Different approaches to enhance the biogas production from the anaerobic digestion of lignocellulosic materials

Gabriele Mancini

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Gabriele Mancini

Thesis Committee

Thesis Promoter

Prof. Giovanni Esposito

Department of Civil and Mechanical Engineering
University of Cassino and Southern Lazio,
Cassino, Italy

Thesis Co-Promoter

Prof. Piet N.L. Lens

Department of Environmental Engineering and Water Technology
UNESCO-IHE, Institute for Water Education
Delft, the Netherlands

Thesis Co-Promoter

Prof. Eric D. van Hullebusch

Laboratoire Géomatériaux et Environnement
University of Paris-Est
Marne-la-Vallée, France

Thesis Co-Supervisor

Dr. Stefano Papirio

Department of Civil, Architectural and Environmental Engineering
University of Napoli Federico II
Napoli, Italy

Reviewer

Prof. Francesco Fatone

Department of Materials, Environmental Sciences and Urban Planning
Marche Polytechnic University
Ancona, Italy

Reviewer

Prof. Massimiliano Fabbricino

Department of Civil, Architectural and Environmental Engineering
University of Napoli Federico II
Napoli, Italy

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Gabriele Mancini

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In front of the PhD committee

Prof. Francesco Fatone	Reviewer
Prof. Massimiliano Fabbricino	Reviewer
Prof. Giovanni Esposito	Promoter
Prof. Piet N.L. Lens	Co-Promoter
Prof. Eric D. van Hullebusch	Co-Promoter
Dr. Stefano Papirio	Co-Supervisor

Erasmus Joint doctorate programme in Environmental Technology for Contaminated Solids, Soils and Sediments (ETeCoS^3)

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List of Abbreviations

AD	Anaerobic digestion
CHP	Combined heat and power
COD	Chemical oxygen demand
COP21	21st yearly session of the Conference of the Parties on climate change
[BMIM][OAc]	1-buthyl-3-methylimidazolium acetate
BMP	Biochemical methane potential
DMAc	N,Ndimethylacetamide
DMSO	Dimethyl sulfoxide
[EMIM][OAc]	1-ethyl-3-methylimidazolium acetate
EU	European Union
FID	Flame ionization detector
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography coupled to mass spectrometry
GHG	Greenhouse gas
HAc	Acetic acid
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
ICP-MS	Inductively coupled plasma mass spectrometry
IL	Ionic liquid
LM	Lignocellulosic material
LOI	Lateral order index
NMMO	N-methylmorpholine-N-oxide
OPEFB	Oil palm empty fruit bunch
ORL	Organic loading rate
RS	Rice straw
SEM	Scanning electron microscopy
TE	Trace element
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
WRV	Water retention value

Abstract

Biogas production via anaerobic digestion (AD) is a long-standing renewable technology and a continuously growing bioprocess worldwide. Lignocellulosic materials (LMs) present several features that make them especially attractive among the organic substrates commonly employed in anaerobic bioreactors. In particular, LMs under the form of agricultural residues have been acknowledged as the most suitable feedstock for biomethane production due to their high availability, low cost, sustainability and no direct competition with food and feed production. However, their recalcitrance to biological conversion hinders their application for full-scale production of biogas and requires a pretreatment step to improve the LM microbial degradability. In addition to the challenges posed by the lignocellulosic structure, the supply of trace elements (TEs) has often been found insufficient within biogas digesters. The microbial growth depends on the availability and optimal amount of several specific TEs, which are essential constituents of cofactors in enzyme systems involved in the biochemistry of methane formation.

Different chemical pretreatments, namely the solvent N-methylmorpholine-N-oxide (NMMO), the organosolv process, and an alkaline pretreatment using NaOH, were investigated during several batch experiments to enhance the biogas production yields from different LMs (i.e. rice straw, hazelnut skin, cocoa bean shell and wheat straw). Changes in the cellulose crystallinity, water retention value and chemical composition were assessed to better evaluate the effect of the different pretreatments studied on the lignocellulosic structure. Furthermore, the addition of different doses of Fe, Co, Ni and Se on the AD of rice straw was studied, evaluating the influence of the inoculum origin, as well as the performance and synergistic effect of combining an alkaline pretreatment with the addition of trace elements prior to the AD of rice straw. The bioavailability of TEs during batch biochemical methane potential tests was also evaluated applying a sequential extraction technique.

The three pretreatments investigated were effective methods for enhancing the biomethane production from the employed LMs. The biomethane yield from the AD of rice straw increased by 82 and 41% after the NMMO and organosolv pretreatment, respectively. When compared within the same experiment, the NMMO, organosolv and NaOH pretreatment were able to improve the AD of wheat straw, differently affecting the chemical composition of the raw LM. The cumulative biomethane production yield of 274 mL CH_4/g VS obtained with the untreated wheat straw was enhanced by 11% by the NMMO pretreatment and by 15% by both the organosolv and alkaline pretreatment. Hazelnut skin and cocoa bean shell, which were never

investigated before as AD substrates, showed a good potential for biogas production, with cumulative biomethane yields of 223-261 and 199-231 mL CH_4/g VS, respectively, for the untreated feedstocks. However, both NMMO and organosolv pretreatments did not lead to a significant enhancement of the biomethane production yields from these two LMs. The TE supplementation had only a minor effect compared to the pretreatment methods. The addition of Fe, Co, Ni and Se did not result in a significant improvement of the AD of rice straw, whereas the use of the NaOH pretreatment, during the same batch experiment, caused a considerable enhancement of the AD, increasing the biogas production yield by 21%. The negligible effect observed after TE supplementation on the AD of rice straw could be linked to its complex lignocellulosic structure, which requires an enhancement of the hydrolysis, which, rather than the methanogenesis, is the rate-limiting step.

Sommario

La produzione di biogas attraverso il processo di digestione anaerobica (DA) è una tecnica consolidata ed una biotecnologia in continua espansione a livello globale. I materiali lignocellulosici (ML) presentano alcune caratteristiche che li rendono particolarmente attraenti tra i substrati organici impiegati comunemente nei digestori anaerobici. Sotto forma di residui agricoli in particolare, i ML sono considerati tra i substrati maggiormente idonei per la produzione di biometano grazie alla loro abbondante disponibilità, basso costo, sostenibilità e al fatto che non sottraggano terreno coltivabile a prodotti destinati all'alimentazione umana. Tuttavia, la recalcitranza dei ML verso la conversione biologica ostacola un loro maggiore utilizzo per la produzione di biogas su larga scala e rende essenziale l'applicazione di un pretrattamento a monte della DA per migliorarne la degradabilità microbica. Oltre alle difficoltà causate dalla struttura lignocellulosica, una concentrazione insufficiente di micro-elementi (ME) è stata spesso rilevata nei digestori anaerobici che utilizzano ML. La crescita microbica è streattamente collegata alla disponibilità e alla quantità ottimale di diversi specifici ME, che svologono un ruolo essenziale come cofattori nei sistemi enzimatici coinvolti nella biochimica della formazione del metano.

Tre diversi pretrattamenti chimici, ovvero utilizzando il solvente N-ossido di N-metilmorfolina (NMMO), il processo organosolv e un pretrattamento alcalino usando NaOH, sono stati studiati durante una seria di esperimenti in condizioni batch al fine di migliorare il rendimento di produzione di biogas da alcuni ML (paglia di riso, buccia di nocciola, guscio della fava di cacao e paglia di grano). Le variazioni della cristallinità della cellulosa, del valore di ritenzione idrica e della composizione chimica sono state determinate per meglio valutare l'effetto dei diversi pretrattamenti sulle strutture lignocellulosiche dei materiali utilizzati. Inoltre si è investigato l'effetto dovuto all'integrazione di ME quali Fe, Co, Ni e Se sulla DA della paglia di riso, utilizzando dosaggi differenti e valutando l'influenza del tipo di inoculo utilizzato, nonché l'efficacia e l'effetto sinergico ottenuto nel combinare un pretrattamento alcalino con l'aggiunta di ME a monte della DA della paglia di riso. Anche la biodisponibilità dei ME è stata valutata durante i test di biometanazione (BMP), applicando una tecnica di estrazione sequenziale.

I tre pretrattamenti studiati si sono rivelati metodi efficaci per aumentare la produzione di biometano dai ML utilizzati. La produzione di biometano ottenuta dalla DA della paglia di riso è aumentata dell'82 e 41% dopo il pretrattamento NMMO e organosolv, rispettivamente. Quando confrontati all'interno dello stesso esperimento, l'NMMO, l'organosolv e il

pretrattamento alcalino con NaOH sono stati tutti in grado di migliorare la DA della paglia di grano, andando però ad influenzare in modo differente la composizione chimica della paglia, rispetto a quella non trattata. La resa cumulativa della produzione di biometano ottenuta con la paglia di grano non trattata, pari a 274 mL di CH_4/g SV, è stata aumentata dell'11% attraverso pretrattamento NMMO e del 15% sia applicando un pretrattamento alcalino che organosolv. La buccia di nocciola e il guscio della fava di cacao, che non erano mai stati studiati prima come substrati per la DA, hanno mostrato un buon potenziale per la produzione di biogas, con rese cumulative di biometano di 223-261 e 199-231 mL di CH_4/g SV, rispettivamente, ottenuti utilizzando i due ML grezzi. Tuttavia, entrambi i pretrattamenti NMMO ed organosolv non hanno apportato un aumento significativo dei rendimenti di produzione di biometano da questi due ML. L'integrazione di ME ha avuto un effetto marginale rispetto ai metodi di pretrattamento utilizzati. L'aggiunta di Fe, Co, Ni e Se non ha portato ad un miglioramento significativo della DA della paglia di riso, mentre l'utilizzo del pretrattamento di NaOH, effettuato durante lo stesso esperimento, ha causato un notevole miglioramento della DA, aumentando la resa di produzione di biogas del 21%. L'effetto trascurabile ottenuto nella DA della paglia di riso aggiungendo ME potrebbe essere legato alla complessa struttura lignocellulosica della paglia, che richiede un miglioramento della fase limitante del processo, l’idrolisi, piuttosto che della metanogenesi.

Résumé

La production de biogaz par digestion anaérobie (DA) est une technologie renouvelable à long-terme et un bioprocédé en continuelle croissance à travers le monde. Les matières lignocellulosiques (MLs) présentent plusieurs caractéristiques qui les rendent attractives parmi les substrats organiques utilisés communément dans les bioréacteurs anaérobiques. En particulier, les MLs sous la forme de résidus agricoles ont été reconnues comme la matière première de biométhane la plus adaptée du fait de leur grande disponibilité, faible coût, durabilité et de l'absence de compétition directe avec la production alimentaire animale et humaine. Cependant, elles sont aussi récalcitrantes à la conversion biologique, ce qui freine leur application à grande échelle et nécessite la mise en place d'une étape de prétraitement pour améliorer la dégradation microbienne des MLs. En plus des défis posés pas la structure lignocellulosique, l'apport d'éléments traces (ETs) a souvent été observé être insuffisante dans les digesteurs à biogaz. La croissance microbienne dépend de la disponibilité et de la quantité optimale de plusieurs ETs spécifiques, qui sont des constituants essentiels des cofacteurs dans les systèmes enzymatiques impliqués dans la biochimie de la formation de méthane.

Différents prétraitements chimiques, tels que l'utilisation du solvant N-oxyde de N-methylmorpholine (NMMO), le procédé Organosolv ou l'utilisation d'un prétraitement à la soude ont été explorés au cours de plusieurs expériences en batch afin d'améliorer les taux de production de biogaz à partir de différentes MLs (paille de riz, de blé, coquille de noix de coco, peau de noisette). Les changements dans la cristallinité de la cellulose, les valeurs de rétention d'eau et la composition chimique ont été analysés pour mieux évaluer les effets des différents prétraitements sur la structure lignocellulosique. De plus, l'addition de différentes doses de Fe, Co, Ni et de Se a été étudié, en évaluant l'influence de la nature de l'inoculum ainsi que la performance et les effets synergétiques de la combinaison d'un prétraitement alcalin avec l'addition d'ETs en amont de la DA de paille de riz. La biodisponibilité des ETs au cours de tests de production de biométhane en batch a aussi été analysée en appliquant une technique d'extraction séquentielle.

Les trois prétraitements étudiés ont permis d'améliorer la production de biométhane à partir des MLs utilisés. Le rendement de production de biométhane lors de la DA de paille de riz a augmenté de 82% et 41% grâce au prétraitement par ajout de NMMO et par le procédé Organosolv, respectivement. Par comparaison au cours d'expériences similaires, les prétraitements par ajout de NMMO, de soude ou par le procédé Organolv ont été capables d'améliorer la DA de la paille de blé, en affectant de manière différente la composition

chimique de la ML brute. Le taux de production cumulée de biométhane de 274 mL CH4/g VS obtenu avec la paille de blé brute a été augmenté de 11% par l'ajout de NMMO et par 15% par l'ajout de soude et le procédé Organsolv. La peau de noisette et la coquille de noix de coco, qui n'avaient jamais été explorés avant comme substrat pour la DA, ont montré un bon potentiel pour la production de biogaz, avec des taux cumulés de production de biométhane à partir des matières premières non-traitées de 223-261 et 199-231 mL CH4/g VS, respectivement. Cependant, ni l'ajout de NONM, ni le prétraitement Organosolv n'ont pu mener à une amélioration significative des taux de production de biométhane à partir de ces deux MLs. L'apport d'ETs a eu un effet mineur par comparaison aux méthodes de prétraitement. L'addition de Fe, Co, Ni et Se n'a pas mené à une amélioration significative de la DA de la paille de blé, alors que le prétraitement par ajout de soude, au cours de la même série d'expérience, a permis une amélioration considérable de la DA en augmentant le taux de production de biogaz de 21%. L'effet négligeable sur la DA observé après l'apport d'ETs pourrait être lié à la structure complexe des MLs. L'étape d'hydrolyse étant cruciale, celle-ci serait, plutôt que la méthanogénèse, l'étape limitante

Samenvatting

Biogasproductie via vergisting (AD) is een langdurige hernieuwbare technologie en een voortdurend groeiende bioproces wereldwijd. Lignocellulosische materialen (LM's) presenteren verschillende eigenschappen die hen voraal aantrekkelijk maken tussen de organische substraten die gewoonlijk in anaërobe bioreactoren worden gebruikt. Voraal, zijn LM's in de vorm van landbouwresiduen bekend als het meest geschikte grondstof voor biomethaanproductie door hun hoge beschikbaarheid, lage kosten, duurzaamheid en geen directe concurrentie met voedsel- en voederproductie. Hun recalcitrantie voor biologische coversie belemmert echter hun toepassing voor full-scale productie van biogas en vereist een voorbehandelingstap om de LM-microbiële afbreekbaarheid te verbeteren. Naast de uitdagingen die door de lignocellulose-structuur worden veroorzaakt, is het aanbod van sporenelementen (TE's) vaak onvoldoende gevonden in biogas-digesters. De microbiële groei hangt af van de beschikbaarheid en optimale hoeveelheid van verschillende specifieke TT's, die essentiële bestanddelen zijn van cofactoren in enzym systemen die betrokken zijn bij de biochemie van methaanvorming.

Verschillende chemische voorbehandelingen, namelijk het oplosmiddel N-methylmorfoline-N-oxide (NMMO), het organosolv-proces en een alkalische voorbehandeling met NaOH, werden onderzocht in verscheidene batchexperimenten om de opbrengst van biogasproductie uit verschillende LM's te verbeteren (d.w.z. rijststro, hazelnoot huid, cacaoboonschil en tarwe stro). Veranderingen in de cellulosekristalliniteit, waterretentie en chemische samenstelling werden bestudeerd om het effect van de verschillende voorbehandelingen op de lignocellulose structuur beter te evalueren. Bovendien werd de toevoeging van verschillende doses van Fe, Co, Ni en Se voor AD van rijststro bestudeerd, de invloed van de oorspronkelijk inoculum geëvalueerd, evenals het prestatie- en synergistische effect van het combineren van een alkalische voorbehandeling met toevoeging van sporen elementen vóór AD van rijststro. De biologische beschikbaarheid van TE's tijdens biochemische methaan potentiaal batch tests werd ook geëvalueerd met toepassing van een opeenvolgende extractietechniek.

De drie onderzochte behandelingen waren effectieve methoden voor het verhogen van de biomethaan productie van de gebruiken LM's. De opbrengst van biomethaan uit de AD van rijststro steeg met respectievelijk 82 en 41% na de NMMO- en organosolv-voorbehandeling. Waneer vergeleken met hetzelfde experiment, waren de NMMO, organosolv en NaOH voorbehandeling in staat om de AD van tarwestro te verbeteren, waardoor de chemische

samenstelling van de ruwe LM is beïnvloed. De opbrengst van de cumulatieve productie van biomethaan van 274 ml CH4/g VS verkregen met het onbehandelde tarwestro werd met 11% verhoogd door de NMMO-voorbehandeling en met 15% door zowel de organosolv- als alkalische voorbehandeling. Hazelnoothuid en cacaoboonschil, die nooit eerder als AD-substraten werden onderzocht, vertoonde een goed potentieel voor biogasproductie, met cumulatieve biomethaanopbrengsten van respectievelijk 223-261 en 199-231 ml CH4/g VS voor de onbehandelde voedingsstoffen. Echter, zowel NMMO als organosolv voorbehandelingen leidden echter niet tot een significante verbetering van de opbrengst van biomethaanproductie uit deze twee LM's.

De TE-aanvulling had slechts een klein effect vergeleken met de voorbehandelingsmethoden. De toevoeging van Fe, Co, Ni en Se resulteerde niet in een significante verbetering van de AD van rijststro, terwijl het gebruik van de NaOH-voorbehandeling tijdens hetzelfde batchexperiment een aanzienlijke verbetering van de AD veroorzaakt, de biogasproductie verhoogde opbrengst met 21%. Het verwaarloosbare effect waargenomen na de toevoeging van TE op de AD van rijststro, zou kunnen worden gekoppeld aan zijn complexe lignocellulose-structuur, die een verhoging van de hydrolyse vereist, die in plaats van de methanogenese de snelheidsbeperkende stap is.

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CHAPTER 1

General introduction and thesis outline

1.1 Background and problem statement

In the last years, major challenges such as climate change, population growth, food security and natural resources exploitation have caused increasing concerns worldwide. In order to tackle these issues and protect the environment in a sustainable way, a shift from the mere use of natural resources to their recovery and reuse has been observed [1].

Anaerobic digestion (AD) is a mature technology used to stabilize organic wastes while producing renewable energy under the form of biogas [2]. AD is carried on by different communities of microorganisms in four stages. During the first one, hydrolysis, hydrolytic bacteria secrete enzymes able to decompose complex organic polymers to soluble monomers. Then, during acidogenesis, microbes convert the soluble monomers to a mixture of volatile fatty acids (VFAs) and other products, which, during the following acetogenesis stage, are then converted to acetate, carbon dioxide and hydrogen by acetogenic bacteria. These products are finally used for methane production by methanogenic archaea, during the last stage of methanogenesis. The final product of the process is biogas, a mixture of two main gases, methane (CH_4) and carbon dioxide (CO_2), which can be used to generate heat and electricity or, after an upgrade, as a transportation fuel [3]. In addition to the production of a clean biofuel, such as biogas, AD has other advantages. Odors and greenhouse gases (GHG) emissions are reduced; furthermore, a nutrient rich digestate is obtained at the end of the AD process, which can be used as a fertilizer in agricultural lands.

Lignocellulosic materials (LMs), namely agricultural residues such as crop residues, grass, fruit and vegetable waste, forest residues and other byproducts of different agro-industrial processes represent the most abundant raw source of organic matter available on earth. LMs are defined as ‘feedstock for the second generation of biofuel’, since the dead plant tissue is employed [4]. Compared to first generation feedstocks, such as food crops, LMs do not compete for food production, thus no ethical concerns arise because of the food chain being threatened. Furthermore, LMs collected as a waste material from agricultural, municipal or industrial activities, generally have a low cost. Despite the clear advantages of being largely available, sustainable and inexpensive, LMs are highly underused as feedstocks for renewable energy production [5]. This is primarily due to the chemical composition of LMs, consisting of three main polymers, i.e. cellulose, hemicellulose and lignin, which create a complex and resistant structure (Fig. 1.1), highly recalcitrant to microbial degradation. As a consequence, the hydrolysis of LMs becomes the AD rate-limiting stage [6].

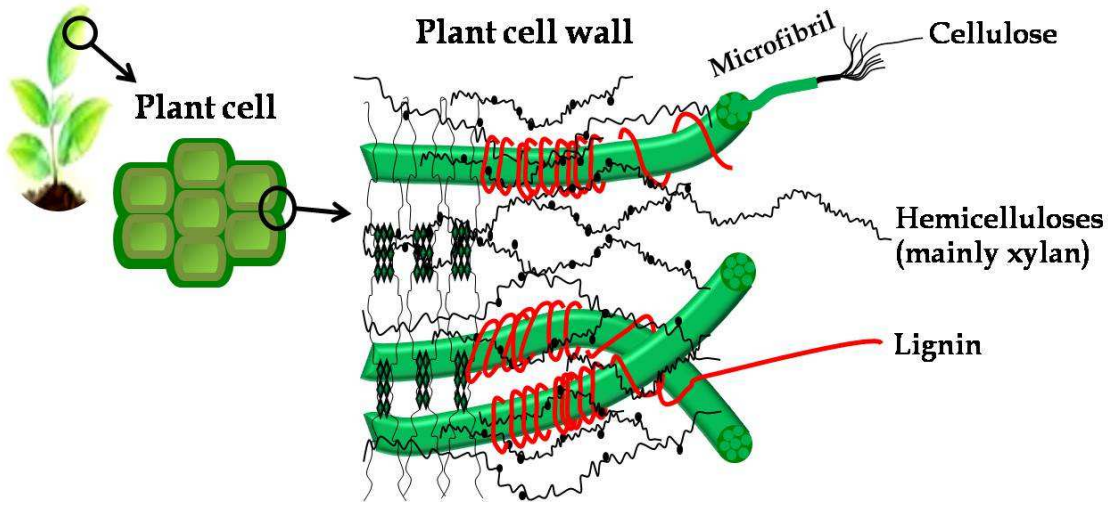

Fig. 1.1. Structure of the lignocellulosic material [7].

1.1.1 State of the art of biogas in Europe

The 2009 EU Directive on the "promotion and use of energy from renewable sources" established the overall policies to achieve the target of at least 20% of the European energy needs from renewable sources by 2020 [8]. In November 2016, the EU Commission proposed a revision of the previous directive [9], in order for the EU to stand as a global leader in renewable energy production, by increasing the target to at least 27% by the year 2030. Furthermore, European countries are required to fulfill at least 10% of their transport fuels share with renewable energy sources by 2020. These measures are strictly connected with the delivery of another EU target, which is the reduction of GHG emissions by at least 40% by 2030, in line with the agreements reached at the Paris climate conference (COP21) in December 2015 [10]. During the United Nation summit in the French capital, world political leaders reached a global agreement on climate change, aimed at reducing GHG emissions, in an attempt to keep the world temperature from rising more than 2°C by 2100, ideally aiming to keeping the temperature increase below 1.5°C. Thereby, a transitional switch from a fossil fuel based economy to a sustainable carbon-neutral bioeconomy was targeted [11].

In this context, the biogas market can play a major role for the achievement of the EU objectives. Currently, biogas production represents about 8% of the overall renewable energy production in the EU [12]. The number of biogas plants in Europe has increased steadily in the last ten years, reaching 17376 plants in 2015, corresponding to 8728 MW of installed electric capacity. The agricultural sector is largely the main raw organic source for European AD plants, representing 69% of the total, followed by sewage sludge with the 16% [13]. However, half of the biogas production in the EU is provided by the AD of energy crops, mainly maize, which cannot be considered a sustainable feedstock in the years to come. The largest growth potential

for the biogas production in the EU can be found in animal manure and organic wastes from agricultural and municipal activities. If the potential of these feedstocks will be properly deployed, the current biogas production could be more than doubled by 2030 [12].

1.2 Aim and objectives of the thesis

The overall goal of this project was to enhance the AD of LMs, in order to achieve higher biomethane production yields. Two main approaches were used through a series of experiments in batch mode. Accordingly, the specific objectives of the research were:

i) To investigate the effects of three chemical pretreatment methods (i.e. N-methylmorpholine-N-oxide, organosolv and NaOH pretreatment) on the AD of different LMs (i.e. rice straw, hazelnut skin, cocoa bean shell and wheat straw). In particular, the extent of each pretreatment effectiveness was evaluated by looking at the changes in the LM chemical composition, porosity and crystallinity, as well as the effect of each pretreatment on the biomethane production yield and kinetics, and the VFA production.

ii) To investigate the effects of different trace elements (TE) supplementation on the AD of rice straw, by evaluating the effect of Fe, Co, Ni and Se addition, at different dosages, on the VFA production and biomethane production yields. The influence of the inoculum origin and the bioavailability of the selected TEs were also among the studied parameters.

1.3 Thesis outline

This PhD thesis is divided into eight chapters. Chapter 1 describes the background and motivation of the research and outlines the structure of the PhD thesis, as shown in Fig. 1.2.

Chapter 2 reports a critical literature review about solvent pretreatment methods, since these novel techniques have shown great effectiveness in enhancing the AD of LMs, thus received the major focus during this work.

Chapter 3 describes the experimental results obtained pretreating three different LMs, namely rice straw, hazelnut skin and cocoa bean shell, with the organic solvent N-methylmorpholine-N-oxide (NMMO). The enhancement of the biogas production yields from the three LMs, together with the analysis of their crystallinity are reported in this chapter.

Chapter 4 discusses the results obtained pretreating the same LMs with a different technique, called organosolv. The biomethane production rates were modelled applying two

different kinetic approaches. Moreover, the influence of organosolv pretreatment on the amount of carbohydrates and lignin of the three LMs was investigated by a compositional analysis.

Chapter 5 presents a comparison of three different chemical pretreatments (i.e. NMMO, organosolv and alkaline pretreatments) in enhancing the AD of wheat straw, assessing the effects of each pretreatment on the biogas production yields, the methane production kinetics and the changes caused in the wheat straw chemical composition.

Chapter 6 investigates the improvement of the biogas production obtained from the AD of rice straw by TE addition and alkaline pretreatment.

Chapter 7 explains the relevance of assessing the bioavailability in studying trace elements dosing strategies to improve the AD of organic substrates.

Chapter 8 highlights the major findings and the implications of the research and provides perspectives and recommendations for future works.

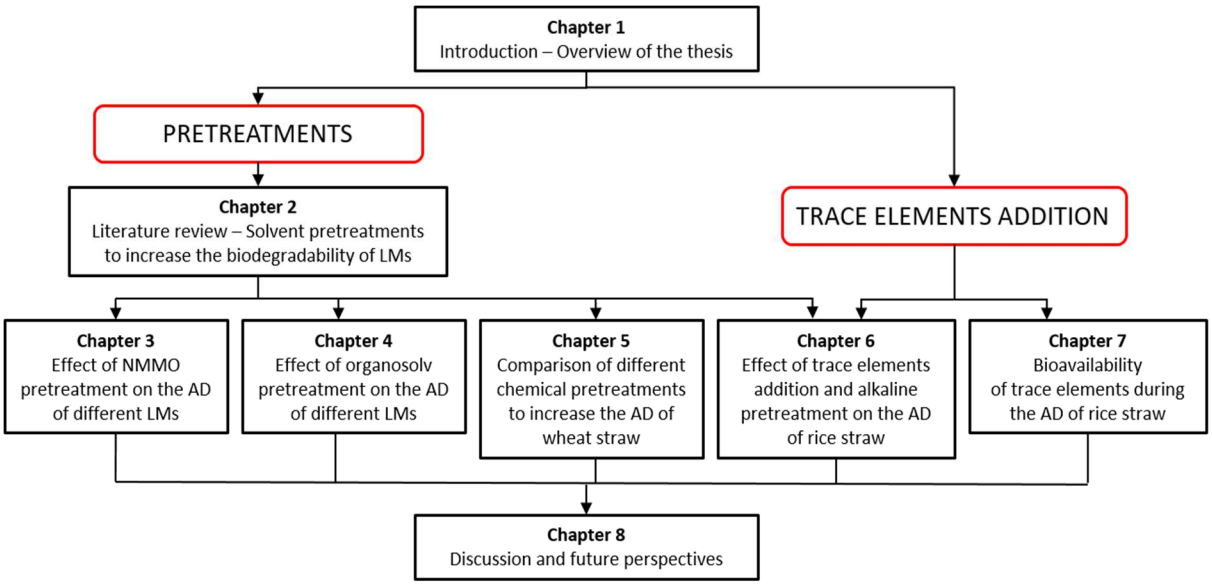

Fig. 1.2. Overview of the PhD thesis structure.

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CHAPTER 2

Solvent pretreatments to increase the biodegradability of lignocellulosic materials

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2.1 Introduction

Anaerobic digestion (AD) is a well-established technology used to convert a variety of organic substrates into renewable liquid and gaseous biofuels. AD is performed by different groups of anaerobic bacteria along a series of biochemical reactions, including hydrolysis, acidogenesis, acetogenesis and methanogenesis. Most AD applications are aimed to the production of a methane-rich biogas [1].

Biogas from the AD of lignocellulosic materials (LMs) has shown great potential as an alternative to fossil fuels, mostly because of the abundant availability of lignocelluloses, which constitutes 50% of the total amount of biomass worldwide [2]. However, application of AD to LMs is still limited, due to their resistance to microbial degradation, and the general absence of low-cost technologies to overcome the difficulties of converting these feedstocks to biomethane [3].

LMs can be used for anaerobic digestion in the form of agricultural residues (i.e. rice straw, corn stover and sugarcane bagasse), dedicated energy crops (i.e. switchgrass, maize and sorghum), and forest products (hardwood and softwood). LMs are mainly composed of cellulose, hemicellulose and lignin, with their amounts varying both qualitatively and quantitatively according to the lignocellulosic species and origin [4]. One feature common to every kind of lignocellulosic substrates is the recalcitrance towards enzymatic hydrolysis, which limits the biological degradation and consequently the biomethane yield [5]. Therefore, a pretreatment step is required in order to achieve a substantial conversion of the lignocellulosic matter into biomethane. Several technologies are currently available, with research focusing on defining the most suitable method for each kind of feedstock, with the objective of increasing the substrate accessible area for the bacteria, to decrease its crystallinity, as well as to reduce the resistance towards biodegradation of the lignin present in the substrate [6, 7].

The production of biomethane through the anaerobic digestion of LMs presents some important advantages over other bioconversion processes, such as the production of bioethanol and biohydrogen. While bioethanol can be used only as liquid fuel, biomethane (after a refining step) may be injected into the gas grid and exploited for residential uses or as vehicle fuel, or employed in a combined heat power unit for the production of heat and electricity through a cogeneration process [8].

Biogas and biohydrogen can be obtained converting both cellulose and hemicellulose, whereas bioethanol is mainly produced through cellulose conversion. The biohydrogen yield obtainable is only 10 to 20% of the energy potential of a feedstock, with the remainder

converted to organic acids and other products that need to be further degraded, making biohydrogen less convenient than methane [9]. Finally, unlike the residues of biohydrogen and bioethanol production that need further treatment, the residue of the anaerobic digestion aimed at biomethane production is a valuable product, namely digestate, made of stabilized organic matter that can be used as a fertilizer in agriculture [10]. In recent years, the biorefinery concept for the production of energy along with several products and by-products is gaining major importance and scientific interest [11, 12]. In a biorefinery, LMs can be converted to biofuels and biomaterials in an integrated manner, e.g. merging biogas and bioethanol production, reducing the waste stream produced as well as increasing the economic value of the feedstocks used.

Pretreatments of LMs are commonly classified into three main categories: physical, chemical and biological, but often a combination of them is employed [13]. For biomethane production, the goal of the pretreatment is to break down the hydrogen bonds between the polysaccharides and lignin, in order to make the carbohydrate portion (i.e. cellulose and hemicellulose) available for the anaerobic bacteria [6]. Hydrolysis of LMs is considered as the rate limiting step [14]. Hence, the pretreatment can accelerate this stage, resulting in shorter hydraulic retention times, in addition to improved biogas yields [9].

Despite the efforts of researchers in testing the effects of different pretreatments on the wide range of LMs, there is not a definite consensus about the effectiveness of a specific pretreatment on a particular substrate, not even when similar substrates are compared. This is mainly due to the high range of possible pretreatment conditions as well as the huge differences found within different varieties of the same agricultural residue [15].

Cellulose solvent-based pretreatments offer several advantages but a “perfect” cellulose solvent for LM pretreatment has still to be found. In recent years, a technique called organosolv has been employed to pretreat LMs, resulting effective in enhancing the biogas production yields. Differently from cellulose solvents, this process acts on the lignin and hemicellulose fractions of the LMs, dissolving the first and part of the latter, and leaving cellulose in the solid phase [7].

In order to be effective, a cellulose solvent is required to have several features: being able to dissolve cellulose at low temperatures and without a previous biomass drying step, being inexpensive, highly recyclable (hence non-volatile, for an easy recycling), thermostable, non-toxic to the subsequent microbial fermentation and having a fast diffusion rate in the solid LMs [16].

Among the pretreatments described in the literature, cellulose solvents have shown a higher efficiency in disrupting the bonds among cellulose, hemicellulose and lignin, compared to conventional pretreatments for LMs, such as steam explosion, hot water or dilute acid pretreatment [17, 18]. After being dissolved, cellulose can be easily regenerated by adding an anti-solvent, such as water, which leads to a self-association of cellulose. The resulting amorphous cellulose has a highly increased surface and thus more accessible than prior to the pretreatment, which can greatly affect the hydrolysis rate [19].

This review aims to present the last advances within the cellulose solvent and organosolv pretreatment techniques currently available for lignocellulosic substrates, attempting to recommend their use according to the different types of feedstock employed.

2.2 Parameters in assessing LMs pretreatment efficiency

The main variables that affect pretreatment efficiency are the accessible surface area and pore size of cellulose, its crystallinity and the protection by lignin and hemicellulose [7, 20]. Recently, these parameters have been critically reviewed [21], as well as the most common analytical techniques used for evaluating the LMs pretreatment efficiency [22].

The surface area of cellulose accessible to microorganisms and the pores size among the main constituents of lignocelluloses are particularly important parameters for the degradation of LMs [23]. Higher surface area and pore size result in an improvement of the hydrolysis, which is carried out by a variety of different enzymes. In particular, cellulases are enzymes produced by cellulolytic microbes in order to hydrolyze the β-(1-4) glycosidic bonds in cellulose [24]. Moreover, certain anaerobic bacteria are able to produce a multi-enzyme complex, called cellulosome, which is specialized in cellulose degradation.

Cellulose crystallinity is defined as the ratio of the crystalline to amorphous portions of cellulose. Solvents are used as pretreatments to produce a less crystalline cellulose and increase the amount of the amorphous region which adsorbs enzymes and water faster, resulting in a better hydrolysis [25].

An improvement of cellulose hydrolysis has been observed after hemicellulose and lignin removal resulting in an increased accessible surface area of lignocelluloses [26]. In particular, lignin is the most recalcitrant constituent of the LMs [27]. Besides indirectly reducing cellulose accessibility, lignin significantly reduces enzyme effectiveness by its hydrophobic binding to the cellulase enzymes [28]. Therefore, the content of lignin and the biodegradability of LMs are considered inversely proportional [27].

2.3 Cellulose solvents affecting anaerobic digestion of LMs

The cellulose dissolution/regeneration process has attracted much attention because of its importance within several production processes and applications [29]. The mechanism of dissolution is, however, not completely understood yet, with the generally accepted picture that considers the cellulose dissolution resulting from the solvent ability to disrupt the inter- and intra-molecular hydrogen bonds among the lignocellulosic components [29]. Recently, other aspects have been emphasized, such as the amphiphilic behavior of cellulose, with some authors suggesting that eliminating hydrophobic interactions could be more important than eliminating hydrogen bonds [30, 32].

In the following sections, recent advances in cellulose solvent-based pretreatments for LMs will be reviewed, analyzing the possible dissolution mechanisms involved, as well as the main advantages and disadvantages (Table 2.1) for their application as pretreatment to enhance the biogas production from LMs.

It is worth to remind that milling and grinding are commonly employed processes in order to improve the subsequent treatment with solvents [33]. These physical pretreatments can increase the LMs surface area as well as reduce the cellulose crystallinity [7]. However, energy requirement remains a limiting factor [33].

Table 2.1. Advantages and disadvantages of using cellulose solvents and organosolv as LM pretreatment

Solvent	Advantages	Disadvantages	Ref.
Ionic liquids	• Low melting point • High thermal stability • High reaction rates • Low volatility • Recyclable	• High cost • High amount of water required for washing step • Corrosive effects showed by some ILs • Possible inhibitory effects on hydrolysis and fermentation	18, 26, 34-38
Concentrated NaOH	• Relatively inexpensive • Alkali remaining with the treated solids can be useful in subsequent AD for pH control	• Possible formation of phenolic compounds • High costs of downstream processing • Effective dissolution of cellulose only at reduced temperatures (e.g. 0°C), which results in high costs.	39-42

Sodium carbonate	• Low cost • Significant delignification while keeping most of the carbohydrates portion • Mild conditions required	• Formation of alkali-stable end groups may lead to low biogas yields	43-44
Concentrated phosphoric acid	• Short pretreatment times • Mild temperatures required • No inhibitory effects of residual phosphoric acid on the following hydrolysis step	• Corrosive effects • High volumes of solvent required • High costs of downstream processing	16, 45-47
NMMO	• High recovery of carbohydrates • Minor degradation of raw material	• High cost • High amount of water required for washing step • Side reactions of the solvent itself without using an anti-solvent • Potentially low recyclability for some LMs	2, 48-54
Organosolv	• A pure lignin fraction, which can be used for several applications, is obtained • Very effective pretreatment for high-lignin LMs • Less energy intensive, as no significant size reduction of feedstocks is required • Recovery of solvents easier than for other pretreatment methods	• Side reactions might occur, producing inhibitory compounds to methanogens • Explosion hazards • Environmental and health and safety concerns associated with the use of volatile organic liquids at high temperatures.	55-57

2.3.1 Ionic Liquids

Ionic liquids (ILs) are a relatively new class of solvents, made of organic salts with low melting point (i.e. below 100°C), high thermal stability, high polarity and negligible vapor pressure [18]. ILs are often regarded as “green solvents” because of their low volatility, hence potentially minimal environmental impact [18, 36, 37]. Compared with other cellulose solvents, ILs present fast reaction rates and some of them are even effective at room temperature [34, 35].

In the last decade, the effectiveness of ILs as cellulose solvents has been investigated, almost exclusively for producing bioethanol [58]. Besides the enhancement of enzymatic hydrolysis and the fermentable sugars yield for bioethanol production, ILs pretreatment is expected to improve biogas production from anaerobic digestion of LMs as well [14, 55, 56]. It was found that ILs having imidazolium or pyridinium cations, when paired with certain

anions (e.g. Cl^-, $CF_3SO_3^-$, $CF_3CO_2^-$, $CH_3CO_2^-$, $HCOO^-$, HSO_4^-, $R_2PO_4^-$, etc.), are able to dissolve cellulose through strong hydrogen bond basicity [18]. The dissolution mechanism involves the oxygen and hydrogen atoms of cellulose hydroxyl groups, which form electron donor – electron acceptor complexes interacting with the ILs [59]. The breaking of hydrogen bonds leads to an opening of the lignocellulose network of linkages, resulting in cellulose dissolution. Subsequently, using an anti-solvent (e.g. ethanol, acetone, methanol, or water), the solubilized cellulose can be quickly precipitated, with the recovered cellulose showing significantly different macro- and micro-structures, in particular increased porosity and decreased crystallinity [60].

The main drawbacks of ILs are their high cost and their inhibitory effect on the hydrolytic enzymes, even at low concentrations. Furthermore, a complete removal of ILs after the pretreatment step entails the usage of high amounts of anti-solvent (generally water), and complex recycling systems, which could make the process economically infeasible [61]. As a consequence, researchers have started to investigate the effects of mixtures containing ILs and small amounts of catalysts in order to lower the energy requirements and obtain a better fractionation of cellulose as well [62]. To the authors' knowledge, only two papers have reported the effect of cellulase cocktails, containing ILs, on the enhancement of the biogas yields from the anaerobic digestion of lignocellulosic substrates [63, 64].

The effect of a mixture of 1-N-butyl-3-methyimidazolium chloride ([Bmim]Cl)/dimethyl sulfoxide (DMSO) for 2 h at 120°C on the pretreatment of water hyacinth (*Eichhornia crassipes*) was investigated by Gao et al. [63]. The lignocellulosic structure and composition were largely altered by the pretreatment with the IL, resulting in a 27.9% increase of the cellulose content in the regenerated water hyacinth and a 49.2% removal of lignin, with a final biogas production of 170 mL/g VS, enhanced by 97.6% compared with the untreated feedstock.

Another IL, namely n-alkyl-3-methylimidazolium chloride ($[C_n mim]Cl$, n = 2, 4, 6), was employed for four LMs (i.e. water hyacinth, rice straw, spruce and mango leaves) at different pretreatment temperatures (from 100 to 140°C) and times (from 2 to 8 h) [64]. Analyses of the pretreated substrate structures and morphologies showed significant decreases of crystallinity and lignin content in all four LMs investigated, with higher efficiencies achieved at higher temperatures and longer incubation times. Moreover, lower crystallinity indexes were obtained treating the feedstocks with $[C_2mim]Cl$, pointing out that the LMs solubility decreased at increasing IL molecule complexity. The biogas yields of the four LMs pretreated with $[C_4mim]Cl$ for 2 h at 120°C were 153, 86, 91 and 122 mL/g VS for rice straw, water hyacinth, mango leaves and spruce, respectively, with cumulative biogas productions increased from

23.4 to 97.6%, compared to the untreated LMs. Finally, important results were observed in the recycling of the ILs, with a recovery of over 90% of [C_4mim]Cl. The ILs were recovered through the formation of an aqueous biphasic system with an upper IL-rich phase and a lower salt (i.e. K_3PO_4) rich phase, based on the recycling strategy developed by Blanch et al. [65].

In all the previous studies, cellulose was dissolved with ILs having the imidazolium cation coupled to chloride. However, chloride can cause problems of corrosion and toxicity. Furthermore, IL are in the solid form at room temperature and have a high viscosity after melting, resulting in difficult handling and processing [66]. Therefore, ILs with carboxylic acid anions, such as 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) and 1-buthyl-3-methylimidazolium acetate ([BMIM][OAc]), may be preferable due to their lower melting points and viscosities, and higher cellulose solvation capacity, due to their ability to react with hydroxyl groups [66].

Several studies have been conducted using [BMIM][OAc] and [EMIM][OAc] to pretreat LMs for bioethanol production, showing a significant improvement of the enzymatic hydrolysis of the tested LMs [61, 67, 68]. However, to the authors' knowledge, only Papa et al. [69] used an IL with a carboxylic acid anion to enhance the production of biomethane. In that study, the effect of pretreating corn stover and switchgrass with [EMIM][OAc] was investigated at 100°C for 3 h. Biomethane production from corn stover was only increased by 6.6%, whereas the use of switchgrass resulted in a 5% decrease of biomethane production [69]. The low performance of the pretreatment was ascribed to the fact that the non-pretreated substrates had high biogas yields (i.e. 283 and 269 mL CH_4/g VS for corn stover and switchgrass, respectively), inferring that the IL pretreatment is only effective for more recalcitrant substrates.

2.3.2 Concentrated sodium hydroxide

NaOH has been extensively used as pretreatment for lignocellulosic biomass because of its ability in removing or modifying the lignin content by disrupting the ester linkages between xylan and lignin, thus increasing the porosity of the material [41]. However, alkaline pretreatment is a complicated process, which involves many reactive and non-reactive phenomena. Hence, the efficiency of this pretreatment highly depends on the process conditions, such as the NaOH concentration, the operating temperature and the treatment time [70].

Depending on the NaOH concentration, the pretreatment can be performed at low (0.5 – 4% (*w/w*) NaOH) or high (6 – 20% (*w/w*) NaOH) concentrations [41]. Generally, when a low

NaOH concentration is used, the pretreatment aims at lignin and hemicellulose removal from the LMs and is performed at high operating temperature and pressure, without NaOH recycling. On the contrary, the high NaOH concentration process takes place at atmospheric pressure and low temperature and only results in cellulose dissolution, without significant delignification [41]. By using high NaOH concentrations, the solvent can be recovered and reused with lower economic and environmental impact.

A tentative explanation of the dissolution power of the NaOH/water solution, made by Egal et al. [71], highlighted the key role played by the NaOH concentration. If the amount of water is too high (i.e. above 94% (*w/w*)), the amount of NaOH is insufficient to dissolve cellulose, or the size of the NaOH hydrate is too large to penetrate the cellulose fibers. On the other hand, when the concentration of NaOH exceeds 20%, NaOH hydrates penetrate into the fibers and form crystals with the cellulose chains. The role of the temperature was explained by the same authors inferring that the strength of the hydrogen bonds among the network of NaOH hydrates increases with the lowering of the temperature. Thus, when cellulose is dissolved at low temperatures, such a network prevents the cellulose chains from forming hydrogen bonds with each other.

As shown in Fig. 2.1, cellulose interacts with NaOH in different ways, in order to form several complexes. In particular, in a small triangular region where the NaOH concentration is in the range of 6 – 10% and the temperature ranges between -10 and 4°C, NaOH causes a huge swelling of cellulose, up to dissolving it, depending on the degree of cellulose polymerization [72].

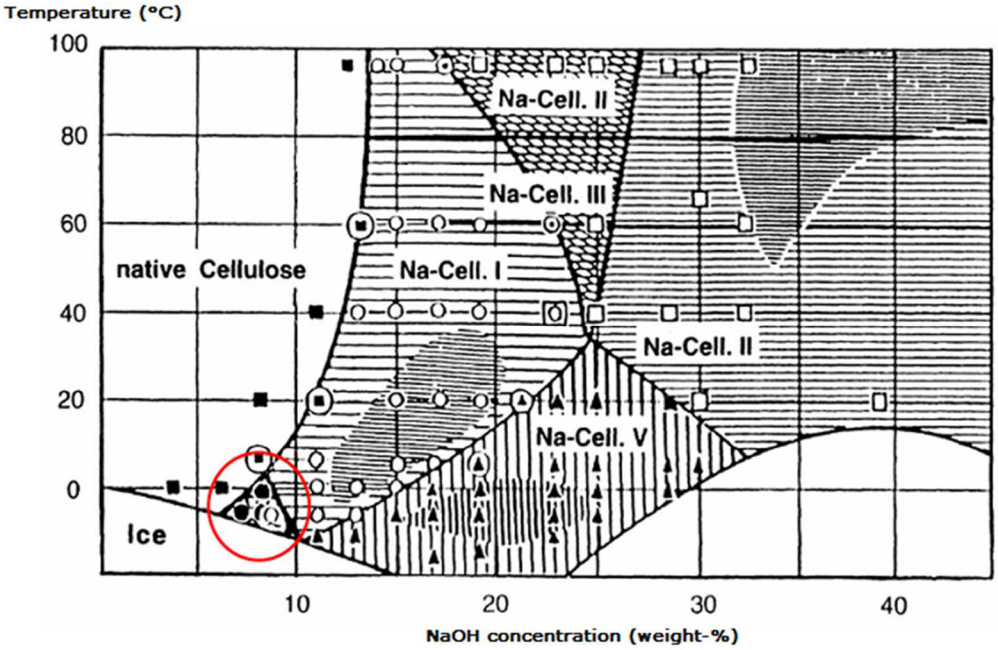

Fig. 2.1. Phase diagram of the ternary system cellulose/NaOH/water [72].

Several substrates have been tested for concentrated NaOH pretreatment, such as rice straw, [42, 73, 74] oil palm empty fruit bunch [47], corn straw [75], spruce [41], birch [41], pine [76], wheat straw [74, 77, 78], corn stover [79], and sorghum [74, 80, 81], as reported in Table 2.2. The NaOH concentrations investigated in the aforementioned studies were all in the range of 4 – 10%. However, not all the tested temperatures were in the optimal range for cellulose dissolution. Nonetheless, the NaOH pretreatment was able to significantly improve the hydrolysis of the LMs under all operating conditions, resulting in higher biogas yields than those obtained with the untreated substrates.

NaOH pretreatment has been shown to improve the biogas production from hardwood birch and softwood spruce, achieving higher efficiencies with the former substrate [41]. The effect of a wide range of temperatures, from -15 to 100°C, was investigated with a 7% NaOH concentration and 2 h of pretreatment time. All the tested temperatures resulted in increasing biogas production, but each temperature differently affected the final composition of the LMs. In particular, the pretreatment did not significantly alter the lignin content, while the hemicellulose content decreased by 27.4 – 33.2% and 46.6 – 71.3% for spruce and birch, respectively, with the highest removal efficiency achieved at the highest temperatures. As a result, the cellulose portion in the woods increased, with a decreasing crystallinity index.

The effects of different temperatures (0 and 100°C) and pretreatment times (10, 30 and 60 min) using 8% NaOH pretreatment were investigated to improve the biogas production from softwood pine [76]. Results showed different trends for the two analyzed temperatures. The pretreatment at 100°C was more effective at shorter incubation time (10 min), with increased content of glucan and lower amount of lignin. On the contrary, at 0°C the pretreatment had a greater impact with a longer retention time (60 min). Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) were used to assess the structural and morphological changes of the LM [76]. The pretreatment at 0°C resulted in a decrease of cellulose crystallinity, whereas 100°C led to a disintegration of the wood structure.

The higher ability of NaOH pretreatment to decrease the crystallinity of cellulose at 0°C than at 100°C could be explained by the stronger binding capacity of Na^+ and OH^- at lower temperatures, which enables the alkali solution to break the hydrogen bonds within the lignocellulosic structure, converting cellulose I (i.e. native cellulose) to cellulose II (i.e. regenerated cellulose). On the other hand, at high temperatures, a breakdown of the ester linkages among cellulose, hemicellulose and lignin in the LM occurs, although a prolongation of the process can result in the degradation of sugars and formation of alkali-stable-end groups that have negative effects on the bioconversion yields [26].

Rice straw was pretreated with NaOH at different concentrations (from 4 to 10%) for 3 weeks at 20°C by He et al. [42]. A 6% NaOH concentration was the most efficient in increasing the biogas yield. The analyses of the lignocellulosic composition showed a considerable decomposition of cellulose, hemicellulose and lignin; significant fractions of them were converted to more readily biodegradable substances, favoring the enhancement of the biogas production. The same concentration was tested by Pang et al. [79] for the pretreatment of corn stover. With the pretreated substrate, anaerobic digestion was shortened from 60 – 75 days to 40 – 60 days, resulting in higher digestion rates and efficiencies. Furthermore, when different organic loading rates (OLRs) were investigated, the highest biogas production was observed at the highest OLR. This result implies that the NaOH pretreatment was able to increase the loading capacity of the AD digesters, in addition to significantly enhance the biogas production from corn stover.

Clear positive effects of NaOH pretreatment were observed on the biogas production from oil palm empty fruit bunches (OPEFB) [47]. The pretreatment with 8% NaOH at 100°C for 60 min resulted in a reduction of the cellulose crystallinity, as well as in organic and inorganic material loss.

Sambusiti et al. [74, 77, 80, 81] conducted several investigations on the effect of NaOH pretreatment at different concentrations on sorghum forage and wheat straw. The studies confirmed the positive effect of the pretreatment on fiber reduction, total organic carbon (TOC), proteins solubilization, and, consequently, the microbial degradability of the lignocelluloses. However, at increasing NaOH dosage, the pretreatment time and temperature did not significantly improve the methane production. Furthermore, contrasting results with different hybrids of sorghum forage suggested that the impact on the biogas yields of the NaOH pretreatment mainly depends on the type and variety of the substrate used [81].

Table 2.2. Biogas yields enhancement by NaOH pretreatment

Raw material	Pretreatment conditions			Yield after pretreatment (mL CH_4/g VS)	Improvement from untreated (%)	Ref.
	Temperature (°C)	NaOH concentration (%)	Duration			
Birch	100	7	2 h	460	84	46
Corn stover	20	4, 6, 8, 10	3 weeks	466	48.5	79
Corn straw	25	4, 6, 8, 10	7 days	163.5	60	75
OPEFB	100	8	1h	404	100	47
Pine wood	0, 100	8	10, 30, 60 min	178.2	118.6	76

Rice Straw	20	6	3 weeks	520	44.4	42
Rice Straw	20	4, 6, 8, 10	3 weeks	520	44.4	73
Sorghum sudanense hybrid	40, 55	4, 10	12, 24 h	316	19	80
Sorghum sudanense hybrid	40, 100	1, 10	1 h	356	32	77
Sorghum sudanense hybrid	40	1, 10	24 h	345	27	74
Spruce	5	7	2 h	50	67	41
Sweet sorghum	55	4, 10	12 h	334	negligible	81
Wheat straw	40, 100	1, 10	1 h	302	48	77
Wheat straw	40	1, 10	24 h	289	28	74

Song et al. [75] compared the effects of seven chemical pretreatments, among which NaOH, on the composition and biogas production of corn straw. NaOH was the most effective solvent in degrading the lignocellulosic structure of corn straw because of the high alkalinity which breaks down the matrix of lignocellulose and changes the straw composition. However, other chemicals, such as H_2O_2 and $Ca(OH)_2$ resulted in higher methane yields than NaOH, thus being the most suitable with respect to effectiveness and economic performance [75].

2.3.3 Aqueous solvents with sodium hydroxide and urea/thiourea

NaOH is able to swell cellulose and, when highly concentrated, even dissolve it by penetrating the amorphous region of cellulose and destroying the neighboring crystalline areas. Addition of other chemicals to the NaOH solution, such as urea and thiourea or a combination of them, as well as polyethylene glycol has shown to significantly improve the dissolution performance of LMs [32, 82]

A combination of alkali/urea dissolves cellulose even at low (-15 to -5°C) temperatures [83]. The dissolution process by cooling is related to complex formation induced by the network of hydrogen bonds between cellulose and solvent components [83]. The complexes formed by cellulose, alkali and urea are relatively stable at low temperature, bringing the cellulose macromolecules into the aqueous solution. The NaOH hydrates are more easily attracted to cellulose chains, forming new hydrogen-bonded networks, whereas the urea hydrates can be self-assembled at the surface of the network between NaOH and cellulose, in order to form an inclusion complex [83]. In the case of NaOH/thiourea pretreatment, the stability of the cellulose solution is even higher than that obtained with NaOH/urea, since the thiourea hydrate is able to create more stable inclusion complexes than urea, leading to an enhancement of the solubility [84]. Recent studies have concluded that urea works in synergy with NaOH to separate the cellulose chains from each other and prevent cellulose from

regenerating the hydrogen bonds. However, urea has no direct interactions with cellulose, but helps NaOH to penetrate the crystalline region of cellulose [85, 86]. Furthermore, urea has a stabilizing effect because it hinders the hydrophobic association of cellulose.

Mohsenzadeh et al. [82] tested the effect of a NaOH/thiourea aqueous solution (7% NaOH (*w/w)*, 5.5% thiourea (*w/w*)) pretreatment at -15°C for 16 h on softwood spruce and hardwood birch. The biomethane production was 180 and 360 mL CH_4/g VS, respectively, and 600% and 56% higher than that achieved with the untreated substrates. The NaOH/thiourea mixture was able to reduce the crystallinity of both birch and spruce species, confirming an improved hydrolysis yield at decreasing crystallinity index [28].

2.3.4 Sodium carbonate

Similarly to other alkaline pretreatments, sodium carbonate (Na_2CO_3) is capable of effectively breaking the ester bonds between lignin, hemicellulose and cellulose, increasing the accessibility of carbohydrates to microorganisms and reducing the lignin content of the pretreated LMs [7]

Moreover, the sodium ions can change the polyionic character of the lignocellulosic matter by diffusing into the structure of the LMs and acting as a countercharge to the carboxylate ions. As a consequence, swelling of the substrates is promoted, leading to an increased accessibility of carbohydrates for bacterial attack [26].

Dehghani et al. [47] achieved a 125% improvement of methane yield by pretreating rice straw with 0.5 M Na_2CO_3 at 110°C for 2 h. The analysis of the pretreated material showed that the improvement was due to a lower cellulose crystallinity, lignin removal and structural modifications. Harsher pretreatment conditions (i.e. pretreatment at 130 °C for 3 h) led to a lower biomethane yield. This was ascribed to peeling reactions of carbohydrate end groups, with the formation of alkali-stable end groups [43].

2.3.5 Concentrated phosphoric acid

When phosphoric acid is concentrated beyond a critical value, it induces a phase transition from cellulose swelling to cellulose dissolution. The cellulose regenerated after dissolution in concentrated phosphoric acid has the dual benefit of possessing high reactivity to cellulase and having an amorphous form [16].

Nieves et al. [47] achieved 40% enhancement of the methane yield by employing concentrated phosphoric acid for the pretreatment of oil palm empty fruit bunches (OPEFB). A mixture of OPEFB and 85.7% (*w/w*) phosphoric acid was incubated at 50°C for 30 min, achieving a cumulative production of 283 mL CH_4/g VS in the following 30 days of AD.

However, a pretreatment of the same initial substrate with 8% NaOH (*w/w*) resulted in a 100% improvement (i.e. 404 mL CH_4/g VS) of the methane production compared with that of the untreated material.

Isroi Ishola et al. [87] studied changes in OPEFB composition after phosphoric acid (85.7% (*w/w*)) pretreatment at 50°C for 5 h. FTIR and SEM analyses showed significant structural and morphological changes. The OPEFB fiber structure was completely damaged by the pretreatment, which reduced the hydrogen bonded stretching absorption, the crystalline cellulose portion, the amount of hemicellulose and the particle size.

The main disadvantage of employing acid pretreatment is that it implies the use of a corrosive reagent, which requires special materials for the reactor construction and downstream neutralization [45].

2.3.6 N,Ndimethylacetamide/lithium chloride

N,Ndimethylacetamide/lithium chloride (DMAc/LiCl) is a solvent for cellulose which has been widely employed in the fields of analysis, shaping and chemical modification of cellulose [58]. DMAc/LiCl is able to dissolve cellulose by forming strong hydrogen bonds between the hydroxyl protons of cellulose and the Cl^- of the solvent (Fig. 2.2). During this process, the intermolecular hydrogen bonding network of cellulose is broken with simultaneous splitting of the $Li^+ - Cl^-$ ion pairs. At the same time, the Li^+ cations form tight linkages with the free DMAc molecules, which accompany the hydrogen-bonded Cl^- to meet the electric balance. The cellulose chains are consequently dispersed in the solvent system forming a homogeneous solution [58]. A different dissolving pattern has been suggested by Medronho and Lindman [29], based on the polyelectrolyte effect. Cl^- interacts with hydroxyl groups of cellulose, as well as the DMAc in the lithium co-ordination sphere. A negatively charged polymer is produced by the accumulation of Cl^- along the cellulose chain, with the macro-cation [Li-DMAc] as the counterion. Therefore, the polymer molecules are forced apart because of charge repulsion. Despite its well-known cellulose solvation ability, to the authors' knowledge, DMAc/LiCl has never been tested for the pretreatment of LMs in order to enhance the biogas production.

One of the main drawbacks of the application of DMAc/LiCl for LM pretreatment is represented by the high temperatures (i.e. 150°C) required to readily swell and dissolve cellulose [32].

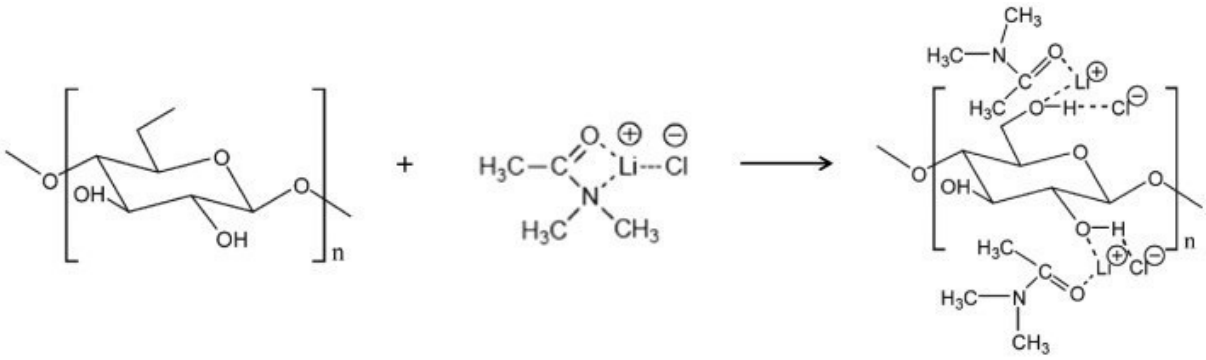

Fig. 2.2. The mechanism of H-bond breaking for cellulose dissolution in the LiCl/DMAc solvent system [29].

2.3.7 N-methylmorpholine-N-oxide

N-methylmorpholine-N-oxide (NMMO) is a cellulose solvent that has been commonly employed in the Lyocell process within the fiber-making industry [88]. This environmentally friendly solvent has the ability to efficiently dissolve cellulose and consequently decrease its crystallinity [53]. Moreover, NMMO can be recovered by more than 98%, without producing toxic waste pollutants and with no chemical derivatization [50]. NMMO belongs to the family of aliphatic, cyclic, tertiary amine oxides (Fig. 2.3). It has a highly polar N-O group, which results in high solubility in water and a tendency to form hydrogen bonds. The N-O bonds can be readily broken, releasing a relatively large amount of energy. This feature entails three main consequences: NMMO is a strong oxidant, thermally labile and sensitive towards all kinds of catalysts that induce N-O bond cleavage [89].

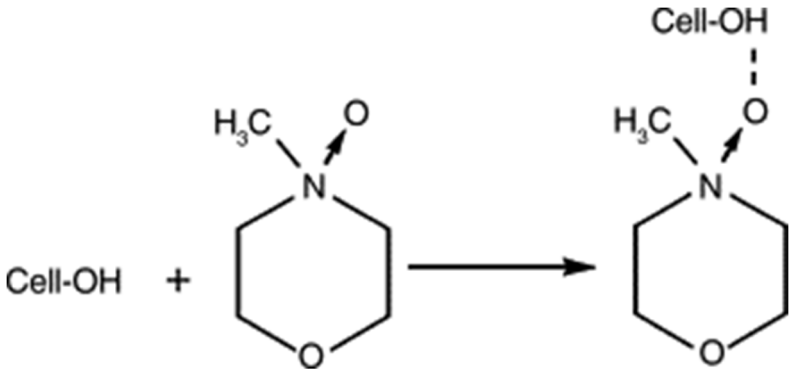

Fig. 2.3. Mechanism of cellulose dissolution by NMMO [90].

Many studies agree that the NMMO solvation power comes from the ability of the solvent in disrupting the hydrogen bond network of the cellulose, with new bonds between the solvent and the macromolecule being formed [89]. Cellulose and water exhibit competitive behavior in forming hydrogen bonds with NMMO, with water being preferred. Therefore, in order to dissolve cellulose, the water content of the mixture has to be lower than 17% (*w/w*),

corresponding to the 1.2 NMMO hydrate. After the pretreatment, the dissolved cellulose can be recovered by adding an anti-solvent, with boiled distilled water being commonly employed to the purpose [51].

In the past few years, the effects of using NMMO on the degradation of LMs have been investigated by several authors, who have tested this pretreatment at lab-scale on different types of lignocelluloses, namely rice straw [53, 91], wheat straw [48], triticale straw [53], barley straw [51], straw fraction of manure [92], forest residues [49, 51, 93], birch [50], spruce [2, 53] oil palm empty fruit bunch [1], pinewood [25], textile wastes [94], and cotton linter [95]. In all these studies, NMMO pretreatment showed a positive impact on the biogas production enhancement (Table 2.3). Besides the different nature of the substrates, other parameters influence the NMMO pretreatment efficiency as below reported.

2.3.7.1 NMMO concentration

Although most of the studies used NMMO concentrated at 85%, the effects of lower concentrations have also been evaluated [1, 2, 49]. Dissolution of cellulose only occurs when the NMMO concentration is 85%, whereas swelling and ballooning of the fibers is observed at concentrations of 73% and 79%, respectively. In order to increase the ethanol production, NMMO pretreatment was found to be more efficient at concentrations of 85%, which is able to dissolve cellulose, whereas lower concentrations of NMMO (79% – ballooning mode and 73% – swelling mode) were found suitable for the enhancement of biogas production [95]. This result was explained pointing out the difference between the mechanism of bacterial digestion and the enzymatic hydrolysis. During anaerobic digestion of cellulose, hydrolysis is carried out by cellulosomes, which are unique extracellular multi-enzyme complexes of anaerobic bacteria. Since these complexes attach both to the cells and the substrate, the hydrolysis is a synergistic action of the different components of the enzyme. This means that the accessible surface area, which several studies consider a more important parameter than the crystallinity, increased more with the swelling-ballooning mode than with the dissolution mode [95].

In contrast with the previous study, Kabir et al. [2] observed a higher biogas production at 85% than 75% NMMO when using a mixture of native forest residues as lignocellulosic substrate under the same operating conditions.

2.3.7.2 Temperature of NMMO pretreatment

The melting point of NMMO is 78°C and it decomposes at temperatures above 130°C [89]. Hence, all the studies concerning NMMO pretreatment have been performed within this range. Most of the investigations have been performed at temperatures of 90, 110, 120 and 130°C, with higher temperature generally corresponding to increased methane yields [1, 4, 96,

97], although the NMMO pretreatment efficiencies at 120 and 130°C have never been compared.

2.3.7.3 Duration of NMMO pretreatment

Different pretreatment times, ranging from 20 min to 30 h, have been investigated. This parameter varies according to the type and size of the raw material, with pure cellulose or cellulose pulp generally requiring shorter pretreatment time compared to wood material [26]. A study by Teghammar et al. [53] examined the effects of different pretreatment times (i.e. 1, 3 and 15 h) on three LMs (i.e. spruce chips, rice straw and triticale straw). While tests on spruce and triticale straw showed that the methane yield was enhanced by increased pretreatment times, rice straw behaved differently, with longer pretreatment times resulting in lower methane yields. Since traditional inhibitors, such as phenols or furans, were not detected, no clear explanation was found for this unexpected behavior of rice straw. However, the straw used in the study of Teghammar et al. [53] contained more than 10% silica, which could have made the straw behave differently from the other lignocelluloses analyzed.

Table 2.3. Biogas yield enhancement by NMMO pretreatment of LMs

Raw material	Pretreatment conditions				Yield after pretreatment (mL CH_4/g VS)	Improvement from untreated (%)	Ref.
	Solid loading (% *w/w*)	Temperature (°C)	NMMO concentration (%)	Duration (h)			
Barley Straw	6	90	85	3, 30	230	92	51
Barley straw	6	90	85	3, 30	450	67	54
Birch wood	7.5	130	85	3	232	46	50
Cotton linter	3	90, 120	73, 79, 85	0.5, 1, 2.5, 5, 15	412	17	95
Forest residues	6	90, 120	75, 85	3, 15	100	141	49
Forest residues	6	90	85	3, 30	150	114	
Forest residues	-	90	85	15	130	37	93
Mixture of woods	6	120	75, 85	3, 15	170	143	2
OPEFB	6	90, 120	73, 79, 85	1, 5	408	48	1
Pinewood chips	7.5	120	85	1, 3, 15	143	580	33
Pinewood powder	7.5	120	85	1, 3, 15	224	240	25
Rice straw	7.5	130	85	1, 3, 15	157	614	53
Spruce, chips (10mm)	7.5	130	85	1, 3, 15	125	1036	20
Spruce, milled (<1mm)	7.5	130	85	1, 3, 15	245	271	20
Straw fraction of cattle manure	7.5	90	85	5, 15	350	22	89

Straw fraction of horse manure	7.5	90	85	5, 15	422	51	92
Textile wastes	5	120	85%	2	258	1158	94
Triticale straw	7.5	130	85%	1, 3, 15	203	577	20
Wheat straw	6	90	85%	7, 15, 30	470	47	48

2.4 Organosolv pretreatment

Organosolv is based on the pretreatment of LMs with an organic or aqueous-organic solvent at high temperatures (i.e. 100 – 250°C). The process relies on the chemical breakdown of the lignin macro-molecule by cleavage of ether linkages and its subsequent dissolution [97]. The solvents most commonly employed within this process are methanol, ethanol, acetone, high boiling point alcohols and organic acids (such as formic and acetic acid) [55]. Methanol and ethanol are often preferred because of their low boiling points and ease of recovery by simple distillation, which implies low energy requirements for their recycling [98].

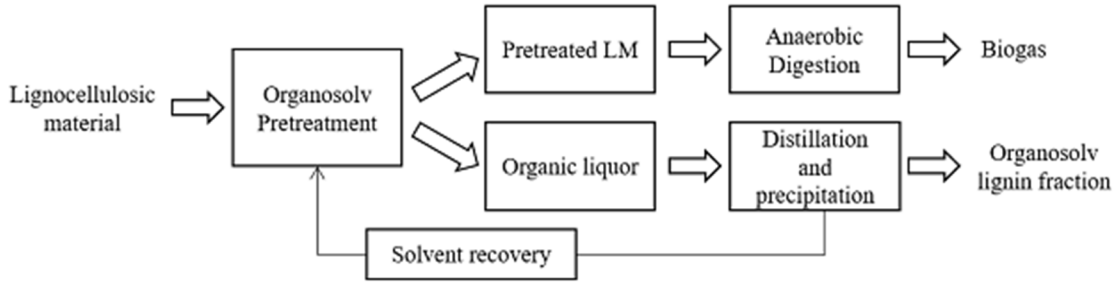

Fig. 2.4. Schematic flow chart of organosolv pretreatment prior to AD process.

The goal of organosolv pretreatment is to fractionate the LM into its individual main fractions, by depolymerizing hemicellulose and delignifing the lignocellulosic matrix. The internal lignin bonds are hydrolyzed by the solvent, as well as the ether and ester linkages between lignin and hemicellulose, with the delignification kinetics varying according to the solvent employed for the pretreatment [56]. After organosolv pretreatment, three separate components are obtained: a pure cellulose fraction, an aqueous hemicellulose stream and a highly pure lignin fraction [99]. Similarly to the other pretreatments described in the previous sections, organosolv causes a decrease in the crystallinity of the LMs and enhances the accessibility of carbohydrates for microbial degradation. However, the major advantage of this process is the recovery of a purified lignin fraction, which is obtained by filtration of the solvent-evaporated liquor in which the lignin fraction was precipitated (Fig. 2.4) [98]. Lignin can then be used for several applications other than combustion, e.g. as additive in inks, paints and varnishes; matrix material in bio-based composites; radical scavengers or component for

phenolic resins and polyurethane foams, significantly improving the economics of a lignocellulosic biorefinery [57].

Recently, a few studies about the effects of organosolv pretreatment on the enhancement of biogas production yields from LMs have been conducted. Kabir et al. [100] used three different organic solvents (i.e. ethanol, methanol and acetic acid) to pretreat forest residues (Table 2.4). The mixture of bark, pine and spruce was added to the 50% (*v/v*) aqueous organic solvents, with a solid to liquid ratio of 1:10, and heated at 190°C for 1 h. Subsequently, anaerobic digestion of the pretreated and washed LM mixture was performed in batch reactors under thermophilic conditions (55°C) for 40 days. The final cumulative biomethane production was 300, 230 and 330 mL CH_4/g VS for ethanol, methanol and acetic acid pretreated LM, respectively, while the production of the untreated biomass reached only 50 mL CH_4/g VS. Therefore, higher methane yields were achieved by using acetic acid and ethanol, compared to methanol. However, the economic analysis performed within the same study showed that the methanol cost and recovery was cheaper than the other two solvents, ultimately leading to a more profitable process.

Organosolv pretreatment of three different LMs (i.e. hardwood elm, softwood pine and rice straw) was conducted using 75% ethanol at 150 and 180°C for 30 and 60 min [101]. After 55 days of solid state anaerobic digestion at mesophilic conditions (39°C), 54.6, 79.5 and 135.2 mL CH_4/g VS were obtained with pinewood, elmwood and rice straw, respectively. However, the organosolv pretreatment was more efficient in terms of methane production enhancement at increasing initial lignin content. Methane production increased by 84, 73 and 32% with pine-wood, elm-wood and rice straw, respectively, compared to the corresponding untreated substrates. Pretreatment temperature was also a factor affecting the methane yields, with a higher temperature (i.e. 180°C) required for hardwood elm, while the optimum temperature for both rice straw and softwood pine was 150°C.

Ostovareh et al. [102] used ethanol to perform organosolv pretreatment at 100% – 160°C for 30 min on sweet sorghum stalks. The cumulative methane production, obtained after 50 days of mesophilic (37°C) anaerobic digestion was 278 mL CH_4/g VS, resulting in a 270% improvement compared to the untreated stalks.

Sunflower stalks were pretreated with 50% (*v/v*) aqueous isopropanol at 140% – 200°C for 30 min and 1 h, resulting in a methane production up to 264 mL CH_4/g VS and 113% higher than that obtained with the untreated material (124 mL CH_4/g VS) after 45 days of mesophilic (37°C) anaerobic digestion [103]. The highest methane production yield was achieved when the organosolv pretreatment was performed at 180°C for 1 h. A slight decrease of the methane

yield was observed increasing the pretreatment temperature above 180°C, probably because of an enhanced solubilization of the hemicellulose fraction occurred at 200°C.

Catalysts, such as HCl or H_2SO_4, can be added to the solvent/lignocellulose mixture during the pretreatment and have been reported to increase the final biogas production [55]. An addition of 1% (*w/w*) H_2SO_4 resulted in a 5% increase of the final methane production (i.e. 278 mL CH_4/g VS) by using sunflower stalks as substrates pretreated with 50% isopropanol [103]. The use of the catalyst also resulted in lower optimal pretreatment temperature (160°C) and time (30 min). Although the use of acidic catalysts lead to slightly higher methane yields and more cost-effective pretreatment operating conditions, several expenses must be taken into account such as the cost for the acid itself and the maintenance costs caused by the highly corrosive and hazardous mineral acids Moreover, contrasting results were obtained in the study of Ostovareh et al. [102], where the addition of sulfuric acid as a catalyst was beneficial only at the lower pretreatment temperatures (i.e. 100% – 120°C), while above 140°C a lower methane yield was obtained in presence of the catalyst.

Table 2.4. Biogas yield enhancement by Organosolv pretreatment of LMs

Raw material	Pretreatment conditions			Yield after pretreatment (mL CH_4/g VS)	Highest improvement from untreated (%)	Ref.
	Temperature (°C)	Solvent used	Duration (min)			
Forest residues	190	Acetic acid	60	330	560	100
		Ethanol		300	500	
		Methanol		230	360	
Elm wood	150, 180	Ethanol	30, 60	79	73	101
Pine wood				55	84	
Rice straw				135	32	
Sweet sorghum stalks	100, 120, 140, 160	Ethanol	30	279	270	102
Sunflower stalks	140, 160, 180, 200	Isopropanol	30, 60	278	124	103

2.5 Conclusion

LMs are promising feedstocks for anaerobic digestion due to their abundance, low-cost and sustainability. However, LMs recalcitrance to microbial degradation still limits their large application in full-scale plants and requires the use of a pretreatment step. The use of solvents as pretreatments has gained scientific interest due to their capability of enhancing the biomethane potential of many LMs. Among the cellulose solvents mostly investigated, NMMO significantly increases the microstructure porosity and decreases the crystallinity of cellulose,

while causing minimal compositional changes and no cellulose loss. Moreover, the high solvent recycling efficiency of NMMO for many LMs makes the use of NMMO economically feasible and applicable at commercial scale.

Similarly to NMMO, ILs effectively decrease the crystallinity and increase the porosity of LMs as well as producing a reduction of the lignin content. However, IL toxicity to hydrolytic bacteria can have detrimental effects on anaerobic digestion by repressing the biochemical pathways. Concentrated NaOH and Na_2CO_3 have also been successfully applied to pretreat LMs, showing significant enhancement of the biogas production yields. Coupling urea or thiourea to NaOH mixtures results in noticeable synergistic effects improving the hydrolysis of LMs and decreasing cellulose crystallinity.

Improvement of the biogas production can be obtained by removing the higher content of lignin through organosolv pretreatment. The recovered lignin fraction is highly pure and can be employed as a functional, high-quality additive within many applications.

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CHAPTER 3

Effect of NMMO pretreatment on the anaerobic digestion of different lignocellulosic materials

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3.1 Introduction

Lignocellulosic materials (LMs) constitute about half of the total amount of biomass worldwide [1]. These abundant feedstocks have shown great potential as possible substrates in the anaerobic digestion (AD) process, leading to the production of biogas and other biofuels [2]. In particular, agricultural residues have been acknowledged as the most suitable biomass for biomethane production [3]. The high availability, low cost and sustainability of these substrates make them particularly appropriate for sustaining the economy of developing countries [4]. The compact and complex structure of LMs is, however, particularly difficult to degrade by microbial enzymes during the hydrolysis stage. Therefore, the use of LMs in AD applications is hindered by their resistance to microbial degradation, and a pretreatment step prior to performing AD is often required [5].

Several pretreatment techniques, generally classified into physical, chemical and biological, have been developed. When compared to the chemical pretreatments, the physical and biological methods are not cost competitive at full scale [6], since the biological pretreatment rate is generally slower [7], while the costs of physical technologies are usually higher [8]. Among the chemical methods investigated, the solvent pretreatments based on cellulose dissolution present many advantages [9]. They are particularly effective in increasing biogas yields from a wide range of LMs. After being dissolved, cellulose is regenerated by adding an antisolvent and presents a more amorphous structure, which results in a higher accessible surface area to the bacteria, enhancing the cellulose hydrolysis rate [10].

N-methylmorpholine-N-oxide (NMMO) is an organic solvent commercially produced by oxidizing N-methylmorpholine with H_2O_2, with the main world producers being based in Germany and United Kingdom [11]. NMMO has shown a great potential for the pretreatment of LMs, given its ability to efficiently dissolve cellulose by disrupting the intermolecular bonds network among polysaccharides [12]. NMMO is considered an environmentally friendly solvent, since it does not produce toxic waste pollutants, is fully biodegradable, and can be recovered by more than 98%. Moreover, NMMO pretreatment of LMs can be conducted under milder conditions than other techniques (i.e. hydrothermal pretreatments) as temperature remains in the 90-130°C range, and atmospheric pressure is used [13].

These advantages make NMMO particularly appropriate to be used for LM pretreatment in developing countries. Other pretreatment methods, such as alkali or acid pretreatment entail high costs due to downstream neutralization and the use of withstanding equipment to avoid

corrosion. Furthermore, a part of the carbohydrates fraction is lost during these pretreatments, while NMMO pretreatment does not change the composition of the LMs [14].

NMMO is the only solvent that has already been used for directly dissolving cellulose in full-scale plants within the textile industry (i.e. the Lyocell process) in the past 20 years [15]. Simulations of a full-scale NMMO pretreatment for wood materials, aimed to enhance the biogas production, were attempted by Teghammar et al. [16] and Shafiei et al. [17]. The raw substrate was fed to a stainless-steel vessel where it undergoes the NMMO pretreatment. The required temperature was achieved by both externally providing and recovering heat from the steam produced in other parts of the process. After the pretreatment, the LM is washed with water and filtered in a vacuum filtration unit, where the NMMO solution is evaporated back to 85% for further use in the pretreatment unit. A minimum amount of washing water is used, in order to reduce the energy demands in the sequent evaporation unit with mechanical vapor recompression.

In this study, NMMO pretreatment was tested on three different types of LMs, namely rice straw, hazelnut skin and cocoa bean shell. Rice straw is a very abundant feedstock, as rice is the third most cultivated crop worldwide [18]. 90% of the rice production is distributed in the developing countries of Eastern and Southeast Asia, with the straw largely being unused [19] or disposed by open field burning [20] resulting in severe pollution and serious environmental damage [21].

Hazelnut is among the most cultivated tree nuts internationally. Turkey is the top producer, accounting for approximately three fourths of the global supply [22]. The skin of the hazelnut kernels, which approximately constitutes 2.5% of the total weight, is usually removed by roasting, and the disposal of this byproduct represents both an environmental and economic problem for the hazelnut industry [23].

The cocoa bean production was estimated to be roughly 4 million tons in 2012, with Africa accounting for over 70% of the world production [24]. Cocoa bean shells are the skin of cocoa nibs, which are the edible portion. Cocoa shells represent more than 10% of the weight of the beans and are separated by roasting [25]. During cocoa processing, about 75% of the raw cocoa fruit constitutes a waste product, which could be employed for energy production [26].

Currently, the leftovers of the hazelnut and cocoa roasting process are disposed as a waste in landfills or incinerated. Both byproducts have also been used as animal feed [23]. However, cocoa shells contain theobromine, which is an undesirable substance in animal diets [27]. Additionally, cocoa shells are used as mulching materials on farmlands or as low-cost precursor for the production of activated carbon [28].

The potential of rice straw for AD has been investigated by several researchers [18], and different types of pretreatment were adopted to enhance biogas yields from this LM [29]. On the other hand, to the authors' knowledge, the biomethane yields from AD of hazelnut skin have not yet been reported, whereas only one study investigated the potential of cocoa shell for biogas production through AD [30]. Therefore, in this study the biogas potential of untreated cocoa shell and hazelnut skin, in addition to rice straw, were initially investigated in bio-methane potential (BMP) batch tests. Subsequently, the effect of a 3 h NMMO pretreatment of the three LMs was evaluated in order to assess the potential enhancement of the biogas production yields. Changes in the structure of the LMs were analyzed by Fourier transform infrared spectroscopy (FTIR). Moreover, the anaerobic degradation process was investigated by studying changes in volatile fatty acids (VFAs) composition and concentration in the digestate.

3.2 Materials and Methods

3.2.1 Raw materials

Rice (*Oryza sativa*) straw, hazelnut (*Corylus avellana*) skin and cocoa (*Theobroma cacao*) bean shell were used as lignocellulosic substrates for AD. Their physicochemical characterization is reported in Table 3.1. The rice straw was harvested from rice fields located in Pavia (Italy) and then cut down to a particle size smaller than 4 mm before the pretreatment. The hazelnut skin and cocoa bean shell were obtained as by-products of the conventional industrial roasting process from a company manufacturing chocolate and confectionery products in Italy.

Table 3.1. Characteristics of the LMs

	Rice straw (% *w/w*)	Hazelnut skin (% *w/w*)	Cocoa shell (% *w/w*)
TS[a]	91.75 ± 0.09	91.58 ± 0.11	89.08 ± 0.08
VS[a]	77.19 ± 0.25	89.94 ± 0.27	79.81 ± 1.58
Total carbon[b]	41.88 ± 3.38	62.03 ± 2.62	37.64 ± 6.01
Total nitrogen[b]	0.92 ± 0.04	2.03 ± 0.03	3.35 ± 0.46

a: on wet weight basis
b: on dry weight basis

3.2.2 NMMO pretreatment

The LMs were pretreated using an 85% (*w/w*) NMMO solution. The commercial 50% (*w/w*) NMMO (Sigma-Aldrich, Germany) aqueous solution was concentrated to 85% (*w/w*) by using a rotary evaporator (Büchi Rotavapor R-114, Switzerland). The pretreatment was

performed in 500 mL Erlenmeyer flasks containing 7.5 g of LM soaked in 92.5 g of NMMO solution. The flasks were placed in an oil bath under atmospheric pressure conditions and heated at 120°C for 3 h. The mixture in each flask was manually stirred with a glass rod every 10 min. In order to prevent oxidation of the solvent during the pretreatment, 0.625 g propyl gallate per kg NMMO was added [31].

After the pretreatment, 150 mL of boiling deionized water was added as antisolvent in order to stop the reaction. The pretreated LMs were then placed on textile bags and repeatedly washed with boiling deionized water until a clear filtrate was achieved. The pretreated LMs were stored at 4°C until further investigations in the subsequent BMP tests.

3.2.3 BMP tests

BMP batch tests were carried out under controlled mesophilic (37 ± 2°C) conditions using 125 mL sealed serum glass bottles (Wheaton, USA). The biomethane production was measured by the water displacement method, according to the procedure described by Esposito et al. [32]. The alkaline solution used to entrap CO_2 was 12% NaOH. The inoculum was collected from a full-scale AD plant treating buffalo manure and milk whey generated from a mozzarella factory, located in Capaccio (Italy). The TS and VS content of the inoculum was 2.72 (± 0.04) and 1.84 (± 0.03)% (*w/w*), respectively. Each bottle contained 33 mL of inoculum and 0.3 g VS of untreated or NMMO pretreated LMs. The VS inoculum to substrate ratio was 2:1. Tap water was added to adjust the final volume to 50 mL in all bottles. In order to create anaerobic conditions, each bottle was flushed with helium gas for 5 min.

Control bioassays, containing only inoculum and tap water, were simultaneously carried out in order to evaluate the biomethane production obtained with the inoculum, which was subtracted from its production achieved in the other bioassays. All the experiments were performed in triplicate and the partial biomethane production was recorded along 40 d. During this period, the bottles were manually shaken once per day. The liquid phase of each bottle was sampled 3 times on the first week and twice on the following two weeks for VFA analysis.

3.2.4 Determination of methane production kinetics

The methane production kinetics were evaluated using a first-order kinetic model. In particular, the following equation was used:

$$G = G_m\left[1 - exp^{(-k_0 t)}\right] \quad (3.1)$$

which becomes:

$$ln\left[\frac{G_m}{G_m - G}\right] = k_0 t \quad (3.2)$$

where G (mL) is the cumulative volume of methane after a time t (d); G_m (mL) is the maximum cumulative volume at an infinite digestion time; t (d) is the digestion time and k_0 (1/d) is the specific rate constant. The specific rate constants are determined as the slopes of the linear part of the plot displaying $ln[G_m/(G_m-G)]$ vs t. The values of G_m were considered as the cumulative volumes of methane at the end of the experimental run.

3.2.5 Energy balance of NMMO pretreatment

A rough estimate of the energy balance of the process was performed for the NMMO pretreatment of rice straw. The energy input of the pretreatment unit was calculated using equation (3.3) [33]:

$$H = \left\{\frac{[m \cdot C_p \cdot \Delta T]}{3600}\right\} \quad (3.3)$$

where H is the heat energy requirement expressed in kWh kg^{-1} VS; m is the mass of NMMO required to treat 1 kg VS of rice straw expressed in kg; C_p is the NMMO specific heat capacity, which was considered equal to 2.92 kJ/kg·°C [34]; ΔT is the difference between the initial temperature of the NMMO-water mixture (assumed as 25°C) and the final temperature required for the pretreatment (i.e. 120°C); 3600 is the conversion factor between kJ and kWh.

The energy produced after the AD of the NMMO pretreated rice straw was calculated from the results obtained in the BMP tests. In particular, it was assumed that the biogas produced is employed in a combined heat and power (CHP) unit with a thermal efficiency of 50% and an electrical efficiency of 35%. The biomethane production, expressed in m^3 CH_4/kg VS was converted to kWh/kg VS, considering a conversion factor of 10 kWh/m^3 [33].

3.2.6 Statistical analysis

Statistically significant differences between the biomethane production of the pretreated and the untreated substrates in the batch assays were determined by a paired t-test, using the software package Minitab (version 17.0).

3.2.7 Analytical methods

Total solids (TS) and volatile solids (VS) of both untreated and pretreated LMs were determined by drying the samples to constant weight at 105 (± 5)°C in a drying oven and igniting at 575 (± 10)°C in a muffle furnace, respectively [35]. The carbon and nitrogen content in the LMs was determined using an elemental analyzer (2400 CHNS, Perkin Elmer, USA). VFAs were analyzed using a HPLC (Prominence LC-20A Series, Shimadzu, Japan) equipped with a Rezex ROA-Organic Acid H^+ column of 300 x 7.8 mm (Phenomenex, USA), heated at 40°C, and a SPD-20A UV detector set at 220 nm. A 0.0065 mM H_2SO_4 solution was used as

mobile phase at a flowrate of 0.6 mL/min. The detection limit was 0.1 mM for each compound analyzed. Before being analyzed, the samples for VFA analysis were centrifuged for 5 min at 8000 rpm and filtered with 0.22 μm Millex cellulose membranes (Merck Millipore, USA).

The untreated and NMMO pretreated LMs were analyzed using a FTIR spectrometer (Spectrum 1000, Perkin Elmer, USA). The spectra were obtained in the range from 600 to 4000 cm^{-1}, with an average of 64 scans and a resolution of 4 cm^{-1}.

3.3 Results and discussion

3.3.1 Effect of NMMO on biogas production from the LMs

3.3.1.1 Methane production yields

The biogas potential of rice straw, hazelnut skin and cocoa shell was investigated in batch digestion experiments under mesophilic conditions (37 ± 2°C). Table 3.2 reports the cumulative methane production obtained with the three LMs, as the average of the triplicates sampled during 40 d.

The biomethane potential of the 3 h NMMO pretreated rice straw was significantly enhanced compared to that obtained with the untreated substrate. The NMMO pretreatment resulted in an 82% higher bio-methane yield with a cumulative CH_4 production increasing from 206 (± 22.1) to 374 (± 22.5) mL CH_4/g VS (Fig. 3.1a). This enhancement was most likely due to the higher accessibility of cellulose and hemicellulose to the bacteria after the pretreatment. NMMO has the ability of breaking the hydrogen bonds holding the cellulose chains together, allowing the formation of new bonds between the solvent and the cellulose, thus inducing the cellulose dissolution [11]. The regenerated cellulose obtained after stopping the reaction with water showed an improved biodegradability. An increase of the biogas production by more than 600% was observed by Teghammar et al. [2] for rice straw pretreated with NMMO. However, the biomethane yield of the untreated straw in their study was only 30 mL CH_4/g VS, probably due to a high silica content (i.e., more than 10% of dry weight) in the straw tested. Silica can limit rice straw digestibility by forming a physical barrier around the carbohydrate portion [36].

Unlike rice straw, the NMMO pretreatment hardly affected the biogas potential of cocoa shell (Fig.1b) and hazelnut skin (Fig.1c). The total methane production was 226 (± 27.0) mL CH_4/g VS for the 3 h pretreated cocoa shell, i.e. only increased by 14% from 199 (± 22.4) mL CH_4/g VS achieved with the untreated substrate. Conversely, the cumulative biomethane yield of hazelnut skin did not increase after the pretreatment and was 223 (± 25.1) and 220 (± 18.4) mL CH_4/g VS for the untreated and pretreated substrate, respectively.

Table 3.2. Specific methane yields, specific rate constants, and lateral order index (LOI) of the untreated and NMMO pretreated LMs

LMs	Pretreatment conditions	Specific methane yield (mL CH_4/g VS added)	Specific rate constant k_0 (d^{-1})[a]	LOI (A1420 cm^{-1}/A898 cm^{-1})
Rice straw	Untreated	207±22.1	0.098	1.21±0.02
	3h, 120°C, 85% NMMO	374±22.5	0.133	1.19±0.02
Hazelnut skin	Untreated	223±25.1	0.136	1.52±0.18
	3h, 120°C, 85% NMMO	220±18.4	0.384	1.39±0.08
Cocoa shell	Untreated	199±22.4	0.269	1.23±0.02
	3h, 120°C, 85% NMMO	226±27.0	0.163	1.42±0.01

a: k_0 obtained during the first 9 days of AD.

In order to verify whether the biomethane yields obtained from the NMMO pretreated LMs were significantly different from those of the untreated ones, a statistical analysis using a paired t-test was performed [37]. The results showed that the increase of the biogas production was significant only for the NMMO pretreated rice straw, with a p-value of 0.001 (i.e. below the 5% significance level). The pretreatment did not cause a statistically significant biomethane production enhancement for cocoa shell and hazelnut skin, with p-values of 0.375 and 0.300, respectively.

To the authors' knowledge, only one study in the literature [30] reported the biomethane production from cocoa shells, whereas the biogas production from hazelnut skin has not yet been reported. Rico et al. [30] obtained a biomethane yield of 223 mL CH_4/g VS of cocoa shell, in line with that obtained with the untreated substrate in this investigation. Despite the NMMO pretreatment was not effective in enhancing the biogas production of hazelnut skin and cocoa shell, Fig. 3.1b and Fig. 3.1c show that these two waste products have a significant potential as AD substrates, even without undergoing a preliminary treatment stage.

Although the operating conditions are less demanding than other pretreatment methods, the high temperatures required and the cost of the raw solvent can limit the applicability of NMMO pretreatment in full-scale applications, especially in developing countries. The enhanced biogas production for rice straw and the high NMMO recyclability can nevertheless make the process economically feasible, as demonstrated in a techno-economic study by Teghammar et al. [16], who evaluated the economic viability of a full-scale plant codigesting NMMO pretreated forest residues and the organic fraction of municipal solid waste in Sweden. This is especially applicable in those countries where rice straw and other agricultural residues are significantly abundant and the development of the economy encourages to use increasing amounts of energy.

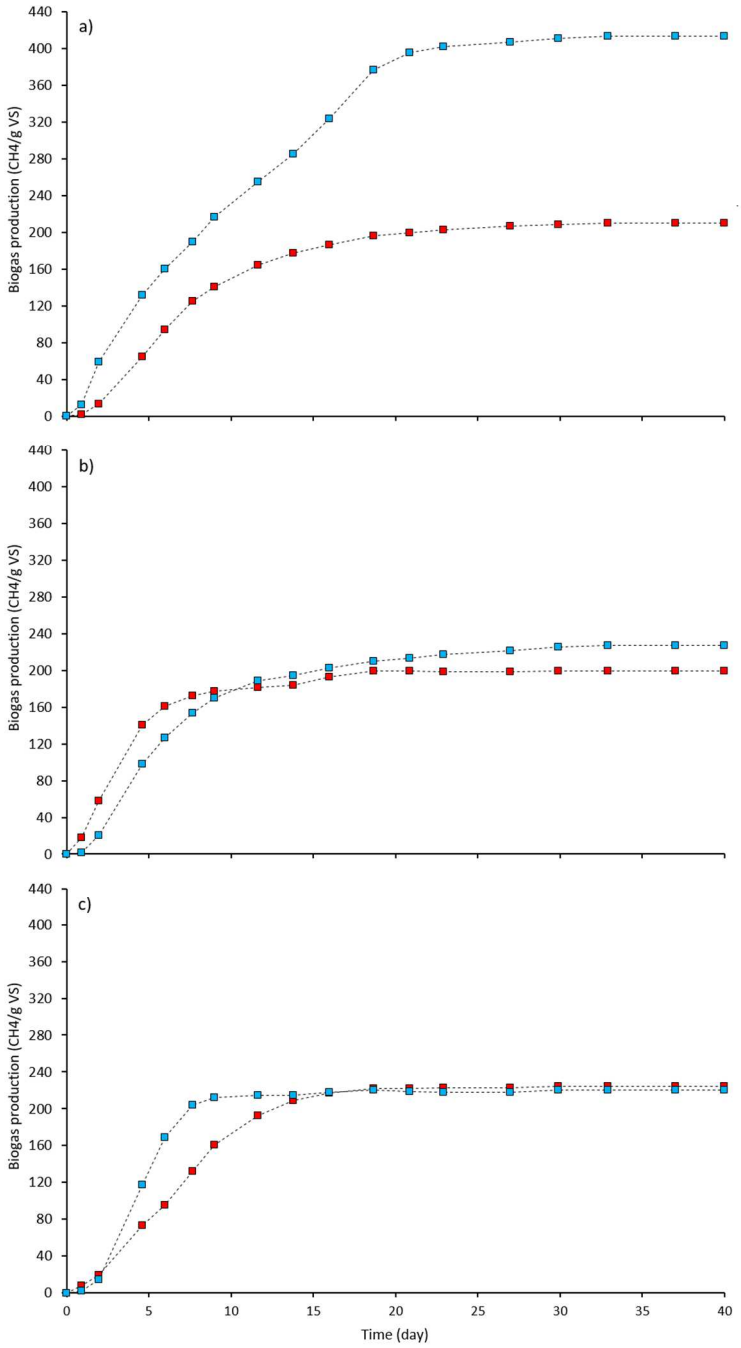

Fig. 3.1. Cumulative methane production from rice straw (a), cocoa bean shell (b) and hazelnut skin (c) (■: NMMO pretreated; ■: untreated).

3.3.1.2 Methane production kinetics

The specific rate constants (k_0) for the untreated and NMMO pretreated LMs are reported in Table 3.2. The results showed that the degradation process was improved by the NMMO pretreatment for both rice straw and hazelnut skin during the first 9 d of AD. The specific rate constants increased from 0.098 to 0.134 d^{-1} and from 0.135 to 0.384 d^{-1} for the untreated and NMMO pretreated rice straw and hazelnut skin, respectively. On the other hand, k_0 decreased from 0.269 to 0.164 d^{-1} for the NMMO pretreated cocoa shell compared to the untreated

substrate, most probably because a washing out of readily available compounds present in the raw cocoa shell occurred during the NMMO pretreatment.

The NMMO pretreatment resulted in an initial faster biomethane production from the pretreated hazelnut skin (Fig. 3.1c). After 7 d, the cumulative methane yields of the pretreated and untreated hazelnut skin were 204 and 132 mL CH_4/g VS, respectively, i.e. 93 and 59% of the corresponding final production. A higher hydrolysis rate was therefore achieved by pretreating hazelnut skin with NMMO, which resulted in a shorter AD time and, hence, an optimization of the overall process. In contrast, the methane production rate of the untreated cocoa shell was higher compared to the pretreated substrate during the first 9 d of AD.

3.3.1.3 Energy balance assessment

An energy balance assessment study was conducted in order to assess whether the additional biogas produced from the pretreated rice straw would justify the surplus energy input for performing the NMMO pretreatment. In particular, the thermal energy for heating the pretreatment unit was related to the thermal energy produced in a common CHP system [33].

Initially, the methane produced by the untreated rice straw was subtracted to that obtained using the pretreated LM. Then, the net biomethane amount produced due to the pretreatment was converted to thermal energy considering an efficiency of 50%. The thermal energy gain obtained was 0.840 kWh/kg VS. The heat energy required in the pretreatment unit was equal to 1.231 kWh/kg VS, considering a NMMO/VS of rice straw ratio of 15.98 (kg/kg). Therefore, the net heat energy, which is the difference between the thermal energy increase and the heat energy requirement, had a negative value (i.e. -0.391 kWh/kg VS). Nevertheless, assuming that 80% of the required heat could be recovered by heat exchangers [33,38], the energy input would decrease to 0.246 kWh/kg VS, and the net heat energy of the total process would achieve a positive value of 0.594 kWh/kg VS.

3.3.2 Effect of NMMO on LM crystallinity

A FTIR analysis was performed in order to assess changes in the LM crystallinity, which is reported to have a close relation with the bioconversion of LMs [39]. In particular, an increase in the biodegradability of lignocellulosic materials can be partly explained by a decrease in the crystallinity of the cellulose fibers. FTIR allows to evaluate the changes in the cellulose crystalline structure by determining the lateral order index (LOI), which is defined as the ratio between the absorbance values obtained at 1420 and 898 cm^{-1}, corresponding to cellulose I and II, respectively [40].

The values of the crystallinity index obtained for the investigated LMs are reported in Table 3.2. The LOI of rice straw was 1.21 and 1.19 prior to and after the pretreatment, respectively. This implied a possible breakdown of the rice straw structure, which resulted in an enhancement of the hydrolysis and, as a consequence, biogas yield. A decrease of the LOI of hazelnut skin from 1.52 to 1.39 was observed after the pretreatment. As a result, the hydrolysis and the biomethane production rates of the pretreated hazelnut skin were much faster during the first 7 d of the BMP tests. In contrast, the NMMO pretreatment of cocoa shell resulted in an increase of the LOI from 1.23 to 1.41. Both peaks at 1420 and 898 cm^{-1} were lower for the pretreated cocoa shell. However, a higher decrease was observed at the wavelength of 898 cm^{-1}, inducing an increase of the LOI of pretreated cocoa shell compared to the untreated one. This was likely due to a reduction in the hemicellulose content of the cocoa shell, which was more pronounced than the increase of the cellulose II concentration in the matrix.

3.3.3 Production of VFAs

VFAs are intermediary products of AD and their concentration is generally used to monitor the extent of AD [41]. During the AD process, VFAs are produced from the fermentation of carbohydrates, deamination of amino acids and hydrolysis of long chain fatty acids [42].

Comparing the trends of VFA composition and concentration with the trends of the methane production yield can provide a better understanding of the AD development for the investigated substrates. In this study, the evolution of VFAs was monitored during the first 20 d of AD (Fig.3.2). The highest VFA production was observed for the 3 h pretreated rice straw, with a total VFA concentration ranging between approximately 1200 and 1400 mg HAc/L from day 4 to day 14. Afterwards, the VFA concentration dropped to 62 mg HAc/L on day 19, when 95% of the cumulative biomethane production was reached.

Acetic acid, which is one of the main precursors of methane formation through the reduction of the methyl group, accumulated also at higher concentrations in the pretreated rice straw along the whole incubation period. In the NMMO pretreated rice straw, the acetogenic bacteria were able to convert propionic, butyric, isobutyric and isovaleric acids to acetate at a faster rate, stimulating a higher biomethane production. An accumulation of acetic acid above 2000 mg/L with an overall VFA concentration above 8000 mg/L was reported to negatively affect the methane yields [43]. This indicates that most likely no inhibition occurred during the AD of the pretreated rice straw in this study, as acetic acid and total VFA concentrations constantly remained below the inhibitory levels (Fig. 3.2).

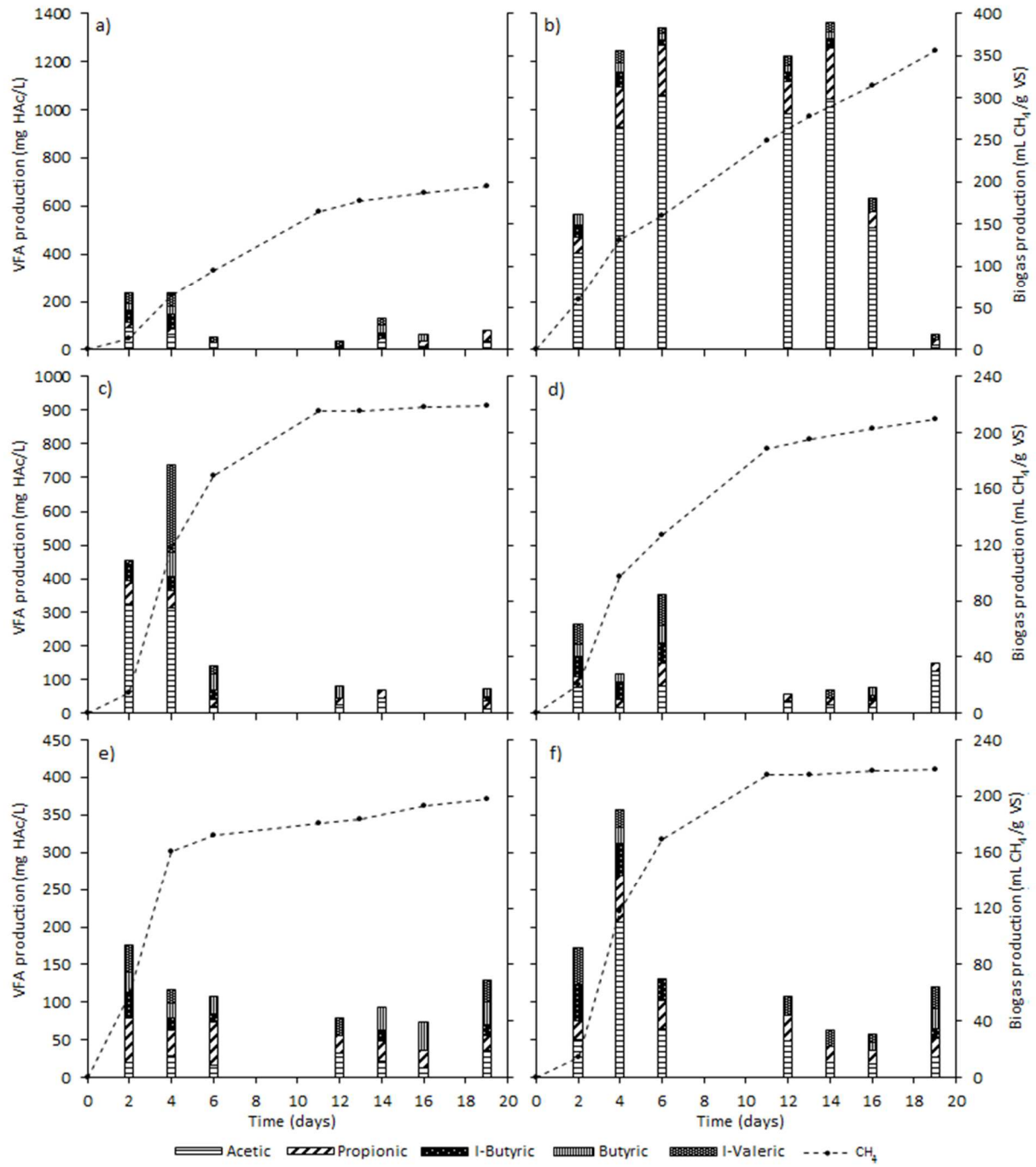

Fig. 3.2. Evolution of VFA concentration during the AD of untreated (a) and pretreated (b) rice straw, untreated (c) and pretreated (d) cocoa shell, and untreated (e) and pretreated (f) hazelnut skin. (*Note that the Y-axis scales are different among the graphs*).

For the untreated and pretreated hazelnut skin, the highest total VFA concentration of 199 and 356 mg HAc/L was observed on days 2 and 4, respectively. In particular, the higher production of VFAs during the first 7 d of pretreated hazelnut skin was most likely due to an increased amount of readily available organic matter, caused by the enhanced hydrolysis after the NMMO pretreatment.

VFA production had an initial peak of approximately 900 mg HAc/L on day 4 for untreated cocoa shells, probably due to a larger portion of the easily biodegradable fraction compared with the other LMs investigated. On the other hand, the total VFA amount was lower in the first week of AD of the pretreated material, confirming that NMMO pretreatment did not have a positive effect on the hydrolysis of the LM cocoa shell.

3.4 Conclusion

NMMO pretreatment was an effective pretreatment method for rice straw, leading to an 82% enhancement of biomethane production, which reached approximately 375 mL CH_4/g VS. A preliminary energy assessment showed that a positive energy balance can be achieved employing the NMMO pretreatment for rice straw when 80% of heat is recovered during the pretreatment stage. Untreated hazelnut skin and cocoa bean shell showed a good potential for biogas production, with cumulative biomethane yields of 223 and 199 mL CH_4/g VS, respectively. Their pretreatment with NMMO further increased the biogas production of cocoa shell by 14%, despite that the crystallinity index and the methane production rate were negatively affected by the pretreatment. For hazelnut skin, NMMO pretreatment resulted in a decrease of the crystallinity index and a higher initial biomethane production rate.

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CHAPTER 4

Effect of organosolv pretreatment on the anaerobic digestion of different lignocellulosic materials

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4.1 Introduction

Biogas is a renewable energy source that can be obtained from the anaerobic digestion (AD) of various organic substrates. Compared to other biofuels, biogas results in a higher energy efficiency, which makes its commercial production advantageous [1]. Lignocellulosic materials (LMs) have the potential to represent the main feedstock for biogas production worldwide, because of their abundance, relatively low cost and sustainability [2]. However, to date LMs are not commonly employed in anaerobic digesters [3].

Due to the recalcitrant structure of LMs towards biodegradation, the biogas yields are generally low [4]. In particular, the presence of lignin in the LM matrix represents the main limitation to the bacterial hydrolysis by hindering the access of microorganisms to the carbohydrate portion [5]. To overcome this drawback, a pretreatment step is required in order to enhance the digestibility of LMs [6]. Several pretreatment methods have been investigated in recent years, including steam explosion, alkali, acid, ammonia fiber explosion, and organic solvents [7]. Among these techniques, organosolv is emerging as one of the most promising in efficiently removing lignin from the lignocellulosic structure [8,9].

The organosolv method is based on the pretreatment of LMs with organic solvents, such as methanol, ethanol, acetone, glycerol and organic acids, at temperatures in the range from 100 to 250°C [10]. This treatment leads to the breakdown of the lignin molecules by cleavage of ether linkages and their subsequent dissolution [11]. By employing low boiling point alcohols, several advantages can be obtained with the organosolv method compared to other conventional pretreatments, such as acid or alkaline [12]. After the pretreatment, the solvent can be easily recovered and recycled through a distillation stage [4]. Moreover, the recovery of a highly pure lignin fraction as an economically viable byproduct can be obtained at the end of the process [13]. Therefore, the organosolv technique represents a promising pretreatment within the development of LM biorefineries [14].

Up to now, the organosolv pretreatment has mostly been investigated as a technique to improve the enzymatic hydrolysis for bioethanol production [15]. In the last couple of years, however, organosolv has also been tested as a method to improve the biogas production from different LMs, including forest and agricultural residues [10].

In this research, the effect of organosolv pretreatment was evaluated on the biomethane production yields from three different LMs, namely rice straw, hazelnut skin and cocoa bean shell. Rice straw represents the main crop residue in the world, with a yearly production of around 700 million tons [16]. 90% of rice cultivation is concentrated in the developing

countries of Eastern and Southeast Asia, where open field burning is still a common practice to dispose of the unused straw, thus causing serious pollution cases [17]. Differently, employing rice straw to produce biogas through the AD process is considered as one of the most environmentally friendly methods for converting this LM into renewable energy [18].

Environmental and economic problems can also arise in the food industry from the disposal of hazelnut skin [19] and cocoa bean shell [20]. These byproducts, which represent the skin and the shell of hazelnut and cocoa fruits, respectively, are obtained after the roasting process [21].

In this study, biochemical methane potential (BMP) tests were carried out to assess the methane production yields in batch assays containing the three untreated and organosolv pretreated LMs. The effect of different pretreatment temperatures (i.e. 150 and 180°C) was evaluated, employing 50% (*v/v*) ethanol as the organic solvent. The biomethane production yields were recorded for all the analyzed LMs and the experimental results were fitted using a first order and a modified Gompertz model [22], providing information about the rates of methane production of the untreated and organosolv pretreated LMs. The development of AD was further monitored by analyzing the volatile fatty acids (VFA) concentration profiles. Moreover, the composition of the three LMs was investigated in order to assess the extent of the pretreatment on the lignin and carbohydrate portions.

4.2 Materials and methods

4.2.1 Feedstocks and inoculum

Rice (*Oryza sativa*) straw was harvested from agricultural fields in Pavia (Italy) and cut down to a particle size smaller than 4 mm. Hazelnut (*Corylus avellana*) skin and cocoa (*Theobroma cacao*) bean shell were obtained as by-products of the conventional industrial roasting process. The two substrates, received from an Italian company manufacturing chocolate and confectionery products, were sieved through a 4 mm sieve. The physicochemical characterization of the three LMs is reported in Table 4.1.

Table 4.1. Characteristics of the raw LMs

	Rice straw	Hazelnut skin	Cocoa bean shell
TS (%)a	93.1 ± 0.1	91.8 ± 0.2	90.6 ± 0.3
VS (%)a	76.8 ± 1.1	89.1 ± 0.8	81.2 ± 0.1
TKN (% TS)	0.7 ± 0.0	1.3 ± 0.2	3.3 ± 0.5
Protein content (% TS)	4.9 ± 0.3	8.3 ± 1.5	20.3 ± 3.2

a: TS and VS are expressed in terms of fresh matter.

The digestate from a full-scale AD plant treating buffalo manure and milk whey from a mozzarella factory located in Capaccio (Italy) was used as the inoculum. The inoculum was degassed for 2 d at 37°C before using it in the following experiments. The total solid (TS) and volatile solid (VS) content of the inoculum was 2.62 (± 0.14)% and 1.67 (± 0.03)%, respectively.

4.2.2 Organosolv pretreatment

The organosolv pretreatment was performed using a high-pressure stainless-steel vessel (Sigma-Aldrich, Germany) with a working volume of 300 mL. An identical procedure was applied to pretreat all the three LMs. The reactor was first loaded with 15 g of LM and soaked in 150 mL of 50% (*v/v*) ethanol. The reactor was sealed and placed in a TCF 50 PRO convection oven (ArgoLab, Italy), equipped with a ramping program. The oven was heated at a rate of 3°C/min to the desired temperature (i.e. 150 or 180°C), which was finally held for 60 min. Afterwards, the reactor was cooled in an ice bath. The pretreated LM was removed, washed with 100 mL fresh 50% (*v/v*) ethanol and subsequently with distilled water until pH 7.0 was obtained in the liquor. The LMs were left overnight to air dry, and finally stored in plastic bags at room temperature until further use.

4.2.3 BMP tests

BMP batch tests were carried out in 125 mL sealed serum bottles (Wheaton, USA) under mesophilic conditions (37 ± 1°C). Biomethane production was measured by the water displacement method, according to the procedure described by Esposito et al. [23], modified as in Mancini et al. [21]. Each bottle was loaded with 50 mL of inoculum and 0.5 g VS of untreated or pretreated LM in order to obtain an inoculum to substrate ratio of 1.5. All the experiments were performed in triplicate and the daily biomethane production was recorded until it was lower than 1% of the cumulative volume of the produced biomethane (i.e. on day 43). For VFA analysis, 0.5 mL of the liquid phase was daily sampled from each bottle during the first ten days of the experiment, except for day 1, 3, 8 and 9.

The biomethane production kinetics were evaluated for each BMP test to quantify whether the organosolv pretreatment caused an enhancement of the AD rates. Two models were applied, namely a first order kinetic model and a modified Gompertz model [22], using Eq. (4.1) and (4.2), respectively:

$$G = G_m\left[1 - \exp^{(-k_0 t)}\right] \quad (4.1)$$

$$G = G_m \exp\left\{-\exp\left[\frac{R_m e}{G_m}(\lambda - t) + 1\right]\right\} \quad (4.2)$$

where G (mL CH_4/g VS) is the cumulative volume of methane after a time t (d); G_m (mL CH_4/g VS) is the maximum cumulative volume at the end of the experimental run; k_0 (1/d) is the first order kinetic constant; t (d) is the digestion time; e is the Euler's number (i.e. 2.7183); λ is the lag phase (d) and R_m (mL CH_4/g VS·d) is the maximum biomethane production rate. k_0, λ and R_m were determined by curve-fitting using the software GraphPad Prism 6.0 (GraphPad Software Inc., USA), based on the experimental data of cumulative methane production obtained in the BMP tests. The coefficient of determination r^2 was calculated for both the adopted models to evaluate the accuracy of the predictions.

4.2.4 Water retention value

The water retention value (WRV), also known as the water swelling capacity, is the ability of a substrate to keep water molecules in the cell wall pores [24]. WRV is used as an indication of the accessible interior surface area and the consequent suitability of the LM to enzymatic hydrolysis. The WRV analysis is based on the principle that no enzyme can enter the pores of LMs if water cannot [25]. After the centrifugation of a water-saturated sample, the WRV is defined as the amount of water that can be retained per unit weight of dry material.

Approximately 1.0 g of each LM was mixed with deionized water in a bottle agitated at 150 rpm for 60 min. The mixture was then filtered using a 0.45 µm filter (Merck Millipore, USA). The obtained cake was transferred into a nonwoven fabric material, which was soaked in deionized water for 2 h at room temperature. The fabric was wrapped, placed into a centrifuge tube with support to make space for water accumulation, and centrifuged at 3000 g for 15 min. The substrate was collected and weighed before and after drying at 105°C for 24 h. The WRV was calculated as follows [24]:

$$\mathrm{WRV} = \frac{W_{wet} - W_{dry}}{W_{dry}} \quad (4.3)$$

where W_{wet} and W_{dry} are the wet and oven dry mass of the LM, respectively.

4.2.5 Analytical methods

Total solids (TS) and volatile solids (VS) of both untreated and pretreated LMs were determined by drying the samples to constant weight at 105°C and by igniting at 575°C, respectively [26]. Total Kjeldahl nitrogen (TKN) was measured using the Kjeldahl method [27]. Total proteins were calculated multiplying the TKN values by a correction coefficient of 6.25 [28]. VFAs were analyzed as reported by Mancini et al. [21].

The content of structural carbohydrates and lignin of the pretreated and untreated LMs was analyzed according to the procedure described by Sluiter et al. [29]. A two-step acid hydrolysis

was applied using first concentrated (i.e. 72%) and then diluted (i.e. 4%) H_2SO_4. The acid-soluble lignin content of the LMs was determined using UV spectroscopy (Lambda 365 UV/Vis, Perkin Elmer, USA), whereas the acid-insoluble lignin content was determined gravimetrically after drying the samples at 575°C. The structural carbohydrates of the LMs were analyzed using a HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with a refractive index detector (RID-20A, Shimadzu, Japan) and a Rezex RPM-Monosaccharide Pb^{2+} (8%) column (Phenomenex, USA), heated at 90°C. HPLC-grade water was used as the mobile phase at a flow rate of 0.6 mL/min.

4.2.6 Statistical analyses

The statistically significant difference between the biomethane production of the pretreated and the untreated substrates in the batch assays was determined by a paired t-test [30] using the software package Minitab 17.0 (Minitab Inc., State College, USA). The results were considered statistically significant when the *p*-value obtained was below 0.05.

4.3 Results and discussion

4.3.1 Effect of the organosolv pretreatment on the LM composition

Rice straw, hazelnut skin and cocoa bean shell were pretreated using 50% (*v/v*) ethanol prior to anaerobic digestion, in order to improve the biogas production yield. The untreated and pretreated materials were characterized in terms of carbohydrates and lignin content, and the results obtained are presented in Table 4.2. The composition of the raw substrates was significantly different between rice straw, hazelnut skin and cocoa bean shell. Nevertheless, the pretreatment had similar impacts in changing the original composition of the three LMs.

The total lignin content, expressed as the sum of acid insoluble lignin and acid soluble lignin, was 34.4 and 29.9%, in terms of dry matter, for hazelnut skin and cocoa bean shell, respectively. The untreated rice straw had a much lower lignin content (i.e. 17.3%). Depending on the temperature adopted, the pretreatment was able to reduce the lignin content by 14 – 15% for rice straw, 5 – 6% for hazelnut skin, and 8 – 12% for cocoa bean shell. A higher pretreatment temperature (i.e. 180°C) corresponded to a higher delignification compared to the milder operative condition (i.e. 150°C). The lignin removal observed in the three pretreated LMs was likely linked to both the cleavage of the bonds between lignin and carbohydrates and the solubilization of lignin [31]. The carbohydrate content was significantly lower for hazelnut skin and cocoa bean shell than for rice straw. The glucan content, which refers to the cellulose amount in the LMs, was 28.6, 11.4 and 13.5% for the raw rice straw, hazelnut skin and cocoa

bean shell, respectively (Table 4.2). The sum of the other polysaccharides, constituting the hemicellulose portion, was 19.5% for rice straw, 5.9% for hazelnut skin, and 7.0% for cocoa bean shell (Table 4.2). The total carbohydrate content of hazelnut skin and cocoa bean shell was similar to that reported by Zeppa et al. [32] and Martínez et al. [20], respectively. Differently from rice straw, the protein and fat content of hazelnut skin and cocoa bean shell are not negligible: the amount of proteins constituted 8.3 and 20.3% of the raw hazelnut skin and cocoa bean shell, respectively (Table 4.1). The fat content was not assessed in this study. However, the amount of fat for cocoa bean shell has been reported in the range 4 – 18% [33], whereas Zeppa et al. [32] recorded a total fat content between 11 and 19% for hazelnut skin.

As a result of the pretreatment, xylan and arabinan (i.e. the main constituents of hemicellulose) decreased for all the three LMs (Table 4.2). On the other hand, after the organosolv pretreatment the glucan content increased by 4 – 12%, 1 – 10% and 3 – 11% for rice straw, hazelnut skin and cocoa bean shell, respectively. Although the cellulose hydrolysis would benefit from a decreased amount of hemicellulose in the matrix, a loss of hemicellulosic sugars can result in a lower biogas production [34].

Table 4.2. Chemical composition of the untreated and organosolv pretreated LMs

Lignocellulosic material	Pretreatment conditions	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Mannan (%)	Lignin (%)
Rice straw	Untreated	28.6 ± 0.2	14.3 ± 0.9	0.4 ± 0.1	4.8 ± 0.2	N.D.	17.3 ± 0.3
	1 h, 150°C, 50% ethanol	29.7 ± 0.8	12.8 ± 0.1	0.3 ± 0.0	4.4 ± 0.3	N.D.	14.8 ± 0.4
	1 h, 180°C, 50% ethanol	32 ± 0.1	11.3 ± 0.2	0.3 ± 0.0	4.4 ± 0.8	N.D.	14.1 ± 0.1
Hazelnut skin	Untreated	11.4 ± 0.4	2.9 ± 0.2	0.9 ± 0.2	2.1 ± 0.1	N.D.	34.4 ± 1.6
	1 h, 150°C, 50% ethanol	11.5 ± 0.1	2.6 ± 0.2	0.6 ± 0.2	1.7 ± 0.1	N.D.	32.6 ± 0.6
	1 h, 180°C, 50% ethanol	12.5 ± 0.1	2.5 ± 0.2	0.6 ± 0.2	1.6 ± 0.0	N.D.	32.5 ± 0.2
Cocoa bean shell	Untreated	13.5 ± 0.1	0.9 ± 0.2	3.1 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	29.9 ± 0.3
	1 h, 150°C, 50% ethanol	13.9 ± 0.7	0.6 ± 0.1	3.0 ± 0.2	0.8 ± 0.1	1.7 ± 0.1	27.6 ± 0.2
	1 h, 180°C, 50% ethanol	15.0 ± 0.3	0.5 ± 0.1	2.7 ± 0.1	0.5 ± 0.1	2.1 ± 0.2	26.3 ± 0.1

N.D. = Not detected.

4.3.2 Effect of the organosolv pretreatment on the LM water retention value

The values of the water retention capacity obtained for the three LMs are reported in Table 4.3. The WRV of the untreated rice straw, hazelnut skin and cocoa bean shell was 1.52, 1.51 and 3.22, respectively. An increase of the WRV was observed in all the pretreated materials. In particular, for rice straw and cocoa bean shell the enhancement was significant. The WRV of cocoa bean shell increased by 63 – 70% at the pretreatment temperature of 150 and 180°C, respectively. For rice straw, the WRV was enhanced by 11 – 16%. A lower increase, in the range of 3 – 4%, was recorded for hazelnut skin. The increase of the WRV was probably related to the effectiveness of the organosolv pretreatment in removing lignin and hemicellulose, thus causing an increase of the accessible surface area and the pore volume [11].

4.3.3 Effect of the organosolv pretreatment on the biogas production from LMs

The cumulative methane production curves obtained from the AD of the three untreated and pretreated LMs are shown in Fig. 4.1, while the final production yields are reported in Table 4.3, as the average of triplicates. The organosolv pretreatment was particularly effective for rice straw, with a significant enhancement of the biomethane potential (Fig. 4.1a). A 29% increase of the cumulative biogas production was obtained upon pretreating rice straw with the organosolv method at 150°C for 1 h, i.e. from 235 (± 12) to 303 (± 19) mL CH_4/g VS. An increase of the pretreatment temperature to 180°C had a further beneficial effect on the methane production yield, which increased to 332 (± 6) mL CH_4/g VS, corresponding to a 41% increase compared to the untreated straw. The enhancement obtained was associated with the dual benefit of the pretreatment in achieving a delignification of the straw (Table 4.2), together with an increase of the accessibility of the material to the microorganisms assessed by the WRV (Table 4.3). Previous studies showed an inverse linear relationship between the lignin content of a substrate and its biomethane potential [35,36]. At the same time, an increase in the LM accessibility improves the hydrolysis stage [37,38].

A slight improvement in the biogas production from hazelnut skin was obtained after the organosolv pretreatment at 180°C (Fig. 4.1b). A biomethane production of 288 (± 6) mL CH_4/g VS was achieved, which represented a 10% enhancement compared to the untreated LM. On the other hand, the cumulative methane yield obtained by pretreating hazelnut skin at 150°C was similar to that obtained with the untreated substrate, i.e. 264 (± 3) and 261 (± 4) mL CH_4/g VS, respectively.

Table 4.3. Specific methane yield, specific rate constant and water retention value of the untreated and organosolv pretreated LMs

Lignocellulosic material	Pretreatment conditions	Specific methane yield (mL CH_4/g VS_{in})	Water retention value (%)	Specific rate constant k_0 (1/d)	Correlation coefficient[a] r^2	Specific rate constant R_m (mL CH_4/g VS·d)	Lag phase time λ (d)	Correlation coefficient[b] r^2
Rice straw	Untreated	235 ± 12	1.52 ± 0.03	0.217 ± 0.031	0.961	23.68 ± 0.97	1.8	0.990
	150°C organosolv	303 ± 19	1.68 ± 0.02	0.247 ± 0.035	0.958	26.99 ± 0.97	1.5	0.991
	180°C organosolv	332 ± 6	1.77 ± 0.12	0.249 ± 0.034	0.960	30.43 ± 0.78	1.3	0.995
Hazelnut skin	Untreated	261 ± 3	1.51 ± 0.04	0.099 ± 0.013	0.970	21.93 ± 0.25	1.6	0.999
	150°C organosolv	264 ± 4	1.57 ± 0.05	0.135 ± 0.019	0.954	31.67 ± 0.71	1.5	0.997
	180°C organosolv	288 ± 6	1.56 ± 0.04	0.136 ± 0.017	0.963	35.14 ± 0.49	1.4	0.999
Cocoa bean shell	Untreated	231 ± 6	3.22 ± 0.04	0.190 ± 0.011	0.993	31.30 ± 0.71	0.6	0.995
	150°C organosolv	181 ± 2	5.26 ± 0.72	0.184 ± 0.019	0.977	26.23 ± 0.79	1.0	0.992
	180°C organosolv	219 ± 4	5.47 ± 0.13	0.185 ± 0.017	0.983	29.97 ± 0.88	0.8	0.992

a: first order model coefficient of determination.

b: modified Gompertz model coefficient of determination.

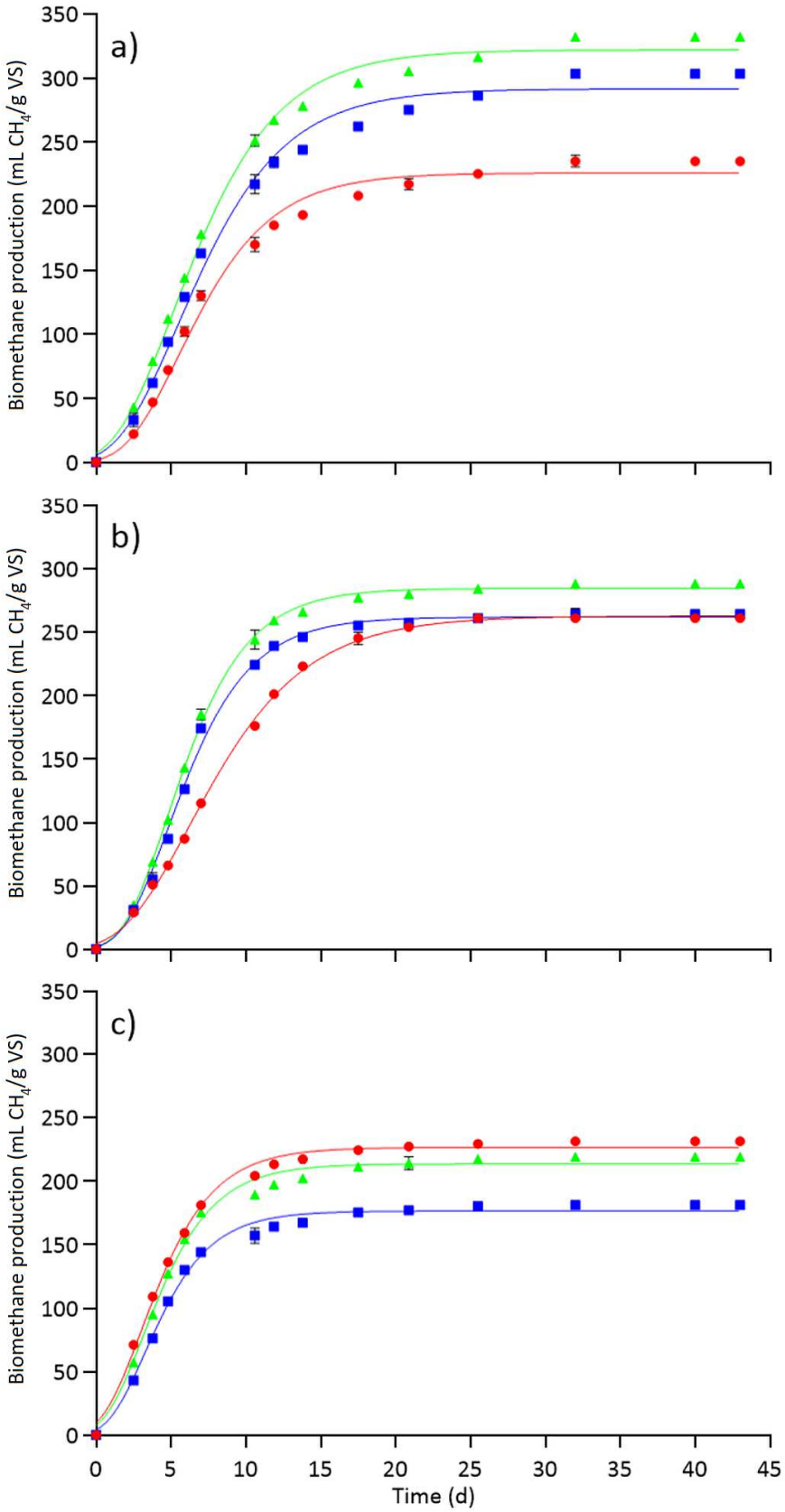

Fig.4.1. Cumulative methane production from the AD of rice straw (a), hazelnut skin (b) and cocoa shell (c) (●: untreated; ■: organosolv at 150°C; ▲: organosolv at 180°C) and modified Gompertz model fit with experimental data (—: untreated; —: organosolv at 150°C; —: organosolv at 180°C).

A negative impact of the organosolv pretreatment on the biogas yields from the cocoa bean shell was noticed during the BMP test (Fig. 4.1c). At both pretreatment temperatures, the final biogas production yield was lower than that achieved using the untreated LM, i.e. 231 (± 16) mL CH_4/g VS. This adverse result might be attributed to a potential loss of biodegradable matter that occurred during the pretreatment, namely proteins and fats, which constitute a significant part of cocoa bean shell. Moreover, despite the cocoa bean shell was repeatedly washed after the pretreatment, it is likely that the ethanol was not completely removed from the pretreated substrate, due to its higher WRV (Table 4.3). This could have resulted in a partial inhibition of the microorganisms, in particular the hydrolytic bacteria, which are known to be susceptible to inhibition by organic solvents [39]. This could represent a potential disadvantage in employing organosolv for the pretreatment of certain LMs such as cocoa bean shell, since the consumption of high amounts of water for washing the feedstock could make the whole process economically inconvenient.

In order to verify whether the biomethane production yields achieved after pretreating the LMs with organosolv were significantly different from those obtained with the untreated LMs, a statistical analysis was conducted using a paired t-test [30]. The increase of the biogas production obtained after pretreating the rice straw with organosolv was statistically significant at both pretreatment temperatures (i.e. p-value was 0.023 and 0.026 at 150 and 180°C, respectively). On the other hand, the cumulative values obtained for the pretreated hazelnut skin were not significantly different from the untreated material (p-value > 0.05). The inhibition observed in the biomethane production yield using the cocoa bean shell pretreated at 150°C was statistically significant, with a p-value of 0.006.

4.3.4 Methane production kinetics

First order kinetic models have been used to determine the methane production rates during AD for decades, resulting particularly appropriate for complex substrates such as LMs, where hydrolysis is considered the limiting step [40]. Recently, the Gompertz equation has been employed successfully to model the biomethane production, with the assumption that the biomethane production rate is proportional to the microbial activity in the AD reactor [41]. In this study, the specific rate constants k_0 and the maximum biomethane production rates R_m were obtained by fitting the experimental results of the BMP tests with a first order and a modified Gompertz model, respectively. The values of k_0 and R_m, estimated with a 95% confidence interval, are reported in Table 4.3. The fitting of the cumulative biomethane production curves by the Gompertz model is shown in Fig. 4.1.

The degradation process of rice straw was improved by employing a pretreatment temperature of 150°C. An additional increase of the k_0 was obtained for the rice straw pretreated at 180°C. The specific rate constant was 0.217, 0.247 and 0.249 1/d, for the rice straw untreated and pretreated at 150 and at 180°C, respectively. R_m was also enhanced by the pretreatment, increasing from 23.68 mL CH_4/g VS·d for the raw straw to 26.99 and 30.43 mL CH_4/g VS·d with the 150 and 180°C pretreated straw, respectively. The significant acceleration of the AD process caused by the organosolv pretreatment was mainly due to the increase of the accessible surface area (Table 4.3) and the partial removal of lignin and hemicellulose (Table 4.2).

The highest enhancement of the kinetic rate was observed for hazelnut skin, with the k_0 increasing from 0.099 1/d for the untreated LM to 0.135 and 0.136 1/d for the pretreated LM at 180 and 150°C, respectively (Table 4.3). After 11 d, 91 and 90% of the final biomethane production yields were achieved at these temperatures, whereas the methane production obtained from the untreated hazelnut skin was 77% on the same day. The removal of the lignin and hemicellulose fraction from the pretreated hazelnut skin, which was similar at both pretreatment temperatures (Table 4.2), led to a faster biomethane production (Fig. 4.1b), entailing a shorter AD time. In continuous systems, this result can be of major importance as it can lead to an optimization of the overall process and a decrease of the reactor volume sizes. Similarly to k_0, the pretreated hazelnut skin showed higher *Rm* values (i.e. 31.67 and 35.14 mL CH_4/g VS·d at 150 and 180°C, respectively) compared to the maximum biomethane production rate of the untreated LM, which was 21.93 mL CH_4/g VS·d.

Contrarily to hazelnut skin and rice straw, the k_0 decreased from 0.190 1/d for the untreated cocoa bean shell to 0.184 – 0.185 1/d for the pretreated LM. Analogously, the *Rm* of the untreated cocoa bean shell (i.e. 31.30 mL CH_4/g VS·d) was higher compared to those of the pretreated LM (Table 4.3). This could indicate that an inhibition of the AD process occurred as a result of the organosolv pretreatment, as shown in Fig. 4.1c.

4.3.5 Volatile fatty acids production

VFAs are intermediates of the AD process, which are produced from the fermentation of carbohydrates, hydrolysis of long-chain fatty acids and deamination of amino acids. VFAs are commonly used as an indicator of the system performance. A deeper understanding of the AD process can be obtained by monitoring the VFA concentration and speciation [42]. In this study, the trend of VFA production was followed during the first 10 d of each BMP test in order to assess the impact of the organosolv pretreatment on their accumulation (Fig. 4.2).

The pretreated rice straw (Fig. 4.2b, 4.2c) showed a higher VFA production than the untreated substrate (Fig. 4.2a). When the organosolv pretreatment was performed at 180°C, the total VFA concentration ranged from about 800 to 1,000 mg HAc/L from day 4 to 7. Subsequently, the VFA concentration dropped to 60 mg HAc/L on day 10, when more than 70% of the cumulative biomethane yield was produced.

Karthikeyan and Visvanathan [43] reported that the biogas production might be inhibited when acetic acid accumulates above 2000 mg/L and the overall VFA concentration exceeds 8,000 mg/L. Both the acetic acid and the total VFA concentration analyzed in this study remained constantly below this level (Fig. 4.2), probably entailing that no inhibition of the methanogenic stage occurred for any of the employed LMs.

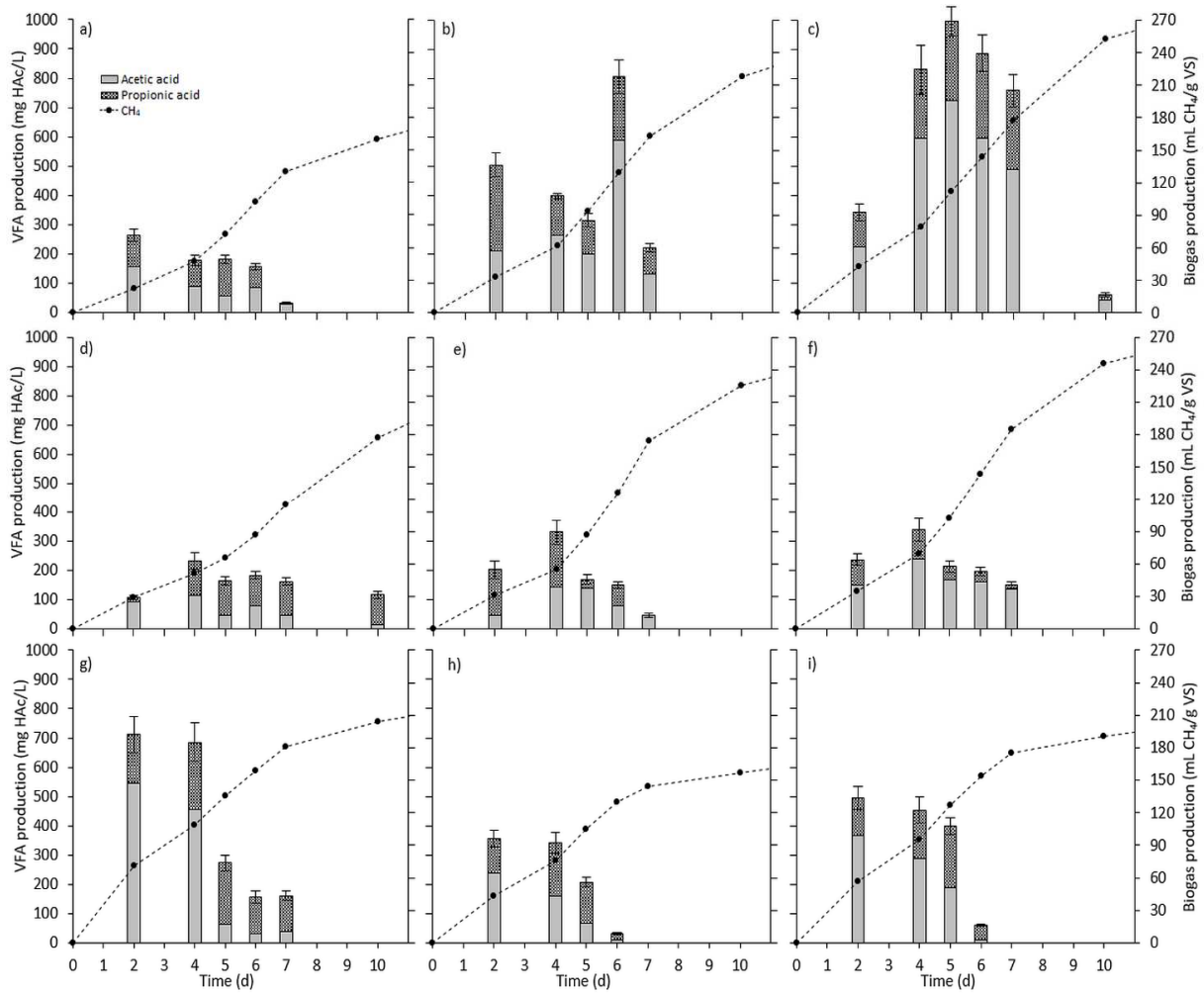

Fig.4.2. Evolution of VFA concentrations during the first 10 days of AD of rice straw (a: untreated; b: pretreated at 150°C; c: pretreated at 180°C), hazelnut skin (d: untreated; e: pretreated at 150°C; f: pretreated at 180°C) and cocoa bean shell (g: untreated; h: pretreated at 150°C; i: pretreated at 180°C).

A correlation between the VFA production and the hydrolysis rate can be observed for all the LMs (Fig. 4.2). In particular, the organosolv pretreatment slightly improved the hydrolysis of hazelnut skin at both pretreatment temperatures, thus the VFA production of the pretreated hazelnut skin was moderately higher during the first 4 d of AD compared to that of the raw LM (Fig. 4.2d – 4.2f). Notwithstanding, the total VFA production from the untreated hazelnut skin was 120 mg/L on day 10 (Fig. 4.2d), whereas on the same day it dropped to below the detection limit for the pretreated hazelnut skin, with consequent repercussions on the methane production obtained (Fig. 4.1). On the contrary, the total amount of VFA was lower in the first days of AD of the pretreated cocoa bean shell (Fig. 4.2g – 4.2i), especially at 150°C. This could indicate that the hydrolysis of cocoa bean shell was slightly hindered by the organosolv pretreatment.

4.4 Conclusion

The organosolv pretreatment was effective for rice straw, enhancing its biomethane production yield by 29 to 41%. These results were corroborated by the VFA and WRV analyses, which showed an increase of the VFA production and the accessible surface area by the pretreated rice straw. The compositional analyses showed that the organosolv pretreatment changed the chemical composition of the three LMs, causing a decrease of the lignin and hemicellulose and an increase of the cellulose content, which was more significant at the highest pretreatment temperature. A 10% increase of the biomethane production was obtained for the hazelnut skin pretreated at 180°C, whereas the pretreatment at 150°C did not enhance the cumulative biomethane yield, which remained at 261 mL CH_4/g VS. The biomethane potential of the raw cocoa bean shell, i.e. 231 mL CH_4/g VS, was negatively affected by the organosolv pretreatment, most likely due to an inhibition of the hydrolysis step.

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CHAPTER 5

Comparison of different chemical pretreatments to increase the anaerobic digestion of wheat straw

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5.1 Introduction

In response to the increase of the global demand for renewable energy, the anaerobic digestion (AD) technology has attracted wide attention in the last few years. The biogas generated from the AD of lignocellulosic materials, such as agricultural waste, has the potential to address the energy needs while providing multiple environmental benefits [1]. Wheat is among the three most cultivated crops worldwide, with a global production of 729 Tg in 2014 [2]. Considering a straw to grain ratio of 1.5 [3], more than one billion tons of wheat residues are produced annually. The large availability, together with the relative low-cost, makes wheat straw a sustainable feedstock for the production of biofuels [4]. However, similarly to any other lignocellulosic substrate, the conversion of wheat straw to biogas is hampered by the complex structure of this material. Specifically, the accessible surface area, the crystallinity of the cellulose and the lignin content limit the digestibility of the lignocellulosic matter [5]. Therefore, a pretreatment prior to AD is required in order to overcome the limitation posed by the hydrolysis rate [6].

An extensive number of techniques have been investigated to pretreat lignocellulosic materials, based on physical, chemical, and biological approaches, with the main goal of increasing the biogas yields [7]. Compared to physical and biological methods, chemical pretreatments have received larger attention because they are usually less expensive and result in faster rates and better efficiencies in enhancing the degradation of complex organic materials [8,9]. Recently, novel pretreatment methods employing organic solvents, such as N-methylmorpholine N-oxide (NMMO) and the organosolv technique, have been tested, proving to be successful in increasing the biogas yields from lignocellulosic materials [10]. On the other hand, alkaline pretreatments have been studied for many years for their ability to increase the accessibility of the carbohydrate portion to the microorganisms, thus enhancing the methane yields [11,12].

Several cellulose solvents have been investigated in the last years, since they offer advantages such as a decrease of the cellulose crystallinity, a minimum loss of fermentable sugars, short pretreatment times and relatively low energy requirements [13]. However, the effectiveness of different pretreatments on a specific substrate has rarely been compared within the same study in terms of enhancement of the biomethane production yields and variation of the lignocellulosic composition of the original feedstock. Due to the high variability in the chemical composition of lignocellulosic materials [4], comparing results from different studies is particularly challenging.

Among the different cellulose solvents, NMMO is particularly attractive since it is already employed in industrial processes for the production of cellulose fibers. Beyond being non-toxic, it is fully biodegradable and recyclable up to 98% [14]. The organosolv method is based on the pretreatment of the lignocellulosic materials with organic solvents, such as low boiling point alcohols, in order to achieve the chemical breakdown of the lignin fraction by cleavage of ether linkages and its subsequent dissolution [15]. The main advantages of this method are the easy recycling of the pretreatment solvent by distillation and the recovery by precipitation of a highly pure lignin fraction, which is an economically valuable byproduct with various applications in the chemical industry or as a fuel [16]. The alkaline pretreatment uses bases, with NaOH being the most popular, to render the lignocellulosic matrix easily degradable for the microbes, through the removal of portions of lignin and hemicellulose [7]. The main mechanism of this method is the saponification and cleavage of the linkages between lignin and carbohydrates [17]. Thus, the alkaline pretreatment leads to an increase of the accessible surface area and porosity, structural swelling, a decrease of the cellulose crystallinity and the disruption of the lignin structure [7].

This work aimed to compare the effects of NMMO, organosolv and NaOH pretreatment on the lignocellulosic composition of wheat straw and the biomethane yields from its subsequent AD. While alkaline pretreatments have been successfully applied to pretreat wheat straw, NMMO and organosolv have up to now not yet been tested for the enhancement of AD of this agricultural residue. Biochemical methane potential (BMP) tests and compositional analyses were performed on the untreated and pretreated wheat straw to evaluate the effectiveness of the three different pretreatment methods. The AD process was further assessed by evaluating the kinetics of biomethane production and analyzing the trends of volatile fatty acids (VFA) production along the AD process.

5.2 Materials and methods

5.2.1 Substrate and inoculum

Wheat (*Triticum aestivum*) straw was harvested from agricultural fields in the province of Salerno (Italy). Before use, the straw was cut into pieces with a size smaller than 4 mm. The inoculum was a digestate from a full-scale AD plant located in the same province, treating buffalo manure and residues from a dairy factory. The main physicochemical characteristics of both the wheat straw and the inoculum are reported in Table 5.1, while Table 5.2 gives the carbohydrates and lignin content of the raw wheat straw.

Table 5.1. Characteristics of the raw wheat straw and the inoculum

	Wheat straw	Inoculum
TS (%)a	93.1 ± 0.1	5.1 ± 0.1
VS (%)a	76.8 ± 1.1	3.4 ± 0.0
TKN (g N/kg TS)	11.2 ± 0.2	51.0 ± 0.4
COD (g/kg TS)	1169.6 ± 71.4	1312.3 ± 47.6

a: Based on wet weight

5.2.2 Pretreatments of wheat straw

Three different chemical pretreatment methods were conducted in this study, namely NMMO, organosolv and alkaline pretreatment. The chosen pretreatment conditions were based on previous studies performed on lignocellulosic materials [11,18,19]. Fig. 5.1 shows the experimental flow adopted.

The NMMO pretreatment was performed using an NMMO aqueous solution (Sigma-Aldrich, Germany), which was concentrated to 85% (*w/w*) from the commercial 50% (*w/w*) solution by using a rotary evaporator (Büchi Rotavapor R-114, Switzerland). 92.5 g of the obtained NMMO solution were added to 7.5 g of wheat straw in a 500 mL Erlenmeyer flask, which was placed in an oil bath and heated at 120°C for 3 h, while stirring every 10 min. Propyl gallate (0.625 mg per g NMMO) was added to prevent oxidation of the solvent. Immediately after the pretreatment, boiling deionized water was added as antisolvent in order to stop the reaction. The pretreated straw was then placed on textile bags and repeatedly washed with boiling deionized water until a clear filtrate was achieved.

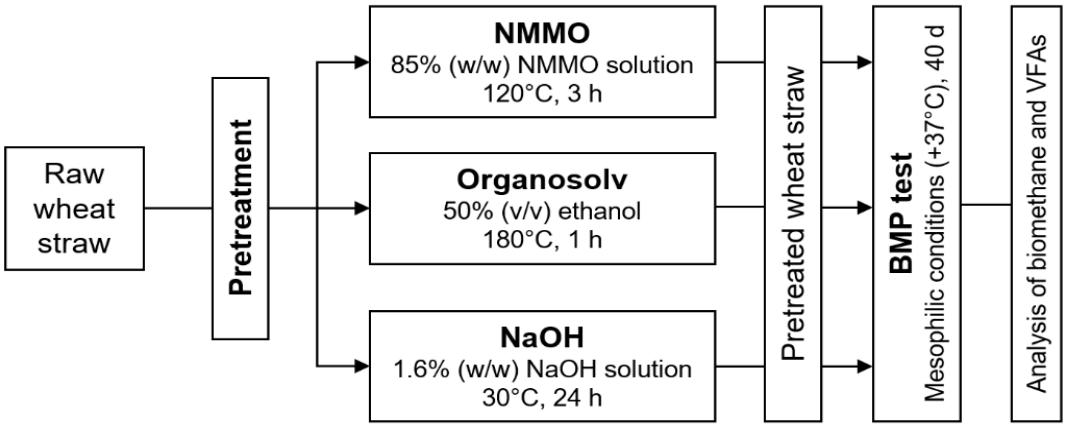

Fig. 5.1. Schematic of the experimental flow used in this study.

The organosolv pretreatment was performed using ethanol as organic solvent. 150 mL of 50% (v/v) ethanol were added to 15 g of wheat straw in a high-pressure stainless-steel vessel (Sigma-Aldrich, Germany) with a working volume of 300 mL. The vessel was then sealed and

placed in a TCF 50 PRO convection oven (ArgoLab, Italy), where it was heated at a rate of 3°C/min up to 180°C, which was maintained for 60 min. Afterwards, the vessel was cooled in an ice bath. The pretreated straw was then removed and washed with 100 mL ethanol and subsequently with deionized water until obtaining pH 7.0 in the liquor.

The alkaline pretreatment was performed using sodium hydroxide. In a 500 mL bottle, 16 g of wheat straw were soaked in 100 mL of 1.6% (*w/w*) NaOH solution. The bottle was then incubated at 30°C for 24 h. After the pretreatment, the straw was filtered and air-dried until further use.

5.2.3 BMP tests

BMP tests were performed in triplicate under mesophilic (37 ± 2°C) conditions. The biomethane production was measured by the liquid displacement method, as described by Esposito et al. [20] and modified as in Mancini et al. [19]. Serum bottles (Wheaton, USA) of 125 mL were loaded with 45 g of inoculum and 1 g of untreated or pretreated wheat straw to obtain an inoculum to substrate ratio of 1.5 g VS/g VS. To reach 70 mL of working volume, 20 mL of tap water were added into each bottle. One triplicate of blank samples containing only inoculum and tap water was also prepared in order to determine the biomethane production of the inoculum, which was then subtracted from the production of the straw samples. For VFA analysis, 0.5 mL of the liquid phase was sampled daily from each batch bottle during the first 10 d of BMP test, except for day 1 and 6.

5.2.4 Kinetic study of biomethane production

To better understand the influence of the employed chemical pretreatments on the efficiency of anaerobic degradation, two different models were applied to fit the biomethane curves obtained during the BMP tests: a first order model and a model based on the modified Gompertz equation [21], using Eq. (5.1) and Eq. (5.2), respectively:

$$G = G_m\left[1 - \exp^{(-k_0 t)}\right] \quad (5.1)$$

$$G = G_m \exp\left\{-\exp\left[\frac{R_m\, e}{G_m}(\lambda - t) + 1\right]\right\} \quad (5.2)$$

where G (mL) is the cumulative volume of biomethane at the digestion time t (d); G_m (mL) is the maximum cumulative volume at an infinite digestion time, which was considered equal to the volume of accumulated biomethane at the end of the BMP test; k_0 (d^{-1}) is the specific rate constant; e is the Euler's number, i.e. 2.7183; λ is the lag phase (d); and R_m (mL CH_4/d) is the maximum biomethane production rate.

5.2.5 Water retention value

The water retention value (WRV) is the ability of a substrate to retain water within its pores and is often used as an indication of the porosity of lignocellulosic materials [22]. The WRV is defined as the amount of retained water per unit weight of dry material, and is measured by subjecting a water-saturated material to a centrifugal force [23].

One gram of each sample of wheat straw was put in bottles containing deionized water and agitated at 150 rpm for 60 min. Each mixture was filtered using a 0.45 µm filter (Merck Millipore, USA), and the obtained filter cake was moved into a nonwoven fabric material, which was immersed in deionized water for 2 h. Thereafter, the fabric was wrapped and placed into a centrifuge tube, which was centrifuged at 3000 g for 15 min. The collected straw samples were weighed before and after drying at 105°C for 24 h, and the WRV was consequently determined as:

$$\text{WRV (g/g dry material)} = \frac{W_{wet} - W_{dry}}{W_{dry}} \quad (5.3)$$

where W_{wet} and W_{dry} are the wet and oven dry mass of the wheat straw samples, respectively.

5.2.6 Analytical methods

Total solids (TS) and volatile solids (VS) were determined according to the method described by Sluiter et al. [24]. Total Kjeldahl nitrogen (TKN) was measured according to the Kjeldahl method [25]. The chemical oxygen demand (COD) was determined according to the procedure described by Noguerol-Arias et al. [26].

The untreated and pretreated wheat straw was analyzed for its structural carbohydrates and lignin content according to the procedure described by Sluiter et al. [27]. A high-performance liquid chromatography (HPLC) system (Prominence LC-20A Series, Shimadzu, Japan) equipped with a UV/vis (SPD-20A, Shimadzu Japan) and a refractive index detector (RID-20A, Shimadzu, Japan) was used to determine sugars and VFAs. Sugars were determined using a Rezex RPM-Monosaccharide Pb^{2+} (8%) column (Phenomenex, USA), heated at 90°C, using HPLC-grade water as the mobile phase at a flow rate of 0.6 mL/min. VFAs were determined using a Rezex ROA-Organic Acid H^+ column (Phenomenex, USA), heated at 40°C, using 0.0065 mM sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min. The acid-soluble lignin content of the wheat straw was determined by UV spectroscopy (Lambda 365 UV/Vis, Perkin Elmer, USA).

5.2.7 Statistical analysis

Statistically significant differences between the biomethane production from the BMP tests with untreated and pretreated wheat straw were determined by a paired t-test. The Microsoft Excel 2016 (Microsoft Corporation, USA) statistical package was used, applying a 95% confidence interval.

5.3 Results and discussion

5.3.1 Effect of the pretreatments on the composition of wheat straw

The results of the chemical characterization of the untreated and pretreated wheat straw are reported in Table 5.2. Glucan, which is the main component of cellulose, constituted 31.0% (*w/w*) of the untreated wheat straw. The other polysaccharides, with xylose being the most abundant, accounted for 18.4%. The lignin content, given by the sum of acid insoluble and acid soluble lignin, was 18.3%. The overall composition was similar to that reported by Dumas et al. [28] and Liew et al. [29], who respectively reported values of cellulose of 32.0 and 32.3%, hemicellulose of 20.5 and 17.9%, and lignin of 17.4% both, of the raw wheat straw they analyzed. Although the composition of the original feedstock can be influenced by many factors, such as the variety of wheat, the growth conditions, the maturity and the harvesting methods [30], it usually has average polysaccharide and lignin values among the different lignocellulosic substrates that are considered for AD [28].

Table 5.2. Carbohydrates and lignin composition of the untreated and pretreated wheat straw

Pretreatment conditions	Glucan (%)[a]	Xylan (%)[a]	Galactan (%)[a]	Arabinan (%)[a]	Lignin (%)[a]
Untreated	31.0 ± 1.0	15.5 ± 0.3	0.3 ± 0.0	2.6 ± 0.7	18.3 ± 0.1
NMMO	31.7 ± 0.2	15.1 ± 0.1	0.3 ± 0.1	2.3 ± 0.1	17.9 ± 0.2
Organosolv	36.3 ± 0.9	6.1 ± 0.2	0.2 ± 0.0	3.1 ± 0.9	15.8 ± 0.5
Alkaline	36.0 ± 0.1	9.7 ± 0.1	0.2 ± 0.1	2.0 ± 0.3	11.6 ± 0.2

[a] Based on dry weight

Each pretreatment had a different impact on the composition of the wheat straw. The NMMO pretreatment did not significantly affect the original composition (Table 5.2). In accordance with previous studies investigating the effects of NMMO on barley [31] and rice straw [32], only a negligible change in the overall amount of carbohydrates (i.e. less than 0.5%) present in the straw was noticed after the pretreatment (Table 5.2). The main target of NMMO within the lignocellulosic matrix is the hydrogen bond network of cellulose. Once disrupted, the solvent can dissolve cellulose, which is then regenerated with a more amorphous structure

by adding boiling water as an antisolvent after the pretreatment [10]. Moreover, NMMO did not result in a significant removal of lignin, which only decreased by 2% (Table 5.2). Since lignin is the most recalcitrant component towards microbial degradation, its presence is not beneficial for the subsequent AD process [29].

Differently from NMMO, both the organosolv and alkaline pretreatment had a major impact on the composition of wheat straw (Table 5.2). A partial removal of lignin was obtained with both pretreatments. After organosolv the amount of lignin decreased by 14%, whereas the alkaline pretreatment reduced the lignin fraction by 36%. Moreover, the hemicellulose portion was significantly affected by the organosolv pretreatment, decreasing by 51%. Likewise, the alkaline pretreatment had a considerable impact on the hemicellulose of wheat straw, which was decreased by 35% after the pretreatment. Furthermore, the compositional analysis showed that both organosolv and alkaline pretreatment resulted in an analogous enrichment of the cellulose content of the original wheat straw, i.e. 17 and 16%, respectively.

The structural changes caused by the organosolv pretreatment can be attributed to the action of the solvent on the ether and ester linkages between hemicellulose and lignin. Due to the breaking of these internal bonds, both hemicellulose dissolution and lignin removal can be achieved [15]. Similarly, the ester bonds that form cross-links between xylan and lignin are targeted by the alkaline pretreatment [33]. By saponification and cleavage of these linkages, alkaline pretreatment leads to a breakdown of lignin

Table 5.3. Specific methane yield, water retention value and parameters of first order and modified Gompertz model

Pretreatment conditions	Specific methane yield (mL CH_4/g VS)	Water retention value (%)[a]	Specific rate constant k_0 (d^{-1})	Correlation coefficient[b] r^2	Specific rate constant R_m (mL CH_4/d)	Lag phase λ (d)	Cumulative methane production[c] (mL CH_4/g VS)	Correlation coefficient[d] r^2
Untreated	274 ± 3	1.30 ± 0.29	0.011	0.863	21.45	0.4	263	0.996
NMMO	304 ± 3	1.90 ± 0.01	0.020	0.959	32.34	0.8	297	0.996
Organosolv	316 ± 1	1.71 ± 0.20	0.018	0.955	32.85	0.8	309	0.997
Alkaline	315 ± 2	2.65 ± 0.02	0.028	0.972	46.72	0.7	314	0.998

[a] Based on dry weight
[b] Correlation coefficient of the first order model
[c] Predicted using the modified Gompertz model
[d] Correlation coefficient of the modified Gompertz model

and hemicellulose, with a consequent increase of the porosity and internal surface area of the lignocellulosic material [7].

5.3.2 Effect of the pretreatments on the water retention value of wheat straw

The WRV of the untreated and pretreated wheat straw is reported in Table 5.3. A WRV of 1.30 was observed for the untreated wheat straw. An enhancement of 46% in the water retention capacity was obtained after the NMMO pretreatment. Despite the chemical composition was not significantly changed by this pretreatment, the porosity of the straw increased, most likely due to a decrease of the cellulose crystallinity. Previous studies have shown that the amorphous region of cellulose increases after an NMMO pretreatment, resulting in an increased porosity of lignocellulosic materials [31,34].

The highest increase (i.e. 104%) of WRV was obtained after the alkaline pretreatment, likely due to the high delignification obtained after the NaOH pretreatment. Organosolv pretreatment enhanced the WRV of wheat straw by 32%. In spite of a partial delignification of the original material, a significant fraction of hemicellulose was also removed from the material. Since the swelling capacity of lignocellulosic materials can be positively influenced by the presence of xylan [35], its large removal could explain the lower increase of the WRV obtained after organosolv compared to the two other pretreatments applied.

5.3.3 Effect of the pretreatments on the biogas production of wheat straw

The three chemical pretreatments were all effective in enhancing the biogas production yields of wheat straw. The final cumulative methane production obtained from the BMP tests are reported in Table 5.3, while Fig. 5.2 shows the cumulative methane production curves. The untreated wheat straw had a cumulative biomethane production yield of 274 (± 3) mL CH_4/g VS. The NMMO pretreatment was effective in enhancing the biomethane production by 11% (Fig. 5.2), achieving a cumulative production of 304 (± 3) mL CH_4/g VS (Table 5.3). This statistically significant enhancement (p-value: 0.003) of the biomethane production was most likely caused by the increase of the accessible surface area, as indicated by the higher WRV than that of the untreated straw (Table 5.3).

Both organosolv and alkaline pretreatment caused an enhancement of the biomethane production by 15%, reaching a cumulative yield of 316 (± 1) and 315 (± 2) mL CH_4/g VS, respectively. Such improvements were most likely associated with the partial removal of lignin achieved after the pretreatment (Table 5.2). Due to its encasing effect, lignin hampers the accessibility of carbohydrates for enzymatic hydrolysis, leading to a low cellulose and hemicellulose degradation and thus a lower biogas production yield [36]. Moreover, an

increase of the accessible surface area for microbial degradation occurred, as confirmed by the obtained WRV (Table 5.3).

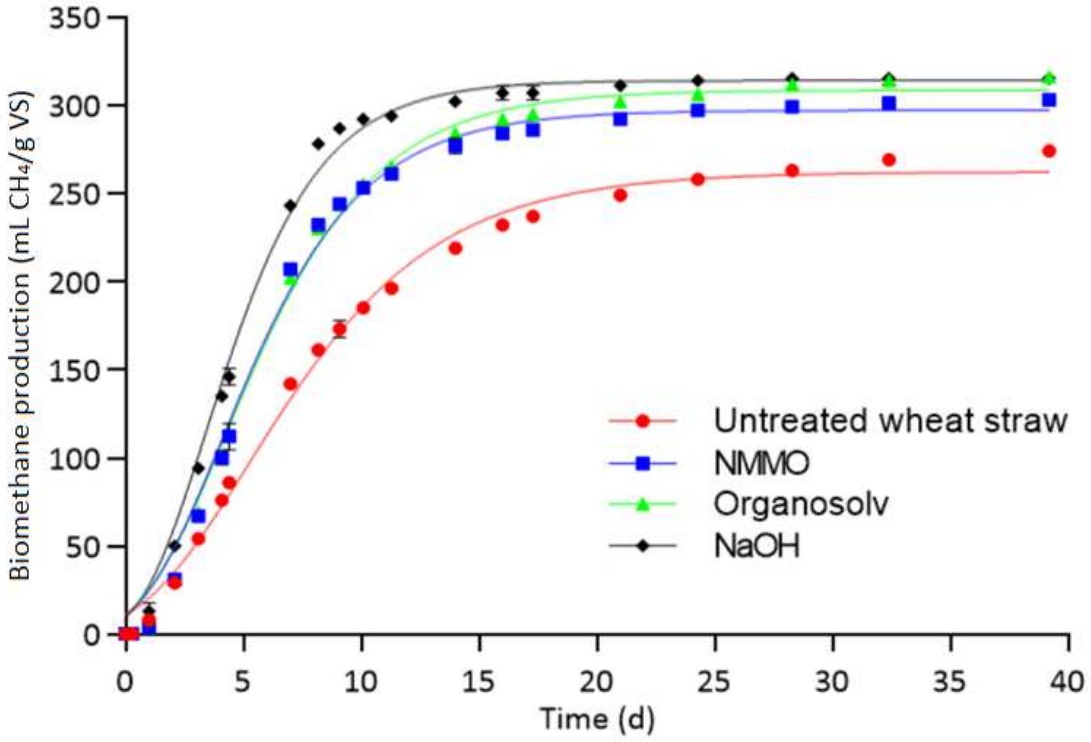

Fig. 5.2. Modified Gompertz model fit with experimental data of methane production from the AD of untreated and pretreated wheat straw.

A technical-economic analysis would be of great interest to evaluate other factors than the final biogas production yield, and assess which pretreatment has the best potential for full-scale applications. Each of the three investigated pretreatments has advantages and limitations, and their efficiency cannot be based solely on their impact on the AD process. The economic performance should be taken into account as well. For instance, the recovery and reuse of NMMO could represent a crucial factor for an economically feasible process [31]. On the other hand, organosolv has the main advantage of allowing the recovery of a valuable purified lignin fraction, which can be used for several industrial applications [37]. Both the NMMO and the ethanol used for the organosolv pretreatment can be recovered almost entirely, with a 99.5% [38] and 96% [18] recovery, respectively. In particular, the use of low-boiling point alcohols (e.g. ethanol or methanol in the organosolv pretreatment) is advantageous, since it allows to save energy in the evaporation unit for the solvent recovery. Finally, the alkaline pretreatment does not require the use of temperatures higher than 30°C, resulting in a considerable energy saving, despite the NaOH used for the pretreatment can generally not be recovered and reused [5].

5.3.4 Methane production kinetics

Despite BMP tests are mainly designed to evaluate the maximum methane yield of a substrate, the methane generation kinetics can be a useful indicator in the operation of continuous systems. The biomethane production curves were fitted with two different models. A classical first order model was used to obtain the specific rate constants (k_0), whereas the modified Gompertz model was used to evaluate the maximum biogas production rate (R_m). The values of k_0 and R_m are reported in Table 5.3.

During the first 10 d of AD, the kinetic constant of the untreated wheat straw was 0.011 d^{-1}. All the pretreatments had a significant impact on the methane production kinetics. The constant k_0 was enhanced by 82, 64 and 155% for NMMO, organosolv and alkaline pretreatment, respectively, whereas R_m increased by 51, 53 and 118%. As shown in Fig. 5.3, after 10 d, the AD of untreated wheat straw resulted in a production of 185 mL CH_4/g VS, which corresponded to 68% of the final cumulative production. At the same time, the methane production reached 84 and 81% of the final production yield for the straw pretreated with NMMO and organosolv, respectively. The alkaline pretreated wheat straw had the fastest methane production kinetics. After 10 d, 93% of the final methane was already produced (Fig. 5.3). These results were in a good correlation with the obtained WRV (Table 5.3) and lignin removal efficiency (Table 5.2). The higher accessible surface area and delignification were the main causes for the acceleration of the AD process when the pretreated substrate was used.

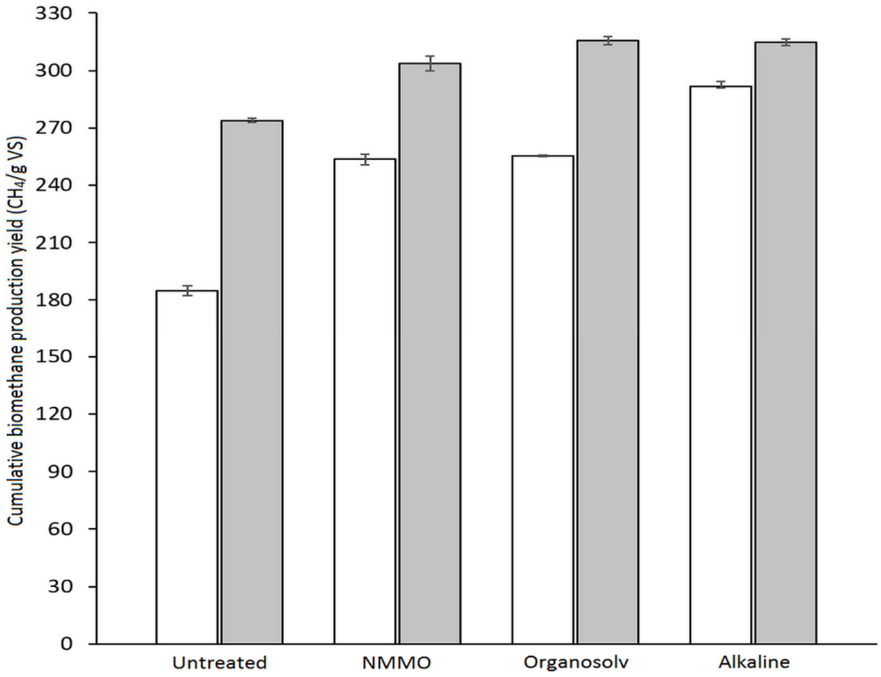

Fig. 5.3. Cumulative methane production yields of untreated and pretreated wheat straw after 10 (white columns) and 40 (grey columns) days of AD.

To validate the modified Gompertz model, the predicted cumulative curves of the biomethane production were plotted against the experimental values obtained in the BMP tests (Fig. 5.2). An adequate fit was observed between the modelled and experimental data, with all the coefficients of determination (r^2) higher than 0.99. The AD of the alkaline pretreated wheat straw displayed the highest maximum production rate, i.e. 46.72 mL CH_4/d, and was more than double compared to the untreated straw, which had an R_m of 21.45 mL CH_4/d (Table 5.3).

5.3.5 Volatile fatty acids production

The VFA distribution released in the BMP medium was followed during the first 10 d of AD in order to better assess the extent of the chemical pretreatments on the VFA production. The concentration of the VFAs, together with the biomethane production evolution, are reported in Fig 5.4.

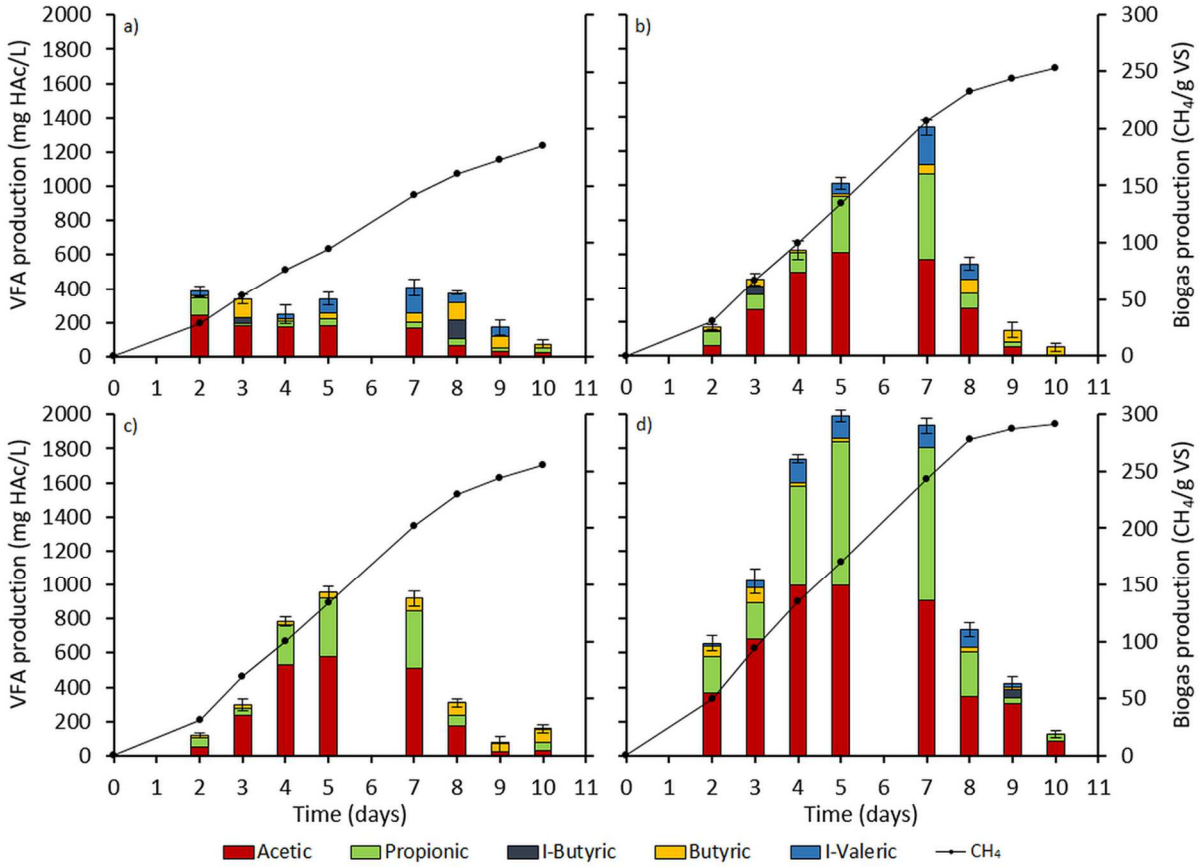

Fig. 5.4. VFA and cumulative methane production patterns during the first 10 days of AD (a: untreated; b: NMMO pretreatment; c: organosolv pretreatment; d: alkaline pretreatment).

Both the acetic acid and total VFA concentration remained constantly below the inhibition level for the methanogenic archaea, which is reported to be in the order of 2,000 and 8,000 mg/L, respectively [39]. Acetic and propionic acid were the main VFAs produced during the BMP test. Higher values of the total VFA production were observed for all the pretreated

samples (Fig. 5.4b – 5.4d) compared to the VFA production from the raw wheat straw (Fig. 5.4a), indicating that the hydrolysis of the straw was enhanced by the pretreatment. In particular, a peak of the total VFAs of approximately 2,000 mg/L was recorded for the alkali pretreated wheat straw (Fig. 5.4d). Besides leading to the highest methane production rate, the alkaline pretreatment also resulted in the highest VFA production.

The highest VFA concentrations for the NMMO (Fig. 5.4b) and organosolv (Fig. 5.4c) pretreated wheat straw were observed from day 4 to 7, when the total VFA production ranged from 620 to 1,360 mg/L and from 780 to 960 mg/L, respectively. On day 8, the VFA concentration dropped for all the pretreated samples, with values decreased by 60 to 66% compared to the previous day (Fig. 5.4b – 5.4d). This high consumption of VFAs demonstrates that methanogenesis was not the limiting step during the AD of wheat straw.

5.4 Conclusion

NMMO, organosolv and alkaline pretreatment are effective techniques to improve the AD of wheat straw. The three pretreatments enhanced the cumulative biomethane potential from 11 to 15%, and the kinetics of biomethane production were increased as well. The alkaline pretreatment resulted in the highest enhancement of the specific rate constant k_0 and the maximum biogas production rate R_m, which were increased by 155 and 118%, respectively. WRV and VFA analysis validated the results obtained during the BMP tests, by showing an increment of both the accessible surface area and VFA production after the pretreatment. The compositional analysis showed that NMMO did not significantly alter the native feedstock composition. Organosolv mainly affected the hemicellulose fraction, whereas the alkaline pretreatment gave the highest delignification.

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CHAPTER 6

Effect of different trace elements addition on the anaerobic digestion of rice straw

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6.1 Introduction

In recent years, the search for alternative renewable energy sources to fossil fuels has caused a growing interest in anaerobic digestion (AD). This process combines the dual benefits of generating biogas and reducing greenhouse gases emissions and landfill waste [1]. Methane-rich biogas can be obtained from several organic substrates and used to produce electricity and heat, or as a transport fuel after an upgrade to biomethane [2]. Among the wide range of feedstocks, lignocellulosic materials are particularly attractive, due to their high carbohydrate content and large abundance worldwide [3]. In particular, the AD of agricultural residues, such as rice straw, can be a sustainable process for future energy generation, despite the limitation caused by its recalcitrant structure to biodegradation, which can be overcome by a pretreatment step [4]. Alkaline pretreatment, using NaOH, has been applied to pretreat different lignocellulosic materials [5,6]. This improves the biodegradability of the raw material due to the removal of lignin and increased porosity, which leads to enhanced hydrolysis and thus higher biogas production yields.

In addition to the complex nature of the material, the biogas production from lignocellulosic residues might be restricted by the lack of bioavailable essential trace elements (TEs) [7]. Lignocellulosic residues usually contain low concentrations of TEs and limitations of any required TE could disturb the overall AD process [8]. TEs such as iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), molybdenum (Mo), selenium (Se) and tungsten (W) are fundamental components of enzymes and cofactors involved in the biochemistry of methane formation, and their role in anaerobic processes has been investigated extensively [7,9–13]. Adequate dosing of TEs is required to maintain an effective AD process by sustaining the growth and metabolism of anaerobic microorganisms [14].

One of the major effects of TE addition on the AD process is the decrease of the level of volatile fatty acids (VFAs) within the anaerobic reactor, which is generally associated with a consequent increase of the biogas production [15]. Choong et al. [9] highlighted that substrates such as food waste have a greater response to TE supplementation than complex feedstocks, such as lignocellulosic materials. However, information about TE requirements of anaerobic digesters fed with lignocellulosic residues is scarce [10]. Leaving aside silages from energy crops, the effects of TE dosing on the AD of agricultural byproducts have so far been investigated only by a few studies in the literature (Table 6.1).

This study aimed to assess the effect of adding different TEs, i.e. Co, Ni, and Se, on the AD of rice straw. Two different inocula were employed to evaluate the effect of different TE

background levels. The effect of the TE addition was also studied in combination with an alkaline pretreatment with NaOH to evaluate potential synergistic effects on the biogas production yields. Biochemical methane potential (BMP) tests were conducted under mesophilic conditions to determine the cumulative biogas production from each configuration adopted. The production of volatile fatty acids was monitored along the AD process to further assess the impact of TE addition on the anaerobic biodegradation of rice straw.

Table 6.1. Trace elements dosing in anaerobic digesters loaded with lignocellulosic residues

Lignocellulosic residue	TEs added	Concentration	Inoculum used	Experimental configuration	Methane yield enhancement	Ref.
Corn stover	Fe, Co, Ni	1.0 (Fe) + 0.4 (Co) + 0.4 (Ni) mg/L	Activated sludge	Batch, 35°C	+62.0%	[43]
Maize straw	Fe	50.0, 200.0, 1000.0, 2000.0 mg Fe/L	Chicken manure	Batch, 37°C	+15.0% (with 1000.0 mg Fe/L)	[44]
Mango waste	Fe, Co, Ni	4000.0 (Fe), 125.0 (Co), 125.0 (Ni) mg/L	Cattle dung	Semi-continuous, 15 to 36°C	+ 120.0% (with 4000.0 mg Fe/L)	[45]
Napier grass	Co, Ni, Mo, Se	0.25 (Ni) + 0.19 (Co) + 0.30 (Mo) + 0.062 (Se) mg/L/d	Rumen fluid and grass leachate	Continuous, 35°C	+ 40.0%	[46]
Phragmites straw	Fe	0.5 to 10.0 mg Fe/L	Cow dung	Batch, 35°C	+ 18.1% (with 10.0 mg Fe/L)	[47]
Phragmites straw	Ni	0.2 to 2.0 mg Ni/L	Cow dung	Batch, 35°C	+ 18.0% (with 0.8 mg Ni/L)	[48]
Phragmites straw	Cu	30.0 to 500.0 mg Cu/L	Cow dung	Batch, 35°C	+43.6% (with 30.0 mg Cu/L)	[49]
Rice straw	Co, Ni, Se	9.0 to 45.0 (Co), 9.0 to 45.0 (Ni), 1.0 to 5.0 (Se) mg/kg TS straw	Buffalo manure, anaerobic granular sludge	Batch, 37°C	+11.6% (with 45.0 mg Ni/kg TS straw	This study

6.2 Materials and methods

6.2.1 Substrate and inocula

Rice (*Oryza sativa*) straw, obtained from agricultural fields in Pavia (Italy), was used as the sole substrate in this work. After collection, the straw was manually cut down to a particle size smaller than 4 mm. Part of the rice straw was pretreated with sodium hydroxide by soaking 16 g of rice straw in 100 mL of 1.6% (*w/w*) NaOH solution inside a 500 mL bottle. The bottle was incubated at 30°C for 24 h. Then, the rice straw was filtered and air dried, until further use.

Two types of inocula, with different background levels of TEs, were used in the BMP tests. The first inoculum was an anaerobic granular sludge, collected from a paper mill wastewater treatment plant located in Eerbeek (the Netherlands), with its characteristics described by Roest et al. [16]. The second inoculum was a digestate from a full-scale AD plant treating buffalo manure and milk whey from a mozzarella factory located in Capaccio (Italy), with its characteristics detailed in Ariunbaatar et al. [17]. Both inocula were degassed by incubating them at mesophilic conditions (37 ± 2°C) for 4 d before starting the experiments. The physicochemical characterization of the rice straw and inocula is reported in Table 6.2.

Table 6.2. Characteristics of the raw rice straw and the two inocula used

	Rice straw	Inoculum 1 (granular sludge)	Inoculum 2 (buffalo manure)
TS (%)	93.1 ± 0.1	16.1 ± 0.9	5.1 ± 0.1
VS (%)	76.8 ± 1.1	11.0 ± 0.6	3.4 ± 0.0
TKN (g N/kg TS)	11.2 ± 0.2	51.0 ± 0.4	27.1 ± 1.3
Fe (µg/g TS)	476.9 ± 81.3	25476.2 ± 833.1	623.0 ± 4.9
Cu (µg/g TS)	16.7 ± 4.6	318.9 ± 13.1	19.5 ± 0.2
Zn (µg/g TS)	61.9 ± 25.3	323.7 ± 0.3	69.7 ± 2.0
Co (µg/g TS)	< 1.0	10.0 ± 0.3	< 1.0
Ni (µg/g TS)	2.0 ± 0.0	28.1 ± 0.3	10.4 ± 0.1
Se (µg/g TS)	< 1.0	< 1.0	< 1.0
Cellulose (%)	28.6 ± 0.2	-	-
Hemicellulose (%)	19.5 ± 1.2	-	-
Lignin (%)	17.3 ± 0.3	-	-

TS: total solids; VS: volatile solids; TKN: total Kjeldahl nitrogen.

6.2.2 Trace elements dosing strategy

Table 6.2 presents the representative concentrations of Co, Ni and Se, together with some other TEs found in the rice straw, the granular sludge and the buffalo manure. The TE concentrations observed in the rice straw were compared with the recommended values from the literature [18]. The differences between the amount of Co, Ni and Se in the rice straw used

in this study and the recommended values were then used to calculate the amount of each TE to be added in the BMP tests, representing the 100% of the calculated requirement. In addition to this optimal concentration, two other amounts were tested, i.e. 200 and 500% of the calculated requirement (Table 6.3). The amount of TEs present in the two inocula was not taken into account in the calculations, in order to evaluate if different TE background levels could result in different effects on the biogas production yields.

The selected TEs were individually supplemented in different serum bottles, injecting different amounts of stock solutions prepared using the following salts: $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$ and Na_2SeO_3 (analytical grade, Sigma-Aldrich, Germany).

Table 6.3. Determination of trace elements addition in the BMP tests

Trace element	Recommended supplementation (Hinken et al. [18])* (µg/g TS)	TE in the used rice straw (µg/g TS)	TE addition used in this study (µg/g TS)		
			100% dosage	200% dosage	500% dosage
Co	9	0	9	18	45
Ni	11	2	9	18	45
Se	1	0	1	2	5

*These values are based on the recommendation by Hinken et al. [18], who reviewed the TE requirements of anaerobic microorganisms

6.2.3 BMP tests

BMP tests were performed in triplicate under mesophilic conditions (37 ± 2°C). The biomethane production was measured by the liquid displacement method, as described by Mancini et al. [19]. During the measurement, each bottle was connected via a capillary tube to a 1 L vessel containing an alkaline solution (3N NaOH), which entrapped the CO_2, allowing only CH_4 to be measured.

The inoculum to substrate ratio was maintained at 2.0 g VS/g VS. Therefore, the 250 mL glass bottles employed in the BMP tests were loaded with 2.5 g of rice straw and 36.0 g of granular sludge or 142.0 g of buffalo manure inoculum, respectively. Tap water was added to reach 150 mL of working volume into each bottle. Triplicates of blank samples containing only inoculum and tap water were also prepared in order to determine the biomethane production of the two inocula, which was then subtracted from the production of the rice straw. For VFA analysis, 1.0 mL of the liquid phase was sampled daily from each bottle during the first 10 d of the BMP tests.

6.2.4 Analytical methods

Total solids (TS) and volatile solids (VS) were determined according to the method described by Sluiter et al. [20]. Total Kjeldahl nitrogen (TKN) was measured according to the Kjeldahl method [21]. The rice straw was analyzed for its structural carbohydrates and lignin content according to the procedure described by Sluiter et al. [22].

TEs analysis was carried out by using inductively coupled mass spectroscopy (ICP-MS) (X-Series, Thermo Fisher Scientific, USA). The total TEs content was determined after drying the samples at 105°C and digesting 0.5 g TS with 10.0 mL HNO_3 in a microwave accelerated reaction system (MARS5, CEM Corp., USA). VFAs were determined by gas chromatography (GC) (Varian 430-GC, Varian Inc., USA) equipped with a CP WAX-58 CB column (25 m × 0.32 mm × 0.2 μm) and a flame ionization (FID) detector. Helium was used as the carrier gas.

6.2.5 Statistical analysis

Statistically significant differences between the biomethane production from the BMP tests with and without addition of TEs were determined by a paired t-test. The same method was used to assess differences between the VFA concentrations recorded in the BMP tests. The Microsoft Excel 2016 (Microsoft Corporation, USA) statistical package was used, applying a 95% confidence interval.

6.3 Results and discussion

6.3.1 Substrate and inocula characterization

The total Ni content in the rice straw was 2.0 μg/g TS, while Co was below the ICP-MS detection limit of 1.0 μg/g TS (Table 6.2). Se was not detectable in the rice straw, nor in the two inocula used. The concentrations of TEs found in the rice straw, granular sludge and buffalo manure were comparable to those determined by Mussoline et al. [23], Zandvoort et al. [24] and Ariunbaatar et al. [25], respectively.

The characteristics of the two inocula used were appreciably different. The granular sludge had a higher TS and VS concentration than the buffalo manure. At the same time, the background concentration of TEs was higher in the granular sludge than in the buffalo manure (Table 6.2). The factor of TE background level should be taken into account before supplementing TEs, in order to avoid overdosing that can inhibit the AD process. This aspect was elucidated by Facchin et al. [26], who showed that TE supplementation to food waste inoculated with a sludge having a high TE background level negatively impacted the AD process, decreasing the methane production yield.

Determining the total TEs concentration is considered the first step to evaluate possible deficiencies that could hinder the AD process [13]. However, the TE bioavailability, defined as the degree to which TEs are available for metabolic activities [27], is more representative than the total TE content, since it allows to consider only the TE fractions that can be directly taken up by microorganisms [7]. Despite a sequential extraction technique was not applied in this study to assess bioavailability, the soluble fraction, which is considered highly bioavailable, was analyzed in the two inocula used to determine the amount of dissolved TEs. The amount of Co in the supernatants of the granular sludge and buffalo manure inoculum was 0.5 (± 0.1) and 0.2 (± 0.1) µg/L, respectively. The Ni concentration was 2.5 (± 0.1) and 3.3 (± 0.2) µg/L, whereas Se was below the detection limit for both inocula supernatants.

6.3.2 Effect of TE addition and pretreatment on the biomethane production

Two consecutive runs of BMP tests were carried out at mesophilic temperature (37 ± 2°C). In the first run, the rice straw was inoculated with granular sludge and Co and Ni were individually added at 3 different concentrations, representing 100%, 200% and 500% of the recommended dosage reported in Table 6.3. Fig. 6.1 shows that an enhancement of the final biomethane production yields was obtained when supplementing Co and Ni, compared to the rice straw without TE addition.

The soluble concentrations of Co, Ni and Se in the two inocula were comparable, despite the amount of total TEs in the granular sludge was considerably higher than in the buffalo manure (Table 6.2). This resulted in a similar bioavailable fraction of Co, Ni and Se for the microorganisms and, consequently, comparable biogas production yields. The biomethane production yield was from 5.0 to 11.6% higher (Table 6.4) when Co and Ni were supplemented to the rice straw inoculated with granular sludge. Similarly, in the second run of BMP tests, Co and Ni were separately added to the rice straw inoculated with buffalo manure, using 100% of the recommended dosage (Fig. 6.2a). The cumulative methane production yield increased by 7.6 and 5.7%, when Co and Ni were respectively added (Table 6.4). However, the differences between the controls and the BMP tests with supplemented TEs were not statistically significant in all the investigated configurations ($p>0.05$).

The role of Co and Ni in the AD process has been studied extensively in the literature [28–30]. These two TEs are considered essential cofactors of several enzymes involved in both the acetoclastic and hydrogenotrophic methanogenesis pathways, such as acetyl-CoA decarbonylase, CO dehydrogenase, methyl-CoM reductase and methyl-H4SPT:HS-CoM methyltransferase [12]. Co is a fundamental constituent of a corrinoid, namely vitamin B_{12}, that

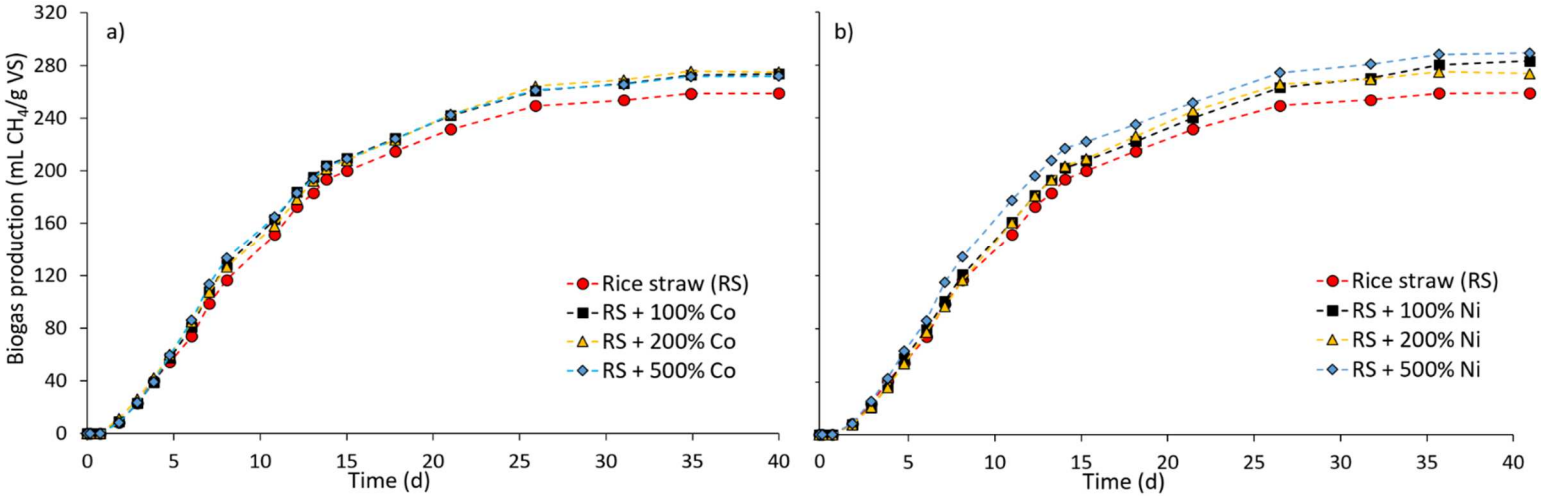

Fig. 6.1. Effect of different Co (a) and Ni (b) concentrations on the cumulative biomethane production from the anaerobic digestion of rice straw inoculated with anaerobic granular sludge.

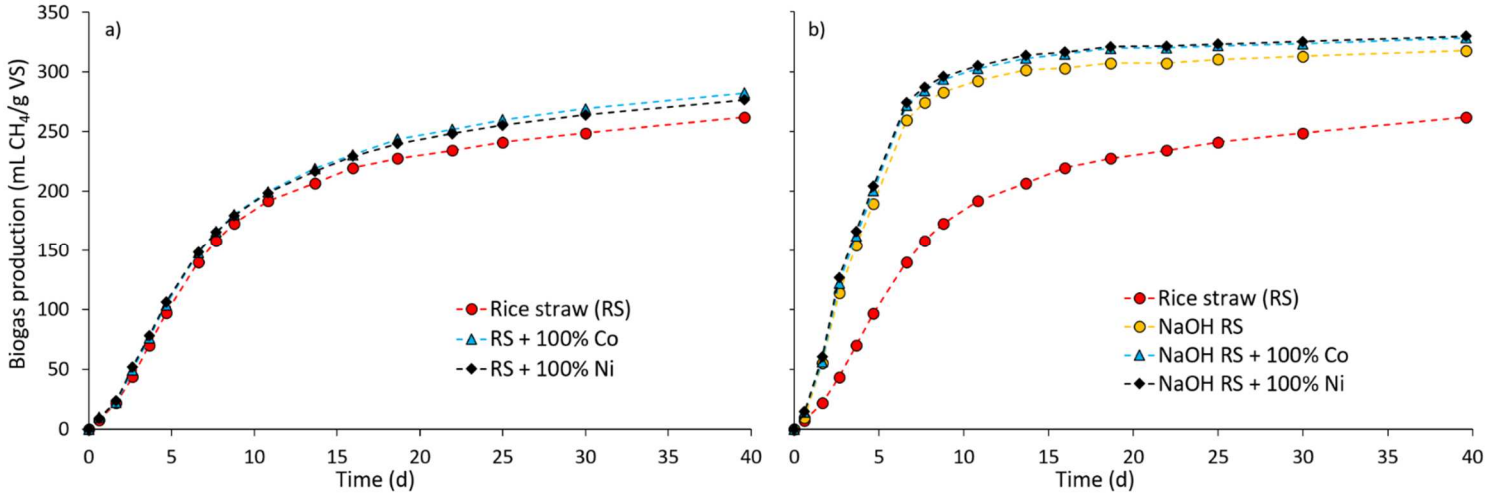

Fig. 6.2. Effect of recommended Co and Ni concentrations on the cumulative biomethane production from the anaerobic digestion of untreated (a) and NaOH pretreated (b) rice straw inoculated with buffalo manure.

binds to the coenzyme methylase, catalyzing the methane formation [31]. Likewise, Ni is required in substantial amounts within the coenzyme F_{430}, which is always present in methanogenic archaea, and involved in the generation of methane through all methanogenic pathways [32]. When Se was added to the rice straw inoculated with granular sludge (Fig. 6.3a), among the three tested concentrations, only the recommended dose of 1.0 µg/g TS (Table 6.3) enhanced the final production (i.e. 7.8% higher than the control). In contrast, doubling the recommended dose led to a negligible increase by 0.8%. A negative response (i.e. – 0.4%) was obtained with the highest dose of 5.0 µg of Se/g TS (Table 6.4). Thus, increasing the Se dosage had a slightly inhibitory effect on the AD of rice straw inoculated with granular sludge. This finding was in agreement with the study of Lenz et al.[33], who noticed that the range between Se stimulation and toxicity in methanogenic anaerobic granular sludge is narrow. While an amount of 6.3 µM Se/g COD_{fed} was stimulatory for the methanogens, a loading of 61 µM Se/g COD_{fed} partially inhibited the methanogenic activity, causing a 50% decrease of the biomethane production [33]. On the other hand, when buffalo manure was used as the inoculum (Fig. 6.3b), the addition of Se resulted in a considerable improvement of the cumulative production yield with all the selected dosages (Table 6.4).

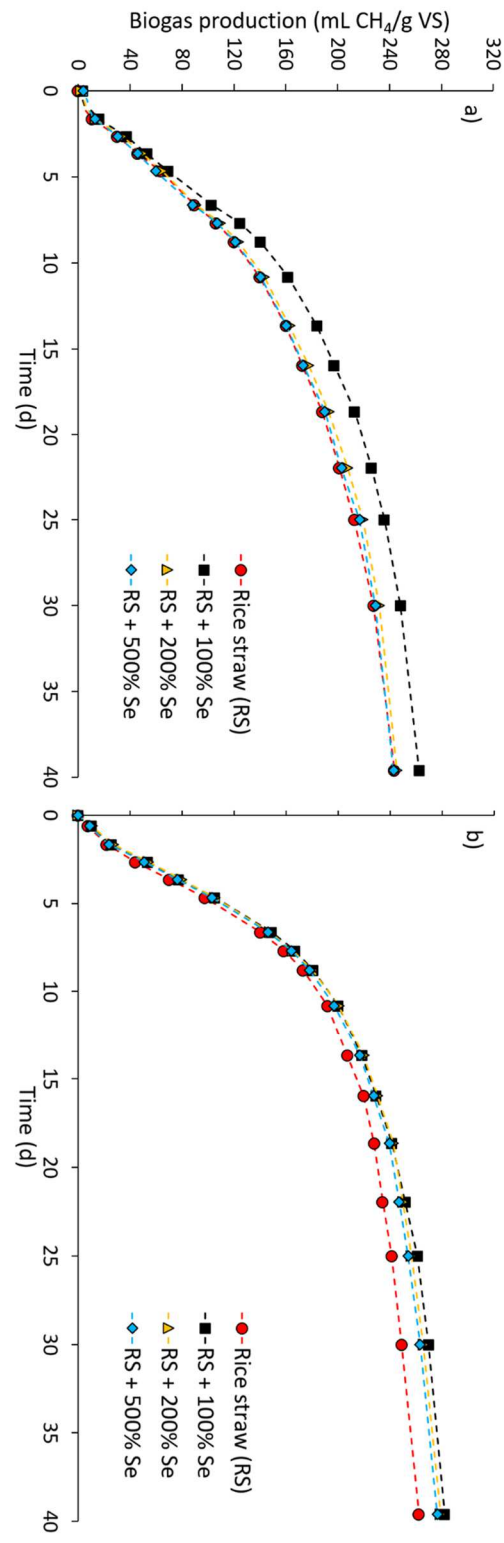

Fig. 6.3. Effect of different Se concentrations on the cumulative biomethane production from the anaerobic digestion of rice straw inoculated with anaerobic granular sludge (a) and buffalo manure (b).

The importance of Se for microbial growth has long been recognized, due to the fundamental catalytic role of selenoproteins in bacteria and archaea [34]. Nonetheless, limited information about the effect of Se addition on the AD process is available in the literature.

Studies showed that a lack of Se in anaerobic processes leads to a decrease of microbial activities [35,36]. During the AD of food waste, the addition of Se enhanced the biomethane production by more than 30% [25,26].

In this study, rice straw was pretreated using NaOH and inoculated with buffalo manure. The final biomethane production yield (i.e. 318 mL CH_4/g VS) obtained using the pretreated rice straw showed a significant (i.e. $p = 0.018$) enhancement, equal to 21.4%, compared to that achieved with the untreated substrate (Table 6.4, Fig. 6.2b). Furthermore, in other batch tests Co and Ni were separately supplemented to the pretreated rice straw (using the 100% dosage, Table 6.3), in order to investigate possible synergistic effects of combining the alkaline pretreatment with TE addition. The specific biomethane production yield of the pretreated rice straw was further increased by only 3.5 and 3.8% through Co and Ni dosing, respectively (Table 6.4, Fig. 6.2b). This extra enhancement due to TE addition was, however, not statistically significant compared to the result obtained with the pretreatment alone. The increase of the biogas production yield caused by the NaOH pretreatment remained the only statistically significant enhancement (i.e. $p = 0.018$) observed in this study.

Table 6.4. Specific methane production obtained from the BMP tests aimed at studying the effect of TE supplementation and pretreatment of rice straw

TE added	TE concentration (µg/g TS_{straw})	Inoculum	Specific methane production (mL/g VS)	Increase from control (%)
Control run 1	0	Granular sludge	259 ± 5	-
Co	9	Granular sludge	274 ± 22	+ 5.8
Co	18	Granular sludge	275 ± 16	+ 6.2
Co	45	Granular sludge	272 ± 7	+ 5.0
Ni	9	Granular sludge	283 ± 7	+ 9.2
Ni	18	Granular sludge	274 ± 20	+ 5.8
Ni	45	Granular sludge	289 ± 29	+ 11.6
Control run 2	0	Granular sludge	244 ± 11	-
Control run 2	0	Buffalo manure	262 ± 26	-
Co	9	Buffalo manure	282 ± 8	+ 7.6
Ni	9	Buffalo manure	277 ± 10	+ 5.7
Se	1	Granular sludge	263 ± 39	+ 7.8
Se	1	Buffalo manure	282 ± 31	+ 7.6
Se	2	Granular sludge	246 ± 26	+ 0.8
Se	2	Buffalo manure	279 ± 14	+ 6.5
Se	5	Granular sludge	243 ± 17	– 0.4
Se	5	Buffalo manure	276 ± 7	+ 5.3
Pretreated rice straw	0	Buffalo manure	318 ± 9	+ 21.4
Pretreatment + Co	9	Buffalo manure	329 ± 11	+ 25.6 (+ 3.5)
Pretreatment + Ni	9	Buffalo manure	330 ± 12	+ 26.0 (+ 3.8)

Alkaline pretreatment proved to be successful in increasing the biodegradability of lignocellulosic materials, thus enhancing the biogas yields [6]. It is known that, breaking the linkages between carbohydrates and lignin, the alkaline pretreatment provokes an increased porosity and the delignification of the raw lignocellulosic materials [37].

6.3.3 Effect of TE addition and pretreatment on the volatile fatty acids production

The effect of TE addition and alkaline pretreatment was further assessed by monitoring the VFA evolution during the first 10 d of AD. The concentrations of the total VFAs recorded during the 2 runs of BMP tests are reported in Fig. 6.4. The total VFA concentration, expressed as mg HAc/L, was given by the sum, in terms of acetic acid equivalents, of acetic, propionic, iso-butyric and butyric acids, with the first two being the predominant VFAs produced during the BMP tests. No significant accumulation of butyric and iso-butyric acids was observed, probably because of the sufficient butyric-degrading syntrophs in the inocula, which rapidly converted these acids to acetic acid.

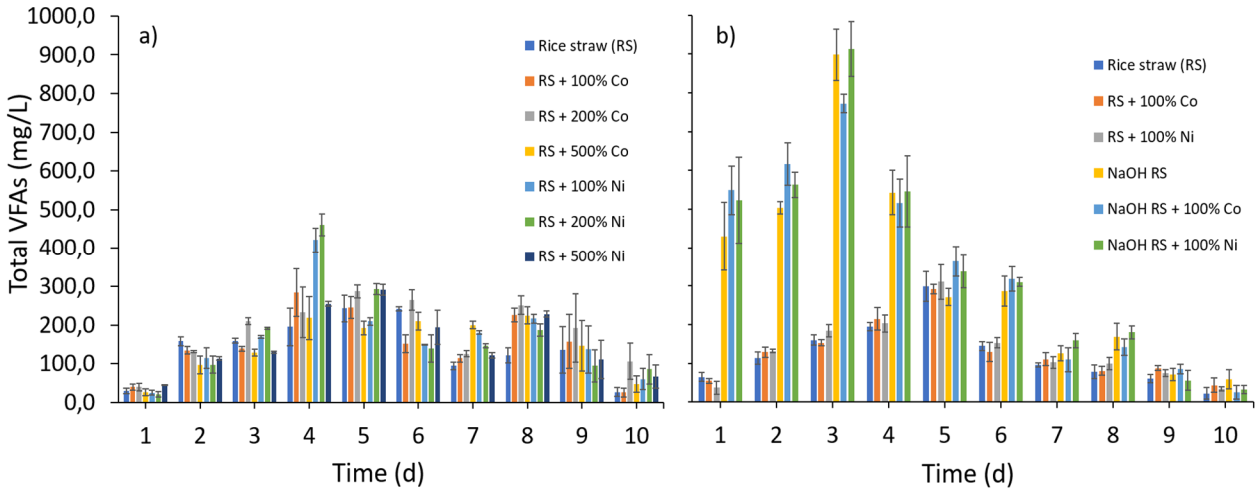

Fig. 6.4. Total volatile fatty acids production during the first 10 d of anaerobic digestion of rice straw inoculated with anaerobic granular sludge (a) and buffalo manure (b).

The total VFA concentration was constantly below 500 mg HAc/L during the AD of rice straw inoculated with granular sludge (Fig. 6.4a), and the addition of Co or Ni did not cause statistically significant differences ($p > 0.05$) in the total VFA concentrations. On the other hand, the production of VFAs was markedly enhanced by the alkaline pretreatment (Fig. 6.4b), with peaks of total VFAs around 900 mg HAc/L recorded on the third day of AD. After that, the VFA consumption rate was higher than the production rate, resulting in a gradual decrease of the VFA concentration until day 10.

The relevant increase of the total VFA concentration, when the pretreated rice straw was used, indicated that the alkaline pretreatment enhanced the hydrolysis of the rice straw, which

consequently caused a higher methane production yield from the pretreated straw (Table 6.4). Despite the larger amount of available VFAs after the NaOH pretreatment, the addition of Co and Ni to the pretreated straw induced only a slight supplementary increase of the final methane production. The VFA production achieved with the pretreated straw was similar with and without TE addition, resulting in no synergistic effects.

Previous studies showed the importance of TE addition to stabilize the AD process in case of VFA accumulation. Se is particularly important to prevent propionic acid accumulation by providing the co-enzymes necessary for the oxidation of formate, which is a breakdown product of propionate [38]. Pobeheim et al.[29] reported that Co and Ni addition during the AD of maize silage stabilized the performance of a continuous digester, where a deficit of the two TEs had caused VFAs accumulation. An increase of the VFA utilization rate by TE addition was also observed by Espinosa et al. [39] and Ariunbaatar et al. [25] during the AD of cane molasses stillage and food waste, respectively. In those studies, the total VFA accumulated to concentrations of 1500 – 10000 mg HAc/L in the absence of TE supplementation, whereas a reduction to levels around 500 – 1000 mg HAc/L was observed when TEs were provided. Similarly, Lindorfer et al. [15] added a TE mixture into 60 full-scale AD plants, which had shown TE deficiency. The major effect was observed in plants operating at high organic loading rates (i.e. 2.5 kg VS/m^3/d), where the TE addition resulted in a considerable decrease of the VFA concentrations from approximately 3000 to 500 mg HAc/L, in a few days from the supplementation. The enhanced consumption of acetic acid by methanogens consequently increased the biogas production yields.

In contrast to the above mentioned works, the VFA concentrations never exceeded 500 mg HAc/L during the AD of raw rice straw in the present study. This could be attributed to the hydrolysis being the limiting step for lignocellulosic materials, rather than methanogenesis as for more easily degradable substrates [40]. The pretreatment was more effective than the TE addition in increasing the hydrolysis of rice straw and this resulted in a higher production of VFAs (i.e. up to 900 mg HAc/L). Alkaline pretreatments are effective methods to enhance the hydrolysis of lignocellulosic materials and the subsequent acidogenesis stage [41,42]. In some cases, an excessively enhanced hydrolysis rate can lead to a failure of the process, due to an excessive acidification caused by VFA accumulation, with a consequent inhibition of the methanogenic population [42]. In particular, the inhibition might occur when the inoculum to substrate ratio is low, i.e. below 0.12 g VS/g VS [41]. In this study, an inoculum to substrate ratio of 2.0 g VS/g VS was applied, thus no acidification occurred, despite the increased VFA production caused by the NaOH pretreatment.

The methanogenic archaea populations present in both the inocula were able to efficiently convert the acetic acid produced to methane. Even when the VFA concentration was almost doubled as a result of the pretreatment, the addition of TEs was not necessary to achieve a complete acetate conversion.

6.4 Conclusion

The addition of Co, Ni and Se did not result in a significant improvement of the AD of rice straw. On the contrary, the use of an alkaline pretreatment with NaOH caused a considerable enhancement of AD, increasing the biogas production yield by 21.4%. The marginal effect observed after TE supplementation on the untreated rice straw could be linked to its complex lignocellulosic structure, which required an enhancement of the hydrolysis, rather than the methanogenesis. This observation was also supported by monitoring the VFA concentration, which was significantly increased by the pretreatment, whereas negligible effects were obtained after TE addition.

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CHAPTER 7

Bioavailability of selected trace elements during the anaerobic digestion of rice straw

This chapter has been submitted as:

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7.1 Introduction

In the last two decades, pressing issues such as world population increase, global warming, rise of energy demand, and the need for national energy security and rural economic development have been the driving forces urging for a switch from traditional fossil fuels towards sustainable energy sources [1]. Biomethane, produced through the anaerobic digestion (AD) process, has the potential to yield more energy than any other available biofuel, such as bioethanol or biodiesel [2]. Biomethane can be produced from a wide range of lignocellulosic materials (LMs), such as various agricultural and horticultural wastes [3]. The term LM refers to any plant dry matter, which is mainly composed of carbohydrate (i.e. cellulose and hemicellulose) and aromatic (i.e. lignin) polymers [4]. Waste LMs represent the most promising renewable organic feedstock for biogas generation, since their production does not compete for arable land [5].

More than 3 billion tons of LMs are available from agricultural sources every year [6] and methane yields from the AD of crop residues are in the range of 3,200 to 4,500 cubic meters per hectare per year [7]. Rice straw is one of the most common agricultural wastes and its biogas production potential is appealing to both developed and developing countries [8]. However, after the rice harvesting, the straw is commonly left unused or burnt in the fields, causing serious environmental problems [9]. Methane (CH_4) and carbon dioxide (CO_2) are considered as the major gases responsible for global warming [10], thus it is essential to reduce the self-decomposition or field burning of rice straw and employ the generated biogas for useful purposes without releasing it into the atmosphere.

In the last years, several studies have focused on the role of trace elements (TEs) within anaerobic processes [11]. The impact of different TEs on the AD of food waste, wastewater sludge and energy crops has been largely investigated, and positive effects of TE addition on the biogas production have been observed [12–14]. In contrast, the TE requirements of biogas reactors operating with agricultural residues have rarely been reported in the scientific literature, despite these types of feedstock usually show a low content of essential TEs [15,16]. The microbial growth and the whole anaerobic fermentation process depend on the optimal supply of TEs and their availability to the microorganisms [17]. The requirement of methanogenic archaea for TEs such as Fe, Ni, Co, Mo, Se and W has been documented [18,19]. However, the recommended values are spread over a wide range of concentrations, suggesting that the presence of a certain TE does not necessarily imply that the microorganisms are able to take up the TE and incorporate it into the catalytic center of their enzymes [20].

A deficiency in the required supply of TEs can lead to AD process imbalances, mainly resulting in the accumulation of volatile fatty acids (VFAs). An acidification of the reactor leads to suboptimal biogas yields or, in the worst cases, a complete failure of the AD process [21]. On the other hand, supplying an excessive amount of TEs could provoke inhibitory or toxic effects to the microorganisms, and eventually to the environment through the application of high TE content digestates in agricultural fields [22]. The total and the bioavailable amount of each TE can be substantially different from each other [23]. Thus, the determination of the total TEs concentration is insufficient to fully evaluate the effect of a lack or excess of TEs on the AD process [22].

In addition to a detailed compositional analysis of the feedstock and inoculum employed in the anaerobic reactor, the bioavailability of the TEs, defined as the degree to which TEs are available for metabolic activity [24], should be determined as well. The bioavailable TE fractions can be approximated with sequential extraction techniques to assess the distribution pattern of each analyzed TE, since the distribution of TEs over different fractions can be an indicator of the TE bioavailability [25]. During a chemical sequential extraction, the pool of bioavailable TEs decreases after each extraction step [23]. The distribution of a specific TE among the different fractions indicates its availability for metabolic activities, and an appropriate knowledge of the bioavailability of the considered TEs is essential for an adequate supplementation strategy [20]. To date, only a few studies have investigated the effect of TEs within AD reactors fed with LMs using sequential extraction methods [22].

The effect of different combinations of TEs on the AD of rice straw were investigated. In particular, iron (Fe) was added alone and in combination with both cobalt (Co) and nickel (Ni) in batch bottles containing rice straw inoculated with buffalo manure. A sequential extraction technique was applied to assess the bioavailability of the supplemented TEs. The main goal of this work was to investigate whether the bioavailability of Fe and Co could be a limiting factor during the AD of rice straw. Biochemical methane potential (BMP) tests were conducted under mesophilic conditions to determine the biogas production yields from each supplied TE configuration.

7.2 Materials and methods

7.2.1 Feedstock and inoculum

Rice (*Oryza sativa*) straw, obtained from agricultural fields in Pavia (Italy), was used as the sole substrate in this work. After collection, the straw was manually cut down to a particle size smaller than 4 mm. The inoculum used in this work was a digestate from a full-scale AD

plant treating buffalo manure and milk whey from a mozzarella factory located in Capaccio (Italy). The inoculum was degassed by incubating it at mesophilic conditions (37 ± 2°C) for 4 d before starting the experiments. The physicochemical characterization of the rice straw and the inoculum are reported in Table 7.1.

Table 7.1. Characterization of the rice straw and inoculum used in the BMP tests

	Rice straw	Inoculum
TS (%)	94.2 ± 0.2	4.3 ± 0.2
VS (%)	80.0 ± 0.7	2.8 ± 0.1
TKN (g N/kg TS)	11.2 ± 0.2	27.1 ± 1.3
Fe (µg/g TS)	477 ± 81	4,634 ± 57
Cu (µg/g TS)	17 ± 5	19 ± 0.2
Zn (µg/g TS)	62 ± 25	69 ± 2.0
Co (µg/g TS)	< 1.0	2 ± 0.1
Ni (µg/g TS)	2 ± 0.0	10 ± 0.1
Se (µg/g TS)	< 1.0	< 1.0
Na (mg/g TS)	0.4 ± 0.0	13.8 ± 1.4
Mg (mg/g TS)	1.1 ± 0.0	12.7 ± 0.4
K (mg/g TS)	14.5 ± 0.4	83.2 ± 7.1
Ca (mg/g TS)	9.1 ± 0.6	33.5 ± 1.6
Cellulose (%)	28.6 ± 0.2	-
Hemicellulose (%)	19.5 ± 1.2	-
Lignin (%)	17.3 ± 0.3	-

TS: total solids; VS: volatile solids; TKN: total Kjeldahl nitrogen.

7.2.2 Trace elements dosing strategy

The representative concentrations of Fe, Co and Ni, together with some other TEs present in the rice straw and the buffalo manure are detailed in Table 7.1. Based on the results of previous studies [26–28], the rice straw was inoculated with buffalo manure and Fe was added in a concentration of 205 µg/g TS of rice straw. Analogously, another set of batch bottles containing rice straw was supplemented with a cocktail of Fe, Co and Ni. The amount of Co and Ni supplemented in the BMP tests was 18 and 45 µg of, respectively, Co and Ni per g TS of rice straw, determined on the basis of the recommended values from the literature [28], adjusted according to the results obtained by Mancini et al. [16]. Stock solutions were prepared using the $FeCl_3 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$ and $NiCl_2 \cdot 6H_2O$ salts (analytical grade, Sigma-Aldrich, Germany), and the required amount of each TE was injected in the batch bottles at the beginning of the experiment.

7.2.3 BMP tests

The biochemical methane production (BMP) was measured at 37 (± 2)°C using the liquid displacement method, as described by Mancini et al. [9]. Glass bottles of 250 mL were loaded with 2.5 g of rice straw and 142.0 g of the buffalo manure inoculum, in order to maintain an inoculum to substrate ratio of 2.0 g VS/g VS. Deionized water was added to reach a working volume of 150 mL into each bottle. Blank samples containing only inoculum and deionized water were also prepared in order to determine the biomethane production of the inoculum, which was then subtracted from the production of the BMP tests containing rice straw. Five bottles were prepared for each configuration, sacrificing two of them at day 5 for sequential extraction analysis and leaving a triplicate until day 40. VFA analysis was performed by daily sampling 1.0 mL of the liquid phase from each bottle during the first 10 d of the BMP tests and on day 15.

7.2.4 Sequential extraction protocol

To investigate the chemical speciation of the TEs in the BMP tests containing only inoculum, rice straw, rice straw supplemented with Fe; and rice straw supplemented with Fe, Co and Ni, a sequential extraction technique was used following the procedure used by Ortner et al. [23]. The applied technique is founded on the principle of the Tessier extraction method, which uses different extraction solvents in order to solubilize specific fractions of metals [29]. In particular, the total TE amount was divided into five different fractions, which were determined following the scheme reported in Table 7.2.

Table 7.2. Extracting agents and conditions applied in the sequential extraction protocol

Fraction	Extracting agent	Extracting conditions[a]		
		pH	Temperature	Shaking time
F1 – water soluble fraction	-	-	-	-
F2 – exchangeable fraction	10 mL 1M NH_4CH_3COO	7.0	25°C	60 min
F3 – carbonate fraction	10 mL 1M CH_3COOH	5.5	25°C	60 min
F4 – organic matter and sulfide fraction	10 mL H_2O_2 (30% *w/w*)	2.0	35°C	180 min
F5 – residual fraction[b]	10 mL HNO_3	-	-	-

a: Samples were centrifuged at 3,000 rpm for 15 min.
b: The residual fraction was determined using the same procedure adopted for the total concentration.

The bioavailability of TEs decreases in each subsequent fraction from F1 to F5, with the water soluble (F1) and exchangeable (F2) fractions being considered highly bioavailable [30]. The fractions F3-F5 are either bound to particulate matter or precipitates and the TE mobilization depends on the aqueous solubility, which is generally poor in the case of sulfides

and carbonates, while the residual fraction is a non-extractable and non-dissolvable fraction which is commonly considered not bioavailable [23].

Fresh material with a TS content of about 1 g was collected from two bottles of each different configuration adopted in the BMP tests at day 5. This digestate was placed in centrifugation tubes, which were filled up with deionized water to a total weight of 50 g. After centrifugation at 3,000 rpm (1841 x g) in a Rotina 420 centrifuge (Hettich, Germany) for 15 min, the supernatant, representing the water soluble fraction (F1), was collected and stored at 4°C until further analysis. As reported in Table 7.2, 1 g of dried material was extracted with ammonium acetate to determine the exchangeable fraction (F2), which was analyzed in the supernatant. The pellet obtained from this extraction step was further extracted with acetic acid, in order to quantify the TEs bound as carbonate (F3). Then, the pellet was extracted with hydrogen peroxide, and the TEs in the supernatant represented the fraction bound to the organic matter and the sulfide precipitates (F4). Finally, the pellet remaining at the end of the extraction process was digested with nitric acid in the microwave in order to determine the residual fraction (F5).

7.2.5 Analytical methods

Total solids (TS) and volatile solids (VS) were determined according to the method described by Sluiter et al. [31]. Total Kjeldahl nitrogen (TKN) was measured according to the Kjeldahl method [32]. The rice straw was analyzed for its structural carbohydrates and lignin content according to the procedure described by Sluiter et al. [33]. The TE content and VFA production were determined by inductively coupled mass spectroscopy (ICP-MS) (X-Series, Thermo Fisher Scientific, USA) and gas chromatography (GC) (Varian 430-GC, Varian Inc., USA), respectively, as described in Mancini et al. [9]. The samples for TE determination were prepared by digesting 0.5 g of dried material with 10 mL of 65% concentrated HNO_3 in a microwave (MARSXpress, CEM, USA) at 175°C for 20 min. Ni remained below the ICP-MS detection limit, thus the fractionation of this TE cannot be reported.

7.3 Results and discussion

7.3.1 Effect of TE addition on the AD of rice straw

The addition of Fe to the AD of rice straw did not result in increased biomethane production yields (Fig. 7.1a). The cumulative biogas production of the rice straw inoculated with buffalo manure was 264 mL CH_4/g VS in both the BMP tests with and without Fe dosing. Despite the Fe addition, the VFA production also remained unchanged compared to the batch

bottles where no TE was supplemented (Fig. 7.1b). Previous studies [27,34] observed the main effect of Fe dosing in preventing VFA accumulation and thus process imbalance, especially when the organic loading rates were high. In such cases, TE addition was beneficial to enhance the acetic acid consumption by the methanogens, consequently increasing the biogas production yields. Differently, the VFAs never accumulated to inhibitory levels in this study. On the contrary, the total VFA production was particularly low, exceeding 250 mg HAc/L only on day 5 (Fig. 7.1b). Hence, the methanogenic archaea population present in the buffalo manure used as the inoculum was able to efficiently convert the acetic acid produced to methane, without requiring external supplementation of TEs.

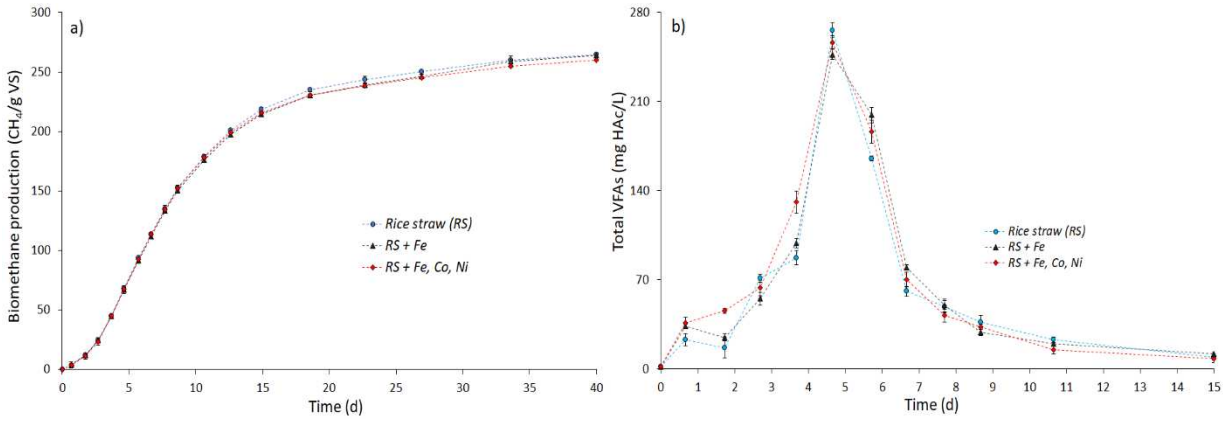


Fig. 7.1. Influence of different trace elements addition on the biomethane production (a) and VFA accumulation (b) during the AD process.

The stimulatory effect of Fe on biogas production has long been reported. Hoban and van den Berg [26] observed a marked increase in the conversion of acetic acid to methane after adding Fe at a concentration of 250 mg/L to a methanogenic culture utilizing acetic acid. Similar Fe concentrations were used to stabilize digesters loaded with cow dung and poultry litter waste, observing an increased methanogenesis by 40%, linked to an enhanced utilization of VFAs and an increased number of methanogens in the reactor [27]. A reduced VFA concentration linked to Fe addition was reported also by Moestedt et al. [34] during the anaerobic codigestion of the organic fraction of municipal solid waste and slaughterhouse residues, supported by an increase of methanogenic communities. Hansen et al. [35] counteracted the sulfide inhibition in a reactor loaded with swine manure by supplementing 4.4 mM of $FeCl_2$, which precipitated as ferrous sulfide, with a subsequent benefit for the biogas production yields. The possibility of enhancing the biomethane production in AD reactors utilizing lignocellulosic waste was investigated by Khatri et al. [36] and Zhang et al. [37], who reported a similar increase in the biomethane production yield (i.e. 15-18%) by supplementing

different amounts of Fe (i.e. 1,000 and 10 mg Fe/L, respectively). A recent study by Cai et al. [38] investigated the bioavailability of Fe in an AD reactor operated with rice straw. The authors reported that 50 mg Fe/L were appropriate to improve the digester performance and increase the amount of Fe in bioavailable fractions to the microorganisms.

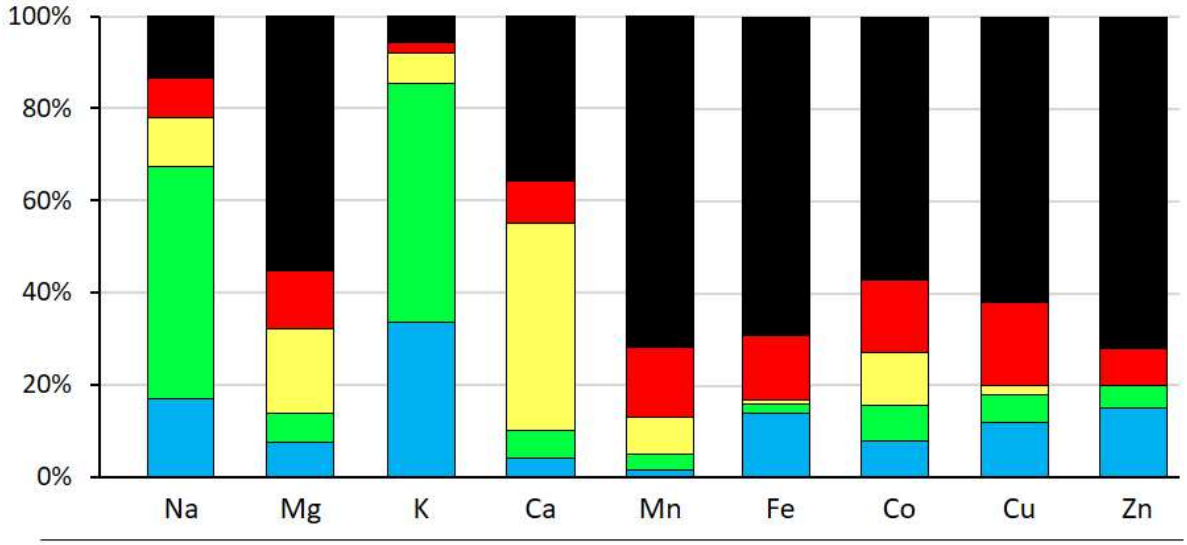
Supplementing Co and Ni together with Fe in the BMP tests also led to negligible effects in terms of enhancement of the biogas yield. Despite the recognized importance of Co and Ni as cofactors for several enzymes involved in both the acetoclastic and hydrogenotrophic methanogenesis pathways (i.e. acetyl-CoA decarbonylase, CO dehydrogenase, methyl-CoM reductase and methyl-H4SPT:HS-CoM methyltransferase) [21], the addition of the two TEs in the cocktail with Fe did not produce any significant improvement of the AD process. The final biomethane production was 260 mL CH_4/g VS, i.e. not statistically different ($p>0.05$) from those observed in the other BMP tests (Fig. 7.1a). This result could again be linked to the marginal effect of the TE addition in increasing the consumption of VFAs, with the concentration of these AD intermediates in the same range of those observed in the BMP test where TEs were not provided (Fig. 7.1b). During the AD of LMs, hydrolysis is the limiting step, rather than methanogenesis as for more easily degradable substrates [16]. Rapidly biodegradable materials have thus more benefits from TE addition than complex lignocellulosic substrates such as rice straw.

An analysis of the enzymatic activity was attempted during this study (data not shown) to evaluate the effects of TE addition on the activity of the enzyme cellulase, which is responsible for the hydrolysis of the cellulose contained in the rice straw [39]. However, the concentration of the enzyme cellulase in the supernatant was too low and could not be detected using traditional protocols [40]. Zhang et al. [37,41] supplemented, respectively, Fe and Ni during the AD of cow dung and *Phragmites* straw. Their studies showed a positive effect of the two TEs in enhancing the biogas production yields, although the cellulase activities were not significantly increased by Fe addition [37]. Similarly, Ni supplementation had its major impact on the methanogenic stage, whereas it stimulated the cellulase activities only secondarily [41].

7.3.2 Sequential extraction

An optimum supply of TEs is essential for the growth and enzymatic activities of the microorganisms involved in the AD process in order to increase the biomethanation yields and rates. Quantifying the bioavailable amount, rather than the total amount, provides better information about the sufficient supply of TEs to the microbial consortium [20]. A sequential extraction technique was performed in order to rule out the possibility that the supplemented

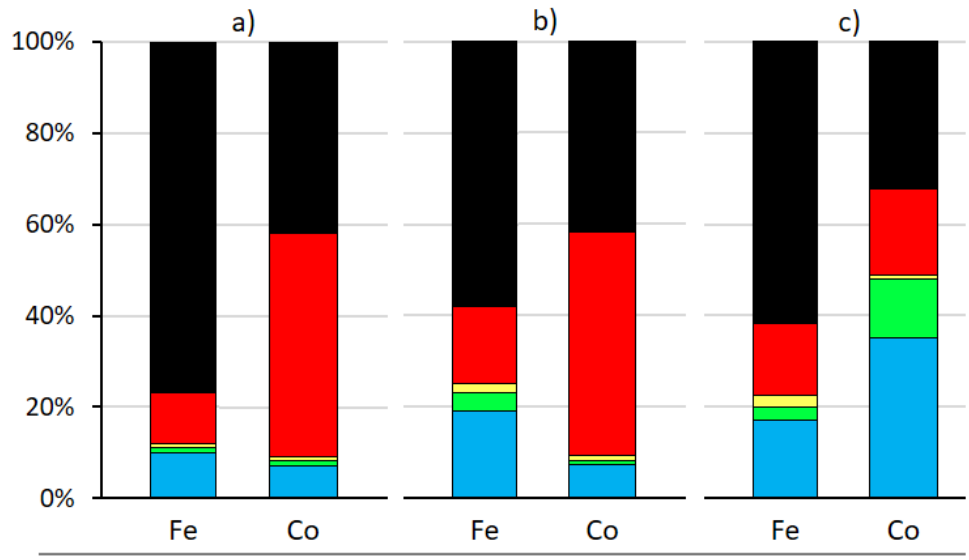
TEs were present under forms not directly bioavailable to the microbial population in the AD bioassays. The TEs already present in the buffalo manure inoculum used in this study, fractionated into 5 categories, are shown in Fig. 7.2.



	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn
F1	2354	964	27970	1342	7	649	0,2	7	48
F2	6952	805	42972	2012	15	93	0,2	3	16
F3	1452	2329	5565	15093	35	46	0,2	1	0
F4	1188	1603	1932	3122	66	650	0,3	10	25
F5	1865	7028	4718	11974	314	3196	1,1	35	229

Fig. 7.2. Sequential extraction of the trace elements present in the inoculum (values are expressed in µg/g TS).

The fractionation of Fe and Co in the inoculum (Fig. 7.2) showed that the highly bioavailable amount of the two TEs, given by the sum of F1 and F2 [23], was approximately only 16% for both metals. The percentage decreased to 11 and 8% for Fe and Co, respectively, analyzing the rice straw inoculated with buffalo manure (Fig. 7.3a), due to a lower aqueous phase because of the presence of straw. When Fe was externally provided, the highly bioavailable amount of Fe (F1+F2) in the bottles increased to 23% (Fig. 7.3b) when Fe was supplemented as the sole TE, and 20% (Fig. 7.3c) when Fe was supplemented with Co and Ni. Similarly, Co supplementation resulted in an increase of the sum F1+F2 up to 48% (Fig. 7.3c).



	Fe	Co	Fe	Co	Fe	Co
F1	216	0,1	531	0,1	480	3,9
F2	25	0,0	112	0,0	81	2,0
F3	22	0,0	56	0,0	69	0,0
F4	241	0,7	475	0,7	450	2,3
F5	1689	0,6	1621	0,6	1752	4,2

Fig. 7.3. Sequential extraction of Fe and Co present in the BMP tests (a: rice straw without TE addition; b: rice straw with Fe addition; c: rice straw with Fe, Co and Ni addition) (values are expressed in µg/g TS).

A direct correlation between the increase of Fe and Co bioavailability (Fig. 7.3) and improved AD performance (Fig. 7.1) was not observed in this study. Despite the amount of bioavailable Fe and Co was higher in the batch tests where the two TEs were externally provided (Fig. 7.3), the biogas production remained unchanged compared to the BMP tests without TE dosing (Fig. 7.1a). This suggests that the amount of Fe and Co required by the methanogens for the conversion of acetate in the AD of rice straw was sufficiently high in the inoculum and an additional supplementation was not required, despite the low amount of the two TEs in the buffalo manure and rice straw (Table 7.1) compared to the recommended dosages from the literature [15,28]. For recalcitrant lignocellulosic materials such as rice straw, hydrolysis should thus be first improved and if VFAs start to accumulate after an enhanced hydrolysis, TEs supplementation may be considered to stabilize the AD process. When the organic loading of the reactor does not lead to VFA accumulation, such as in this study, the

methanogenesis phase cannot be enhanced by TE dosing, regardless of the presence of TEs in a bioavailable form.

7.4 Conclusions

The sequential extraction performed in this study showed that the Fe and Co supplemented to the AD of rice straw were present in bioavailable forms for microbial metabolic activities. The biomethane production was, however, not enhanced by the TE addition. The VFA concentration was similar in all the BMP tests performed, regardless the presence of bioavailable TEs, suggesting that the hydrolysis of rice straw is the AD rate limiting step. In the case of recalcitrant lignocellulosic materials such as rice straw, a TE dosing strategy should be applied only after the enhancement of the hydrolysis, when VFAs start accumulating during the AD process. In contrast, when the VFA concentration is low, TE addition is not required to stimulate the acetate conversion to methane, independently whether the TEs are supplemented in a bioavailable form.

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CHAPTER 8

General discussion and future perspectives

8.1 Introduction and objectives

The global energy consumption has increased significantly in the last decades, as a result of the world population growth and the rising of human living standards [1]. The use of non-renewable fossil fuels, which still represent more than 80% of the global energy supply, is causing devastating effects on the environment, contributing to major issues such as global warming and climate change [2]. Supplying energy using renewable resources, such as lignocellulosic materials (LMs), is regarded as an optimal solution to overcome these problems, due to the wide availability and relatively low cost of LMs. In fact, about 10% of the current energy demand is met by utilizing LMs as primary source [3]. However, a large share of this percentage results from the direct combustion of LMs, thus not representing a solution to cutting down the emissions of greenhouse gases [4]. Therefore, alternative pathways to take the best out of LMs have been widely considered, with biogas production via the anaerobic digestion (AD) process, resulting as one of the favored options [5].

The use of starch-based materials, such as crops, to produce biogas, often raises ethical concerns, linked to the conflict "food vs fuel". Therefore, most of the research is currently focusing on other types of LMs, such as agricultural residues, which represent the most promising type of renewable raw biomass, since it does not compete for arable land [6]. The main limitation in the AD of LMs is represented by the complex recalcitrant structure of the raw material, which requires a pretreatment step in order to enhance the rate-limiting hydrolysis and, consequently, achieve higher biogas production yields [7].

Several LM pretreatments have been developed in recent years to break down the rigid structure of LMs, thus allowing an enhanced degradation by the microorganisms involved in the AD process. Among the many physical, biological and chemical processes investigated, the latter showed the highest efficiency in altering effective parameters such as cellulose crystallinity, carbohydrate accessibility, degree of delignification, etc. [8,9].

Another key factor for a stable AD process is to provide an adequate concentration of trace elements (TEs) for the microorganism requirements [10]. Despite their acknowledged importance within anaerobic bioreactors, the exact role and dosage of each TE is still under discussion [11]. In particular, information about TE requirements of anaerobic digesters fed with LMs is scarce, with the effects of TE dosing on the AD of agricultural residues having been investigated only by a few studies so far [12].

The overall goal of this work was to enhance the biomethane production from the AD of various LMs in batch conditions using different approaches. Three chemical pretreatments,

namely NMMO, organosolv and NaOH pretreatment were separately investigated, assessing the improvements obtained in the AD process, by analyzing parameters such as the LM crystallinity, water retention value and chemical composition, as well as the VFA production, the kinetics of biomethane production and the biomethane production yield. Furthermore, the impact of TE supplementation on the AD of rice straw was evaluated by adding different concentrations of Fe, Co, Ni and Se and studying the effect generated on the VFA and biomethane production. The effect of NaOH pretreatment and TE addition were also compared within the same study by assessing the effect of pretreatment and TE dosing on the AD of rice straw.

8.2 Major research findings

8.2.1 Effect of pretreatments on the AD of lignocellulosic materials

The main goal of pretreating LMs is to break down the linkages between the polysaccharides and lignin, in order to make the carbohydrate portion (i.e. cellulose and hemicellulose) available for the anaerobic bacteria [13]. Hydrolysis of LMs is considered as the rate limiting step. Thus, the pretreatment can accelerate this stage, resulting in enhanced biogas yields [14]. An extensive review of the chemical pretreatments involving the use of solvents was conducted in Chapter 2, presenting advantages, limitations and the main results, from the existing literature, obtained pretreating LMs with several cellulose solvent-based methods and organosolv. Furthermore, the possible dissolution mechanisms were analyzed.

The solvent N-Methylmorpholine-N-Oxide (NMMO) is able to dissolve cellulose by breaking the hydrogen bonds in the LM network, with new bonds between the solvent and the cellulose being formed. After the pretreatment, the dissolved cellulose can be recovered by adding an antisolvent, with the pretreated LMs presenting a decreased cellulose crystallinity. During this research, the NMMO pretreatment was investigated to pretreat four different LMs, namely rice straw, hazelnut skin, cocoa bean shell and wheat straw. The pretreatment was particularly effective for rice straw, leading to an 82% enhancement of the biomethane production, which reached 374 mL CH_4/g VS (Table 3.2). A preliminary energy assessment was conducted to evaluate whether the additional biogas produced from the pretreated rice straw would justify the surplus energy input for performing the NMMO pretreatment, which requires a temperature of 120°C for a duration of 3h. The results showed that a positive energy balance can be achieved employing the NMMO pretreatment for rice straw. Assuming an 80% of heat recovery during the pretreatment stage, a positive net heat energy value of 0.594 kWh/kg VS can be obtained at the end of the whole AD process. This result was corroborated

by the recent findings of Khoshnevisan et al. [2], who showed that the NMMO pretreatment for biogas production outperformed other pretreatment processes (i.e. steam explosion) and other final products (i.e. bioethanol), in terms of energy balance and environmental performance. This latter aspect is particularly important, since the economic benefits associated with actions aimed to cut GHG emissions (also known as social cost of carbon) should be considered in any economic evaluation.

Hazelnut skin and cocoa bean shell, which were never investigated before as AD substrates, showed a good potential for biogas production, with cumulative biomethane yields of 223-261 and 199-231 mL CH_4/g VS, respectively, for the untreated feedstock (Table 3.2 and 4.3). However, both NMMO and organosolv pretreatments did not lead to significant enhancement of the biomethane production yields from these two LMs. This was likely due to the higher content of protein and fat in the hazelnut skin and cocoa bean shell, compared to the rice straw (where the protein and fat content is negligible), with the two pretreatments having a negative effect on these components.

The chemical composition of the untreated and organosolv pretreated rice straw, hazelnut skin and cocoa bean shell was detailed in terms of polysaccharides and lignin content (Table 4.2). The organosolv pretreatment resulted in a delignified material. Depending on the temperature adopted, the pretreatment was able to reduce the lignin content by 14–15% for rice straw, 5–6% for hazelnut skin, and 8–12% for cocoa bean shell (Table 4.2). Other than being advantageous for the subsequent AD process, the removal of lignin can be a relevant aspect for full-scale applications of the organosolv process. In fact, the recovery of a purified lignin fraction can be obtained after the organosolv pretreatment by filtrating the solvent-evaporated liquor in which the lignin fraction precipitates (Fig. 2.4). Lignin can then be used for several applications other than direct combustion (e.g. as additive in inks, paints and varnishes; matrix material in bio-based composites; radical scavengers or component for phenolic resins and polyurethane foams) significantly improving the economics of a lignocellulosic biorefinery [15]. In a recent techno-economic analysis of the organosolv pretreatment process [16], it was highlighted that, despite the high energy consumption, the organosolv process results in a better usage of the whole feedstock, due to the efficient removal of lignin, compared to other methods, such as diluted acid pretreatment.

Due to the high variability in the chemical composition of lignocellulosic materials [17], comparing results from different studies can be challenging. In particular, the effectiveness of different pretreatments on a specific substrate has rarely been compared within the same study in terms of enhancement of the biomethane production yields and variation of the

lignocellulosic composition of the original feedstock. In this work, the effects of NMMO, organosolv and NaOH pretreatment on the lignocellulosic composition of wheat straw and the biomethane yields from the subsequent AD of the pretreated material were evaluated and compared. The three investigated pretreatments were able to improve the AD of wheat straw. The cumulative biomethane production yield of 274 mL CH_4/g VS obtained with the untreated feedstock (Table 5.3) was enhanced by 11% by the NMMO pretreatment and by 15% by both the organosolv and alkaline pretreatment (Fig. 5.2). The three pretreatment methods had a different impact on the chemical composition of the wheat straw (Table 5.2). NMMO hardly changed the amount of carbohydrates and lignin present in the original feedstock. Organosolv had a major impact on dissolving the hemicellulose component, whereas the alkaline pretreatment was the most effective in removing the lignin fraction. In addition to the increased biogas yields, the applied pretreatments enhanced the kinetics of biomethane production (Table 5.3). Despite the need for further studies, NMMO, organosolv and alkaline pretreatment present bright future perspective to enhance the AD of LMs.

8.2.2 Effect of trace elements dosing on the AD of lignocellulosic materials

The effect of TE addition on the anaerobic digestion of rice straw was investigated in batch tests using inocula of different origin, such as buffalo manure and anaerobic granular sludge. Fe, Co, Ni and Se were added to the raw rice straw at different dosages. An NaOH pretreatment was applied to the rice straw both alone and in combination with the addition of TEs, in order to evaluate potential synergistic effect of the pretreatment and the TE supplementation on the biogas production yields. The results (Table 6.4) showed that the alkaline pretreatment was more effective than the TE addition in increasing the cumulative biogas production, causing a 21.4% enhancement of the final biomethane yield (Fig. 6.2), whereas the increase due to the TE supplementation was not statistically significant. The analysis of VFAs confirmed that the NaOH pretreatment resulted in a higher production of VFAs, indicating an increased hydrolysis, while TE addition did not cause significant changes in the VFA concentrations (Fig. 6.4).

Several studies concluded that TE dosing have the major effect in avoiding VFA accumulation, thus maintaining a stable AD process [11,18,19]. Inhibition is likely to occur when the reactor is working at low inoculum to substrate ratios (i.e. below 0.12 g VS/g VS) [20]. In this work, an inoculum to substrate ratio of 2.0 g VS/g VS was applied, thus no acidification occurred, despite the increased VFA production caused by the NaOH pretreatment. The methanogenic archaea populations present in both the inocula were able to

efficiently convert the acetic acid produced to methane. Even when the VFA concentration was almost doubled as a result of the pretreatment, the addition of TEs was not necessary to achieve a complete acetate conversion. Eventually, the results obtained during this work demonstrated that TE dosing is not a proper enhancement strategy for the AD of LMs, in comparison with a chemical pretreatment. For recalcitrant AD substrates such as agricultural residues, the rate limiting hydrolysis should be improved by breaking the linkages in the lignocellulosic network through a pretreatment. Enhancing the methanogenesis stage via TE addition is not required if no VFA accumulation occurs, such as in the AD of the investigated LMs.

8.3 Future research perspectives

Based on the results obtained during the various batch experiments conducted, NMMO, organosolv and alkaline pretreatment showed an excellent potential to enhance the AD of agricultural residues such as rice and wheat straw. Further studies for their implementation at pilot scale level are significantly recommended. Improvements are especially needed on the energy saving mechanisms, in particular during the solvent recovery stage, to make the process more economically competitive. Detailed life cycle assessment (LCA) and techno-economic analysis of the whole process, including an environmental impact assessment, need to be performed in order to better evaluate the real efficiency of the AD process of LMs following the selected pretreatment.

A significant uncertainty remains on the different environmental and sustainability factors on the performance of second generation biofuels in a commercial scale. Despite the current expectations and efforts made in improving the pretreatment processes, uncertainty related to the feedstock availability and revenue stability remains an issue, also subject to political risks [3]. Several aspects still remain to be properly optimized before an efficient full-scale configuration is acquired. Nevertheless, the advances in the scientific understanding will help in scaling-up these novel pretreatments for the AD of agricultural residues a feasible option in the near future.

Moreover, each pretreatment investigated during this work faces some challenges. For instance, the use of recycled NMMO after 5 cycles has been found not to be as effective as using fresh NMMO for the pretreatment of LMs containing high lignin and bark, such has forest residues [21]. Thus, further studies are required to have a full understanding of the reaction mechanisms between the substrate and NMMO. The main challenge faced by the organosolv pretreatment is that the process should be integrated within a biorefinery structure, in order to exploit the different fractions obtained from the pretreated LMs. Therefore, rather

than optimizing the pretreatment conditions, such as the pretreatment temperature and time and the solvent concentration in terms of maximum product yield and lower cost, the production of lignin-based products should be optimized at the same time in order to make the organosolv pretreatment competitive in the market [22]. Although a great progress has been made in the alkaline pretreatment of LMs, further improvements in process parameters and selectivity are still needed to become a commercially viable option at full-scale level [17].

Unlike acetogenesis and methanogenesis, the role of TEs in the stages of hydrolysis and acidogenesis during the AD process is lacking of research studies [11], especially for LMs. Thus, the impact of TEs such as Fe, Co and Ni on the hydrolytic step of the AD should be assessed using cellulose as a model substrate for hydrolysis. Moreover, the effect of TEs on the methanogenesis of LMs should be studied at high TS content (i.e. 15-20%) in order to stimulate VFA accumulation, which could benefit of TE addition to avoid the reactor failure. Motte et al. [23] observed a threshold of 29% TS during the AD of wheat straw. Above this concentration, the AD process was inhibited by excessive VFA accumulation. However, at high TS content, the low liquid content within the reactor could also limit the bioavailability of TEs. Therefore, the presence of free liquid should also be taken into account.

Another aspect, which was not evaluated during this work, though represent a major barrier for the expansion of AD technology is the proper management of the large quantities of digestate produced. Digestate has a low TS content, thus its storage and transportation are costly. Moreover, digestate could also cause some environmental problems due to a potentially high content of pathogens, heavy metals and unbalanced nutrients [24]. Therefore, alternative methods for digestate processing and management are needed in the short term for a wider adoption of the AD technology. In the long run, the development of AD biorefineries should include the processing of the digestate in order to transform it into higher value products, such as biochar or bio-oil.

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