flow chart of overall PhD project

2. The anaerobic digestion process

The AD process is carried out by several groups of microorganisms that have two main end products: biogas and digestate. In contrast to aerobic treatment (in presence of oxygen) like in the case of composting, very little heat is generated during AD. The produced energy, which is chemically bounded in the feedstock, remains mainly in the produced biogas, in the form of methane (Di Maria et al., 2014). Based on the chemical reactions carried out by these organisms, AD process can be simplified into four major steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

2.1 Degradation pathway during anaerobic digestion

These four steps are often used to illustrate the sequence of microbial events that occur during the AD process leading to the production of biogas. These steps are illustrated schematically in Figure 2.

Hydrolysis

The main components in biomass are polymeric compounds such as carbohydrates, protein and fats. The hydrolysis step is an extra-cellular process in which these polymeric compounds or particulate organic matter are broken down to soluble oligomers and monomers. Hydrolysis of these compounds into smaller units is the first step in the AD process. As the fermentative (acidogenic) bacteria cannot adsorb complex organic polymers directly into their cells, hydrolysis is an important step prior to the acidogenesis step. Hydrolytic enzymes include cellulase, cellobiase, xylanase and amylase for degrading carbohydrates into sugars (monosaccharides), protease for degrading protein into amino acids, and lipase for degrading lipid into glycerol and long-chain fatty acids (Schön, 2010).

Hydrolysis is often consideref a rate-limiting step in the AD of particulate feedstock such as lignocellulosic biomass because digestible hemicelluloses are covered by a sheath of insoluble lignin and because of the crystalline nature of cellulose. The chemical and physical composition of lignocellulose indicate that

this complex polymer is very hard for microorganisms to degrade (Taherzadeh & Karimi, 2008b). Therefore a pre-treatment is often required to open up the structure and reduce the cellulose crystallinity (Hendriks & Zeeman, 2009).

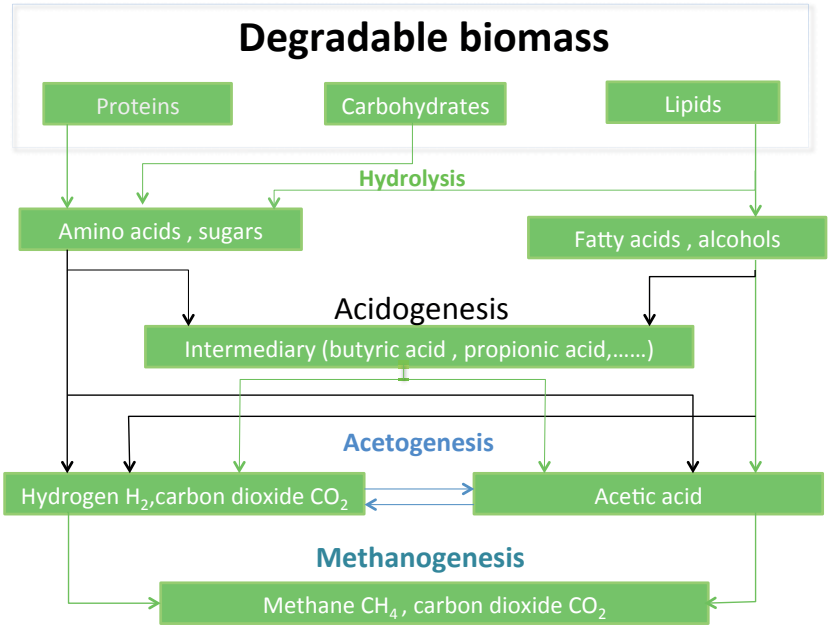

Figure 2. Schematic of anaerobic digestion process

Acidogenesis

In this stage, acidogenic bacteria, also known as acid formers, convert the products of the first phase to VFAs (e.g. acetate, propionate, butyrate,etc.), alcohols, carbon dioxide, hydrogen, ketones, aldehydes etc. This degradation pathway is often the fastest step and also gives a high-energy yield for the microorganisms (Vavilin et al., 2008).

Acetogenesis

The VFA and alcohol produced during by acidogenic fermentation are oxidized to acetate, formate, carbon dioxide and hydrogen by obligate hydrogen-producing acetogenic bacteria. The production of hydrogen increases the hydrogen partial pressure in the AD process or reactor. Hydrogen production by acetogens is

generally energetically unfavorable due to high Gibbs free energy requirements ('G0 > 0). However, with a combination of hydrogen consuming Archeae (methanogens), the co-culture systems provide favorable conditions for acetogenic reactions ('G0 < 0). Thus, these reactions can only occur within a narrow range of extremely low levels of partial pressure of hydrogen. Because of the low-energy yield from this degradation step, acetogens are very slow-growing and sensitive to changes in organic load, flow rate and environmental changes.

Methanogenesis

The final step of the biogas process is carried out by two groups of methanogens (Archeae) i.e. acetotrophic and hydrogenotrophic. Methanogens are strict anaerobes and different methanogens can utilize different substrates to produce methane. Under stabilized conditions the acetoclastic methanogens contribute to 70 % of the methane production while 30 % comes from the hydrogenotrophic methanogens (Angelidaki et al., 2011). However, in the presence of high concentration free ammonia, there could be an inhibition of acetotroclastic methanogens and methane is produced via synthrophic acetate oxidizing bacteria and hydrogenotrophic methanogens pathway instead (Schnürer & Nordberg, 2008). Methanogenesis is a crucial step in the entire AD process, as it is the slowest biochemical reaction of the process. In well-balanced anaerobic microbial communities, all products of the previous metabolic stage are converted into the next product, resulting in nearly complete conversion of the anaerobically biodegradable organic material into biogas without significant accumulation of reduced intermediates.

2.2 Factors affecting the anaerobic digestion process

Like any other biochemical process, the AD process is influenced by various environmental factors and process parameters which, depending on how optimized they are, may accelerate, retard, or even stall the overall process (Angelidaki et al., 2003). The factors described in this section include environmental factors (temperature, VFAs, pH and alkalinity, inhibitors/nutrients, water content), and some operating parameters (mixing, organic loading rate, residence time, inoculum to substrate ratio) etc.(Chen et al., 2008; Weiland, 2010). In the study presented in this thesis, some of these factors were closely monitored and in some cases fine-tuned with the goal of improving process performance and stability. Factors that have been studied in the current study include pH, VFAs, alkalinity,

mixing, water content, temperature, ammonia, and inoculum to substrate ratio (degree of inoculum).

Temperature

Temperature is an important factor that influences the growth of the microorganisms in the AD process as well as the partition of the gaseous products (e.g. CH_4 and CO_2) between the liquid and gaseous phases (Gerardi, 2003). The AD process can be operated over a wide temperature range i.e. psychophilic (11-25 °C), mesophilic (35 to 40 °C), thermophilic (50 to 55 °C) and hyperthermophilic (≥ 55 °C) wherein mesophilic and thermophilic have gained the most attention and recognition (Takashima et al., 2011; Weiland, 2010). Traditionally, AD was generally applied in the mesophilic temperature range. Thermophilic process was once believed to be more susceptible to inhibition, less stable and more rapidly lead to process failure (Labatut et al., 2014). The advantages and disadvantages of these two temperatures ranges are debatable though thermophilic AD is increasingly promoted especially in lignocellulosic SS-AD processes (Shi et al., 2013)**.** This may be because the high operating temperature can facilitate degradation or hydrolysis of recalcitrant cellulose (Frigon & Guiot, 2010a; Shi et al., 2013).

pH, volatile fatty acids and alkalinity

The AD process functions within a pH range from 6.5 to 8 wherein the microbial consortium can replicate and so degrade the available substrates. Below or above this range, the AD process may be inhibited as a results of the proliferation of toxic intermediates (Weiland, 2010). The pH value in the AD process increases when there is ammonium accumulation (Calli et al., 2005), while the accumulation of VFA decreases the pH value (Gerardi, 2003). VFAs are precursors for methane production but their accumulation may inhibit methanogenesis as a result of the drop in pH (Chen et al., 2008). The accumulation of VFA will not always result in a pH drop, due to the buffer capacity (alkalinity) of the system (Nges et al., 2012b). At low pH (3~5), the unprotonated VFAs may enter the microbial cell leading to the acidification of the cytoplasm (Wang et al., 2016). The pH in AD is usually governed by the liquid alkalinity, where feedstock characteristics directly influence the alkalinity through the formation of degradation products such as ammonium, bicarbonate, sulfides and phosphates (Gerardi, 2003). Ammonium has a large influence on pH and alkalinity, hence protein rich biomass are associated with high alkalinity (Weiland, 2010).

Ammonia

Ammonium is produced during the degradation of protein rich biomasses. While protein-rich materials are considered as desirable substrates for biogas production as they have high methane potentials. However, they are often associated with process disturbances. The amounts of ammonia and sulfide in a reactor liquid depend on the substrate composition.

Free ammonia at concentrations as low as 100-150 mg/L have proved to be inhibitory to an unadapted AD process, whilst an adapted process can tolerate up to 1g/L free ammonia (Chen et al., 2008; Hansen et al., 1998). The degree of inhibition is influenced by pH and temperature wherein the inhibition increases as the pH and temperature increases (Yenigün & Demirel, 2013). Free ammonia exerts its inhibitory action by passively entering the cell and distorting the proton balance or elevating the pH of the cytoplasm (Chen et al., 2008). Recent studies have shown that the acetoclastic methanogens are more prone to free ammonia toxicity wherein acetate is instead converted to CO_2 and H_2 by syntrophic acetate oxidizers to ensure continuance of methane production via the hydrogenotrophic pathway (Manzoor et al., 2016; Yenigün & Demirel, 2013).

Mixing

Mixing is essential to achieving an optimal AD process. It has been reported to improve the contact between microorganisms and the organic biodegradable material. Mixing can create a homogeneous digester content preventing stratification and formation of a surface crust, and prevention of sedimentation of solid material (Kaparaju et al., 2008). Furthermore, mixing also enables heat transfer, particle size reduction as digestion progresses and the release of gas bubbles trapped in the digester (Wang et al., 2017). However, it is important that the mixing is not too vigorous, since this may break up colonies of microorganisms that are essential for efficient decomposition (Gerardi, 2003). German standard, VDI 4630 recommends single vigorous mixing on workdays to encourage degassing of the biogas produced and to prevent the formation of dry and inactive layers (VDI 4630, 2016). In batch SS-AD processes, leachate recycling is often employed to serve the mixing need for improving mass transfer (Li et al., 2011).

Moisture content

The moisture content is an important environmental factor in enhancing biogas production. Water is essential to the AD process, since water is the media of all biochemical reactions, and the nutrients for the microorganisms must dissolve in

water before they can be assimilated (Lay et al., 1997). Conversion of biomass to biogas with help of anaerobes takes place in the liquid phase and then transferred to the gas phase.

High moisture content may also assist microbial movement in the reactor matrix. Moreover, moisture content is well known to influence the mass transfer in SS-AD as well as the balance between acidogenic bacteria and methanogenic bacteria (Ghosh, 1985; Yang et al., 2015). Increasing the total solid (TS) content means a decrease in moisture content; this proportionately affects concentrations of other variables such as alkalinity and free ammonia. Additionally, process improvement such as the addition of fresh water to the inoculum or inoculum recirculation (to also aid mixing) have been reported in some studies (Yang et al., 2016).

Inoculation

The inoculum is of great importance as it contains active microbes, macro- and micro-nutrients, which are needed for the AD process. Different factors related to inoculum can affect the process, i.e. source, time of sampling and storage, concentration, activity, adaptation etc. The digestates (degraded substrate) produced in pilot-scale or full-scale biogas plants have served as sources for these inocula (Heiske et al., 2015; Li et al., 2011; Ye et al., 2013). In addition to using inoculum with high methanogenic activity, bioaugmentati with high populations of hydrolytic microbes may also improve methane yield, especially for lignocellulosic biomass (Peng et al., 2014). Supplementation of hemicellulolytic bacteria which is enriched and immobilized in zeolite was found to increase xylanase activity by up to 162%, and increase methane production by 53% (WeiB et al., 2010).

“Degree of inoculation” is often referred to as the inoculum to substrate ratio (ISR). The ISR is often presented in terms of volatile solid (VS) and has been shown to affect batch fermentation tests in lab-scale in regards to the lag phase, consumption rate of intermediates and methane production (Raposo et al., 2011; Xu et al., 2003). It is commonly accepted in many standards protocols that the ISR should be maintained at 2 or above so as to provide an adequate an active microbial population to engineer the overall degradation process (VDI 4630, 2016). Additionally, when the effect of different ISR has been studied, increasing the amount of inoculum is generally recommended (Boulanger et al., 2012; Dechrugsa et al., 2013). However, it has been proven that an ISR of 2 is sufficient (Boulanger et al., 2012) unless the substrate is laden with highly toxic intermediates or compounds (Chen et al., 2008). The work presented in this thesis was performed mainly with an ISR of 2.

Under SS-AD conditions, the degree of inoculation is often presented in terms of substrate or feedstock to inoculum ratio (S/I or F/I) (Yang et al., 2016). Addition of inoculum to the fresh feedstock is usually required to speed up the initiation of a new SS-AD process (Forster-Carneiro et al., 2007). It has been recommended that a 30% (w/w) inoculation is needed for an effective process (Sans et al., 1995). Fresh water addition to the inoculum in batch SS-AD has also be recommended as a means to ease process (NH_3 and VFAs) inhibition (Yang et al., 2015).

Nutrients

For an effective AD process, there must be sufficient macro and micronutrients to feed the nutritional needs of the dedicated anaerobes. Micro nutrients or trace elements are also essential co-factors or parts of co-enzymes which are directly linked to the biochemistry of methane production(Schnürer & Jarvis, 2010). Nutrient augmentation in the AD of lignocellulosic biomasses and simply balancing the carbon to nitrogen (C/N) ratio has been shown to substantially improve the methane yield (Lebuhn et al., 2008; Nges et al., 2012a; Takashima et al., 2011). Nutrient imbalance can also be resolved through co-digestion especially when it leads to an optimal C/N ratio. Higher methane yields have been reported in synergetic co-digestion processes as a result of improved C/N ratios (Brown & Li, 2013; Nges et al., 2012b).

3. Feedstock analysis

A wide range of biomass types can be used as substrates (feedstock) for the production of biogas via AD. The input substrate is often measured or presented in term of TS, VS, total chemical oxygen demand (COD) or even fresh/wet weight. Particulate feedstock such as lignocellulosic biomasses are often presented in terms of TS or VS, while soluble organic matter such as wastewater or diluted industrial waste streams can be presented in terms of COD. The biochemical methane potential (BMP) test (section 3.2) is increasingly being use as a tool for feedstock analysis. In general, the feedstock can be presented as composed of carbohydrates, proteins, lipids, minerals (nutrients) and water. Carbohydrates can be divided into easily degradable and slowly degradable fractions, while lipid and protein are considered as easily degradable.

3.1 Feedstock used in anaerobic digestion

Feedstocks which are generally accepted and widely used as substrate for biogas production via AD are herein termed conventional feedstock (i.e., agricultural residues, sewage sludge, food waste and manure). On the other hand, emerging and not generally accepted or widely use substrates (due to accessibility or availability) are termed non-conventional feedstocks (i.e., *Miscanthus* and seagrass).

In this study, crop or lignocellulosic biomasses (*Miscanthus*, seagrass and rice straw) and waste biomasses (chicken manure and chicken processing waste) were used as (co-) feedstock in the AD process. In particular, the use of *Miscanthus* and seagrass, here termed non-conventional feedstock is related to a full-scale, ambitious biogas production scheme in China, the Minhe biogas project. The Minhe biogas project is the largest biogas plant network in Asia for waste management and biogas production from chicken manure. As the Minhe biogas project aims to be expanded in terms of its treatment capacity during a second phase construction, there is a need for additional and continuous feedstock supply. In fact, proximal non-conventional feedstocks such as *Miscanthus* (grows on marginal land, low nutrient requirement etc.) and seagrass (readily available in the

vicinity) are being envisaged as possible candidates. This section gives an overview of feedstocks commonly used for biogas production as well some non-conventional feedstocks (*Miscanthus* and seagrass).

Energy crops

In recent years, a new category of AD feedstock has been tested and introduced in many countries, the dedicated energy crops (EC). These are crops grown specifically for energy (biogas, bioethanol, biohydrogen) production (Frigon & Guiot, 2010b). EC can be herbaceous (*Miscanthus*, maize, raps) but also woody crops (willow, poplar, oak), although the woody crops need special delignification pre-treatment before AD.

In Germany, maize is the most important biogas energy crop, contributing 73% of crop-derived biomass (FNR, 2014). The monoculture of maize prevails is often criticized as the environmental impact may be high, due to high erosion, nitrate leaching risks and the negative impacts of pesticide use on biodiversity (Vogel et al., 2016). However, the use of maize fosters competition between energy crops and food crops, which may result in increased food commodity prices. On the other hand, *Miscanthus,* which is a non-food and self-adaptable crop, grows rapidly, is high yielding and has low nutrient requirements, has recently been placed in the spotlight. One such species is *Miscanthus lutarioriparius* (*M.lutarioriparius*), a highly promising herbaceous perennial grass that produces cane-like stems with high biomass productivity. Studies have shown that the average annual dry biomass yield of *M.lutarioriparius* can reach as high as 32t ha^{-1} or 33.4t ha^{-1} (Li et al., 2017b; Liu et al., 2013). These values are comparable to those of maize but higher than those for hemp and Pennisetum, or elephant grass (Figure 3).

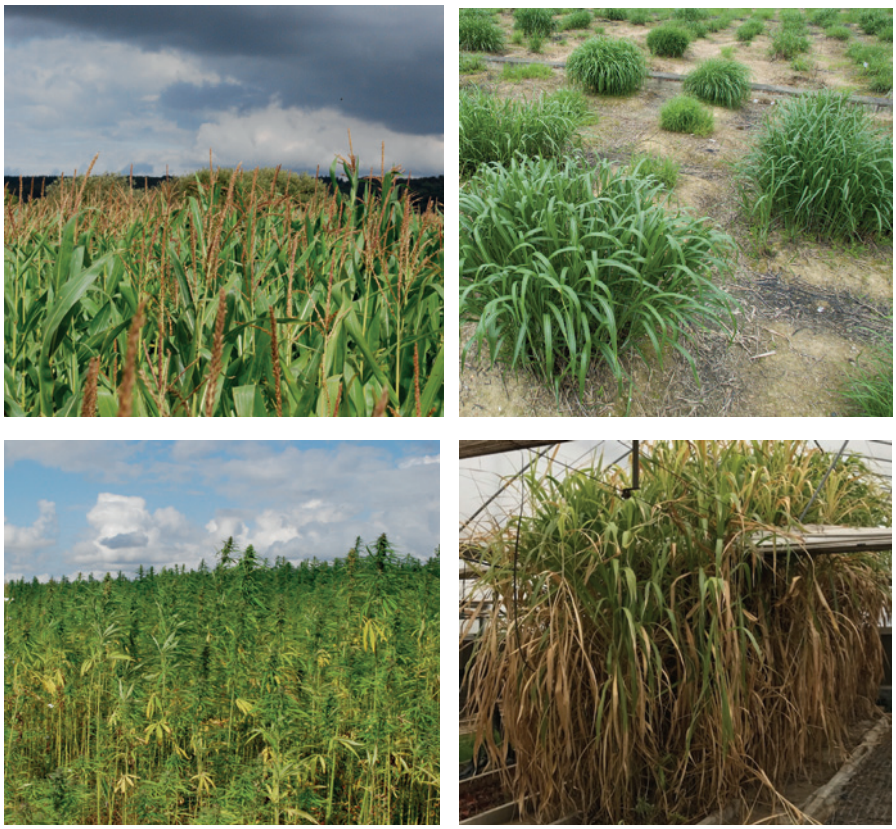

Figure 3. Energy crops: **Upper left:** Maize culture, most frequently used as energy crop, with a wide range of biomass yields per hectare from 9 up to 30 tons DM; **Upper right:** Miscanthus, a perennial plant, producing hectare yields in a wide range of 17–33 tons DM; **Lower left:** Hemp in early cultivation stage, used for oil production as well as an energy crop in anaerobic digestion. Hectare yields of 18-23 tons DM are possible; **Lower right:** Pennisetum hybrid is frequently used as energy plant, enabling 4–7 harvests per year under moderate climate conditions. Yields of 12–16 tons DM per hectare are obtained.

Rice straw

A large quantity of agricultural waste is produced every year in China and the main types of this waste are rice straw, wheat straw, and corn stover. These types of waste account for approximately 47%, 25%, and 28% of total crop residue, respectively (Chen, 2016). The waste management of rice straw via incineration or returning to farmland can raise serious environmental problems such as greenhouse gas emissions (MOA, 2011). Rice straw is mainly composed of cellulose and hemicellulose (around 30% and 18%, respectively) and a silicified

surface layer (Zhou et al., 2017). The main problem with the AD of rice straw as with most agricultural residues (lignocellulosic biomass) is the recalcitrance to hydrolysis and low nutrient (nitrogen) content. The methane production of untreated rice straw reached 306.2 ml/gVS which is closed to the methane yield of rice straw (340 ml CH_4/gVS) in another study (Paepatung et al., 2009). To improve the digestibility of crop residues, pre-treatment methods such as size reduction, pre-aeration, heat treatment, fungal treatment etc. are required. For optimizing the C/N ratio of agricultural residues, co-digestion with sewage sludge (Abudi et al., 2016), animal manure or kitchen waste (Ye et al., 2013) is recommended.

Seagrass

Seagrasses is considered as one of the most rapid growing sources of lignocellulosic biomass, with a growth rate estimated to be 10 times higher than most terrestrial plants (Brudecki et al., 2015). It is considered as relatively unexplored and underdeveloped as a biomass for the sustainable production of biogas and can be found along the beaches or floating near coast lines. Seagrasses are so-named because most species have long green, grass-like leaves. They are often confused with seaweeds, but are more closely related to the flowering plants that one sees on land. Although they often receive little attention, seagrasses play a dominant role in the most productive ecosystems in the world (Zhou et al., 2015).

Zostera marina (Zosteraceae), or eelgrass (Figure 4), is the most common and widespread seagrass species throughout the temperate northern hemisphere of the Pacific and Atlantic (Olsen et al., 2016). *Z. marina* is also an important representative seagrass in north China, and is distributed throughout the Liaoning, Hebei and Shandong provinces. Studies on methane production from seagrass are scarcely reported in scientific literature, it was used as co-feedstock in AD for the first in this study.

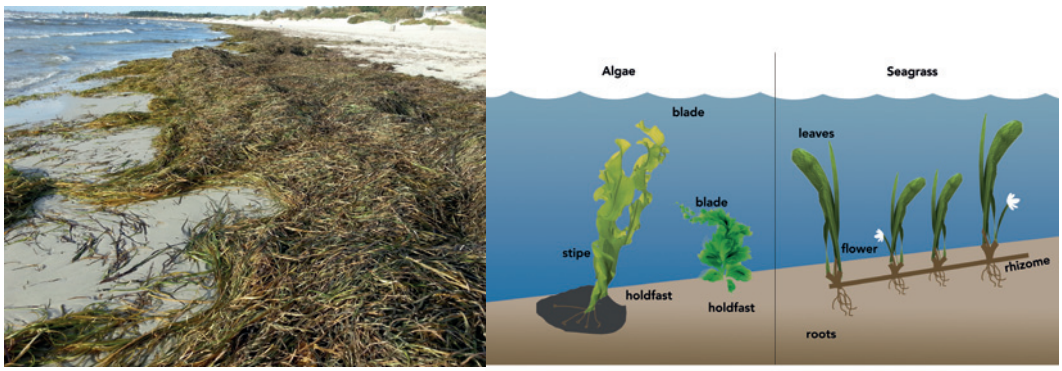

Figure 4. Left: Cast seagrass on the beach at Lomma, southern Sweden, September 2014; **Right:** The difference between seagrasses and algae or "seaweeds".

Manure

With the increase in large scale mechanized poultry breeding industries, huge amounts of animal manure are generated each year in the China. Manure, which contains high water and recalcitrant fractions, has lower methane yield per VS or COD than easily degradable substrate such as food waste (Fang & Zhang, 2015). The methane yields of manure are in the range of 100-400 L CH4/kg VS. Chicken manure is a plentiful source of biomass for biogas production via AD, which has not been fully utilized so far. Since chicken manure has a higher fraction of biodegradable organic matter than other animal wastes, AD of uric acid and undigested proteins may result in the production of high amounts of unionized ammonia and ammonium ions (Abouelenien et al., 2009). The methane potential of chicken manure is exceptionally high and shows even higher nitrogen content compared to manure from other farm animals (Ahn et al., 2010; Li et al., 2017a; Li et al., 2017b; Zhang et al., 2013).

Pig manure can also contain high concentrations of ammonia, but this depends on the content of solids. Pig manure has been reported to show higher methane yield than cow manure probably due to a higher protein and lipid content, less lignin, and less slowly degradable carbohydrates (Møller et al., 2004). In the study presented in this thesis, chicken manure was used as a main feedstock and co-digested with *Miscanthus* and seagrass.

Sewage sludge

Sewage sludge management is a cornerstone of public health and environmental protection; there is concern about the large quantity, potentially environmental risk and the high cost for disposal which accounts for up to 50% of the total wastewater treatment expense (Appels et al., 2008). AD is the most widely employed process for sludge stabilization because it has the advantage of not only reducing organic content, but also producing renewable energy (Neumann et al., 2016). Sewage treatment generates primarily two parts: primary or raw sludge and activated or secondary sludge. In comparison with activated sludge, primary sludge is rather easily degradable as it generally contains more fat, protein and carbohydrates as opposed to recalcitrant bacterial cells which are in the activated sludge (Lu & Ahring, 2006). The methane yields of sewage sludge are in the range of 224-381 L CH4/kg VS (Davidsson, 2007) in Sweden.

Fat- and protein-rich substrates

Substrates with high fat and protein contents that often originate from the food industry (slaughter houses and food-processing industries), are attractive substrates for methane production with specific yields between 360 and 600 L/kg VS (Hejnfelt & Angelidaki, 2009; Maya-Altamira et al., 2008). However, these kinds of wastes are generally regarded as difficult substrates for the AD process, mainly because their degradation products (ammonia and long chain fatty acids) are inhibitory to an un-adapted process. The free ammonia concentration is believed to be the active component that causes ammonia inhibition (Hansen et al., 1998). Additionally, lipids may also cause problems in AD because of the hydrolytic, acidogenic and methanogenic bacteria can be inhibited by accumulation of long chain fatty acids produced during hydrolysis of lipids (Palatsi et al., 2009). This therefore warrants fat- and protein-rich substrates to be added in low amounts into the digester as a co-substrate, which may be a feasible way to alleviate the product inhibition and use these substrates' potential energy (Pagés-Díaz et al., 2014). An example of a fat/protein rich waste is the chicken process waste used as a co-substrate for biogas production in the study reported in Paper IV.

3.2 Biochemical Methane Potential Test

One of the primary aspects of anaerobic digestion is the selecting and screening feedstock. The energy content (methane potential) and/or biodegradability of the feedstock are often determined via the batch AD test procedure commonly called

biochemical methane potential (BMP) test. The volume of methane that can be generated per unit substrate via AD and it often obtained via the BMP test. On the other hand, the theoretical methane potential of a substrate can be calculated via the stoichiometric elemental composition based on the Buswell formula (Symons & Buswell, 1933). However, as well as the predictive models (see modeling below) not all the elements are viable candidates for methane generation during AD (at least within the applied retention time) and portion of the substrate is also used up for microbial growth and replication. Therefore, BMP test have been used widely to achieve the practical methane potential of a substrate (Holliger et al., 2016; Weiland, 2010).

The BMP test is batch AD process which is often used to determine, experimentally the amount of methane that can be produced from a unit substrate defined as TS, VS or COD under predefined and un-limiting conditions (Raposo et al., 2011). The BMP has emerged as the method of choice for determining both the degradation kinetics profile and the methane yield of any substrate (Hansen et al., 2004). This test procedure involves, basically, the exposing of a pre-determined amount of substrate to an inoculum obtained from an active anaerobic digester, and then measuring either the total gas (cumulative gas production), or carbon dioxide (CO_2) and methane (CH_4) content to calculating the methane volume or direct measurement of methane gas after removing other gas components, e.g. CO_2, H_2S. The gas measurements are determined as a function of time typically 30 days or longer for poorly degradable substrates and the residual substrate is determined at the termination of the test. The BMP assay, therefore, can serve as a tool to assess the ultimate or extent and rate of anaerobic transformation of a given substrate to energy rich methane. Most of the work presented in this thesis was performed via the BMP test.

The BMP test process was first established in 1979 (Owen et al., 1979) as a simple and inexpensive procedure to monitor the relative anaerobic biodegradability of different organic substrates. More recently, BMP tests have also been applied to digestates, to assess their residual biogas yield and evaluate the efficiency of a full-scale anaerobic digestion process (Li et al., 2017a; Schievano et al., 2011). In the current study, anaerobic batch test was carried out to evaluate the effects of co-digestion, impact of harvest time and inoculation of the lignocellulosic biomasses.

3.3 Modelling of BMP test

Two types of mathematical models are often applied to describe the AD process in BMP tests. Firstly, using empirical models to predict the ultimate methane yield based on the chemical composition of the substrate (Dandikas et al., 2015; Rath et

al., 2015; Thomsen et al., 2014). These types of models allow only for the prediction of the methane yield of a well-defined substrate group with high prediction accuracy, or applicable to a broad range of substrates at the expense of accuracy e.g. models based on near infrared data (Triolo et al., 2011). Secondly, kinetic models have been developed to describe the methane production during a BMP test (Angelidaki et al., 2009; Koch & Drewes, 2014; Strömberg et al., 2015). These models allow for both early prediction of the ultimate methane yield, and therefore downsize the required duration of a BMP test to a few days (Strömberg et al., 2015). The most commonly applied model for BMP tests is the simple first-order kinetics developed by Hashimoto (Hashimoto, 1989) and suggested in the protocol by Angelidaki (Angelidaki et al., 2009) **(Papers II and IV).** While the first-order approach is applicable to most substrates, a Monod-type alternative based on the approach by Eastman and Ferguson (Eastman & Ferguson, 1981) has been developed by Koch and Drewes for slowly degradable material (Koch & Drewes, 2014). Other commonly used models which are extensions of the these two models; for example the modified Gompertz model which is a typical sigmoid function allowing for the modeling of an initial lag phase (Strömberg et al., 2015), **Papers I and IV**. The output in the models consists of two parameters, namely the ultimate methane yield and the rate constant k. The ultimate methane yield can be considered as the methane potential of the substrates, as it describes the methane yield obtained after infinite digestion time. In all, models are recommended for double-checking the data's quality of an experimental BMP test, provide a parameter that describes the rate of AD, and gives insights that cannot be taken from the raw data (i.e., ultimate methane yield after infinite digestion time).

4. Technologies for improving degradation of lignocellulosic biomass

Lignocellulosic biomass is one of the most abundant organic matter on earth but its bioconversion to methane via AD is hampered by the cell wall as well as the shielding of cellulose and hemicellulose by impermeable, ingestible lignin (Weiland, 2010). Therefore, the methane yield of lignocellulosic biomasses are often low, which in some cases can be as little as 10% of the theoretical methane yield (Yang et al., 2015). These low or poor yields can be associated with and not limited to: (i) poor hydrolysis, (ii) resistant to enzymatic and microbial attacks, (iii) nutrient imbalance, (iv) the degree of lignification and (v) poor methanogenesis. Steps towards overcoming these hurdles to improve the methane yield of lignocellulosic biomasses were investigated in the work presented in this thesis. The specific goals, work performed and results are presented below.

4.1 Composition of lignocellulosic biomass

Lignocellulosic biomasses generally consist of three main polymers: cellulose, hemicellulose, and lignin together with traces of proteins, pectin, extractives and ash (minerals). Depending on the type, species and growth age (harvest time), these polymers' form a hetero-matrix to different degrees and varying composition (Bajpai, 2016; Kreuger et al., 2011). The relative abundance of these polymers are a key determinant of the amount of energy (methane) that can be harnessed from a given biomass via AD (Kreuger et al., 2011).

Cellulose is the single largest constituent of lignocellulosic biomass (35-50%) especially in the cell wall where it grants structural support. It is a linear polymer of beta-D-glucopyranose (glucose) moieties linked via beta-(1,4) glyosidic bonds. The cellulose polymers are linked together by hydrogen and Van der Waals bonds, which cause the cellulose to be packed into microfibrils which are then covered by hemicelluloses and lignin. The cellulose polymer is of high molecular weight with a degree of polymerization ranging from 1500 to 10000 (Bajpai, 2016; Willför et al., 2008).

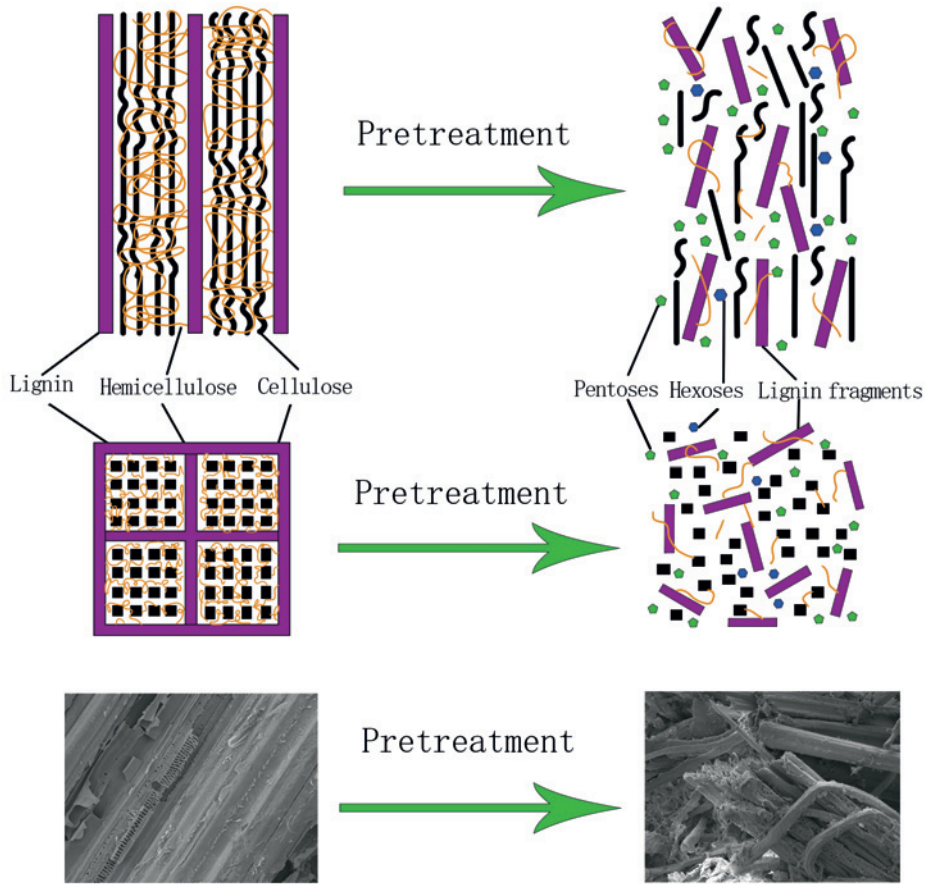

Figure 5. Pre-treatment of lignocellulosic biomass for enzymatic hydrolysis (Chapple et al., 2007; Li et al., 2016).

Hemicellulose, made up of a group of polysaccharides, is the second most abundant polymer and comprises approximately 25% of the biomass. These carbohydrate polymers are of lower molecular weight than cellulose (100-200 degree of polymerization). Hemicellulose is composed of both hexoses and pentose. The hexoses are glucose, mannose, and galactose, while the pentoses are xylose and arabinose (Bajpai, 2016). The hemicellulose is built from (1→4)-linked β-D-glucopyranosyl and (1→4)-linked β-D-mannopyranosyl units (Willför et al., 2008). Hemicellulose is thought to bind non-covalently to the surface of cellulose fibrils (Bajpai, 2016). It acts as an amorphous matrix material, holding the stiff

cellulose fibrils in place. It has been reported that substitution with hydrophobic groups such as acetyl and methyl groups in hemicellulose enhances the affinity of hemicellulose to lignin and thus aids the cohesion between the three major lignocellulosic polymers (El Hage et al., 2009). As a result of its non-crystalline nature, hemicellulose is more susceptible to depolymerization than cellulose (Brandt et al., 2013).

Lignin is an aromatic polymer of coniferyl, sinapyl and *p*coumaryl alcohols moieties (Bajpai, 2016). It is water-insoluble and becomes part of the composite after plant growth has ceased. It provides water-proofing, structural reinforcement and resilience to biological and physical attack greater than that of the carbohydrate cell walls of immature plant tissues (Brandt et al., 2013). Lignin is generally viewed as a 'glue' that binds the other components of lignocellulosic biomass together making the biomass impermeable to water (Bajpai, 2016). The lignin polymer contains a wide range of linkages, the most prevalent of which is the β-O-4 ether bond making up over 50% of all inter-subunit bonds (El Hage et al., 2009). Hence, the lignin crust has been reported to physically shield cellulose and hemicellulose and has been blamed for poor biomass deconstruction hindering an efficiency bioenergy generation process (Taherzadeh & Karimi, 2008a).

Though the process and technology of lignocellulosic biomass AD has been a key topic in most engineering laboratories and even extensively operated commercially, there is still room for improvement in terms of process yield and speed especially in case of novel, non-conventional feedstocks. Feedstock characteristics or quality used in the AD process has been reported to play a great role in both process efficiency and stability. Also, the characteristics of the inoculum as well as process configuration may have a significant impact on the biogas process. As has been mentioned earlier, lignocellulosic biomass such as *Miscanthus*, seagrass and rice straw as feedstock for AD may be limited, amongst others, by poor hydrolysis or high cellulose crystallinity, poor nutrient content and poor mass transfer especially in SS-AD processes. In general, there is a plethora of methods aimed at improving the AD process. The researches presented in this thesis delve into (a) feedstock optimization for improved biogas production via various pre-treatments, (b) balancing the C/N ratio and other nutritional needs via co-digestion, (c) optimization of crop harvest for improved biogas production via harvesting time/frequency and (d) inoculum optimization via liquor supplementation.

4.2 Pre-treatment of lignocellulosic biomass

The principal feedstocks used as substrate in the AD process in the work presented herein are lignocellulosic in nature. The bioconversion of lignocellulosic biomass to bio-energy in the form of methane via AD may be limited by its hydrolysis as the digestible cellulose and hemicelluloses are covered by a sheath of insoluble lignin (Weiland, 2010). The ultimate goal of any pre-treatment technology is to change the structure and composition of the feedstock to remove the obstacles of hydrolysis and therefore improve the rate of enzymatic hydrolysis and increase the yields of fermentable sugars from cellulose and hemicelluloses (Taherzadeh & Karimi, 2008a). Various state-of-art pre-treatments for enhanced methane production from lignocellulosic biomass have been reviewed and presented succinctly in a number of studies (Karimi & Taherzadeh, 2016b; Taherzadeh & Karimi, 2008a). In the same way, the advantages and disadvantages of these pre-treatments have also been carefully reviewed (Karimi & Taherzadeh, 2016a). It is worth mentioning that some hydrolytic substances produced via certain pre-treatments methods may be too toxic to the enzymatic biocatalyst and the anaerobic consortium which can lead to poor process yields, and even cessation of the AD process (Jönsson & Martín, 2016). This is why it is paramount to have sufficient research either in selecting the best pre-treatment method or versed with strategies to alleviate such inhibition. The pre-treatment methods employed during this thesis work were chosen with the aim to improve the overall process yield and rate of AD, while at the same time minimizing the risk of inhibition.

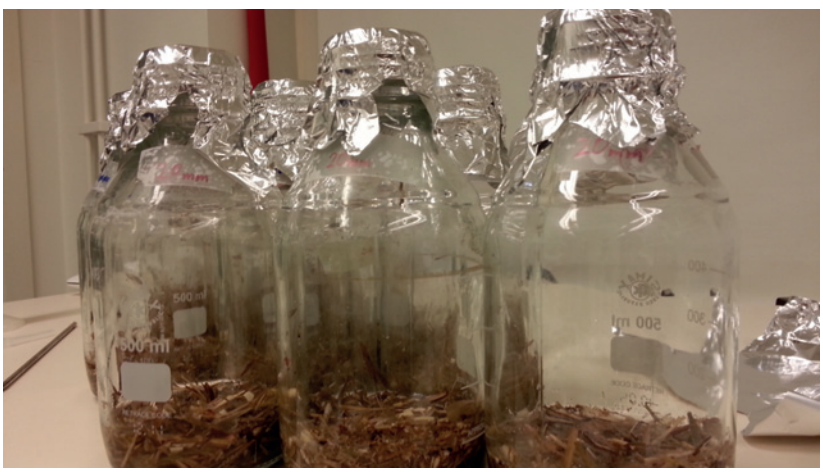

Figure 6. Chemical pre-treatment to *Miscanthus lutarioriparius* in Paper II.

In **Papers I and II**, various and physio-chemical (steam explosion, size reduction, alkali pre-treatment and acid pre-treatment) pretreatments of *Miscanthus lutarioriparius* were performed for the improvement of biogas production. Meanwhile in **Paper V**, aerobic pre-treatment at various temperatures (25 to 45°) and durations (0 to 8 days) was employed to improve the methane yield of rice straw. The effects of pre-treatments were all evaluated using the BMP test.

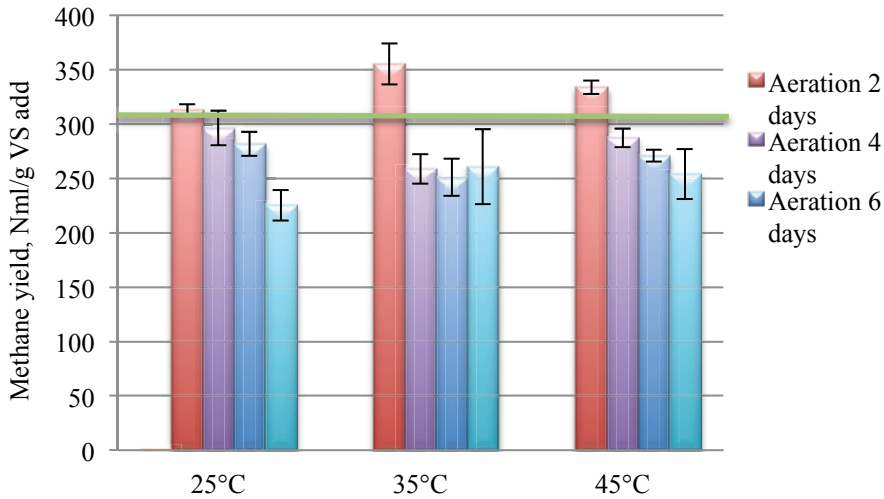

Figure 7. The cumulative methane yields of untreated/aerated rice straw at various incubation temperatures (The green line stands for methane yield of untreated rice straw).

Results show that the pre-treatments led to enhanced hydrolysis as evident by higher hydrolytic rate constants, destruction of the lignocellulosic structure and increased dissolved COD (**Papers I and II**). Steam explosion seemed the most effective pre-treatment method as there was a much as a 35% increase in methane yield after pre-treatment. This result is comparable to the 27% increase methane improvement in the aqueous ammonia soaking pre-treatment of Miscanthus reported in another study (Jurado et al., 2013). The scanning electron microscopy (SEM) technique demonstrated that the exposed fiber surface area increases the accessibility to anaerobic microbes, which may improve the hydrolysis process and as a result methane production. The aerobic pre-treatment of rice straw (**Paper V**) led to lignin degradation evident by reduction in lignin content and improved hydrolysis. The results presented in Figure 7 showed that the aeration treatment at 35 °C for 2 days resulted in the highest increase in methane yield up to 16% higher than the control. Low temperature, aerobic pre-treatment may be recommended opposed to high temperature, chemical pre-treatments due to the lower energy

requirement and low environmental degradation. However, high temperature (130 °C), chemical (N-methylmorpholine-N-oxide) pre-treatment of rice straw has been shown to results in far higher methane yields (above 400% increase)(Teghammar et al., 2012).

4.3 Influence of harvesting time/frequency on digestibility

Bioconversion of energy crops has recently attracted much attention as the bioprocess produces a diverse number of biofuel products. The quality of lignocellulosic biomass can be improved by adjusting the harvesting time, which can be an efficient and cost-effective measure. Studies have shown that the cumulative specific methane yields from *M. giganteus* decreased significantly as plant maturity increased as a result of higher lignin concentrations at later harvest time (Wahid et al., 2015). Another major factor that may thwart methane potential gains is due to research that found that the degree of polymerization and crystallinity of cellulose were noted to increase during the growth period (Peng et al., 2017). This suggests that a green harvest would be preferable; however, earlier harvest will lead to higher moisture content (Kiesel et al., 2017) which may incur higher transportation costs and a higher risk of decomposition.

Miscanthus can be harvested twice or more within a year in tropical and subtropical regions. However, the yield of the following year's first cut appeared less at the early harvest of green biomass. This phenomenon is cutting tolerance and scientists must consider the ability of the energy crop to recover from an early green harvest without yield reductions in the following year (Kiesel & Lewandowski, 2017) .

In the **Paper III**, the changes in composition, cellulose degradability and the BMP of four *Miscanthus* species during the growing season have been investigated concurrently. The results showed that during the growing season from 60 to 180 days, there was only a slight change in the compositional contents. At the same time, there were significant changes in the BMP and the cellulose degradability. For all four species, the BMP was positively correlated to the cellulose degradability. The data obtained in this work are valuable for the determination of suitable harvest times for biofuels production from *Miscanthus*. Moreover, the results of this work suggested that along with the biomass yield, the cellulose degradability of the lignocellulosic biomass may also be a considerable factor for improving the economic benefit of biofuel production from energy crops. This is

especially important in tropical and subtropical regions where the energy crops can be harvested twice or more within a year.

4.4 Co-digestion of multiple feedstock

With the increasing popularity of the AD process, there is a need for 'new' and continual substrate supply throughout the year to feed the anaerobic digester. Co-digestion has been described as a technology to ease feedstock shortage (Li et al., 2009), alleviate the inhibitory effect of high ammonia and sulfide concentrations (Mata-Alvarez et al., 2000) by providing nutrient balance and a buffer capacity (Zhang et al., 2013), and improve the process stability and methane yields of the biogas process (Bayr et al., 2014).

The selection and characterization of the co-substrate that is added to the pre-existing digester is often (or should be) determined via the BMP test. Furthermore, beneficial synergetic effects are observed in co-digestion as a practical disposal route. Studies using whey as a model co-substrate into the co-digestion of municipal sewage sludge with solid organic waste have used BMP tests to show the synergistical enhancement in methanization (Aichinger et al., 2015). This means that the mixture of the two substrates results in a considerably higher yield of methane than would be expected from the individual substrates proportionately.

In **Paper IV**, the study presents new information on using chicken manure co-digested with three regional biomass substrates: chicken processing waste, seagrass and four species of *Miscanthus*. The study used BMP tests to investigate the synergistic or antagonistic impacts of co-digestion, by comparing the methane production rate from the mono-digestion of each substrate with expected production from the co-digestion of the substrates, as well as the effect from various inoculum to substrate ratios.

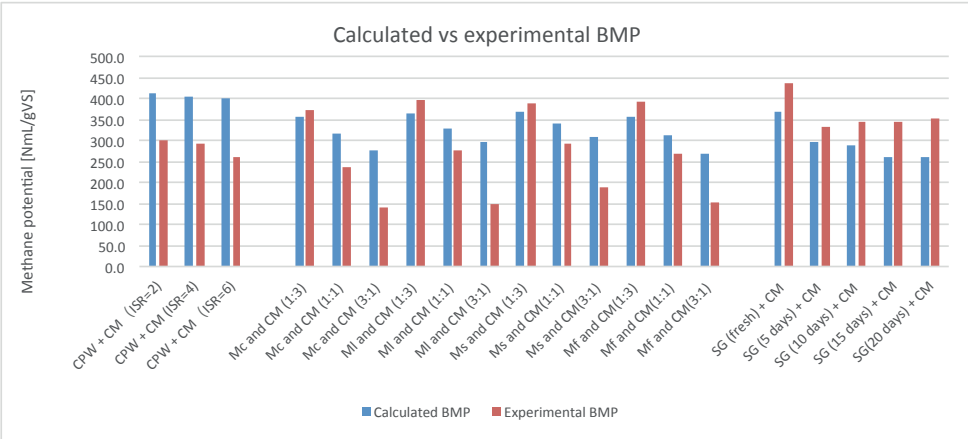

Figure 8. BMP of calculated and of the experimental co-digestion of different mixture ratios in Paper IV. CM = chicken manure; CPW = chicken processing waste; Ms = Miscanthus sinensis; Mf = Miscanthus floridulus, Mc = Miscanthus sacchariflorus; Ml = Miscanthus lutarioriparius; SG = seagrass.

The results showed that using seagrass as a co-digestion substrate with chicken manure increased methane yield with 11.8-34.6% higher than the yield achieved from mono-digestion (Figure 8). *Miscanthus* methane production demonstrated that, if the added quantity of *Miscanthus* exceeds 25% (VS based), there is no longer a positive co-digestion effect and that it can even be expected to exhibit antagonistic effect. While a reduction in methane production (27-35%) was seen when co-digesting chicken processing waste and chicken manure, this fact was expected since high content of protein was converted to ammonia. It is therefore imperative to choose co-substrate with care to avoid a case of antagonism as demonstrated in the co-digestion of chicken manure and the fat/protein rich chicken processing waste (Figure 8 and Paper IV).

4.5 Solid-state anaerobic digestion (SS-AD)

The SS-AD process is a promising technology often employed in methane production from various kinds of solid organic wastes (Yang et al., 2015). The SS-AD is increasingly gaining recognition worldwide as an environmentally friendly and cost-effective technology for extracting energy from lignocellulosic biomass (Ge et al., 2016). As compared to wet or liquid AD technologies such as the CSTR, the SS-AD is hence particularly suited for the treatment of coarse lignocellulosic biomass. Wastes with high moisture content, such as manure, food processing

wastewater, and sewage sludge, are often treated via CSTR. On the other hand, high solids content biomass such as energy crops and crop residues are suitable for the SS-AD process which operates at a TS content of more than 15 % (Weiland, 2010). Compared to the wet AD, SS-AD may need longer digestion time, due to slower mass transfer (Ge et al., 2016). However, SS-AD does not have stratification problems and thus is generally more robust in treating floatable and fibrous material (Yang et al., 2015). Typical advantages of the SS-AD process include a higher volumetric loading capacity, higher volumetric productivity (volume of CH4 produced/ unit digester volume), and reduced energy needs for mixing and heating (Angelonidi & Smith, 2015; Lin et al., 2014).

4.5.1 Substrate characterization for SS-AD process

A wide range of organic solid materials, such as the organic fraction of municipal solid waste (OFMSW), agriculture waste, industrial waste and energy crops are all commonly used as feedstocks in the SS-AD process (Li et al., 2011). The selection of feedstocks is mainly influenced by the feedstock availability and suitability. Varied feedstocks differ in characteristic (composition and structure) thus the handling of each feedstock in SS-AD process varies.

The main composition of OFMSW is kitchen waste which in China has the typical characteristics of high water content and high lipids content (Liu et al., 2012). In case of OFMSW, the strategy of feedstock preparation is to homogenize waste with adequate structured biomass. Input OFMSW that tend to form clumps or silt in the fermentation process are mixed with structural material, like shrubs or energy crops, to increase structural content. Although inert substances do not interfere with the fermentation process, large structures such as sheets or plastic bags can result in dead zones that the percolate cannot penetrate.

In addition to OFMSW, lignocellulosic biomass has lately gained more attention as a suitable substrate for SS-AD. For batch operational mode, structure-rich biomasses, like rice straw and green cut or dried energy crops, are especially advantageous choice when considering the SS-AD process. An additional benefit is that the process retains nitrogen by establishing a permanent liquid phase in the bottom of the digester (Cuzin et al., 1992). Thus, nitrogen deficiency can be avoided when utilizing lignocellulosic biomass with a high C/N ratio and thereby the dependency on nitrogen-rich substrates such as animal manure is reduced (Heiske et al., 2015).

4.5.2 SS-AD process configuration

Many batch and continuous SS-AD operating systems have been developed and marketed over the years wherein the Bekon, Valorga, Kompogas, or Dranco SS-AD systems are the most prevalent (Li et al., 2011). Continuous processes function on the principal of adding waste to the reactor at regular intervals and removing an equal amount of finished product (plug-flow). The batch processes function as a stackable column wherein the waste is inoculated with finished digestate from the previous batch (Li et al., 2011; Xu et al., 2013). The Bekon 'garage-type' percolation SS-AD is probably the most common batch reactor and most suited for the treatment of lignocellulosic biomass. It is filled with a mixture of new raw material and digestate (to provide inoculum) and opened and emptied only at the end of a cycle to restart the cycle with completely new filling (Liew et al., 2012). Percolation or leachate recycling ensures the microbial colonization of the reactor content by providing a passive transport mechanism (Li et al., 2011).

The batch SS-AD may also include a pre-aerobic process that acts as a pre-treatment to enhance the actual SS-AD process. The same principle was adopted and studied in the research presented in **Paper V**. The process configuration adopted and tested was a sequential aerobic and AD process under solid-state conditions wherein the aerobic phase acted as a pre-treatment method (section 4.5.2). The aerobic phase was conducted under various low temperature regimes (25, 35 and 45 °C) and durations (0, 2, 4, 6 and 8 days). Results showed that aerated rice straw at 35°C for 2 days led to the highest hydrolytic efficiency and BMP. The pre-aeration was also noted to enhance the initiation speed of the SS-AD process resulting in high methane production as high as 75% of the achieved BMP. The combined aeration and SS-AD was therefore established as viable option to improve methane production from a lignocellulose biomass.

4.5.3 Inoculum optimization and liquor supplementation

One of the main obstacles to an efficient SS-AD process is the poor moisture content. This exacerbates the problem with mass transport limitations. Moisture or water provides a medium wherein the microorganisms 'live and replicate' as well as enhances the contact between the substrate and the microbial consortium in the AD process (Yang et al., 2016). Water or liquid can also serve as a dilutant, lessening the toxic effects of inhibitory intermediates in the reaction broth. Leachate or digestate supplementation and recirculation have been employed in SS-AD processes to boost process performance and stability via improved inoculation and dilution of inhibitors (Li et al., 2011). However, there are conflicting results as to the validity of leachate recirculation as some findings have

shown negatives effects (Yang et al., 2015). One possible reason is the accumulation of ammonia, VFAs, and other metabolic products in leachate which may inhibit microbial activities (Nordberg et al., 2007). Therefore, it has been recommended that leachate recycling usually should be coupled with fresh water addition to aid mass transport and dilute inhibitors (Shahriari et al., 2012). Digestate supplementation has also been employed successfully in failed SS-AD processes in a bid to alleviate inhibition and boost process performance (Yang et al., 2016). Meantime, inoculum dilution with recycled water has been shown to improve the SS-AD of rice straw through mitigation of VFA accumulation and enhanced buffering capacity **(Paper V).** Therefore, it is plausible to state inoculum choice and optimization (addition of water) plays a critical role in the SS-AD process and care should be taken in the duration of inoculation to assure percolation and therefore minimize the VFA accumulation.

The batch SS-AD may be operated under thermophilic or mesophilic conditions (Li et al., 2015b); however, there are conflicting viewpoints in literature as to which is the most effective (Li et al., 2014; Shi et al., 2013). In this aspect, the research in **Paper VI** explores and compares the process performance and stability in liquor supplemented thermophilic and mesophilic SS-AD processes fed with rice straw.

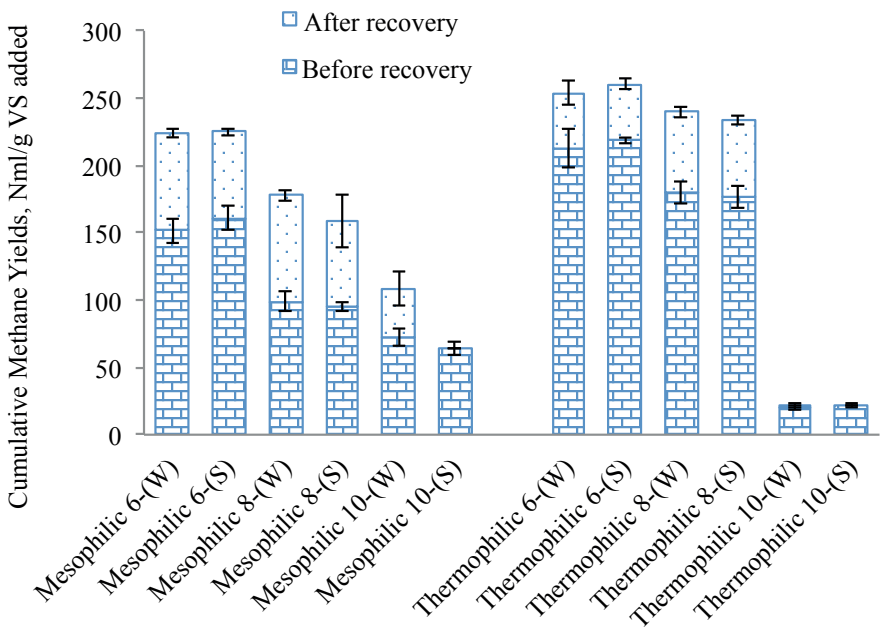

Figure 9. Specific methane yields under mesophilic and thermophilic conditions for the various processes. W = recycled water; S = sludge supernatant.

In the research work presented in Paper VI, two different kind of liquor (recycled water and sludge supernatant) were used as supplements to inhibited or failed rice straw-fed SS-AD processes under mesophilic and thermophilic conditions. The S/I ratios were 6, 8 and 10 (VS bases) giving TS contents in the reactors of 17%, 21% and 24% under the mesophilic scenario and 16%, 19% and 22% under the thermophilic scenario. The hypothesis tested was that liquor supplementation in failed or inhibited process could alleviate the high toxicity through dilution, improve moisture content, and reestablish a medium for microbial growth which would boost the biogas process. Results showed that liquor supplementation was especially effective under mesophilic conditions and a high substrate load (S/I ratio) as significant improvement was seen in process performance (over 94% increment in methane yield) and process stability (low volatile fatty acid to alkalinity ratio) (Figure 9). The thermophilic processes generally showed higher and lower methane yields at low and high substrate loads respectively as compared to the mesophilic processes. This was most likely because of enhanced hydrolysis which was also detrimental at high loads as high VFAs concentrations could not be ‘diluted’ by the liquor supplementation. Overall, the study revealed that the energy yield in failed or inhibited SS-AD process can be doubled by simply adding waste or recycled water to the process.

5. Conclusions and Perspectives

The research presented in this PhD study evaluated the feasibility of utilizing lignocellulosic biomass in the AD. Potential energy crops, agriculture residues and co-substrates were screened and selected according to their biodegradability, biochemical methane potential and synergistic effect. In addition, batch solid-state anaerobic digestion was studied on rice straw as per methane production and general process performance.

The major findings from this study are summarized here below.

- Pre-treatment of feedstock prior to AD was demonstrated to enhance biogas production and VS reduction via improved hydrolysis which was evident from a higher hydrolytic rate constant after the pre-treatments.

- Steam explosion pre-treatment seems to be the most effective method for the pretreatment of lignocellulosic materials (*Miscanthus*). It showed the highest impact on methane yield wherein as much as 35% more methane was produced after the pre-treatment.

- Harvest time had a significant impact on different varieties of Miscanthus in regards to composition, cellulose degradability, and methane potential. In particular, cellulose degradability was highest at early harvest translating to higher methane yield.

- Co-digestion by adding lignocellulosic biomass to a waste-based anaerobic digester may improve methane production. This was most likely due to the synergistic effects established in the digestion process of providing needed micro and macronutrients and diluted inhibition. However, co-digestion of fat- or protein-rich substrates with high nitrogen content led to inhibition or operation failure. The blend ratio between the chicken manure and lignocellulosic biomass was an important factor for synergism or antagonism evaluation.

- Batch solid-state anaerobic digestion was demonstrated to be a particularly well-suited state-of-the art technology for treating lignocellulosic biomass (rice straw). The combined aeration and SS-AD was therefore established as a viable option to improve methane production from a lignocellulose biomass. Moreover, to reduce the risk of VFAs accumulation, the addition of recycled water could

improve the buffering capacity, and mitigate the accumulation of toxic intermediates which can lead to improved process performance and stability. The outcome from this study could further the commercialization of the solid-state anaerobic digester.

To further improve the utilization of lignocellulosic biomass, operational methods associated to feedstock pre-treatment and co-digestion need to be further optimized. It is important to choose the suitable treatment method in relation to energy consumption and effects achieved. Finally, are the methods verified in the laboratory feasible, and the most effective for lignocellulosic biomass treated in full-scale applications? There is therefore a need for pilot studies in full-scale operations to test the sequential aerobic and SS-AD process to further validate this concept.

An area that is still in its infancy and needs further clarification is the interactions between the microbial consortium in the solid-state treatment of lignocellulosic substrate. Also, as the availability of lignocellulosic biomass is likely to be seasonal, storage methods and other feedstock alternatives that may be co-digested with lignocellulosic biomass should be further explored.

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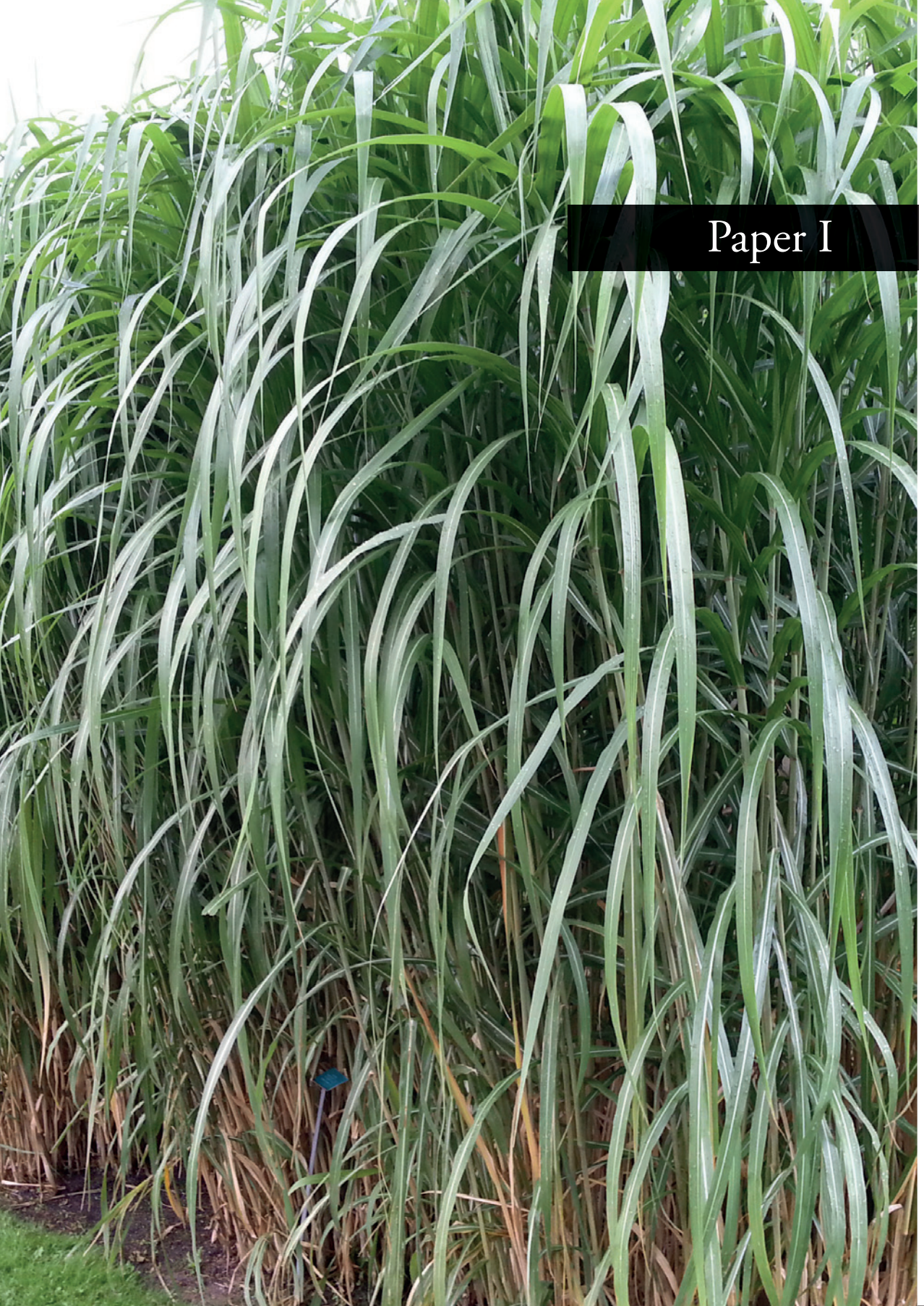
Paper I

Fuel 179 (2016) 267–273

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Enhanced biomethane production from *Miscanthus lutarioriparius* using steam explosion pretreatment

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HIGHLIGHTS

- Miscanthus is a potential feedstock for biomethane production.
- Five different steam explosion conditions were studied on Miscanthus.
- Resulted indicated hemicellulose degradation and increased crystallinity.
- Over 49% enhancement in BMP was achieved as compared with the raw substrate.

ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form 22 March 2016
Accepted 23 March 2016
Available online 30 March 2016

Keywords:
Anaerobic digestion
Miscanthus lutarioriparius
Biochemical methane potential
Steam explosion

ABSTRACT

Steam explosion pretreatments was used to improve the anaerobic biodegradability of *Miscanthus lutarioriparius* (*M. lutarioriparius*). The pretreatments were carried out under five different conditions: L1 (0.5 MPa 153 °C 5 min), L2 (1.0 MPa 180 °C 5 min), L3 (1.5 MPa 198 °C 3 min), L4 (1.5 MPa 198 °C 5 min) and L5 (1.5 MPa 198 °C 10 min). The biochemical methane potential (BMP) of raw *M. lutarioriparius* sample was 181.7 ± 10.7 mLCH_4/gVS. After applying the steam explosion pretreatments under the five aforementioned levels, the BMP value of pretreated samples increased by 5.9%, 19.9%, 51.3%, 49.7% and 49.8% respectively. The Fourier Transform Infrared Spectroscopy (FTIR) of raw and pretreated *M. lutarioriparius*, revealed the disintegration of the biomass structure. Furthermore, scanning electron microscopic (SEM) images revealed the apparent disruption of the recalcitrant structure of the *M. lutarioriparius*. The structural changes in *M. lutarioriparius* observed via FTIR and SEM after steam explosion therefore led to the improvement in biomethane potential and gas production rate. BMP results indicate that *M. lutarioriparius* could be effectively converted to bioenergy in forms of biogas after steam explosion pretreatment.

1. Introduction

China is one of the world's largest energy consumer. China imported 2.8×10^8 tons of crude oil in 2013, and its dependency on foreign oil reached a record 55.6% in the same year [1]. This trend is unfortunately not only a threat to energy security, given the limited petroleum resources China holds, but also a serious barrier to tackling global climate change. Biogas or biomethane represent one secure, competitive and low carbon energy alternative which is highly attractive and with the potential to give good contribution to China's renewable energy mix. In order to accelerate the level of interest and investment in biogas production, growing non-food dedicated bioenergy crops on marginal land can be an option. This can open up the possibility for increasing biogas production by using co-digestion at agricultural central farm, which the main substrate is manure. Co-digestion of bioenergy crops with manure can result in stable and higher biogas output due to the improved nutrient balance [2].

The perennial C4 grass (inhabits hot moist, acid and non-saline areas) Miscanthus originating from East Asian has been considered as a high potential crop biomass that can be converted into bioenergy (CH_4) and bio-fertilizers [3]. Compared to the first generation of energy crops (e.g. wheat, sugar or oil seed rape) and the second generation of energy crops, known as advanced bioenergy crops (e.g. lignocellulose feedstock), Miscanthus, as a highly self adaptability plant, has been placed in the spotlight. One such species is *Miscanthus lutarioriparius* (*M. lutarioriparius*), a highly promising woody perennial grass that produces cane-like stem with high

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http://dx.doi.org/10.1016/j.fuel.2016.03.087
0016-2361/

biomass productivity. *M. lutarioriparius* have relative low nutrients requirement and ecological adaptability to environments, currently grows along the middle and lower reaches of the Yangtze River. *M. lutarioriparius* produced the highest biomass compared with other three Miscanthus species, even when transplanted to the semiarid Loess Plateau [4]. It also has a high level of genetic diversity and a low level of genetic differentiation that showed its outstanding adaptability under different environmental conditions [5]. These new findings implied that *M. lutarioriparius* has a great potential for bioenergy production considering its outstanding properties as an energy crop [6,7].

All dedicated energy crops primarily contains hemicelluloses, cellulose glued together by network of lignin that is resistant to enzymatic and microbial attacks. This makes the hydrolysis stage the rate-limiting step in anaerobic digestion (AD) process and therefore a pretreatment is required to open up the structure and reduce the crystallinity of lignocelluloses [8]. For an effective and efficient AD of *M. lutarioriparius*, a pre-treatment may lead to more biogas production and/or increase the conversion of biomass to biogas, i.e. more biogas can be produced from the same amount of biomass. There have been several pretreatments applied on lignocellulosic material, such as mechanical treatment (milling, grinding and ultrasonic), chemical treatment (oxidation, alkali treatment), biological pretreatment methods (partial aerobic pretreatment, bacterial hydrolysis and enzymatic treatment) and a combination of these methods [9–13].

Steam explosion (SE) is generally considered as one of the most cost-effective pretreatment technologies [14,15]. The intensive pretreatment is applied with hot steam within a few minutes followed by an explosive decompression of the biomass that results in a breakage of the fibrous rigid structure. Compared with some chemical or biological pretreatments methods, steam explosion is deemed to show remarkably low environmental impact because it does not require the use of external catalyst or chemicals [16]. However, steam explosion pretreatment also produces inhibitors which are divided into three categories: weak acids, furan derivatives and phenolic compounds [17]. The manipulating process parameters of steam explosion are residence time, temperature and steam pressure [18]. Therefore, it is important to balance the improvement in biodegradability, energy requirements and production of inhibitor during steam explosion. Several pretreatment methods have been well investigated on Miscanthus to obtain more desired products or precursors for bio-energy production [13,19,20]. However, little is reported about steam explosion on Miscanthus, in particular on *M. lutarioriparius*, a species with extremely rich in cellulose [7].

In this study, *Xiangnandi* 2, a new interspecific hybrids genotype of *M. lutarioriparius*, has been identified as one of the promising lignocellulosic energy crops in the central-south part of China due to its rapid growth and high biomass yields compare to *Miscanthus sinensis*, *Miscanthus floridulus* and *Miscanthus sacchariflorus* [3]. This paper attempts to evaluate the impact steam explosion conditions on the yield and rate of methane production from batch AD of *M. lutarioriparius*. Five steam explosion regimes were adopted and their impact was studied on AD of *M. lutarioriparius*. The techniques of scanning electron microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR) also used to investigate the change of physical structure and chemical content of raw and pretreated *M. lutarioriparius* prior to AD.

2. Materials and methods

2.1. Substrates and inoculums

Rhizomes of *M. lutarioriparius* was collected from different regions in China and used recurrent selection and polycross from 2005 to 2014 for 9 years. This new genotype *M. lutarioriparius* named *Xiangnandi* 2 has high productivity, high cellulose content and strong adaptability, collected from *Miscanthus* nursery at Hunan Agricultural University (China) (28°10′54″N 113°05′06″E) in November 2014 was used as substrate in the present study.

Mass and size reduction of the samples was performed immediately after sampling. The sample was spread out on a large paper and was chopped manually into pieces of 30 mm. A grinder Grindomix 200 (Retsch, Germany) was used to grind a small part of samples to pass through 20 mm sieves for size reduction as a control. All the samples were well packed and sent to Sweden by air express-mail service guarantying delivery within 24 h and stored at +4 °C prior to use.

The inoculum used in the study was provided by Källby wastewater treatment plant in Lund (Skåne, Sweden). It consisted of anaerobic digested sludge from mesophilic biogas process treating dewatered sewage sludge. The particulate matter (>1 mm) was removed from the inoculum by passing through a sieve. Prior to the batch assay tests, the inoculum was incubated at 37 °C in thermostatic bath for 5 days to decrease the endogenous biogas production. The pH of the inoculum was 7.4 with total solid (TS) of 4.9% and volatile solid (VS) of 3.0%.

2.2. Steam explosion and experimental design

Steam explosion pretreatment of *M. lutarioriparius* was performed in a 5.0 L batch vessel equipped with a reactor chamber, a reception chamber and a steam generator. About 200 g biomass (water content, 42.0 ± 5.5%) with 30 mm sized was fed into the reactor batch-wise. High-pressure steam supplied by the steam generator was then injected into the reactor until the pressure reached the set values (0.5, 1.0 and 1.5 MPa). After reaching the desired time (3, 5 and 10 min), the sample was suddenly exploded into the reception chamber by opening the ball valve. After the pretreatment, the moisture content increased substantially and all pretreated samples were dried at 35 °C for 8 h until further use.

In order to evaluate the effect of pretreatment, the range and intensity of the following factors: steam pressure, temperature and retention time, were selected as independent variables denoted by the letter 'L' as illustrated in Table 1. Furthermore, both the untreated 20 mm and 30 mm size *M. lutarioriparius* was used as control reference.

2.3. Biochemical methane potential tests

BMP assays were performed in an automatic methane potential test system (AMPTS II, Bioprocess Control AB, Sweden) under mesophilic (37 ± 0.5 °C) conditions. The trials were conducted in 15 reactors of 500 mL each, in which the substrates and inoculum was mixed at a ratio of 1:2 in terms of VS amount in grams [21]. Each reactor was sealed with rubber stoppers and connected to a mechanical agitator to provide complete mixing.

The biogas produced in reactors was relayed into carbon dioxide (CO_2) absorption unit where 3.0 M NaOH solution was used for absorbing CO_2 from the raw gas. The remaining gas after scrubbing was transported to ultra-low gas flow meters which are connected to the data analytical and acquisition system. In

Table 1
Stem-explosion pretreatment experimental design.

Level	Steam pressure (MPa)	Temperature (°C)	Retention time (min)
L1	0.5	153	5
L2	1.0	180	5
L3	1.5	198	3
L4	1.5	198	5
L5	1.5	198	10

order to eliminate the background gas production from inoculum, reactors containing only inoculum in the absence of substrates were included in each batch as blanks. Microcrystalline cellulose was also tested during the process as a positive control. All the reactors in this study were prepared in triplicates for statistical analysis.

2.4. Analytical methods

The TS and VS contents of all the prepared samples were measured in triplicates using standard methods [22]. The pH was measured by the automatic titrator Titroline Easy (Schott Instrument, Germany). The content of cellulose, hemicellulose and lignin of *M. lutarioriparius* were determined by the standard analysis of biomass composition described by the National Renewable Energy Laboratory (NREL) [23]. A 0.3 g dried sample was treated with 3.0 mL 72% H_2SO_4 at 30 °C for 2 h, then diluted to 4% and autoclaved at 121 °C for 45 min. The hydrolysis solution was filtered and analyzed for sugar content. The sugar content was determined by HPLC (1200 series, Agilent Technologies) equipped with a refractive index detector (RID) and an organic acid analysis column (Aminex® HPX-87H Ion Exclusion Column). The column was operated at 65 °C and eluted with 5 mM H_2SO_4 solutions at a flow rate of 0.5 mL/min. The solid residue was dried at 105 °C for 12 h and further placed in the muffle furnace at 550 °C for 2 h. The weight of ash was recorded and the content of Klason lignin was calculated by deducting the ash content from the solid residue.

Biogas composition was analyzed by gas chromatography on a Perkin Elmer autosystem (Clarus 400 GC) equipped with thermal conductivity detector and a stainless steel column packed with molecular sieve 5A (80/100 Mesh).

The scanning electron microscopy of *M. lutarioriparius* was examined with a JEOL JSM-5600 LV scanning electron microscope (Tokyo, Japan) under high vacuum and at an accelerating voltage of 5.0 kV (10 mm, 500× magnification). Structural changes during the various regimes of steam pretreatments were evaluated with the aid of Fourier Transform Infrared Spectroscopy (FTIR-8400S, SHIMADZU). The region between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1} and 40 scans were recorded. Each sample was prepared according to the potassium bromide technique.

2.5. Model stimulation

By assuming methane production rate in batch test corresponds to specific growth rate of microbial biomass, degradation kinetics was studied by using the modified Gompertz equation (Eq. (1) [24].

$$P = P_{max} \times exp\left\{-exp\left[\frac{R_{max} \times e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (1)$$

In this equation, P is the cumulative specific methane yield ($mLCH_4\ g^{-1}VS$) for a given time t; P_{max} is the maximum methane potential ($mLCH_4\ g^{-1}VS$) at the end of digestion time; R_{max} is the maximum methane production rate ($mLCH_4\ g^{-1}VS\ d^{-1}$); λ is the lag phase (d); t is time (d) and e is exp (1), i.e. 2.71828. "Matlab 2014a" software package was used for estimating the value of the model parameters (P, R_{max} and k) Eq. (1). Coefficient R^2 was generated automatically during the process.

3. Results and discussion

3.1. The chemical composition of M. lutarioriparius after different steam explosion

The composition of the *M. lutarioriparius* before and after pretreatment are shown in Table 2. The VS content of the raw sample was 86.5 ± 1.4% which was increased by the steam explosion pretreatment. However the VS increase is relatively small and did not follow any particular pattern (Table 2).

The untreated sample showed 39.3% glucan (cellulose), 19.6% xylan (hemicellulose) and 19.4% lignin. The glucan and lignin content increased by 1–2% while the xylan decreased about 1–2% when the steam pretreatment severity were 0.5 MPa and 1.0 MPa for 5 min compared to untreated sample. When the pressure of steam pretreatment was increased to 1.5 MPa, the increase of retention time led to the increase in glucan and lignin contents to 6.6% and 7.1% respectively, while xylan decreased about 7% (Table 2). The relative increase of glucan and lignin and decrease of xylan were caused by hemicellulose degradation which is known to occur when the temperature is over 150 °C [25], while steam explosion is usually carried out at relatively high temperature (140–220 °C). The elevated temperature during steam explosion decreased the water p*K*w and the releases of organic acids from biomass components create a mild acidic condition. A series of hydro-thermal reactions can be triggered in high-temperature and acidic environment where the hemicellulose can be hydrolyzed into monomeric and oligomeric sugars while partial soluble sugars will subsequently be degraded into small molecular products by the catalysis of acidic water, acetic and other acids derived from acetyl groups [26].

The FTIR spectra of *M. lutarioriparius* before and after steam explosion are demonstrated in Fig. 1. The absorbent intensities of typical peaks compared with 1426 cm^{-1} originated from the C–H stretching of cellulose and lignin are also provided in Table 3. The broad peak at 3388 cm^{-1} and the lateral peak at 2925 cm^{-1} were attributed to hydroxyl stretching vibration and the C–H stretching of methyl and methylene groups, respectively [27]. All the *M. lutarioriparius* samples showed similar absorption at the two peaks and the main differences were concentrated in the region of 1750–850 cm^{-1}. The bands at 1515 cm^{-1} and 1373 cm^{-1} represented the frame vibration and C–H stretching of aromatic ring which originated from lignin, while the band at 1125 cm^{-1} was associated with syringyl units in lignin molecules [28]. These characteristic bands of lignin were not significantly changed under different steam explosion conditions, thereby revealing no dramatical damage of the aromatic ring structure during the pretreatment process. However, all the three bands were enhanced relatively (Table 3), suggesting the increase of lignin content after steam explosion, which corresponded to the data on compositional analysis determination presented in Table 2. The repolymerization of sugar degradation products and polymerization with lignin to form a lignin-like material termed pseudo-lignin or condensed lignin fragments might be the reasons for apparent increase in lignin content [29–32].

The absorption at 1726 cm^{-1} belonged to the non-conjugated carbonyl group which was probably originated from acetyl of the xylan [27] and its intensity decreased after steam explosion, indicating the removal of acetyl groups during the pretreatment. The absorption of conjugated carbonyl group at 1645 cm^{-1} mainly corresponded to phenolic acids which possessed an unsaturated double bond and a carbonyl group [33]. Decreasing peak intensity at 1645 cm^{-1} with the increasing of steam pretreatment severity indicated the instability of phenolic acids at higher temperature. The peak at 896 cm^{-1} was associated with the characteristic absorption of β-glycosidic bonds between glucose units [34] and its intensity increased after steam explosion which suggested the enhancement of cellulose content in the pretreated samples. Thus, the FTIR analysis demonstrated that the hemicelluloses of the *M. lutarioriparius* samples were partially degraded due to the removal of acetyl groups during steam explosion resulting in the relative increase of cellulose content and lignin content. It has been demonstrated in other studies that the sudden pressure release

Table 2
Components analysis of *M. lutarioriparius* before and after steam explosion treatment.

Steam explosion conditions	Glucan (%)[a]	Xylan (%)[a]	Klason lignin (%)[a]	Ash (%)[a]	TS (%)[b]	VS (%)[b]
Raw	39.3 ± 1.1	19.6 ± 0.7	19.4 ± 0.4	5.1 ± 1.1	90.5 ± 1.2	86.5 ± 1.4
L1	40.1 ± 0.2	18.4 ± 0.0	20.3 ± 0.7	3.4 ± 0.3	91.9 ± 0.1	87.6 ± 0.1
L2	40.5 ± 0.7	18.2 ± 0.4	21.4 ± 0.5	3.5 ± 0.3	92.9 ± 0.2	89.2 ± 0.2
L3	41.6 ± 0.3	17.2 ± 0.2	21.9 ± 2.2	3.0 ± 0.3	94.0 ± 0.1	89.8 ± 0.7
L4	43.7 ± 1.4	15.2 ± 1.0	23.6 ± 1.5	2.7 ± 0.5	92.4 ± 0.9	88.2 ± 1.3
L5	45.9 ± 0.6	12.6 ± 0.5	26.5 ± 1.3	2.4 ± 0.2	93.8 ± 0.1	88.9 ± 0.1

[a] Mass percentage.
[b] Solid content-as wet weight.

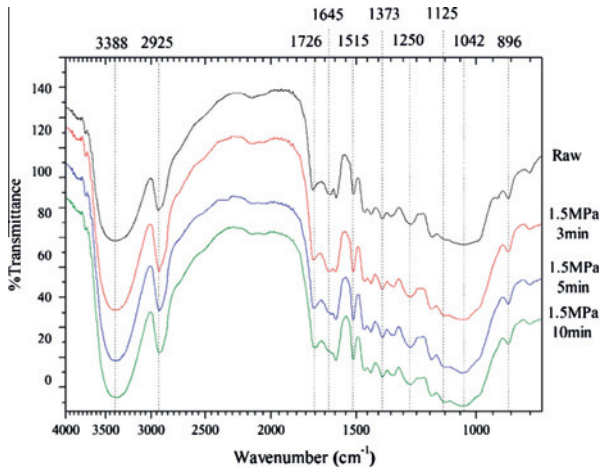

Fig. 1. FTIR spectra of raw and steam explosion pretreated *M. lutarioriparius* under three condition: L3 (1.5 MPa 3 min), L4 (1.5 MPa 5 min), L5 (1.5 MPa 10 min).

Table 3
Relative absorption intensity of untreated and steam exploded *M. lutarioriparius* FTIR.

	Raw	1.5 MPa, 3 min	1.5 MPa, 5 min	1.5 MPa, 10 min
A_{1726}/A_{1426}	0.75	0.75	0.72	0.72
A_{1645}/A_{1426}	0.79	0.77	0.75	0.76
A_{1515}/A_{1426}	0.79	0.84	0.84	0.85
A_{1373}/A_{1426}	1.06	1.07	1.06	1.08
A_{1125}/A_{1426}	1.53	1.59	1.63	1.77
Crystallinity index(A_{1426}/A_{896})	1.21	1.39	1.44	1.46
Total crystallinity index (A_{1375}/A_{2900})	1.11	1.22	1.37	1.43

during steam explosion defibrillates the cellulose bundles with further destruction of the hemicellulose structure [12] which in line with the findings reported in the present study.

The relative absorption intensity and crystallinity of untreated and steam exploded *M. lutarioriparius* FTIR are showed in Table 3. Crystallinity is used to describe the ratio of the amount of crystalline cellulose to the total amount of sample which include crystalline and amorphous cellulose [35]. The *M. lutarioriparius* exhibited considerable changes after steam explosion and its relative absorption density changed with different steam severity. The results also showed that the crystallinity increased during steam explosion pretreatment, which is consistent with other studies [32,36,37]. This is attributable to the melting amorphous cellulose reaching the glass transition temperature then rearranging to a crystalline state. Crystallinity was also considered as an important factor indicating biomass digestibility [38]. However, it did not affect the biomass biodegradation much because cellulose accessibility and enzymes hydrolysis increase will be balanced out by steam explosion pretreatment [39].

3.2. Effect of pretreatment on M. lutarioriparius structure

The surfaces of raw *M. lutarioriparius* and after the steam explosion pretreatments at different severities are showed in SEM micrographs (Fig. 2). As observed in Fig. 2a, the untreated *M. lutarioriparius* portrays the ordered structure and vascular bundles of the cell wall. After low-pressure steam explosion (0.5 MPa, 5 min, 153 °C), the rectangular cell wall boundaries were observed clearly because the lignocellulosic components on the surface was removed (Fig. 2b). Increasing the steam pressure to 1.0 MPa with retention time of 5 min at 180 °C resulted in the destruction of the plant cell wall and the internal structures were clearly displayed (Fig. 2c). Similar treatment effect has been reported for steam explosion of medicinal plants [40] and corn stover [41]. When the steam pressure increased to 1.5 MPa with retention of 3 min at 198 °C, the surface of the pretreated sample was greatly destroyed and the plant cells were hardly observed, instead the sample demonstrated some blurry tissue attached on the fibers surface (Fig. 2d). Under the condition with the steam pressure of 1.5 MPa, the increasing retention time to 5 min removed the blurry

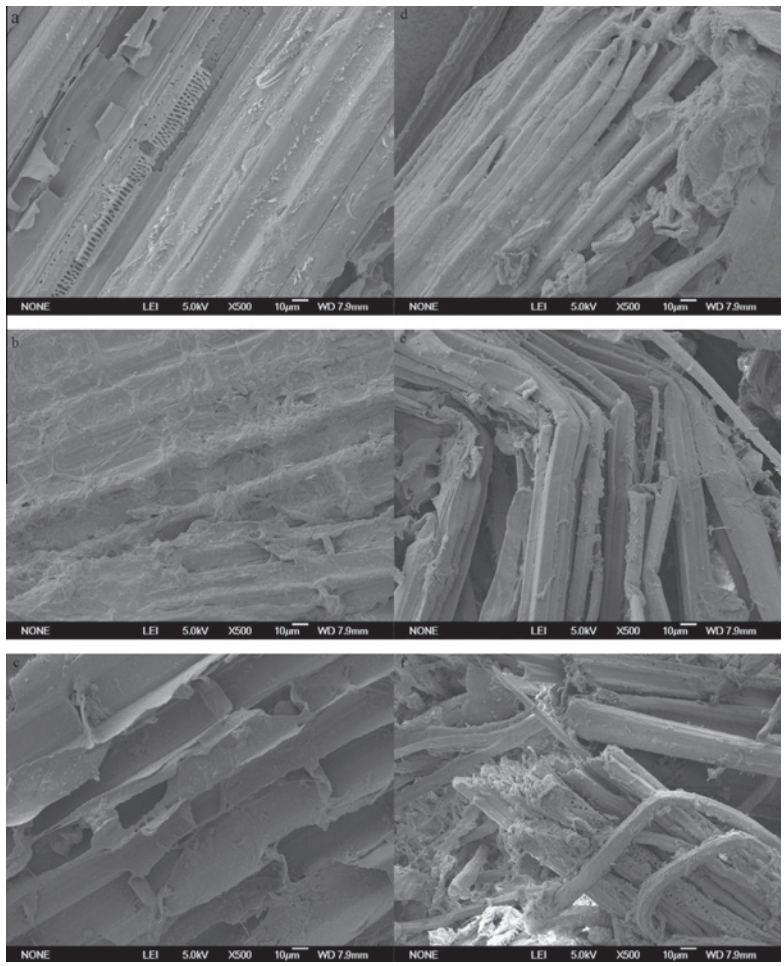

Fig. 2. SEM micrographs of raw (a) and steam explosion pretreated *M. lutarioriparius* under the five conditions: L1 (b), L2 (c), L3 (d), L4 (e), L5 (f).

tissue attached on the fibers surface then the fiber bundles were exposed (Fig. 2e). When the retention time was increased to 10 min, the fiber bundles were well separated and most of them appeared as individual fractions (Fig. 2f). As a result, the large specific surface area of fibers was observed in the *M. lutarioriparius* sample after pretreated by the increased steam explosion condition. It is plausible therefore to sate that the exposed fiber surface area will increase the accessibility of the *M. lutarioriparius* sample to anaerobic microorganisms, which might increase the hydrolysis process and hence methane production.

3.3. Methane potential and degradation dynamics

Fig. 3 shows the methane production and methane content of raw and pretreated *M. lutarioriparius*. The BMP of raw_{30mm} and raw_{20mm} *M. lutarioriparius* were 181.7 ± 10.7 and 181.2 ± 10.7 $mLCH_4/gVS$, respectively, which did not differ significantly ($P < 0.05$). The BMP of *M. lutarioriparius* after steam explosion pretreatment under 5 conditions were 192.4, 217.9, 274.1, 272.1 and 272.3 $mLCH_4/gVS$, respectively. Compared to the raw *M. lutarioriparius* under the two particle sizes of 20 and 30 mm, there was a significant ($P < 0.05$) increased in methane yield in the order of 5.9%, 19.9%, 51.3%, 49.7% and 49.8% respectively. The methane content of pretreated *M. lutarioriparius* ranged from 69.9% to 73.2%, which was in the same range as the raw sample (69.4%).

The BMP value of raw_{20mm} *M. lutarioriparius* indicated that the size-reduction method alone could hardly remove or destroy the hardly-degradable components to improve the BMP on such occasion. However, in contrast, the BMP of steam exploded

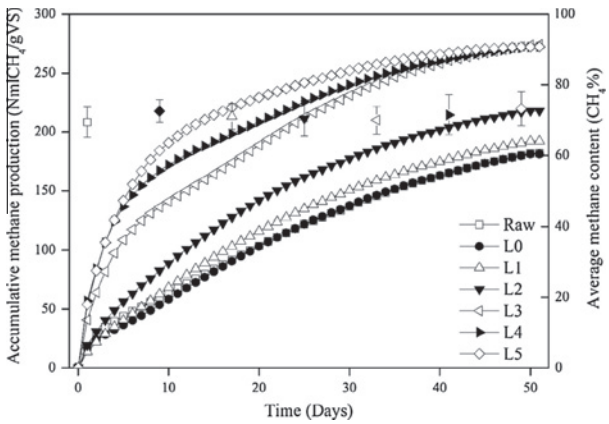

Fig. 3. The accumulative methane production and methane content of raw and steam explosion pretreated *M. lutarioriparius*.

M. lutarioriparius increased with the severity rising of steam explosion condition. Increasing severity in the steam explosion pretreatment led to an increasing contentment of cellulose (glucan), which correlated positively with that is the obvious reason for the increase in the BMP (Table 2). The increase in methane yield also correlated positively with the destruction of biomass cell wall as evident by the SEM images. The sudden thermal expansion opens up the substrate structure to result in particle size reduction and pore volume augmentation [14]. In addition, the increasing of BMP may be attributed to the fact that the cellulose will be easily accessible by bacteria with the increasing severity of steam explosion. Also, the increasing steam pressure can degrade part of hemicellulose to water-soluble oligomers or to individual sugars which contribute to the increase in BMP [14]. Thus, the structure damages and hemicellulose dissolving caused by steam explosion were the main reason for the increase in the BMP of *M. lutarioriparius*. Fig. 3 also showed the unvarying BMP of *M. lutarioriparius* at steam explosion level 3, 4, 5, which implied that the increase in retention of steam explosion may not affect structure and effective chemical composition of pretreated *M. lutarioriparius* ($P < 0.05$), that is accordance with the observation from FTIR analysis. On the other hand, the longer retention times during steam explosion the faster the methane production rate or hydrolysis rate (Table 4) of *M. lutarioriparius*.

Table 4 shows the simulation of modified Gompertz model to the methane production process. The parameter of R_{max} increased with the steam explosion severity. That means the pretreatment increased hydrolysis rate and hence the methane production rate; the higher the severity of the SE condition the higher the rate of hydrolysis which translated to a higher methane production rate. At the same steam explosion pressure, the methane production of *M. lutarioriparius* at L3–L5 were almost the same, while the methane production rate/hydrolysis rate increased with the retention time. Especially at L5 the methane production rate/hydrolysis rate increased by 88.1% compared to L3. It is plausible to state that the increase of steam explosion severity not only enhance the methane production of *M. lutarioriparius* but also the methane production rate/hydrolysis rate. Meanwhile, the increase in retention time at the same pressure might only increase the methane production rate/hydrolysis rate. Also, many studies have showed that the increase of steam explosion severity in a specific range will increase the BMP, however, an exceptionally high steam explosion severity will lead to the decrease of the BMP [14,42,43]. This might be due formation of inhibitory from cellulose and hemicellulose degradation such as phenolics and furfural [14,44]. Thus, the appropriate steam explosion condition should be chosen considering the improvement of methane production rate or hydrolysis rate.

4. Conclusions

This study demonstrated that the steam explosion pretreatment could significantly enhance the anaerobic digestion of *M. lutarioriparius*. Steam explosion can remove a large part of hemicellulose and destroy the structure of cellulose and lignin in *M. lutarioriparius*. Under the optimal pretreatment conditions with steam pressure of 1.5 MPa at 198 °C, similar methane yield were achieved irrespective of the retention time. Longer retention time did significantly improve the hydrolysis rate. From this perspective, it would make sense to further optimize pretreatment with economic and environmental consideration. *M. lutarioriparius* may therefore represent a promising candidate as an energy crop for biogas production after steam explosion pretreatment.

Table 4
Summaries of estimated parameters from modified Gompertz equation and experimental methane yields for raw and pretreated miscanthus.

Sample	P_{max} (mLCH_4 g^{-1}VS)	R_{max} (mLCH_4 g^{-1}VS d^{-1})	λ (d)	R^2	CH_4 yield (mLCH_4 g^{-1}VS)
Raw	200.4	4.34	−4.02	0.994	181.7 ± 10.7
L0	195.8	4.67	−2.11	0.997	181.2 ± 11.5
L1	200.1	5.324	−2.14	0.995	192.3 ± 5.1
L2	222.7	6.36	−3.20	0.993	217.9 ± 12.9
L3	280.4	7.55	−7.33	0.974	274.1 ± 10.9
L4	265.7	9.35	−7.47	0.947	272.0 ± 9.1
L5	262.0	14.21	−3.83	0.959	272.2 ± 15.2

Acknowledgements

This research was financially supported by the EU-China SME Innovation Funding from the China Ministry of S&T (SQ2013ZOC500005) and Swedish Research Links Program (348-2011-7347). The authors are grateful to Dr. Liang Xiao at Hunan Agricultural University for his kind collaboration during this project.

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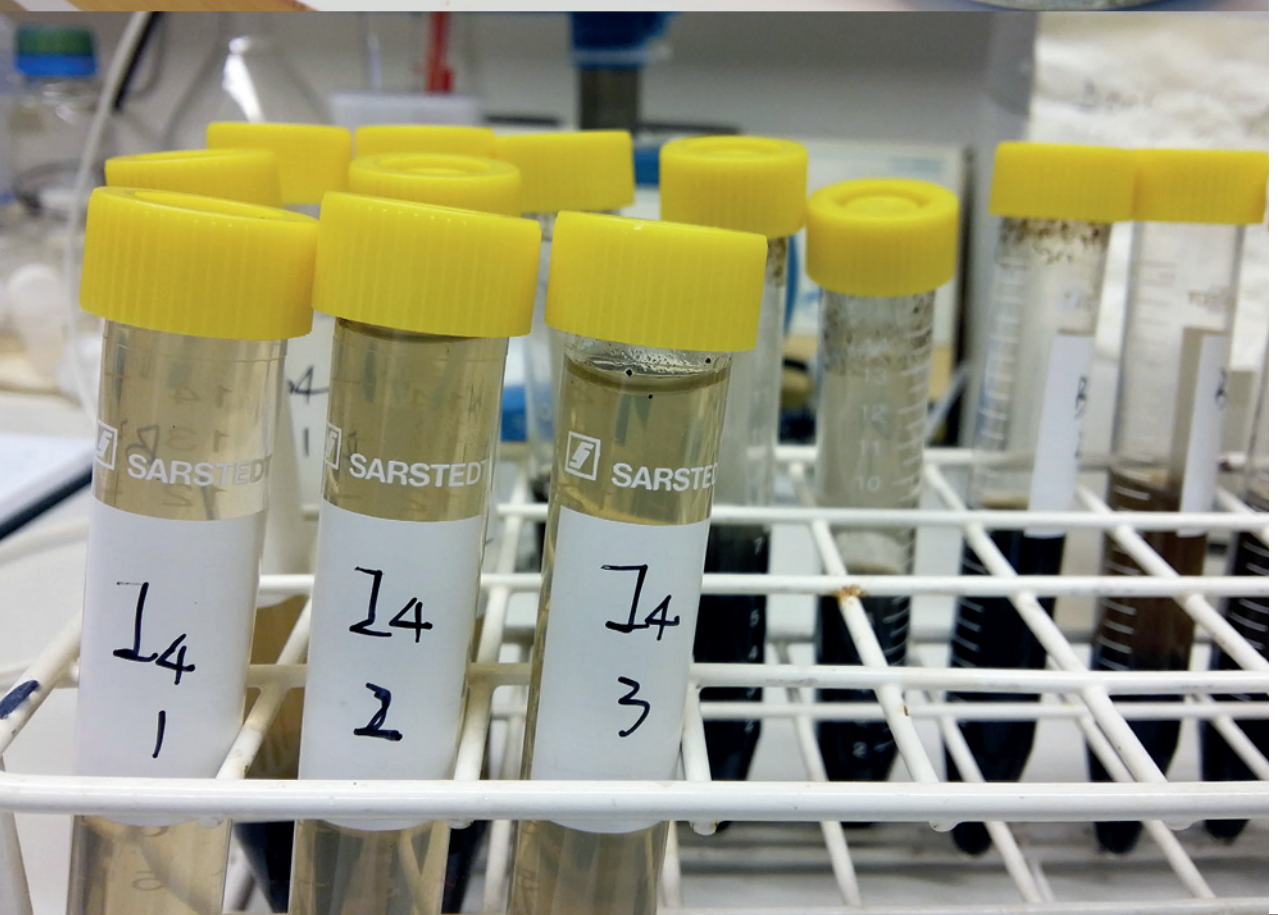
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Fuel 166 (2016) 29–35

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Physio-chemical pretreatments for improved methane potential of *Miscanthus lutarioriparius*

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HIGHLIGHTS

- Miscanthus is a potential feedstock for methane production.
- Methane yields from Miscanthus waste increased with decreasing particle sizes.
- Pre-treatments led improvements in both sCOD and methane yields.
- Mild alkaline pre-treatment was most efficient in enhancing methane yield and rate.
- Competition for biomass for food/feed is avoided by converting Miscanthus to methane.

ARTICLE INFO

Article history:
Received 24 June 2015
Received in revised form 23 October 2015
Accepted 27 October 2015
Available online 31 October 2015

Keywords:
Anaerobic digestion
Lignocelluloses
Methane yield
Miscanthus
Pretreatment
Steam explosion

ABSTRACT

Bio-energy production from lignocelluloses biomass has gained a lot of interest in recent years. *Miscanthus* sp. are high lignocelluloses biomass yielding with relatively high carbohydrate content cultivable on different soils and under climatic conditions. However, the lignocellulosic nature means that cellulose and hemicellulose are cover by lignin network that might limit hydrolysis. In this study, physical pretreatments (size reduction), physiochemical (steam expulsion) and chemical pre-treatments (mild acid and alkaline) were investigated in a view to improve the anaerobic biodegradability of *Miscanthus lutarioriparius* for biogas production. Prior to the pretreatment and methane potential test, the compositional analyses of *M. lutarioriparius* was performed in order unveil the carbohydrate (cellulose and hemicelluloses), protein and lignin contents. From these analyses, the maximum theoretical methane potential was estimated. All pretreatments led to solubilisation of organic matter as was evidenced by increase dissolve COD and ammonium nitrogen. There was a positive correlation between dissolved COD and methane yields meanwhile a negative correlation was observed for reducing sugar and methane yields. The achieved methane yields ranged from 121 to 238 ml CH_4/g VS. Steam explosion, 0.3 M NaOH treatment and 0.5 mm size reduction led to the highest increases in methane yields, which was in the order of 57% with regard to the untreated samples. These improvements resulted in 71% of theoretical methane yield of *M. lutarioriparius*. Alkaline pretreatment in particular also improve the rate of methane production as was evidenced by the fact that as high 15% of the final methane yield that was achieve on the first day as compared to only 3% for the untreated sample. Indeed, the time to reach 90% of the ultimate methane yield was reduced by 13 days following 0.3 M NaOH treatment. *M. lutarioriparius* may therefore represent an interesting candidate as a lignocellulosic feedstock for biogas production after suitable pretreatment.

1. Introduction

With the increasing popularity in the anaerobic digestion (AD) process for biogas production and shortages of organic waste as substrate for biogas production, there is need for alternative substrates, easily accessible and cheap with no conflict with food or

Abbreviations: AD, anaerobic digestion; ADF, acid detergent fibre; ADL, acid detergent lignin; AMPTS, automatic methane potential test system; BMP, biochemical methane potential; COD, chemical oxygen demand; sCOD, soluble chemical oxygen demand; TS, total solids; VS, volatile solids; WW, wet weight.

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http://dx.doi.org/10.1016/j.fuel.2015.10.108
0016-2361/

feed. Biogas production through anaerobic digestion has emerged as one of the renewable energy production technology of choice because through AD biogas as a renewable fuel as well as a biofertilizer can be harness while at the same acting as waste treatment technology. Various forms of organic substrates such as food waste, organic fraction of municipal solid waste, sewage sludge and energy crops can be use as feedstock for biogas production.

Miscanthus sp. are high yielding crop biomass which grows rapidly; to heights as tall as 3 m. It produces a crop every year without the need for replanting with very low nutrient requirements. These attributes has made Miscanthus an energy crop of choice over other energy crops such as maize and triticale. In fact the biomass yield of Miscanthus averages 45 tons dry matter/ha/year [1,2] as compared to an average of 16 tons dry matter/ha/year for maize [3,4]. Amongst the different species of Miscanthus, *Miscanthus lutarioriparius* has been singled out to have outstanding properties as an energy crop of choice. *M. lutarioriparius* has been shown to produce the highest biomass and had the highest photosynthetic rates as compared its other relatives such as *M.* × *giganteus*, *M. sacchariflorus* and *M. sinensis* [5].

Miscanthus is a lignocellulosic energy crop and its conversion to biogas through anaerobic digestion may be limited by its hydrolysis because digestible hemicelluloses are covered by a sheath of insoluble lignin [2,6]. It is worth mentioning though that in some feedstock such as rice, cell wall polymer levels i.e. lignin were not the main determinant for an efficient and effective biomass hydrolysis [7]. Nonetheless, for an effective and thorough conversion of Miscanthus to biogas through AD, an appropriate pre-treatment must be effectuated. Different types of pre-treatments ranging from chemical, physical, physio-chemical and biological have been reported in literature with the aim of improving the biogas or methane yield of a substrate [2,6,8]. Though biological pre-treatment by enzymes has been praised mainly because of environmental benefits [9], the high cost of enzymes remain an essential barrier [10] to implementation of enzyme pre-treatment in full scale operation. Aggressive and vigorous pre-treatments using high concentrations of catalyst (chemicals) at high temperature may improve the methane yield of a feedstock [11]; however these pre-treatment methods are expensive and detrimental to our environment through the wasting of these chemicals in our surroundings.

Concerning the different approaches for improving methane production, this work aims to investigate the effects of pre-treatments of small energy demand, low pollutions levels, lower environmental impact and easy full-scale applicable [12]. In this light, steam explosion, size reduction and low-concentrated chemicals, low temperatures pre-treatments in view to single out the most appropriate pre-treatment as per the AD of *M. lutarioriparius* was studied for the first time. The achieved methane yields and methane production rates were correlated with released sugars and substrate dissolution during pre-treatment. The methane yields/methane production rates of the raw and pretreated samples were studied under mesophilic conditions, with the aid of the automatic methane potential test system (AMPTS II).

2. Materials and methods

2.1. Source and description of material

The *M. lutarioriparius* was collected from Miscanthus nursery at Hunan Agricultural University (28°10′54″N 113°05′06″E) Eastern Changsha, China at the end of November 2014. This new species of *Miscanthus* with high productivity, high cellulose content and strong resistance to pest has been successfully cultivated from 2005 to 2014. The original sample had total solids (TS) of 88.9% and volatile solids (VS) of 84.4%. Part of the crop was immediately subjected to steam explosion while the rest was transported to the Department of Biotechnology, Lund University, Sweden (test facility) and store at +4 °C prior to use. Anaerobically digested sewage sludge from Källby wastewater treatment plant (Lund, Sweden) was used as inoculum. The inoculum was pre-incubated at 37 °C under anaerobic conditions for 5 days to reduce residual or background methane production. The inoculum had an average TS of 4.7%, VS of 2.8% and pH of 7.5. Other characteristics of substrate and inoculum are presented in Table 1.

2.2. Pre-treatments

2.2.1. Steam explosion

Steam explosion pre-treatment test was performed in a 5.0 L batch vessel equipped with a reactor chamber, a reception chamber and a steam generator. The sample was steam heated at a temperature of 198 °C and a pressure of 1.5 MPa for 5 min. The sample was thereafter discharged through restricted orifices and exploded at atmospheric pressure into a pulp. The samples were dried at 60 °C prior to use for methane production.

2.2.2. Size reduction

For size reduction, a grinder Grindomix 200 (Retch USA) with different orifices was used to grind the samples to pass through 0.5 mm, 5 mm, 10 mm and 20 mm sieves. For simplicity, the four different particle sizes were termed a, b, c and d respectively (Fig. 1). These samples were thereafter investigated without further treatment in a subsequent methane production process as describe below.

2.2.3. Chemical pre-treatment

The four crop particle sizes (a, b, c and d, Fig. 1) were chemically pre-treated at 60 °C for 24 h with mild acid and mild alkaline, i.e. 0.1 M HCl and 0.1 M NaOH respectively. In another line of chemical pre-treatments, the biggest particle size (sample d) which had shown low methane yields and extended lag phase as compared with the other particle sizes was subjected to various concentrations of acid and alkaline. The acid and alkaline concentrations were varied from 0.05 to 0.3 through 0.15 and 0.2 M while the temperature was maintained at 60 °C for 24 h. As a control and to evaluate the effect of heat treatment on the samples and subsequent methane production, all the pre-treatments for the above particle sizes were performed at 60 °C for 24 h while replacing the acid or alkaline solution with tap water, here termed 'hot water' pre-treatment.

Table 1
Characteristics of *Miscanthus lutarioriparius* and inoculum.

	Miscanthus	Inoculum
TS (% ww)	88.9 ± 0.5	4.8 ± 0.4
VS (% ww)	84.4 ± 0.3	2.8 ± 0.1
T COD (g/kg)	922 ± 9	nd
sCOD	4.6 ± 0.1[a]	nd
pH	Nd	7.8
NH_4-N (mg/L)	8.9 ± 0.2[a]	2300 ± 12
Alkalinity (mg/L)	Nd	6500 ± 25
Cellulose (% ww)	41.2	nd
Hemicellulose (% ww)	24.2	nd
Lignin (% ww)	9.5	nd
Crude protein (% ww)	3.2	nd

[a] Are values determined from the 0.5 mm samples, nd = not determined.

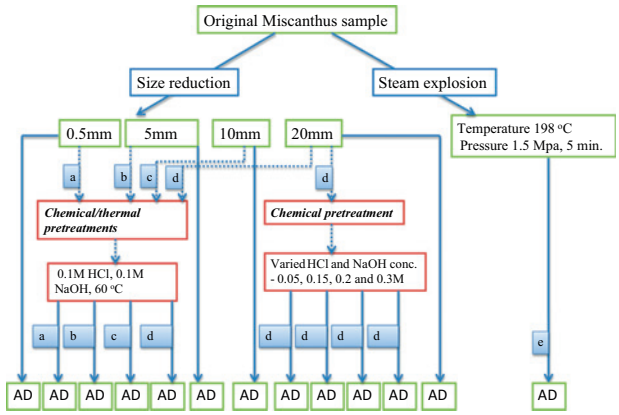

Fig. 1. Experimental protocol and pretreatments performed during the entire study.

2.3. Biochemical methane potential test

The biochemical methane potential test (BMP) was performed as was previously reported by Shen et al. [6]. The BMP tests were performed in an Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control AB, Sweden) under mesophilic conditions for 50 days. Specifically, the VS based inoculum to substrate ratio was set at 2:1 and two controls were included in the tests. The first control was the inoculum only and the methane production from the inoculum was subtracted from the test samples. The second control was AD of a known substrate, cellulose (Avicel PH-101, Sigma–Aldrich, St. Louis, MO, USA) which was used to test the activity of the inoculum and evaluate the experimental protocol. The steam pre-treated sample (e) was used directly for AD (Fig. 1). The differences sizes (Section 2.2.2) were used directly for AD and twelve chemical (HCl and NaOH) and thermal (60 °C) pre-treated samples. Sample d which was pre-treated with varying concentrations of acid and alkaline produced eight AD experiments (Fig. 1). All AD experiments were carried out in triplicates totalling 81 lines of AD experiments.

2.4. Analytical methods and calculations

Standard methods [13] were used for the analyses of TS, VS and pH. Total proteins, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were be determined sequentially with the aid of the filter bag system (Ankom Technology, New York) by an accredited food/feed laboratory (Eurofins Food and Feed Testing, Lidköping, Sweden). Total nitrogen (N) was gotten by dividing the total protein concentration by a factor of 6.25. The carbon (C) was assumed to be 48% of TS as recommended by Klimiuk et al. [14] Percentage cellulose in the sample was computed as the difference between ADF and ADL while the percentage of hemicellulose was computed as the difference between NDF and ADF with recommendation from Wolfrum et al. [15]. Total and soluble chemical oxygen demand (COD), sCOD and tCOD respectively were determined by Dr. Lange test cuvette LCK 914 (HACH LANGE GmbH, Germany) after appropriate dilutions and filtration with 0.45 μm nylon membrane filter to fall within the detection range. COD solubilisation was calculated according to the following equation:

$$\text{COD } solubilisation = (\text{sCOD} \div \text{tCOD}) \times 100 \quad (1)$$

Soluble nitrogen or ammonium nitrogen (NH_4-N) was determined with Dr. Lange cuvette CLK 303(HACH LANGE GmbH, Germany). Reducing sugar released during pre-treatments were analysed by the 3,5-dinitrosalicylic acid (DNS) technique according to Moshi et al. [16]. Hourly or daily methane volume and methane production rate, normalised at 0 °C, 1 atm and zero moisture content was down-loaded directly from AMPTS software.

2.5. Kinetics

The rate of degradation of the different samples was assumed to follow a first-order rate of degradation, and thus the following formula was used to describe the methane production of each category of treatment: A traditional first-order model (Eq. (1)) with a lag-time parameter [17] was used to evaluate the kinetic degradation profiles.

$$B(t) = B_0(1 - \exp(-k \cdot (t - \theta))) \quad (2)$$

where $B(t)$ is the cumulative methane yield (ml CH_4/gVS) after 50 days of incubation, B_0 is the maximum theoretical or ultimate methane yield (ml CH_4/gVS) of the substrate, k is the rate constant or hydrolysis rate constant (d^{-1}), t is the time (d) and θ is the lag time (d). The cumulative methane yield of each waste material was calculated by dividing its average cumulative methane production by the amount of VS added to each reactor.

2.6. Statistical analysis

Dixon's test ($P \leqslant 0.05$) was used to eliminate outliers in the replicate-BMP tests. One way analysis of variance (ANOVA) was performed with the statistical package SPSS, version 16 to assess statistical differences in methane yield between the various sample sizes and pre-treatments at a 95% confidence level to accept or reject the null hypothesis. Therefore, "significant" was used only when a statistical analysis has been performed.

3. Results and discussions

3.1. Feedstock characteristics and changes after pre-treatments

M. lutarioriparius was characterised regarding the TS and VS, COD, total, and ammonium nitrogen (total-N and NH_4-N)

concentration as well as cellulose, hemicelluloses and protein and lignin contents. The TS and VS of Miscanthus were on average 88.9% and 88.4% ww respectively. The percentages of cellulose, hemicelluloses and lignin were 41.2%, 24.2% and 9.5% ww respectively (Table 1). The above values are the same range as those reported by Menardo et al. [18] for Miscanthus with the exception of lignin which was much lower in the present study i.e. 15.6% as compared to 9.5% ww. From these results, it could be seen that *M. lutarioriparius* was less fibrous (low hemicelluloses content of 24% ww) as compared to *M.* × *giganteus* (28%) and *M. sacchariflorus* (30%). The C/N of Miscanthus in the present study was 82.9 which was close to the C/N of 103 reported for a different species i.e. *M. sacchariflorus* [14]. The total COD was 922 g/L, which was in total agreement with VS values of 88.4% or 884 g/L considering the fact that the VS was mostly carbohydrate in nature (Table 1). Theoretically, a unit of VS in form of carbohydrate should yield 1.07 units of COD. The dissolved COD and nitrogen (NH_4-N) of the original sample were however very low as compared to the total COD and nitrogen.

In most cases, except for acid and 'hot water' treatments, the pre-treatments led to an increase in COD dissolution as seen by the increase in sCOD and NH_4-N (Table 2). Increase in sCOD and NH_4-N indicated some of organic nitrogen and organic matter (in general) was solubilised. The low NH_4-N for steam explosion sample as compared to the raw sample and other pre-treatments could be explained as probably the lost of nitrogen in the form of ammonia gas during the extended transport and storage time. Alkaline pre-treatment led a significant increase in sCOD and reducing sugars with regard to acid treatment. In addition, both parameters increased with deceasing particle size. When the largest particle size (20 mm) was pre-treated with varying concentration of acid and alkaline, the NH_4-N, sCOD and reducing sugars showed an increasing trend with increasing acid and alkaline concentration though alkaline pre-treatment showed the highest increase in both NH_4-N and sCOD. The degree of increase in sCOD was not proportionate to the increase in reducing sugars meaning that the carbohydrates were hydrolysed to other products than reducing sugars for alkaline pre-treatment. At best, the highest COD solubilisation i.e. 3.3% was achieved for 0.3 M NaOH pre-treatment of the 20 mm particle size sample. With regard to the untreated 20 mm sample, the 0.3 M NaOH led to a 4.8 fold increase in sCOD. This in agreement with studies on alkaline pre-treatment of bamboo [6] where alkaline pre-treatment was demonstrated to lead to higher COD solubilisation with regard to acid and enzyme (cellulase) treatments. Steam explosion showed a rather poor impact as per the release of sugars and dissolution of COD. Similar findings have been reported by Menardo et al. [18]. It would seem therefore that under certain steam expulsion regimes (different temperature, pressure durations) the cellulose bundles may be defibrillated [18] without actual hydrolysis of the cellulose and hemicelluloses structure.

3.2. Methane yields

The 50-day methane yields of all the samples are presented in Fig. 2. The methane yields achieved in the present study ranged from 121 to 238 ml CH_4/g VS added. The yields were in some cases higher than those reported for different species of pre-treated or ensiled Miscanthus. Methane yield of ensiled *M. sacchariflorus* has been reported to be 190 ml/gVS while that for ensiled *Miscanthus* × *giganteus* was 100 ml/gVS [14]. Size reduction from 10 mm down to 0.5 mm did not lead a significant difference ($p \leqslant 0.05$, $n = 3$) in methane yield though the 0.5 mm samples showed highest methane of 237.8 ± 15.3 ml CH_4/gVS. On the other hand, the methane yield at particle size of 20 mm was significantly lower ($p \leqslant 0.05$, $n = 3$) as compared to the rest (Fig. 2A). It has been reported that size reduction can only be significant if it down to a few mm [19] where under such conditions there is an increase in surface area warranting a proper and effective enzymatic attack and eventual methanation. In fact, it has been reported that size reduction down to a few mm can decrease the crystallinity of cellulose and also its degree of polymerisation [20]. The control experiments, i.e. pre-treatment at 60 °C while replacing acid and alkaline with water (hot water) of the various particle sizes led to significantly lower ($p \leqslant 0.05$, $n = 3$) methane yields (Fig. 2B). Acid pre-treatment (0.1 M HCl) of the different particle sizes also

Table 2
Dissolved nitrogen, dissolved COD, reducing sugars, methane productivity and hydrolysis rate constant (k) determined after various pretreatments and BMP.

	NH_4-N (mg/kg ww)	SCOD (g/kg ww)	Sugar (g/kg ww)	k (d^{-1})	ml CH4/gVS·d day 1
Raw (0.5 mm)	8.9 ± 0.2	4.6 ± 0.1	1.6 ± 0.0	0.05	18.2
5 mm	nd	nd	nd	0.04	15.3
10 mm	nd	nd	nd	0.03	17.7
20 mm	nd	nd	nd	0.03	12.9
Steam explosion	7.1 ± 0.5	13.6 ± 0.1	1.8 ± 0.0	0.03	29.6
0.5 mm 0.1 M HCl	11.2 ± 0.1	6.2 ± 0.1	5.2 ± 0.2	0.07	19.1
5 mm 0.1 M HCl	11.2 ± 0.0	5.6 ± 0.5	5.2 ± 0.2	0.05	17.4
10 mm 0.1 M HCl	11.5 ± 0.1	5.9 ± 0.1	6.2 ± 0.0	0.04	18.3
20 mm 0.1 M HCl	11.45 ± 0.4	5.3 ± 0.1	5.2 ± 0.1	0.03	14.9
0.5 mm 0.1 M NaOH	11.1 ± 0.1	16.3 ± 0.4	8.3 ± 0.2	0.09	32.4
5 mm 0.1 M NaOH	9.6 ± 0.1	15.5 ± 0.4	7.1 ± 0.2	0.09	27.4
10 mm 0.1 M NaOH	10.9 ± 0.1	15.5 ± 0.1	10.8 ± 0.2	0.09	24.7
20 mm 0.1 M NaOH	11.3 ± 0.0	14.0 ± 0.1	5.6 ± 0.1	0.08	22.6
0.5 mm 60 °C	16.3 ± 0.0	5.4 ± 0.1	0.3 ± 0.1	0.06	6.5
5 mm 60 °C	12.1 ± 0.0	4.5 ± 0.3	0.6 ± 0.0	0.05	4.3
10 mm 60 °C	19.0 ± 0.1	5.5 ± 0.1	2.5 ± 0.0	0.04	4.6
20 mm 60 °C	16.3 ± 0.2	4.4 ± 0.2	1.2 ± 0.0	0.03	1.5
20 mm 0.05 M HCl	6.5 ± 0.1	4.2 ± 0.2	1.0 ± 0.0	0.04	12.4
20 mm 0.15 M HCl	8.2 ± 0.0	4.9 ± 0.1	2.9 ± 0.1	0.13	17.7
20 mm 0.2 M HCl	8.9 ± 0.1	6.5 ± 0.2	4.9 ± 0.1	0.14	18.9
20 mm 0.3 M HCl	9.9 ± 0.1	8.2 ± 0.1	6.0 ± 0.1	0.15	23.3
20 mm 0.05 M NaOH	9.9 ± 0.2	11.9 ± 0.4	0.9 ± 0.0	0.03	16.1
20 mm 0.15 M NaOH	12.2 ± 0.4	22.1 ± 0.1	0.7 ± 0.0	0.04	23.7
20 mm 0.2 M NaOH	18.0 ± 0.2	26.3 ± 0.4	0.9 ± 0.0	0.04	22.5
20 mm 0.3 M NaOH	21.7 ± 0.0	30.8 ± 0.1	0.8 ± 0.0	0.06	22.3

nd, not determined.

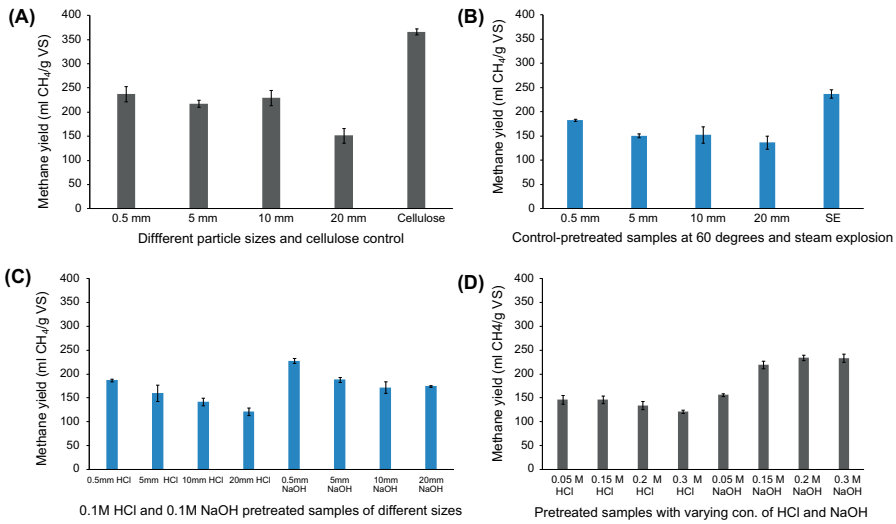

Fig. 2. Methane yields of *M. lutarioriparius* after the various treatments. (A) Shows the methane yields of the different particle sizes. (B) shows the methane yield of 'hot water' pretreated samples. (C) Shows the methane yields of different particle sizes after acid and alkaline pretreatments while (D) shows the methane yields of sample 20 mm after pre-treatment with increasing concentration of alkaline and acid (SE stands for steam explosion).

led to significantly lower methane yields (Fig. 2C). Lower methane after acid and 'hot water' treatments have been reported in other studies [6,20]. It is probable that during these pre-treatments, the crystallinity of cellulose and re-polymerisation of amorphous subunits occurred leading to the low methane yields as well as low sCOD and reducing sugar concentrations (Table 2). It is often so that acid pre-treatment is performed either at high temperature, low acid concentration or low temperature and high acid concentration [20]. However, in the present study, low temperature and low acid concentrations were investigated at an extended residence time of 24 h. It is plausible therefore to state that the destruction of lignin network that is normally reported for acid pre-treatment [21] was not achieved in the present study. Instead, lignin may have just been relocated as reported by Bruni et al. [22]. This postulation was also supported by the low sCOD and poor reducing sugar release (Table 2).

High methane yield (237.2 ± 8.2 ml CH_4/g VS) comparable to those achieved at 0.5 mm particle size and after 0.2–0.3 M NaOH pre-treatment of 20 mm particle sized samples was achieved after steam expulsion (Fig. 2A–C). It has been reported that the sudden pressure release during steam expulsion defibrillates the cellulose bundles and this may result in a better accessibility of the cellulose for enzymatic hydrolysis and fermentation [18]. Alkaline pre-treatment had been reported to impart a similar outcome. Alkaline pre-treatment may lead to swelling of the fibres (cellulose and hemicelluloses) to enhance the accessibility of hydrolytic enzymes [23]. Alkaline treatment can also saponify ester bonds in lignin leading to partial or total disruption of lignin thereby exposing cellulose and hemicelluloses for eventual enzymatic attack [24]. Alkaline pre-treatment (0.1 M NaOH) of the different particle sizes led to a significant improvement in methane yield (Fig. 2A and C). Increasing the alkaline concentration in the pre-treatment of the 20 mm sample led to significant increase in methane yield when the alkaline concentration was greater than 0.05 M (Fig. 2D). It should be noted that alkaline (0.2 and 0.3 M NaOH) pre-treatment as mentioned above led to a similar methane yield (234.4 ± 5.5 ml CH_4/g VS) as steam exploded samples (Fig. 2B and D). This translated into a 54.7% improvement as compared with untreated, 20 mm sample. Though no enzymes were added during the pre-treatment and fermentation (AD process), the inoculum used in the present study has earlier been showed to possess cellulotytic properties [6]. It is probable as reported by Bruni et al. [22] that alkaline pre-treatment also resulted in the conversion of lignin to acetic acid which was readily converted to methane during methanogenesis. The methane yields achieved in this study were in the same range as those recorded for other lignocelluloses biomass (170–390 ml CH_4/gVS) cultivated on marginal land [25]. The yields are also in line with those of various grasses (253 – 394 ml CH_4/gVS) achieved under different harvesting times and year of cultivation [26]. On the other hand, sugar and starch based energy crops have been reported to have higher methane yields of about 450 ml CH_4/gVS [25].

In general, there was a positive correlation between methane yield and COD solubilisation (sCOD). As with methane yield, the highest sCOD was noted for pre-treatment of the 20 mm sample with 0.3 M NaOH. Lowest methane yield (121.4 ml CH_4/g VS) of the 20 mm 0.1 M acid pre-treatment was also mirrored by its low sCOD which was comparable to acid pre-treatments and the raw sample (Table 1). Similar conclusions have been documented in other studies [6,27]. On the contrary high reducing sugar yields did not necessary translates to high methane yields. Increasing the concentration of the alkaline led to high methane yields (Fig. 2D) however the reducing sugar concentrations followed an opposite trend. This in line with observation from a pre-treatment experiment using bamboo as substrate [6]. This observation may also confirm the school of thought that alkaline pre-treatment do no necessary lead to complete destruction of the lignocelluloses structure of biomass but rather lead to its swelling, increase pore size and breakage of hydrogen bonds [28,29] making it readily accessible to hydrolytic enzymes and eventual methanation [6].

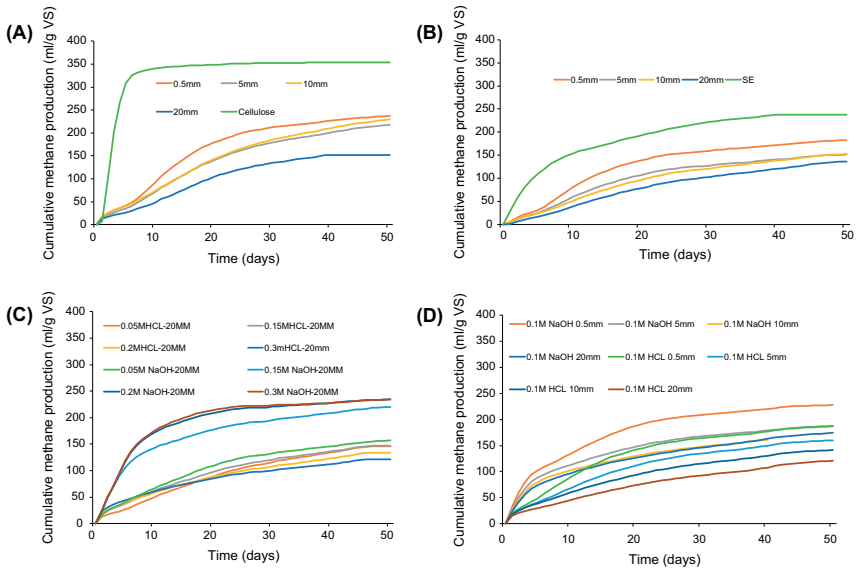

Fig. 3. The 50-day cumulative methane production of various samples of pre-treated *M. lutarioriparius* (SE stands for steam explosion).

The cellulose control showed a methane yield of 366.5 ± 6.9 ml CH4/g VS (Fig. 2A), 88% of the theoretical methane yield of cellulose (415 ml CH_4/g VS) which is the same range as those reported in other studies [6,30]. The theoretical methane yields of *M. lutarioriparius* from the chemical composition with and without lignin (Table 1) [6] were computed from the theoretical methane yields of the respective molecules to be 333.8 and 402.3 ml CH_4/g VS respectively. These empirical values are in agreement with those reported in another study. The theoretical methane yield of Miscanthus (species not defined) using Boyle's formula has been reported to be 450.8 ml CH_4/gVS when all carbon (plus lignin) was considered to be assimilated [18]. Without considering lignin, as high as 71% of the theoretical methane was achieved when the samples were steam exploded, reduced sample size to 0.5 mm and 0.2–0.3 M NaOH alkaline of 20 mm sample size. However, when lignin was considered only 59% of the theoretical yield was achieved during this present study. Nonetheless, it has been proven that in various species of ensiled Miscanthus lignin was not degraded during anaerobic digestion; the lignin content in the digestate was reported to be in same range as that in the feedstock [14]. It should be noted that with more aggressive and vigorous pre-treatments, lignin can be degraded under anaerobic conditions to produce methane [31,32]. It is plausible therefore to state that though the present study led to more than 50% improvement in methane yield, there are still room for further improvement harness even more energy from *M. lutarioriparius* probably with the aid of more vigorous pre-treatments.

3.3. Methane production rate

From an economic point of view, it is vital to speed up the production capacity of a (bio) process. Pre-treatments are normally performed with a view to improve digestibility or the ultimate yield but also importantly to improve the rate or speed of production (volumetric productivity, ml CH_4/g VS·day). Fig. 3 shows the 50-days cumulative methane production of *M. lutarioriparius*. Methane production began within the first 24 h (day 1) of incubation for all samples. The highest methane production rate on the first day (32 ml CH_4/g VS·day which is about 15% of final methane yield) was achieved for the 0.1 M NaOH pre-treatment of the 0.5 mm samples while the lowest (1.5 ml CH_4/g VS·day, which is only about 1% of the final methane yield) was achieved for the 'hot water' or 60 °C pre-treatment of the 20 mm samples (Table 2, Fig. 3). It is interesting to note that the 20 mm sample showed an initial rate of 13 ml CH_4/g VS·day (about 9% of the final methane yield) as opposed to the 1.5 ml CH_4/g VS·day when pre-treated with hot water (Table 2). This observation is in line with the postulation that 'hot water' (60 °C) might actual increase the crystallinity or recrystallinity of cellulose [20]. In fact, the highest percentage of methane produced on day one for the 'hot water' pre-treatment was only 3% of the final or 50-day yield for the 0.5 mm samples, the lowest as compared to the other treatment option in the present study. The time for 90% methane production was as short as 5 days for the cellulose control and as high as 42 days for 'hot water' 20 mm sample (Fig. 3). As best, 90% methane production was achieved after 17 days for the 20 mm samples after 0.3 M NaOH pre-treatment. It is therefore plausible to state that alkaline pre-treatment of *M. lutarioriparius* does not only speed up the methane production process but it may also lead to shortening of the residence time which would be of economic interest especially in commercial biogas processes.

The calculated hydrolysis constants (k) values in present study ranged from 0.03 to 0.15 d^{-1} and 0.33 d^{-1} for the cellulose control (Table 2). These values are in the same as those reported for carbohydrates such as cardboard and newsprints [17,33]. The k values appeared to increase as sample particle size was decreased wherein the same trend was observed when the different particle sizes were pre-treated (Table 2). This can be explained by the process acceleration as a result of the reduction in particle size. Also, increasing the alkaline or acid concentration seemed to increase

the k constant (Table 2) which correlated in general with increasing methane yields (Fig. 2). The highest k value was recorded for cellulose control (0.33 d^{-1}) followed by 0.15 d^{-1} for the 0.3 M HCl, 20 mm pre-treated sample meaning these samples produced methane at a much higher rate as compared to the other treated (HCl in this same case) or untreated samples [33]. That notwithstanding, the k values obtained in this study are in general less than those of carbohydrate, protein and lipid meaning that the hydrolysis rate may be neglected as recommended by Feng et al. [34]. It has been reported that hydrolysis rate should be described by first-order kinetics if the hydrolytic enzyme concentration which is supposed to control hydrolysis exceeds the available amount of adsorption sites of the particulate substrate [17]. Other factors such as the origin and pre-handling of inoculum; inoculums to substrate ratio, and temperature mixing may also greatly affect the rate constant [33], reason why these values to should be treated with caution.

4. Conclusions

M. lutarioriparius is a lignocellulosic biomass with high content of cellulose and low lignin content which can be converted to bioenergy in form of biogas. Pre-treatments led to an improvement in COD solubilisation and reducing sugar concentrations. Size reduction of biomass led to significant improvement in methane yield and methane production rate. Mild alkaline pre-treatment and steam explosion also led to significant improvement in methane yields. These pre-treatments could aid the harness of up to 71% of the theoretical methane yield of *M. lutarioriparius*. Alkaline pre-treatment was of particular interest as it not only led to a significant improvement in methane yield but the up to 90% of the methane was produced within two weeks of digestion. This could be of interest to commercial biogas plant as shortened resident time without jeopardizing process performance may significantly improve profits.

Acknowledgements

This research was conducted in the framework of Co-digestion of Multiple Feedstocks Project (SQ2013ZOC500005) supported by the EU-China Innovation Funding for Small and Medium size Enterprise from the China Ministry of S&T and Swedish Research Links Program. The corresponding author thanks for the funding support from Beijing Overseas Talents Program.

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Paper III

Bioresource Technology 235 (2017) 389–395

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Changes in composition, cellulose degradability and biochemical methane potential of *Miscanthus* species during the growing season

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HIGHLIGHTS

- *Miscanthus* composition changed slightly during growing season.
- The BMP correlated positively to the cellulose degradability.
- Prolonged growth time led to a decrease in BMP and the cellulose hydrolysis rate.
- Cellulose degradability impacts on the economic benefit from *Miscanthus*.

ARTICLE INFO

Article history:
Received 15 January 2017
Received in revised form 21 March 2017
Accepted 22 March 2017
Available online 24 March 2017

Keywords:
Miscanthus
Methane potential
Growing season
Cellulose
Biofuels

ABSTRACT

The composition, cellulose degradability and biochemical methane potential (BMP) of *M. sinensis*, *M. floridulus*, *Miscanthus* × *giganteus* and *M. lutarioriparius* were investigated concomitantly at different growth/harvest times during their growing season. For all the four species, there was only a slight change in the compositional content. Meanwhile there was a huge change in the BMP values. At the growth time of 60 days the BMPs ranged from 247.1 to 266.5 ml g^{-1} VS. As growth time was prolonged, the BMPs decreased by 11–35%. For each species, the BMP was positively correlated to the cellulose degradability with the correlation coefficients (R^2) ranging from 0.8055 to 0.9925. This suggests that besides the biomass yield, it is justifiable to consider cellulose degradability when selecting the suitable harvest time for biofuels production from *Miscanthus*, especially in tropical and subtropical regions where *Miscanthus* can be harvested twice or more within a year.

1. Introduction

The perennial C4 grass *Miscanthus*, native to subtropical and tropical regions originating from Asia (Brosse et al., 2012) has been considered as a high potential energy crop because of its high photosynthetic rate and low levels of land, water and nutrient requirements (Lewandowski et al., 2003; Tubeileh et al., 2016). It was brought to Europe (temperate or cold zones) in 1935 by Aksel Olsen (Wahid et al., 2015) and cultivated throughout Europe as an ornamental plant. Since the 1980s the potential of *Miscanthus* as a bioenergy crop has been investigated (Wahid et al., 2015). In Asia, *Miscanthus* is often used as animal feed and for roofing material but it was not considered as an energy crop until the end of 20th century (Wahid et al., 2015). This plant, containing more than 20 species, can grow widely under various climate conditions, including tropical, subtropical, temperate and even cold climates (Głowacka, 2011; Li et al., 2016b). It has been cultivated widely as an energy crop in many countries especially in Asia, Europe and North America (Brosse et al., 2012; Kim et al., 2012; Tubeileh et al., 2016; Wang et al., 2010).

There are three main routes of *Miscanthus* biomass conversion to energy: direct combustion, bioconversion (saccharification and fermentation) into biofuels such as ethanol and methane, and thermochemical conversion (pyrolysis) into bio-oils (Tubeileh et al., 2016). Among these routes, bioconversion of *Miscanthus* has recently attracted the most attention as this bioprocess produces diversity biofuel products without the need of special (high-temperature and high-pressure) equipments. Many of these bioconversion technologies, and their near- and long-term commercial potentials, have been discussed in literature (Hayes,

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http://dx.doi.org/10.1016/j.biortech.2017.03.128
0960-8524/

2009, 2013b). During the bioconversion process, carbohydrates (cellulose and hemicellulose) are often degraded to sugars which are then consumed by microorganism for biofuels production. The biomass yield, the composition and the biodegradability of the carbohydrate in *Miscanthus* plant are important factors that affect the biofuels production efficiency and cost. Selecting a suitable harvest time is very important for improving the economic benefit for biofuels production from *Miscanthus* because the biomass yield, biochemical composition and structure are known to change during the growth and decline seasons of the plant (Purdy et al., 2016; Godin et al., 2013a,b).

In temperate zones, such as in Europe, *Miscanthus* shoots start to emerge during spring (April) and accumulate rapidly through summer with the highest yield around September (Wahid et al., 2015). The yield then starts to decline around October until February as results of the shedding of dead leaves and translocation of nutrient to the rhizomes (Beale and Long, 1997). It is harvested once a year during the harvest time from August (green) to the following April (matured and dried in the field) (Frydendal-Nielsen et al., 2016; Hayes, 2013a). The effects of the harvest time over this harvest window on biomass yield, composition and methane yield were investigated in some previous literature. For example, it has been reported that early harvest (October to early December) had up to 38.4% more biomass and 29.3% biofuel per hectare than the late harvests (the following March and April) (Hayes, 2013a). Also, another study (Frydendal-Nielsen et al., 2016) demonstrated that Green biomass (September harvest) showed higher methane yield than biomass harvested in the following February and April in Denmark. However, in the tropical and some subtropical areas, such as in south of China, the temperature is high enough for *Miscanthus* growth in early spring and later autumn; and even all throughout the year (Chou, 2009). In these areas, *Miscanthus* may be harvested twice or more within a year. It is plausible therefore to state the harvest window is all round the whole year. In such areas, selection of suitable harvest times is more important to obtain a higher income for biofuels production from *Miscanthus*. Nonetheless, to our knowledge, there are just a few literature that can be consulted to this effect (Arundale et al., 2015).

In the present work, four representative *Miscanthus* species, *M. sinensis*, *M. floridulus*, *M. lutarioriparius* and *Miscanthus* × *giganteus* were harvested at different growth times during their growing season (early March to October). The biochemical compositions, enzymatic degradability and methane production potential (BMP) of each sample were analyzed for the purpose of generalizing their changes during the growing season. The data obtained in this work may be valuable for the determination of suitable harvest times for biofuels production from *Miscanthus*, especially in tropical and subtropical regions.

2. Materials and methods

2.1. Sample preparation and inoculum

The samples of *M. sinensis*, *M. floridulus*, *Miscanthus* × *giganteus* and *M. lutarioriparius* were harvested from the *Miscanthus* nursery at Hunan Agricultural University (Changsha City, Hunan Province, China) (28°10′54″ N 113°05′06″ E) during four sampling times from May 2015 to September 2015 namely, 10th May, 20th June, 31st July and 9th September. In the *Miscanthus* nursery at Hunan Agricultural University, the *Miscanthus* shoots start to emerge on about the 10th March and start to wither around late October. Therefore, in this paper the growth times of the samples collected at 10th May, 20th June, 31st July and 9th September were respectively defined as 60, 100, 140 and 180 d respectively. The economic benefit for biofuels production from *Miscanthus* depends on many factors such as biomass yield, fertilizer input, and harvest cost etc. In our practices, if the harvest/growth time is less than 60 days, the total benefit from the *Miscanthus* would be low. Therefore in this manuscript we selected 60 days as the minimal harvest/growth time for investigation. After harvested by a reaping hook the fresh stem together with leaves was cut into 3–5 cm and grinded by a Grindomix 200 (Retsch, Germany) to pass through 20 mm sieves for size reduction. The grinded samples were immediately used for volatile solids (VS), total solids (TS) determination and BMP test. The samples were also used for biochemical compositional analyses and cellulose degradability test. Prior to the composition and cellulose degradability tests the grinded samples were dried in a 60 °C oven till constant weight. The inoculum was collected from a mesophilic anaerobic digester in Gaobeidian Wastewater Treatment (Beijing, China).

2.2. Determination of BMP

The BMP of the *Miscanthus* sample was evaluated in batch automatic methane potential test system (AMPTS II, Bioprocess Control AB, Sweden). The test was conducted in triplicates, under mesophilic conditions (37 ± 0.5 °C) and the inoculum to substrate ratio was set at 2:1 in terms of grams of VS. The experimental protocol was performed as was previously reported in a previous study (Li et al., 2017; Peng et al., 2016). Two sets of controls were included in the test. Firstly, the inoculum only was used, to measure its intrinsic methane production. A second control containing cellulose (Avicel PH-101, Sigma-Aldrich) was used to validate the experimental setup and procedure. The experiments were terminated after 39 days of incubation when the daily methane production was less than 1% of the total production (Zhou et al., 2017). The methane produced by the inoculum was subtracted from the results obtained from the test samples.

2.3. Determination of compositions

The compositions of each plant sample were determined by the standard analysis of biomass composition described by the National Renewable Energy Laboratory (NREL) (A Sluiter et al., 2007). 0.3 g of dry sample was treated with 3.0 mL 72% H_2SO_4 at 30 °C for 2 h, then diluted to 4% and autoclaved at 121 °C for 60 min. The hydrolysis solution was filtered and analyzed for sugar content. Glucose, xylose and arabinose concentrations of the hydrolysate were measured by HPLC system equipped with a Hi-Plex Ca column (7.7 × 300 mm, Agilent Technology, USA), LC-20AT pump (Shimadzu, Japan) and RID-10A refractive index detector (Shimadzu, Japan), using water at a flow rate of 0.6 ml min^{-1} as mobile phase. The amounts of released glucose, xylose and arabinose were used for calculating the cellulose, xylan and araban contents, respectively. The solid residue was dried at for 12 h and weighted. The dried solid residue was further placed in a 550 °C muffle furnace for 2 h. The weight of ash was recorded and the content of Klason lignin was calculated by deducting the ash content from the solid residue. The TS and VS of the plant samples and the inoculum were conducted according to standard methods (APHA, 2005).

2.4. Determination of cellulose enzymatic hydrolysis rate

The reaction system for enzymatic cellulose hydrolysis was prepared in 50 mM sodium acetate, pH 5.0 with the dry plant sample of 2% (w/v). The loading rate of the commercial cellulase CTec2 (http://www.bioenergy.novozymes.com/) for hydrolysis was 20 mg g^{-1} cellulose. The reaction volume was 1000 μL in a 2 mL Eppendorf tube, which was sealed by winding parafilm after closing the lip, and put in a water bath at 50 °C for 72 h. After hydrolysis the glucose concentrations were measured by HPLC

described in the segment of 2.3. The cellulose enzymatic hydrolysis rate was calculated according to the following formula:

$$\text{Cellulose hydrolysis rate (\%)} = \frac{\text{mass of released glucose} \times 0.9}{\text{mass of cellulose in the sample}} \times 100\% \quad (1)$$

2.5. Statistical analysis

Data are expressed as the means ± standard deviations of the triplicate measurements. Differences between mean values were examined by T-test based on the ANOVA analysis using Origin 8.0, and statistical significance was assumed at a level of $P < 0.05$. The correlation analysis based on the orthogonal regression between the BMP and the cellulose enzymatic hydrolysis rate was performed using Microsoft Excel.

3. Results and discussion

3.1. Biochemical methane potential (BMP)

The BMPs of the four *Miscanthus* species harvested at different growth times (60, 100, 140 and 180 d) analyzed with the aid of the AMPTS II, are presented in Fig. 1 (The data shown in Appendix Table 1). The results showed that the growth time significantly affected the BMP of all the four *Miscanthus* species. The samples harvested at the growth time of 60 d showed higher BMPs as compared to those harvested at longer growth time especially noted for *Miscanthus* × *giganteus* and *M. floridulus*, wherein the BMPs were seen to decrease from 266.5 and 247.1 to 172.4 and 184.6 ml g^{-1} VS (reduction of 35.3% and 25.3%) respectively when the growth time was prolonged from60 to180 d. In fact, with a short prolongation of growth time from 60 to 100 d, the BMPs of all the four *Miscanthus* species deceased, with the reduction degree of 20.9% (from 255.6 to 202.2 ml g^{-1} VS) for *M. sinensis*, 11.1% (from 247.1 to 219.8 ml g^{-1} VS) for *M. floridulus*, 21.2% (from 266.5 to 210.1 ml g^{-1} VS) for *Miscanthus* × *giganteus* and 12.5% (from 254.9 to 223.0 ml g^{-1} VS) for *M. lutarioriparius*. At the growth times of 100 and 140 d, the BMPs were kept relatively stable except the BMP of *M. sinensis* slightly increased from 202.2 to 217.9 ml g^{-1} VS (increasing 7.8%). When the growth time prolonged from 140 to 180 d, the changes of BMPs for the four *Miscanthus* species were different. For *M. floridulus* and *Miscanthus* × *giganteus* the BMPs decreased by 18.6% (from 226.8 to 184.6 ml g^{-1} VS) and by 18.1% (from 210.4 to 172.4 ml g^{-1} VS) respectively. While for *M. sinensis* and *M. lutarioriparius* the BMPs increased slightly by 3.6% (from 217.9 to 226.0 ml g^{-1} VS) and by 2.0% (from 222.6 to 227.1 ml g^{-1} VS) respectively.

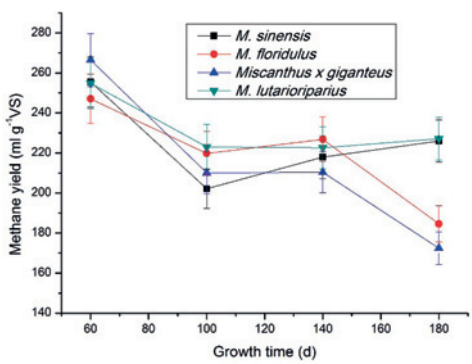

Fig. 1. BMP of the four *Miscanthus* species harvested at different growth times. Error bars indicate the standard deviation.

Fig. 2 shows the 39-day accumulated specific methane yield of all the *Miscanthus* species employed in the present study. At the growth time of 60 d (Fig. 2 A) the digestion durations for reaching 90% of the BMPs of each sample were 17 to 20 days. While at the growth times of 100, 140 and 180 d (Fig. 2 B–D) the digestion durations for reaching 90% of the BMPs of each sample were 26 to 31 days. These results indicated that for all of the examined *Miscanthus* species, the methane yields and production rate will decrease when the growth time prolonged for more than 60 days. In order to decipher the mechanism for this change in BMP during the various growth (harvest) times, the compositions and the cellulose enzymatic degradability of these *Miscanthus* samples were examined concomitantly.

3.2. Compositions of Miscanthus species

Table 1 shows the compositions of the four *Miscanthus* species harvested at the growth times of 60, 100, 140 and 180 d. The cellulose contents of these samples ranged from 45.2% to 54.2%. For *Miscanthus* × *giganteus*, increasing the growth time from 60 to 180 d, the cellulose content increased from 47.2% to 54.2%. While for the other three species, the cellulose content seemed to decrease with increasing growth time wherein the farthest growth time (180 d) showed the lowest cellulose contents. This result indicates that *Miscanthus* × *giganteus*, the sterile hybrid genotype from *M. sacchariflorus* and *M. sinensis*, has a higher ability of cellulose accumulation than the other species. The xylan contents of these samples ranged from 16.6% to 20.7%. There were no obvious differences in xylan contents between each *Miscanthus* species. The xylan content of *M. lutarioriparius* decreased from 20.6% to 16.6% when prolonging the growth time from 60 to 180 d. For the other species, the xylan contents changed slightly according to different growth times. The araban contents of all the four *Miscanthus* species were low, ranging from 1.8% to 3.4%. Totally, the lignin contents of these *Miscanthus* samples ranged from 11.5% to 17.8%. For all *Miscanthus* species, the lignin content was observed to increase when prolonging the growth time. This demonstrated that the *Miscanthus* species were lignifying during the growth season. The ash contents of the *Miscanthus* species were all low, ranging from 3.5% to 5.6%, which were similar to what the literature reported (Hodgson et al., 2011).

The high content carbohydrate and low contents of lignin makes *Miscanthus* an excellent candidate for biofuel production (Brosse et al., 2012; Li et al., 2016a). However, from the detailed analyses of the data of BMP (Fig. 1) and the compositional content of the four *Miscanthus* species (Table 1) it is worthwhile to note that the BMP was not in proportional to the carbohydrate content. For example, the carbohydrate (cellulose + xylan + araban) content of *Miscanthus* × *giganteus* increased from 68.7% to 74.0% with prolonging the growth time from 60 to 180 d, while there was decrease in BMP from 266.5 to 172.4 ml g^{-1} VS. The BMP also depends on other chemical compounds such as proteins, starch and soluble sugars. However the total content of these compositions are below 8.2% in *Miscanthus* (Godin et al., 2013a). The changes of these compositions are not the important factor for the changes of BMP. This indicated that besides the carbohydrate content, the degradability of the *Miscanthus* is yet another important factor influencing the BMP.

3.3. Degradability of cellulose in the Miscanthus species

Cellulose and hemicellulose, which can be hydrolyzed to fermentable sugars, are the actual substrates for bioconversion of

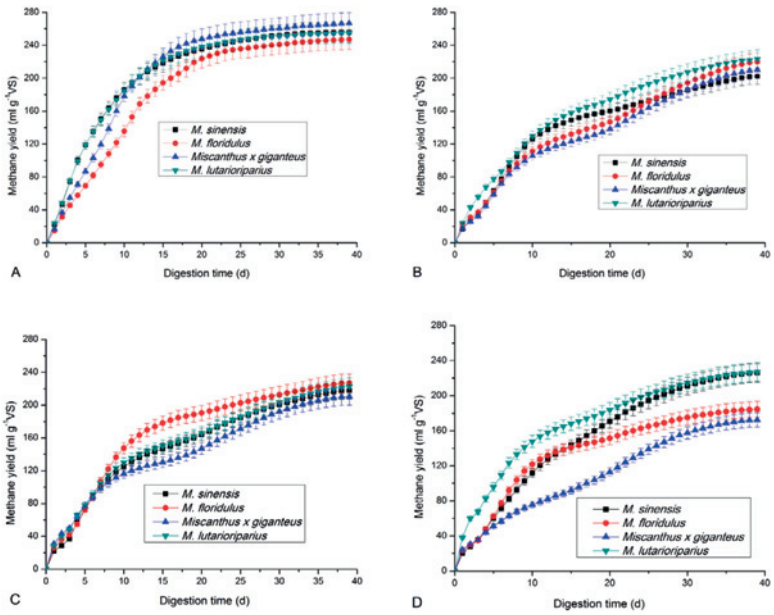

Fig. 2. Accumulated specific methane yield for the four *Miscanthus* species harvested at the growth times of (A) 60 d, (B) 100 d, (C) 140 d and (D) 180 d. Error bars indicate the standard deviation.

Table 1
Compositional contents of *Miscanthus* species at different growth times.

Species	Growth time d	Compositional content %[a]				
		Cellulose	Xylan	Araban	Lignin	Ash
M. sinensis	60	51.9 ± 2.44^{a}	19.6 ± 1.07^{a}	2.9 ± 0.12^{a}	13.6 ± 0.67^{b}	5.6 ± 0.25^{a}
	100	48.6 ± 2.35^{b}	19.3 ± 1.11^{a}	2.4 ± 0.11^{a}	15.5 ± 1.12^{a}	5.4 ± 0.27^{a}
	140	50.7 ± 2.51^{a}	19.8 ± 0.86^{a}	2.6 ± 0.13^{a}	16.2 ± 1.06^{a}	3.6 ± 0.16^{b}
	180	46.2 ± 2.37^{c}	18.8 ± 0.75^{a}	3.2 ± 0.13^{a}	17.8 ± 1.05^{a}	4.3 ± 0.19^{b}
M. floridulus	60	49.1 ± 2.23^{a}	19.5 ± 0.91^{a}	3.2 ± 0.14^{a}	12.2 ± 0.51^{c}	5.1 ± 0.22^{a}
	100	51.6 ± 2.51^{a}	20.3 ± 1.02^{a}	2.6 ± 0.12^{b}	13.9 ± 0.63^{b}	3.4 ± 0.17^{b}
	140	50.7 ± 2.42^{a}	20.7 ± 1.12^{a}	3.5 ± 0.12^{a}	14.8 ± 0.69^{a}	3.8 ± 0.19^{b}
	180	45.2 ± 2.17^{b}	18.7 ± 0.69^{b}	2.6 ± 0.13^{b}	15.1 ± 0.78^{a}	3.9 ± 0.18^{b}
Miscanthus × *giganteus*	60	47.2 ± 2.18^{c}	18.3 ± 0.84^{a}	3.2 ± 0.14^{a}	11.5 ± 0.53^{c}	5.6 ± 0.27^{a}
	100	51.4 ± 2.49^{b}	17.9 ± 0.87^{a}	2.3 ± 0.11^{a}	13.1 ± 0.59^{b}	4.5 ± 0.21^{b}
	140	52.7 ± 2.53^{b}	17.8 ± 0.77^{a}	2.3 ± 0.11^{a}	13.9 ± 0.68^{a}	4.0 ± 0.18^{b}
	180	54.2 ± 2.65^{a}	18.1 ± 0.79^{a}	1.8 ± 0.10^{b}	14.4 ± 0.72^{a}	3.6 ± 0.16^{b}
M. lutarioriparius	60	51.4 ± 2.44^{a}	20.6 ± 1.14^{a}	3.4 ± 0.15^{a}	13.2 ± 0.57^{a}	5.5 ± 0.27^{a}
	100	51.2 ± 2.32^{a}	18.4 ± 0.91^{b}	2.3 ± 0.12^{b}	13.9 ± 0.65^{a}	3.7 ± 0.17^{b}
	140	52.4 ± 2.53^{a}	17.3 ± 0.63^{b}	2.3 ± 0.12^{b}	14.4 ± 0.64^{a}	3.5 ± 0.16^{b}
	180	50.4 ± 2.46^{b}	16.6 ± 0.88^{b}	2.3 ± 0.11^{b}	14.6 ± 0.72^{a}	3.8 ± 0.18^{b}

[a] All data are presented as means ± standard deviations (n = 3). Values with different letters in a column of each species mean significant differences at $P < 0.05$ as determined by ANOVA.

Miscanthus to methane via AD. Hemicellulose is relatively easily to be saccharified due to its amorphous structure with lower degree of polymerization, usually between 50 and 300 (Pu et al., 2008). The degradability of *Miscanthus* is essentially determined by the degradability of cellulose which forms crystal structure and is hard to be degradation (Brosse et al., 2012). In this study, the *Miscanthus* samples were hydrolyzed by the commercial cellulase CTec2. Table 2 shows the enzymatic degradability of various *Miscanthus* species under the different growth/harvest times. The cellulose hydrolysis rates of the samples at the growth time of 60 d ranged from15.3% to 16.4%. They were higher than those (ranged from 10.6% to 14.7%) of the samples at the growth times of 100, 140 and 180 d. This result was in according with the result of BMP analysis (Figs. 1 and 2) which showed that the BMPs of the samples at the growth time of 60 d were higher than those of 100, 140 and 180 d. The reason is possibly due to the fact that the degree of polymerization and crystallinity of cellulose were increased during *Miscanthus* species growth, leading to the reduction in cellulose

Table 2
Enzymatic degradability of cellulose of *Miscanthus* species at different growth times.

Growth time d	Enzymatic hydrolysis rate of cellulose %[a]			
	M. sinensis	*M. floridulus*	*Miscanthus* × *giganteus*	*M. lutarioriparius*
60	15.3 ± 0.67[a]	16.4 ± 0.82[a]	15.9 ± 0.76[a]	15.5 ± 0.74[a]
100	13.5 ± 0.64[c]	12.2 ± 0.61[c]	11.3 ± 0.54[b]	11.3 ± 0.56[b]
140	14.1 ± 0.69[b]	14.7 ± 0.72[b]	11.5 ± 0.54[b]	11.3 ± 0.53[b]
180	13.6 ± 0.63[c]	10.9 ± 0.49[d]	10.6 ± 0.51[c]	11.5 ± 0.57[b]

[a] All data are presented as means ± standard deviations (n = 3). Values with different letters in a column mean significant differences at P < 0.05 as determined by ANOVA.

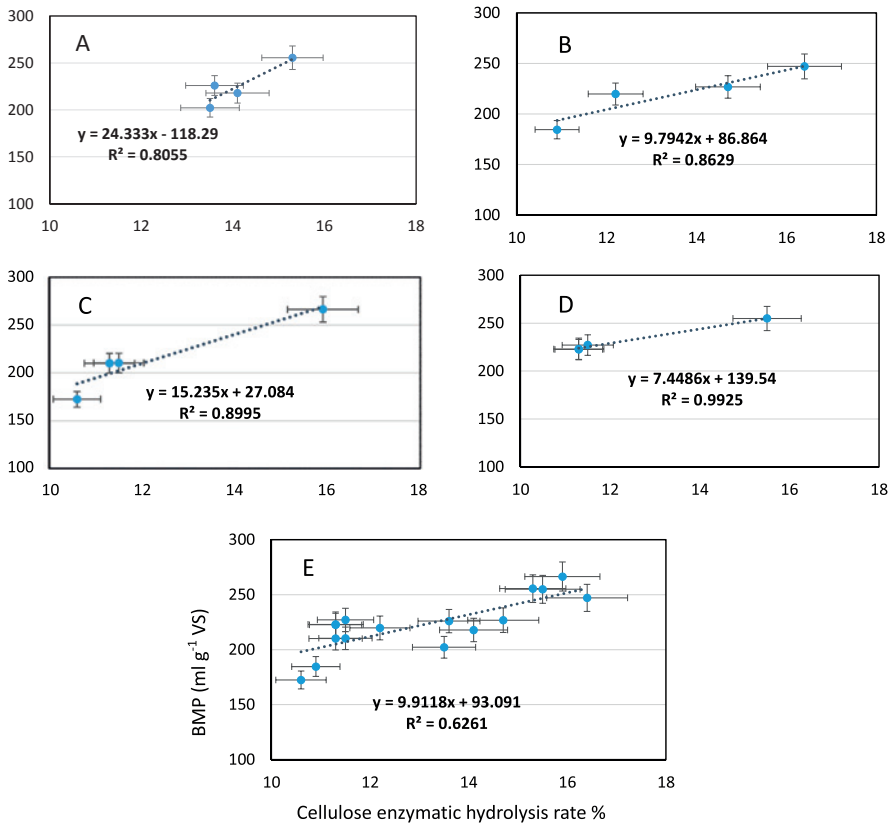

Fig. 3. The relationship between the BMP and the cellulose enzymatic hydrolysis rate of *Miscanthus* samples. The data of A, B, C and D were respectively from the samples of *M. sinensis*, *M. floridulus*, *Miscanthus* × *giganteus* and *M. lutarioriparius*. The data of E were from all of the *Miscanthus* samples. Error bars indicate the standard deviation.

degradability. It seems that the degradability of cellulose is much more important that highly affects the BMP, rather than the composition of the *Miscanthus* plant. The relationship between the BMP and the cellulose degradability of *Miscanthus* samples is shown in Fig. 3. The BMP was positively correlated to the cellulose enzymatic hydrolysis rate. The correlation coefficients (R^2) of the BMP and the cellulose hydrolysis rate for each *Miscanthus* species (Fig. 2 A–D) ranged from 0.8055 to 0.9925. While using all of the samples as a statistical set (Fig. 2E), the correlation coefficient (R^2) of the BMP and the cellulose hydrolysis rate was low as 0.6261. This indicated that the BMP of the *Miscanthus* sample could be preliminarily estimated by examining its cellulose degradability (enzymatic hydrolysis rate of cellulose) after establishing the calibration curves between the BMP and the cellulose degradability for each *Miscanthus* species. This could be a time-saving method for BMP preliminary examination in practice.

In the recent years, a lot of research on *Miscanthus* as an energy crop has been carried out based on the precondition that the *Miscanthus* is harvest once a year (Li et al., 2016a, 2017; Nges et al., 2016). However, in the tropical and subtropical regions, such as North Asia, *Miscanthus* can be harvest twice or more within a year. For example, at the *Miscanthus* nursery at Hunan Agricultural University

(Changsha City, Hunan Province, China) (28°10′54″ N 113°05′06″ E), the *Miscanthus* shoots starts to emerge at early March and starts to wither around October. Our previous research (Li et al., 2017; Yi, 2012) showed that when *M. lutarioriparius* was harvest once a year at the end of October the yield was 33.4 to 42.0 tons per hectare. When *Miscanthus* was harvest at early May the yield was about 20 tons per hectare. That is to say that about 50% of the biomass was accumulated within the first two months of growth season. If the *Miscanthus* was harvested before 1st August, the new shoots would come out soon and the biomass yield for the second time will exceed 70% of the yield harvested once a year. Hence, it is plausible to state that during the growth season the *Miscanthus* can be harvested once, twice or even three times within a year. There is yet limited knowledge for instructing to improve the income for bioconversion of *Miscanthus* based on the condition of harvest twice or more within a year. The results from the present research demonstrated that at the short growth time of 60 d, the examined four *Miscanthus* species all showed the highest BMP and cellulosic degradability. This information is therefore valuable for selecting the suitable harvest times for *Miscanthus* in a view to enhance its bioconversion and the overall economic profitability.

The economic benefit for biofuels production from the energy crop *Miscanthus* depends on many factors such as biomass yield, fertilizer input, chemical composition and degradability. This study has shown that the BMPs of the *Miscanthus* samples were positively correlated to their cellulose enzymatic hydrolysis rates which were different up to more than 30% between the samples harvested at different growth times. Considering the fact that the process(es) of pretreatment and/or enzymatic hydrolysis of lignocellulosic biomass cover significant cost and conversion of lignocellulosic biomass to fermentable sugars represents a major challenge in lignocellulosic biomass refinery (Lynd et al., 2008), the degradability of cellulose should therefore be projected as an important factor that affects the economic benefit for biofuels production from the energy crop *Miscanthus*.

4. Conclusions

The changes in composition, cellulose degradability and the biochemical methane potential of four *Miscanthus* species during the growing season have been investigated concurrently. For all four species, the BMP was positively correlated to the cellulose degradability. During the growing season from 60 to 180 d, there was only a slight change in the compositional contents. Meantime, there were significant changes in the BMP and the cellulose degradability. Besides the biomass yield, the cellulose degradability of the lignocellulosic biomass will be a considerable factor for improving the economic benefit for biofuels production from the energy crops such as *Miscanthus*.

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (No.21676279), the National High Technology Research and Development Program of China (863 Project No. 2014AA021905), and the 100 Talents Program of institute of process engineering, Chinese Academy of Sciences. The authors are grateful for experimental support from Nova Skantek Environmental Technology (Beijing) Co., Ltd. The authors would like to thank the lab work support of Mr. Liang Xiao.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.03.128.

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Paper IV

Biochemical Engineering Journal 118 (2017) 1–10

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Assessment of regional biomass as co-substrate in the anaerobic digestion of chicken manure: Impact of co-digestion with chicken processing waste, seagrass and Miscanthus

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ARTICLE INFO

Article history:
Received 27 July 2016
Received in revised form 11 October 2016
Accepted 8 November 2016
Available online 10 November 2016

Keywords:
Anaerobic digestion
Biochemical methane potential
Chicken manure
Co-digestion
Energy crops

ABSTRACT

The biochemical methane potential (BMP) assays were used as a tool to investigate methane potential of chicken manure (CM) and three co-substrates (chicken processing waste, *Miscanthus* and seagrass) in mono-digestion and co-digestion studies for selecting regional biomass in a bid to support the expansion of a full-scale biogas plant. Two types of kinetic models (first order and modified Gompertz models) were also applied to study the kinetics of the degradation process. The results show that all feedstock were converted to methane. The experimental methane production of chicken processing waste (CPW) and CM decreased about 27–35% compared to calculated methane production. However, the methane production rate/hydrolysis rates of mono digestion of chicken processing waste and co-digestion with CM were above 2 times quicker under the inoculum to substrate (I/S) ratio of 6 than that at the I/S ratio of 2 and 4. *Miscanthus* co-digestion effect was influenced by its composition and seagrass (SG) showed synergetic effect evidenced by high methane yield (which was 11–34% higher than the yield achieved from calculated BMP).

1. Introduction

Anaerobic digestion (AD) is one amongst the renewable energy production technologies, doubling also as a waste treatment method, and the residue (digestate) from the process can be return to farmland as a bio-fertilizer. Agricultural residues (manure and straws) have been used, as a feedstock of choice for biogas production through AD [37]. By using manure as feedstock in AD, the bulk of waste from animal husbandry and dairy farming can be converted to a renewable energy while at the same time 'cleaning up' the waste.

With the growing in intensive and mechanized poultry breeding industries, huge amounts of chicken manure (CM) are generated each year in the world. AD of CM is considered the best method to minimize waste and recover bioenergy [13]. However, AD of manure (pig manure, cow manure and chicken manure) is often hampered by free ammonia inhibition resulting in poor methane yields and consequently long retention times [6]. It is therefore not surprising that many studies have reported on the benefits of using manure as a co-substrate in co-AD processes [12,23]. Co-digestion has been described as a technology to ease feedstock shortage, dilute toxicants, balance feedstock composition and augment much needed micro and macronutrients in the biogas process [25]. Co-digestion of local available biomass such as, protein rich, high nitrogen-content chicken processing waste (CPW) and lignocellulosic biomass *Miscanthus* and seagrass (SG) is therefore worth investigating.

The Minhe biogas plant Minhe, which consists of eight continuous mixed digesters (with volumes of 3300 m^3 each) divided into two independent production lines that can treat all chicken manure and wastewater from the farms, has been in operation since 2009 [13]. Process parameters (e.g., annual loaded, daily loaded, loading rate, Hydraulic retention time (HRT)) of Minhe were listed (Table SI, Supplementary data). <As Minhe wants to expand their plant during the second phase construction by adding12 new biogas digesters, there will be a dramatic increase in feedstock demand by expansion for the future operation, which can only be solved by co-digestion. It is essential for plant treating CM to produce as much biogas as possible in order to increase the economic viability. Long

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http://dx.doi.org/10.1016/j.bej.2016.11.008
1369-703X/

transportation of substrates could become a major cost for the biogas plant and thus the transportation distance of the co-substrates and the seasonal availability are of utmost importance for the economic viability of the plant. Thus, three regional biomasses i.e. CPW, SG and *Miscanthus* were chosen as co-substrates to evaluate which are suitable for co-digestion of CM. In addition, the high water content of CM could act as solvent for the more dry types of wastes, resolving problems of pumping and mechanical handling of solid wastes.

Chicken processing industry generates a large amount of waste, mostly in the form of skin, feathers, heads, legs, blood, bones and viscera, in addition to whole carcasses when the animal is dead on arrival herein termed CPW. CPW, which consists of significant amounts of fat and protein with high potential to recover energy and nutrient resources through waste management, is an attractive substrate for AD and should be properly managed to avoid environmental damage. The mono-digestion of CPW may risk inhibition or operation failure if the concentrations of free ammonia (NH_3), optimal value of 200 mg/L, and/or long chain fatty acids (LCFA), optimal value 1000 mg/L, exceed certain levels or concentrations[10].

Miscanthus is a woody perennial grass native to the East Asian region. About 20 species of the genus *Miscanthus* have been listed by various researchers and in particular four species, namely *Miscanthus sinensis* (Ms), *Miscanthus floridulus* (Mf), *Miscanthus sacchariflorus* (Mc), and *Miscanthus lutarioriparius* (Ml) have been assessed for their biomass production, chemical composition, saccharification efficiency and are considered as ideal candidates for bioenergy production [22]. On the other side, the economic feasibility of *Miscanthus* used as co-substrate for biogas production partly depends on the biomass yield per hectare by different genotypes. Owing to the intrinsic characteristics of *Miscanthus*, including high biomass production potential, low input requirements and its ability to grow in marginal conditions, there have been an increase used of *Miscanthus* as feedstock for biogas [21,27,33].

SG is considered as one of the most rapid growing sources of biomass, with a growth rate estimated to be 10 times higher than most terrestrial plants [7]. It remains relatively unexplored and underdeveloped for the sustainable production of biofuel feedstock and can be found along the beaches or floating near coasts lines. Utilization of SG for biogas generation could mitigate the emission of the greenhouse gas, and would economically benefit the local communities [24]. Natural drying of SG by sun and wind is still the most common and economic way of conserving this type of biomass. However, the high concentration of salinity in SG may be toxic to the methanogens [9]. It is worth mentioning that energy crops, especially maize (silage) are often used as feedsctocks of choice in co-digestion AD processes in large biogas producing countries such as Germany. However, the use of food crops for energy purposes is an important, ongoing debate [11].

Batch test is a simple, effective and commonly used method to evaluate the effect on digester performance of adding a co-substrate [17]. Besides the methane production potential, a batch tests also provide information regarding the degradation kinetics of a material [2,26]. The dynamic degradation profile is something that should be considered in the selection of the most efficient co-digestion mixture, as it indicate how much of the feedstock that can be treated and what retention time is necessary for the material to be sufficiently degraded. The degradation kinetics is normally evaluated by fitting a kinetic model to the gas production data and studying the model parameters.

In this study, three proximal co-substrates derived from chicken farm and coastal marginal lands were investigated as potential co-substrates of CM in the AD process. Therefore, the objective was to select the best alternative regional biomass by investigating the methane potential and biodegradablity of CPW, *Miscanthus* and SG. Effect of inoculum to substrate ratio, species difference and storage time were tested respectively to evaluate the feasibility of co-substrates selection, based on the practical production needs. Additionally the feasibility of co-digesting CM with biomass containing a low content of proteins (*Miscanthus* and SG) and a readily available CPW was studied. The use of non-conventional lignocellulose biomass such as *Miscanthus* and SG in manure co-digestion AD is rarely discussed in literature. These co-substrates may be presented as cheap, readily available substitutes to food-type crop biomasses such as maize (silage). A detailed kinetic analysis, including model fitting with a first order and a modified Gompertz model, was performed to determine which substrates or combination of substrates had the most positive effect on the degradation rate and ultimate methane yield. The overall aim was to compare methane potential of the three different regional biomass around biogas plant regarding the synergism with co-digestion carried out in batch experiments in a bid to provide fundamental information for further continuous research.

Table 1
Characteristics of chicken manure and inoculum used in the study.

Variable	Units	Chicken manure	Inoculum
TS	% (w/w)	7.40 ± 0.11	3.30 ± 0.05
VS	% (w/w)	5.26 ± 0.06	1.58 ± 0.03
NH_4^+-N	mg L^{-1}	3482 ± 72.10	4583 ± 72.00
TN	mg L^{-1}	4145 ± 7.2	5101 ± 72.0
ON	mg L^{-1}	559 ± 72.0	505 ± 102.0
TOC	mg L^{-1}	12288 ± 32.0	1914 ± 26.0
COD	mg L^{-1}	33700 ± 565.0	5075 ± 176.0
VFA	mg L^{-1}	21891 ± 21.00	52 ± 1.00
TA	g L^{-1}	9.4 ± 0.1	15 ± 0.3
TK	mg L^{-1}	3323 ± 180.0	3147 ± 61.0
TP	mg L^{-1}	294 ± 5.0	325 ± 3.0
pH	mg L^{-1}	6.4 ± 0.2	7.9 ± 0.1

TS, total solid; VS, volatile solid; BMP, biochemial methane potential; NH4+-N, ammonium nitrogen; TN, total nitrogen; ON, organic nitrogen; TOC, total organic carbon; COD, chemical oxygen demand; VFA, volatile fatty acids; TA, total alkalinity; TK, total potassium; TP, total phosphorous; pH, hydrogen ion concentration.

2. Materials and methods

2.1. Biomass and inoculum

Characteristics of substrates and inoculums are listed in Tables 1 and 2. CM and inoculum used in this study were collected from the Minhe biogas plant (Shandong, china), while the CPW was collected from the chicken processing factory (Penglai, Shandong province, China) and stored at 4 °C prior to use. The inoculum was kept under anaerobic conditions at 37 °C for 7 days to reduce endogenous biogas production. Prior to the AD process, CPW was sanitized at 130 °C and 2 bar for 60 min. Then it was made into to a slurry mixture by chopping with a Waring blender for 2 min (JJ-2B, Changzhou, China). The final product after this pretreatment was a lipid-rich pulp-like substrate.

The four species of *Miscanthus* (Ms, Mf, Mc and Ml) were transplanted from Hunan *Miscanthus* nursery to Yantai city, China in 2011, and cultivated over a 3-year period in the surrounding farmlands (37°44′45.3″N 120°42′43.1″E). The farm site has a warm temperate continental monsoon climate with a mean annual rainfall of 651.9 mm and temperature of 11.8 °C. Each plot was composed of 10 separate plants which were planted in a 1.5 × 0.75 m area. The aboveground biomass was hand harvested from late December to early January each year. The *Miscanthus* samples were milled (Grindomix 200, Retch USA) to pass through a 20 mesh.

SG (*Zostera marina*) was collected on the beach close to the Minhe biogas plant. The SG samples were used fresh as (co)-substrate in AD and after nature storage in open plastic tanks in a well ventilated area at room temperature (32 ± 5 °C) for 5, 10, 15

Table 2
Characteristics of co-digested feedstock used in the study.

	TS(%)	VS(%)	Cellulose (%)	Hemicellulose (%)	Liglnin (%)	Oil and grease (%)
CPW	27.8 ± 0.14	26.09 ± 0.21	nd	nd	nd	11.5 ± 5.17
Ms	95.37 ± 0.12	81.32 ± 0.50	35.51 ± 0.37	28.76 ± 0.18	8.58 ± 0.35	nd
Mc	95.0 ± 0.02	83.49 ± 0.36	42.21 ± 0.18	30.98 ± 0.75	12.79 ± 0.98	nd
Mf	95.5 ± 0.06	88.98 ± 0.91	47.22 ± 0.24	27.44 ± 0.12	13.2 ± 0.52	nd
Ml	95.41 ± 0.08	89.52 ± 1.06	49.03 ± 0.29	26.8 ± 0.72	14.44 ± 0.13	nd
SG_{fresh}	15.07 ± 0.32	11.85 ± 0.34	26.81 ± 4.72	9.82 ± 3.05	10.72 ± 2.62	nd
SG_{5day}	66.84 ± 2.81	52.18 ± 2.56	nd	nd	nd	nd
SG_{10day}	65.86 ± 2.20	53.27 ± 1.05	nd	nd	nd	nd
SG_{15day}	97.78 ± 0.17	79.9 ± 0.38	nd	nd	nd	nd
SG_{20day}	98.82 ± 0.14	52.98 ± 4.72	nd	nd	nd	nd

CPW, chicken processing waste: Ms, Miscanthus sinensis; Mc, Miscanthus sacchariflorus; Mf, Miscanthus floridulus; Ml, Miscanthus lutarioriparius; nd, not determined.

and 20 days. Except the fresh SG sample, all samples were milled (Grindomix 200, Retch USA) to pass through a 20 mesh.

2.2. Experimental design

The entire experiment was divided into two phases: biochemical methane potential (BMP) test of mono-substrate and substrate mixes for co-digestion. Fig. 1 shows a flow diagram of the experimental protocol.

2.2.1. Mono-substrates study

CM, being the main substrate has been used as a mono-substrate in the AD process. The four species of *Miscanthus* and the five types of SG stored under different conditions (Section 2.1) were studied in terms of biomethane potential and degradation kinetics. Due to the high protein content of CPW, three different inoculums to substrate ratios (ISR) based on volatile solids (VS) content were tested in order to ensure batch test can be performed without risk of ammonia inhibition, that is 2, 4 and 6 (also in co-digestion of CPW with CM) were adopted and tested.

2.2.2. Co-digestion study

The amount of co-substrates added in the AD of CM was based on availability and accessibility in the region. In order to simulate the co-digestion based on accessible quantity in the biogas plant, CM was mixed with CPW in a ratio of 20:1 on a wet weight basis. Similarly, CM was mixed with SG in a ratio of 10:1 based on wet weight. In total, five lines AD experiments were performed in the co-digestion of CM and SG. For each of the four *Miscanthus* species, three different mix ratios were tested, i.e. 1:1, 3:1 and 1:3 for investigation CM and *Miscanthus* respectively. This leads to twelve lines of AD experiments were conducted for the co-digestion of CM and *Miscanthus*. For co-digestion of CM and CPW, three different ISR (2:1; 4:1; 6:1) were also adopted and investigated. For each of the five samples of SG and twelve samples of *Miscanthus*, the ratio of inoculum to substrate was chosen to be 2:1 on a volatile solids basis. In all, thirty-three lines of experiments were performed in triplicates totalling ninety-nine lines of AD experiments (Fig. 1).

2.3. Biochemical potential test

The BMP tests were conducted with the Automatic Methane Potential Test System (AMPTS II, Bioprocess Control AB, Sweden) was has been reported in a previous study [20]. The volume of methane automatically normalized to standard condition (dry gas, T = 0 °C, P = 1013.25 hPa) according to the following equation [31].

$$V_{STP} = \frac{P_{gas}}{P_{STP}} \cdot \frac{T_{STP}}{T_{gas}} \cdot V_{gas} \quad (1)$$

In Eq. (1), V_{STP} is the volume adjusted to standard temperature and pressure, P_{STP} is the standard pressure (hPa), P_{gas} is the pressure for the measured gas at the time of reading (hPa), T_{gas} is the temperature of ambient space in Kelvin (K), T_{STP} is the normal temperature (0 °C) and V_{gas} is the recorded gas volume.

All test were carried out under mesophilic conditions (37 °C) until the daily gas production was less than 1% of the total gas production VDI, 4630, 2006). Two sets of controls were included in the experimental protocol, i.e. a blank and a positive control consisting of pure microcrystalline cellulose. The control protocol was performed as has been reported in other studies [5,27].

Besides, to quantitatively determine the synergistic or antagonistic impacts of co-digestion, the methane production in each condition was estimated according to the proportions of main substrate and co-substrate and their separate methane production during mono-substrates batch test (Eq. (2)).

$$BMP_{calculated} = f_{main} \times BMP_{main} + f_{co} \times BMP_{co} \quad (2)$$

BMP_{main} is the measured methane potential of main substrate alone. BMP_{co} is the measured methane potential of co-substrate alone. The f_{main} and f_{co} values correspond to the proportions of main substrate and co-substrate in each co-digestion mixture.

2.4. Analytical methods and calculations

Total solids (TS), VS, chemical oxygen demand (COD), total organic carbon (TOC) total nitrogen (TN), organic nitrogen (ON), and ammonium-nitrogen (NH_4-N) were analyzed according to standard method [3]. Total alkalinity (TA) was determined in liquid phase by titration with HCl to a pH endpoint of 4.3, as recommended in standard methods. Total phosphorus (TP) concentrations were determined by an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Thermo, USA). The pH values were measured by TitraLab™ 80 titrator (Radiometer, Copenhagen, Denmark). The concentration of volatile fatty acid (VFA) was determined using a GC-FID (Agilent 7890A, Agilent Technologies Inc, USA). The content of hemicellulose, cellulose, and lignin were determined according to the neutral detergent fiber (NDF), acid detergent fiber and lignin (ADF/ADL) analyses as described by Van Sowest et al. [32]. Oil and grease were determined by InfraCal TOG/TPH analyser (Wilks Enterprise Inc, USA), where S-316 was used as extraction solvent.

2.5. Kinetics analysis

Two types of kinetic models were applied to study the kinetics of the degradation process, i.e. a first order rate model and a modified Gompertz model. For samples where the hydrolysis is limiting the anaerobic degradation process, a first order rate model (Eq. (3)) is commonly used to describe this process (Vavelin et al., 2008). In Eq. (1), $B(t)$ is the methane yield at a given time t, B_0 is the value of the ultimate methane yield or maximum value at infinite diges-

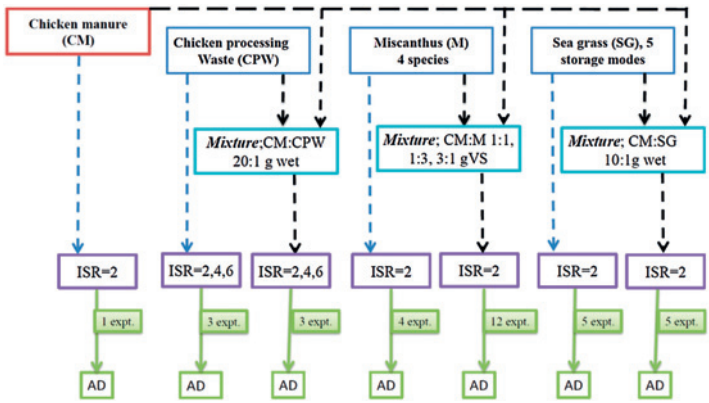

Fig. 1. Experimental protocol showing substrate mixtures, handling and the number of anaerobic digestion (AD) batch test processes (experiments, expt).

tion time, k is the rate or hydrolysis constant and θ is the lag time constant.

$$B(t) = B_0 \cdot \left(1 - exp\left(-k \cdot \left(t - \theta\right)\right)\right) \quad (3)$$

If the degradation process follows a more traditional bacterial growth curve, with a sigmoidal profile, a modified version of the Gompertz model (Eq. (4)) have been shown to be suitable [15,38]. Besides the symbols already presented, R_{max} represents the maximum methane production rate and e is the base of the natural logarithm (2.7183).

$$B(t) = B_0 \cdot \exp\left(-exp\left(-\frac{R_{max} \cdot e}{B_0} \cdot \left(\theta - t\right) + 1\right)\right) \quad (4)$$

2.6. Statistical analysis

Outliers in the BMP test were determined and eliminated with the aid of the Grubb test ($P \leq 0.05$). Statistical difference in methane yields were determined by one-way analysis of variance (ANOVA) at a 95% confidence interval. Statistical differences were also established between the experimental and calculated yields in the co-digestion experiments.

3. Results and discussions

3.1. Characteristics of chicken manure and co-substrates

The characteristics of CM and inoculum are listed in Table 1. The C/N ratio of CM was 3 indicating that CM had very high nitrogen content. The NH_4-N concentration of CM was high (over 3000 mg L^{-1}) which also explains the high NH_4-N content of the inoculum (4583 mg L^{-1}). The high NH_4-N concentration led to a high NH_3 concentration 350 mg/L (according to Eq. (5)), which may result in an unstable AD process due to loss of methanogenic activity [18].

$$\frac{[NH_3]}{[TNH_3]} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729,92}{T(K)}\right)}}\right)^{-1} \quad (5)$$

where $[NH_3]$ is the concentration of free ammonia, $[TNH_3]$ is the concentration of total ammonia nitrogen, pH is the pH value determined in the reactor, and T(K) is the temperature (Kelvin).

High nitrogen substrates can pose an inhibitory effect in the AD process through NH_3 accumulation. As for unadapted methanogenic cultures, NH_4-H inhibition have been reported to start at concentrations of 1700 mg L^{-1} [10]. However, there are other reports wherein tolerance of up to 4000 mg L^{-1} NH_4-H has been demonstrated by adaptation of the biogas process [1]. In the present study, using CM as substrate, it is plausible to state that the process was adapted to high NH_4-N concentrations evidenced by the high process stability and performance (methanogenic conditions and high methane yields as discussed below). Also, as can be seen from Table 1, high concentration of VFA may have resulted in the low pH value in CM. This may theoretically counteract the adverse effect of ammonia due to a decrease in the free ammonia concentration. This phenomenon have been described in another study 'inhibited steady-state' which is characterized by partial inhibition of AD process [14].

Table 2 shows the characteristics of the co-substrates used in the present study. The four *Miscanthus* species showed a high content of cellulose and hemicelluloses while the content of lignin was rather low. SG was also characterized by low lignin content of 10.72%; however, the cellulose and hemicelluloses contents were lower than that in *Miscanthus*. Similar characterization of *Miscanthus* and SG have been reported in literature [7,21].

3.2. Methane potential of chicken manure

Fig. 2 shows a typical methane cumulative curve in the BMP of CM. It can be observed that the accumulated methane production reached 395 ml g $^{-1}$ VS after 13 days of incubation. The methane production rate was high in the first 12 days and reaching a peak value of 46.2 ml g^{-1} VS d^{-1} on the 7th day. Thereafter came the flattening of the methane production curve that may be indicative of the easily degradable part of CM. Though CM showed a rather low C/N ratio of 3 (optimal range for an effective AD process is between 20 and 30) and high NH_4-N concentration, its plausible to state that the inoculum was well adapted to elevated levels of NH_3, as discussed above, which should have otherwise inhibit the AD process [10]. The methane yield of CM (400 ml g $^{-1}$ VS) achieved in the present study was higher than those reported in literature i.e. 291–370 ml g^{-1} VS [28,38]. It should be noted that the VS-based BMP of CM might have been overestimated probably because volatile compounds such as VFAs and ammonia which abound in the present CM (Table 1) might have been lost during standard VS determination procedure [19].

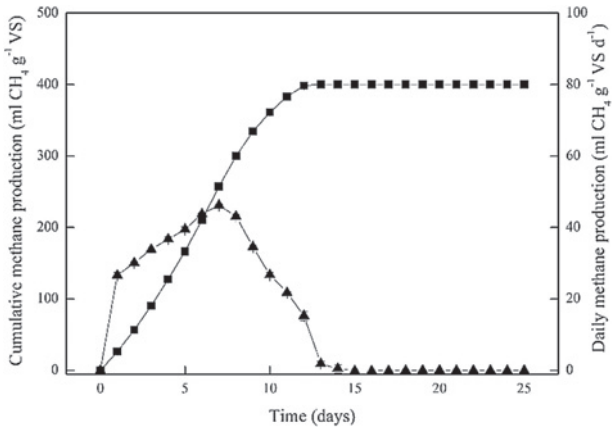

Fig. 2. The methane potential and daily methane production of chicken manure.

3.3. Methane potential of chicken processing waste and co-digestion with chicken manure

The relationship between the inoculum quantity and amount of CPW or CM (based on the wet weight content) need to be investigated to understand the appropriate inoculum to substrate ratio (ISR). The 50-day BMP performance from the CPW and co-digestion with CM is presented in Fig. 3. CPW was characterized by a high degree of easily degradable parts whose digestibility was likely increased by the prior sanitation process. Results showed that the BMP value of CPW as a mono-substrate decreased with the increasing ISR ratio (Fig. 3a). The maximum volume was 464 ml g^{-1} VS at ISR of 2. However, the yield decreased by 6.9% and 10.6% at ISR 4 and 6 respectively which were significantly lower ($P \leq 0.05$) than the yield at ISR of 2. A suitable S/I ratio, adjusted for the specific feedstock and inoculum, is important to provide the necessary microorganisms and allow for a well-balanced startup of the anaerobic digester. This findings are also in line with other studies were higher methane yields have been achieved with increasing substrate loadings i.e. lower ISR [36]. It was found that the highest daily methane production of 46 ml g^{-1} VS d^{-1} was achieved on the 9th day at the ISR 6, which was earlier than the production obtained at ISR 4 (14th day) and ISR 2 (27th day).

Similar results were obtained in the co-digestion of CM and CPW, where the maximum value of 302 ml g^{-1} VS was achieved at ISR 2 (Fig. 3b). At ISR of 4 and 6, the methane yield decreased by 3.3% and 13.6%, respectively. The degradation period was shorter with the increasing ISR, and the earliest maximum daily methane yield of 32.3 ml g^{-1} VS d^{-1} occurred on the 5th day at ISR 6. In addition, the BMPs in the co-digestion of CPW and CM were significantly lower ($p < 0.05$) than the calculated yield from the mono-digestion of CPW and CM.

It should be noted that mono-AD of CPW showed a lag phase of 10 days at ISR of 2 whereas there was no lag phase at ISR of 4 and 6. The CPW is proteinaceous and lipid-rich in nature (Table 2), and its degradation products, in particular NH_3 and LCFAs, could have initially inhibited the AD process[10]. However, this kind of inhibition did not hinder the AD process owing to the possible resilience of the inoculums, which was adapted to high NH_3 concentrations. The amount of microorgansims in the digester was increased with higher ISR, which was beneficial for a fast start of the anaerobic reaction [29]. As a result, the reaction period was shorter and the maximum daily methane production was reached much earlier. At the same time, the lowered substrate concentration could lead less inhibition of the microbial consortium. The co-digestion production curves showed a two-peak curvature or a diauxic growth pattern. A possible explanation is that the anaerobic microorganisms consumed a preferred substrate type first, presumably the proteinaceous (inoculums is adapted to high nitrogen or protein content) fraction before consuming the other faction(s) which require longer degradation time.

Evaluation of co-digestion effect is provided (Fig. 4). Antagonistic effects occurring in all the batch assays and an increment in NH_4-N were observed after the batch test (Table SII, Supplementary data). The experimental methane production decreased about 27–35.2% compared to theoretical methane production. This fact was expected since high content of protein was converted to ammonia during AD. Meanwhile, it can be seen from Fig. 2 that the 15 days incubation period that was enough for the digestion of CM, while it took 40–50 days for the AD of CPW. However, the digestion of CPW and co-digestion of CPW with CM at high ISR (4 and 6) were much faster than that at low ISR. This therefore warrants the low content of CPW to be added into digester as a co-substrate to CM, which might be a feasible way to use its potential energy.

3.4. Methane potential of Miscanthus and co-digestion with chicken manure

The accumulated methane production and daily methane generation from the four species of *Miscanthus* are presented in Fig. 5b. The methane yields (ml gVS^{-1}) were 227, 234, 262 and 281 ml g^{-1} VS for Mf, Mc, Ml and Ms, respectively. The methane yields are in line with the yields of 190 for Mc [16], 238 for Ml [27] and 307 for Ms [35]. All four *Miscanthus* samples registered the maximum daily methane production on the first day with Ms showing the highest value of 25.9 ml g^{-1} VS d^{-1} followed by Mf, Mc and Ml with 12.1, 11.1 and 9.8 ml g^{-1} VS d^{-1}, respectively. The long degradation time of 50 days was indicative of a relatively difficult degradation of *Miscanthus* [27] compared to CM (Section 3.2).

The comparison of BMP between the four *Miscanthus* species is not the only determining criteria. The methane yield (m^3 ha^{-1}) of these energy plants could be an important parameter to decide which species of *Miscanthus* that should be use in the biogas process. The average annual dry biomass yields of *Ms*, *Mf*, *Mc*, and

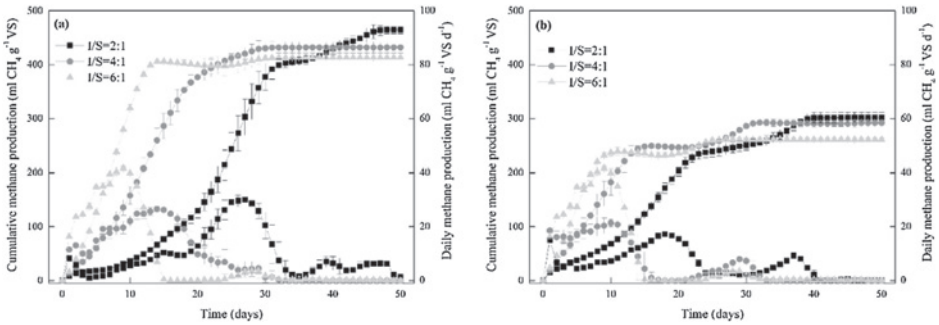

Fig. 3. Methane potential and daily methane production of chicken processing waste (a) and co-digestion of chicken manure and chicken process waste (b).

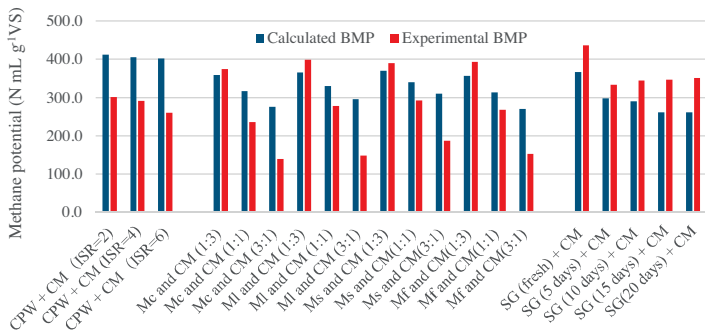

Fig. 4. BMP of calculated and of the experimental co-digestion of different mixture ratios.

Ml were 17.1, 24.0, 17.2, and 33.4 t ha^{-1} over 3 years, respectively (Fig. 5a). Therefore, the highest methane yield (m^3 ha^{-1}) was obtained in the AD of *M.lutarioriparius* which was 8211 m^3 ha^{-1} followed by *M. floridulus* (5076), *M.sinensis* (4097) and *M.sacchariflorus* (3537). Based on the BMP and biomass yields, *M.lutarioriparius* showed the high potential to be use as (co)-substrate in the full-scale biogas plant.

The digestibility should also be evaluated if *Miscanthus* is to be used as a co-substrate with CM. Fig. 5c–f shows the methane production and the production rate of the co-digestion of CM and *Miscanthus*. Three mixing regimes of CM and *Miscanthus* were investigated, i.e. 1:1, 1:3 and 3:1 based on VS. The mixtures with high proportion of CM showed the highest methane yields ($p < 0.05$) of 375, 399.1, 390.1 and 393.8 ml g^{-1} VS in the co-digestion with Mc, Ml, Ms and Mf, respectively. It was reasonable that the high methane potential of CM and the high proportion of CM will contribute to the high methane yields during the co-digestion. There was no significant difference in the methane yields ($p > 0.05$) between the four species in the mixtures with the high proportion of CM (3:1). Also, there was no difference ($p > 0.05$) between the methane yields when the ratio was 1:3. However, a significant difference ($p < 0.05$) was observed at a ratio of 1:1. Concerning the methane production rate, some disperse peaks denotes the discrepancy in fermentation process. The earlier peaks might from easy-to digest substances, then the later period peaks represented the further solubilization and methanisation of tightly affiliated biodegradable substances and even recalcitrant biodegradable compounds[39]. This demonstrated that *Miscanthus* co-digested with chicken manure induced a multi-phase degradation process.

In addition, a synergistic effect between the four species of *Miscanthus* and CM was observed when the proportion of CM:M was set at = 3:1, while antagonism effect was exhibited in other mix ratios (Fig. 4). Many studies [4,30] have shown that the blend ratio between the co-substrates was an important considering factor for synergism or antagonism. A possible explanation is that the added material can lead to an adjustment of the C/N ratio to an optimum level (20–30), balance the buffer capacity and or provide the lacking nutritional elements. It is plausible therefore to state that optimal mixing of CM (C/N ratio of 3) and Miscanthus led to a favourable C/N ratio that translated into high methane production. Consequently, *M.lutarioriparius* can be considered as a promising feedstock to be used as a substrate in the full-scale biogas plant because of its high biomass yields and ideal methane production.

3.5. Methane potential of seagrass and co-digestion with chicken manure

Fig. 6 shows the methane potential and methane production rates from different samples of SG and co-digestion with CM. It would seem the cumulative methane yield decreased with increasing storage time, as the maximum yield was 222.4 ml g^{-1} VS for the SG_{fresh} group, which was 1.4 times higher than that of the SG_{20day} (161.7 ml g^{-1} VS). The other yields were 196.1, 182.9 and 170 ml g^{-1} VS for SG_{5day}, SG_{10day} and SG_{15day}, respectively. As compared to dry *Miscanthus* which have similar characteristics, the methane pro-

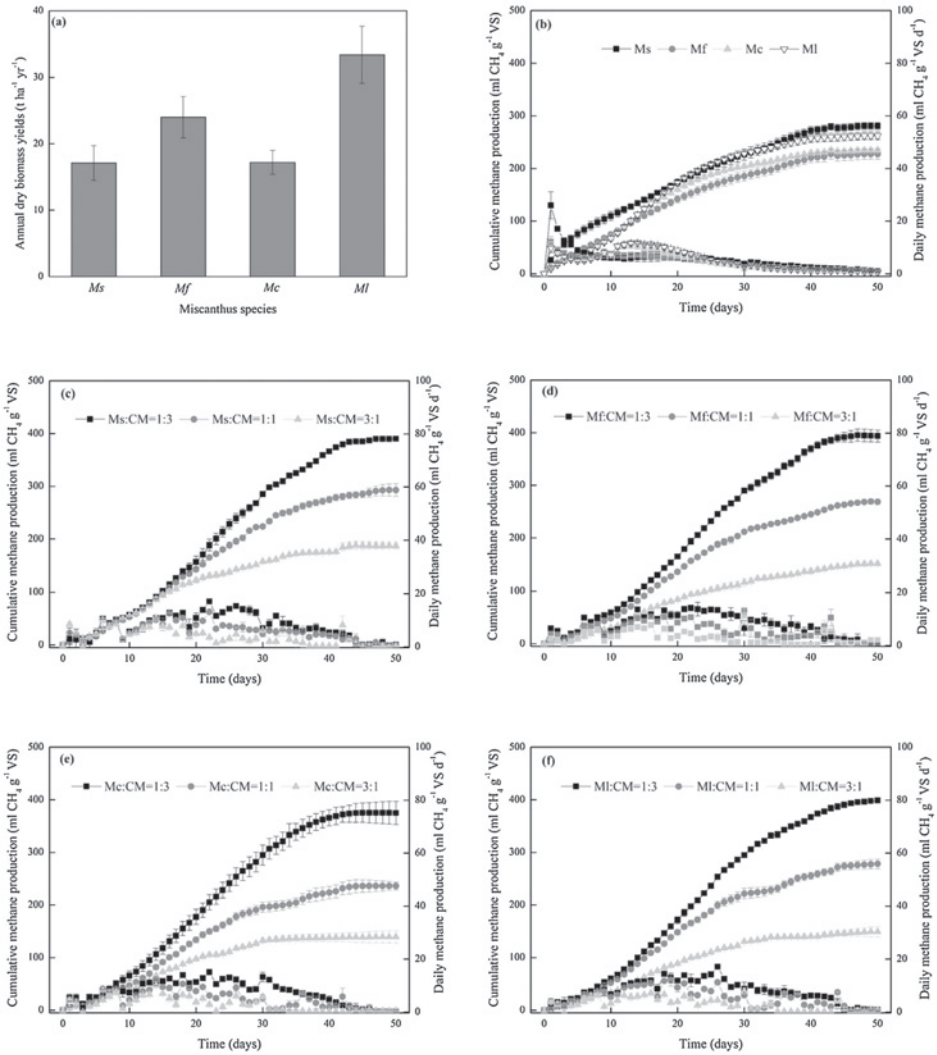

Fig. 5. The annual dry biomass yields (a), BMP and daily methane production of four species of Miscanthus (b), and co-digestion of chicken manure with Ms (c), Mf (d), Mc (e) and Ml (f) at ISR = 2.

duction from fresh seagrass is lower. It is hypothesized that sodium ions occurring in seagrass originating from culture systems based on salt water may have inhibited the process of methanogenesis [10]. The daily methane generation increased significantly in the first 5 days of each mono-digestion group. The maximum value was 37 ml g^{-1} VS d^{-1} at first day for SG_{5day}, while other samples ranged from 31 to 33 ml g^{-1} VS d^{-1}. After day 11 the methane production maintained a relatively stable generation at 2.6 ml g^{-1} VS d^{-1}. The water content decreased with increasing storage time, TS of SG_{fresh} was 15.07%, while it was 98.82% in the SG_{20day} (Table 2). The volatile solids to total solids ratio (VS/TS) showed a decreasing trend with increasing storage time coupled with longer storage time would bring about easier converted part of organic matter loss and further lower methane potential.

A similar curve of daily methane yield was found in the co-digestion groups with an increasing methane production rate in the initial 5 days and a stable production after 11 day in each group. Cumulative methane production in the four co-digestion groups (SG_{5day} + CM, SG_{10day} + CM, SG_{15day} + CM, SG_{20day} + CM,) ranged from 333.84 to 352.16 ml g^{-1} VS. These values did not show any sig-

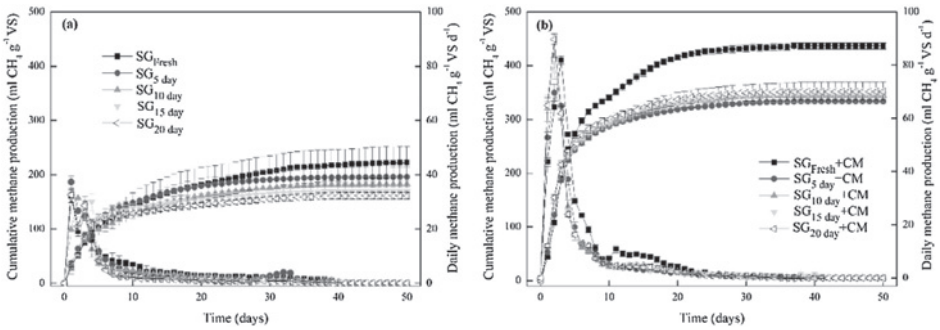

Fig. 6. The BMP and methane production rates of sea grass (a) and co-digestion with chicken manure (b).

nificant difference and were 76.4 to 80.6% that of the SG_{Fresh} + CM group which differed significantly ($P < 0.05$). It is possibly that the natural drying might lead floatation of biomass; constricting, shrinkage and loss of porosity in the cells making it inaccessible to enzymatic attack through decreased surface area to volume ratio. The maximum daily methane value in SG_{20day} + CM group reached 89.6 ml g^{-1} VS d^{-1}, which was about 1.3 times higher than the SG_{5day} +CM (69.6 ml g^{-1} VS d^{-1}) group, while other samples ranged from 81.1 to 83.4 ml g^{-1} VS d^{-1}. Importantly, these co-digestion tests carried out with higher substrate concentrations could induce positive effect on degradation rate [36] (Table SV, Supplementary data). The performance of the AD of SG and the mixture of SG and CM implied that SG is a good substrate for a biogas plant because of the exhibition of a significant ($P < 0.05$) synergistic effect when the different SG samples were digested with CM (Fig. 4). As a result, SG_{Fresh} + CM group represented the highest methane performance in this study.

Based on the results, collecting of seagrass accumulating on the beach and potential usage of this material for biogas production showed this macrophyta biomass can be considered a proper choice for co-digestion substrate with chichekn manure. Air-drying or natural storage could bring about organic matter loss. Nonetheless, the fresh seagrass can be preserved via ensiling. Furthermore, co-digestion seagrass will decrease nutrient reserves in the sea to counteract eutrophication and the increase in greenhouse effects from the environmental point of view [8]. However, it should be mentioned that AD of high-solid content lignocelluloses biomasses such *Miscanthus* and sea grass (in wet processes) may be prone to floatation and limited hydrolysis. Such conditions may lead to poor methane yields, hence neccesitating a good degree of meticulous operation vis a vis various pre-treatments.

3.6. Kinetic analysis

The accumulated methane potential was fitted with a first order model and a modified Gompertz model and the results are presented in Table 3. Looking at the model accuracy it is rather clear that the modified Gompertz model is more suitable to describe the degradation profiles of CM, CPW and the *Miscanthus* samples, while the first order model is more suitable for SG. For that reason, the performance of each sample type will be discussed based on the parameter data from the most suitable model.

For *Miscanthus* samples, the coefficient of determination (r^2) is 0.997 or above for all except one sample (i.e. Ms). This shows that the modified Gompertz model is excellent for this kind of sample, which can be hinted by the sigmoidal shape of the cumulative methane production profiles. The poor fit for Ms, is explained by a deceleration of the production at the start of the test where a profile following a sigmoidal shape normally accelerates. Most likely the deceleration at the start for Ms is an anomaly as all other co-digestions involving Ms as well as all other *Miscanthus* samples showed an acceleration at the start. Chicken manure and chicken processing waste also showed a rather typical sigmoidal curve, which is why the modified Gompertz model was more suitable to describe the degradation profile of this sample type. The sea grass was characterized by a more typical first order kinetic degradation profile with continuous deceleration of the gas production. However, as the deceleration was rather uneven and a few samples showed two or more stages in their gas profile, the accuracy was still rather low for the first order model. For that reason, the model data for the sea grass samples should be regarded with caution.

The maximum methane production rate for the mono digestion of CM was significantly higher (139 vs. 16.6 and 19.2 ml g^{-1} VS d^{-1}) compared to what was obtained by Li et al. [38] and Kafle et al. [15]. This together with the high methane potential indicate that the CM employed in this study consists of high amounts of fast and easy degradable energy-rich material.

Co-digestion with CM seems to increase the degradation rate of SG, demonstrated by higher first order rate constants when co-digested with CM. Interestingly, co-digestion rate constants are even higher compared to monodigestion of CM. These indicate that there is a positive effect on the degradation rate from the co-digestion. The storage time has a positive effect on the degradation rate, but due the correlated negative effect on the methane yield a long storage time has to be considered a poor option. Due to the poor fit of the model parameters for SG this information should be regarded with caution and further tests is needed for confirmation.

For all *Miscanthus* samples, the highest maximum methane production rate and the synergetic effect (4.5–10.3%) were observed at a mixing ratio of 1:3 (M:CM), which further support that this is the most suitable mixing ratio of the studied substrates. This shows that co-digestion with CM increase the degradation rate, but as the increases are very small, it has a rather limited effect. All co-digestions between the *Miscanthus* samples and CM at 3:1 (M:CM) actually has lower maximum methane production rates compared to monodigestion of the respective *Miscanthus* sample. This, in combination with the fact that the maximum methane production rate for all co-digestions are considerably lower than with monodigestion of CM, indicate that the co-digestion of these two samples has a negative effect on the degradation rate. The lag times are also longer for all co-digestions, which furthermore suggests that co-digestion of these two samples has a negative impact on the overall degradation time. Looking at the four different *Mischantus* species,

Table 3
Calculated model parameters and accurary of the first order model and the modified Gompertz model. *RMSE* – Root Mean Square Error; r^2 – Coefficient of determination.

	First order model					Modified Gompertz model				
Sample	k	t_{lag}	B_0	*RMSE*	r^2	R_m	t_{lag}	B_0	*RMSE*	r^2
Chicken manure	0.22	2.0	413	20.0	0.978	139.0	1.5	408	9.6	0.995
CPW ISR 2	0.10	17.2	486	33.7	0.964	57.7	12.7	487	18.0	0.990
CPW ISR 4	0.11	5.0	451	18.7	0.984	73.8	3.8	439	7.6	0.997
CPW ISR 6	0.21	3.0	413	17.4	0.979	126.3	2.3	412	10.0	0.993
CPW + CM ISR 2	0.06	5.8	335	14.7	0.979	32.6	3.8	311	9.3	0.992
CPW + CM ISR 4	0.12	1.8	292	11.4	0.981	52.5	0.5	285	11.2	0.981
CPW + CM ISR 6	0.20	1.0	264	11.2	0.970	92.3	0.8	255	9.3	0.979
Mc	0.06	4.2	255	8.9	0.986	25.1	2.0	239	2.5	0.999
Ml	0.06	5.4	286	10.7	0.985	28.4	3.1	269	2.9	0.999
Ms	0.04	0.0	339	8.2	0.989	25.0	0.0	290	11.5	0.979
Mf	0.03	0.6	289	4.0	0.997	19.6	0.0	239	3.5	0.997
Mc + CM (1:3)	0.04	7.2	496	15.4	0.987	35.6	5.9	415	5.6	0.998
Mc + CM (1:1)	0.05	6.2	276	7.7	0.991	23.7	4.7	245	3.0	0.999
Mc + CM (3:1)	0.07	5.4	151	5.3	0.988	17.2	3.8	143	2.4	0.997
Ml + CM (1:3)	0.03	7.3	568	13.2	0.991	35.6	6.6	439	3.2	0.999
Ml + CM (1:1)	0.04	6.0	282	6.9	0.992	22.6	4.6	245	3.0	0.999
Ml + CM (3:1)	0.06	5.7	163	5.3	0.989	16.2	4.4	149	2.2	0.998
Ms + CM (1:3)	0.03	7.4	607	14.4	0.989	34.2	6.8	445	4.9	0.999
Ms + CM (1:1)	0.04	6.2	380	9.5	0.991	27.0	5.2	314	2.7	0.999
Ms + CM (3:1)	0.05	3.3	215	4.8	0.994	18.4	1.8	193	3.4	0.997
Mf + CM (1:3)	0.03	7.1	609	14.1	0.990	34.0	6.3	450	4.3	0.999
Mf + CM (1:1)	0.04	6.3	332	8.0	0.992	25.1	5.0	281	2.8	0.999
Mf + CM (3:1)	0.04	4.7	188	2.7	0.997	13.9	3.9	156	3.0	0.997
SG (fresh)	0.10	0.0	222	8.4	0.975	40.5	0.0	210	13.2	0.938
SG (5 days)	0.15	0.0	196	8.2	0.963	56.1	0.0	188	10.7	0.937
SG (10 days)	0.13	0.0	183	7.5	0.967	42.6	0.0	175	11.1	0.928
SG (15 days)	0.19	0.0	170	9.0	0.934	65.7	0.0	162	8.9	0.936
SG (20 days)	0.17	0.0	162	9.6	0.915	60.6	0.0	152	9.6	0.915
SG (fresh) + CM	0.17	0.0	437	11.4	0.987	131.5	0.0	426	19.1	0.963
SG (5 days) + CM	0.23	0.0	334	12.0	0.969	142.0	0.0	324	13.5	0.961
SG (10 days) + CM	0.23	0.0	345	14.9	0.953	154.8	0.0	334	16.7	0.941
SG (15 days) + CM	0.23	0.0	347	14.7	0.955	150.4	0.0	336	16.7	0.942
SG (20 days) + CM	0.24	0.0	352	15.0	0.954	160.4	0.0	341	16.7	0.943

the differences in parameter values between the four *Miscanthus* species are small, but given the increase in maximum methane yield (B_0) when Mf is co-digested with CM, it is suggested that this *Miscanthus* type is the most suitable selection for an efficient co-digestion mixture.

Co-digestion of CM with CPW seemed to have a negative influence on the maximum methane production with higher values for mono digestion of both sample types. However, as the lag time parameter is reduced significantly, the overall effect on the overall degradation time does not have to be negative. As both of these samples showed higher maximum specific methane production when digested individually, it can be concluded that the combination of CM and CPW is a poor option as a co-digestion mixture.

Overall it can be concluded that SG_{fresh} is the most suitable option as co-substrate for CM from a kinetic and modelling perspective. It both has a high degradation rate and methane yield parameter. The worst co-substrate would be CPW as it showed a negative effect on both the methane production rate and yield. However, it should be pointed out that a kinetic analysis of batch tests should mainly be regarded with some caution as anaerobic digestion is such a dynamic process influenced by several parameters (e.g. inoculum source, pretreatment and storage condition, heating, mixing, addition of nutrients or not). It also believed that the kinetics would change significantly after continuous operation of the respective co-digestions. Partly due to adaption of the microorganisms, but also due to the change in available nutrients that comes with a new substrate type.

4. Conclusions

The results from the present study showed that *Miscanthus* and seagrass could be utilized as co-feedstocks with chicken manure for biogas production, while chicken processing waste might need more attention even it showed a high methane production rate at high I/S ratio. The mixing ratio is an essential parameter to achieve synergistic effect for *Miscanthus*/sea grass and chicken manure. *Miscanthus* and sea grass therefore replace conventional food-type energy crops in manure-based AD process. The batch test for biochemical methane potential is only the first step that has to be expanded by long-term anaerobic digestions experiments. But it provides fundamental information to verify whether the chosen feedstocks are suitable for co-digestion, also an efficient way for evaluating the feasibility of utilisation different regional biomass around biogas plants.

Acknowledgement

This study was financially supported by Nova Skantek Environmental Technology (Beijing) Co., Ltd. through the EU-China SME Innovation Funding (SQ2013ZOA000002, SQ2013ZOC500005)from the China Ministry of S & T. This research was also financially supported National "Twelfth Five-Year" Plan for Science & Technology Support (2014BAD24B01).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.11.008.

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Paper V

Bioresource Technology 224 (2017) 78–86

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

The effects of pre-aeration and inoculation on solid-state anaerobic digestion of rice straw

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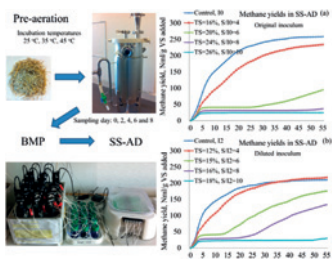
HIGHLIGHTS

- Impact of pre-aeration on the degradation of rice straw in SS-AD was investigated.
- Pre-aerated straw for 2 d/35 °C showed the highest hydrolytic efficiency and BMP.
- VFAs accumulation at high S/I ratio and TS content led to a reduced methane yield.
- Lower inoculum concentration was priority choice for rapid initiation in SS-AD.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 October 2016
Received in revised form 24 November 2016
Accepted 27 November 2016
Available online 29 November 2016

Keywords:
Solid-state anaerobic digestion
Rice straw
Pre-aeration
Substrate to inoculum ratio
Inoculum concentration

ABSTRACT

Pre-aeration was investigated for enhancing biodegradation of recalcitrant lignocellulosic structure of rice straw under various low temperatures regimes (25, 35 and 45 °C) and aeration durations (0, 2, 4, 6 and 8 days). It was demonstrated aerated rice straw for 2 days at 35 °C resulted in highest hydrolytic efficiency and biochemical methane potential (BMP) (355.3 ± 18.7 ml CH_4/gVS). Furthermore, both methane yields and initiation speeds of the solid-state anaerobic digestion (SS-AD) were inversely proportional to substrate-to-inoculum ratios due to the accumulation of volatile fatty acids (VFAs) and poor mass transfer. The highest methane yield achieved under SS-AD was 234 ml CH_4/gVS at TS of 16% which 72% of the BMP. Inoculum dilution with recycled water improved buffering capacity and mitigated accumulation of VFAs, resulting in an improved SS-AD performance. The combined pre-aeration and SS-AD was therefore established as a viable option to accelerate methane production for lignocellulosic biomass.

1. Introduction

Nowadays, increasing attention has been devoted on various strategies for the bioconversion of biomass into methane-rich biogas due to increased global warming, the need for sustainable waste management and high energy costs (Li et al., 2016b). The anaerobic digestion (AD) or biomethanation is an attractive approach to biodegradable waste treatment which has a dual-advantage of volumetric reduction of organic wastes in the oxygen-free condition and renewable energy generation such as biogas, containing 60–70% of methane (Yan et al., 2015). AD of biowaste and sludge is a well-developed technique in European countries. In Germany, which is the leading country in this field, >50% of the biogas potential results from energy crops treated in over 7000 biogas plants (Li et al., 2011). Furthermore, it was reported by Swedish Energy Agency that there are over 260 biogas plants in

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http://dx.doi.org/10.1016/j.biortech.2016.11.104
0960-8524/

Sweden, which facilitates the utilization of sewage sludge, manure, agricultural crops and food waste (IGR, 2015). Compared to liquid-state anaerobic digestion (L-AD), solid-state anaerobic digestion (SS-AD) has several predominances including smaller digester volume, higher solid loading capacity with total solids (TS) content of 15%–35%, free of floating and stratification of fibrous materials, reduced energy requirements for agitation, minimal material wear by fewer detachable machine parts to handle. SS-AD may also be less susceptibility to detrimental substances and over acidification especially when the new substrate is mixed with the digestate from the bottom of the reactor (Weiland, 2010). SS-AD has been used to manage municipal solid waste since the 1990s and preferred over conventional L-AD (Karthikeyan and Visvanathan, 2013). Moreover, various types of organic wastes with lignocellulosic structure of high solid contents are also treated by SS-AD to produce biogas, avoiding the subsequent slurry treatment (Li et al., 2011). It has also been reported that there is no significant difference in methane yields in both SS-AD and L-AD when treating lignocellulosic biomasses such as wheat straw, switch grass and corn stover (Brown et al., 2012).

Rice straw is one of the major agricultural wastes and the dry content of global rice straw reached approximately 741 million tons in 2014 (FAO, 2016). The waste management of rice straw via incineration or landfilling can raise serious environmental problems such as greenhouse gas emission. Several studies have reported the co-digestion of rice straw with animal manure or waste water to produce biogas (Mussoline et al., 2012; Ye et al., 2013). However, one of the drawbacks for rice straw as co-substrate is that pre-processing may be required, including material particle size reduction and pre-mixing prior to digestion. Also, it is complicated to adjust substrates ratio in co-digestion and the outcome can either be synergetic, leading higher methane yields, or antagonistic, leading to even lower yields (Li et al., 2017). It is noteworthy that L-AD is not preferred to anaerobically digest rice straw since rice straw cannot be pumped or homogenized with conventional digester without grinding due to its high TS contents. AD of rice straw in L-AD is often plague with clogging of tubing, stratification and scum formation, and floatation of biomass (Li et al., 2011). On the other hand, the recalcitrant lignocellulosic structure and nutritive deficiency are the major problem of digestion using rice straw as the mono-substrate in SS-AD. The reason is that hydrolysis becomes the limiting step of biogas production due to the highly crystalline and compact structure (Li et al., 2016b).

Pre-treatment is a critical step for improving the biodegradation of recalcitrant structure of lignocellulosic feedstocks. Numerous types of pretreatments ranging from physical (eg. size reduction), biological (eg. enzyme) to chemical (eg. alkaline) have been reported in literatures with the aim of improving the biogas or methane yield (Dehghani et al., 2015; Li et al., 2016a; Mussoline et al., 2012). Among biological methods, pre-aeration can be considered as a simple and easy operated pretreatment. Pre-aeration was introduced in a number of lab-scale studies to improve the start-up condition and initial performance in SS-AD. Yan et al. (2015) used composting pretreatment to facilitate the bioconversion efficiency. Nguyen's research showed a positive effect in methane production through micro-aeration (2007). The reason for this might be derived from better hydrolysis/acidification during the start-up of AD process and the provision of substrate for methanogens. Additionally, short time aeration is also used as the pre-step in the industrial scale in biogas plants which have numerous dry fermentation techniques and brands such as BEKON and GICON by using lignocellulosic biomass. A major concern with pre-aeration though is the finding a balance as to avoid the toxic effect of oxygen on the slow growing, fastidious methanogens. It is thus worthy to study the effect of different pre-aeration regimes on hydrolysis and subsequent methane generation.

Compared to L-AD, SS-AD usually has inadequate mass transfer and tends to be more difficult to start up and control, thus inoculation is a principal factor for this process (Le Hyaric et al., 2012). A primary parameter that drives SS-AD is substrate to inoculum (S/I) ratio. S/I ratio has been reported to have significant impact on the methane yield (Di Maria et al., 2012; Motte et al., 2013a; Xu et al., 2016). These studies showed that increasing the quantity or concentration of inoculum could strengthen the active microorganisms for a quick start-up, shortened digestion time and improved efficiency of the SS-AD. Comparison of inoculum from solid anaerobic digesters and dewatered effluent from liquid anaerobic digesters with different S/I ratios was studied by using yard trimmings with high TS content (Xu et al., 2016). This study concluded that dewatered effluent as inoculum reduced the start-up time due to higher concentration of methanogens which reduced the risk of volatile fatty acids (VFAs) accumulation in the initial stage of SS-AD, but prolonged lag phase and even inhibition was observed with high S/I ratio. Inoculum with proper concentration provides sufficient beneficial microorganisms and prevents process inhibition from VFAs accumulation (Schievano et al., 2010). It should be observed, though, that these studies were conducted in batch reactor by mixing the substrate and effluents from liquid or SS-AD process, without comparing the impact from inoculum concentration. The inoculum concentration has also been considered as a critical factor for performing effective SS-AD. At low inoculum concentration, diluted microbial community exhibits lower metabolic activity and weakens methane production. On the other hand, lower inoculum concentration ameliorates mass transfer of solutes in the solid matrix (Bollon et al., 2013).

From the above, it is clear that the effects of pre-aeration and the role of inoculum in SS-AD require further investigation. For example, Yan et al. (2015) used composting pretreatment as a prelude to SS-AD of rice straw. However, the impact of composting on the biodegradability of the rice straw mixture was not investigated. In this study, low temperature pre-aeration pretreatment regimes (temperatures of 25, 35, 45 °C and duration of 0, 2, 4, 6, 8 days) were evaluated to improve the biodegradability of rice straw. To facilitate a process study of SS-AD, the effects of inoculum concentration via dilution using recycled water and S/I ratio were evaluated. The study was performed via a three phase configuration: (1) pre-aeration to improve hydrolysis of rice straw (2) biochemical methane potential assay to evaluate the effects of the pre-treatment on methane production and (3) SS-AD to test the feasibility of digestion of rice straw under the solid-state mode.

2. Material and methods

2.1. Substrates and inoculum

The rice straw was collected from Caisang Lake Village (29°53′60″N 112°75′32″E) in Yueyang, China where it is prevalently cultivated with high productivity. It was packed outdoor and air-dried for 2 months after harvest, then transported to Lund University, Sweden at the end of October 2015 and stored at 4 °C prior to use. The TS of the rice straw was 93.0% and a volatile solid (VS) was 80.2%. The rice straw was ground with a grinder (Grindomix 200, Retch USA) to pass through a 2-cm for homogeneity. The aerobic sludge was collected from the secondary sedimentation tank at a wastewater treatment plant (WWTP, Källby, Lund, Sweden). The aerobic sludge was statically placed for 24 h and the supernatant was collected to mix with rice straw in order to adjust moisture content and enrich microbial diversity for aeration treatment.

The anaerobic sludge (inoculum) was collected from the anaerobic digester at the same WWTP. The inoculum was pre-incubated

at 37 °C under anaerobic conditions for 5 days to reduce background interference of methane production. The inoculum had an average TS of 4.8%, VS of 3.2% and pH of 8.0. The dilution liquid (recycled water) was collected from the final clarification tank at the same WWTP. The recycled water had COD of 27 mg/L, total nitrogen of 0.3 mg/L and total phosphorus of 10 mg/L. Its character showed nontoxic effect as the dilution liquid instead of tap water, which is a precious resource. Other characteristics of the substrate and inoculum are listed in Table 1.

2.2. Experimental design

2.2.1. Pre-aeration process

The reactors set-up for pre-aeration consisted of three wall-jacketed stainless steel tanks (CSTR-10S, Bioprocess Control AB, Sweden) with 10-L working volume which was designed to have high flexibility for either composting (aeration) or AD in a single closed vessel. The overhead motor for stirring was removed from the main lid while keeping the feeding funnel open. The schematic diagram of the experiment is presented in Fig. 1a. Rice straw was mixed with the supernatant of the aerobic sludge at a ratio of 1:2 (fresh wt. basis). Five hundred grams (500 g) of rice straw and 800 mL supernatant of the aerobic sludge were mixed plus 500 g plastic beads addition as bulking agent. The supernatant of the aerobic sludge was used to adjust C/N ratio to ~25 and moisture content (60%) of matrix to optimize the initial condition of short-time aeration. It was also expected to enrich the microbial diversities and quantities in the system by buffering low pH and augment nutrients in the mixture. Wood chips were distributed at the bottom of the tank before loading substrates to secure the ventilation by its coarse structure. The reactors were incubated at different temperatures (25 °C, 35 °C and 45 °C) by recycling heated water in the wall-jacket space of the reactor. The mixture was aerated with constant air flow (0.5 L/min/kg substrates, dry wt. basis) for 8 days and samples were collected on day 0, 2, 4, 6 and 8 h as recommended by (Zhou et al., 2014).

2.2.2. Methane potential assessment of pre-aerated substrate

To determine the biochemical methane potential (BMP) and compare the efficiency from the rice straw treated under different regimes (incubation temperatures and aeration times), the batch-mode Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control AB, Sweden) was used to perform BMP tests. Inoculum to substrate (I/S) ratio was set at 2:1 (based on VS). Cellulose (Avicel PH-101, Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control to validate the inoculum activity and evaluate the experimental protocol. The system was mixed intermittently (160 rpm, stirrers were on for 5 min and off for 25 min continuously throughout the test) to ensure a good mass transfer (Fig. 1b). Test bottles were incubated under mesophilic condition (37 °C) in triplicates until the daily methane production was less than 1% of the accumulated methane volume. Detail information of the AMPTS system has been reported previously reported by Li et al. (2017). The daily methane volume and methane production rate (adjusted to standard conditions i.e. 0 °C, 1 atm and dry conditions) was calculated by subtracting the methane production of the inoculum in blank reactor from the test samples which contained exclusively rice straw and the methane yield was gotten by dividing the volume with the amount of VS added.

Table 1
Characters of rice straw and anaerobic sludge.

	Rice straw	Inoculum
TOC (mg/l)[a]	398,000 ± 22	13,230 ± 19
TKN (mg/l)[a]	14,070 ± 20	1930 ± 9
C/N[a]	28.3 ± 1.1	6.9 ± 0.5
TS (%)[a]	93.0 ± 2.3	4.8 ± 0.6
VS (%)[a]	80.2 ± 0.7	3.2 ± 0.3
TS/VS[a]	1.2 ± 0.4	1.5 ± 0.2
sCOD (mg/l TS)[a]	33.3 ± 0.5	5865.5 ± 17.0
pH	6.0 ± 0.1	8.0 ± 0.1
PA (mg/l)	299 ± 11	4427 ± 15
VFAs (mg/l)	ND	1131 ± 11

ND = not determined.
[a] Based on dry weight of samples; the others are based on wet weight of samples.

2.2.3. Evaluation of pre-aeration and inoculum effects on solid-state anaerobic digestion

In order to compare the effect of inoculum concentration on the pre-aerated rice straw, two inoculum concentrations were adopted: (1) the original inoculum (I_0) and (2) I_0 was diluted with the recycled water from the same wastewater treatment plant by a factor of two (I_2). The pre-aerated rice straw which showed the highest BMP value was loaded into 1 L SS-AD reactor (with working volume of 700 ml) and the inoculum mixed at S/I ratios of 2–10 (VS basis). The pre-aerated rice straw was mixed with the original inoculum (I_0) to obtain S/I ratios of 4, 6, 8 and 10 (based on VS) with corresponding TS contents of 16, 20, 24 and 28%. For I_2, the S/I were 6, 8 and 10 which corresponded to TS contents of 15, 16, and 18% respectively (Fig. 1c). Low S/I ratio of 2 for both inoculums was set as control and L-AD performance was compared by I_2 with S/I ratio of 4. The pre-aerated rice straw was loaded into 1 L glass reactors first and followed by inoculum addition to avoid floating problem with high S/I ratios. Methane production was monitored with aid of the AMPTS II for 55 days, the period of which was agreed to Xu et al's study, using lignocellulosic biomass in SS-AD (Xu et al., 2016). Triplicate reactors were run for each condition. Inoculum without any substrate addition was used as a blank. The daily methane volume and methane production rate (adjusted to 0 °C, 1 atm and dry condition) were calculated following the description in previous section.

2.3. Analytical methods

Core temperature of the aerated rice straw was monitored daily with the use of temperature probes (Rubicson, Sweden). The TS and VS contents of inoculum and substrate were determined according to standard protocols (APHA, 1998). The pH and partial alkalinity (PA) were measured using a TitraLab™ 80 titrator (Radiometer, Denmark) as reported in another study (Nges and Liu, 2009). Total organic carbon (TOC) and dissolved organic carbon (DOC) of rice straw and the supernatant of aerobic sludge were determined by Walkey-Black method as described by Nelson and Sommers (1982). Ammonium, total Kjeldahl nitrogen (TKN), total phosphorus, and chemical oxygen demand (COD) contents were determined by Lange test cuvette kits (HACH LANGE Gmbh, Germany). The hydrolysis efficiency was calculated based on the ratio between DOC of hydrolysate and TOC of the aerated rice straw. The electrical conductivity (EC) and cress (*Lepidium sativum* L.) seed germination index (GI) were determined as described in HKORC (2015). The changes of cellulose, hemicellulose and lignin contents were determined by the standard analysis of hydrolysis process described by the National Renewable Energy Laboratory (NREL) Analytical Procedure (Sluiter et al., 2008).

For VFAs analysis, the pH of the digestate samples were adjusted to 1–4 with 0.5 M NaOH and filtered through 0.22 μm filter by HPLC (1200 series, Agilent Technologies) equipped with a refractive index detector (RID) and an organic acid analysis column (300 mm × 7.8 mm, Aminex® 160 HPX-87H Ion Exclusion Column). The column was operated at 55 °C and eluted with 5 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml/min. The injection volume was 10 μL.

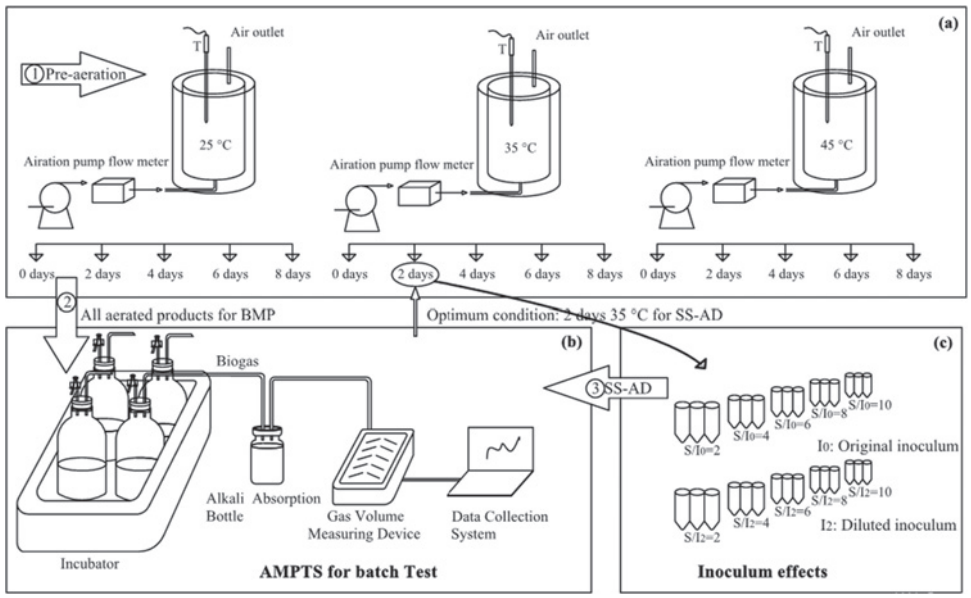

Fig. 1. Experimental processing flow diagram. (a) Pre-aeration of rice straw by various aeration times and incubation temperatures; (b) methane production of different S/I ratios by using Automatic Methane Potential Test System; (c) biochemical methane potential (BMP) analysis by using Automatic Methane Potential Test System.

2.4. Statistical analysis

All the analyses were performed in triplicate sets and the mean values and standard deviations are presented. The data were processed using SigmaPlot 11.0 and IBM SPSS statistics 19 while the significance of differences were tested using Duncan multiple rage test at $p < 0.05$.

3. Results and discussion

3.1. Characterization of rice straw and impact various pre-aeration regimes

Table 1 showed the basic characteristics of rice straw and the inoculum wherein a C/N ratio of 28 for rice straw is most prominent as per a feedstock for aerobic degradation. The cellulose, hemicelluloses and lignin content are shown in Table 2. Rice straw shows a rather high content of lignin and ash which is in agreement with figures published in a another study (Weerachanchai et al., 2012). The lignin content is especially high as compared to lignocellulosic biomasses such as *Miscanthus*, corn stover, switch grass and wheat straw (Brown et al., 2012). It has been reported that lignin is the compound that physically shields hemicelluloses and cellulose thereby limiting their hydrolysis (Taherzadeh and Karimi, 2008). Bioconversion of lignin to biogas is also problematic though some advances in pre-treatment technology have yielded fruit in the direction of the bioconversion of lignin (Weerachanchai et al., 2012) and ultimately increased biogas production (Shen et al., 2014). During pre-aeration, aerobic microorganism hydrolyze organic solids by producing exoenzymes to solubilize the solid substrate (López et al., 2002). Thereafter, the dissolved substrates enter the cell to be degraded by endoenzymes, resulting in microbial growth and heat generation. As with easily putrescible substrates such as food waste, the core temperature of matrix generally increased as a results of rapid heat generation (>55 °C) within 48 h of aeration (Kumar et al., 2010). In this study, the temperature was maintained at incubation temperature during the 8 days aeration process for all three reactors. This indicated that microbial access to cellulose (a major biodegradable component of rice straw) was inhibited by recalcitrant structure during the decomposition process probably due to the high lignin content.

That notwithstanding, pre-aeration led to some changes in the structural composition of the rice straw. The basic characteristics of the untreated and aerated rice straw were listed in Table 2. The VS/TS values decreased along with aeration days under all incubation temperatures. VS reduction (basis on TS) at 35 °C was 4% which was slightly higher than the change at 45 °C (2.4%) and 25 °C (1.7%). The result was in agreement with the decomposition rates of lignocellulose since around 75% of rice straw was constituted of cellulose, hemicellulose and lignin. The decomposition rates of lignocellulose after aeration (16.2% at 25 °C, 17.7% at 35 °C and 11.1% at 45 °C) indicated that mesophilic temperature was more efficient on its degradation. More reduction of lignin content was found on the 2nd aeration day at 35 °C. It has been reported that aerobic microorganisms which are capable of mineralizing lignin cannot survive under higher temperature (Tang et al., 2007). These findings are confirmed in another study Vikman et al. (2002), wherein it is stated that mesophilic temperatures are more effective in aerobic lignin degradation. Therefore, the reason for the reductions of lignocellulosic content of rice straw may be due to the relatively higher production of hydrolytic enzymes, and the increased specific microbial growth is mainly form incubation temperatures other than self-heat generation.

Table 2
Component analysis of the rice straw, substrates with different incubation temperatures and aeration days.

Incubation temperatures _Aeration days	VS/TS (%)[a]	Cellulose (%)[a]	Hemicellulose (%)[a]	Lignin (%)[a]	Ash (%)[a]	Total C (%)[a]	Total N (%)[a]
Untreated rice straw	85.4 ± 0.2	30.2 ± 0.3	18.3 ± 0.1	23.3 ± 1.1	14.6 ± 0.2	46.3 ± 0.2	1.85 ± 0.2
25 °C_ 2 days	84.6 ± 0.1	29.6 ± 0.1	16.9 ± 0.1	21.6 ± 0.0	15.4 ± 0.1	47.2 ± 0.1	1.96 ± 0.2
25 °C_ 4 days	83.9 ± 0.3	25.2 ± 1.6	14.1 ± 1.2	24.6 ± 1.7	16.1 ± 0.3	47.3 ± 0.3	1.38 ± 0.4
25 °C_ 6 days	82.9 ± 0.2	26.2 ± 0.6	15.6 ± 0.2	17.7 ± 0.4	17.1 ± 0.2	46.3 ± 0.2	1.42 ± 0.2
25 °C_ 8 days	82.8 ± 0.4	26.4 ± 0.8	16.4 ± 0.7	17.4 ± 0.8	17.2 ± 0.4	46.8 ± 0.4	1.24 ± 0.4
35 °C_ 2 days	84.5 ± 0.2	28.8 ± 2.1	14.8 ± 3.0	15.9 ± 2.0	15.6 ± 0.1	45.3 ± 0.2	1.80 ± 0.2
35 °C_ 4 days	83.6 ± 0.4	26.7 ± 3.2	16.0 ± 2.6	19.3 ± 2.7	16.4 ± 0.2	49.1 ± 0.4	1.30 ± 0.4
35 °C_ 6 days	81.3 ± 0.1	24.5 ± 0.5	14.6 ± 0.4	22.1 ± 2.9	18.8 ± 0.5	45.4 ± 0.1	1.53 ± 0.1
35 °C_ 8 days	80.5 ± 0.1	23.9 ± 0.0	16.8 ± 1.4	18.4 ± 2.0	19.5 ± 0.1	46.1 ± 0.1	1.59 ± 0.1
45 °C_ 2 days	85.3 ± 0.1	27.5 ± 0.5	14.2 ± 0.0	24.8 ± 2.0	14.7 ± 0.2	47.2 ± 0.1	1.49 ± 0.2
45 °C_ 4 days	84.6 ± 0.3	28.6 ± 0.6	15.3 ± 1.0	23.3 ± 0.6	15.4 ± 0.3	47.1 ± 0.3	1.39 ± 0.3
45 °C_ 6 days	83.7 ± 0.2	28.2 ± 0.5	18.2 ± 0.1	17.5 ± 1.0	16.3 ± 0.2	46.8 ± 0.2	1.52 ± 0.2
45 °C_ 8 days	82.9 ± 0.3	26.7 ± 0.2	15.7 ± 0.1	21.4 ± 2.1	17.1 ± 0.3	47.3 ± 0.3	1.45 ± 0.3

[a] Mass percentage.

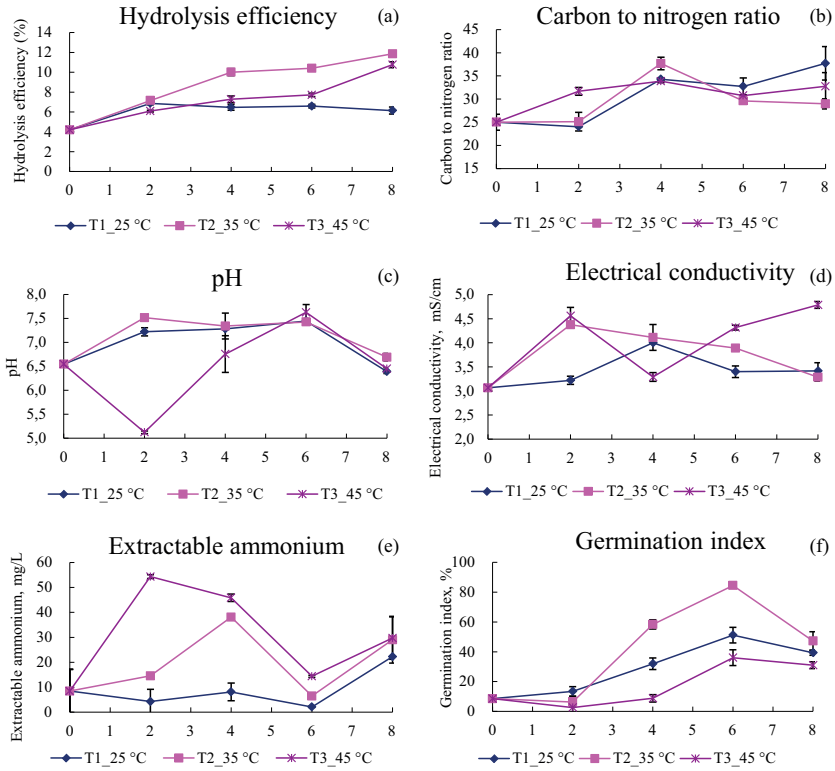

Fig. 2. Changes of rice straw treated by various incubation temperatures and aeration days. (a) hydrolysis efficiency; (b) carbon to nitrogen ratio; (c) pH; (d) electrical conductivity; (e) extractable ammonium; (f) germination index.

Concurrent with the decomposition of lignocellulose, different hydrolysis efficiencies ($p < 0.05$) were compared with various incubation temperatures (Fig. 2). The hydrolysis efficiencies values at 35 °C and 45 °C increased slightly by 11.9% and 10.8% respectively due to effective degradation, while a smaller increase of 6.1% was observed at 25 °C. The hydrolysis efficiency can be explained by the ratio between DOC of hydrolysate and TOC in rice straw mixture, which illustrated the extent to which readily biodegradable products can be converted for methane production. After 8 days pre-aeration, the lowest C/N ratio was observed at 35 °C, and it has been reported that C/N ratio decreases along with degradation of organic substances during composting (Fig. 2b) (Epstein, 1996).

From these findings, it is plausible to state nitrogenous compounds are poorly degraded as compared to cellulose and hemicellulose during aeration of lignocellulosic biomasses.

The variations in pH and EC are useful parameters for monitoring the pre-aeration process (Hosseini and Aziz, 2013). As shown in Fig. 2c, pH values was maintained stably around 7.0 ± 0.5 at 25 °C and 35 °C. Nevertheless, the pH value decreased rapidly on Day 2 at 45 °C. This can be attributed to fast conversion of the substrate to acidic compounds (Epstein, 1996). In contrast, the concentration of extractable ammonium increased rapidly on Day 2 at 45 °C, probably as a result of the decomposition of organic acids by microorganisms as well as by the release of ammonia. These free ions (free acids and ammonium ion) led to fluctuated change in EC values, and even contrary trends in different treatments (Fig. 2d-e). Generally, EC value below 4 mS cm^{-1} is considered as the stable status of organic substance conversion and has inhibition of plant growth (good GI performance) (Zhou et al., 2014). Thus, as shown in Fig. 2f, during the initial days, the treatments incubated at 35 °C and 45 °C had low GI values, probably was due to the phytotoxic effect of ammonium release during the early stage of composting. After aerated for 4 days, GI values of all treatments suddenly increased, indicating that huge quantities of organic substances which are phytotoxic were consumed by microbes and transformed into inorganic forms (Epstein, 1996).

3.2. Biodegradability of pre-aerated rice straw

Fig. 3 shows the evolution of methane production after pre-aeration under the various regimes. It is well documented that a C/N ratio of between 20 and 30 is optimal for an efficient and effective biogas process (Weiland, 2010). The methane production of untreated rice straw reached 306.2 ml/gVS which is close to methane yield of rice straw (340 ml CH_4/gVS) in another study (Paepatung et al., 2009). A major observation is the decrease in methane yields with increasing aeration times which was more profound at 25 °C wherein the yield after 8 days of incubation was significantly low ($p \leqslant 0.05$). At 35 °C and 45 °C, the yields from 2 days incubation were significantly high ($p \leqslant 0.05$). However, the yields after 4, 6 and 8 days incubation did not differ significantly ($p \leqslant 0.05$). These findings are in line with another study by Charles et al. (2009) wherein increased duration of aeration led to a decrease in methane production. The rice straw aerated for 2 days at 35 °C showed the highest methane yield of 355.3 ± 18.7 ml CH_4/gVS which was significantly higher ($p \leqslant 0.05$) than the rest. The highest methane yield corresponded to the highest decrease in lignin content and secondary minimal degradation of cellulose (Table 2). It would appear therefore that for an effective conversion of lignocellulosic biomass to methane, degradation of lignin is a most called upon prerequisite. As has been mentioned earlier, lignin is hardly converted to methane and it has been blamed as the compound responsible for the poor hydrolysis of lignocellulosic biomass (Taherzadeh and Karimi, 2008).

It should be noted that the methane yields after 4, 6 and 8 days incubation under the various temperatures did not differ significantly ($p \leqslant 0.05$) except for the yield at 25 °C. In all, the lowest methane yields were recorded after 8 days incubation (Fig. 3) which tallied with the highest reduction or degradation of cellulose (Table 2). Increased aeration may therefore lead to the proliferation of microorganisms which may use the easily degradable organic matter resulting in CO_2 production. Therefore, the operation of pre-aeration should be implemented prudently in order to improve the hydrolysis rate while conserving methane potential as much as possible.

The methane yields in this study are higher than other pre-treated rice straw sample e.g. extrusion pre-treated rice straw (Chen et al., 2014) and those reported for different lignocellulosic substrates such as wheat straw, corn stover and switch grass (Brown et al., 2012). This can be explained by the fact that hydrolytic exoenzymes produced by aerobic microorganism provided methanogenic metabolites which were feasibly converted methane (Capela et al., 1999; Kumar et al., 2010).

3.3. Effect of substrate load and inoculum concentration on SS-AD performance

Based on the aforementioned analysis, pre-aerated rice straw with optimum BMP (2 days at 35 °C) showed favorable functions on the substrate biodegradation. Thus, this pretreated sample was used as substrate for SS-AD with different S/I ratios and inoculum concentrations to facilitate methane yields. The treatment at S/I_0 of 2 (TS of 12%) was used as control to elucidate the AD performance without limitation of substrate diffusion and compared with treatments with high substrate loads (TS > 15%). Fig. 4a illustrates the cumulative methane along the digestion time calculated at standard temperature and pressure. The control treatment (S/I_0 of 2) showed highest methane yield of 238.5 ± 1.4 ml CH_4/gVS, which reached 72.8% of the optimum methane potential achieved in the BMP assays. For the SS-AD (TS > 15%), the methane yields

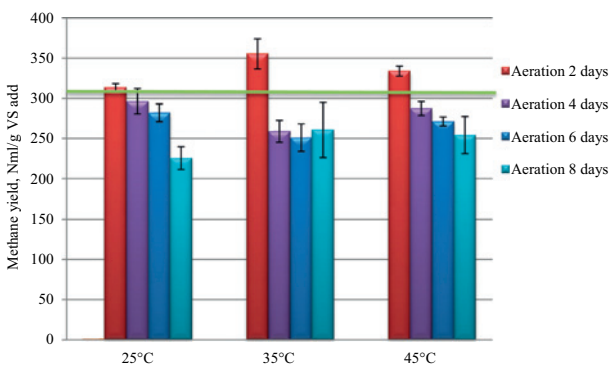

Fig. 3. The cumulative methane yields of untreated/aerated rice straw at various incubation temperatures (The green line stands for methane yield of untreated rice straw). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

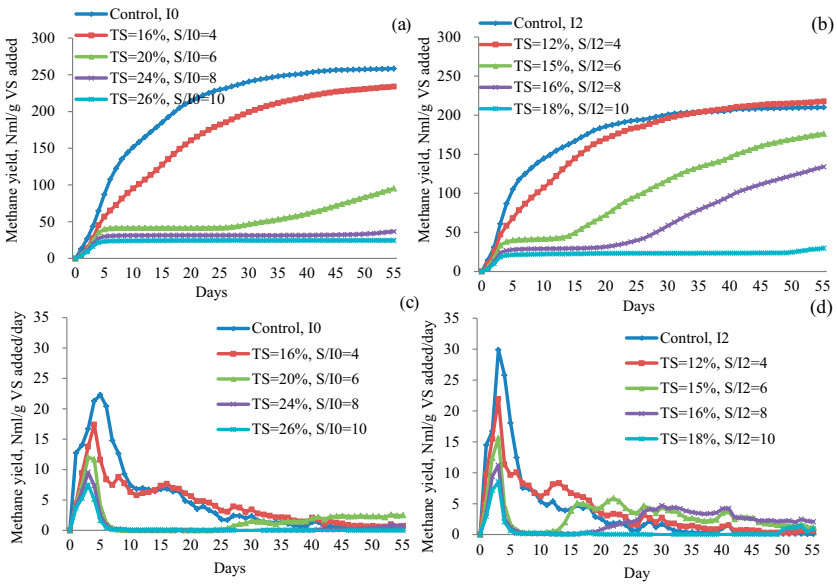

Fig. 4. Effects of inoculum concentration and S/I ratio on methane yields of pre-aerated rice straw. (a) Cumulative methane yields of I_0; (b) cumulative methane yields of I_2; (c) daily methane yields of I_0; (d) daily methane yields of I_2.

were found to be significantly lower ($p \leqslant 0.05$) than control and were inversely proportional to S/I_0 ratios. Amongst the SS-AD experiments using the original inoculum (I_0), the S/I_0 of 4 showed the highest methane production of 190.4 ± 10.8 ml CH_4/gVS which was significantly higher ($p \leqslant 0.05$) than others. A sharp decrease in methane production was observed at S/I_0 of 6, 8 and 10 (Fig. 4a) wherein all the cumulative CH_4 yields were below 50 ml CH_4/gVS. There seemed to be some sort of inhibition (prolonged lag phase) with the test at S/I_0 ratio of 6 showing some degree of recovery after 30 days of incubation. It is plausible therefore to state that at S/I_0 ratio higher than 4, digester failure occurred which was likely because of poor hydrolysis and mass diffusion limitation (Xu et al., 2014a). The decreased disintegration coefficient and hydrolysis rates at high TS content has also been reported in another study where wheat straw was used as substrate (Motte et al., 2013b). In addition, other studies have confirmed the decreasing enzymatic conversion of lignocellulosic biomass with increasing TS contents (Kristensen et al., 2009). The prolonged lag phase was concomitant with VFAs accumulation in the reactors with S/I_0 of 8 and 10. Fig. 5 shows the pH, partial alkalinity and VFAs in the various processes. The pH was fairly constant for the control and S/I of 4 and 6 (averaging 8) except at S/I of 8 and 10 which dropped below 7. The alkalinity showed a decreasing trend with increasing substrate load (increasing S/I ratio) The acetic acid played the dominant role in excessive VFAs inhibition in this AD process and showed an increasing trend with increasing substrate loads (Fig. 5c). It was revealed that VFAs concentration higher than 6 g/L led to the collapse of the reactor in this study. The concentration of propionic acid in treatments with limited methane production (1.7 g/L in $S/I_0 = 8$, 1.8 g/L in $S/I_0 = 10$) also exceeded the inhibitory level 1 g/L for methanogenic activities (Xu et al., 2014b).

The original inoculum (I_0) was diluted two times with recycled water to obtain a diluted inoculum (I_2) and as with the I_0 experiments; S/I_2 of 2 showed TS content below 15% and was kept as a control. It should be noted that the TS for S/I_2 of 4 was also below 15% and was hence the experiment was not considered as SS-AD. Treatments at S/I_2 of 2 and 4 stimulated the methane production resulting in 193.4 ± 0.4 ml CH_4/gVS and 187.1 ± 22.2 ml CH_4/gVS, respectively. S/I_2 of 2 achieved 61.2% of the optimum methane potential achieved in the BMP assays. Compared with I_0, significant quantity of methane was produced as S/I_2 of 6 and 8 (176.3 ± 15.7 ml CH_4/gVS and 133.9 ± 24.0 ml/gVS, respectively), though the lag phase was prolonged from 15 days to 20 days as substrate content increased. Methane production was significantly increased 3.6 folds as compared to I_0 (36.7 ± 5.5 ml/gVS) by using I_2 when the S/I ratio was set at 8. It was therefore evidence that I_2 had a better dilution capacity on acidic compounds and mass transfer performance by the additional water, both of which improved methanogenic activity. The high substrate load at S/I_2 of 10 led to reactor failure on the 4th day due to acid inhibition (Fig. 5c). On the contrary, although VFAs content exceeded the inhibitory level for methanogenic activities (1 g/L), the methane suppression at S/I_2 of 8 was controlled with better self-buffering capacity (Fig. 5b). Stability of AD system can be judged by VFA/alkalinity ratio and 0.1–2.5 is considered as optimum range. The increment in the ratio indicates higher risk of failure in the digester (Ertem, 2011). In this study, VFA/alkalinity ratio of below 2 indicated no process inhibition or excess acidification.

As shown in Fig. 4c-d, it took almost 5 days to reach the peak methane production at I_0 under all S/I ratios whereas it took only 3 days to reach the peak at I_2 under all the S/I ratios investigated . The optimum daily methane yield was 22.3 ml/gVS at I_0 while it was 29.9 ml/gVS at which were all for the controls. The daily methane yields decreased (as with the ultimate methane yield) with increasing substrate load. For S/I of 2 and 4 under both inoculums, 90% of the methane was produced within 20 days of incuba-

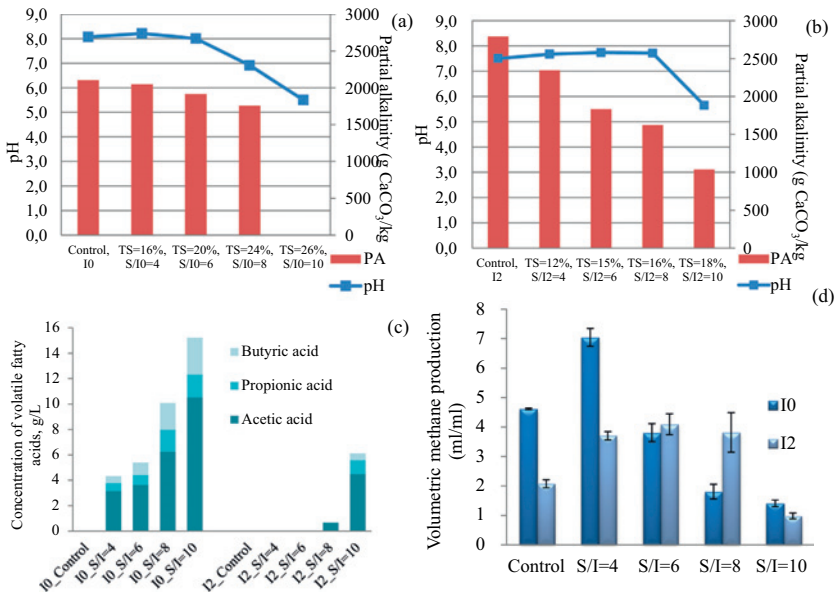

Fig. 5. Effects of inoculum concentration and S/I ratio on methane yields of pre-aerated rice straw. (a) pH and PA of I_0; (b) pH and PA of I_2; (c) composition of VFAs in final digestates; (d) volumetric methane production.

tion. However, for the higher S/I or load, less than 25% of the total methane was produced after 20 days of incubation. The prolonged lag phase meant that methane production could continue to be significant after the 55 days incubation period. The low methane yield achieved in this especially at high loads or TS are not uncommon. It has been reported that high TS can lead to slow mass transfer between microbes and substrate, hindrance in gas-liquid transfer (accumulation of CO_2 and H_2 in the liquid matrix) resulting in slow methane production and low methane yield (Yang et al., 2015). A total cessation in methane production has been reported at TS content higher than 28% in SS-AD (Xu et al., 2016). This is in agreement with the findings in this study wherein methane production ceased on the 3rd day of incubation when the TS content was higher than 24%. This can be the reason explained by lack of free water for microbial activity resulting in poor microbial metabolism (Yang et al., 2015). The low free water content in AD may also lead to a shift in the metabolic pathways of anaerobic microbes towards hydrogen production (Motte et al., 2013b) thereby rendering poor methane yields.

3.4. Volumetric methane production

The volumetric methane production (VMP) is the parameter explaining the efficiency of methane yields in per unit digester volume in order to optimize the economic value. The VMP values of various S/I ratios are shown in Fig. 5d. For I_0, VMP had a bell-shaped performance and significantly reduced when S/I ratios was higher than 4. As for I_2, no significant difference of VMP between S/I of 4, 6 and 8, while the VMP value was much lower at S/I of 10. The highest VMP was obtained at S/I of 4 for I_0 (7.0 ml/ml) which was 79.9% higher than the lowest S/I of 10. As for I_2, S/I of 6 showed to the maximum VMP (4.1 ml/ml), 76.0% higher than the lowest S/I of 10. For S/I ratios of 6 and 8, diluted inoculum showed priority on methane yields and VMP. Thus, in SS-AD, high inoculum concentration showed no advantage as concerns methane yield or methane productivity at high S/I ratios. On the contrary, the studied demonstrates feasibility of using diluted inoculum with high substrate loads for better process performance.

4. Conclusions

Pre-aeration (optimized for 2 days at 35 °C) led to improved hydrolysis of rice straw. Diluted inoculum showed priority for faster initiation in SS-AD especially at high substrate loads. It was demonstrated that high concentrated inoculums led to acidification and drop in reactor pH while diluted inoculums showed less acidification which translated to a better process performance at high substrate loads. The methane yields achieved under stable solid state conditions were between 60% and 75% of the specific methane yield of rice straw achieved in BMP assays. The experimental results provide insights into SS-AD of aerobic pre-treated rice straw.

Acknowledgements

Financial support from the China-EU Innovation Funding for Small and Medium Size Enterprise from the China Ministry of S&T (SQ2013ZOA000002) is gratefully acknowledged. This research was also financially supported by National "Twelfth Five-Year" Plan for Science & Technology Support Program (2014BAC24B01). The authors would like to thanks Mr. Mingxing Zhao (Jiangnan University, China) for the laboratory support.

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Paper VI

Enhancement of the solid-state anaerobic digestion of rice straw by liquor addition under mesophilic and thermophilic conditions

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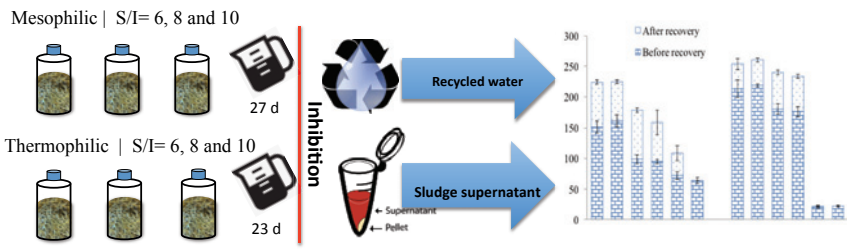
Abstract

Solid state anaerobic digestion (SS-AD) has gained attention in recent years because of it is robust, cheap and easy to operate. However, at high loads, the SS-AD is often plagued by acidification and poor methane yields. Mesophilic and thermophilic SS-AD at the different substrate to inoculum ratios (S/I) aided by liquor (recycled water and sludge supernatant) supplementation was studied in batch mode. Results demonstrated that the methane yields decreased with increasing S/I while the highest methane (260.3 ± 5 ml CH_4/g VS) was achieved under thermophilic conditions at a S/I of 8. This yield was 85% of the biochemical methane potential achieved under non-limiting conditions. Recovery of the inhibited or failed reactors with recycled water and sludge supernatant significantly improved process performance i.e. over 95.7% increment in methane yield under mesophilic conditions and process stability i.e. low volatile fatty acid (VFA) content and low VFA to alkalinity ratio. This study, therefore, revealed that the energy yield in an SS-AD of lignocellulosic biomass can be doubled at high loads by simply adding recycled water or sludge supernatant especially in an inhibited SS-AD process.

Graphic abstract



Keywords: Solid-state anaerobic digestion; Inhibition; Rice straw; Mass transfer; Methane yield.

1. Inroduction

Rice straw is a major agricultural waste and the amount (dry content) of global rice straw reached approximately 741 million tonnes in 2014 (FAO, 2016). Rice is a staple food in many parts of the world and increasing population growth and standard of living will eventually lead to an increase in production and hence rice straw. Waste management of rice straw is often done via landfilling or incineration. However, these methods are not environmentally friendly as they may be a cause of global warming via greenhouse emissions. On the other hand, rice straw can be converted to energy- rich biogas via the low cost, energy efficient anaerobic digestion (AD)(Weiland, 2010).

AD as a means of renewable energy production, waste management and nutrient recycle (creation of a bio-fertilizer) is gaining increasing recognition all over the world. These positive aspects coupled with rapid population growth, increasing energy demand, and global warming has promoted further research in the AD process development, improvement and wide application (Ariunbaatar et al., 2014). The sustainable production of renewable energy has received national as well as international attention wherein various energy policies have been enacted. For example, the EU Directive 2009/28/EC advocated for a 20% energy consumption from renewable sources and a binding minimum of 10% of renewable energy in domestic transport (European and Commission, 2009).

For the most part, solid state anaerobic digestion, SS-AD (>15 total solids or TS), is increasingly replacing the conventional wet AD, W-AD, (0.5-15% TS) especially in the treatment of lignocellulosic biomass (Yang et al., 2015). As opposed to wet AD, SS-AD has been reported to show resilient in handling feedstocks with higher TS content, relatively stable, requires less energy input and therefore, performs more effectively at higher organic loads and shows higher volumetric biogas productivity (Yang et al., 2015; Zhu et al., 2010).
In general, the disadvantages in using a lignocellulosic biomass such as rice straw as a substrate in the AD process may include its poor nutrient content, need for a pre-treatment to improve hydrolysis, poor moisture content etc (Zhou et al., 2017). In the same light, the challenges facing SS-AD of lignocellulosic biomass are primarily related poor methane yield and potential instability which either may be due to the inherent limits of SS-AD (e.g. retarded mass transfer caused by high solid content) or the recalcitrant nature of lignocellulosic biomass to hydrolysis or poor nutrient content (Nges et al., 2012; Yang et al., 2015). Amongst others, the moisture of the biomass and nutrients are essential for the activities of the waste-decomposing anaerobes and hence for effective SS-AD process (Hilkiah Igoni et al., 2008; Nges et al., 2012). Temperatures, fluidity, prompt acclimation of the microbial community in the seed inoculum may also impact on the SS-AD process (Abbassi-Guendouz et al., 2012; Di Maria et al., 2012; Li et al., 2011; Li et al., 2015; Liotta et al., 2016).

The AD process can be operated over a wide temperature range i.e. psychrophilic (11-25 °C), mesophilic (35 to 40 °C), thermophilic (50 to 55 °C) and hyperthermophilic (≥ 55 °C) wherein the SS-AD process is widely performed under mesophilic and thermophilic (Takashima et al., 2011; Weiland, 2010). Thermophilic conditions may be favourable for AD of lignocellulosic feedstocks since the high operating temperature can facilitate degradation or hydrolysis of recalcitrant cellulose (Frigon and Guiot, 2010; Li et al., 2015; Shi et al., 2013). However, hydrolytic products such as volatile fatty acids (VFAs) may turn to accumulate, thereby retarding the AD process, especially if there is the balance between hydrolytic (and acetogenic) bacteria and methanogens is not established timely (Weiland, 2010). On the other hand, mesophilic AD processes are often acclaimed as being stable and have been widely employed in (commercial) full-scale AD processes (FNR, 2010; Weiland, 2010). From an economic point of view, the mesophilic AD is highly promising and will be promoted in the future because of the lower heating requirements (Yan et al., 2015). The increased high heating energy demand under thermophilic conditions may, however, be settled by an increase in energy- rich methane production. It is therefore worth pursuing a comparative study of mesophilic and thermophilic SS-AD.

Inoculum or leachate recirculation of different regimes are commonly reported as a means of improving SS-AD and even recovering of failed (inhibited) SS-AD of lignocellulosic biomasses (Di Maria et al., 2012; Yang et al., 2015). In a recent study, different methods of inoculum addition were employed to reignite the performance of failed SS-AD processes (Yang et al., 2016). Nonetheless, inoculum often contains significant amounts coarse, particulate, material which may not adequately percolate through the near-tight structural arrangement of an SS-AD reactor. Therefore, there have been recommendations about the addition of fresh water to the inoculum during the batch SS-AD process of food waste (Shahriari et al., 2012). Moreover, knowledge about mesophilic or thermophilic SS-AD aided by liquor supplementation of lignocellulosic biomass such as rice straw is scared or still in its infancy in scientific literature even though the SS-AD process has received worldwide acclamation.

Therefore, the hypothesis investigated in the present study is that the process performance and stability in inhibited or failed thermophilic and mesophilic SS-AD of rice straw can be enhanced by liquor supplementation. Paying attention to the fact that SS-AD of a lignocellulosic biomass such as rice straw may be limited by its hydrolysis and poor moisture content, the experimental protocol was designed to include (i) comparison of the mesophilic and thermophilic SS-AD processes with the view that hydrolysis and microbial growth may be enhanced under thermophilic conditions (ii) investigate the impact of recycled water and sludge supernatant addition to alleviate the poor moisture content, improve mass transfer and dilute inhibitors (VFAs). It was therefore hypothesised that supplementation of inhibited or failed processes with recycled water or sludge supernatant would boost biodegradation in the SS-AD of rice straw under both mesophilic and thermophilic conditions. This study will add to the canon of knowledge and help close the knowledge gap in enhanced SS-AD processes of lignocellulosic biomasses especially rice straw which is still in its infancy.

2. Material and methods

2.1 Substrates and inoculum

The rice straw was collected from Luofang Town (27°85'19''N 115°11'89''E) in Xinyu, Jiangxi Province, China where rice it is prevalently cultivated with high productivity. It was packed outdoor and air-dried for 2 months after rice harvest and threshing or winnowing. It was then transported to the Department of Biotechnology, Lund University, Sweden at the end of October 2016 and stored at 4 °C prior to use. The rice straw was cut with a grinder (Grindomix 200, Retch USA) to pass through a 2-cm sieve to keep the substrates homogenous. The TS of the rice straw was determined to be 90.1% and volatile solids (VS) was 77.6%.

The inoculums were collected from Källby wastewater treatment plants, WWTP (Lund, Sweden) which consisted of an anaerobically digested sludge from the biogas process treating dewatered sewage sludge and Ellinge wastewater treatment plants (Eslöv, Sweden) which consisted of an anaerobically digested sludge from the biogas process treating agricultural residues. The inoculums were mixed in a ratio 4:1 (w/w) respectively to ameliorate its microbial diversity. The final inoculum was pre-incubated at 37 °C for 1 week and 55 °C for 2 weeks in a thermostatic bath for degassing and adaptation purposes. The pH of the mixed inoculum was 8.0, the TS of mesophilic and thermophilic inoculums were 3.7% and 3.4%, while the VS values were 2.4% and 1.6% respectively. The sludge supernatant used as one of the dilutants was collected from NSR AB (Helsingborg, Sweden) and it had TS of 1.0% and VS of 0.5%. The recycled water was collected from the final clarification tank of the Källby WWTP (Lund, Sweden). The recycled water had a pH of 7.3, PA of 197 mg/L, COD of 22 mg/L, NH_4-N of 0.28 mg/L and total phosphorus of 9 mg/L. Other characteristics of the substrate and inoculum are listed in Table 1.

Table 1 Characters of the rice straw, inoculum and sludge supernatant

	Rice straw	Inoculum (mesophilic)	Inoculum (thermophilic)	Sludge supernatant
TOC (mg/l)	398000 ± 22	-	-	-
TKN (mg/l)	14070 ± 20	-	-	-
C/N	28.3 ± 1.1	-	-	-
Cellulose (%)	30.2 ± 0.3	-	-	-
Hemicellulose (%)	18.3 ± 0.1	-	-	-
Lignin (%)	23.3 ± 1.1	-	-	-
Ash (%)	14.6 ± 0.2	-	-	-
TS (%)	90.1 ± 1.3	3.7 ± 0.6	3.4 ± 0.5	1.0 ± 0.0
VS (%)	77.6 ± 0.3	2.4 ± 0.1	1.6 ± 0.3	0.5 ± 0.0
TS/VS	1.2 ± 0.4	1.5 ± 0.2	1.6 ± 0.2	2.0 ± 0.0
sCOD (mg/l)	-	1116 ± 28	2140 ± 19	2828 ± 21
NH_4-N (mg/l)	-	866 ± 25.5	1002 ± 93	672 ± 91
pH	-	8.0 ± 0.1	8.0 ± 0.1	8.1 ± 0.1
PA (mg/l)	-	1319 ± 13	1022 ± 22	961 ± 9
VFAs (mg/l)	-	250 ± 9	561 ± 13	114 ± 5

"-" means not detected.

2.2 Solid-state anaerobic digestion of rice straw

The rice straw was loaded into 1 L SS-AD glass reactors (with working volume of 700 ml) and the inoculum mixed at the substrate to inoculum (S/I) ratios of 6, 8 and 10 (based on VS). The TS contents in the reactors were hence 17%, 21% and 24% under the mesophilic scenario and 16%, 19% and 22% under the thermophilic scenario. The rice straw was loaded into the glass reactors first then followed by inoculum addition in a bid to avoid floating problems. After a prolonged low methane production for 3 days, the recycled water and sludge supernatant (100 ml) were added on day 27 and day 23 with an apparent change the TS contents (based on the original TS values) to 13%, 16% and 18% under the mesophilic scenario and 12%, 14% and 17% under the thermophilic conditions. Methane production was monitored by the batch-mode Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control AB, Sweden) for 47 days at 37 ^{o}C (mesophilic conditions) and 37 days at 55 ^{o}C (thermophilic conditions) respectively i.e till when the daily methane production was less than 1% of the total methane produced. Cellulose (Avicel PH-101, Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control to validate the inoculum activity and evaluate the experimental protocol. Triplicate reactors were run for each condition. Inoculum without any substrate addition was used as a blank. The methane potential of the sludge supernatant was also investigated considering that it showed an organic content of about 2 g/L in terms of COD. The methane production from the sludge was further deducted from the process supplemented with the sludge supernatant. The daily methane volume and methane production rate (adjusted to standard conditions i.e. 0 ^{o}C, 1 atm and dry conditions) was

calculated by subtracting the methane production of the inoculum in blank reactor from the test samples which contained exclusively rice straw and the methane yield was gotten by dividing the volume with the amount of VS added.

2.3 Analytical methods

The TS and VS contents of the rice straw, inoculum, sludge supernatant and digested rice straw were determined according to standard protocols (APHA, 2005). The percentage TS and VS reductions were calculated as the difference between the TS/VS of the influent and effluent, then divided by the TS/VS of influent. The pH and partial alkalinity (PA) were measured using a TitraLab™ 80 titrator (Radiometer, Denmark) as reported in another study (Nges and Liu, 2009). Total organic carbon (TOC) of the rice straw was determined by the Walkley-Black method as described by Nelson and Sommers (Nelson and Sommers, 1982). The cellulose, hemicellulose and lignin contents were determined by the standard analysis of hydrolysis process described by the National Renewable Energy Laboratory (NREL) Analytical Procedure (Sluiter et al., 2008). For VFAs analysis, the pH of the digested rice straw was adjusted to 1-4 with 0.5 M NaOH and filtered through 0.45 μm filter. The filtrate was analysed for VFAs by HPLC (1200 series, Agilent Technologies) equipped with a refractive index detector (RID) and an organic acid analysis column (300 mm ×7.8 mm, Aminex® 160 HPX-87H Ion Exclusion Column). The column was operated at 55 °C and eluted with 5 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml/min. The injection volume was 10 μL.

2.4 Statistical analysis

The data were processed using SigmaPlot 11.0 and the IBM SPSS statistical 19 package. The significant differences were tested using Duncan multiple range test at $p \leq 0.05$. All the analyses were expressed as the mean of the triplicates with standard deviation (STD). The multiple comparison tests were used to determine the statistical difference between the mesophilic and thermophilic processes.

3. Results and discussion

3.1 Feedstock, inoculum and sludge supernatant characterization

Table 1 shows the basic characteristics of the rice straw, the mesophilic and thermophilic inoculums and the sludge supernatant. The C/N ratio of 28.3 for rice straw is most prominent as per a feedstock for microbial activities in AD which falls within the range demonstrated as optimal for an efficient process (Weiland, 2010). Rice straw also shows an appreciable high content of cellulose, which is higher than that in other similar feedstock such as wheat straw though it shows a rather high content of lignin and ash. The lignin content is especially high as compared to lignocellulosic biomasses such as *Miscanthus*, corn stover, switchgrass and wheat straw (Brown et al., 2012). It should be noted that lignin can negatively impact the biogas process by acting as a shield on cellulose and hemicellulose components (Taherzadeh

and Karimi, 2008). The sludge supernatant and inoculums showed appreciable buffering capacity, nitrogen content (NH_4-N) and the pH values (also for the recycled water) were all within the range often reported to promote methanogenic activity (Weiland, 2010).

3.2 Methane production under mesophilic conditions

Figure 1 shows the cumulative methane production (1a) and methane production rate (1b) where the dotted lines indicate the time of liquor supplementation. The methane production increased steadily and seemed to level off on day 27 especially at the high S/I ratios. The same increasing trend was noted for all the processes after the recovery phase (recycled water and sludge supplementation) except for the processes supplemented with sludge supernatant at S/I ratio of 10. It is plausible therefore to state that the liquor addition boosted the biogas processes via the dilution of inhibitors such as VFAs, improved mass transfer and improved reaction surface area by bringing into contact reaction intermediates and methanogens. Comparatively, recycled water proved to be a be a better dilutant as compared to the sludge supernatant as evidenced by the higher methane production especially at high loads or S/I ratio. The sludge supernatant at S/I ratio of 6 improved methane yields on the 2nd day after addition, resulting in the maximum recovery speed but low methane production. On the other hand, the recycled water addition showed a 3-day delay peak but a 10.1% higher methane yield. As substrate loads increased, the addition of recycled water was more efficient than supernatant, for example 16.8% more methane was achieved at S/I ratio of 8 with recycled water addition. It is probably that recycled water could easily percolate through the coarse structure of feedstock in the reactor as opposed to the sludge supernatant. It should be noted that sludge supernatant had a rather high organic content (2 g/L COD) which could have further exacerbated the inhibitory process through an even higher organic load in the reactors. However, the methane production from the sludge supernatant was infinitesimally small (≤5 mL) as compared to the methane production from the inoculum and hence rice straw.

The methane production rate (Figure 1b) showed the first peak on day 1 for all the processes which could be ascribed to the degradation readily available, water soluble organic components of the feedstock (rice straw). The highest methane production rate (ml CH_4/g VS·d) was 9.4, achieved at the S/I of 6 and the lowest was 6.1 which was achieved at S/I of 10. Thereafter, there was a sharp decrease in the production rate on day 3, to values as low as 2.4 ml CH_4/gVS·d, which could be related to the slow degradation of the recalcitrant components of rice and the synthesis of enzymes or enzyme system to aid the hydrolysis of the feedstock (adaptation). The methane production rate increased afterwards from day 3 to day 7 and maintained a fairly stable production with rates as high as 7.5 ml CH_4/g VS·d. The relatively stable methane production from 7 to day 19 could be referred to a state wherein the hydrolytic products (VFAs) were readily converted to methane or the system buffering capacity was resilient as to ward off VFAs accumulation and its toxicity. The decreasing methane production from day 20 which levelled off from day 25 and beyond could be ascribed to VFAs inhibition i.e. the rate of hydrolysis surpassed the rate of methanogenesis leading to an adverse imbalance in the whole SS-AD process (inhibition). The addition of the liquid media on day 27 to led an immediate increase in methane production rate to the pre-

inhibition stage, except for the S/I ratio of 10 sludge supernatant supplemented process, which eventually decrease over time, till end of the process, probably as the accessible feedstock components were used up.

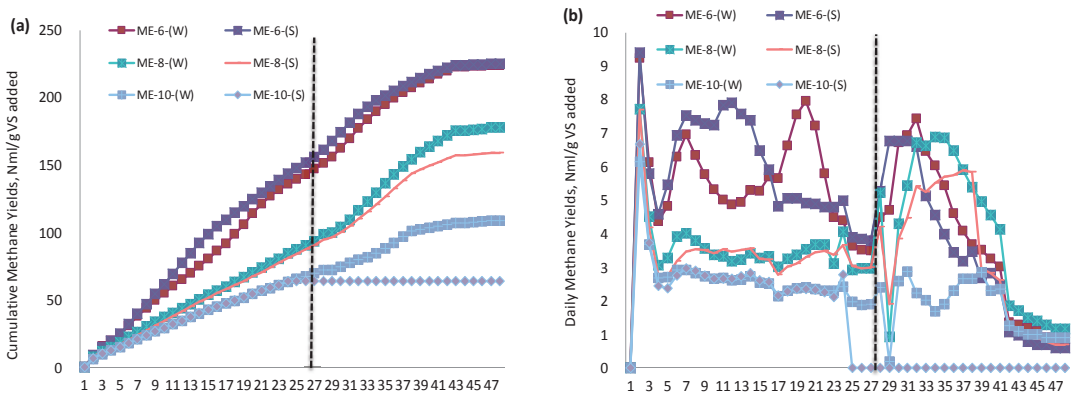


Figure 1. Effects of different liquors addition on methane yields of various S/I ratios at mesophilic temperature. (a) cumulative methane yields; (b) daily methane yields (ME: mesophilic, W: recycled water, S: sludge supernatant)

3.3 Methane production under thermophilic conditions

Figure 2 demonstrates the cumulative methane production (Figure 2a) and methane production rate (Figure 2b) where the dotted lines indicate the time of liquor supplementation. The cumulative methane production curves (Figure 2a) seemed to show diauxic curvatures pre-supplementation and another curvature after supplementation. However, the S/I of 10 processes showed a steady inhibited state even after water and sludge supernatant addition. In general, the cumulative methane production decreased with increasing substrate load (S/I). The first curvature can be explained by the fact that higher temperature enhanced the biodegradation of the rice straw, efficiently producing intermediates (VFAs) which initially, partially inhibited methane production. Thereafter, the processes adapted to high VFAs milieu through adequate buffering and growth of microbial consortium or factors thereof for the efficient continuance of methane production (Nges and Björnsson, 2012). The addition of recycled water and sludge supernatant (third curvature) may have led to an improved mass transfer, dilution of VFAs which gave rise to a further increase in methane production. On the contrary, at S/I ratio of 10, an even higher concentration of VFAs may have led to total inhibition of the methanogenic microorganisms wherein the addition of the recycled water and sludge supernatant could not sufficiently dilute the VFAs or improve mass transfer for a proper recovery of the processes (Yang et al., 2016). The methane production rates, ml CH_4/g VS^{-}d, (Figure 2b) showed three distinct peaks. The first peak was noted on day 1 for all processes and rates ranged from 11 to 14 ml CH_4/g VS^{-}d. The second peaks appeared on 10 and 14 for the S/I ratios 6 and 8 respectively wherein the highest rate was achieved at the S/I ratio of 6. The third peak appeared on day 27, four days after recycled water and sludge supplementation with values as high as 11.2 ml CH_4/g VS^{-}d which eventually dropped and

levelled out at the end of the processes on day 37. It should be noted that recycled water and sludge supplementation at S/I ratio of 10 led to a small but significant surge in methane production rates on day 24 i.e. 0.4 ml CH_4/gVS and 2.1 ml CH_4/gVS·d respectively, which was however not sustained as they dropped almost immediately on day 25 and levelled out till the end of the process. These findings suggest therefore that the processes were high yielding, faster but also (easily) prone to process imbalance especially at high S/I. As earlier mentioned, the thermophilic conditions could have provided a milieu for increased hydrolysis which led to the production of VFAs that were converted to methane (methanogenesis) especially the lower S/I ratios (6 and 8). This was further aided by the recycled water and sludge supernatant supplementation except for the S/I ratio of 10 processes wherein the high VFAs accumulation (Figure 4) could not be buffered and or diluted even with the addition of the recycled water or sludge supernatant.

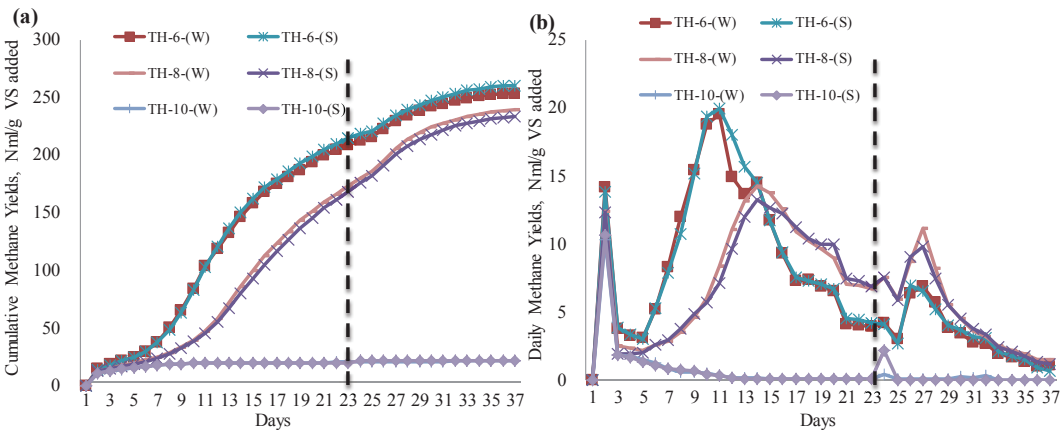


Figure 2. Effects of different liquors addition on methane yields of various S/I ratios at thermophilic temperature. (a) cumulative methane yields; (b) daily methane yields (TH: thermophilic, W: recycled water, S: sludge supernatant)

3.4 Comparison of SS-AD under mesophilic and thermophilic temperatures

3.4.1 Methane yields

Figure 3 demonstrates the methane yields under both mesophilic and thermophilic conditions for the various investigated process configurations. A main difference between the mesophilic and thermophilic processes was the speed or time to reach negligible methane production in the test. The mesophilic yields were achieved after 47 days while it was only 37 days under thermophilic conditions, a difference of 10 days (Figures 1 and 2). This should have significant economic value, especially in full-scale commercial processes, though the higher temperature may also translate to a higher investment cost in terms of heating requirements.

A general trend of decreasing methane yields was noted when the S/I was increased from 6 to 10. The diluting liquid seemed to impact on the methane yields wherein the recycled water proved to a better dilutant with regards to the higher methane yields in the water diluted processes at the same S/I ratio. The decreasing methane yield with increasing S/I ratio pattern was profound under mesophilic conditions whereas the methane yields under thermophilic conditions for S/I of 6 and 8 did not show any significant difference i.e. the diluting liquid did not impart any noticeable effect on the methane yields.

A multi-comparison of the methane yields under both mesophilic and thermophilic conditions divulged that thermophilic SS-AD performed better than mesophilic SS-AD under all the S/I ratios investigated. The highest methane yield achieved during the present study was 260.3 ± 5 ml CH_4/g VS added at S/I ratio of 6 under thermophilic conditions, diluted with the sludge supernatant, which was however not significantly different from that at S/I ratio of 6 diluted with recycled water. This methane yield was 15.6% higher than that achieved under mesophilic conditions. However, at the high S/I ratio of 10, the mesophilic SS-AD outperformed the thermophilic process wherein the mesophilic process showed over 55.9% increment especially in the recycled water diluted process. The recycled water diluted process (recovery) could bring about 36% additional methane yield at S/I ratio of 10 under mesophilic whereas no additional methane was achieved with the recovery action (water and sludge supernatant supplementation) under thermophilic conditions. It should be reiterated that the recovery action highly impacted mesophilic processes as compared to the thermophilic processes wherein as high as 48.9 % methane yield was achieved after the recovery action i.e. at S/I ratio of 8 diluted with recycled water. In a previous study (Zhou et al., 2017), the biochemical methane potential (BMP) of the same sample (rice straw) achieved under non-limiting conditions was demonstrated to be 306.2 ± 13.4 ml CH_4/g VS. The methane yields achieved under thermophilic SS-AD could, therefore, reach 76.6% and 85.0% of the BMP before and after recovery respectively.

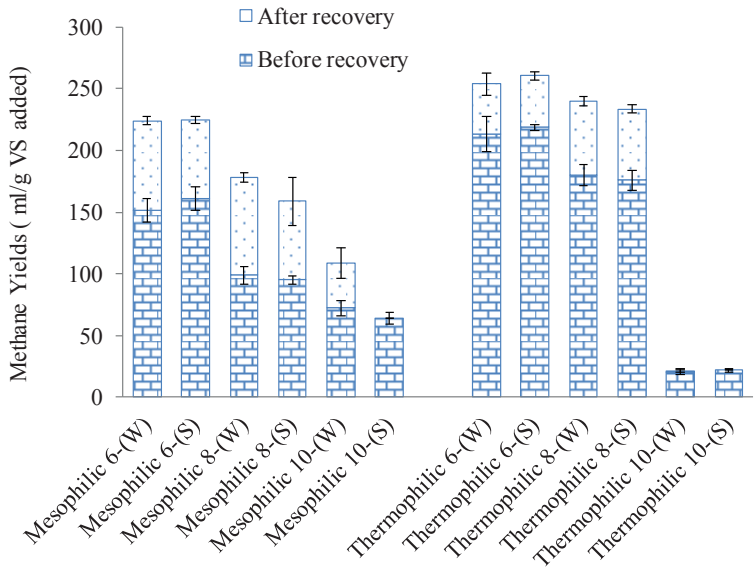


Figure 3. Specific methane yields under mesophilic and thermophilic conditions for the various processes.

The most interesting finding in the present study is that liquor supplementation as a means of recovery was prevalently more efficient under mesophilic than the thermophilic temperature. For instance, the additional methane yields after recovery (recycled water and sludge supplementation) could reach 84 ml CH_4/g VS which was 95.7% of yield before recovery (i.e. under mesophilic conditions, S/I ratio of 8). It is plausible therefore to state that the methane yields in an inhibited SS-AD of lignocellulosic biomass (rice straw) can be (nearly) doubled by merely adding recycled water or sludge supernatant. It is common knowledge that the SS-AD is often plagued by acidification problems especially at high solid loads (Yang et al., 2015). The increment in methane production could have been as results of restored process stability because of the dilution of inhibitors (VFAs) and addition of alkalinity or buffering capacity. In a related study wherein addition of inoculum was employed to alleviate or recover failed SS-AD process fed with corn stover, only about 40% additional methane was achieved (Yang et al., 2016). The near double (95.7%) additional methane achieved in the present proved therefore that recycled water or sludge supernatant is a better dilutant of VAFs (inhibitors) as compared to an inoculum. This is probably because water or the sludge supernatant can easily penetrate the nooks and crannies of the reactor thereby also enhancing mass transfer and reaction area. This thesis has been confirmed in another study wherein fresh water addition to inoculum was recommended as a means to improve process performance and stability in SS-AD (Shahriari et al., 2012).

3.4.2 VS reductions

Another parameter often used to gauge process performance is TS or VS reduction. The percentage TS and VS reduction are presented in Table 2 together with pH, PA and VFAs/PA ratio. The TS and VS reduction were noted to decrease with ascending S/I ratios under both mesophilic and thermophilic conditions. The VS reduction under stable operation (after recovery of the inhibited or failed reactors) ranged from 34 to 57% which are in line with reported VS reduction values during the AD of lignocellulosic biomass (Nges and Björnsson, 2012). However, VS reduction values are low as 2.7% under thermophilic conditions, S/I ratio of 10. It should be reiterated that the lowest methane yields were achieved under these scenarios. As a matter of fact, no additional methane was achieved after recovery under these thermophilic scenarios. In addition to poor methane yield (poor substrate utilisation), the extremely low VS reduction may also be explained by underestimation of VS values commonly encountered during VS determination of volatile compounds e.g. VFAs laden sample (Kreuger et al., 2011). As presented in Figure 4, the S/I of 10 thermophilic SS-AD processes showed the highest concentrations in VFAs with values was high as 9.5 g/L.

Table 2. Process parameters (pH, partial alkalinity or PA, VFA/alkalinity ratio) and VS-reduction at the end of the SS-AD process

	TS reduction (%)		VS reduction (%)		pH		PA (mg/l)		VFA/PA ratio	
	Mesophilic	Thermophilic	Mesophilic	Thermophilic	Mesophilic	Thermophilic	Mesophilic	Thermophilic	Mesophilic	Thermophilic
Inoculum	-	-	-	-	7.95 ± 00	7.96 ± 0.01	13192 ± 16	10220 ± 7	-	-
6W	49.88 ±0.13	31.40 ± 0.19	55.70 ±0.22	42.14 ±0.33	7.98 ±0.00	8.33 ±0.02	9972 ±16	14721 ±20	0.01± 0.00	0.01± 0.00
6-S	50.22 ± 0.13	27.45 ± 0.25	57.64 ± 0.10	37.40 ± 0.16	8.02 ± 0.01	8.29 ± 0.04	10961 ± 18	9961 ± 32	0.00 ± 0.00	0.07 ± 0.00
8-W	47.56 ± 0.06	28.13 ± 0.07	53.02 ± 0.12	34.40 ± 0.15	8.05 ± 0.02	8.32 ± 0.02	10500 ± 25	9250 ± 19	0.03 ± 0.00	0.07 ± 0.00
8-S	42.30 ± 0.16	27.39 ± 0.17	50.45 ± 0.06	35.05 ± 0.00	8.05 ± 0.00	8.34 ± 0.06	15172 ± 22	10746 ± 11	0.01 ± 0.00	0.08 ± 0.00
10-W	41.90 ± 0.21	10.35 ± 0.26	46.71 ± 0.17	11.54 ± 0.15	7.87 ± 0.02	5.27 ± 0.03	17018 ± 19	-	0.24 ± 0.02	-
10-S	57.98 ± 0.09	0.64 ± 0.19	47.79 ± 0.20	2.67 ± 0.20	7.99 ± 0.02	5.33 ± 0.03	13536 ± 17	-	0.01 ± 0.00	-

3.4.3 Process stability

The process stability was judged in terms of pH, VFAs contents, VFAs/PA ratios (Figure 4 and Table 2). The pH values under both mesophilic and thermophilic conditions were within the reported methanogenic range i.e. from 7.9 to 8.3 (Gerardi, 2003; Weiland, 2010) except at S/I ratio of 10 under thermophilic conditions wherein the pH values were as low as 5.3. The pH values correlated positively with VFAs accumulation. The VFAs concentrations under the methanogenic conditions as per the pH values were < 0.24 g/L and they were mainly acetic and propionic acids. Nonetheless, at the S/I ratio of 10, mesophilic process supplemented with sludge supernatant showed a rather high VFAs concentration (> 4 g/L), which correlated with the poor methane yields and VS reduction. This observation may further buttress the thesis that the sludge supernatant could not easily penetrate the reactor matrix as compared to recycled water due to its particulate nature. It has been reported that acetic acid which is one of the main precursors for methane production is usually present at low concentrations in stable biogas processes, while the accumulation of propionic and butyric acid are more severe indicators of the inhibition of methanogenesis or the biogas process as a whole (Weiland, 2010). In all the stabilised processes (processes with final pH within the methanogenic range), the PA ranged from 9.3 to 17.0 g/L wherein the VFAs/PA ratio remained below 0.24 (Table 2). As have been reported in other studies, the VFAs/PA ratios within this range are suitable for the smooth functioning of the anaerobic consortium, thereby leading to high process stability and efficiency (Bouallagui et al., 2009).

The failed thermophilic processes which could not be recovered showed VFAs concentrations as high as 8.4 g/L (recycled water supplemented) and 9.5 g/L (sludge supernatant supplemented) wherein butyric acid was 69.8% and 69.1% of the total VFAs respectively (Figure 3). The accumulation of the VFAs led to a drastic drop in pH (5.3) with a concomitant destruction of the buffering capacity of the system as evidenced by near zero PA. This phenomenon should translate to rather high VFAs/PA ratio i.e. as high as 9.6.

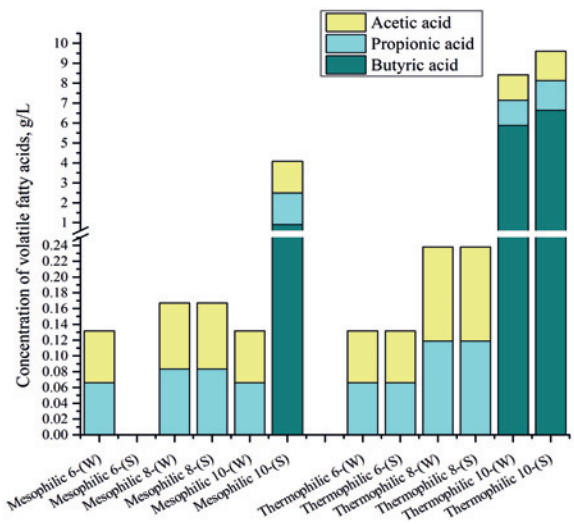


Figure 4. Individual and total VFAs concentrations under both mesophilic and thermophilic SS-AD at the end of the various processes.

The high concentrations of propionic and butyric acids and hence high VFAs/PA ratio in the failed thermophilic are in agreement with the scientific literature (Bouallagui et al., 2009; Weiland, 2010; Yang et al., 2015). It is evident therefore that the propionate and butyrate degrading acetogens or the hydrogenotrophic methanogens involved in the interspecies hydrogen transfer under the thermophilic conditions at S/I of 10 were severely inhibited in the present study. The lower VFAs concentrations in the corresponding mesophilic process (Figure 4) and hence higher stability and performance (methane yield) could be attributed to slower or gentler hydrolysis (reaction rate) commonly observed at lower temperatures as opposed to higher temperatures (Shi et al., 2013). In all, independently of the system pH, VFAs have been reported to as a cause of the inhibition of the cellulolytic activity at concentrations ≥2 g/L, and therefore the hydrolysis of lignocellulosic biomass (rice straw) (Siegert and Banks, 2005). This may also explain the poor methane yields in processes with high VFAs concentrations.

4. Conclusions

In the thermophilic and mesophilic solid-state anaerobic digestion of rice straw boosted in-between by liquor supplementation, it was demonstrated that the thermophilic processes were superior to the mesophilic process in terms of methane yield, especially at lower solid loads. However, at higher loads (S/I >8), the mesophilic processes outperformed the thermophilic ones mainly due to higher process stability as results of low production of inhibitors such as propionic and butyric acids. The highest energy yield (methane yield) achieved during the study was 260.3 ml/g VS which was over 85% of the biochemical methane potential achieved under non-limiting conditions. Supplementation of the inhibited reactors with mere recycled water or sludge supernatant could double the methane yield and boost process stability. This study, therefore, provides evidence for a simple and practical means of improving process performance in solid-state anaerobic digestion for an enhanced waste management or treatment.

Acknowledgements

Financial support from the Regional Science & Technology Program from Beijing Dongcheng District (2017-1-012) is gratefully acknowledged.

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Printed by Media-Tryck, Lund 2017 NORDIC ECOLABEL, 3041 0903

It was a long journey from Copenhagen to Kuala Lumpur; flying on flight LH782 April 26, 2015 to attend the Asia Biogas Forum. I generally fly back and forth between Sweden and other Asian cities seven to eight times per year. However, even during my travels I do not have time to rest. As a PhD student who is also an entrepreneur, I spend every hour balancing the requirements of a rigorous PhD program with those of a business owner. So, on that flight, I had no time to rest, but instead did what I would do on any other day: reviewing my calculations, reading research papers, making graphs for my next manuscript and for the presentation to my next client. Nevertheless, at that instant, on that very plane, I envisaged the endless horizon that lays before my eyes, the infinite possibilities that the future holds for me.

What value could a PhD, that pinnacle of academic snobbery, bring to entrepreneurship? To me, the PhD is fundamentally a problem-solving degree. The most important aspect of a PhD is that you define your own experience. The topic, the collaborators, even the points of emphasis are all shaped and molded from your own ideas. It was clear to me that this kind of research education opened the door to exploration and to impart advanced process knowledge. Ultimately, this could help me become part of biogas industry to find innovative solutions that moves ahead of the global sustainable energy curve.

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ISBN 978-91-7422-540-2
ISRN LUTKDH / TKBT – 17 / 1168 – SE

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