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Physical, chemical and biological pretreatments to enhance biogas production from lignocellulosic substrates

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Abstract

Methane production through anaerobic digestion of lignocellulosic substrates, as renewable energy source, can offer a potential to replace energy produced from fossil fuels. However, the anaerobic degradation of lignocellulosic substrates can be limited by their compositional and structural features (i.e. the presence of lignin, the crystalline structure of cellulose and its accessible surface area…). Thus, various methods of pretreatment (physical, chemical and biological), originally investigated to bioethanol production, have been quite applied more recently in order to alter the structure of lignocellulosic substrates, facilitating their enzymatic hydrolysis during anaerobic digestion, and consequently enhancing their methane production. Nevertheless, the high variability of pretreatment conditions, methods and results, even when similar substrates are compared, suggest that no definite consensus on their effectiveness for the improvement of the anaerobic biodegradability of agrowastes and energy crops has yet been attained. Thus, this thesis aims: 1) to evaluate the effect of different pretreatment strategies (physical, chemical and biological) on chemical composition and methane production from two lignocellulosic substrates; 2) to evaluate the influence of sorghum varieties on the pretreatment efficiency, evaluated in terms of chemical composition, structural structure and anaerobic digestion performances; 3) to evaluate the applicability and implementation of the pretreatment step prior to a semi-continuous anaerobic digester. For this purpose, different pretreatment categories, such as mechanical, alkaline, thermal, biological (both with commercial enzymes and fungal extracts) and their combinations, were tested on ensiled sorghum forage and wheat straw. Alkaline pretreatment was then performed on six sorghum varieties (one variety of biomass sorghum, two forage sorghum varieties and three varieties of sweet sorghum). Then the alkaline pretreatment of ensiled sorghum forage, found as the best pretreatment strategy for this substrate, was tested prior to a semi-continuous anaerobic digester. Results about the different pretreatment categories suggest that, both chemical and physical structure of ensiled sorghum forage was not influenced by the particle size reduction (between 2 and 0.25 mm). On the contrary, alkaline, thermal, thermo-alkaline, mechanical-alkaline, biological (i.e. enzymatic and fungal) and alkaline-biological pretreatments led to a solubilisation of cellulose, hemicelluloses and lignin for both ensiled sorghum forage and wheat straw, with more or less success. As for anaerobic digestion performances, the mechanical pretreatment did not enhance methane potentials nor anaerobic digestion kinetics of ensiled sorghum forage, between 2 and 0.25 mm. By combining the mechanical and the alkaline pretreatment, an increase in both methane yield (20%) and kinetic constants (by 31%) was observed, due to the effect of the alkaline reagent (10 gNaOH $100g^{-1}$TS), but these results were not significantly influenced by the particle size reduction. Among alkaline, thermal and thermo-alkaline pretreatments, the best results in terms of methane production increase were observed by treating wheat straw at 40 and 100°C with 10 gNaOH $100g^{-1}$TS for 24 h (43% and 67%, respectively) and ensiled sorghum forage with the same conditions (29% and 32%, respectively). Biological pretreatments, performed with commercial enzymatic preparations (i.e. xylanase, endo and eso-glucanase) under anaerobic conditions, led to an increase of methane production of both substrates (15% and 55%, for sorghum and wheat straw, respectively). On the contrary, biological pretreatments performed with the enzymatic extract of one fungal strain (*Polyporus Tulipiferus*), did not improve methane production. The combination of alkaline and biological pretreatment did not led to satisfactory results if compared to the sole alkaline pretreatment, for both substrates. Thus, according to previous results, sodium hydroxide pretreatment (10 gNaOH $100g^{-1}$TS, 40°C, 24 h), which was found as the best pretreatment strategy to treat ensiled sorghum forage, was chosen in order to evaluate its influence on six sorghum varieties. In the case of five varieties sorghum, different from ensiled sorghum forage, alkaline pretreatment had a positive effect in increasing anaerobic digestion kinetics (by 31%), but it did not affect methane production of untreated substrates. For this reason, ensiled sorghum forage was chosen to apply the alkaline pretreatment prior to a semi-continuous anaerobic reactor. Results showed that an alkaline pretreatment step, prior to the anaerobic digestion of ensiled sorghum forage, can have a benefit effect both in enhancing methane production (an increase of 25% on methane production was observed, if compared to that of untreated sorghum) and in giving more stability to the anaerobic digestion process.

List of abbreviations

AGR: Agricultural anaerobic sludge
BMP: Biochemical Methane Potential
BMP_{theo}: Theoretical Biochemical Methane Potential
$BMP_{t\rightarrow\infty}$: Ultimate Biochemical Methane Potential
CEL: Cellulose
CHP: Combined Heat and Power
CMC: Carboxymethylcellulose
COD: Chemical Oxygen Demand
CODs: Soluble Chemical Oxygen Demand
CSTR: Continuous Stirred Tank Reactor
DRX: X- Ray Diffraction
F/M: Substrate to Inoculum ratio
FTIR: Fourier Transformed Infared Spectroscopy
GA: Galacturonic acid
GR: Granular anaerobic sludge from a chemical industry
GR2: Granular anaerobic sludge from a sugar factory
H-CEL: Hemicelluloses
HMF: Hydroxylmethylfurfural
HPLC: High Performance Liquid Chromatography
HRT: Hydraulic Retention Time
IL: Ionic Liquid
LHW: Liquid Hot Water
K-LIG: Klason lignin
LiP: Lignin Peroxidase
LOI: Lateral Order Index
MIM: Microbiologia Industriale, Milano
MIX: Anaerobic mix inoculum
MnP: Manganese Peroxidase
MPR: Methane Production Rate
OLR: Organic Loading Rate
SA: Accessible Surface
SSF: Solid State Fermentation
TKN: Total Kjeldhal Nitrogen
TOC: Total Organic Carbon
TS: Total Solids
UASB: Upflow Anaerobic Sludge Blanket
VFA: Volatile Fatty Acid
Vp: Pore Volume
VP: Versatile Peroxidase
VS: Volatile Solids
WW: Anaerobic sludge from a waste water treatment plant
$Y_{CH4,th}$: Theoretical methane yield

General introduction and specific objectives

Nowadays some 85% of the world's overall energy supply is derived from fossil fuels (IEA, 2010; Edenhofer et al., 2011), which contributes for many environmental damages, the main being global warming (Nigam and Singh, 2010; Saidur et al., 2011). Current energy policies address the use of renewable energy sources (i.e. wind, solar, hydraulic, geothermal, and biomass) in order to reduce greenhouse gas emissions, as well as to increase energy security. In this context, biomasses offer a huge potential for the production of biofuels, and their use could be beneficial to reduce the world's dependency on oils and reduce the global emissions of greenhouse gases (Naik et al., 2010).

Biofuels, referred to as liquid (bioethanol, vegetable oil and biodiesel) or gaseous (biogas, biosyngas and biohydrogen) fuels, that are predominantly produced from biomass, can be categorized into three generations (1st, 2nd and 3rd generations), according to the origin of the biomasses used (Dragone et al., 2010). First generation biofuels are produced from the edible part of the plant (i.e. sugars, grains and seeds), second generation ones come from the non-edible part of the plants (i.e. lignocellulosic substrates) and the third generation ones come from microalgae (Nigam and Singh, 2010). The production of first generation biofuels is in an advanced state with mature technologies and relatively well-understood processing and production pathways. However, the production of first generation biofuels is controversial due to both considerable economic and environmental limitations. The major limitations include the impact that they may have on biodiversity and competition with agriculture arable land used for food production (Schenk et al., 2008). Therefore, lignocellulosic biomasses (i.e. agricultural residues and energy crops) can offer the potential to provide novel "second generation" biofuels (i.e. bioethanol, biogas and biohydrogen), due to the fact that they do not create competition for lands used for food production (Ohman et al., 2006; Kleinert and Barth, 2008).

Among second generation biofuels, the production of methane through anaerobic digestion of lignocellulosic substrates has different advantages as compared to bioethanol production or biohydrogen through dark fermentation. Firstly, contrarily to bioethanol, derived only from cellulose fermentation, biohydrogen and methane can be produced through the conversion of both cellulose and hemicelluloses fractions. However, only 10-20% of the energy potential of an organic substrate is obtained from biohydrogen production, with the remainder converted to organic acids and other

products which need to be degraded, thus rendering methane more advantageous than biohydrogen. Secondly, compared to bioethanol (used only as liquid fuel), methane, once purified and compressed, can be injected in natural gas grid to be used as vehicle fuel or for municipal uses; it can be also used to produce heat and electricity, through cogeneration (Combined Heat Power) systems. Finally, bioethanol and biohydrogen production processes generally produced an amount of residues (such as wastewaters streams form bioethanol conversion or organic acids from biohydrogen production), which need to be treated and valorised. One alternative to treat and to valorise these residues is to convert them into methane, through anaerobic digestion process. The residue of the anaerobic digestion process, called digestate, is mainly composed of stabilised organic materials which can be enriched in nitrogen and phosphorus and can thus be used as an environmentally-friendly fertiliser for the growth of agricultural plants.

Lignocellulosic biomasses mainly consist of cellulose (40-50%), hemicelluloses (25-35%) and lignin (15-20%), which vary quantitatively and qualitatively according to the plant origin (Aman, 1993). However, some compositional and structural features (i.e. the presence of lignin, the crystalline structure of cellulose and its accessible surface area) can limit their enzymatic degradation (Tong et al., 1990; Chang and Holtzapple, 2000; Monlau et al., 2012a). Therefore, in order to achieve the enzymatic hydrolysis for bioethanol and biohydrogen production, as well as in order to improve the production of biogas from some lignocellulosic substrates more difficult to degrade (e.g. sunflower stalks, wheat straw, maize stalks,…), a pretreatment step is necessary (Chang and Holtzapple, 2000; Taherzadeh and Karimi, 2008). In particular, if a pretreatment is actually necessary for bioethanol and biohydrogen production, it is not always necessary for methane production. Indeed, methane production from ensiled maize or some sorghum varieties is possible without pretreatment. Thus, pretreatment of lignocellulosic substrate is rather an option to improve its conversion into methane. At present, due to the necessity of a pretreatment step, the production of second generation fuels is not cost effective because there are a number of technical barriers that need to be overcome before their potential can be realized, although pilot and demonstration facilities are being developed (IEA, 2008). Some authors consider that pretreatment of lignocellulosic substrates is among the most costly steps in the biochemical conversion of lignocellulosic biomass. For instance, Aden and Foust (2009) stated

that pretreatment accounts for more than 16-19 % of the total cost equipment of a lignocellulosic biorefinery, which includes (1) the feedstock or raw material cost, (2) the capital equipment (upfront investment) costs, and (3) operating costs, including utilities and chemicals/supplies consumed (Dale and Ong, 2012).

Pretreatments, originally investigated for the production of second generation bioethanol, are normally classified into three categories: physical (i.e. milling, irradiation, microwaves, steam explosion, liquid hot water…), chemical (i.e. alkaline, acidic, oxidative, ionic liquids, wet oxidation, inorganic salts…) and biological (enzymatic, fungal) or their combination (Mosier et al., 2005). The best method and conditions of pretreatment depend greatly both on the type of substrate and on the final end-products (bioethanol, biohydrogen or methane). In the case of bioethanol production, the key parameter is to convert cellulose into fermentable soluble sugars, by using *Sacharomyces cerevisiae* yeast strain; for biohydrogen production, an increase of the soluble carbohydrates trough dark fermentation using mixed cultures, by hydrolysis of cellulose and hemicelluloses content is required (Monlau et al., 2012b); to enhance methane production the aim of pretreatment is mostly to break down the linkage between polysaccharides and lignin thus making cellulose and hemicelluloses, degradable by anaerobic microorganisms, more accessible to hydrolytic enzymes during anaerobic digestion (Chang and Holtzapple, 2000; Mosier et al., 2005; Taherzadeh and Karimi, 2008). Since, as for complex substrates, the hydrolysis of lignocellulosic substrates is considered the rate limiting step during anaerobic digestion, pretreatments could accelerate the hydrolysis process and improve the final methane production. In a full scale anaerobic digestion plant, this means that the hydraulic retention time of digesters could be decreased. In literature, pretreatment categories were applied with more or less success to enhance methane production of a wide range of lignocellulosic substrates. Nevertheless, the high variability of pretreatment conditions, methods and results, even when similar substrates are compared, suggests that no definite consensus on their effectiveness for the improvement of the anaerobic biodegradability of agro-wastes and energy crops has yet been attained. Thus, a comparison between many different types of pretreatment applied on the same substrate could be useful in order to define the best pretreatment strategy. Moreover, due to the high variability of methane potential and pretreatments results, depending not only on substrate type but also in crop

variety, the same pretreatment need to be tested on other lignocellulosic substrates with different chemical and structural composition. Finally, once the pretreatment strategies have been defined, the best pretreatment condition for methane production defined previously in the batch assays has been tested in anaerobic mesophilic semi-continuous reactors in order to evaluate its feasibility for a possible scale-up, regarding not only the benefits in terms of methane production increase but also considering the energetic, economic and environmental assessments.

In this context, the main objectives of this PhD thesis are:

I) To evaluate the effect of different pretreatment strategies (physical, chemical and biological) on chemical composition, physical structure and methane production of two lignocellulosic substrates (Chapters III and IV);

II) To evaluate the influence of the substrate varieties on the pretreatment efficiency, evaluated in terms of chemical composition, structural structure and anaerobic digestion performance (Chapter V);

III) To evaluate the applicability and implementation of the pretreatment step prior to a semi-continuous anaerobic digester (Chapter VII).

According to the main objectives defined previously, this thesis is composed of eight chapters, structured as follows:

In Chapter I, a comprehensive literature review on the application of pretreatment technologies to enhance methane production from lignocellulosic residues is presented.

Chapter II describes the experimental procedures and methods used to perform pretreatments, biological tests for methane production and analytical determinations.

In Chapter III, results about the effect of physical (mechanical and thermal), chemical (alkaline), and combined physical-chemical pretreatments on chemical composition, physical structure and on methane production potentials of ensiled sorghum forage and wheat straw, were evaluated. Among physical arena, thermal (at relatively low temperatures and pressures) and mechanical (milling) pretreatments were chosen because they could be performed in any agricultural biogas plant. Among

the most used chemical pretreatments (sodium hydroxide and sulphuric acid pretreatments), the use of sodium hydroxide was preferred to that of sulphuric acid, mainly due to its efficacy in altering the structure of lignin and increasing the accessibility to cellulose and hemicelluloses, which is one of the major parameters in anaerobic biodegradation of lignocellulosic substrates.

In Chapter IV, the effect of biological and chemical-biological pretreatments on methane production from ensiled sorghum forage and wheat straw was evaluated. Biological pretreatments, both with commercial enzymatic preparations and the use of an enzymatic fungal filtrate were performed as possible alternatives to physical and chemical pretreatments, mainly according to environmental perspectives.

Chapter V describes the influence of the sorghum varieties both on methane yield and on alkaline pretreatment performances. For this purpose, the effect of the alkaline pretreatment was evaluated in terms of chemical composition, physical structure changes and methane production of six varieties of sorghum (one variety of biomass sorghum, two varieties of forage sorghum and three varieties of sweet sorghum).

In Chapter VI, general remarks about pretreatment strategies previously investigated at batch mode on ensiled sorghum forage and wheat straw were presented by comparing their effects both on chemical composition and methane production, in order to define the best pretreatment strategy for these substrates with a view to scale-up. Then, information about the parameters which can affect both methane production and anaerobic digestion kinetics were tried to be drawn by correlating them with analytical data determinations performed both on untreated and pretreated sorghum and wheat straw.

In Chapter VII, the best pretreatment condition obtained in batch mode on alkaline pretreatment, described in Chapter III, was performed on ensiled sorghum forage in order to evaluate its feasibility in a mesophilic semi-continuous anaerobic reactor for a possible scale-up of the technology. For this purpose preliminary economic and energetic evaluations were also included.

Finally, Chapter VIII presents an overall conclusion of the work and proposes several perspectives for further research work.

Results obtained in this thesis have already been published in peer-review journals and in conference proceedings (Appendix I).

Chapter I. Literature review

The aim of this chapter is to describe the state of art about the application of different pretreatment technologies to enhance methane production from lignocellulosic residues. The first paragraph introduces a global vision of the world energy consumption, with a special focus on the use of biomass, as renewable energy source to produce biofuels. Then, lignocellulosic residues are described according to their structural and compositional characteristics, and up to date data of specific methane production potential from lignocellulosic residues are presented. Finally, the last part of the chapter describes different pretreatment categories, originally developed for bioethanol production, and currently applied to modify compositional and structural features of lignocellulosic substrates for enhancing their methane production. Furthermore, literature data about energetic and economic assessments of various kinds of pretreatments, performed on lignocellulosic substrates, are discussed.

I.1. Biofuels

I.1.1. Introduction to biofuels

One of the major problems that the world is facing is the dependence on fossil fuels for our energy requirements. It was estimated that current world primary energy demand is around 485 EJ $year^{-1}$ and that almost 85% of the total energy supply is derived from fossil fuels, such as petroleum, coal and natural gas (IEA, 2010; Edenhofer et al., 2011), which use directly relates to global warming issues, mainly due to carbon dioxide (CO_2) gas emissions (Schneider and McCarl, 2003), and it also causes a decline in their availability (Liu et al., 2007). Therefore, the request for sustainable and environmental sources of energy for our industrial economy and society has become urgent (Mabee et al., 2005). In 1998, the Kyoto Protocol fixed the objective to reduce by 5.2% the world greenhouse gas emissions over the 2008-2012 periods (Kyoto Protocol, 1998). More recently, the Renewable Energy Directive adopted in 2009 focuses on achieving a 20% share of renewable energies in the EU's energy mix by 2020 (EU Directive 2009/28/EC, 2009). Energy produced from renewable sources (i.e. wind, solar, hydraulic, geothermal, and biomass) could help to minimize the fossil fuel burning and CO_2 emission, to mitigate the global warming, as well as to increase energy security. On a global basis (Figure I.1), it

is estimated that renewable energy accounted for 12.9% of the total primary energy supply in 2008 (IEA, 2010). In this context, biomass, as the largest renewable energy contributor (10.2%) offers a huge potential for the production of biofuels, and its use could be beneficial to reduce the world's dependency on oils, as well as the global emission of greenhouse gases (Naik et al., 2010).

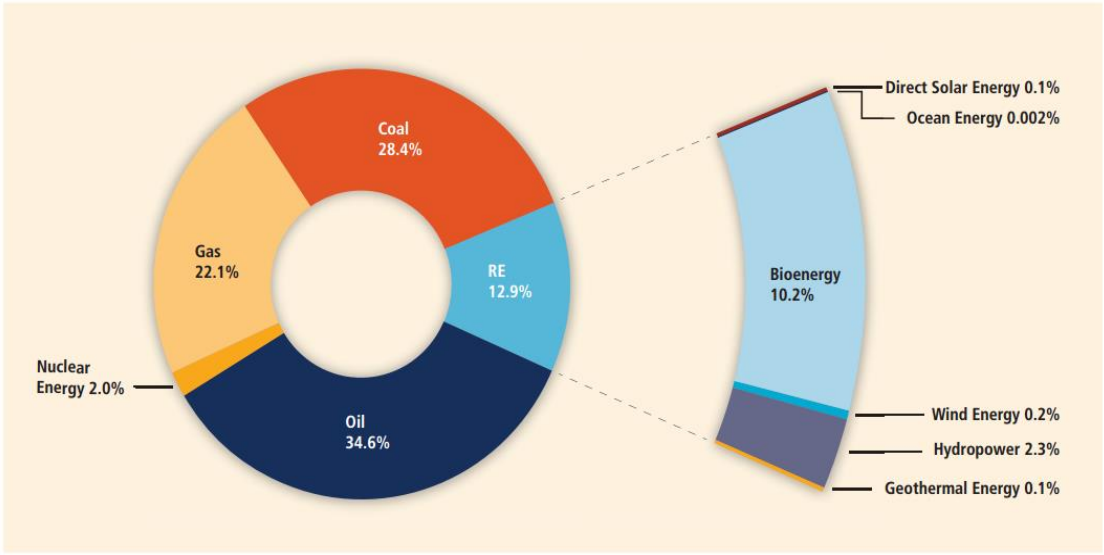

Figure I.1. Shares of energy sources in total global total primary energy supply in 2008 (Edenhofer et al., 2011).

The term biofuel is generally referred to as solid (combustion of pellets, wood chips…), liquid (bioethanol, vegetable oil and biodiesel) or gaseous (biogas, biosyngas and biohydrogen) fuels that are predominantly produced from biomasses (Balat and Balat 2010). Solid biofuels are known as "primary biofuels" and they are used in their unprocessed form like fuelwoods, wood chips and pellets. On the contrary, "secondary" biofuels are liquid and gaseous, produced after biomass processing and transformation (Nigam and Singh, 2010) and they can be categorized into three generations (1st, 2nd and 3rd generations) (Figure I.2), according to the origin of the biomass used (Dragone et al., 2010).

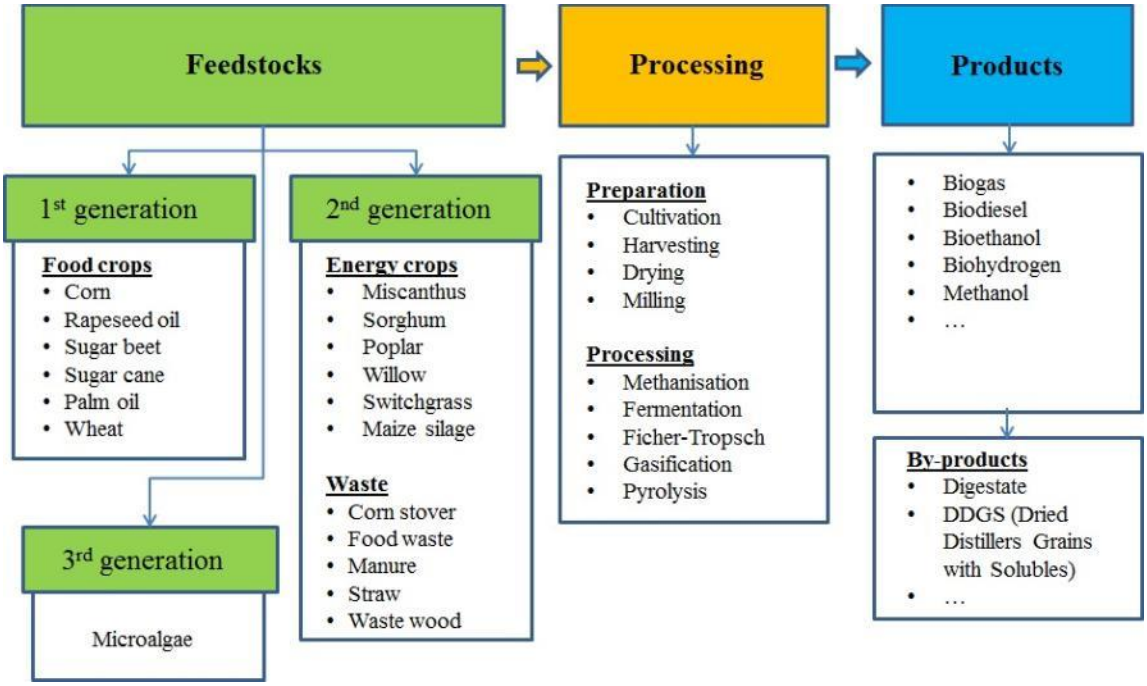

Figure I.2. Classification of "secondary" biofuels (adapted from Azapagic and Stichnothe, 2011).

First generation biofuels are made from agricultural substrates traditionally destined for food and animal purposes (IEA, 2008). These substrates are generally further fermented into bioethanol (rich carbohydrates crops, such as corn and wheat), biodiesel (rich lipid crops, such as soy) and methane (both rich carbohydrates and lipid crops) (IEA, 2008). The production of first generation biofuels is in an advanced state with mature technologies and relatively well-understood processing and production pathways. European Union, United States and some emerging countries (i.e. Brazil, China, Thailand, Indonesia, and Colombia) have successfully implemented a first-generation biofuel industry (as bioethanol and biodiesel). For instance, in USA the production of bioethanol from corn accounted for 1.5 billion L of bioethanol produced from 4.0 billion kg of corn in 2005. As for biodiesel, the largest worldwide producers remain European Union, United States, Brazil and Indonesia. In 2007, 8.6 million tons (t) of edible oil were used by these country for biodiesel production, compared to 132 million tons (t) of edible oil totally produced (Balat, 2011).

However, the production of first generation biofuels is controversial due to considerable economic and environmental limitations. One disadvantage of using the 1st generation biofuels is that they require significant amount of fossil fuels and fertilizers for their own cultivation (IEA, 2008). On the other hand, the production of 1st generation biofuels needs the use of arable land and thus competes with

food, increasing price and lowering availability, thus raising the "food vs fuel" dilemma (Chen and Khanna, 2012). Indeed, the rise in food commodity prices since 2004, which reached high record in 2008, has coincided with the tripling of corn ethanol production from 15 to 50 billion liters over the 2004–2010 period (Chen and Khanna, 2012).

Thus, the wide support that first generation biofuels, enjoyed some years ago, has eroded more recently as new studies began to emerge linking their production to raising food prices, questioning their ability to displace fossil energy, and criticizing their potential contribution to monoculture and deforestation (Searchinger et al. 2008; Fargione et al. 2008; Mitchell 2008). This has stimulated the development of 2nd generation biofuels (i.e. biodiesel, bioethanol, biohydrogen and methane) produced from non-food biomass. Biodiesel can be produced using non edible vegetable oils, such as Jatropha Circus oil (Balat, 2011), while second generation bioethanol, biohydrogen and methane are generally produced from lignocellulosic materials, such as energy crops, cultivated on no arable lands (miscanthus, sorghum, poplar, willow, switchgrass,…) and crop residues (corn stover, manure, straw, waste wood, …). The use of lignocellulosic substrates presents several advantages. Firstly they are abundant nonfood materials, with an estimated worldwide production of 10-50 billion dry tons (t) year^{-1} (Galbe and Zacchi, 2002), which contributes for over 100 EJ year^{-1} (around 20% of the current world primary energy demand), and with production costs in the range of USD 2-3 GJ^{-1} annual (IEA, 2008). Moreover, they do not create competition for lands used for food production, as energy crops can be grown on dedicated non-food lands and crop residues correspond to the waste after food extraction (Ohman et al., 2006; Kleinert and Barth, 2008).

However, at present, contrarily to first generation biofuels, the production of second generation fuels is often not cost effective because there are a number of technical barriers that have to be overcome, such as the necessity of a high cost pretreatment step in order to convert lignocellulosic substrates into bioethanol, biohydrogen or methane. Indeed, pretreatment of lignocellulosic substrates is among the most costly steps in the biochemical conversion of lignocellulosic biomass, which includes: (1) the feedstock or raw material cost, (2) the capital equipment (upfront investment) costs, and (3) operating costs, including utilities and chemicals/supplies consumed (Dale and Ong, 2012). Aden and Foust

(2009) estimated that the pretreatment step accounts for more than 16-19 % of the total cost equipment of a lignocellulosic biorefinery.

Among second generation biofuels produced by biochemical pathways (i.e. bioethanol, biohydrogen and methane), the production of methane through anaerobic digestion of lignocellulosic substrates could be an interesting option for increasing the country biofuel production and achieve the target fixed by the European Union. Methane has some advantages compared to bioethanol. Firstly, contrarily to bioethanol, derived from cellulose fermentation, biohydrogen and methane can be produced through the conversion of both cellulose and hemicelluloses. However, only 10-20% of the energy potential of an organic substrate is obtained from biohydrogen production, with the remainder converted to organic acids and other products which need to be degraded, thus rendering methane more advantageous than biohydrogen. Secondly, methane is a more versatile energy vector than bioethanol and biohydrogen, because once purified and compressed, it can be injected in natural gas grid to be used as vehicle fuel or for municipal uses and also it can be used to produce heat and electricity, through cogeneration (Combined Heat Power) systems. Moreover, compared to most liquid biofuels (i.e bioethanol), methane exhibits far better performances with regard to both agricultural land area efficiency and life cycle emissions (Borjesson and Mattiasson, 2008). Finally, contrarily to the bioethanol residues, which need to be treated, the residue of the anaerobic digestion process (digestate) is mainly composed of stabilised organic materials which can be enriched in nitrogen and phosphorus and can thus be used as an environmentally-friendly fertiliser for the growth of agricultural plants (Frigon and Guiot, 2010). On the other hand, the current bottleneck for the use of biogas is the cost of its upgrading, necessary to inject biogas in natural gas grid.

It has been estimated that within the agricultural sector in the EU, 1500 million tons (t) of biomass could be anaerobically digested each year, half of this potential accounted for by energy crops (Amon et al. 2001). In 2010, the annual contribution of primary energy production that was made by biogas exploitation from landfill, sewage sludges from municipal and industrial wastewater treatment plants, decentralised agricultural plants, municipal solid waste plants and centralised codigestion plants was in the EU of 11 Mtoe, of which 7 Mtoe derived from anaerobic digestion of agricultural crops and

municipal solid wastes. Germany remains the leader with 6.7 Mtoe, while an amount of 1.7 Mtoe and 0.6 Mtoe was registered in United Kingdom and in Italy, respectively (EurObser'ER, 2012). In Italy, it has been estimated an increase of 116% in 2011 (from 150 up to 324 Mtoe) of the energy production derived from agricultural biogas plants and municipal solid waste plants. Positive future trends of electrical energy produced by biogas plants have been also observed. Indeed, the gross electricity production from biogas in the European Union in 2010 was 30.3 TWh and has been estimated an increase up to 65 TWh in 2020 (EurObser'ER, 2012). In the last years the number of anaerobic digesters has increased. For instance in Germany by the end of 2005, there were approximately 3000 farm biogas plants in operation (Weiland, 2005), while in 2010 they were 6000 (IEA, 2010). Also in Italy the number of agro-industrial biogas plants has increased between 86 in 2005 year and up to 511 in 2011 year (Piccinini, 2012).

Third generation biofuels derived from microalgae are considered to be another viable alternative energy source that is devoid of the major drawbacks associated with first and second generation biofuels (Chisti, 2007; Li et al., 2008; Dragone et al., 2010; Nigam et al., 2010). The major advantages of producing biofuels from microalgae are related to the high oil content (around 40% of the dry basis) and they can be used for producing a wide range of biofuels (i.e. biodiesel, biomethane, biohydrogen, bioethanol) (Balat, 2011). Nevertheless, the high water use, the large areas and the high initial costs needed for their cultivation remain the major disadvantages that limit their commercialization (Azapagic and Stichnothe, 2011).

I.1.2. Methane production through anaerobic digestion

Anaerobic digestion is an old and well established biological process originally used to treat a wide range of substrates (i.e. industrial and municipal wastewaters and sludges, municipal solid wastes, manures), especially from the agro-processing industry, containing high concentrations of readily biodegradable organic material in the form of carbohydrates, proteins and fats. Over the last ten years, several studies have focused on the production of biomethane using lignocellulosic residues which

constitute a sustainable source thanks to their abundance and low cost (De Vrije et al., 2002; Panagiotopoulos et al., 2009).

The anaerobic digestion process involves the degradation and stabilization of organic materials under anaerobic conditions by microbial organisms and leads to the formation of biogas which consists mainly of CH_4 (55-75%) and CO_2 (25-45%). The ecology of anaerobic digestion is complex, and involves several microorganisms groups and up to nine steps of conversion of organic matter. However, it is possible to distinguish four main steps (namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis) and four major microorganisms groups (Figure I.3): the hydrolytic-fermentative bacteria that hydrolyze complex organic compounds into simple ones; fermentative bacteria that convert the simple organic compounds into volatile fatty acids with the simultaneous production of hydrogen (H_2) and carbon dioxide (CO_2); the acetogenic bacteria that convert the above-mentioned acids into acetic acid and finally the methanogenic archae that produce methane, either from acetate or from H_2 and CO_2.

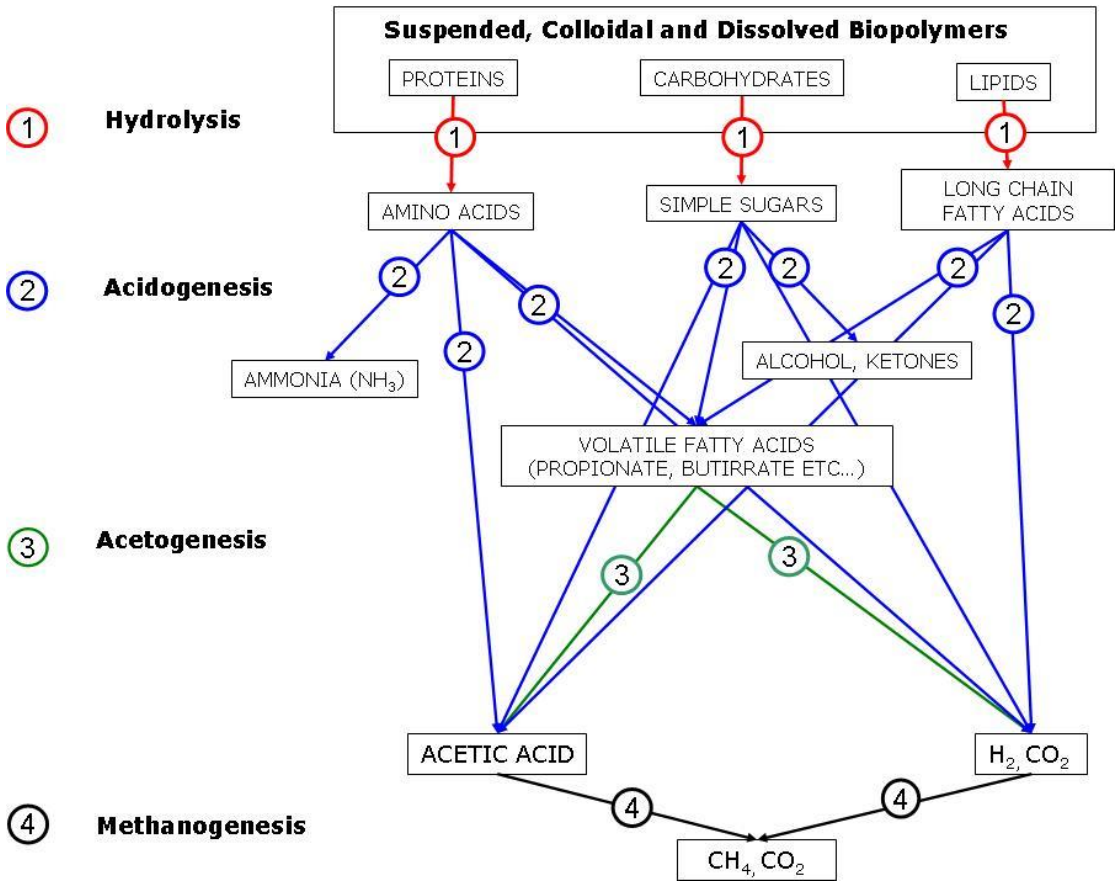

Figure I.3. Anaerobic digestion pathway (hydrolysis, acidogenesis, acetogenesis and methanogenesis steps).

I.1.2.1. Anaerobic digestion pathway

Hydrolysis

During the hydrolysis step organic polymers, such as proteins, lipids and carbohydrates, are hydrolyzed into amino acids, long chain fatty acids, and simple sugars, respectively. Hydrolytic bacteria, known as primary fermenting bacteria, are facultative anaerobes and they hydrolyze the substrate with extracellular enzymes. A wide range of enzymes (cellulases, hemicellulases, proteases, amylases, lipases) can be produced during the biogas process (Taherzadeh and Karimi, 2008). Thus, biogas processes can hydrolyze almost all kinds of substrates (Bruni et al., 2010a). Lignin (Fernandes et al., 2009) is among the exceptions. When the microorganisms can produce the suitable enzymes, hydrolysis is a relatively fast step. On the contrary if the substrate is hardly accessible for the enzymes, as in the case of lignocellulosic substrates, hydrolysis becames the rate-limiting step (Taherzadeh and Karimi, 2008).

Acidogenesis

During acidogenesis, primary fermenting bacteria convert the products of hydrolysis and convert them into volatile fatty acids (e.g. acetic-, propionic- valeric-and butyric acid), alcohols, aldehydes and gases like CO_2, H_2 and NH_3. Acidification is due to a very diverse group of bacteria both obligate and facultative anaerobes. The acidogenic bacteria are able to metabolise organic material down to a very low pH of around 4.

Acetogenesis

Methanogenic microorganisms cannot use directly the acidogenesis products, thus they have to be further transformed, during acetogenic phase, before they can be converted into biogas. During acetogenesis, the conversion of the acidogenic products into acetic acid, hydrogen and carbon dioxide takes place, by secondary fermenting bacteria. In general, two different types of acetogenic mechanisms (hydrogenations and dehydrogenations) can be distinguished. Acetogenic hydrogenations include the production of acetate, as a sole end product either from fermentation of hexoses or from

CO_2 and H_2. Acetogenic dehydrogenations refer to the anaerobic oxidation of long and short (volatile) chain fatty acids. The microorganisms involved are obligate proton-reducing or obligate hydrogen-producing bacteria. They are inhibited by even low hydrogen partial pressures, thus they can survive only in syntrophic association with microorganisms that consume hydrogen, such as acetoclastic methanogens. The acetic acid, hydrogen and carbon dioxide produced during acidogenesis and acetogenesis are the substrates for the methanogenesis step.

Methanogenesis

Finally, methanogenesis is the conversion of acetate, carbon dioxide (CO_2) and hydrogen (H_2) into biogas, which consists mainly of CH_4, (55-75%) and CO_2 (25-45%). The methanogenic microorganisms involved are obligate anaerobic archaea and they can be distinguished into hydrogenophilic and acetoclastic methanogens, which transform the mixture CO_2/H_2 and acetate into methane, respectively. Approximately 65-70% of the methane produced in anaerobic digesters comes from acetate. On the other hand, hydrogenotrophic microorganisms convert the H_2, produced by the secondary fermenting bacteria, and the CO_2 into CH_4, keeping a low hydrogen partial pressure and thus supporting the growth of acetogenic bacteria.

I.1.2.2. Environmental conditions

Anaerobic digestion, as a biological process, is strongly influenced by environmental conditions, such as temperature, pH, and the absence of toxic materials and the availability of nutrients. In particular, methanogens are very sensitive to adverse environmental conditions and for this reason it is necessary to maintain optimal conditions for these microorganisms. For example mesophilic (32-42°C) or thermophilic (50-58°C) conditions are optimal for methanogenesis; pH ranges of 5.2-6.3 and 6.7-7.5 are recommended for hydrolysis/acidogenesis and methanogenesis steps, respectively; a Carbon/Nitrogen (C/N) ratio between 20 and 30 is also optimal for methanogens (Chandra et al., 2012a).

I.1.2.3. Inhibition

It is generally accepted that a wide variety of substances, such as free ammonia (NH_3), H_2S, light metal ions (Na, K, Mg, Ca, and Al), heavy metals (chromium, iron, cobalt, copper, zinc, cadmium, and nickel) have been reported to be inhibitory, at defined concentrations, to the anaerobic digestion processes, causing an adverse shift in the microbial population or inhibition of bacterial growth, and anaerobic reactor upset and failure. In particular, some of them (i.e. NH_3, H_2S, and sodium ion) can be generated after the application of lignocellulosic pretreatments. For instance, free ammonia can be generated by the protein hydrolysis that occurred after chemical pretreatments (i.e. alkaline pretreatment); a high concentration of sodium ions can be obtained after an alkaline pretreatment with sodium hydroxide (NaOH). H_2S can be found by pretreating biomass with sulphuric acid. However, literature data show considerable variation in the inhibition/toxicity concentration levels for most substances, due to the complexity of the anaerobic digestion process where mechanisms such as antagonism, synergism and acclimation, could significantly affect the phenomenon of inhibition. In their review, Chen et al. (2008) reported ammonia concentrations, ranging from 1.7 to 14 g L^{-1} that caused a 50% reduction in methane production. Hydrogenotrofic and acetoclastic methanogens were found inhibited for concentration of H_2S higher than 1 g L^{-1}. Among light metal ions, the toxic concentration for sodium has been reported to be between 5.3 and 53 g L^{-1}.

Moreover, other compounds (i.e. furfural and 5-hydroxymethylfurfural (HMF), originating from the dehydration of pentoses and hexoses; syringaldehyde and vanillin (in their phenolic aldehydes or acids form) originating from the degrading of lignin polymers through the syringyl (S) and guaïacyl (G) units, respectively, generated after thermo-chemical lignocellulosic pretreatments, are well known to inhibit enzymatic hydrolysis (Kim et al., 2011a; Ximenes et al., 2010), glucose fermentation in the case of bioethanol production (Palmqvist and Hahn-Hägerdal, 2000; Klinke et al., 2004) and biohydrogen production through dark fermentation (Quéméneur et al., 2012b). Indeed, such compounds have been found to have negative impact on biohydrogen and bioethanol fermentation processes, or even stop, the fermentation stage at low concentration of 1-2 g L^{-1} for biohydrogen using mixed cultures and bioethanol using *S.Cerevisae* (Delgenès et al., 1996; Laser et al., 2002; Quéméneur

et al., 2012b). The nature and concentration of such byproducts depend on several factors, mainly the nature of lignocellulosic biomass, the kind of pretreatments used and the severity factors for a selected pretreatment (Mussatto and Roberto, 2004; Du et al., 2010). In the case of methane production, data dealing with the possibility of methanogenic inhibition effects caused by these compounds are still limited in literature. According to Barakat et al. (2011) furfural, 5-HMF, syringaldehyde and vanillin concentrations less than 1 g L^{-1} are not expected to inhibit the final methane production of xylose. Park et al. (2011) found that the methanogenic activity was not inhibited by a 5-HMF concentration lower than 5 g L^{-1}. Benjamin et al. (1984) stated that in presence of furfural and 5-HMF, the methanogenics microorganisms require a period of adaptation decreasing the methane production rate but not the final methane yield. Finally, some studies have shown that 5-HMF and furfural at low concentration of 1 g L^{-1} are bioconvertible to methane during anaerobic digestion (Barakat et al., 2011). However, the possibility of methanogenic inhibiting compounds generation has to be taken into account when a pretreatment step is applied prior to anaerobic digestion.

I.2. Lignocellulosic substrates

Lignocellulosic materials, such as agricultural residues (i.e. wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), and dedicated energy crops (i.e. sorghum, switchgrass, salix), represent renewable sources for "second generation" biogas production (Hendriks and Zeeman, 2009; Kumar et al., 2009a).

In this paragraph, chemical composition and structural characteristics of lignocellulosic substrates are presented.

I.2.1. Chemical composition

Lignocellulosic substrates consist of mainly three types of polymers (Figure I.4): cellulose, hemicelluloses and lignin (Hendriks and Zeeman 2009) along with smaller amounts of ash, pectins, proteins and soluble sugars (Jorgensen et al., 2007).

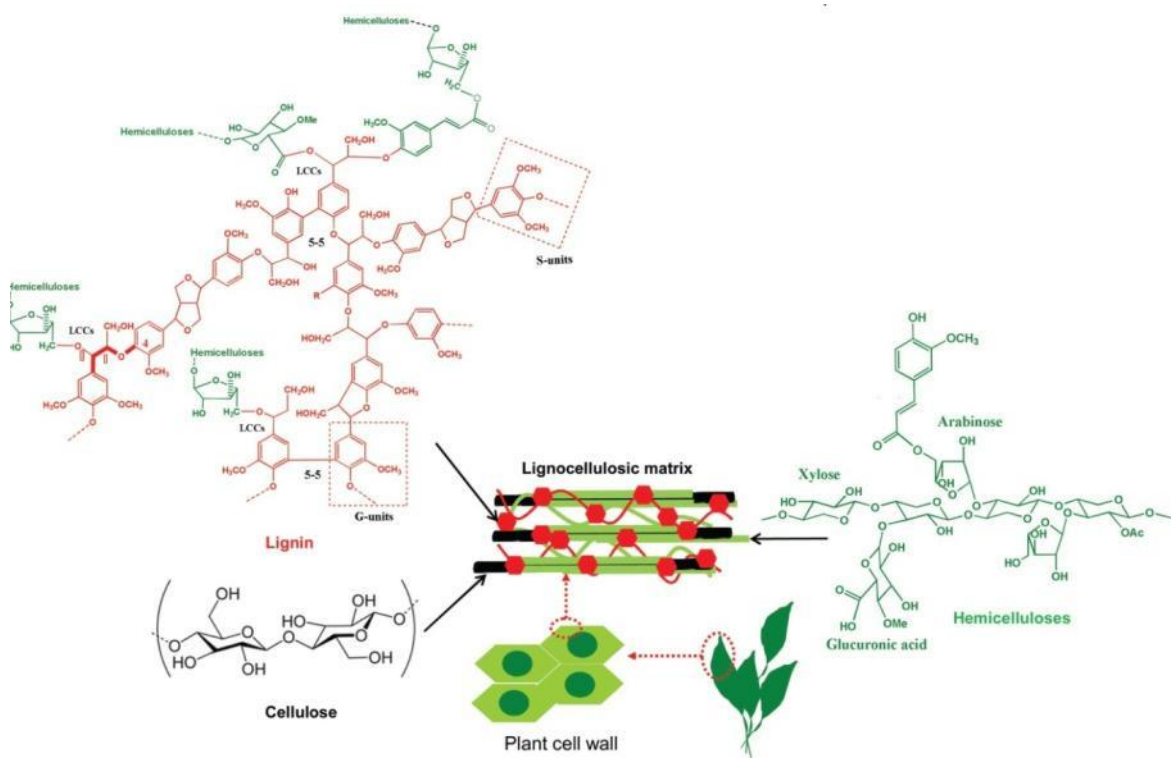

Figure I.4. Scheme of composition of plant cell walls in a lignocellulosic matrix. (adapted from Monlau et al., 2012).

Generally, cellulose is the most abundant component, representing 30-50% of dry matter of lignocellulosic biomass while hemicelluloses and lignin represent 15-30% and 10-25% of the total biomass dry matter, respectively. In addition to these compounds, lignocellulosic biomass can contain non-structural carbohydrates (such as glucose, fructose, sucrose and fructans), proteins and pectins (Monlau et al, 20112a). Ash (3–10% of total feedstock dry matter) is the residue remaining after ignition of herbaceous biomass. It is composed of minerals such as silica, aluminum, calcium, magnesium, potassium, and sodium.

The composition of the three main fractions (cellulose, hemicelluloses and lignin) may vary according to plant type, varieties, part and maturity (Moisier et al., 2005; Vanholme et al., 2010). Table I.1 presents compositions of cellulose, hemicelluloses and lignin encountered in the most common sources of lignocellulosic biomass.

I.2.1.1. Cellulose

Cellulose, the main structural constituent in plant cell walls, is a linear polysaccharide polymer of D-glucose subunits made of cellobiose units, linked by β-(1→4) glycosidic bonds (Fengel and Wegener,

1984; Fengel, 1992). Cellulose strains are 'bundled' together and form, the so-called cellulose fibrils or cellulose bundles. The micro-fibrils are attached to each other by hemicelluloses and bonded together by lignin (Chandra et al., 2012a). Many properties of cellulose depend on its chain length, crystallinity or degree of polymerization (Monlau et al., 2012a). Cellulose in biomass comprises a major proportion of organized crystalline structure and a small percentage of unorganized amorphous structure. Cellulose is more susceptible to enzymatic degradation in its amorphous form. Cellulose is insoluble in water and in most organic solvents, and it can be broken down chemically into its glucose units by treating it with concentrated acids at high temperature.

I.2.1.2. Hemicelluloses

Hemicelluloses can be any of the heteropolymers (matrix polysaccharides) present in almost all plant cell walls along with cellulose (Aman, 1993). Hemicelluloses are composed of five-carbon (C_5) and six-carbon (C_6) sugars. The dominant sugars in hemicelluloses are mannose (C_6 sugar) in softwoods and xylose (C_5 sugar) in hardwoods and agriculture residues (Sun and Cheng, 2002; Lavarack et al., 2002; Emmel et al., 2003; Persson et al., 2006). The backbone of hemicelluloses is either a homo-polymer or a hetero-polymer with short branches linked by β -1,4-glucan bonds and occasionally β -1,3-glucan bonds. Polymers present in hemicelluloses are easily hydrolysable by both dilute acid or base as well as by numerous hemicellulase enzymes (Fengel and Wegener, 1984; Kuhad et al., 1997; Kacurakova et al., 1999; Ebringerová and Heinze, 2000). Hemicelluloses also contain smaller amounts of non-sugars components such as acetyl groups (Kumar et al., 2009a). Kumar et al. (2009a) have noticed acetyl contents of 2.5% and 3.3% respectively for corn stover and poplar. Moreover, Fengel and Wegener (1989) have shown that acetic acid is produced in higher quantities for hardwood feedstocks than for softwood feedstocks.

I.2.1.3. Lignin

Lignin is the third most abundant polymer in nature, after cellulose and hemicelluloses, and it is present in cell walls, in order to give the plant structural rigidity, impermeability and resistance against microbial attack and oxidative stress. It is an amorphous heteropolymer constructed of phenolic

monomer units linked in a three-dimensional structure. Three phenyl propionic alcohols exist as monomers of lignin: (i) coniferyl (G) alcohol (guaiacyl propanol), (ii) coumaryl (H) alcohol (p-hydroxyphenyl propanol), and (iii) sinapyl (S) alcohol (syringyl alcohol). The nature and the quantity of lignin monomers (H, G, S) vary according to species, maturity and the space localization in the cell (Yoshizawa et al., 1993). In general, herbaceous plants, such as grasses, have the lowest lignin content, whereas hardwoods have the highest lignin content (Monlau et al. 2012a). There are three main groups of lignin: lignin from softwoods (gymnosperms) contains mainly guaiacyl units, those from hardwoods (angiosperms) mainly guaiacyl and syringyl units, whereas the lignin from herbaceous plants (non-woody or gramineae) contains all three units (H, G, S) in significant amounts with different ratios (Nimz et al., 1981; Lapierre et al., 1986; Billa and Monties, 1995; Boerjan et al., 2003; Vanholme et al., 2010). Lignin is insoluble in water and optically inactive, which makes its degradation very difficult (Fengel and Wegener, 1984; Grabber, 2005; Akin, 2008). Lignin normally starts to dissolve in water at around 180°C under neutral conditions (Kubikova et al., 1996). The solubility of lignin in acid, neutral or alkaline environments depends, however, on the precursor of the lignin (p-coumaryl, coniferyl, sinapyl alcohol or combinations of them) (Grabber, 2005).

I.2.1.4. Other compounds: pectins, proteins and soluble sugars

As mentioned before, plant cell walls of lignocellulosic biomass are also composed of smaller amounts of pectins, proteins and soluble sugars. Pectins backbone is usually an unbranched chain of D-galacturonic acid units. Galacturonic acids account for approximately 70% of the pectins content (Mohnen, 2008). Other carbohydrates, such as rhamnose, arabinose, galactose, and xylose, may be linked to the backbone, and affect pectins particular properties. Like other polymers, pectins contribute to give physical strength to the plant and to provide a barrier against the outside environment (Harholt et al., 2010). According to Chandel et al. (2011) and Cosgrove et al. (2005), part of pectins may be strongly bound with hemicelluloses, cellulose, and lignin.

Lignocellulosic substrates contain also variable amounts of carbohydrates (mainly starch, sucrose and inulin), which are easily soluble in water and not bound to the solid structure (Chen et al., 2007). High amounts of soluble carbohydrates (16.9% and 28.1% VS) were respectively observed for sweet

sorghum and napiergrass (Gunaseelan et al., 2007). Sugar beets are composed of 67.3% TS of sucrose and only 4.2% and 5.2% respectively of cellulose and hemicelluloses (Panagiotopoulos et al., 2009). Inulin (β-2, 1 fructose) is also an easily soluble sugar present in some lignocellulosic substrates. For instance, Jerusalem artichoke is made up of 70-90% of inulin (Thuesombat et al., 2007). Lignocellulosic substrates are also composed of a small amount of proteins (Cosgrove, 2005). Guo et al. (2012) have evaluated proteins contents of 3.3, 3.4 and 5.1%TS respectively for rice straw, giant reed stalks and giant red leaves. Pakarinen et al. (2012a) have noticed higher proteins contents of 10.6, 16.9 and 18.5%TS for respectively maize, white lupin ad faba bean.

Table I.1. Chemical composition of lignocellulosic substrates (values are referred to %TS).

Subtrate	Celluloses	Hemicelluloses	Klason Lignin	Proteins	Acetyl groups	References
GRASSES / GRAMINAE						
Wheat straw	27-42	11-27	14-21	n.d	1.9	Lee, 1997; Lequart et al., 1999; Sun et al., 2005a; Kaparaju et al, 2009; Akpinar et al., 2009 ; Persson et al., 2009; Gnansounou and Dauriat, 2010; Sathitsuksanoh et al., 2012
Sunflower stalk	34-42	19-21	12-30	2.3-4.8	2	Ruiz et al., 2008; Akpinar et al., 2009; Diaz et al., 2011 ; Monlau et al., 2012c
Barley straw	36	12-29	8-15	n.d	n.d	Sun et al., 2005a ; Persson et al., 2009 ; Park and Kim, 2012
Rice straw	27-44	14-34	13-26	4-5	n.d	Teramoto et al., 2009; Oberoi et al., 2010; Chen et al., 2011; Park and Kim, 2012; Sathitsuksanoh et al., 2012
Maize stems	36-38	10-30	3.5-10.5	7.4	n.d	Sun et al., 2005a; Monlau et al., 2012b
Corn stover	37-39	23-31	14-18	4	n.d	Lee, 1997; Sills and Gossett, 2012; Theerarattananoon et al., 2012; Saha et al., 2013
Switch grass	17-36	20-28	18-26	n.d	n.d	Gnansounou and Dauriat, 2010; Sills and Gossett, 2012
Sweet sorghum bagasse	27-38	15-20	10-20	5-8	n.d	Li et al., 2010; Monlau et al., 2012b
Forage sorghum	32-36	20-23	18-26	n.d	n.d	Li et al., 2010; Manzanares et al., 2012
Mischantus	38-43	24-37	19-25	n.d	n.d	Kurakake et al., 2001; Velasquez et al., 2003; Brosse et al, 2009; Sathitsuksanoh et al., 2012
HARDWOOD						
Hardwood stems	40-55	24-40	18-25	n.d	n.d	Sun and Cheng 2002
Poplar	40-43	12-26	21-28	n.d	n.d	Gonzalez Garcia et al., 2010; Sathitsuksanoh et al., 2012;
Mixed Hardwood	43	15	24	n.d	n.d	Sills and Gossett, 2012
Pinus rigida	43	24	29	n.d	n.d	Park and Kim, 2012
SOFTWOOD						
Larix leptolepis	43	24	29	n.d	n.d	Park and Kim, 2012
Eucalyptus	34-44	18-19	19-30	n.d	3	Romani et al., 2010; Gnansounou and Dauriat, 2010; Park and Kim 2012
Softwood stems	40-50	25-35	25-35	n.d	n.d	Sun and Cheng, 2002

n.d. = not determinated

I.2.2. Compositional and structural characteristics affecting the accessibility and anaerobic biodegradability of lignocellulosic substrates

The physico-chemical characteristics of lignocellulosic materials are highly dependent not only on their constituent properties but also on the organization of their constituents and the interaction between them (Salmen and Olsson, 1998). Many authors tried to determine the substrate characteristics which lead to a decrease in the rate of cellulose hydrolysis and, in many cases, to the incomplete hydrolysis of the lignocellulosic substrates (Koullas et al., 1992; Yoshida et al., 2008; Zhu et al., 2008; Hendriks and Zeeman, 2009). Most of this works concerned bioethanol production and focused on the separation of cellulose from lignin and hemicelluloses, in order to enhance enzymatic cellulose hydrolysis. However, some studies provide useful information in assessing or understanding the anaerobic biodegradability of lignocellulosic materials. Indeed, the anaerobic digestion of such materials has been shown to be limited by the biological hydrolysis step as well as by the accessibility of biodegradable compounds (cellulose and hemicelluloses) (Pavlostathis and Giraldogomez, 1991). Several compositional and structural features can affect the hydrolysis and accessibility of cellulose. Among these parameters, there are the degree of polymerization and crystallinity of the cellulose, the structure of hemicelluloses, the lignin content and composition, the pectins content, the accessible surface area and pore volume.

Thus, to achieve high anaerobic biodegradation yields, lignocellulosic substrates must first be pretreated.

I.2.2.1. Cristallinity of cellulose

The enzymatic hydrolysis of cellulose is mainly influenced by its crystallinity, degree of polymerization, and its accessible surface area. Some authors showed that during the enzymatic hydrolysis of cellulose the readily accessible regions (amorphous regions) are more efficiently hydrolyzed than the crystalline ones (Mooney et al., 1999; Mansfield and Meder, 2003; Hayashi et al., 2005; Jeoh et al., 2007; Gupta and Lee, 2009). A good negative correlation between the crystallinity and the rate of enzymatic hydrolysis of pure cellulose was also found by some authors (Ciolacu et al.,

2008; Gupta and Lee, 2009). However, this relationship is not so clear for lignocellulosic materials, due to their more heterogeneous nature and the contribution of other components, such as lignin and acetyl groups present in hemicelluloses (Chang and Holtzapple, 2000; Koullas et al., 1992). Crystallinity of cellulose is commonly determined by Diffraction Rayon X (DRX) measurement and represents the proportion of cellulose crystalline in the biomass (Driemier and Calligaris, 2011). However, according to other literature results, Fourier Transform Infrared Spectroscopy (FTIR) can be used to compare the indices of cellulose crystallinity from different lignocellulosic materials (Akerholm et al., 2004; Sills and Gossett 2012; Monlau et al., 2012b). Moreover, Marson and El Seoud (1999) have noticed a very good correlation between the indices of crystallinity determined by FT-IR and DRX.

I.2.2.2. Influence of hemicelluloses structure

Hemicelluloses serve as a connection between lignin and cellulose fibers and give rigidity to the whole cellulose–hemicelluloses–lignin matrix (Salmen and Olsson, 1998; Watanabe et al., 2003). In general, the dominant hemicelluloses from all plant cell walls are xylans, which structure is more complex than that of cellulose (Puls, 1997; Izydorczyk and MacGregor, 2000; Saake et al., 2001; Izydorczyk and Dexter, 2008). Xylans structure depends on the degree of substitution of xylose linear chains by arabinose, hydroxycinnamic and uronic acids. All these parameters depend on the species, plant part, and plant maturity (Aman, 1993; Saulnier et al., 1997; Saulnier et al., 1999; Dervilly et al., 2000; Izydorczyk, 2009). The type and the distribution of substitution determine the degree of solubility as well as the capacity to bind the components of the plant cell wall. Hemicelluloses also contain smaller amounts of non-sugars such as acetyl groups that can limit enzymatic hydrolysis (Kumar et al., 2009a; Kim and Holtzapple, 2005). Indeed, Chang and Holtzapple (2000) reported a negative correlation between the enzymatic digestibility and acetyl contents. Kong et al. (1992) have shown that deacetylation increases the yield of sugars obtained from enzymatic hydrolysis of aspen wood. Moreover, Chen et al., 2012 have investigated the effect of corn stover deacetylation by alkaline de-esterification prior to hydrolysis of holocelluloses using dilute-acid pretreatment. Compared to dilute

acid pretreated corn stover controls, the deacetylated corn stover feedstock is approximately 20% more digestible after pretreatment (Chen et al., 2012).

I.2.2.3. Influence of lignin content and composition

As mentioned above, the macromolecular structure of lignin polymer depends mainly on the monomer distribution (G, S and H) and their molecular weight (Nimz et al., 1981; Lapierre et al., 1986; Billa and Monties, 1995; Boerjan et al., 2003; Vanholme et al., 2010). The nature and the quantity of lignin monomers (H, G, S) vary according to species, maturity and the space localization in the cell wall (Yoshizawa et al., 1993). The distribution and composition of lignin are very important for enzyme accessibility and the digestibility of biomass (Adler, 1977; Yuan et al., 2008; Clark et al., 2009; Guo et al., 2010; Ntaikou et al., 2010). Indeed, these factors have been cited as responsible for the higher recalcitrance of softwood-derived substrates (Mooney et al., 1998; Mooney et al., 1999). It was found that substrates containing little or no lignin showed good correlation between initial hydrolysis rates, while substrates with higher lignin content demonstrated a poor correlation (Koullas et al., 1992; Chang and Holtzapple, 2000).

I.2.2.4. Influence of pectins content

Pectins content has been shown to limit the enzymatic accessibility to cellulose (Berlin et al., 2007; Frigon et al., 2012; Pakirinen et al., 2012b;). Indeed, Berlin et al. (2007) and Pakarinen et al. (2012b) suggested that the use of pectinase enzymes can increase the hydrolysis of cellulose, by hydrolizing pectins. Pakarinen et al. (2012b) showed that the hydrolysis of pectin on fiber hemp using commercial pectinase (Pectinex, Novozyme, Denmark, 2.5 mg protein g^{-1}TS substrate) can increase the enzymatic hydrolysis yield by 26% from the theoretical carbohydrates of untreated hemp. However, on the same substrate, hot alkali treatment (121°C, 1h, 1% NaOH (w/w)) and steam explosion (14.5 bar, 200°C, 5min) were found more efficient by increasing the conversion of the total carbohydrates by 60% and 78% (Pakarinen et al., 2012b).

I.2.2.5. Surface area and pore volume

Other parameters such as pore volume (Vp in cm^3 g^{-1}TS) and accessible surface area (SA in m^2 g^{-1}TS) have been shown to affect the biodegradability of lignocellulosic materials. Some authors (Gharpuray et al., 1983; Puri, 1984; Koullas et al., 1992; Chang and Holtzapple, 2000; Laureano-Perez et al., 2005; Park et al., 2007) showed a positive correlation between pore volume, surface area and the enzymatic digestibility of lignocellulosic materials. Among them, Gharpuray et al. (1983) observed that an increase of the accessible surface area from 0.64 to 1.7 m^2 g^{-1}TS, by pretreating wheat straw at 100°C with 10% NaOH (w/w) during 30 min, resulted in higher hydrolysis yield.

I.2.3. Methane production from lignocellulosic substrates

The advantage of producing methane, through anaerobic digestion of lignocellulosic substrates, compared to other biofuels (bioethanol or biohydrogen), is that not only soluble sugars such as pentoses and hexoses, but also polymers (i.e. cellulose, hemicelluloses) are converted into methane. Even potentially inhibiting compounds of bioethanol fermentation (i.e. furfural, HMF, and compounds derived from lignin degradation) can be transformed into methane if not highly concentrated (Benjamin et al., 1984; Barakat et al., 2011).

Methane production and anaerobic biodegradability of lignocellulosic substrates are affected by their chemical composition, which is generally influenced by many factors, including harvest time and frequency, growth stage, plant variety, leaf/stem ratio, growing conditions and fertilisation (Lethomäki 2006; Amon et al., 2007). In literature, many studies were attempted in order to evaluate the methane yield (BMP, LCH_4 kg^{-1}VS) of various agricultural lignocellulosic biomasses, and results are reported in Table I.2.

Table I.2. Methane yields (BMP, $LCH_4\ kg^{-1}VS_{added}$) of lignocellulosic substrates, according to literature data.

Substrate	**BMP ($LCH_4\ kg^{-1}VS_{added}$)**	**References**
Sisal fibre	180	Mshandete et al., 2006
Sunflower stalks	192	Monlau et al., 2012c
Switchgrass	125- 403	Guiot et al., 2009; Jackowiak et al., 2010; Frigon et al., 2008
Wheat grass	160	Romano et al., 2009
Wheat straw	182- 297	Menardo et al., 2012; Amon et al., 2007; Jackowiak et al., 2011; Kaparaju et al., 2009
Barley straw	189- 240	Amon et al., 2007; Dinuccio et al., 2010; Menardo et al., 2012
Rice straw	190-224	Zhang and Zhang, 1999; Dinuccio et al., 2010; Menardo et al., 2012; Ghosh and Bhattacharyya, 1999
Miscanthus	200	Uellendahl et al., 2008
Sugar beet leaves	210	Amon et al., 2007
Sugar beet tops	310	Lehtomaki et al., 2004
Grass hay	230	Lehtomaki et al., 2004
Bagasse	237	Kivaisi and Eliapenda, 1994
Maize stalks	246	Menardo et al., 2012
Cynara stalks	310 - 500	Oliveira et al., 2012
Maize	317-321	Dinuccio et al., 2010; Pakarinen et al., 2011
Potato pulp	332	Kryvoruchko et al., 2008
Winter rye	336	Petersson et al., 2007
Sorghum	270-420	Jerger and Chynoweth, 1987; Chynoweth et al., 1993; Bauer et al., 2009; Hermann et al., 2011
Sunflower (ensiled)	269-300	Bauer et al., 2009; Amon et al., 2007
Maize (ensiled)	370-390	Bruni et al., 2010b; Amon et al., 2007
Barley (ensiled)	375	Bauer et al., 2009
Grass (ensiled)	431	Pakarinen et al., 2009

In some cases the theoretical methane yield is used to predict the methane production of a specific substrate. As generally accepted, the theoretical biochemical methane potential of the substrate (BMP_{theo}, $mLCH_4\ g^{-1}VS$) can be calculated by considering the theoretical methane yield ($Y_{CH4,th}$) of each degradable compound (Symons and Buswell, 1933), the latter calculated by knowing their elemental composition ($C_aH_bO_cN_dS_e$):

$$Y_{CH4,th}\left(mLCH_4\ g^{-1}\right)=\frac{22.4(4a+b-2c-3d-2e)}{8(12a+b+16c+14d+16e)} \quad \text{(Equation I.1)}$$

According to Equation I.1, the theoretical methane yield ($Y_{CH4,th}$) of each biodegradable compound during anaerobic digestion process can be calculated (415 $mLCH_4\ g^{-1}$cellulose $(C_6H_{10}O_5)_n$, 424 $mLCH_4\ g^{-1}$ xylan $(C_5H_8O_4)n$, 288 $mLCH_4\ g^{-1}$uronic acids $(C_6H_{10}O_7)$, 420 $mLCH_4\ g^{-1}$proteins $(C_{14}H_{12}O_7N_2)_n$, 1014 $mLCH_4\ g^{-1}$lipids $(C_{57}H_{104}O_6)$, 727 $mLCH_4\ g^{-1}$lignin $(C_{10}H_{13}O_3)$.

It is also recognized that the theoretical methane yield (BMP_{theo}, $mLCH_4\ g^{-1}VS$) can be calculated by knowing the Chemical Oxygen Demand (COD) and the COD/VS ratio of a substrate. Indeed, at normal condition 350 $mLCH_4$ can be obtained from 1 gCOD removed (McCarty, 1964).

However, experimental methane yields from lignocellulosic biomass can be far lower than the theoretical values, due to their complex structure composed of poorly or not biodegradable compounds (i.e. lignin) which acts as physical barrier preventing the degradation of biodegradable compounds (i.e. cellulose and hemicelluloses). The anaerobic biodegradability of a specific substrate can be calculated by comparing experimental (BMP, $mLCH_4\ g^{-1}VS$) and theoretical ($BMP_{theo,}$ $mLCH_4\ g^{-1}VS$) methane yields as follows (Equation I.2):

$$BD(\%)=\frac{BMP}{BMP_{theo}}\cdot 100 \quad \text{(Equation I.2)}$$

The influence of lignin on the anaerobic biodegradability of lignocellulosic substrates is documented by many authors who showed that lignin content plays a major role in methane production by limiting the access to holocelluloses (cellulose and hemicelluloses). According to Tong et al. (1990), holocelluloses, which are anaerobically-biodegradable compounds in their pure form, appear to be less biodegradable or even completely refractory when combined with lignin, thus limiting the methane production. Kobayashi et al (2004) found a strong negative correlation (R^2 =0.95) between the amount of methane produced and the amount of Klason lignin in bamboo. Negative correlations were also found between the lignin content and biochemical methane potentials for manure and energy crops (R^2=0.88) (Triolo et al., 2011). Monlau et al., (2012c) found a strong negative correlation (R^2 = 0.92) between the biochemical methane potential of sonflower stalks and its lignin content. Furthermore, Buffiere et al. (2006) showed a link between the methane potential of various lignocellulosic residues

and the sum of their cellulose and lignin contents: the higher the sum of cellulose and lignin, the lower the methane potential. Thus, to achieve high anaerobic biodegradation yields, lignocellulosic substrates must first be pretreated, in order to break down the linkage between polysaccharides and lignin to make cellulose and hemicelluloses more accessible to anaerobic microorganisms. This means also an improvement of the anaerobic digestion hydrolysis kinetics. Indeed, Monlau et al. (2012c) showed a good positive correlation ($R^2 = 0.91$) between the sum of solubilised proteins, hemicelluloses, cellulose and uronic acids with the anaerobic hydrolysis kinetic constants, after thermo-chemical pretreatments on sunflower stalks.

These findings confirm that hydrolysis is the limiting step of the anaerobic digestion of lignocellulosic, as complex substrates. Hydrolysis is a complex multi-step process which takes place by extracellular enzymes excreted by the biomass. Batstone (2000) defined three mechanisms for the release of enzymes and consequent hydrolysis of complex substrates: (i) the organisms secrete enzymes to the bulk liquid where they are adsorbed onto a particle or react with a soluble substrate (Jain et al., 1992); (ii) the organisms attach to a particle, produce enzymes in its vicinity and benefit from soluble products released by the enzymatic reaction (Vavilin et al., 1996); (iii) the organism has an attached enzyme that may also acts as a transport receptor to the interior of the cell. This method requires the organism to absorb onto the surface of the particle. Few studies on anaerobic sludge (Hobson, 1987; Philip et al., 1993) showed that enzymes seemed to be cell associated. Mechanisms 2 and 3 seem therefore more likely than mechanism 1 and a good contact between organisms and substrate is an important prerequisite for a good hydrolysis.

In literature authors tried to model the complex hydrolysis mechanisms. In Anaerobic Digestion Model n.1 (Bastone et al., 2002), the hydrolysis rates are taken by assuming a first order kinetic model (Equation I.3):

$$\frac{dS}{dt} = -k_h S \qquad \text{(Equation I.3)}$$

Where:

S is the concentration of the biodegradable substrate, t the time and k_h the first order hydrolysis constant.

Some authors proposed other more complicated models than previous one. For instance, Vavilin et al. (2008) proposed two models: the first one, based on Contois kinetic, that considers the growth of hydrolytic/acidogenic biomass, and the second one which is a two-phase kinetic model, considering surface colonization and biodegradation separately. Ramirez et al. (2009) have proposed a modified ADM1 version using Contois model associated to the growth of hydrolytic bacteria.

However the first order kinetic model, which is not directly coupled to bacterial growth, was found in many cases to be satisfactory representative of the anaerobic degradation process in the case of slowly degradable lignocellulosic substrates for which the disintegration and hydrolysis are the limiting steps. Indeed, Angelidaki et al. (2009) proposed this expression to obtain further information on the substrate studied like the hydrolysis rate, by interpolation of experimental (BMP) curves from BMP tests. By using the first part of the experimental curve built for the determination of the ultimate methane production of a given substrate, it is possible to define the constant k_h for a first order hydrolysis model, as the slope of the linear curve obtained by integrating the equation I.3, as follow (Equation I.4):

$$\ln \frac{BMP_{t\to\infty}-BMP}{BMP_{t\to\infty}} = -k_h \cdot t \qquad \text{(Equation I.4)}$$

Where $BMP_{t\to\infty}$ is the value of the ultimate methane yield (mLCH4 g^{-1}VS) and BMP is the methane yield (mLCH4 g^{-1}VS) at the time t, during the batch tests. Now, the value of the first order hydrolysis constant, kh (d^{-1}) can be determined.

I.3. Pretreatments of lignocellulosic substrates

As described above, several compositional and structural characteristics (i.e. cellulose cristallinity, hemicelluloses structure, lignin content, pectins content, surface area and pore size) provide resistance to biological degradation, limiting the conversion of lignocellulosic substrates into methane. Thus, pretreatment methods became fundamentals in order to break the resistant leyer of lignin, reducing the cristallinity of cellulose, thus increasing the availability of carbohydrates (amorphous cellulose and hemicelluloses), to be converted into methane. Various methods of pretreatment, originally

investigated for the production of second generation bioethanol, were performed and well described in many reviews papers (Mosier et al., 2005; Galbe and Zacchi, 2007; Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009; Alvira et al., 2010). Only some of them have been applied to enhance methane production of lignocellulosic substrates, and they are detailed below. Pretreatments are generally classified into three categories: physical (i.e. chipping, grinding, milling, ultrasound, microwaves, steam explosion, liquid hot water); chemical (i.e. alkali, acidic...); biological (commercial enzymes and fungi), or various combination of them (Mosier et al., 2005).

I.3.1. Physical pretreatment

Physical pretreatments include mechanical (i.e. grinding, chipping, milling, knife mill, scissors…), microwaves, ultrasound, steam explosion, and liquid hot water. **Mechanical** pretreatments generally lead to a reduction of particles size (between 5 cm to few mm), decreasing the degree of crystallinity of cellulose and increasing the accessible surface area and pore size of the substrate (Palmowski and Muller, 2000; Galbe and Zacchi, 2007; Taherzadeh and Karimi, 2008). Therefore the organic matter availability to enzymes or microorganisms is favoured (Hemery et al., 2009; Dumas et al., 2010; Ghizzi d. Silva et al., 2010). For instance, Gharpuray et al. (1983) have shown that ball milling pretreatment was found to be effective in increasing the specific surface area (2.3 m^2 g^{-1}pretreated substrate compared to 0.64 m^2 $g^{-1}$for raw wheat straw) and in decreasing the crystallinity index (23.7 compared to 69.6 for the raw wheat straw). This process has been considered by some authors not cost-effective because it requires too much energy and it has been shown that greater amounts of energy are needed to reduce size when biomass has higher moisture content (Yu et al., 2006; Ghizzi D. Silva et al., 2010). Kratky and Jirout (2011), estimate a consumption of about 33% of the total electricity demand of a biogas plant. Nevertheless, the energy consumption of mechanical pretreatments is strictly related to the final particle size and to the kind of substrate used (structure and moisture content). However, to date, at agricultural farms, shredding equipment is usually installed between the biomass storage tank and the biogas plant (final screen size of 4-6 mm) to avoid clogging of pumps and pipes by large particles. The effect of mechanical pretreatment on methane production

was investigated by many authors (Table I.3) and results are variable, suggesting that the impact on methane production and hydrolysis kinetics may depend on pretreatment methods used (cutting milling, ball milling, chipping, grinding), particle size reductions, and physical structure of substrates. Mshandete et al. (2006) studied the degradation and biogas potential of sisal fiber with sizes ranging from 10 cm to 2 mm. It was shown that the methane yield was inversely proportional to particle size with an increase of 22% when the fibers were cut at 2 mm size (220 $LCH_4\ kg^{-1}VS$ for 2 mm, compared to 180 $LCH_4\ kg^{-1}VS$ for untreated fibers). Recently, Menardo et al., (2012) found an increase of methane potentials after mechanical pretreatment of barley straw (by 54% for particle size of 0.5 cm) and wheat straw (by 83.5% for particle size of 0.2 cm). On the contrary, no significant methane potentials improvement was noticed for maize stalks. Dumas et al. (2010), found no significant differences on the maximum methane production of wheat straw after a cutting mill and centrifugal grinding pretreatment between 804 and 45 µm particle sizes. Nevertheless, they found a significant increase in the first order kinetic constant between 113 and 45 µm. Ficara and Malpei (2011) found that particle size variations between 2 and 5 mm did not have relevant effects on the hydrolysis rate on ensiled maize and sweet corn mixture.

Digestibility of lignocellulosic biomasses can also be enhanced by use of **ultrasounds or microwaves**. These types of pretreatment lead to cleavage of β-1,4-glucan bonds increasing the accessible surface area and reducing the crystallinity of cellulose (Takacs et al., 2000; Chandra et al., 2012a). Microwaves pretreatment was found efficient in increasing the solubilization of cellulose from switchgrass as glucose content in the hydrolyzate of 400 mg L^{-1} and 1 g L^{-1} were noticed after 90°C and 150°C respectively. Nevertheless, ultrasounds and microwaves technologies have several disadvantages, including high energy consumption (Chandra et al., 2012a), production of possible inhibiting byproducts like phenolic acids (Jackowiak et al., 2010), complex operation procedures and strict monitoring of equipment (Pan et al., 2008), that actually limit their commercial applications.

During **steam explosion**, lignocellulosic biomass is heated rapidly to a high temperature (160-260°C) with sufficient pressure (1-7 MPa) to enable water molecules to penetrate the substrate structure for a few minutes. The pressure is then suddenly released to allow the water molecules to escape in an

explosive manner. Steam pretreatment can be improved by using an acid catalyst, such as H_2SO_4 or SO_2 (0.3-3 g_{acid} $100g^{-1}$), which increases the recovery of cellulose and hemicelluloses sugars (Ballesteros et al., 2000; Galbe and Zacchi, 2007). This pretreatment opens up the plant cells, increases surface area and enhances the digestibility of biomass (Ballesteros et al., 2000). Piccolo et al. (2010) have shown that SO_2 steam explosion at 190°C during 2 min increase the accessible surface area of wheat straw from 1.1 m^2 g^{-1} (untreated wheat straw) to 1.9 m^2 g^{-1}. Limitations of steam explosion are the incomplete disruption of the lignin-carbohydrate matrix and the formation of hemicelluloses and cellulose degradation byproducts as water acts as an acid at high temperature (e.g. furfural and hydroxymethylfurfural) (Kumar et al., 2009a).

Steam explosion has been also widely investigated to enhance methane potential from lignocellulosic residues and some results are summarized in Table I.3. When steam explosion at 180°C for 25 min was applied to wheat straw, methane production increased by 31% (Bauer et al., 2009). Teghammar et al. (2009) combined steam explosion with chemical pretreatment. They observed that the combination of steam explosion with 2 gNaOH $100g^{-1}_{substrate}$ and 2 gH_2O_2 $100g^{-1}_{substrate}$ enhanced the methane yield of paper tube residues from 238 LCH_4 kg^{-1}VS to 493 LCH_4 kg^{-1}VS (Teghammar et al., 2009).

Liquid hot water (LHW) is a hydrothermal treatment which does not require rapid decompression and does not employ any catalyst or chemicals. Pressure is generated to maintain water in the liquid state at elevated temperatures (200–240 °C) for few minutes (Kumar et al., 2009a). However, actually liquid hot water pretreatments performed at relative low temperature (90°-170°C) for 0.5-1 h, were also applied. Water under pressure penetrates into the biomass, increasing surface area and hence removing hemicelluloses and lignin (Mosier et al., 2005). Three types of reactor can be used for liquid hot water pretreatment: co-current (biomass and water are heated together for a certain residence time), counter-current (water and lignocelluloses move in opposite directions), and flow-through (hot water passes over a stationary bed of lignocelluloses) (Liu and Wyman, 2005; Mosier et al., 2005). In general, liquid hot water pretreatments are attractive for their cost-savings potential: no catalyst requirement and low-cost reactor construction due to low-corrosion potential. However, water and energetic requirement remain higher (Alvira et al., 2010). Liquid Hot Water pretreatments have been

investigated to enhance methane production from lignocellulosic residues and some results are summarized in Table I.3. After thermal pretreatment at 120°C during 1 h, Menardo et al. (2012) showed 32% and 64% methane increase respectively for rice straw and wheat straw. Monlau et al. (2012c), showed an increase in methane production of 14%, by treating sunflower stalks at 170°C for 1h.

Table I.3. Methane potentials ($LCH_4\ kg^{-1}VS$) of physically pretreated lignocellulosic substrates.

Pretreatment method	Lignocellulosic substrates	Pretreatment condition	CH_4 production after pretreatment ($LCH_4\ kg^{-1}VS$)	Increase CH_4 (%)	References
Food mix cutter	Barley straw	2 cm (**Initial size** : 5cm)	339	41	Menardo et al., 2012
Grinding	Wheat straw	0.5 mm (**Initial size** : not determined)	248	53	Sharma et al., 1988
	Bermuda grass	0.4 mm (**Initial size** : not determined)	228	66	Sharma et al., 1988
Milling	Sisal fibre	2 mm (**Initial size** : 10 cm)	220	22	Mshandete et al., 2006
	Maize silage	2 mm (**Initial size** : 8 mm)	410	11	Bruni et al., 2010b
	Mixture of maize silage and sweet corn	2 mm (**Initial size** : 5 mm)	304	0	Ficara and Malpei, 2011
Cutting mill and centrifugal grinding	Wheat straw	0.045 mm (**Initial size** : 0.8 mm)	302	0	Dumas et al., 2010
Knife milling	Maize stalks	0.2 cm (**Initial size** : 2 cm)	272	0	Menardo et al., 2012
	Wheat straw	0.2 cm (**Initial size** : 5 cm)	334	84	Menardo et al., 2012
	Barley straw	0.5 cm (**Initial size** : 5 cm)	370	54	Menardo et al., 2012
Microwaves	Switchgrass	2450 MHz, 150°C, power range 400 and 1600 W	320	8	Jackowiak et al., 2010
	Wheat straw	2450 MHz, 150°C, power range 400 and 1600 W	345	28	Jackowiak et al., 2011
	Grass	2450 MHz, max. 260°C, power range 0 and 1800 W	-	-18	Li et al., 2012
Ultrasound	Sugar beet leaves	Machine J.P Selecta Ultrasons 110 W, 3 min	572	43	Wang, 2011
	Maize	Machine J.P Selecta Ultrasons 110 W, 3 min	710	41	Wang, 2011

Pretreatment method	Lignocellulosic substrates	Pretreatment condition	CH_4 production after pretreatment ($LCH_4\ kg^{-1}VS$)	Increase CH_4 (%)	References
	Sunflower oil cakes	specific energy 24.000 kJ kg^{-1}TS	187	54	Fernandez-Cegri et al., 2012
Steam Explosion	Japanese cedar chips	4.51MPa, 258°C	365	-	Take et al., 2006
	Bamboo	5min, 243°C	215	-	Kobayashi et al., 2004
	Potato pulp	15min, 107°C	373	12	Kryvoruchko et al., 2008
	Wheat straw	10 min, 170°C	361	31	Bauer et al., 2009
	Paper tube residues	10 min, 220°C, 4 ($gH_2O_2\ 100g^{-1}_{substrate}$) + 4 ($gNaOH\ 100g^{-1}_{substrate}$)	493	122	Teghammar et al., 2009
Liquid Hot Water	Japanese cedar chips	170 °C, 30 min	28	-	Take et al., 2006
	Wheat straw	120°C, 1h	299	64	Menardo et al., 2012
	Rice straw	120°C, 1h	261	32	Menardo et al., 2012
	Barley straw	90°C, 1h	340	42	Menardo et al., 2012
	Maize stalks	120°C, 1h	267	9	Menardo et al., 2012
	Cynara stalks	160°C, 1h	620	24	Oliveira et al., 2012
	Sunflower stalks	170°C, 1h	219	14	Monlau et al., 2012c

I.3.2. Chemical pretreatment

To this group belong pretreatments that are purely initiated by chemical reactions for disruption of the biomass structure. Among chemical pretreatments, in this paragraph were described only those have been applied to enhance methane production. They include oxidative, alkaline, dilute-acid, ionic liquids, wet oxidation and inorganic salts pretreatments. Table I.4 summarizes some recent literature data about methane production from chemical pre-treated agricultural substrates.

Oxidative pretreatment (H_2O_2) is usually used in association with alkali (pH=11.5) (Rabelo et al., 2008). In order to enhance methane production, oxidative pretreatment was carried out at concentration of 4 gH_2O_2 $100g^{-1}_{substrate}$ at high temperature of 190°C and short time of 30 min, or at low temperature of 55°C and high residence time of 24 hours (Teghammar et al., 2009; Monlau et al., 2012c). Oxidative pretreatment can be used to solubilize lignin and hemicelluloses and to increase the surface area of cellulose (Monlau et al., 2012c). Delignification of 66% was observed compared to untreated corn stover by pretreating corn stover with 1 gH_2O_2 $100g^{-1}_{substrate}$ (pH=11.5) at 65°C for 3h (Selig et al., 2009). No generation of furfural and 5-HMF were observed by pretreating sunflower stalks at 4 gH_2O_2 $100g^{-1}$TS during 55°C for 24h (Monlau et al., 2012c). Oxidative pretreatments have been poorly investigated to enhance methane production from lignocellulosic residues (Table I.4). Teghammar et al. (2009) reported a low increase of 5% in term of methane potentials after oxidative pretreatments (4 gH_2O_2 $100g^{-1}_{substrate}$, 190°C, 30min) of paper tube residuals (Teghammar et al., 2009). Monlau et al. (2012c) recently showed an enhancement of 23% of the methane potential of sunflower stalks after oxidative pretreatment (55 °C, 4 gH_2O_2 $100g^{-1}$TS, 24h).

Alkaline pretreatment is usually performed by using bases such as sodium, potassium, and calcium hydroxide**.** To enhance methane production, sodium hydroxide is the main chemical reagent used to perform alkaline pretreatment, as shown in Table I.4. Alkaline dosages, contact times and temperatures of pretreatment are quite variable in literature and depend on the substrate used. Alkaline dosages ranging between 1-30 gNaOH $100g^{-1}_{substrate}$, temperatures ranging between 10°C and 200°C and contact times between few minutes to days, were found in the literature. According to Zhu et al.

(2010a), alkaline pretreatment are efficient in lignin removal, by preserving most of the carbohydrates and in particular cellulose. For instance, pretreatment of miscanthus with 12 gNaOH $100g^{-1}_{substrate}$ at 70°C for 4 h led to 77% of delignification compared to the raw substrate (De Vrije et al., 2002). Monlau et al. (2012c) have shown that alkaline pretreatment (55°C, 4 gNaOH $100g^{-1}$TS, 24 h) removed 36 % of lignin without cellulose solubilization. Lime pretreatment was also found efficient in removing more than 55% of lignin and 90% of acetyl groups of corn stover (Da Costa Sousa et al., 2009). Alkaline pretreatment can also swell the fibers and increases pore size and accessible surface area, facilitating the diffusion of the hydrolytic enzymes (Datta, 1981; Gharpuray et al., 1983). For instance, Gharpuray et al. (1983) noticed an increase of the accessible surface area of wheat straw from 0.64 m^2 g^{-1} (untreated wheat straw) to 1.9 m^2 g^{-1} after alkaline pretreatment at 10 gNaOH $100g^{-1}_{substrate}$ for 129°C during 2 h. In addition, alkali pretreatments can remove acetyl and the various uronic acid substitutions on hemicelluloses, which decrease the accessibility of the enzyme to the hemicelluloses and cellulose surface (Chang and Holtzapple, 2000). Actually, chemical requirement, the possible accumulation of sodium ions and/or the possible formation phenolic compounds, which can inhibit methane production, remain the major drawback of this technology. Alkaline pretreatment have been widely investigated to enhance methane potentials of various lignocllulosic substrates (Table I.4). For instance, the application of a NaOH pretreatment to corn stover led to high methane production increases of 75%, respectively (Zheng et al., 2009). The best enhancement of methane yield (792%) was observed by Neves et al. (2006) after alkaline pretreatment of barley waste, but this pretreatment was performed at high sodium hydroxide concentration of 30 gNaOH $100g^{-1}_{substrate}$ during overnight at 25°C. Dongyan et al. (2003) compared the effects of sodium hydroxide (8 gNaOH $100g^{-1}_{substrate}$) and ammonia (5 gNH_3 $100g^{-1}_{substrate}$) pretreatment on corn stalks during 20 days at room temperature. They found an enhancement of methane yield of 207% and 51%, for sodium hydroxide and ammonia pretreatments, respectively.

Dilute-acid pretreatment can be carried out either at short retention time (1–5 min) and high temperature (120-170 °C) or at long retention time (more than 15 minutes, hours or days) and lower temperature (around 25°C). Among chemical reagents, sulfuric acid is the most applied acid (around 4

g $100g^{-1}$ $_{substrate}$), while other acids have been used, such as hydrochloric acid (Taherzadeh and Karimi, 2008; Fernandes et al., 2009; Kumar et al., 2009a). Acid pretreatment can be successfully used to remove efficiently hemicelluloses by breaking ether bonds in lignin/phenolics-carbohydrates complexes without dissolving lignin (Knappert et al., 1981). Weak acids, such as peracetic acid which is also an oxidant, can also be used for acid hydrolysis. It was shown to lead to a drastic reduction in the crystallinity and increase the accessible surface area of wheat straw (Gharpuray et al., 1983). The major drawback of acid hydrolysis is the possible formation of inhibitors compounds of anaerobic digestion mainly furfural, derived from the hemicelluloses degradation (Larsson et al., 1999). Moreover, H_2S can be found by pretreating biomass with sulphuric acid, which can compete with the biogas process (Zehnder and Stumm, 1988). Other drawbacks are related to the use of a corrosive reagent, with corresponding downstream neutralization, and special materials for reactor construction. Like for thermo-alkaline pretreatment, dilute-acid pretreatment have been well investigated last years to enhance the methane production using lignocellulosic residues (Table I.4). The application of HCl pretreatment to bagasse and coconut fibers led to methane production increases of 32% and 76%, respectively (Kivaisi and Eliapenda, 1994). Badshah et al. (2012) have recently shown the application of dilute-acid pretreatment on sugarcane bagasse at 121°C during 15 min and 0.02 H_2SO_4 g L^{-1} led to an increase of 166% compared to untreated bagasse (Badshah et al., 2012). Dilute acid pretreatment using HCl was also found efficient to enhance the methane potentials of 21% and 48% respectively for sunflower stalks and sunflower oil cakes (Monlau et al., 2012c; Monlau et al, 2012d). On the contrary, dilute-acid pretreatment on maize silage was found unefficient, which can be explained by the nature of the substrate. Indeed, ensiling acts as a pretreatment and can hide the further effect of dilute-acid pretreatment on the methane potentials (Pakarinen et al., 2011).

Treating lignocellulosic biomass with **Ionic Liquids (IL)**, also called "green solvents", has gained attention in the last decade as an aid to dissolve lignocellulosic biomass (Dadi et al., 2007; Nguyen et al., 2010; Samayam and Schall, 2010). Only one study was found (at 130°C, for 1-15 h) to enhance methane production of two lignocellulosic substrates (rice straw, triticale straw) (Table I.4). This pretreatment can dissolve a great number of lignocellulosic biomass (e.g. corn stover, cotton, bagasse,

switchgrass, wheat straw, and wood), thus producing cellulose with a little residual crystallinity (Samayam and Schall, 2010). For instance, ionic liquid at 120°C for 30 min using (Emin)OAc (1-ethyl 3-methyl imidazolium acetate) was found efficient in reducing the crystallinity of switchgrass from 21% to 6% (Samayam and Schall, 2010). Ionic liquids can also remove lignin efficiently with more than 40% of lignin removal on wood (Lee et al., 2009). Ionic liquids have minimal environmental impact due to their low-volatility and can be reused after pretreatment, thus reducing costs of solvents usage (Brodeur et al., 2011; Dadi et al., 2007). However, at the present time, this process is still expensive due, mainly, to the high cost of ionic liquids (Nguyen et al., 2010). Moreover the application of this pretreatment to produce biofuels needs to be tested, in order to verify the ability of microorganisms to ferment sugars in the presence of these solvents (Brodeur et al., 2011). In term of methane potential (Table I.4), promising results have been observed using ionic liquids N-methylmorpholine-N-oxide (NMMO) to enhance methane production of lignocellulosic substrate (Teghammar et al., 2011). Indeed, an increase of 608% of the methane potential from rice straw was observed after ionic liquid (NMMO) pretreatments at 130 °C during 1 hour (Teghammar et al., 2011).

Wet oxidation pretreatment, as alternative to steam explosion, operates with oxygen or air in combination with water at elevated temperatures (above 120°C) and pressure. One study (Table I.4) was found with pretreatment temperature of 195°C and pressure of 12 bar O_2. This process is an effective method for disrupting the crystalline structure of cellulose and for separating the cellulosic fraction from lignin and hemicelluloses (Panagiotou and Olsson, 2007). Originally, this method was applied in order to treat wastes with high organic matter (Jorgensen et al., 2007). Then, it was successfully applied for the treatment of hardwood and wheat straw (Schmidt and Thomsen 1998). Furfural and hydroxymethylfurfural, known inhibitors of microbial growth were not observed following the wet oxidation treatment (Kumar et al., 2009a). High investiment and reagent costs and the potential formation of inhibitors compounds, derived from cellulose, hemicelluloses and lignin degradation at high temperatures, remain the major drawbacks of this technology. Up to date, few studies have investigated the effect of wet oxidation to enhance methane potential from lignocellulosic

residues (Table I.4). Wet oxidation gave interesting results with energy gains of 80% on miscanthus and willow (Uellendahl et al., 2008).

Inorganic salts (NaCl, KCl, $FeCl_3$...) have been tested as catalysts for the degradation of hemicelluloses in corn stover (Liu et al., 2009a; Liu et al., 2009b). In general, such pretreatment is performed at high temperature (140°C-200°C) for few minutes or hours (Liu et al., 2009a; Liu et al., 2009b; Monlau et al., 2012c). This pretreatment can disrupt almost all the ether linkages and some ester linkages between lignin and carbohydrates but had no effect on delignification. $FeCl_3$ significantly increased the solubilisation of hemicelluloses in aqueous solutions heated to between 140°C-200°C, with 90% of hemicelluloses solubilization and only 10% of cellulose solubilisation (Liu et al., 2009a; Liu et al., 2009b). Monlau et al. (2012) have recently tested inorganic salts pretreatment (170°C, 10 g$FeCl_3$ 100g^{-1}TS, 1h) to enhance methane potentials of sunflower stalks. Interestingly, an increase of the methane potentials from 192 LCH_4 kg^{-1}VS (untreated) to 248 LCH_4 kg^{-1}VS. Besides the fact that such technologies seems to be promising in term of methane potentials, the presence of trace elements such as Fe in anaerobic digester can significantly improve the performance of the anaerobic process (Demirel and Scherer, 2011).

Table I.4. Methane potentials ($LCH_4\ kg^{-1}VS$) of chemically pretreated lignocellulosic substrates.

Pretreatment method	Lignocellulosic substrates	Pretreatment condition	CH_4 production after pretreatment ($LCH_4\ kg^{-1}VS$)	Increase CH_4 (%)	References
Oxidative	Paper tube residues	4 gH_2O_2 $100g^{-1}_{substrate}$, 190°C, 30 min	233	5	Teghammar et al., 2009
	Sunflower stalks	4 gH_2O_2 $100g^{-1}$ TS, 55 °C, 24 h	256	23	Monlau et al., 2012c
Alkaline	Paper tube residues	4 gNaOH $100g^{-1}_{substrate}$, 190°C, 30min	269	21	Teghammar et al., 2009
	Grass hay	4 gNaOH $100g^{-1}_{substrate}$, 25°C, 24 h	270	17	Lehtomaki et al., 2004
		3 $gCa(OH)_2$ $100g^{-1}_{substrate}$ + 4 gNa_2CO_3 $100g^{-1}_{substrate}$, 25°C, 72h	270	17	Lehtomaki et al., 2004
	Sugar beet tops	2 gNaOH $100g^{-1}_{substrate}$, 20°C, 24 h	350	13	Lehtomaki et al., 2004
	Corn stover	2 gNaOH $100g^{-1}_{substrate}$, 10; 20; 30; 50°C, 3 days	208; 233; 222; 207	56; 75; 67; 56	Zheng et al., 2009
		1; 2.5; 5 gNaOH $100g^{-1}_{substrate}$, 20°C, 24 h	267; 276; 372	0; 3; 40	Zhu et al., 2010a
	Cynara stalks	1.4 $gNaOH\ L^{-1}$, 160 °C, 20 mins	620	90	Oliveira et al., 2012
	Sunflower stalks	4 gNaOH $100g^{-1}$ TS, 30; 55; 80°C, 24 h	225; 259; 240	17; 35; 25	Monlau et al., 2012c
	Grass sillage	1; 2.5; 5; 7.5 gNaOH $100g^{-1}_{substrate}$, 100°C, 48h	359; 402; 449; 452	10; 23; 38; 39	Xie et al., 2011
	Corn stalks	8 gNaOH $100g^{-1}_{substrate}$, 30 days	472	207	Dongyan et al., 2003
		5 gNH_3 $100g^{-1}_{substrate}$, 30 days	316	51	Dongyan et al., 2003
	Rice straw	5 gNaOH $100g^{-1}_{substrate}$, 200°C, 10 mins	133	122	Chandra et al., 2012b
		2 gNH_3 $100g^{-1}_{substrate}$, 90°C, 10mm	245	29	Zhang and Zhang, 1999
	Barley waste	30 gNaOH $100g^{-1}_{substrate}$, 25°C, overnight	222	792	Neves et al., 2006

Pretreatment method	**Lignocellulosic substrates**	**Pretreatment condition**	**CH_4 production after pretreatment (LCH_4 $kg^{-1}VS$)**	**Increase CH_4 (%)**	**References**
Dilute-acid	Sugarcane bagasse	2 gH_2SO_4 L^{-1}, 121°C, 15 min	173	166	Badshah et al., 2012
	Sunflower oil cakes	1 gHCl $100g^{-1}$ $_{substrate}$, 170°C, 5 min	289	48	Monlau et al., 2012d
	Sunflower stalks	2 gHCl $100g^{-1}$TS, 170°C, 1h	233	21	Monlau et al., 2012c
	Maize sillage	2 gHCl $100g^{-1}$TS, 20°C, 24h	312	0	Pakarinen et al., 2011
	Cassava residues	3 gH_2SO_4 $100g^{-1}$TS, 158°C, 20 min	248	57	Zhang et al., 2011
	Bagasse	1M HCl, 25°C, 30 days	-	32	Kivaisi and Eliapenda, 1994
	Coconut fibers	1M HCl, 25°C, 30 days	-	76	Kivaisi and Eliapenda, 1994
Ionic liquids	Rice straw	N-methylmorpholine-N-oxide (NMMO), 130°C, 1h	328	608	Teghammar et al., 2011
	Triticale straw	N-methylmorpholine-N-oxide (NMMO), 130°C, 15h	362	583	Teghammar et al., 2011
Wet oxidation	Miscanthus	-	360	80	Uellendahl et al., 2008
	Willow	-	360	80	Uellendahl et al., 2008
	Winter rye	2 gNa_2CO_3 L^{-1}, 195°C, 15 min, 12 bar O_2	447	33	Petersson et al., 2007
Inorganic salts	Sunflower stalks	10 g$FeCl_3$ $100g^{-1}$TS, 170 °C, 1h	248	29	Monlau et al., 2012c

I.3.3. Biological pretreatment

Despite the physical and chemical pretreatments have been regarded as the current leading pretreatment technologies, the biological pretreatments, have received considerable attention in recent years, as alternatives to them, due to their environmental benefits (Lee, 1997; Taherzadeh and Karimi, 2008). Biological pretreatments can be performed by applying either **commercial enzymes** or **fungi** to the lignocellulose material. Recently, **ensiling** has been discovered to be suitable for treating crops prior to anaerobic digestion, enhancing methane production.

Commercial enzymes, such as cellulase (endoglucanase, exoglucanase and β-glucosidase), xylanase, pectinases (poly-galcaturonase and pectate-lyase) or lignolytic enzymes (laccase, lignin and manganese peroxidase), are industrially synthesized by liquid cultivation (< 5% as substrate loading) of a variety of living organisms (i.e. fungi and bacteria) grown on a specific organic substrate. They are generally characterized by high enzymatic activities and they can be used to breakdown all components of lignocelluloses, including lignin, the polymer most refractory to microbial attack (Lopez et al., 2007). The use of enzymes has some advantageous properties, such as the high substrate and reaction specificity and the possibility to operate under mild conditions (T°≈50°C and pH≈5) avoiding the formation of by-products (i.e. furfural and 5-HMF), potentially inhibiting methane production (Howard et al. 2003). However, enzymatic activity can be affected by many factors including the substrate, incubation time, system configuration, and environmental conditions (e.g. temperature and pH). More research is needed to determine if and when the addition of enzymes to the anaerobic digestion system will improve digestion rates and biogas yields of lignocellulosic biomass (Romano et al., 2009). To date, potential loss of carbohydrates during the enzymatic pretreatment, due to of consumption of soluble sugars by indigenous microorganisms naturally present on the substrate, and the high cost of industrial enzymes are the major disadvantages for enzymatic pretreatment and constitute a limitation for further application at industrial scale (Alvira et al., 2010; Banerjee et al., 2010; Quéméneur et al., 2012). Some studies (Table I.5) reported that an enzymatic pretreatment, by using different types of enzymes, enzymatic activities, contact times and temperatures, can improve

anaerobic digestion of lignocellulosic substrates. Sonakya et al. (2001) pretreated wheat grains with Trizyme (cellulase, a-amylase, and protease mix) at 37°C for 24 h before anaerobic digestion and observed an increase in methane production by 14%. Lehtomaki et al. (2004) applied enzymatic pretreatment to grass at 35°C for 24 h using two xylanases (GC 320 and Multifect) and two cellulases (IndiAge MAX L and Primafast 200), and an increase of 22% in methane yield was observed. Frigon et al. (2012) noticed that lignolytic enzymes can increase methane production potentials, and an energy gain of 28 and 42% was reached using lignin peroxidase and manganese peroxidase, respectively. They showed also that the use of pectinases, in particular polygalacturonase and pectate-lyase, significantly increased anaerobic digestion from switchgrass of 40 and 72%, respectively. Only one study was found on the combination of acidic and enzymatic pretreatment to enhance methane production of lignocellulosic substrates. Badshah et al. (2012) studied the effect of combined acid with enzymatic pretreatments on sugarcane bagasse, showing a 208% increase of methane yields. The majority of the studies summarized in Table I.5 suggest that the addition of exogenous enzymes can improve the performance of anaerobic digestion systems. However, the high costs of industrial enzymes (around 1000 € kg^{-1}) remain a drawback for further industrial development. To overcome the high cost of industrial enzymes, fungal pretreatment with lignin-degrading fungi (i.e. white, brown and soft rot fungi), has received renewed interest for biogas production. Traditionally, white rot fungi have been employed for biopulping, bioremediation of soil and wastewater, by oxidizing lignin and a wide range of lignin analogous compounds, and in recent years also for bioethanol production (Wesenberg et al., 2003; Winquist et al., 2008; Sanchez, 2009; Wan and Li, 2012). White fungi, but also brown-, and soft-rot fungi, are capable of degrading lignocellulosic biomass. However, due to their unique ligninolytic systems, white rot fungi are most effective for delignification (Eriksson et al., 1990). Thus, white rot fungi such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Coriolus versicolor*, *Cyathus stercoreus*, and *Ceriporiopsis subvermispora*, have been studied for the pretreatment of a wide range of biomass feedstocks for biofuel (bioethanol or methane) production, through solid state or liquid fermentation processes. For example, white-rot fungi (*Ceriporiopsis subvermispora* and *Cyathus stercoreus*) were found effective in delignification of bermuda grass; after incubation with *Ceriporiopsis subvermispora* and *Cyathus stercoreus*, about 23% and 41% of total aromatics were

removed respectively (Akin et al., 1995). Lee et al. (2007) have investigated the effect of three white-rot fungi (*Ceriporia Lacerata, Stereum hirsutum, Polyporus brumalis)* on Japanese red pine. Among the three white-rot fungi, *Stereum hirsutum* selectively degraded the lignin of the wood sample rather than holocelluloses. Indeed loss of 14.5% and 7.8% were respectively noticed for lignin and holocelluloses (Lee et al., 2007). Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase (Lee et al., 2007) synthetized during solid state or liquid state fermentation processes. The following reactions are generally involved: (i) oxidative, (ii) demethylation (or demethoxylation), (iii) sidechain oxidation and (iv) propyl-side-chain cleavage. The remaining lignin is demethylated on arylmethoxy groups and contains a greater number of ring hydroxyl groups. To date, most of researchers are interested in solid state fungal pretreatment prior to anaerobic digestion, due to its advantages compared to thermo-chemical pretreatments, such as low energy requirements, reduced output of waste streams, reduced processing costs and nor or reduced inhibitors to biogas production. Despite the advantages, substantial holocellulose (cellulose and hemicellulose) loss and long pretreatment time (36-90 days), due to low lignin degradation rates (Muller and Trosch, 1986; Dongyan et al., 2003), are the major issues associated with solid state fungal pretreatment. To avoid high holocelluloses loss, selective lignin degrading fungi (i.e. *Polyporus giganteus, Polyporus berkeleyi…*) are preferred compared to non-selective ones (i.e. *P. chrysosporium*), because they degrade larger amount of lignin compared to cellulose (Wan and Li, 2012). As for pretreatment times, using fungal treatment concurrently with on farm wet storage (> 45% moisture content), applying fungal pretreatment in combination with physical and thermo-chemical pretreatments or growing fungi in liquid state processes and then extract enzymatic filtrate to perform pretreatment, can be alternatives to avoid long pretreatment times.

In table I.5 are summarized literature results about solid state fungal pretreatment performed by cultivating different fungal strains directly on the substrate. Results about rice straw, grass hey, wheat straw, maize straw and bagasse, suggest that fungal pretreatment enhanced methane production of all substrates. Ghost and Bhattacharrya (1999) studied the effect of white-rot fungi and brown-rot fungi

on rice straw. Increases in methane of 32% and 46% were observed respectively for rice straw pretreated with brown- and white-rot fungi compared to untreated straw (Ghosh and Bhattacharyya, 1999).

Ensiling, performed during the storage of biomasses, can also be considered as a biological pretreatment (Neureiter et al., 2005). For almost 100 years, ensiling has been the preferential method in mantaining the energy nutrient content of crops, ensuring a good nutritional value when used as feed (Vervaeren et al., 2010). The main objective of ensiling is to induce anaerobic conditions in which the lactic bacteria, which are present in crops, can convert mainly soluble sugars into organic acids, with a decrease of pH around 4. Lactic acid, formed during ensiling, is the main acidifying agent preserving structural carbohydrates and proteins (Pakarinen, 2012a). Additives, such as acids (i.e. formic acid) and bases (i.e. urea) are currently used to accelerate the pH change, to prevent the growth of unwanted microorganisms and limiting the loss of carbohydrates or formation of other acids (Pakarinen, 2012a). Recently, ensiling has been discovered to be suitable for treating crops prior to anaerobic digestion (Neureiter et al., 2005; Lehtomaki, 2006; Bauer et al., 2009; Bruni et al., 2010b; Pakarinen et al., 2012a). Lehtomaki (2006) has shown that ensiling has a positive impact on methane production and suggested that the structural polysaccharides contained in plant material, which are quite resistant to anaerobic degradation, can be partially degraded by lactic bacteria during storage (Lehtomaki, 2006). Thus, ensiling can be considered as a promising cost-effective storage and pretreatment to enhance methane potentials in condition that the loss of carbohydrates is avoided. A 25% increase in methane potential was observed for maize after four months ensiling compared to fresh maize (Neureiter et al., 2005). Similar trend was noticed on hemp ensiled for 4 months with a methane increase over 50% compared to fresh hemp (Pakarinen, 2012a). However, ensiled faba bean led to less methane than the fresh material (Pakarinen, 2012a). Recently, Herrmann et al. (2011) showed that ensiling prolonged storage and biological commercial silage additives have positive effects on methane yields of up to 11%. Indeed, biological silage additives normally inhibit or restrict undesirable silage fermentation or aerobic deterioration (Herrmann et al., 2011).

Table I.5. Methane potentials ($LCH_4\ kg^{-1}VS$) of biological pretreated lignocellulosic substrates.

Pretreatment method	Lignocellulosic substrates	Pretreatment condition	CH_4 production after pretreatment ($LCH_4\ kg^{-1}VS$)	Increase CH_4 (%)	References
Enzymes	Switchgrass	Lignin peroxidase (20 U g^{-1}VS)	202	29	Frigon et al. , 2012
		Manganese peroxidase (20 U g^{-1}VS)	223	42	
		Polygalcaturonase (50 U g^{-1}VS)	240	72	
		Polygalcaturonase (10 U g^{-1}VS)	64.9	-53	
		Pectate-lyase (6313 U g^{-1}VS)	288	40	
		Pectate-lyase (1263 U g^{-1}VS)	205	-7	
	Grass hay	Genencor industrial enymes: Xylanases (GC320, Multifect) + 2 cellulases (Indiage Max L, Primafast) at 0.1% (g g^{-1}TS)	280	22	Lehtomaki et al., 2004
	Sugarcane bagasse	2% H_2SO_4 (g L^{-1}), 121°C, 15 min + Enzyme Accelerase® (mixture of cellulase, hemicellulases and β-glucosidase)	200	208	Badshah et al., 2012
	Wheat grain	37°C, 24 h, Trizyme, cellulase (0.98 U mg^{-1}TS, a-amylase(0.8 U mg^{-1}TS), and protease(0.013 U mg^{-1}TS)			Sonakya et al. (2001)
Fungi	Rice straw	White rot-fungus *Phanerochaete chrysosporium*	328	46	Ghosh and Bhattacharyya, 1999
		Brown rot-fungus *Polyporus ostreiformis*	295	32	
	Grass hay	White rot-fungus *Pleurotus ostreatus*	240	4	Lehtomaki et al., 2004
	Wheat straw	Fungus *Pleurotus florida*, 90days	343	17	Muller and Trosch, 1986
	Japanese cedar chips	Fungus *Cyathus Stercoreus AW 03-72*	43	-	Take et al., 2006
		Fungus *Trametes hirsuta AW 03-72*	30	-	
Ensiling	Maize	Ensiling (4 months)	480	25	Neureiter et al., 2005
	Hemp	Ensiling (4 months)	380	58	Pakarinen, 2012a
	Maize	Ensiling (8 months)	445	14	

I.3.4. Energetic and economic assessments of pretreatments, during anaerobic digestion of lignocellulosic substrates.

I.3.4.1. Energy pretreatments requirements

In this part, the energy requirement into heat or electricity of various kinds of pretreatment technologies used on lignocellulosic substrates is discussed. Most of the studies are based on the production of bioethanol from lignocellulosic substrates. However, the pretreatment technologies used are similar than those applied in the case of anaerobic digestion.

Energy requirement data presented below have to be taken only as a rough estimation, because sometimes the energy inputs of the lab-scale equipment can be far from those of a full-scale implementation (Carlsson et al., 2012). As stated by Carlsson et al. (2012), one of the major difficulty for comparing pretreatment requirements is to analyse the different energetic and economic inputs, such as thermal energy, electrical energy, chemical and/or enzymatic costs.

Several studies investigated the energy requirement for size reduction of lignocellulosic substrates. The energy requirement for size reduction depends on many factors mainly the materials properties, the mass feed rate, the substrate nature, the moisture content and the final particle size (Mani et al, 2004; Tavakoli et al., 2009; Bitra et al., 2009; Zhu et al., 2012). Mani et al. (2004) studied grinding performance on four lignocellulosic substrates (i.e. wheat, barley straws, corn stover and switchgrass) and they found that among the four materials, switchgrass had the highest specific energy requirement (27.6 kWh t^{-1}), and corn stover had the less specific energy requirement (11.0 kWh t^{-1}) at 3.2 mm screen size. Similarly, Adapa et al. (2011) have investigated the energy requirements to mill four lignocellulosic substrates (i.e. barley, wheat, oat, canola straws), previously chopped, at different particle sizes (30, 6.4, 3.2, 1.6 mm) and showed that the energy requirement depends both on the substrate type and on the final size reduction. For a final size of 1.6 mm, they reported that energy requirement of 25.1, 35.7, 41.5 and 42.6 kWh t^{-1} were necessary for barley, canola, oat and wheat straw, respectively. For a same substrate (i.e. wheat straw), they noticed that an increase of the energy

requirement from 2.1 to 42.6 kWh t^{-1} was necessary to reduce the particle size from 30 mm to 1.6 mm. In general, energy requirement for size reduction of woody biomass is higher than that for herbaceous substrate. Indeed, specific energy to mill aspen wood chips, reduced to particle sizes less than 6.4 mm, was five times higher than that required for corn cobs, using a knife mill (Himmel et al., 1985). Cadoche and Lopez (1989) reported that the energy required grinding agricultural straw and corn stover was about 6–36% of the energy necessary to grind wood. Zhu et al. (2010b) reported energy consumptions of 689 kWh t^{-1} for disk milling of wood chips. Another important parameter to take into account in the energy requirement for size reduction process is the mass feed rate. Bitra et al. (2009) have investigated various feed rates ranged from 1 to 11 kg min^{-1} on the energy requirement of milling. They stated that an increase in the mass feed rate reduces the specific energy requirement. Indeed, total specific energy decreased gradually by 55%, 49%, and 75% with an increase in mass feed rate from 2 to 11 kg min^{-1}, 2 to 9 kg min^{-1}, and 2 to 7 kg min^{-1} for switchgrass.

The speed of the device used is another important parameter to be considered for the energy requirement of size-reduction of lignocellulosic substrates. For instance, total specific energy for switchgrass, wheat straw, and corn stover grinding increased by 37, 30, and 45% from 32, 35, and 29 kWh t^{-1}, respectively, with an increase in hammer mill speed from 2000 to 3600 rpm. In the case of pretreatment combination it was shown that performing a thermal or thermo-chemical pretreatment before milling step can reduce the energy requirement due to the size reduction. Indeed, Zhu et al. (2010b) have shown that energy requirement for size reduction as post pretreatment decrease of 13 and 46% after hot water pretreatment and thermo-acid pretreatment, respectively. Similar results were noticed by Adapa et al. (2011) during milling of steam exploded wheat, oat and canola straw.

Thermal (i.e. liquid hot water, steam explosion) and thermo-chemical pretreatments conducted at high temperature required also high amount of thermal energy to raise the water at the work temperature. However, such pretreatments can be interesting solutions when the biogas produced is converted into thermal and electrical energy by a CHP system (Carlsson et al., 2012). Indeed, in most of cases the heat produced through the cogeneration system is considered as a waste stream. Thus, thermal energy remaining after the internal use for heating anaerobic digester, can be used both for the thermal

pretreatment requirement (Pickworth et al., 2006; Carlsson et al., 2012). Nevertheless, such assumption would not be applicable to system when biogas is used as transportation ful or injected into natural gas grid (Carlsson et al., 2012).

Zhu et al. (2010b) stated that the thermal energy consumptions depend on two factors, as the pretreatment temperature and the solid loading or liquid to solid ratio. Zhu et al. (2012) stated that reducing pretreatment temperature had much less effect than increasing solids loading for reducing thermal energy input. By increasing the solid loading from 5%TS to 20%TS, Monlau et al. (2012d) found that the energy requirement for thermal pretreatment at 170°C can be reduced from 3536 kWh t^{-1}TS to 1010 kWh t^{-1}TS. Another aspect to take into account is the energy requirement for mixing, during thermal and thermo-chemical pretreatment. Indeed, Pavlostathis and Gosett (1984) evaluated as 10.5 kWh t^{-1} the energy requirement for 24 h of mixing during thermo-alkaline of wheat straw at a solid loading of 5%TS. Others data on mixing energy requirement can be found in relation with the ethanol production by using a solid state fermentation (SSF). Zhang et al. (2009) have shown that the energy requirement for the mixing steps is highly dependent on the solid loading: the higher the solid loading, the lowest the energy requirement. Indeed, they showed that the energy for mixing step increased when the solid loading of steam exploded corn stover increased from 15% to 30%TS (22, 31, 95 and 280 kWh t^{-1} for the solid loading of 15%, 20%, 25% and 30% (w/w) respectively). Similar results were noticed by Zhu et al. (2011) that reported energy requirement varying from 160 MJ t^{-1} wood (44 kWh t^{-1}wood) to 290 MJ t^{-1} wood (80 kWh t^{-1}wood) during the SSF at a solid loading of 18% according to the pretreatment performed on wood. Finally, in the case of chemical and/or enzymatic pretreatment, the costs of chemicals and/or enzymes have to be considered. For instance, the cost of sodium hydroxide and sulphuric acid which are commonly used to perform thermo-chemical pretreatments are of 412 and 1089 € t^{-1}, respectively (ICIS, 2010). In the case of industrial enzymes, their costs can reach 0.1c€ U^{-1}, 2.5 € U^{-1}, 50 € U^{-1}, 250 € U^{-1} and 5 c€ U^{-1}, for xylanase, cellulase, LiP, MnP and Laccase, respectively (SIGMA-ALDRICH®).

I.3.4.2. Energy balance and cost benefits of pretreatments

Most of the time, the benefit provided by pretreatment technologies are evaluated in terms of increase

in methane potential, without considering energetic and economic aspects. It is important to verify if the net energy and economical gain obtained by the application of a pretreatment, prior to anaerobic digestion step, can cover investment costs, operational costs and energetic pretreatments requirements (i.e. energetic requirement to size reduction and to heat biomass and the cost of chemicals and enzymes).

Until now, few studies made some preliminary energetic and economical assessments on the applications of physical and thermo-chemical pretreatments for enhancing methane production from lignocellulosic residues (Jackowiak et al., 2011; Menardo et al., 2012; Monlau et al., 2012d; Monlau et al., 2012e). Results are presented in Table I.6. Except for the studies of Menardo et al. (2012), the electric energy consumption of the machineries normally used to ground the substrates (straw bale breaker and shredder) was not considered in these analysis as it can be assumed that these machineries be already in use even when untreated substrates are anaerobically digested. Menardo et al. (2012) have shown that the energy requirement for straw bale breaker and shredder was of 15 kWh t^{-1}TS.

Moreover, according to all literature studies, the surplus of methane produced from pretreatment is generally transformed into heat or in a mix of heat and electricity using a combined heat and power (CHP) system. Normally, the electric energy produced by the CHP system is sold to the public grid at a fixed rate. To compare results coming from different studies in term of energetic and economical assessments, the energetic value was expressed in terms kWh t^{-1}TS and the economical assessment was evaluated using the European government incentive of three countries (France, Italy, Germany) with a mean call price of electricity coming from anaerobic digestion of 0.24 € kWh^{-1} electricity.

Table I.6. Preliminary energetic and economical assessments on the applications of physical and thermo-chemical pretreatments for enhancing methane production from lignocellulosic residues.

Substrate	Pretreatments conditions *(Assumptions)*	Methane increase	Conversion into heat and electricity	Energy produced		Pretreatments requirements			Energy gain		References
				Surplus Heat	Surplus Electricity	Heat	Electricity	Chemical Cost	Heat	Benefits[a]	
		$m^3\ t^{-1}TS$		$kWh\ t^{-1}TS$		$kWh\ t^{-1}TS$		€ $t^{-1}TS$	$kWh\ t^{-1}TS$	€ $t^{-1}TS$	
Wheat straw	Microwaves (150°C) Heating rate 5°C / min Amount treated: 2.6 gVS	70 (batch)	100 % (heat and electricity)	701		_	73111	_	_	no benefit	Jackowiak et al., 2011
	Microwaves (150°C) Heating rate 5°C / min Amount treated: 7.8 gVS	70 (batch)	100 % (heat and electricity)	701		_	37977	_	_	no benefit	
	Microwaves (150°C) *Heating rate 15°C / min* *Amount treated: 24 gVS*	70 (batch)	100 % (heat and electricity)	750		_	8333	_	_	no benefit	
	Grinding 0.5 cm	135 (batch)	CHP : 38 % electricity; heat: not defined	_	541	_	30	_	_	124	Menardo et al., 2012
	Thermal (90°C) Solid load: 20% TS	108 (batch)	CHP : 38 % electricity; heat: not defined	_	433	_	115	_	_	76	
	Thermal (90°C) Solid load: 20% TS *Use of thermal energy form CHP to increase water temperature*	108 (batch)	CHP : 38 % electricity; heat: not defined	_	433	_	55	_	_	91	
Barley straw	Grinding 0.5 cm	110 (batch)	CHP : 38 % electricity; heat: not defined	_	442	_	30	_	_	99	
	Thermal (90°C) Solid load: 20% TS	85 (batch)	CHP : 38 % electricity; heat: not defined	_	340	_	115	_	_	54	
	Thermal (90°C) Solid load: 20% TS *Use of thermal energy form CHP to increase water temperature*	85 (batch)	CHP : 38 % electricity; heat: not defined	_	340	_	55	_	_	68	
Sunflower oil cakes	Thermal (170°C; 1h) Solid load: 5% TS	32 (batch)	CHP : 35 % electricity; 50% heat	161	110	3536	_	_	-3375	26	Monlau et al., 2012d
	Thermal (170°C; 1h) *Solid load: 20% TS*	32 (batch)	CHP : 35 % electricity; 50% heat	161	110	1010	_	_	-849	26	

Substrate	Pretreatments conditions *(Assumptions)*	Methane increase	Conversion into heat and electricity	Energy produced		Pretreatments requirements			Energy gain		References
				Surplus Heat	Surplus Electricity	Heat	Electricity	Chemical Cost	Heat	Benefits[a]	
		$m^3\ t^{-1}TS$		$kWh\ t^{-1}TS$		$kWh\ t^{-1}TS$		$€\ t^{-1}TS$	$kWh\ t^{-1}TS$	$€\ t^{-1}TS$	
	Thermal (170°C; 1h) *Solid load: 20% TS* *80% of heat recovery from pretreatment step*	32 (batch)	CHP : 35 % electricity; 50% heat	161	110	152	_	_	9	26	
	Thermo-acid (170°C;1% HCl (w/wTS); 1h) Solid load: 5% TS	78 (batch)	CHP : 35 % electricity; 50% heat	389	265	3536	_	1089	-3147	52	
	Thermo-acid (170°C;1% HCl (w/wTS); 1h) *Solid load: 20% TS*	78 (batch)	CHP : 35 % electricity; 50% heat	389	265	1010	_	1089	-621	52	
	Thermo-acid (170°C;1% HCl (w/wTS); 1h) *Solid load: 20% TS* *80% of heat recovery from pretreatment step*	78 (batch)	CHP : 35 % electricity; 50% heat	389	265	152	_	1089	237	52	
Sunflower stalks	Thermo-alkaline (55°C;4% NaOH (w/wTS); 24h) Solid load: 3.5% TS	36 (continuous)	CHP : 35 % electricity; 50% heat	185	129	1034	_	410	-849	14	Monlau et al., 2012e
	Thermo-alkaline (55°C;4% NaOH (w/wTS); 24h) *Solid load: 20% TS*	36 (continuous)	CHP : 35 % electricity; 50% heat	185	129	210	_	410	-25	14	
	Thermo-alkaline (55°C;4% NaOH (w/wTS); 24h) *Solid load: 20% TS* *80% of heat recovery from pretreatment step*	36 (continuous)	CHP : 35 % electricity; 50% heat	185	129	52	_	410	133	14	

[a] *Calculated by the author considering the cost of NaOH and H_2SO_4 (412 € t^{-1} and 1000€ t^{-1}, respectively) and the call price of electricity (24 c€ kWh^{-1}).*

Jackowiak et al. (2011) and Menardo et al. (2012) provided energetic and economical balances of using physical pretreatments (i.e. microwaves and grinding) during anaerobic digestion of lignocellulosic substrates. The surplus of methane obtained after microwaves pretreatment (150°C, 5°C/min, ramp time: 30min) on wheat straw was totally transformed into heat and electricity (Jackowiak et al., 2011). Pretreatment was performed at different amount of substrate (2.6 and 7.8 gVS) and for both, the energy gain obtained after pretreatment (701 kWh t^{-1}TS) was not sufficient to cover the energy pretreatment requirement (73111, 37977 kWh t^{-1}TS, respectively), even if increasing the amount of biomass treated significantly reduced the energy requirement for the pretreatment. Different assumptions were made to avoid this deficiency of energy as increasing the amount of treated biomass or the heating rate of the microwaves device. By increasing the amount of wheat straw up to 24 gVS (maximum that can be treated using their microwaves devices) and the heating rate (15°C/min), a decrease of energy consumption up to 8333 kWh t^{-1}TS was noticed, but still higher than the 701 kWh t^{-1}TS necessary to cover the entire pretreatment energy requirement. Menardo et al. (2012) have recently investigated the energetic assessment of using mechanical (bale breaker + shredder + hummer grinder; final size: 0.5 cm) pretratments on barley straw and wheat straw. The surplus of methane obtained after pretreatment was converted into heat and electricity using a combined heat and power (CHP) system, with 38% efficiency for electricity. The electric energy demand for mechanical pretreatment was evaluated as 30 kWh t^{-1}TS. Interestingly, Menardo et al. (2012) showed that the energy requirement for mechanical pretreatment is cover by the electric surplus of pretreatment. By the sale of the remaining electricity, economic benefits of 99 € t^{-1}TS and 122 € t^{-1}TS were obtained for barley straw and wheat straw, respectively. Moisan (2012) have also investigated the energy and economical balances of applied ultrasound pretreatments on three lignocellulosic substrates (i.e. wheat straw, ground hay, switchgrass). For all lignocellulosic substrates, negative energetic and economical balances were reported. This result was partially explained by the high energy requirement (≈ 573 kWh t^{-1}TS) of ultrasonic pretreatment performed at a solid loading of 2%TS during 40s.

The energetic and economical assessments of thermal pretreatment have been investigated by some studies (Monlau et al., 2012d; Menardo et al., 2012). Menardo et al. (2012) have shown that the

electric requirement for thermal pretreatment (90°C) was 100 kWh t^{-1}TS and can be reduced to 40 kWh t^{-1}TS if the thermal energy produced by the CHP were employed to raise the water temperature. In this case the electric demand (40 kWh t^{-1}TS) was exclusively associated for steam delivery and materials handling. Thus, an economical gain of 68 € t^{-1}TS and 91 € t^{-1}TS was found for barley straw and wheat straw, respectively. Monlau et al. (2012d) have also investigated the energetic and economical assessment of thermal (170°C, 5min) pretreatment on sunflower oil cakes. In their study, the methane increase obtained by pretreating biomass was converted into heat and electricity through a combined heat and power (CHP), with efficiency of 50% for heat and 35% for electricity. It was shown that by performing pretreatment with an initial solid loading of 50 gTS L^{-1}, the heat energy surplus obtained by digesting thermal was not enough to cover the heat requirement. Several assumptions can be considered to improve the heat balance, as increasing the solid loading and the heat recovery of the pretreatment step or recovering the exhaust gases from the CHP system. Indeed, heat requirement of the pretreatment step is highly dependent on the solid loading during pretreatment and the highest solid loading, the lowest energy requirement. Schell et al. (2003) demonstrated the feasibility of a pilot scale system (1 ton d^{-1}) capable to continuously perform acid pretreatment on corn stover at a solid loading of 20%TS. Moreover, Dhar et al. (2012) have reported 80% heat recovery from thermally pretreated sludge. Other studies have shown that the remaining exhaust gases (400-450°C) produced through CHP can also be used to increase the water temperature (Katta et al., 2008). Thus, by assuming a solid loading up to 200 gTS L^{-1} and with 80 % of heat recovery from the pretreatment step a positive heat balance was obtained after thermal pretreatment. An economical benefit of 26 € t^{-1}TS after thermal pretreatment was obtained by the sale of the surplus of electricity produced.

Monlau et al., (2012d and 2012e) have also investigated the energetic and economical assessment of thermo-alkaline (55°C, 4 gNaOH $100g^{-1}$TS, 24h) pretreatment on sunflower stalks and thermo-acid (170°C, 1 gH_2SO_4 100 g^{-1}TS, 5min) pretreatment on sunflower oil cakes. In these cases, the methane gain (36 m^3 CH_4 t^{-1}TS and 78 m^3 CH_4 t^{-1}TS) obtained by pretreating biomass was converted into heat and electricity through a combined heat and power (CHP), with efficiency of 50% for heat and 35% for electricity. Like previously noticed for thermal pretreatment, by increasing the solid loading to

200g TS L^{-1} and with a 80% heat recovery of the pretreatment step, the heat balance became positive. Considering a cost of sodium hydroxide of 412 € t^{-1} (ICIS, 2010), an economical gain of 14 € t^{-1}TS was obtained by the selling of the electricity surplus for thermo-alkaline pretreatment. An economical benefit of 64 € t^{-1}TS was noticed after thermo-acid pretreatment. However, it is pertinent to notice that the cost of chemical reagent was not taken into account. Considering the cost of acid sulfuric (1089 € t^{-1}; ICIS, 2010) the economic gain of dilute acid pretreatment (52 € t^{-1}TS) remains still positive.

Up to date, no economical and energetic balances were found on the use of biological pretreatments during anaerobic digestion of lignocellulosic residues. However, it seems that some bottleneck such as the sterilization step and the use of expensive industrial enzymes have to be improved for a further industrial feasibility and viability.

For most of these studies, the energetic and economical balances were positive with economical gain varying from 14 € t^{-1}TS to 124 € t^{-1}TS. However, such studies can give only preliminary information, as more of them were realized in batch mode and not continuous mode necessary to validate the industrial feasibility. Furthermore, the increase in methane production should not be the only benefit evaluated when applying pretreatment to lignocellulosic substrates. Indeed, pretreatments can also allow an increase of the methane production rate, leading to a reduction of the anaerobic reactor size.

Nevertheless, to make a global economical and energetic balances of the overall anaerobic digestion process, cost of equipments and facilities, cost of maintenance and other energetic requirements concerning the anaerobic digester (heating and mixing of the digester) have to be integrated. Indeed, Pavlostathis and Gosett (1984) have shown that heating and mixing of the digester represent an energy requirement of 1280 kWh t^{-1} of wheat straw which represent 65.1% of the total energy input from an anaerobic digester at 35°C treating one ton of thermo-alkaline pretreated wheat straw. Recently, Adl et al. (2012) have made global energetic balances of the anaerobic digestion process of one ton of thermally pretreated corn stalks and they stated that the energy required to heat the digester can varied from 266 kWh t^{-1}TS to 671 kWh t^{-1}TS depending on the TS of the digester's influent. Finally, Karellas et al. (2009) have provided an economical evaluation of biogas plant projects based on agricultural feedstock and stated that the installation became profitable after nine years of establishment.

Another aspect which has to be taken into account, to improve the overall economic and

environmental balances of the anaerobic digestion process of lignocellulosic substrate, is the valorisation of digestate. Indeed, digestate can be enriched in nitrogen and phosphorous after anaerobic digestion of such substrates and therefore can be used as a fertilizer for the growth of other lignocellulosic substrates and improve the environmental balance (Frigon and Guiot, 2010). Moreover the digestate rich in lignin can also be burnt to provide some heat for the pretreatment step and thus improve the energetic balance. It has been reported that the heating value of lignin is higher than cellulose and hemicelluloses with a heat value of 25.4 kJ g^{-1} lignin (Lau et al., 2009; Saidur et al., 2011).

I.4. Conclusions

The present literature review reports the state of art of pretreatments applied to enhance methane production from lignocellulosic substrates. Methane has many advantages compared to bioethanol and biodiesel, such as it can be produced from a large range of substrates, it is a versatile energy vector and, in most of cases, the residue (digestate) of the anaerobic digestion can be reutilize directly as fertilizer. However, in the case of anaerobic digestion of lignocellulosic substrates, pretreatments are required in order to overcome the lignocellulosic barriers (i.e. lignin content, pectins content, cellulose crystallinity…) that limit the accessibility and degradation of biodegradable compounds. Therefore, a large range of pretreatments (physical, chemical, and biological or combination of them) have been investigated in order to increase the methane production of different kind of lignocellulosic substrates.

Physical-chemical pretreatments are actually considered as the current leading technologies, but for most of them, the industrial applications are still limited. They were found in most of cases efficient in enhancing the methane potential (from 9% to 608%) by increasing the accessible surface area by gradual removal of lignin, hemicelluloses, and pectins and sometimes by reducing the crystallinity of cellulose. Nevertheless, the high energy consumption, the high cost of chemicals and the possible formation of inhibiting byproducts, derived from the degradation of hemicelluloses, cellulose and lignin, such as furfural, HMF and phenol compounds, are actually the major drawbacks for physical-chemical pretreatments. In a more "environmental-friendly" pretreatment approach, biological

pretreatments, with the use of commercial enzymes or by solid state fermentation of fungi, have been investigated to enhance methane production from lignocellulosic substrates and in most of cases, they were found efficient in enhancing the methane potentials of lignocellulosic residues from to 4% to 200%. Nevertheless, the high cost of commercial enzymes, the potential carbohydrates loss and the long pretreatment time required for fungal pretreatment, remain the major drawbacks of biological treatments. As for enzymatic pretreatment an alternative to avoid holocelluloses loss is to perform the treatment under sterilized conditions or under anaerobic conditions, latter never investigated, while, for fungal pretreatment, the possibility is to use lignin degrading fungi (i.e. *Polyporus giganteus, Polyporus berkeleyi…*) which degrade larger amount of lignin compared to cellulose. Moreover, using fungal treatment concurrently with on farm wet storage (i.e. ensiling), applying fungal pretreatment in combination with physical and thermo-chemical pretreatments or growing fungi in liquid fermentation processes and then extract enzymatic filtrate to perform pretreatment, can be alternatives to avoid long pretreatment times.

Another important aspect to be considered is related to the energetic, economic and environmental assessments. Indeed, at present, due to the necessity of a pretreatment step, the production of second generation biofuels, such as methane through anaerobic digestion of lignocellulosic substrates, is not cost effective because there are a number of technical barriers, such as the cost of the pretreatment, that need to be overcome before their potential can be realized. Until now, only few studies made some preliminary energetic and economic evaluations on the applications of physical and thermo-chemical pretreatments for enhancing methane production from lignocellulosic residues. Therefore, once the pretreatment strategies have been defined energetic and economic assessments have to be taken into account for a future scale-up of the technology.

Chapter II. Materials and methods

Materials and methods

II.1. Lignocellulosic substrates and preparation

II.1.1. Lignocellulosic substrates

Seven lignocellulosic substrates (ensiled sorghum forage, *sudanense hybrid Trudan 8*; Biomass sorghum, *Biomass* 133; sweet sorghum, *hybrid BMR Sisco*; forage sorghum, *Trudent Headless*; sweet sorghum, *sorghum 405*; sweet sorghum, *sorghum 506* and wheat straw, *Aubusson*) were used to perform experimental tests. All substrates were considered as lignocellulosic materials, due to their chemical composition, mainly composed of lignin, cellulose and hemicelluloses fractions. They were chosen for their availability at agricultural farms and because they are often co-digested with animal manure in agricultural biogas plant in the North of Italy.

Sorghum (*Sorghum bicolor [L.] Moench*), is a warm-season, short-day annual grass, which can be cultivated in soils unsuitable for food production and, as a quick-growing crop, it can be rotated as a part of an annual cropping system (Newman et al., 2010). Actually, it occupies a world cultivated land of about 40 million ha and it has an annual hectare yield up to 25 tTS ha^{-1} (FAO, 2012). *Sorghum bicolor [L.] Moench* is a genus with many species and subspecies, generally classified into forage and grain types (Newman et al., 2010). Forage sorghums are grouped into four types, including hybrid forage sorghum, sudangrass, sudan hybrids, and sweet sorghum varieties. Sweet sorghums are attractive for biofuel production because they have a high concentration of soluble sugars in the plant sap and a high hectare yield (18 tTS ha^{-1} $year^{-1}$). Indeed they have generated interest as a feedstock for ethanol production since the 1970s, and more recently also for biogas production (Newman et al., 2010). Hybrids of forage sorghum and sudangrass, with hectare yields of 10-11 tTS ha^{-1} $year^{-1}$, are commonly used for silage (Newman et al., 2010). However, compared with maize, forage sorghum is cheaper to produce, it requires less water to growth, and it has comparable annual hectare yields. Thus, these qualities give to forage sorghum a potential for use in biofuels production (Corredor et al.,

2009). Recently, cultivars of sorghum have been commercialized a new variety of "biomass sorghum" to energy purpose, characterized by a higher hectare yield (22 tTS ha^{-1} $year^{-1}$) than the other varieties.

Wheat straw is an agricultural byproduct and could be an alternative to energy crops in anaerobic digestion plants. Moreover, this substrate presents the advantage to be renewable, and in abundance at agricultural Italian farm. Wheat straw consists in the dry stalks of wheat after the grain has been removed. Actually, wheat plants have a world cultivated land of 217 million ha and an annual hectare yield (referred to grains) ranging between 3.6-11.75 tTS ha^{-1} $year^{-1}$ (FAO, 2012). Among them, the variety *Aubusson* is the most commonly used in Italy, due to its high yield (up to 9 tTS ha^{-1} $year^{-1}$) and to its capacity of adaptation to any types of soil.

II.1.2. Substrate preparation

Ensiled sorghum forage (*Trudan 8*) samples were collected both in November 2010 and May 2011 (Table II.1) and thus they were subjected to different times of ensilage (4 and 9 months of ensilage, respectively). Wheat straw (*Aubusson*) was collected in November 2010. Both sorghum and wheat straw were collected from a farm near Cremona (Lombardy Region, Italy). After collection, samples were oven dried at 60°C for two days to moisture content less than 10%, and then grounded into small particles. Five varieties of sorghum (*Biomass sorghum 133*, sweet sorghum *hybrid BMR Sisco*, forage sorghum *Trudent Headless*, *sweet sorghum 405* and *sweet sorghum 506*) were collected in August 2011, in Saint Thibery in the South of France. After collection, all sorghum varieties were stored at -20°C and dried with a freeze drying (HetoPowerDry PL 3000; ThermoElectron Corporation), before to be grounded by a cutting mill (MF 10.1, IKA). All substrates were conserved into air-tight containers at ambient temperature.

Table II.1 summarizes the substrates used and their preparation, according to pretreatment methods performed.

Table II.1. Substrates used and their preparation according to pretreatment methods performed.

Substrate	Drying	Milling (mm)	Pretreatment method	Result chapter
Ensiled sorghum forage (Trudan 8); Wheat straw (Aubusson)	oven drying at 60°C	1	Alkaline, thermal and thermo-alkaline	III.1
Ensiled sorghum forage (Trudan 8)		2, 1, 0.5, 0.25	Mechanical	III.2
		1, 0.25	Mechanical-alkaline	III.2
Ensiled sorghum forage (Trudan 8)		1	-	IV.1
Ensiled sorghum forage (Trudan 8); Wheat straw (Aubusson)		1	Enzymatic	IV.2
		1	Fungal	IV.3
Ensiled sorghum forage (Trudan 8); Biomass sorghum 133; Sweet sorghum hybrid BMR Sisco; Forage sorghum (Trudent Headless); Sweet sorghum 405; Sweet sorghum 506	freeze drying	1	Alkaline pretreatment	V
Ensiled sorghum forage (Trudan 8)	oven drying at 60°C	0.5		VII

II.1.3. Pretreatment methods

II.1.3.1. Alkaline pretreatment

Alkaline pretreatment tests were performed on all substrates used in this study, according to experimental conditions summarized in Table II.2. Pretreatment conditions were chosen according to literature suggestions reported in Table I.4.

Table II.2. Alkaline pretreatment conditions.

Initial TS (g/L)	Temperature (°C)	Time (h)	Alkaline dosage (gNaOH 100g^{-1}TS)	Stirring	Result chapter
160	40	24	1-10	No	III.1
35	55	12	4-10	Yes, 120 rpm	V
160	40	24	10	No	VII

Tests were carried out in 500 mL digestion flasks, closed with rubber septa, in which samples were soaked in a sodium hydroxide (NaOH) solution. Different alkaline dosages (1, 4, and 10 gNaOH 100g^{-}

[1]TS), initial TS concentrations (35, 160 gTS L^{-1}), contact times (12 and 24 h) and temperatures (40 and 55°C) were tested. To maintain pretreatment at the desired temperature, flasks were put in a thermostatic incubator with or without stirring, by using a thermal heater shaker (INNOVA 43, 120 rpm) or a simple thermostate (MPM Instruments srl, Bernareggio, Italy, M60-TB), respectively. The alkaline pretreatment performed prior to feed semi-continuous anaerobic reactor (see results in Chapter VII) were performed, by manteining the flask in a water bath at desidered temperature.

Only for analytical purposes, after pretreatment, samples were filtered through a sieve of 0.20 mm of pore size. The sieve-separated solid and the liquid fractions were taken for compositional analyses.

II.1.3.2. Thermal and thermo-alkaline pretreatment

Thermal and thermo-alkaline pretreatments were performed on ensiled sorghum forage (*Trudan 8*) and wheat straw (*Aubusson*). Experimental conditions are summarized in Table II.3. Pretreatment conditions were chosen according to literature suggestions reported in Tables I.3 and I.4.

Table II.3. Thermal and thermo-alkaline pretreatment conditions.

Initial TS (g/L)	Temperature (°C)	Time (h)	Alkaline dosage (gNaOH $100g^{-1}$TS)	Stirring	Result chapter
160	40	24	0	No	III.1
	100	0.5	0-1-10		
	160		0-1		

Thermal pretreatment tests were performed at 40°C, 100 and 160°C with an initial Total Solids (TS) concentration of 160 gTS L^{-1}.

Tests at 40°C were carried out by soaking samples in tap water, by using 500 mL digestion flasks closed with rubber septa. Then, each flask was kept in a thermostatic incubator (MPM Instruments srl, Bernareggio, Italy, M60-TB) at 40°C for 24 h, without stirring.

Tests at 100°C and 160°C were carried out by soaking samples in tap water, by using a closed cylindrical steel tank with a total volume of 6.2 L (working volume of 5 L), equipped with a thermal heater, a manometer and a temperature controller (Figure II.1). This apparatus is able to reach maximum temperatures (T_{max}) and pressures (P_{max}) of T_{max} = 160 ±0.5°C and P_{max} = 6± 0.16 bar, respectively.

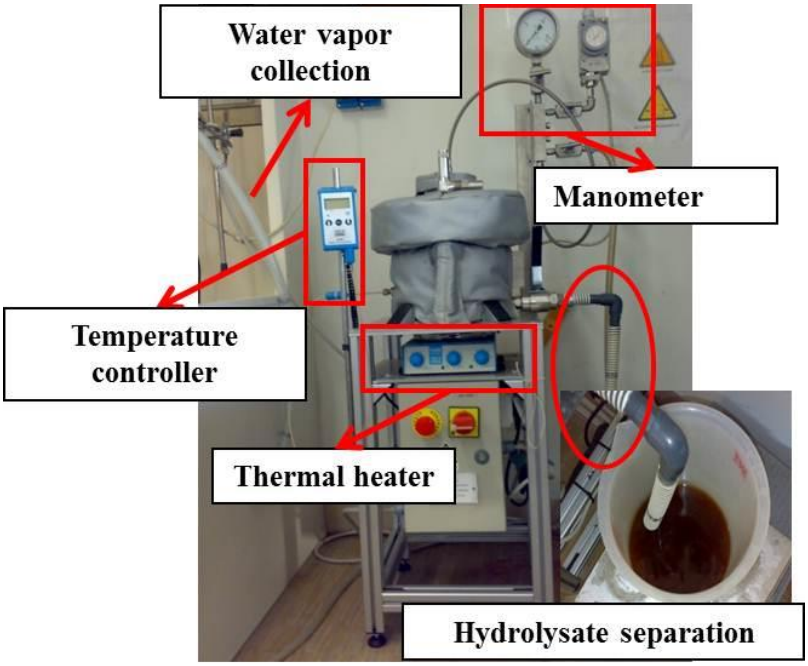

Figure II.1. Thermal pretreatment apparatus.

As for pretreatments, approximately 1 hour was required to reach the desired test temperatures of 100°C and 160°C. Samples were then maintained under this condition for 30 min, without stirring. The pressure reached a maximum value of 6 bar when samples were heated up to 160°C. The water vapors released were cooled and collected.

Thermo-alkaline pretreatments were performed at 100 and 160°C, by using the same protocol of thermal pretreatment. Samples were soaked in a NaOH solution at different concentrations (1, 10 gNaOH $100g^{-1}$TS for pretreatment at 100°C and 1 gNaOH $100g^{-1}$TS at 160°C), with a total solid concentration of 160 gTS L^{-1}.

Only for analytical purposes, after pretreatments, samples were filtered through a sieve of 0.20 mm of pore size. The sieve-separated solid and the liquid fractions were taken for compositional analyses.

II.1.3.3. Mechanical and mechanical-alkaline pretreatment

Mechanical pretreatment was performed on ensiled sorghum forage (*Trudan 8*), by using a cutting mill (SM 100, Retsch) with 2 mm screen (Figure II.2A). Then, it was ground successively by using a cutting mill (MF 10.1, IKA) with 1, 0.5 and 0.25 mm screens (Figure II.2B).

A B

Figure II.2. (A) Cutting mill (SM 100, Retsch) with 2 mm screen and (B) cutting mill (MF 10.1, IKA) with 1, 0.5 and 0.25 mm screens.

Mechanical-alkaline pretreatment was carried out in 500 mL digestion flasks, closed with rubber septa. Sorghum samples milled into 1 and 0.25 mm particle sizes were soaked in a sodium hydroxide solution at 10 gNaOH $100g^{-1}$TS dosage and maintained at 55°C for 12 h in an incubator shaker (INNOVA 43), continuously agitated for complete mixing (120 rpm). After pretreatment, samples were filtered through a sieve of 0.20 mm of pore sizes, only for analytical purposes. The sieve-separated solid fraction was taken for compositional analyses.

II.1.3.4. Biological pretreatments

Selection of commercial enzymatic preparations

Firstly, in order to define the optimal enzymatic mixture to perform pretreatment tests, four commercial enzymatic preparations were characterized in terms of enzymatic activities: Agazym BGL and Ultra L (Garzanti Specialties), Pulpzyme HC (Novo Nordisk), and Primafast 200 (Genencor Inc.).

Agazym BGL is especially formulated to favour the breakdown of plant cell walls to extract tissue components during industrial processing of cereals. It is an enzymatic mix characterized by cellulose, β-glucanase, Hemicellulase, Xylanase from *Aspergillus Aculeatus*.

Ultra L is recommended to perform alcoholic fermentation of red wines, when must is fermented in contact with grape husk, to facilitate pigments and flavours extraction. It is mainly characterized by polygalacturonase and pectinase from *Aspergillus* strains.

Pulpzyme HC is used during the process of bleaching and deinking for the production of recycled paper. It is characterized by endo-xylanases from *Bacillus* strains.

Primafast 200 is recommended for clothes processing such as depilling, softening and to obtain the so-called "stone-washed look". It is characterized by endo-1-4-β-glucanases.

Enzymatic pretreatment with commercial preparations

According to results obtained in Chapter IV, two enzymatic preparations (Agazym BGL and Primafast 200) among those described in previous paragraph, were chosen to perform pretreatments. Enzymatic pretreatments were performed on untreated and alkaline (10 gNaOH $100g^{-1}$TS, for 24 h, at 40°C) pretreated ensiled sorghum forage (*Trudan 8*), and wheat straw (*Aubusson*), not sterilized. Trials were performed in 500 mL digestion flasks, closed with rubber septa. In each flask, the enzymatic preparations were added to each substrate at a final concentration of 0.20 and 0.12 mL g^{-1}TS for BGL and Primafast, respectively. Then, H_2O was added to reach a total solids (TS) concentration of 70 gTS L^{-1}, pH was corrected at appropriate enzyme-specific value (pH = 5) with HCl and N_2 was inflated for 10 min to guarantee anaerobic conditions. Samples were then incubated at 50°C for 72 h in a thermostatic incubator (Thermo Scientific Heraeus, BK800) in stationary condition.

After pretreatment, samples were filtered through a sieve of 0.20 mm of pore size. The sieve-separated solid fraction was taken for compositional analyses.

Selection of fungal strains

Firstly, in order to define the optimal fungal strain to perform pretreatment tests, five fungal strains (Industrial Microbiology Collection of the University of Milan) were characterized, by measuring their induced enzymatic activities: *Irpex lacteus* (MIM 100; MIM: Microbiologia Industriale, Milano), *Irpex lacteus* (MIM 257), *Phanerochaete chrysosporium* (MIM 166), *Polyporus tulipiferus* (MIM 259) and *Daedalea quercina* (MIM 76). They are all classified as white-rot fungi. Strains were maintained on solid PDA (Potato Dextrose Agar, Formedium, Hunstanton- UK), incubated at 25°C for 7 days and then stored at room temperature (Figure II.3).

Figure II.3. Two fungal strains maintained on solid PDA (Potato Dextrose Agar, Formedium, Hunstanton – UK).

Liquid cultures were set up employing the following culture medium, modified according to Reyes et al. (1980) and containing (1 L): soybean peptone 5 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, KCl 0.5 g, yeast extract (Costantino, Favria – Italy) 2 g, Tween 80 (Sigma Aldrich) 2 mL, microelement solution (Na_2BO_7 x 10 H_2O 0.1 g L^{-1}, $ZnSO_4$ x 7 H_2O 0.07 g L^{-1}, $FeSO_4$ x $7H_2O$ 0.05 g L^{-1}, $CuSO_4$ x $5H_2O$ 0.01 g L^{-1}, $MnSO_4$ 0.01 g L^{-1}, $(NH_4)_6MoO_{24}$, 0.01 g L^{-1}) 1 mL, pH 6, sterilized at 118°C for 20 min. Precultures were set-up in 1000 mL Erlenmeyer flasks each containing 180 mL culture medium added with 20 mL of presterilized glucose (200 g L^{-1}) as substrate, inoculated (1 slant per flask) and incubated in an Infors-HT (Basel- Switzerland) shaker incubator at 25°C and 180 rpm for 5 days. Grown cultures were then inoculated (10% v/v) into 1000 mL Erlenmeyer flasks containing 180 mL of production medium containing 4 g (dry weight) of sorghum or cellulose powder as substrates instead

of glucose. These cultures were incubated as mentioned before for up to 15 days. At appropriate intervals, samples were taken and biomass separated from culture filtrate through vacuum–filtration. The obtained filtrates were characterized in terms of enzymatic activities, as reported in Chapter IV.

Enzymatic pretreatment with fungal enzymatic filtrate

According to results obtained in Chapter IV, *Polyporus tulipiferus* (MIM 259) was employed and its obtained filtrate was used as crude enzymatic preparation in pretreatment tests. Trials were performed on untreated and alkaline (10 gNaOH $100g^{-1}$TS, for 24 h, at 40°C) pretreated ensiled sorghum forage (*Trudan 8*), and wheat straw (*Aubusson*), not sterilized. They were performed in 500 mL digestion flasks, closed with rubber septa. In each flask, the fungal filtrate was added to untreated substrates to a final total solid concentration of 70 gTS L^{-1}. pH was corrected at appropriate enzyme-specific value (pH = 5) and N_2 was inflated for 10 min to guarantee anaerobic conditions for avoiding mycelial growth. Samples were then incubated at 37°C for 48 h in a thermostatic incubator (Thermo Scientific Heraeus, BK800) in stationary condition.

After pretreatment and only for analytical purposes, samples were filtered through a sieve of 0.20 mm of pore size. The sieve-separated solid and the liquid fractions were taken for compositional analyses.

II.2. Biological tests for methane production

II.2.1. Biochemical Methane Potential (BMP) tests

Two methodologies (volumetric and manometric) and five types of anaerobic digestion inoculum were employed to perform BMP tests. Test conditions are summarized in Table II.4.

II.2.1.1. Origin and characteristics of anaerobic sludge inocula

To perform BMP tests, five types of anaerobic digested sludge were employed: 1) WW: collected from a digester fed on waste activated sludge; 2) AGR: collected from a digester fed on agro-wastes (cattle manure and corn silage); 3) GR: a granular sludge collected from a Upflow Anaerobic Sludge

Blanket (UASB) reactor treating wastewater from a chemical industry; 4) MIX: a sludge obtained by mixing (50% each on a VS basis) WW and AGR; 5) GR2: a granular sludge collected from a UASB reactor treating wastewater from a sugar factory.

II.2.1.2. Biochemical Methane Production (BMP) tests

All Biochemical Methane Production (BMP) tests were performed under anaerobic mesophilic conditions (35±0.5°C) and in duplicate. Before BMP test, the inoculum was kept under endogenous anaerobic conditions at 35°C for about 7 days to reduce non-specific biogas generation. Samples (untreated or pretreated before sieve-separation) were digested into closed glass bottles with a total volume of 500 mL and a working volume of 400 mL. In each bottle, an amount of sample (at 2.5 or 5 gVS/L) was mixed with the anaerobic digestion inoculum (at 5 gVS/L), obtaining a substrate/inoculum (F/M) ratio of 0.5-1 gVS g^{-1}VS, respectively, as reported in Table II.4. Finally, 50 mL of mineral medium of macronutrients (as suggested by OECD 311, 2006) and tap water were also added. Samples pretreated with 10 gNaOH $100g^{-1}$TS at 40 °C, 55°C and 100 °C had a final pH ranging between 10 and 12 (Chapters III.1 and V). Therefore, they were neutralised to pH = 7 with HCl (37%) solution after adding the inoculum, the mineral medium and water, prior to start BMP tests. On the contrary, both for thermally pretreated samples (40, 100 and 160°C) and samples pretreated with 1 gNaOH $100g^{-1}$TS at 40 °C, 100°C and 160°C (Chapter III.1) and 4 gNaOH $100g^{-1}$TS at 55 °C (Chapter V), no further neutralization was necessary, because they had a final pH ranging between 6 and 8. Once the bottle was prepared, a degasification step with nitrogen gas (N_2) was carried out to obtain anaerobic conditions. A blank sample was performed by mixing the inoculum, the mineral medium, and the deionised water, without the addition of substrate. Methane production was monitored by using two methodologies (volumetric and manometric), as described below. In each case, methane volumes reported in result chapters are referred at normal temperature and pressure conditions (0°C, 1013 hPa).

Volumetric tests were performed by using a commercial laboratory instrument (AMTPS, Bioprocess control, Sweden), as represented in Figure II.4. This is a volumetric device consisting of 15 gas-tight

glass bottles (400 mL of working volume) placed in a water bath at 35±0.5 °C and continuously mixed with a rotary stirrer. The biogas produced passes through a NaOH solution (3M), for CO_2 absorption. Methane flows through a liquid-displacement automated measuring unit with a resolution of 11-13 mL and a data acquisition system allows flow-rate methane data to be recorded continuously.

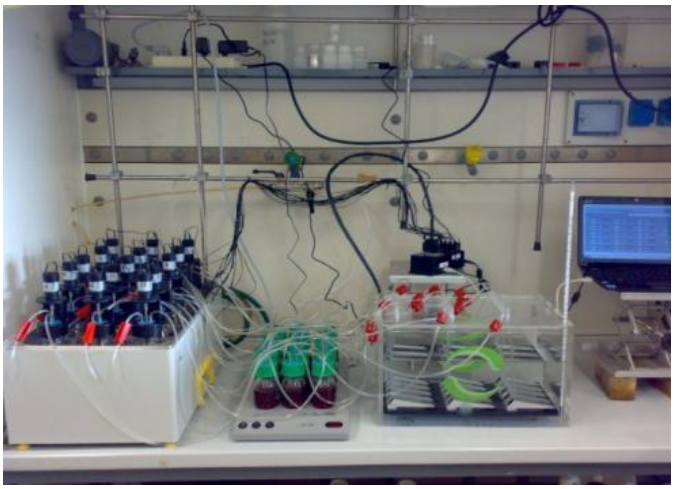

Figure II.4. Commercial laboratory instrument (AMTPS, Bioprocess control, Sweden).

Manometric tests were performed by using glass bottles (400 mL of working volume) closed with rubber septa, continually mixed (120 rpm) in an incubator shaker (INNOVA 43). Biogas volume was monitored with a manometric device (LEO2, KELLER), while biogas composition was determined using a gas chromatograph (Varian GC-CP4900) equipped with two columns: the first (Molsieve 5A PLOT) was used at 110°C to separate O_2, N_2, CH_4, the second (HayeSep A) was used at 70°C to separate CO_2 from other gases. The injector temperature was 110°C and the detector 55°C. The detection of gaseous compounds was done using a thermal conductivity detector. The calibration was carried out with a standard gas composed of 25 % CO_2, 2 % O_2, 10 % N_2 and 63 % CH_4.

Table II.4. Origin of inoculum, substrate to inoculum ratio (F/M) and BMP methodology employed to perform batch tests.

Origin of inoculum	F/M	BMP methodology	Result chapter
MIX	1	Volumetric	III.1
GR2	1	Manometric	III.2
MIX, GR, AGR, WW	1	Volumetric	IV.1
MIX	0.5	Volumetric	IV.2- IV.3
GR2	1	Manometric	V

II.2.2. Semi-continuous anaerobic reactors

Anaerobic digestion process was monitored by using two semi-continuous anaerobic reactors (see results in Chapter VII), whose configurations are schematized in Figure II.5.

The two anaerobic glass reactors had an operating volume of 1.5 L each one, were continuously agitated for complete mixing by using a magnetic stirrer, and maintained at 35°C by an external water recirculation system. Both feeding (inlet) and discharge (outlet) were operated manually once a day (5 days per week), by using a syringe.

Biogas was collected in a gas bag directly connected to the reactor headspace and then its volume was measured twice a week by a liquid displacement method, the liquid being water at pH = 2, by adding HCl, and 10 g L^{-1} of NaCl. The accuracy of this measurement was ±10 mL. Biogas composition (O_2, CO2, CH4, H2 and N2) was analysed by a gas chromatograph (Varian GC-CP4900), twice a week as well.

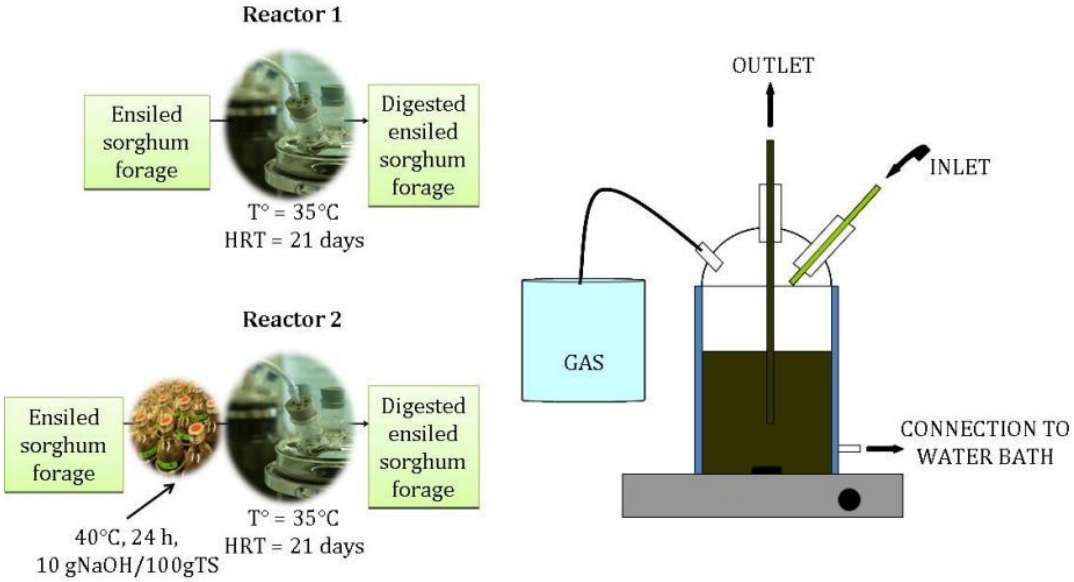

Figure II.5. Reactor configurations.

Both reactors were initially inoculated with a granular sludge from a mesophilic anaerobic digester treating the effluent from a sugar factory (GR2), with an initial Total Solids (TS) and Volatile Solids (VS) concentrations of 61±0.7 gTS L^{-1} and 51±0.7 gVS L^{-1} (Reactor 1) and 63±0.6 gTS L^{-1} and 54±0.3 gVS L^{-1} (Reactor 2). Reactors were fed 5 days per week (once a day) with 100 mL of untreated (reactor 1) and alkaline pretreated (10 gNaOH $100g^{-1}$TS, 40°C, 24h) ensiled sorghum forage

(Trudan 8) (reactor 2), after a dilution with tap water to reached the desired volatile solid content (26.3 gVS L^{-1}) necessary to avoid clogging problems caused by the high solid concentration. As for reactor 2, alkaline pretreatment was performed every day, according to the protocol defined (Paragraph II.1.3.1). Despite the high pH value of the feeding (pH = 10), no pH adjustment was performed. At day 9, 140 mmol of alkalinity (corresponding to 6 $gCaCO_3$ $g^{-1}VS_{in}$) was also added in the reactor 1 (see results in Chapter VII). This amount of bicarbonate alkalinity was requested to compensate the CO_2 partial pressure and to maintain the VFA/alkalinity ratio below the threshold value of 0.3 gVFA $g^{-1}CaCO_3$. During the last 21 days, 0.08 $gN\text{-}NH_4Cl$ $g^{-1}VS_{in}$ was added to the influent preparation once a week, to avoid long term nitrogen limitation. Reactors operated at constant Hydraulic Retention Time (HRT) of 21 days, for 3 HRT, at Organic Loading Rate (OLR) lower than 1.8 kgVS m^{-3} d^{-1} (see results in Chapter VII).

Reactors were monitored by daily measures of pH and Volatile Fatty Acids (VFA), and, two or three times per week by measuring soluble Chemical Oxygen Demand (CODs), ammoniacal nitrogen ($N\text{-}NH_4^+$), VS and biogas volume and composition. Sodium (Na^+) concentration was measured both in the inoculum and in the digestate, while Total Kjeldahl Nitrogen (TKN) was measured only in the digestate.

II.3. Analytical methods

II.3.1. Total solids (TS) and volatile solids (VS)

TS and VS content were measured according to Analytical Standard Methods (APHA, 2005).

II.3.2. Determination of Total Organic Carbon (TOC), Chemical Oxygen Demand (COD) and soluble COD

TOC was analysed with a Carbon TOC-V module (Shimadzu). COD of untreated substrates was determined according to the open reflux method (APHA, 2005). According to this method, an amount of

dried sample (150 mg) was mix with 20 mL of sulphuric acid (H_2SO_4 at 98%) and 20 mL of potassium dichromate ($K_2Cr_2O_7$) 2N. The sample was heated at 148 h for 2 h with a condenser and then cooled up to room temperature and washed with deionized water (to reach 500 mL). The excess of $K_2Cr_2O_7$ (50 mL of sample) was tritate with Ferrous Ammonium Sulfate (FAS) tritant (0.1 N), by using 2 or 3 drops of ferroin indicator.

Soluble COD (CODs) was determined, after 0.45 µm filtration (Glass microfiber GF/C filter, WHATMAN), in the liquid fraction after pretreatment with commercial photochemical test kits (Spectroquant® test kits, Merck, Darmstadt, Germany; LCK514, Hach Lange GmbH, Dusseldorf, Germany) and with a Spectrophotometer (HACH DR/2000 Hach Company, Loveland, CO., USA).

II.3.3. Determination of Total Kjeldahl Nitrogen (TKN) and ammoniacal nitrogen (N-NH_4^+)

TKN was determined according to Kjeldahl method (Kjeldahl, 1883), by using a mineralisator (BUCHI digestion unit K 438) and a BUCHI 370-K distillator/titrator. Ammoniacal nitrogen (N-NH_4^+) was determined by the titrimetric method after distillation using a BUCHI 370-K distillator (Rodier, 1975).

II.3.4. Determination of proteins content

Proteins content was estimated by multiplying the total Kjeldahl nitrogen by a factor of 6.25 (Izhaki, 1993) or determined with a NIRSystem (5000 monochromator, Foss).

II.3.5. Determination of fats content

Fats were measured according to Analytical Standard Methods (APHA, 1992), by using an automated extraction system for accelerated solvent (petroleum ether) extraction (Model ASE 200 Dionex, Germany). Fats were also determined with a NIRSystem (5000 monochromator, Foss).

II.3.6. Determination of sodium ion (Na^+) concentration

After centrifugation of the digestate in 2 mL Eppendorf® tubes, followed by filtration at 0.2 µm (Nylon membrane, Acrodlsc®), 50 µL of supernatant were transferred to a vial prior to the analysis by ICS-3000 Ion Chromatography System.

II.3.7. Determination of total and reducing sugars

The amounts of **total soluble sugars** in liquid samples were detected by the phenol sulphate method (Dubois et al. 1956). Briefly, 1 mL of liquid sample was diluted up to sugars concentration range between 0.01 and 0.04 mg mL^{-1}. Deionized water (1 mL), as blank sample, and 1 mL of a glucose standard solution (0.04 mg mL^{-1}) were also prepared. Then, 1 mL of phenol solution (at concentration of 5% v/v) and 5 mL of sulphuric acid were added to each sample. After 10 minutes, samples were mixed and the amount of total soluble sugars was determined by using a Spectrophotometer (OD 490 nm) (6705 UV/Vis Spectrophotometer, Jenway, UK).

The amount of **reducing sugars** in liquid samples was measured according to Somogyi-Nelson method (Somogyi, 1952). Briefly, an aliquot of liquid sample (1 mL) was diluted up to sugars concentration ranged between 0.03 and 0.09 mg/mL. Deionized water (1 mL), as blank sample, and 1 mL of a glucose standard solution (0.04 mg mL^{-1}) were also prepared. Each sample (1 mL) was then mixed and boiled for 25 minutes with 1 mL of a solution containing (1 L): Na_2CO_3 30 g, $NaHCO_3$ 20 g, Na and K tartrate 15 g, Na_2SO_4 180 g, $CuSO_4$ x $5H_2O$ 20 g and $NaSO_4$ 180 g. To the mixture was then added 1 mL of a solution containing (1 L): ammonium molybdate 56 g, sulphuric acid (5% v/v) and Na_2HAsO_4 x 7 H_2O 6.3 g. After 5 minutes, samples were diluted up to 10 mL with deionized water. The amount of reducing sugars was then measured after 20 minutes by using a Spectrophotometer (OD 560 nm) (6705 UV/Vis Spectrophotometer, Jenway, UK).

II.3.8. Determination of cellulose (CEL), hemicelluloses (H-CEL) and klason lignin (K-LIG) content

Cellulose (CEL), hemicelluloses (H-CEL) and klason lignin (K-LIG) were measured using a strong acid hydrolysis method adapted from Effland et al. (1977). Samples (200 mg) were first hydrolyzed with 12 M H_2SO_4 acid for 2 h at room temperature, then diluted to reach a final acid concentration of 1.5 M and kept at 100°C for 3 h. The insoluble residue was separated from the supernatant by filtration on fibreglass filter (GF/F, WHATMAN). This insoluble residue was washed with 50 mL of deionized water and then placed in a crucible. The crucible and the paper fibreglass were dried at 100°C during 24 h to determine by weighting the amount of klason lignin. The supernatant, after centrifugation of the sample in 2 mL Eppendorf® tubes, was filtrated at 0.2 µm (Nylon membrane, Acrodlsc®). Then an aliquot of supernatant (800 µL) was transferred to a vial prior to the analysis by high-pressure liquid chromatography (HPLC). Structural carbohydrates (i.e. glucose, xylose, arabinose, glucuronic and galacturonic acids) were measured by High Performance Liquid Chromatography (HPLC) coupled to refractometric detection (Waters R410). The components were separated by an Aminex column HPX-87H column (300×7.8 mm, Bio-Rad) equipped with a protective precolumn (Microguard cation H refill cartbridges, Bio-Rad). The eluting solution corresponded to 0.005 M H_2SO_4, and the flow rate was 0.3 mL min^{-1}. The column temperature was maintained at 50°C and the refractometric temperature was fixed at 45°C. A refractive index detector (Waters 2414) was used to quantify the carbohydrates. The system was calibrated with glucose (0-6 g L^{-1}), xylose (0-6 g L^{-1}), arabinose (0-2 g L^{-1}), and uronic acids (0-2 g L^{-1}) (galacturonic and glucuronic) standards (Sigma–Aldrich®). Thereafter, cellulose and hemicelluloses contents were estimated as follows (equation II.1 and II.2):

$$\text{Cellulose (\%VS)} = \text{Glucose (\%VS)} / 1.11 \quad \text{(Equation II.1)}$$

$$\text{Hemicelluloses (\%VS)} = [\text{Xylose (\%VS)} + \text{Arabinose (\%VS)}] / 1.13 \quad \text{(Equation II.2)}$$

Where:

1.11 is the ratio of the molecular weights of glucose to glucan (180/162) and 1.13 is the ratio of the molecular weights of xylose and arabionose to xylan (150/132).

II.3.9. Determination of enzymatic activities

Endoglucanase (CMCase) enzymatic activity was determined by measuring the amount of glucose released from carboxymethylcellulose (CMC) using the Somogyi-Nelson method with glucose as standard (Somogyi, 1952). For this purpose an aliquot of diluted sample (0.5 mL) was mixed with 0.5 mL of a CMC suspension (1% w/v) in citrate buffer (0.05 mol L^{-1}, pH 5). Reaction mixtures were left at 55°C for 30 minutes, and then boiled to stop the enzymatic activity. Sugars release was then determined with glucose as standard (Somogyi, 1952). One unit of enzyme (IU) was defined as the amount of enzyme which hydrolyzes 1 µmol of reducing sugars, expressed as glucose, in 1 minute.

Exoglucanase (Avicelase) enzymatic activity was determined according to Desrochers et al. (1981). An aliquot of diluted sample (1 mL) was mixed with 1 mL of a suspension containing microcrystalline cellulose Avicel® (2% w/v) in acetate buffer (0.1 mol L^{-1}, pH 5). Samples were then incubated at 30°C for 24 h, and then boiled to stop the enzymatic activity. The amount of glucose released from cellulose Avicel®, was measured according to Somogyi-Nelson method with glucose as standard (Somogyi, 1952). One unit of enzyme (IU) was defined as the amount (µmol) of glucose released from 1 mL of sample, in 1 minute.

β-glucosidase enzymatic activity was measured by mixing 0.1 mL of sample with 0.9 mL of p-Nitrophenyl-a-D-Glucopyranoside (0.1% w/v) in citrate buffer (0.025 M, pH 4.4). A blank sample with deionized water (0.1 mL) was also prepared in the same buffer. Samples were incubated at 50°C for 10 minutes and then mixed with 2 mL of Na_2CO_3 (2% w/v). β-glucosidase activity was determined by using a spectrophotometer (OD 405 nm) (6705 UV/Vis Spectrophotometer, Jenway, UK). One unit of enzyme (IU) was defined as the amount of enzyme which hydrolyzes 1 µmol of p-Nitrophenol-a-D-Glucopyranoside in 1 minute.

Xylanase enzymatic activity was determined employing a procedure adapted from Shewale and Sadana (1979), by mixing 0.5 mL of sample with the same volume of a xylan solution (1% w/v) in citrate buffer (0.025 M, pH 5). A blank sample with 0.5 mL of deionized water was also prepared. Samples were incubated at 50°C for 30 minutes and then, boiled to stop the enzymatic activity.

Reducing sugars were determined again through the Somogyi procedure, employing xylose as standard. One unit of enzyme (IU) was defined as the amount which releases 1 µmol of reducing sugar (either glucose or xylose) equivalents per minute under the conditions specified above.

Laccase activity was determined according to Li et al. (2008) by mixing 0.5 mL of sample with 0.5 mL of 2,2'-azino-di-3-ethyl benzthiazoline-6-sulphonic acid (ABTS, Sigma) and 0.5 mL of acetate buffer (0.1 M, pH 5). A blank sample with 0.5 mL of deionized water was also prepared. Samples were incubated at 37°C for 10 minutes and laccase activity determined by using a spectrophotometer (OD 420 nm) (6705 UV/Vis Spectrophotometer, Jenway, UK). One unit of enzyme (IU) was defined as the amount of enzyme which oxidizes 1 µmol of ABTS in 1 minute.

Lignin peroxidase activity was determined by mixing 0.6 mL of sample with 0.3 mL of veratryl alcool (2 mM), 0.3 mL of H_2O_2 (5 mM) and 0.3 mL of tartaric acid (50 mM, pH 2.5). A blank sample with 0.6 mL of deionized water was also prepared. Samples were incubated at 37°C for 10 minutes and then the enzymatic activity was determined by using a spectrophotometer (OD 310 nm) (6705 UV/Vis Spectrophotometer, Jenway, UK). One unit of enzyme (IU) was defined as the amount of enzyme which oxidizes 1 µmol of veratryl alcool in 1 minute.

II.3.10. Determination of Volatile Fatty Acids (VFA), monomeric sugars and byproducts of degradation (Furfural and 5-HydroxylMethylFurfural)

Volatile fatty acids (VFA) were quantified using a gas chromatograph (GC-3900, Varian). The liquid samples were collected in 2 mL Eppendorf® tubes and centrifuged at 5000 g during 10 min using a centrifuge (Eppendorf, Mini spin). Then, 500 µL of the supernatant were transferred in analytical vials where 500 µL of standard internal solution (1 g L^{-1} of Diethylacetic acid ($C_6H_{12}O_2$) acidified to 5% with H_3PO_4) were added. VFA composition of the liquid phase, such as acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids were determined using a gas chromatograph (GC-3900, Varian) equipped with a flame ionization detector (FID).

Concentrations of monomeric sugars (i.e. glucose, xylose, arabinose, ramnose) and hydrolyzate byproducts (furfural and 5-hydroxylmethylfurfural) present in liquid phase were measured by High Performance Liquid Chromatography (HPLC) coupled to refractometric detection (Waters R410). The analytical chain was composed of an automatic sampler (Water 717plus), a pumping system (DIONEX UltiMate 3000), an oven (DIONEX ultimate 3000RS). After centrifugation of the samples in 2 mL Eppendorf® tubes, followed by filtration at 0.2 µm (Nylon membrane, Acrodlsc®), 800 µL of supernatant were transferred to a vial prior to the analysis by high-pressure liquid chromatography (HPLC). The components were separated by an Aminex column HPX-87H column (300 × 7.8 mm, Biorad) equipped with a protective precolumn (Microguard cation H refill cartbridges, Bio-Rad). The eluting solution corresponded to 0.005 M H_2SO_4, and the flow rate was 0.4 mL min^{-1}. The column temperature was maintained at 35°C and the refractometric temperature was fixed at 45°C.

II.3.11. Biochemical changes and crystallinity measurement assessment by FTIR

Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy was used to visualize the physical composition changes induced by physical-chemical pretreatments and to determine the crystallinity of lignocellulosic substrates. FTIR spectra were collected in the 4000–600 cm^{-1} range using a Nexus 5700 spectrometer (ThermoElectron Corp.) with built-in diamond ATR single reflection crystal and with a cooled MCT detector. Spectra were recorded in absorption mode at 4 cm^{-1} intervals with 64 scans, at room temperature. Three spectra were recorded for each sample and all spectra pretreatments (i.e. baseline corrections, smooth, and normalization) were performed by using Omnic v7.3.software. The peaks were assigned as summarized in Table II.5.

Table II.5. Assignments of FTIR absorption bands.

Wavenumbers (cm^{-1})	Assignment	Reference
3300	O-H stretching (indicates rupture of cellulose hydrogen bonds)	Pandey and Pitman, 2003; Kumar et al., 2009b
2900	C-H stretching (indicates rupture of methyl/methylene group of cellulose)	Pandey and Pitman, 2003; Kumar et al., 2009b
1733	C=O ester; strong carbonyl groups in branched hemicellulose	Pandey and Pitman, 2003; Sun and Tomkinson 2005; Kumar et al., 2009b;
1511	C-O absorption of guayacyl rings in lignin	Pandey and Pitman, 2003; Corredor et al., 2009

Wavenumbers (cm^{-1})	Assignment	Reference
1430	C-H deformation (asymmetric) of cellulose	Gastaldi et al., 1998; Pandey and Pitman, 2003; Corredor et al., 2009
1375	C-H deformation in cellulose and hemicelluloses	Yang et al., 2009; Shafiei et al., 2010
1230	C-O-H deformation and C-O stretching of phenolics and C-C-O stretching of esters	Sene et al., 1994; Corredor et al., 2009
1157	C-O-C vibration in holocelluloses	Pandey and Pitman, 2003; Yang et al., 2009; Shafiei et al., 2010
898	Glucose ring stretch, C-H deformation (removal of amorphous cellulose)	Stewart et al., 1995; Pandey and Pitman, 2003; Corredor et al., 2009; Kumar et al., 2009b

The bands ratio H 1430/ H 898 commonly called Lateral Order Indice (LOI) can be used to determine the amount of crystalline cellulose (O' Connor et al., 1958; Hurtubise and Krasig, 1960). Indeed, the bands at 1430 and 898 cm^{-1} are sensitive to the amount of crystalline cellulose and amorphous cellulose respectively (Spiridon et al., 2010). Generally, higher value of LOI indicates that the material has a high crystallinity and ordered structure. H lignin / H carbohydrates ratio shows the relative intensity of lignin peaks at 1511 cm^{-1} as opposed to carbohydrates peaks at 1430, 1375, 1157 and 898 cm^{-1} (Yang et al., 2009).

II.4. Data processing

II.4.1. Kinetic study

A kinetic study was performed in order to quantify the kinetic advantage on anaerobic digestion process obtained after the application of a pretreatment. The anaerobic digestion process was assumed to follow a first order kinetic model, as it is the case of lignocellulosic substrates for which hydrolysis is the limiting steps (Angelidaki et al., 2009). The first order kinetic constants were calculated by using the least-squares fit of methane production data from BMP tests (see results in Chapters III and V) during time (t), according to the following equation II.3:

$$BMP(t) = BMP_{t\to\infty} \cdot \left(1 - \exp\left(-k_h \cdot t\right)\right) \quad \text{(Equation II.3)}$$

Where:

BMP (t) is the cumulative methane yield ($NmLCH_4\ g^{-1}VS$) at the time t (d), $BMP_{t\to\infty}$ is the ultimate methane yield ($NmLCH_4\ g^{-1}VS$) of the substrate, k_h (d^{-1}) is the first order kinetic constant and t (d) is the digestion time. $BMP_{t\to\infty}$ and k_h were determined by using TableCurve 2D software v. 5.01, SYSTAT Software Inc.

II.4.2. Hydrolysis, acidogenesis and methanogenesis rates

The semi-continuous anaerobic reactor was assumed as a Continuous-Stirred-Tank Reactor (CSTR). Thus, hydrolysis ($r_{hydrolysis}$), acidogenesis ($r_{acidogenesis}$) and methanogenesis ($r_{methanogenesis}$) rates (kgCOD $m^{-3}\ d^{-1}$) during the course of the experimentation for the two anaerobic reactors were calculated by the following equations (equations II.4, II.5 and II.6), describing the mass balance of soluble organic matter (CODs) and Volatile Fatty acids (VFA). The hydrolysis ($r_{hydrolysis}$) and acidogenesis ($r_{acidogenesis}$) rates have been considered constant in a discrete and little time interval Δt. The methanogenesis rate was computed, knowing the methane production rate (MPR).

$$\frac{1}{V_R}\frac{\Delta CODs}{\Delta t}=\frac{1}{V_R}\frac{CODs_{out,t+1}-CODs_{out,t}}{\Delta t}=\frac{1}{V_R}\frac{CODs_{IN,t}-CODs_{out,t+1}}{\Delta t}+r_{hydrolysis}-r_{acidogenesis} \qquad \text{(Equation II.4)}$$

$$\frac{1}{V_R}\frac{\Delta VFA}{\Delta t}=\frac{1}{V_R}\frac{VFA_{out,t+1}-VFA_{out,t}}{\Delta t}=\frac{1}{V_R}\frac{VFA_{IN,t}-VFA_{out,t+1}}{\Delta t}+r_{acidogenesis}-r_{methanogenesis} \qquad \text{(Equation II.5)}$$

$$r_{methanogenesis}=\frac{MPR}{V_R} \qquad \text{(Equation II.6)}$$

Where:

CODs and VFA are the mass (expressed as kg COD) of soluble COD and VFA in input (IN) or in output (OUT) from the reactor, respectively; Δt is the time interval (d); V_R is the reactor volume (m^3); MPR is the methane production rate.

II.4.3. Preliminary evaluation of energetic and economic balances

A preliminary energetic and economic analysis was computed by comparing the extra energy and operational costs for the substrate pretreatment with the extra gains due to the improved methane production resulting from the pretreatment (see results in Chapter VII). For this preliminary analysis, investment costs were assumed constants, while operational costs included the sole thermal energy request for the pretreatment step and NaOH cost. Moreover, as a preliminary assumption, the electric energy consumption of the machineries normally used to ground the substrates (straw bale breaker and shredder) was not considered in this analysis, because it is assumed that these machineries are already in use even when untreated substrates are anaerobically digested. As for the cost of the chemical pretreatment, the European cost of the sodium hydroxide was used (412 € t^{-1}, ICIS 2010). The specific heat (H_s) requirement for thermally pretreating the substrate up to 40°C was calculated not considering any dissipation of energy from the reactor, as follows (equation II.7):

$$H_s = \left\{\left[(1/\rho_s)\cdot C_p \cdot (T_{final} - T_{initial})\right]/3600\right\}\cdot 1000 \qquad \text{(Equation II.7)}$$

Where H_s (kWh t^{-1}TS) is the heat required for the thermal pretreatment of the substrate; ρ_s (kgTS m^{-3}) is the solid content of the substrate suspension (160 kgTS m^{-3}); C_p is the specific heat capacity of the substrate, assumed equal to the specific heat capacity of water (4.18 kJ $kg^{-1}°C^{-1}$); $T_{initial}$ (°C) is the initial temperature of the substrate suspension, assumed equal to the mean annual ambient temperature of 13°C; T_{final} (°C) is the final temperature of the substrate suspension, as 40°C; 3600 is the conversion factor between kJ and kWh. Thermal and electric energies for digester heating and mixing were not considered in this analysis, because it is assumed that these consumptions are included even when untreated substrates are anaerobically digested. On the other hand, the electric energy and heat produced after the anaerobic digestion of the pretreated substrate was calculated considering the improved specific methane production assessed in semi-continuous reactors and assuming a CHP electric and thermal efficiency of 40% and 41%, respectively.

Normally, the electric energy produced by the CHP system is sold to the public grid at a fixed rate. As previous assumption, the government incentive policy for biogas energy of three European countries was considered: France (0.17 € kWh_{el}^{-1}), Germany (0.25 € kWh_{el}^{-1}), and Italy (0.28 € kWh_{el}^{-1}).

Chapter III. Effect of physical, chemical and physico-chemical pretreatments on chemical composition and methane production from ensiled sorghum forage and wheat straw

Effect of physical, chemical and physico-chemical pretreatments on chemical composition and methane production from ensiled sorghum forage and wheat straw

In this chapter, physical (thermal and mechanical), chemical (alkaline), and physico-chemical (thermo-alkaline and mechanical-alkaline) pretreatments were performed on ensiled sorghum forage (*Trudan 8*) and wheat straw (*Aubusson*), in order to evaluate their effects on chemical composition, physical structure and methane production.

Alkaline and thermo-alkaline pretreatments were performed at different sodium hydroxide dosages (1 and 10 gNaOH $100g^{-1}$TS), pretreatment temperatures (40, 100 and 160°C), and contact times (0.5 and 24 h), as described in more details in Chapter II.

Mechanical pretreatment was performed alone or in combination with alkaline pretreatment (10 gNaOH $100g^{-1}$TS dosage, 55°C, 12 h) by milling the substrate into small particle sizes (2-0.25 mm).

III.1. Impact of thermal, alkaline and thermo-alkaline pretreatment of ensiled sorghum forage and wheat straw[1]

III.1.1. Chemical composition of untreated substrates

The chemical composition of sorghum and wheat straw is given in Table III.1. Both samples had an average COD/VS value of almost 1.2, which is close to the typical value for carbohydrates (1.19 and 1.21 gCOD g^{-1} for cellulose and hemicelluloses, respectively). Sorghum had higher proteins and fats content, but lower celluloses, hemicelluloses and lignin content than wheat straw.

[1] *Adapted from Sambusiti et al., 2013. A comparison of different pre-treatments to increase methane production from two agricultural substrates. Applied Energy, 104, pp. 62-70.*

Table III.1. Chemical composition of both ensiled sorghum forage and wheat straw. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Parameter	Ensiled sorghum forage	Wheat Straw
TS (% wet weight)	93±4	94±4
VS (%TS)	86.6±0.4	92.7±0.4
COD/VS	1.21	1.15
Proteins (%VS)[a]	9±3	4±1
Fats (%VS)[a]	1.8±0.3	0.9±0.8
Cellulose (%VS)	32.2±1.1	35.0±0.2
Hemicelluloses (%VS)	16.0±0.6	17.5±0.2
Klason lignin (%VS)	25.7±0.2	29.0±0.2

[a] *Proteins and fats content was determined with a NIRSystem (5000 monochromator, Foss).*

Despite the high variability of substrates composition, varying according to plant type and variety, results can be considered in accordance with literature values, for both substrates (see Table I.1, Chapter I). Typical compositional values of wheat straw ranged between 32-42 %TS for cellulose, 11-27 %TS for hemicelluloses and 18-21 %TS for klason lignin. Forage sorghum showed cellulose content between 32-26 %TS, and hemicelluloses and lignin values vary between 20-23 %TS and 18-26 %TS, respectively.

III.1.2. Effect of pretreatment on the fibrous composition

Fibre composition changes induced by alkaline, thermal and thermo-alkaline pretreatments on ensiled sorghum forage (Figures III.1A) and wheat straw (Figures III.1B) were investigated. Values are referred to the chemical composition of the solid fraction separated after the pretreatment and they are expressed in terms of % initial VS.

By treating samples at mild temperature (40°C), no changes in chemical composition (cellulose, hemicelluloses and lignin) of untreated ensiled sorghum forage and wheat straw were observed, while an increasing of pretreatment temperature up to 100°C and 160°C were found effective in solubilising cellulose and hemicelluloses, but not lignin. The highest level of cellulose and hemicelluloses solubilisation, with respect to untreated substrates, was observed after thermal pretreatment performed at 160°C, with a solubilisation of 19% and 17% of cellulose and of 30% and 24% of hemicelluloses

for sorghum and wheat straw, respectively. These findings are accordance with the study of Menardo et al. (2012), who found a slight solubilisation of cellulose (6%) and hemicelluloses (23%) when wheat straw was treated at 120 °C, while lignin remained unaffected.

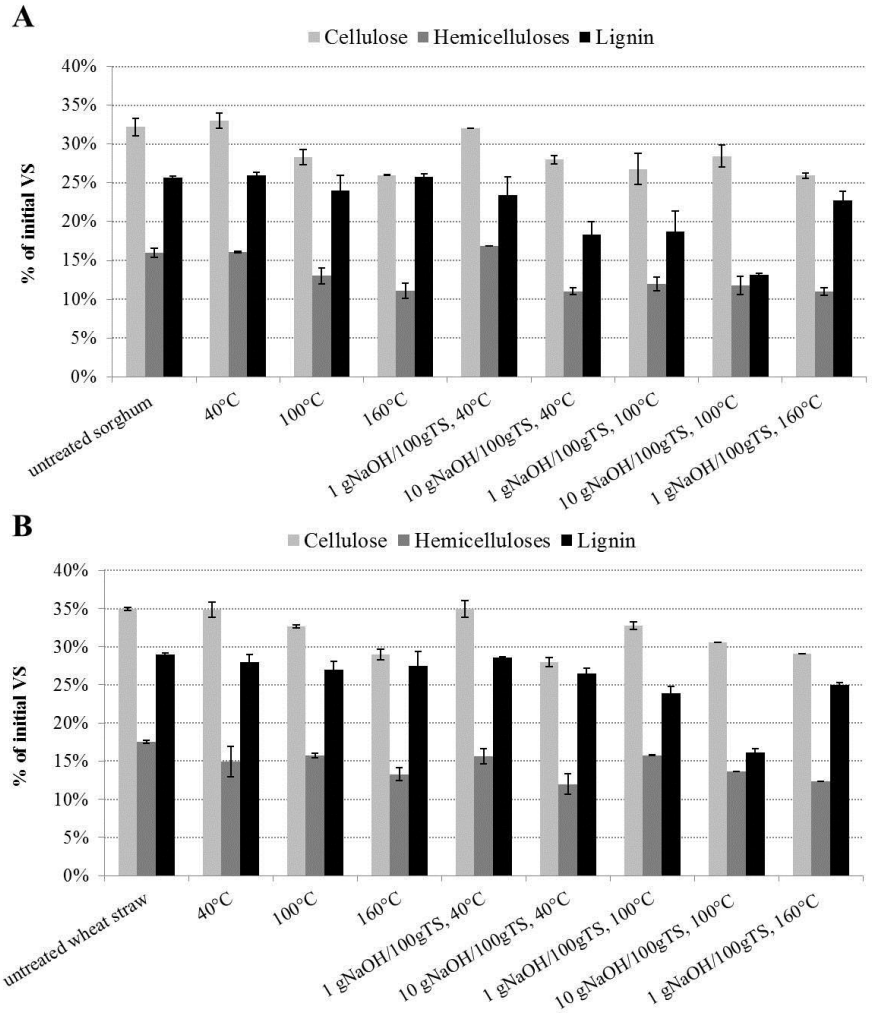

Figure III.1. Fibrous composition of untreated and pretreated ensiled sorghum forage (A) and wheat straw (B) (results are expressed in terms of % initial VS). Values correspond to mean ± standard deviation of measurement performed in duplicate.

By performing alkaline pretreatment at 40°C with 1 gNaOH $100g^{-1}$TS, no significant cellulose, hemicelluloses, and lignin solubilisation was observed, for both substrates, compared to untreated ones. A great effect of alkaline pretreatment on chemical composition was observed by increasing the alkaline dosage up to 10 gNaOH $100g^{-1}$TS. At this pretreatment condition (40°C), a solubilisation of cellulose (13%), hemicelluloses (31%), and lignin (29%) was observed for sorghum. As for wheat

straw, at the same alkaline pretreatment condition, solubilisations of cellulose (20%), hemicelluloses (32%), and lignin (9%), were also observed.

Thermo-alkaline pretreatments at 100°C with 10 gNaOH $100g^{-1}$TS, were found to be more effective in lignin solubilisation than the sole thermal or chemical pretreatments for both substrates. Indeed, for both substrates, the highest lignin reduction, compared to untreated samples, was found at 100°C with 10 gNaOH $100g^{-1}$TS dosage (49% and 44% for sorghum and wheat straw, respectively).

Results about the effect of thermo-alkaline pretreatment on fibrous fractions solubilisation were confirmed by many studies found in literature. Xie et al. (2011) found that an alkaline pretreatment performed on grass silage at 60°C for 24 h, by dosing 5 and 7.5 gNaOH $100g^{-1}$VS, resulted in lignin solubilisations of 38% and 57%, respectively. They also found that an increase of alkaline dosage led not only to a further lignin solubilisation, but also in a further hemicelluloses removal, confirming results, obtained in this study. Recently, Monlau et al. (2012c) found that NaOH pretreatment performed at 55°C for 24 h with 4 gNaOH $100g^{-1}$TS was efficient in lignin solubilisation of sunflower stalks (36%).

III.1.3. HMF and furfural release

The content of furfural and Hydroxylmethylfurfural (HMF), as dehydration products of pentose and hexose sugars (i.e. xylose and glucose), was also determined in the liquid fraction after pretreatment, as potentially inhibiting methane production. After thermal pretreatment at 100°C and 160°C, low concentrations of furfural (0.2-0.4 $g_{furfural}$ $100g^{-1}$VS), coming from the solubilisation of hemicelluloses, were found both for sorghum and wheat straw, while no HMF were detected in the liquid fraction. Such results were also previously observed by Monlau et al. (2012c), who detected a small amount of furfural (0.7 $g_{furfural}$ $100g^{-1}$VS) in the liquid fraction, when sunflower stalks were pretreated at 170°C. Diaz et al. (2011) also noticed a production of furfural lower than 1 $g_{furfural}$ $100g^{-1}$VS and quite no HMF generation during hydrothermal pretreatment (180°C-230°C) of sunflower stalks. According to literature data, at these concentrations such by-products are not expected to inhibit the final methane

production from xylose (Barakat et al., 2011). Contrarily to thermal pretreatment, no furfural or HMF concentrations were generated during alkaline and thermo-alkaline pretreatments. Results are in agreement with Bejamin et al. (1984), who found that, thermo-alkaline pretreatments are less responsible of furfural and HMF generation than thermal and thermal-acid pretreatments, due to different reaction mechanisms.

III.1.4. Effect of pretreatment on COD solubilisation

Soluble COD (CODs) released under each pretreatment condition, referred to the COD of the untreated substrate, are shown in Figure III.2. Results highlighted that similar COD solubilisation was observed for both substrates at each pretreatment condition.

Thermally pretreated samples at 40°C and 100°C released a similar amount of CODs (around 10 and 7% for sorghum and wheat straw, respectively). On the contrary, an enhancement of COD solubilisation (up to 20%) was observed by increasing pretreatment temperature up to 160°C, mainly due to further cellulose and hemicelluloses solubilisations observed for both substrates.

The addition of 1 gNaOH $100g^{-1}$TS did not affect further COD solubilisation for thermally pretreated samples at 40°C, 100°C and 160°C, not changing significantly the fibrous composition of thermally treated samples. In accordance with fibrous composition results, the highest COD solubilisations were achieved with alkaline and thermo-alkaline pretreatments, at 40 and 100°C with 10 gNaOH $100g^{-1}$TS for both substrates (around 30-40% for both substrates), due to a further lignin solubilisation, as previously observed.

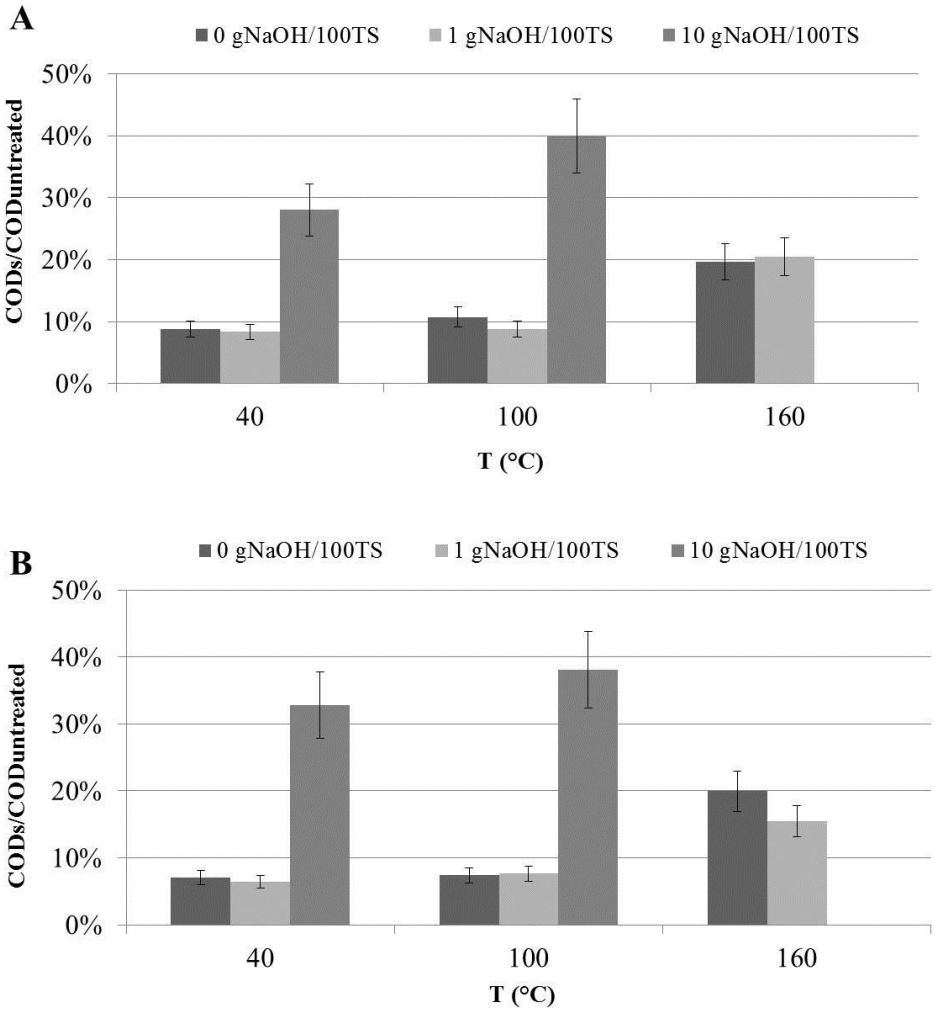

Figure III.2. Soluble COD (CODs) released under each pretreatment condition, referred to the total COD of untreated substrates for (A) ensiled sorghum forage and (B) wheat straw. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Results about COD solubilisation were also observed by Xie et al. (2011), who found an increase of soluble COD up to almost 30%, by soaking grass silage in a NaOH solution (7.5 gNaOH $100g^{-1}$VS) for 12 and 24 h at 60°C.

III.1.5. Biochemical methane potential

Methane yields ($NmLCH_4$ g^{-1}VS) of untreated and pretreated ensiled sorghum forage and wheat straw are represented in Figure III.3.

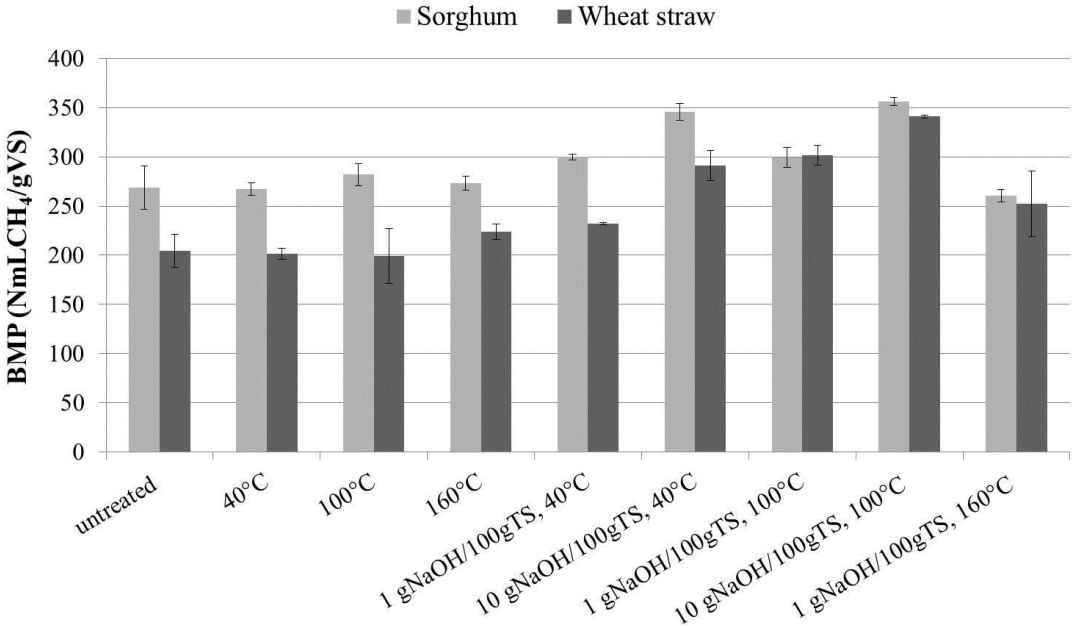

Figure III.3. Methane yields (BMP, $NmLCH_4$ $g^{-1}VS$) at normal temperature and pressure conditions of untreated and pretreated ensiled sorghum forage and wheat straw. Values correspond to mean ± standard deviation of measurement performed in duplicate.

The methane yield of untreated wheat straw (204±17 $NmLCH_4$ $g^{-1}VS$) was lower than that of ensiled sorghum forage (269±22 $NmLCH_4$ $g^{-1}VS$). Despite the high variability of crop methane yields, depending mainly on plant variety, and harvesting time, these experimental data are in agreement with literature values (270-420 $mLCH_4$ $g^{-1}VS$ and 182-297 $mLCH_4$ $g^{-1}VS$ for untreated sorghum and wheat straw, respectively), as reported in Table I.2 (Chapter I). By knowing the COD/VS ratio for each substrate (Table III.1) and considering that, at normal condition, 350 $mLCH_4$ can be obtained from 1 gCOD removed (McCarty, 1964), the corresponding anaerobic biodegradability (equation I.2) has been obtained (63% and 51% for untreated sorghum and wheat straw, respectively).

Results from Figure III.3 showed that no significant differences in methane yields were observed between thermally pretreated samples (at 40°C, 100°C and at 160°C) and both untreated samples, probably due to the fact that lignin was not solubilized at these pretreatment conditions.

The best pretreatment condition for sorghum was observed at 40°C and 100°C with 10 gNaOH $100g^{-1}$TS, enhancing the methane production and anaerobic biodegradability up to 32%.

As for the pretreated wheat straw, substantial increases in methane production were observed at 40°C and 100°C with 10 gNaOH $100g^{-1}$TS (43% and 67%, respectively). Differently from sorghum, the combination of thermal (at 100°C) and chemical pretreatments on wheat straw had a positive synergetic effect (even with 1 gNaOH $100g^{-1}$TS), leading to higher methane yields than those obtained by the application of the sole chemical pretreatment (at 40°C), probably due to a further solubilisation of lignin, which is not sensed by soluble COD results. On the contrary, for both substrates, a further increase in the temperature to 160°C led to a detrimental effect. Under this condition, the methane yield of sorghum decreased, while for wheat straw it remained constant, if compared to the chemically pretreated one (at 40°C with 1 gNaOH $100g^{-1}$TS), thus contrasting fibrous and soluble COD results. Further analysis of the liquid and solid fraction after this pretreatment condition has to be performed in order to give possible explications. The best pretreatment condition for wheat straw was at 100°C with 10 gNaOH $100g^{-1}$TS, enhancing the methane production and anaerobic biodegradability up to 67%.

Much better results than those obtained in this study after thermal pretreatment, were achieved by applying a hydrothermal pretreatment with temperatures ranging between 90-120°C on rice and barley straw, wheat straw, maize stalks (Menardo et al., 2012), sunflower stalks (Monlau et al., 2012c) and Cynara stalks (Oliveira et al., 2012), with methane production increases between 9 and 64%; however, the variability in the initial anaerobic biodegradability of the substrates can be a reason for this difference.

As for alkaline and thermo-alkaline pretreatments, it can be observed that the applied conditions are quite variable in literature and thus a comparison with results of this study is difficult. Results on methane production are also quite scattered even when similar substrates are compared. As reported in Table I.4, mild NaOH pretreatments (1-5 gNaOH $100g^{-1}_{substrate}$) at room or mesophilic temperatures led to interesting increases in the methane yield of corn stover (up to 40%) and sunflower stalks (up to 25%) (Zhu et al., 2010a; Monlau et al., 2012c); positive results were achieved on rice straw (up to 122%) although quite long contact times (5 days) were applied (Chandra et al., 2012b). Even higher values were achieved on corn stover (up to 75%) when longer contact times (from 1 to 3 days) were applied (Zheng et al., 2009). By combining high temperatures (100°C) and mild NaOH dosages (1-7.5

gNaOH $100g^{-1}_{substrate}$), the methane yield of grass silage was improved (up to 39%) and increased with the applied NaOH dosage (Xie et al., 2011).

Experimental results summarized in Table III.2 make it evident the positive effect of thermal (at 100°C and 160°C) and thermo-alkaline pretreatments on the anaerobic degradation of samples in terms of anaerobic digestion kinetics. In order to estimate the kinetic constant k_h (d^{-1}), anaerobic digestion process was assumed to follow a first order kinetic model (equation II.3, Chapter II), as it is the case of substrate where hydrolysis is the limiting steps, such as lignocellulosic residues (Angelidaki et al., 2009). Results (Table III.2) suggest that, for all samples, the first-order kinetic model was successful in modeling the experimental methane production ($R^2 > 0.96$), suggesting that such a simple model is efficient to describe the complex anaerobic of lignocellulosic substrates. First order kinetic constants ($R^2 = 0.97$) were 0.19 and 0.11 d^{-1} for ensiled sorghum forage and wheat straw, respectively. After alkaline, thermal and thermo-alkaline pretreatments, first order kinetic constant increases I_{kh} (d^{-1}) were observed (Table III.2), due to the effect of pretreatment on fibrous solubilisation. As for thermal pretreatments at 100°C and 160°C, an increase in kinetics constant (k_h) was observed up to 13% and 107% for sorghum and wheat straw, respectively, due to a solubilisation of carbohydrate fractions (cellulose and hemicelluloses). Similar results were observed by Monlau et al. (2012c), who found an increase by 64% of the first order kinetics constant after thermal pretreatment of sunflower stalks at 170°C. For alkaline and thermo-alkaline pretreatments a high increase (up to 65 and 161% for sorghum and wheat straw, respectively) of anaerobic digestion kinetics was observed at 40°C with alkaline dosage of 10 gNaOH $100g^{-1}$TS, due to both lignin and carbohydrates solubilisation. Similar trends were observed by Fernandes et al. (2009) who show an increase (30%) in the first order hydrolysis rate constant after an alkaline pretreatment of hay, performed with 10%w/w $Ca(OH)_2$, at 85°C, for 16 h.

Table III.2. Increases of first order kinetic constants I_{kh} (d^{-1}) with respect to the untreated ensiled sorghum forage and wheat straw (data are mean of duplicates, CV = 1-2%).

	Thermal			Thermo-chemical				
	40°C	**100°C**	**160°C**	**1%, 40°C**	**10%, 40°C**	**1%, 100°C**	**10%, 100°C**	**1%, 160°C**
Sorghum								
I k_h (%)	0	18	13	34	65	14	15	27
R^2	0.96	0.99	0.98	0.99	0.99	0.97	0.97	0.97
Wheat straw								
I k_h (%)	0	19	107	52	161	21	3	91
R^2	0.98	0.98	0.98	0.99	0.99	0.98	0.97	0.97

III.2. Effect of particle size on methane production of untreated and alkaline pretreated ensiled sorghum forage[2]

III.2.1. Chemical composition of milled and alkalized samples

The chemical composition of milled and milled-alkalized samples was determined in terms of % initial VS and it is shown in Table III.3. For combined milled-alkalized samples, results of Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), Cellulose (CEL), Hemicelluloses (H-CEL) and Klason Lignin (K-LIG) are referred to the chemical composition of the solid fraction separated after alkaline pretreatment and they are expressed in terms of % initial VS.

Table III.3. Composition of milled and alkaline pretreated ensiled sorghum forage. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Samples	**VS**	**TOC**	**TKN**	**CEL**	**H-CEL**	**K-LIG**
	% TS	**% initial VS**				
2 mm	76.7±0.8	53.9±0.2	1.4±0.0	41.9±1.9	29.4±1.2	29.6±1.0
1 mm	77.8±0.5	52.7±0.2	1.8±0.0	38.5±1.8	28.3±1.1	32.8±4.5
1 mm, 10 gNaOH 100g^{-1}TS	76.0±0.6	47.4±0.0	1.0±0.1	23.4±0.3	16.1±0.2	20.9±0.0
0.5 mm	77.9±0.4	52.6±0.4	1.9±0.0	34.4±2.7	25.8±2.6	31.1±2.3
0.25 mm	78.2±0.8	52.7±0.6	2.1±0.1	33.4±0.6	24.8±0.2	30.8±0.8
0.25 mm, 10 gNaOH 100g^{-1}TS	77.6±0.9	42.9±0.5	1.0±0.0	23.2±0.1	15.5±0.1	20.4±0.0

2 Adapted from Sambusiti et al., 2013."Effect of particle size on methane production of raw and alkaline pretreated ensiled sorghum forage", Waste and Biomass Valorization, DOI: 10.1007/s12649-013-9199-x.

According to Table III.3, no differences of the VS, TOC and lignin contents were observed between 2, 1, 0.5 and 0.25 mm particle sizes. Very small differences between particle sizes were observed in terms of TKN, cellulose and hemicelluloses contents. The TKN content and therefore proteins content (TKN*6.25) appeared smallest for highest size fraction (2 mm), compared to 0.25 mm, while cellulose and hemicelluloses fractions appeared smallest for particle sizes of 0.5 and 0.25 mm compared to highest ones (1-2 mm). Despite not clear indications were found in literature to explicate this phenomenon, some authors confirmed these observations. Chundawat et al. (2007) observed that larger sized particle (> 0.8 mm) had higher cellulose and hemicelluloses (structural carbohydrates) contents and finer fractions (< 0.15 mm) were richer in proteins and water soluble components and had lower hemicellulosic content. Ghizzi D. Silva et al. (2011) and Bridgeman et al. (2007) also showed that a big reduction in particle (< 50-90 µm) caused significant carbohydrate loss (up to 17% and 33% for cellulose and hemicelluloses, respectively). Nevertheless due to these very small variations in results, no firm conclusions can be drawn about this aspect.

As a consequence of the NaOH addition, a solubilisation of lignin (around 34-36%), cellulose (around 30-40%), and hemicelluloses (around 40-45%), was observed for both particle sizes (1 and 0.25 mm). Moreover, the alkaline pretreatment resulted in the solubilisation of TOC, mainly derived from fibrous fractions, and proteins, as confirmed by the reduction of TOC (up to 20%) and TKN (up to 50%) from the solid fraction separated after the pretreatment, for both particle sizes. Proteins solubilisation by alkaline pretreatment was already observed by Sun et al. (1995) who found a solubilisation of about 38%, by soaking 2.5 g of wheat straw in 100 mL of NaOH solution (1.5 %NaOH) at 20°C for 6 h.

III.2.2. Infrared (FTIR) spectroscopy

In this study, FTIR spectra in the 800 to 3000 cm^{-1} region were employed to characterize the structure of milled and alkalized samples (Figure III.4).

Peaks were assigned as follows: 1511 cm^{-1} assigned to aromatic C-O stretching mode for guayacyl ring of lignin; 1430 cm^{-1} assigned to C-H deformation in “crystalline” cellulose; 1375 cm^{-1} assigned to

deformation in cellulose and hemicelluloses; 1157 cm^{-1} assigned to C-O-C vibration in holocelluloses and 898 cm^{-1} assigned to C-H deformation in "amorphous" cellulose. A detailed description about peak assignments is reported in Chapter II.

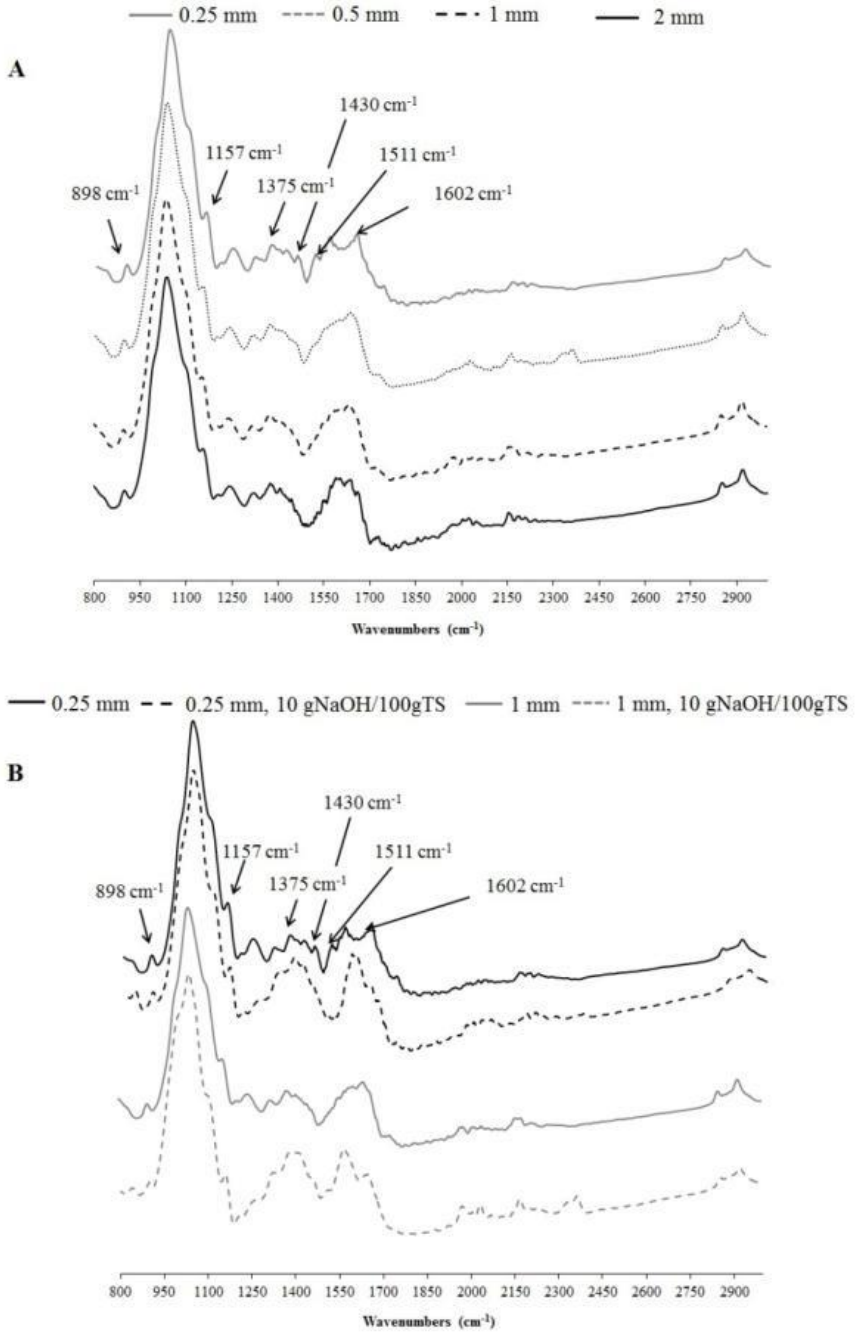

Figure III.4. Fingerprint region (800-3000 cm^{-1}) of the FTIR spectra of milled samples (A) and alkalized samples (B). Values correspond to mean of measurement performed in triplicate.

From the examination of the spectra the Lateral Order Index (LOI, H_{1430}/H_{898}), which is sensitive to the amount of crystalline cellulose versus amorphous cellulose, and H lignin/Hcarbohydrates ratio

($H_{1511}/_{H1430,1375,1157,898}$), which shows the relative intensity of lignin peaks at 1511 cm^{-1} as opposed to carbohydrates peaks at 1430, 1375, 1157 and 898 cm^{-1}, were computed (Table III.4). Both ratios seemed to be unaffected by particle size reduction, with and without the addition of NaOH. No changes in LOI index indicate that pretreatment did not affect significantly the cristallinity structure of cellulose, while no significant changes in H lignin/H carbohydrates ratio were observed after the addition of NaOH, due to a similar solubilisation of cellulose, hemicelluloses, and lignin which affect both particle sizes (1 and 0.25 mm). However, results about the cristallinity of cellulose have to be confirmed by the X-ray diffraction (DRX) analysis, which is actually the most common and appropriate technology to evaluate the crystallinity content of cellulose (Driemier and Calligaris, 2011).

Table III.4. Lateral Order Index (LOI) and H lignin / H carbohydrates ratio for mechanical and alkalized pretreated ensiled sorghum forage. Values correspond to mean± standard deviation of measurement performed in triplicate.

Samples	LOI	H lignin/H carbohydrates
2 mm	1.0±0.1	0.2±0.0
1 mm	1.1±0.1	0.2±0.0
1 mm, 10 gNaOH 100g^{-1}TS	1.2±0.0	0.2±0.0
0.5 mm	1.1±0.1	0.2±0.1
0.25 mm	1.1±0.0	0.2±0.0
0.25 mm, 10 gNaOH 100g^{-1}TS	1.2±0.3	0.2±0.1

III.2.3. Effect of pretreatment on methane production

In Figure III.5 methane yield trends ($NmLCH_4$ $g^{-1}VS$) of milled and alkalinized (10 gNaOH 100g^{-1}TS, 55°C, 12h) samples are represented as a function of the digestion time. The experimental data revealed that ensiled sorghum forage, milled into 1, 0.5 and 0.25 mm particle sizes (Figure III.5A), did not show any methane yield improvement as compared to 2 mm particle size. As expected, after alkaline pretreatment (Figure III.5B), an increase in methane production of 20% was observed for both 1 and 0.25 mm, but also in this case results were not affected by the particle size reduction. In accordance with these results, little variations of particle size ranges (5-2 mm or 0.8-0.045 mm) did not enhanced

methane production after mechanical pretreatment of wheat straw and a mixture of maize silage and sweet corn (Dumas et al., 2010; Ficara and Malpei, 2011).

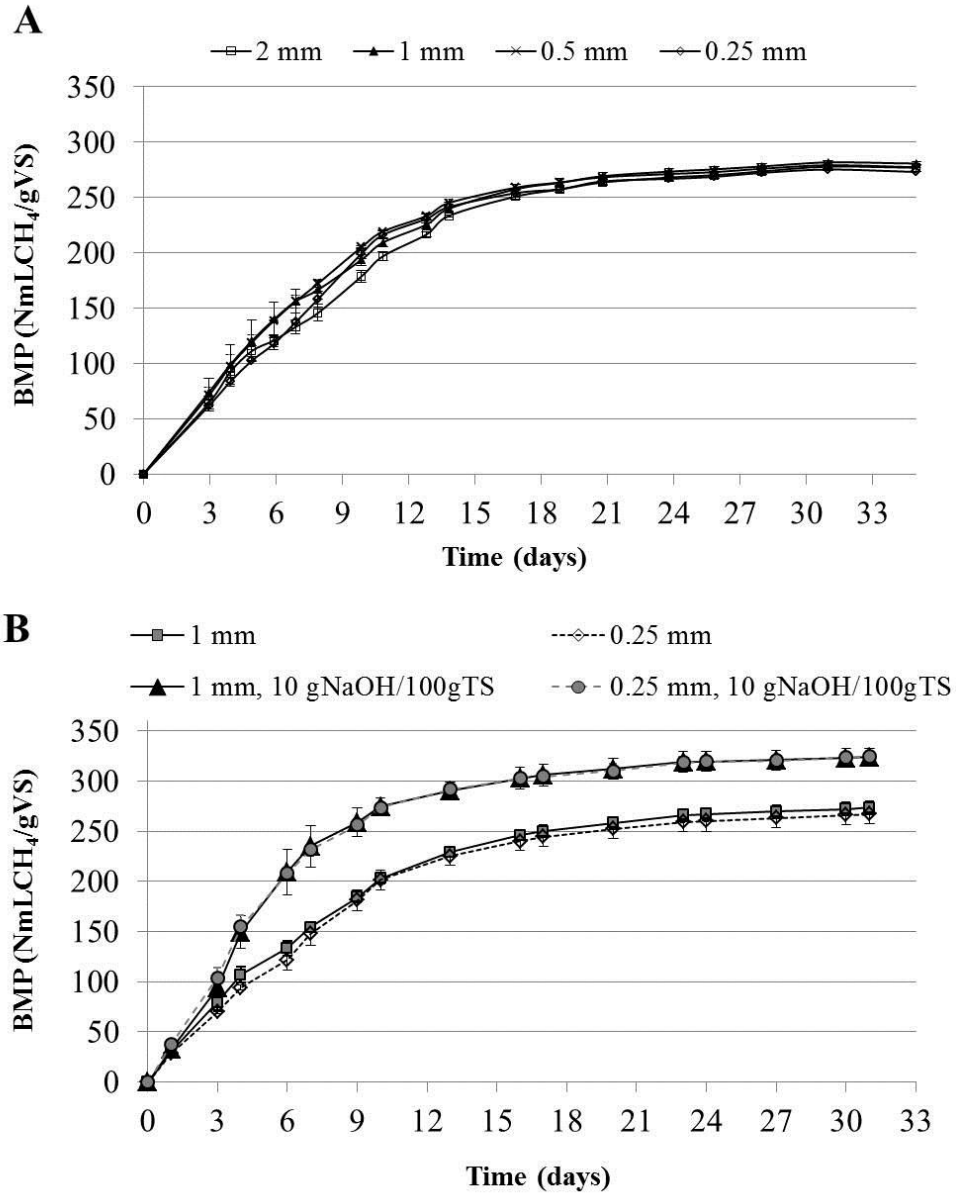

Figure III.5. Methane yields (BMP, $NmL_{CH4}\ g^{-1}VS$), at normal temperature and pressure, of milled samples (A) and alkalized samples (B). Values correspond to mean ± standard deviation of measurement performed in duplicate.

Finally, Table III.5 summarises the kinetic constant (k_h) and the ultimate methane potential ($BMP_{t\rightarrow\infty}$), obtained after milling and combined milling and alkaline pretreatment. Ensiled sorghum forage, milled into 1, 0.5 and 0.25 mm particle sizes, did not show any improvement of the first order kinetic constant as compared to 2 mm, as confirmed by Ficara and Malpei (2011). On the contrary, with the addition of NaOH, an increase in the first order kinetic constants of 31% was observed for both 1 and

0.25 mm, suggesting that, in the range of reduction studied, particle sizes did not affect the anaerobic digestion kinetic, even after the addition of an alkaline reagent.

Table III.5. Ultimate methane potential ($BMP_{t\to\infty}$, $NmLCH_4\ g^{-1}VS$) and kinetic constant (k_h, d^{-1}) values, with 95% confidential limits for each condition tested. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Samples	$BMP_{t\to\infty}$ ($NmLCH_4\ g^{-1}VS$)	k_h (d^{-1})	R^2
2 mm	298±4	0.10±0.00	0.99
1 mm	290±2	0.11±0.01	0.99
1 mm, 10 gNaOH $100g^{-1}$TS	328±5	0.16±0.01	0.97
0.5 mm	291±3	0.12±0.01	0.99
0.25 mm	288±4	0.11±0.00	0.98
0.25 mm, 10 gNaOH $100g^{-1}$TS	327±3	0.16±0.01	0.99

III.3. Partial conclusions

Results revealed that methane production from ensiled sorghum forage and wheat straw is possible, as methane potential around 270 and 200 $NmLCH_4\ g^{-1}VS$, respectively. Nevertheless, values are still lower (as 63% and 51% for sorghum and wheat straw, respectively) than theoretical ones that can be expected if all biodegradable matter was converted, suggesting that some compositional characteristics (i.e. lignin) limit their conversion into methane.

Thermal pretreatment performed at 100°C and 160°C did not have a benefit effect in the increasing of methane yield both for sorghum and wheat straw, but led to an increase of anaerobic digestion kinetics, mainly due to the solubilisation of cellulose and hemicelluloses fractions. On the contrary, as expected, good results on the enhancement of both methane yields and kinetic constants were obtained after alkaline and combined thermo-alkaline, due to the lignin degradation induced by alkaline reagent. The maximum anaerobic biodegradability of wheat straw was obtained by treating the substrate with a thermo-alkaline pretreatment performed at 100°C with 10 gNaOH $100g^{-1}$TS, while sorghum reached its maximum anaerobic degradability at 40°C with the same alkaline dosage of wheat straw.

As for anaerobic digestion kinetic, both thermal pretreatments at 100°C and 160°C and thermo-alkaline pretreatments led to an increase of kinetic constants, mainly due to the solubilisation of fibrous fractions and in particular to cellulose and hemicelluloses fractions. However, highest values were obtained by treating both substrates with 10 gNaOH $100g^{-1}$TS at 40°C for 24 h. The increase in the first order kinetic constant would result in a decreasing of the hydraulic retention time in a full scale anaerobic digestion plant. However, previous results based on batch tests, should be applied in continuous reactor to be closer to full scale anaerobic digestion plant and to evaluate the applicability and benefits to apply a pretreatment step, also in terms of both economic and energetic assessments.

Preliminary results about the effects of particle size, in the same range reduction (mm), on methane production of ensiled sorghum forage, revealed that ensiled sorghum forage, milled into 2, 1, 0.5 and 0.25 mm particle sizes, did not show any significant differences in terms of methane yields and kinetic constants. These results were also confirmed by chemical composition and infrared spectroscopy analysis, which revealed that the chemical and structural composition seemed to be not significantly affected by particle size reduction. Only, after the addition of NaOH (at 55°C for 12 h with 10 gNaOH $100g^{-1}$TS), a solubilisation of lignin, cellulose, and hemicelluloses was observed, but even in this case, results were unaffected by particle size reduction. Indeed, the increase both in methane yield and kinetic constants observed after the pretreatment resulted similar for both the 1 and 0.25 mm particles size. The comparison between these results with those of literature suggests that the impact on methane production and anaerobic kinetics may depend on pretreatment methods used (cutting milling, ball milling, chipping, grinding), on the physical structure of the substrate but also on the range of particle size reduction. Indeed, in the same range of particle size reduction no benefit, in terms of methane production increase, was observed.

Chapter IV. Effect of biological and chemical-biological pretreatments on chemical composition and methane production from ensiled sorghum forage and wheat straw

Effect of biological and chemical-biological pretreatments on methane production from ensiled sorghum forage and wheat straw

In this chapter, enzymatic pretreatments were performed with by using both commercial enzymatic preparations and fungal filtrates in order to evaluate their effect on chemical composition and methane production of ensiled sorghum forage (*Trudan 8*) and wheat straw (*Aubusson*). However, enzymes are also naturally secreted by microorganisms existing in the anaerobic digestion inoculum. Therefore, firstly, a tentative was made to explore and quantify the differences in terms of enzymatic and metabolic behaviour of four types of anaerobic sludge inoculum and to correlate them with results of Biochemical Methane Potential (BMP) tests on ensiled sorghum forage. Then, one type of inoculum (obtained by mixing a municipal anaerobic sludge and an agricultural sludge) was used to perform BMP tests before and after enzymatic pretreatments.

Pretreatments were performed with two commercial preparations on both untreated and alkaline pretreated (10 gNaOH $100g^{-1}$TS, 24 h, 40°C) ensiled sorghum forage and wheat straw, before BMP tests. Enzymatic preparations were chosen according to results obtained by testing four commercial enzymatic mixtures (paragraph IV.2.2). Pretreatments were performed under anaerobic conditions in order to avoid a loss of carbohydrates and thereafter a reduction of methane yield compared to that of untreated substrates.

Pretreatments with a fungal enzymatic filtrate were also performed. Firstly, five fungal strains were individually cultivated in a liquid medium containing ensiled sorghum forage and cellulose powder as carbon and energy sources, in order to induce enzymes secretion. After incubation, culture filtrates, were separated from the grown fungal biomass and then, according to enzymatic activities present in filtrates (paragraph IV.3.1), one fungal strain was chosen to perform the pretreatment both on untreated and alkaline pretreated (10 gNaOH $100g^{-1}$TS, 24 h, 40°C) sorghum and wheat straw, before BMP tests. In this case, pretreatments were performed under anaerobic conditions in order to avoid mycelial growth.

IV.1. Enzymatic and metabolic activities in four anaerobic sludges and their impact on methane production from ensiled sorghum forage

IV.1.1. Inoculum and sorghum characteristics

Table IV.1 summarizes Total Solids (TS) and Volatile Solids (VS) concentration of each inoculum used (WW: collected from a digester fed with waste activated sludge; AGR: collected from a digester fed with agro-wastes (cattle manure and corn silage); GR: a granular sludge collected from a UASB reactor treating wastewater from a chemical industry; MIX: a sludge obtained by mixing WW and AGR, as 50% each on a VS basis).

Table IV.1. TS and VS (g L^{-1}) concentration of four sludge inocula (AGR, WW, MIX and GR). Values correspond to mean ± standard deviation of measurement performed in duplicate.

	AGR	WW	MIX	GR
TS (g L^{-1})	54±3.6	18±0.3	27±3.8	32±0.2
VS (g L^{-1})	35±2.3	12±0.1	18±2.5	21±0.1
VS/TS (%)	65	65	65	65

TS concentration was different for the four inocula. Nevertheless, similar VS/TS ratios were found for all sludge samples.

As for ensiled sorghum forage, TS content was 93±4 gTS $100g^{-1}$ wet weight, while VS was 86.6±0.4 gVS $100g^{-1}$TS. Cellulose, hemicelluloses and lignin contents of sorghum were: 32.2±1.1 g $100g^{-1}$ VS, 16±0.6 g $100g^{-1}$ VS, and 25.7±0.2 g $100g^{-1}$ VS, respectively, as reported and discussed in Chapter III.

IV.1.2. Enzymatic activities of sludge inoculum

The enzymes are naturally secreted by microorganisms existing in anaerobic digestion inoculum but the enzymatic activities trend can vary during the course of the Biochemical Methane Potential (BMP) tests, as a consequence of the presence or absence of substrates that induce or not the secretion of enzymes. In order to characterize the sludge inocula in terms of their enzymatic behavior, BMP tests were performed both in presence and in absence of sorghum, with a substrate to inoculum ratio of 1

(see Chapter II). Then, at appropriate intervals, liquid samples were taken from each BMP bottle and characterized in terms of hydrolytic enzymatic activities expressed as IU $g^{-1}VS_{inoculum}$ (Figure IV.1).

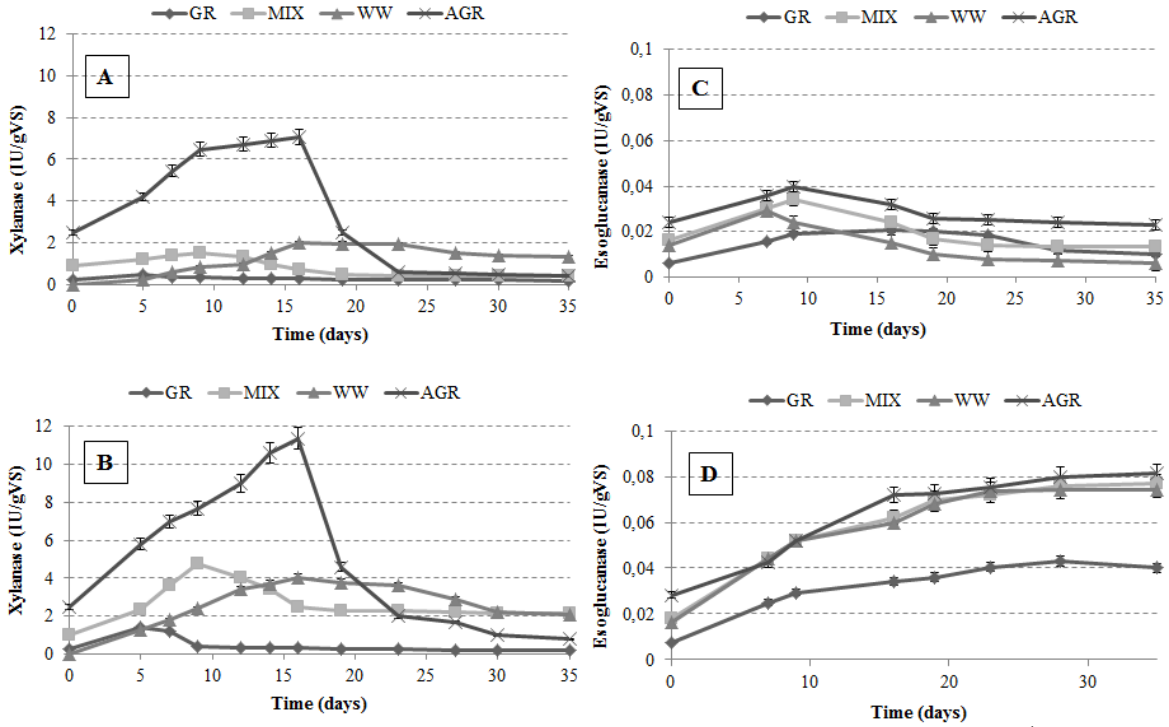

Figure IV.1. Time course of xylanase and exoglucanase enzymatic activity (expressed as IU $g^{-1}VS$) in BMP samples employing four different digested sludges in absence (A, C) and in presence (B, D) of sorghum.

Figures IV.1A and IV.1B report xylanase enzymatic activity (IU $g^{-1}VS$) trends detected in sludge samples during biochemical methane production (BMP) tests. In absence of sorghum (Figure IV.1A), xylanase activity was detected in all inocula since the beginning of the test. During the course of the following endogenous methane production, xylanase activity significantly increased in the first 17 days for the AGR sludge (max. around 7 IU $g^{-1}VS$), to decrease later on. As for the GR sludge, no dynamic evolution was observed. These data suggest that this enzymatic activity is physiologically present in all sludge inocula. The presence of sorghum increased the xylanase activity in all samples (Figure IV.1B) suggesting that xylanase production is induced by the substrate, i.e. by hemicelluloses concentration.

Exoglucanase activity (Figures IV.1C and IV.1D) was found in lower (two order of magnitude) levels than the xylanase one. Under endogenous condition (Figure IV.1C), an increasing followed by a

decreasing trend was again observed. In presence of sorghum, exoglucanase activity was found to increase in all samples, even if levels remained modest, with a maximum of 0.08 IU g^{-1}VS. This behavior is reasonable, due to the increase of cellulose hydrolysis which may favour exoglucanase expression.

No significant endoglucanase activities were observed for AGR, GR and MIX slugdes. Only at the beginning of incubation (5-7 days), WW sludge showed an activity up to 1.24 and 1.96 IU g^{-1}VS in absence and in presence of sorghum, respectively.

Laccase activity was always found in traces (max. 2.5 x 10^{-4} IU g^{-1}VS at 17 days) for AGR inoculum and in presence of sorghum.

On the overall, xylanase was the prevailing enzymatic activity for all inocula. The agricultural (AGR) inoculum showed always the highest enzymatic activities, while granular sludge (GR) showed the lowest values, with municipal (WW) sludge in-between. The mixed (MIX) sludge did not have an intermediate behaviour between WW and AGR as expected from the MIX composition, suggesting that enzyme expression is not additive and it is difficult to predict.

IV.1.3. Metabolic activities of sludge inocula

In order to define the metabolic activities of the four sludge inocula, tests were performed by measuring the methane production rate after addition of specific substrates. According to Angelidaki et al. (2009), acetic (1 g L^{-1}), propionic (1 g L^{-1}) and butyric (1 g L^{-1}) acids were dosed to assess acetoclastic, methanogenic and acetogenic activities, respectively. For the determination of hydrolytic and acidogenic activities, 1 g L^{-1} of carboxymethylcellulose (CMC) and glucose were used as substrate, respectively. Metabolic activities measured on the sludge inocula are summarized in Figure IV.2.

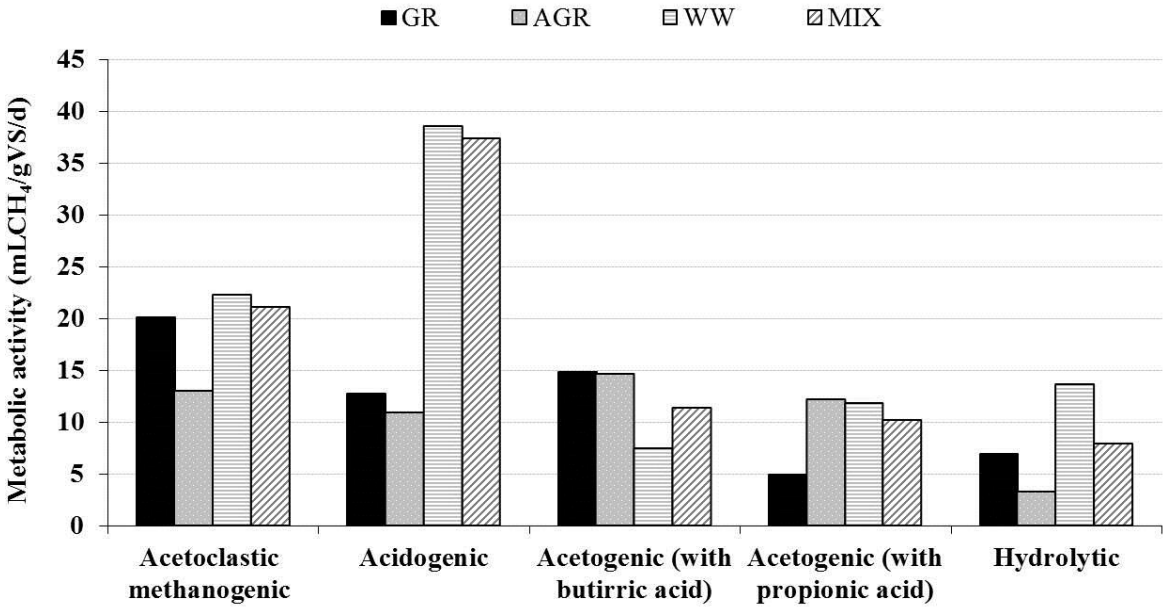

Figure IV.2. Results of the metabolic (acetoclastic methanogenic, acidogenic, acetogenic and hydrolitytic) activities, measured on the four sludge inocula.

Results suggest that the methanogenic activity was similar for both GR and WW inocula and lower for AGR. The AGR sludge showed a balanced and high acetogenic activity; a higher capacity for degrading butyric than propionic acid was observed for the GR sludge, while an opposite behaviour was found for the WW sludge. As for glucose degradation (acidogenic activity), the methane production rate observed for the WW sludge is surprisingly higher than the acetoclastic-methanogenic activity, suggesting that hydrogenotrophic methanogenesis was contributing to the observed methane production rate. This is not the case of the GR and AGR sludge, probably due to a different microbial consortia present in the different inocula. Again, the MIX inoculum did not necessarily show an intermediate behaviour between WW and AGR inocula, showing synergistic (on acetate and glucose) or slightly antagonistic (on propionic acid) effects. Interestingly, hydrolytic activity (measured by using CMC as substrate), is higher for WW than for the other inocula and the MIX inoculum showed an intermediate behaviour between WW and AGR inocula. This result is in accordance with the endoglucanase behaviour resulted high only for WW sludge at the beginning of the incubation. Indeed, as detailed in Chapter II, endoglucanase activity is determined by measuring the amount of glucose released from the CMC substrate.

IV.1.4. Results of Biochemical Methane Production (BMP) tests

The methane production of ensiled sorghum forage was monitored by performing BMP tests by using the four anaerobic sludges as inocula (GR, MIX, AGR and WW sludges), with endogenous methane yields of 23, 43, 41 and 42 $mLCH_4/gVS$, respectively. Results obtained are reported in Figure IV.3.

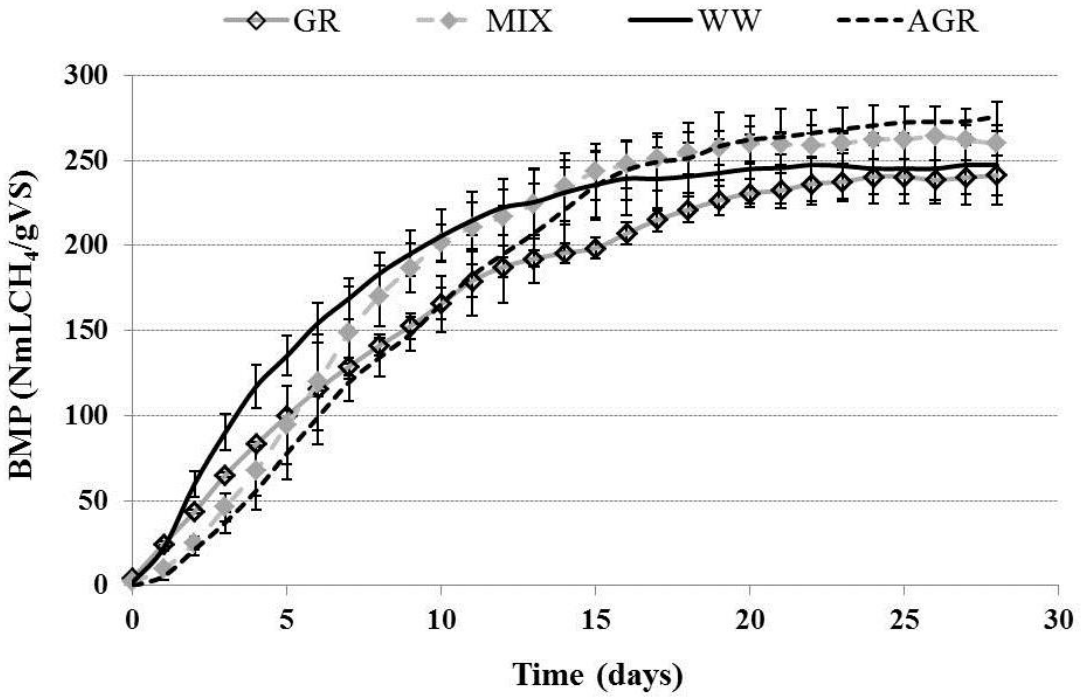

Figure IV.3. Methane yields (BMP, $NmLCH_4\ g^{-1}VS$) trends at normal temperature and pressure conditions, using ensiled sorghum forage as substrate and four anaerobic sludges as inocula. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Low differences were obtained in terms of methane yields (257±15 $NmLCH_4\ g^{-1}VS$) measured at the end of digestion batch test. However a slight higher mean value of methane yield was obtained from the agricultural sludge (AGR) than from the other inocula, possibly due to the adaptation of the bacterial consortium to similar agricultural wastes and to the highest increase in xylanase and exoglucanase enzymatic activities, measured both in present and in absence of sorghum, during the course of BMP. The lower enzymatic activities measured on GR sludge may also explain the lower methane yield obtained at the end of the BMP test. In the first 15 days, the fastest methanization occurred when using the urban sludge (WW) while the slowest was obtained from the agricultural sludge (AGR), with MIX sludge in-between. These results are in agreement with the observed

hydrolytic and acidogenic metabolic activities that were highest for the WW inoculum, lowest for the AGR sludge, with MIX sludge in-between.

In conclusion, the inoculum type may influence both the results of methane yield, although slightly, and the methane production rates during BMP tests, suggesting that the origin of the inoculum is another key parameter, with the substrate/inoculum ratio, which has to be taken into account, performing BMP tests. Despite, only few studies on the effect of different inocula on BMP of organic substrates were found in literature (Marchetti et al., 2009; Keating et al., 2011), they are in accordance with those of this study.

IV.2. Impact of enzymatic and combined alkaline-enzymatic pretreatment on ensiled sorghum forage and wheat straw

IV.2.1. Chemical composition of untreated substrates

The chemical composition of ensiled sorghum forage (*Trudan 8*) and wheat straw (*Aubusson*) is the same as reported and discussed in Chapter III and it is given here for completeness.

As for ensiled sorghum forage, TS content was 93±4 gTS $100g^{-1}$ wet weight, while VS was 86.6±0.4 gVS $100g^{-1}$TS. Cellulose, hemicelluloses and lignin contents of sorghum were: 32.2±1.1 g $100g^{-1}$VS, 16±0.6 g $100g^{-1}$VS, and 25.7±0.2 g $100g^{-1}$VS, respectively.

As for wheat straw, TS content was 94±4 gTS $100g^{-1}$ wet weight, while VS was 92.7±0.4 gVS $100g^{-1}$TS. Cellulose, hemicelluloses and lignin contents of sorghum were: 35.0±0.2 g $100g^{-1}$VS, 17.5±0.2 g $100g^{-1}$VS, and 29.0±0.2 g $100g^{-1}$VS, respectively.

IV.2.2. Characterization and selection of commercial enzymatic preparations

Enzymatic preparations were first characterized for their xylanase, endoglucanase (CMCase) and exoglucanase (avicelase) contents, according to the methods described in Chapter II. Results are

reported in Table IV.2 and Figure IV.4. BGL preparation was found to contain 235.7 ± 24.3 IU mL^{-1} endoglucanase activity and 126.5 ± 10.6 IU mL^{-1} xylanase. Agazym Ultra L was composed mainly by endoglucanase 613.2 ± 42.9 IU mL^{-1}, while Pulpzyme by xylanase 106.8 ± 1.9 IU mL^{-1}. Primafast is a highly concentrated preparation containing 2063.4 ± 0.8 IU mL^{-1} endoglucanase and 282.8 ± 5.7 IU mL^{-1} xylanase. Exoglucanase activity in all samples was found in traces (max 3.5 IU mL^{-1} in Primafast).

Table IV.2. Xylanase, endoglucanase (CMCase) and exoglucanase (avicelase) enzymatic activities (IU mL^{-1}) of the employed commercial preparations. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Enzymatic preparation	Xylanase	Endoglucanase	Exoglucanase
		IU mL^{-1}	
AGAZYM BLG	126.5 ± 10.6	235.7 ± 24.3	0.3 – 1.7
AGAZYM ULTRA L	108.0 ± 12.1	613.2 ± 42.9	1.7 – 6.1
PULPZYME HC	106.8 ± 1.9	10.9 ± 2.7	4 E-4 –7 E-3
PRIMAFAST 200	282.8 ± 5.7	2063.4 ± 0.8	1.9 – 3.5

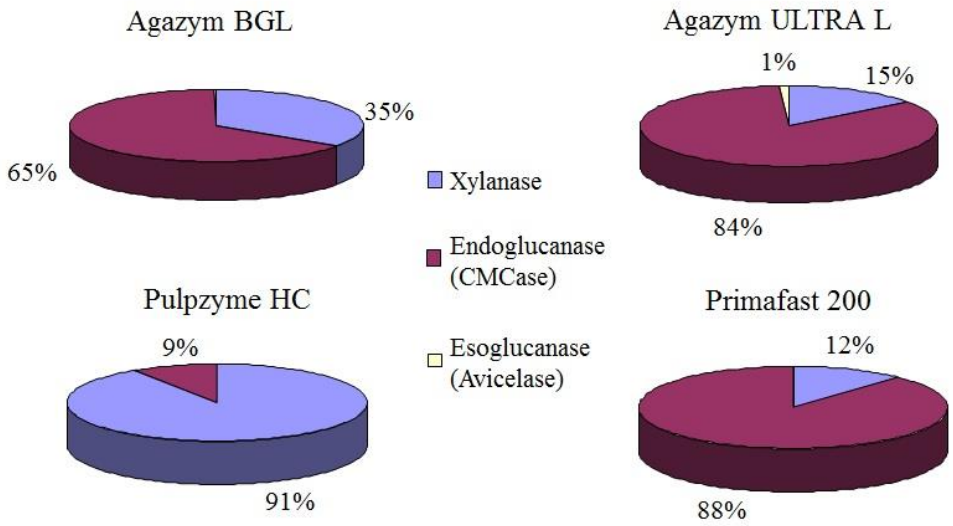

Figure IV.4. Graphic representation of the investigated activities of the four enzymatic preparations: xylanase, endoglucanase (CMCase) and exoglucanase (avicelase).

In order to choose the most proper preparation to perform enzymatic pretreatment, alkaline (with 10 gNaOH $100g^{-1}$TS, at 40°C, for 24 h) treated sorghum samples (not sterilized) were added with the four commercial enzymatic preparations (Table IV.3). Concentrations tested were chosen according to the technical instructions provided by the supplier, in particular Agazym BGL was tested at 0.04 - 0.10 - 0.20 mL g^{-1}TS, Primafast 200 at 0.12 mL g^{-1}TS, Pulpzyme HC at 0.04 mL g^{-1}TS and Agazym Ultra L

at 0.04 g^{-1}TS. After 24 h incubation at each appropriate pH (7.0 for Pulpzyme HC, Primafast 200 and Agazym Ultra L, 4.5 for Agazym BGL) and temperature (50°C for Pulpzyme, Primafast and BGL, 20°C for Ultra), liquid fractions were analyzed for their monomeric sugars content. Results are reported in Table IV.3.

Table IV.3. Monomeric sugars content (g L^{-1}) after 24 h incubation in samples of alkaline pretreated sorghum (10 gNaOH 100g^{-1}TS, at 40°C, for 24 h) added with different commercial enzymatic preparations (data are mean of three different analyses, CV in the range 5-8%).

Sample	Dosage	Glucose	Mannose-Galactose-Xylose	Ramnose	Arabinose	Glucuronic acid	Cellobiose
	mL g^{-1}TS	g L^{-1}					
BGL	**0.04**	0.28	0.37	0.21	0.32	< 0.01	0.09
BGL	**0.10**	1.21	2.43	0.18	0.49	0.03	< 0.01
BGL	**0.20**	3.49	2.46	< 0.01	0.53	< 0.01	0.04
Primafast	**0.12**	8.24	3.12	< 0.01	0.46	0.53	< 0.01
Ultra	**0.04**	< 0.01	< 0.01	0.09	< 0.01	< 0.01	0.12
Pulpzyme	**0.04**	< 0.01	< 0.01	0.14	0.09	< 0.01	< 0.01

The best result was found with Primafast (0.12 mL g^{-1}TS) and BGL (0.20 mL g^{-1}TS) with a total monomeric sugars content of 12 and 6.5 g L^{-1}, respectively, while Pulpzyme and Ultra L preparations were not active on the studied substrate.

Because of the higher degree of hydrolysis observed, the mix of BGL (0.20 mL g^{-1}TS) and Primafast (0.12 mL g^{-1}TS) was chosen to perform the enzymatic pretreatment both on sorghum and wheat straw.

Xylanase, endoglucanase and exoglucanase activities were expressed in terms of IU g^{-1}VS of the substrate, in order to compare the specific enzymatic activity of the MIX inoculum (Figure IV.1) with those added to perform enzymatic pretreatment prior to BMP tests.

As detailed in Chapter II, enzymatic pretreatments were performed with an initial total solid substrate concentration of 70 gTS L^{-1}, corresponding to an initial volatile solids concentration of 61 and 65 gVS $L^{-1}$for sorghum and wheat straw, respectively. As shown in Table IV.2, BGL preparation was found to contain 126.5 ± 10.6 IU mL^{-1} xylanase, 235.7 ± 24.3 IU mL^{-1} endoglucanase and 0.3-1.7 IU mL^{-1} exoglucanase activity, while primafast contained 282.8 ± 5.7 IU mL^{-1} xylanase, 2063.4 ± 0.8 IU mL^{-1} endoglucanase and 1.9-3.5 IU mL^{-1} exoglucanase activity.

Thus, the corresponding specific enzymatic activities of the commercial enzymatic preparations used for sorghum and wheat straw pretreatment were reported in Table IV.4. All activities resulted hundred or thousand times higher than those naturally found in the MIX inoculum during the course of BMP test (Figure IV.1). Indeed, maximum values of xylanase activity as 5 and 1.5 IU g^{-1}VS were found in the MIX inoculum at day 9 of BMP tests in presence and in absence of sorghum, respectively. Maximum value of exoglucanase activity was found at day 9 of BMP tests in absence of sorghum (0.04 IU g^{-1}VS) and at day 35 in presence of sorghum as substrate (0.08 IU g^{-1}VS). Endoglucanase was found in trace for the MIX inoculum during all the time of BMP tests.

Table IV.4. Enzymatic activities (IU g^{-1}VS) of the commercial enzymatic preparations used for the pretreatment of sorghum and wheat straw.

	Ensiled sorghum forage			**Wheat straw**		
	Xylanase	**Endoglucanase**	**Exoglucanase**	**Xylanase**	**Endoglucanase**	**Exoglucanase**
	IU g^{-1}VS					
BGL	2.08 E+03	3.87 E+03	4.9-27.9	1.94 E+03	3.62 E+03	4.6-26.1
Prima	4.64 E+03	3.39 E+04	30.4-57.5	4.34 E+03	3.17 E+04	28.4-53.8

IV.2.3. Effect of enzymatic pretreatment on the fibrous composition

Fibre composition changes induced by enzymatic and combined alkaline (10 gNaOH 100g^{-1}TS, 40°C, 24 h) and enzymatic pretreatments on ensiled sorghum forage (Figure IV.5A) and wheat straw (Figure IV. 5B) were investigated, by analysing the solid fraction separated after pretreatments. Results about sole alkaline pretreatment (see Chapter III) were reported for comparative purposes.

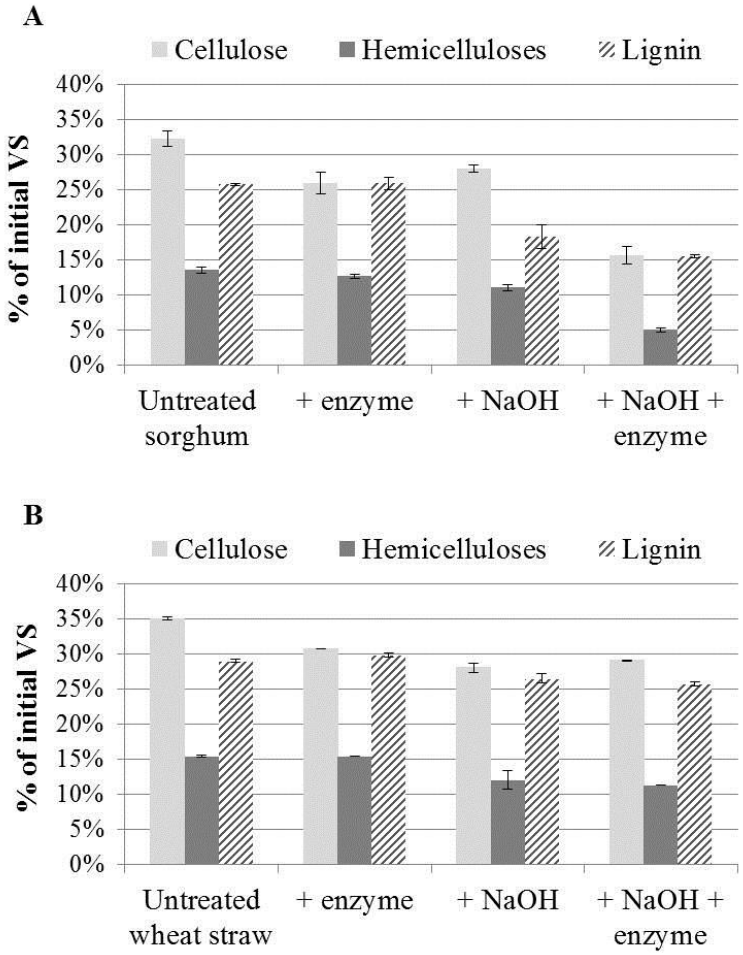

Figure IV.5. Fibrous composition of untreated and pretreated ensiled sorghum forage (A) and wheat straw (B) (results are expressed in terms of % initial VS). Values correspond to mean ± standard deviation of measurement performed in duplicate.

As shown in Figure IV.5, cellulose solubilisation (as 20 and 12 % for ensiled sorghum forage and wheat straw, respectively), was observed after enzymatic pretreatment, due to the action of endoglucanase (CMCase) and exoglucanase (Avicelase) activities. On the contrary, the sole enzymatic pretreatment led to neither hemicelluloses nor lignin solubilisation. Indeed, the absence of lignin degrading enzymes into commercial preparations did not permit the solubilisation of lignin, thus probably avoiding the subsequent solubilisation of hemicelluloses not promptly available for the enzymatic attack.

As found in Chapter III, alkaline pretreatment led to solubilisation of lignin (29% and 9% for sorghum and wheat straw, respectively), thus permitting a subsequent solubilisation of both cellulose and hemicelluloses fractions. As expected, for both substrates, no further solubilisation of lignin, compared

to that obtained after alkaline pretreatment alone, was observed after the combined alkaline-enzymatic pretreatment, due to the absence of lignin degrading enzymes into commercial preparations.

A further solubilisation of celluloses (32% and 5%) and hemicelluloses (56% and 27%) fractions, compared to enzymatic pretreatment alone, was observed for sorghum and wheat straw respectively, after the combination of pretreatments. However, by comparing these results with those of alkaline pretreatment alone, it is possible to observe that in the case of wheat straw carbohydrates solubilisation was only caused by the effect of sodium hydroxide pretreatment, while, for sorghum, a further solubilisation of both cellulose (39%) and hemicelluloses (44%) was favored by the combination with enzymatic pretreatment.

This is probably due to a different physical structure between wheat straw and sorghum. Indeed as observed by Barakat et al. (2007), both physical distribution and composition of lignin can play an important role for enzyme accessibility and the digestibility of the substrate. During alkaline pretreatment, a physical redistribution of lignin could occur and the composition of lignin could change, but this is strictly related to the type of substrate (Barakat et al., 2007).

As observed by some authors, other factors can explain the increase of enzymatic hydrolysis of cellulose and hemicelluloses after alkaline pretreatment. First of all, the increase of accessible surface area and pores volume observed after alkaline pretreatment. Gharpuray et al. (1983), observed an increase of the accessible surface area from 0.64 to 1.7 m^2 g^{-1}TS by pretreating wheat straw at 100°C with 10% NaOH (w/w) during 30 min. Gharpuray et al. (1983) have shown that specific surface area can affect the digestibility of biomass: an increase in accessible surface area resulted in higher hydrolysis yield. However, the small increases of both accessible surface area (2.5%) and pores volume (27%), observed by Monlau et al., (2012c), between untreated and alkaline pretreated (55°C, 24 h, 4 gNaOH $100g^{-1}$TS) sunflower stalks suggest that other factors may affect the enhancement of hydrolysis yield. Among them, removal of uronic acids observed after alkaline pretreatment could improve the enzymatic hydrolysis. Indeed, Pakarinen et al. (2012b) have shown that the removal of

pectins (polymer of galacturonic acids) present in hemp can increase the enzymatic hydrolysis by 26%.

IV.2.4. Biochemical methane production tests on ensiled sorghum forage and wheat straw

To assess the effect of the pretreatment on methane production of both sorghum and wheat straw, BMP tests were performed on untreated, enzymatic, and combined alkaline-enzymatic pretreated samples (Figure IV.6). Results of the BMP tests performed after alkaline pretreatment were also reported for comparative purposes.

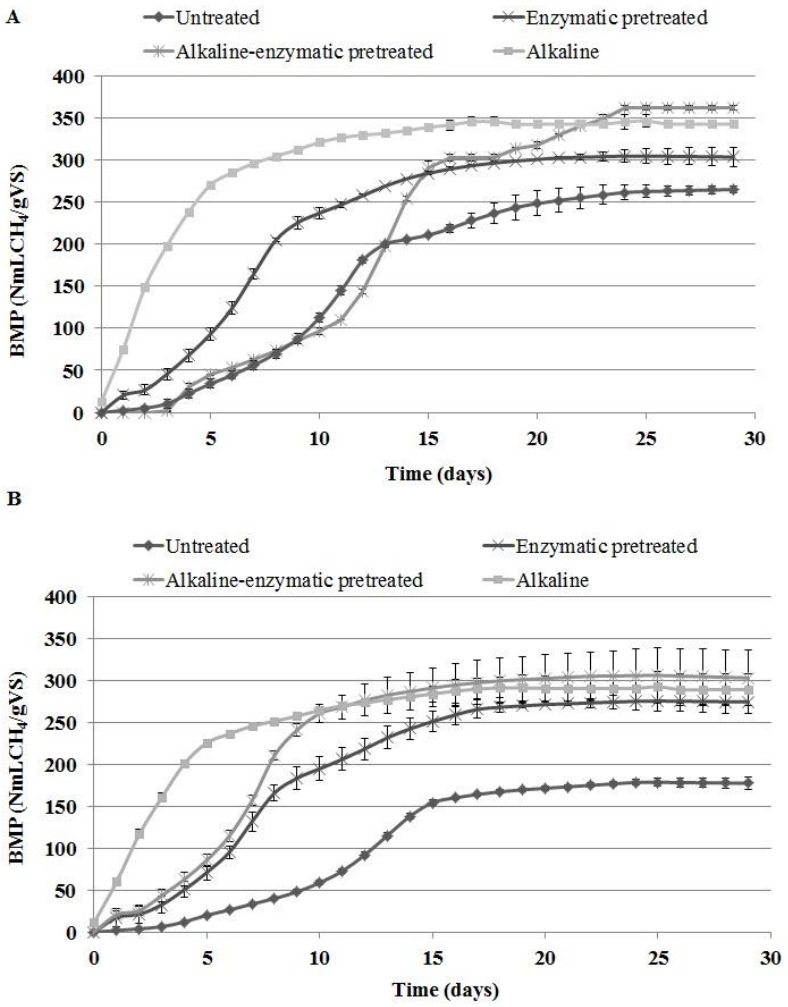

Figure IV.6. Methane yields (BMP, $NmLCH_4\ g^{-1}VS$) trends, at normal temperature and pressure conditions, of untreated, enzymatic, alkaline and combined alkaline-enzymatic pretreated ensiled sorghum forage (A) and wheat straw(B). Values correspond to mean± standard deviation of measurement performed in duplicate.

Similarly to results described in Chapter III, methane yields of untreated sorghum and wheat straw were 265±4 $NmLCH_4$ $g^{-1}VS$ and 178±7 $NmLCH_4$ $g^{-1}VS$, corresponding to an anaerobic biodegradability (according to McCarty, 1964 and equation I.2, Chapter I) of 63% and 42%, respectively.

By performing enzymatic pretreatment, increases in methane yield of 15% and 55% were observed for sorghum and wheat straw, respectively. According to fibrous composition analysis, this result is probably due both to the effect of endoglucanase enzymes which were able to attack and solubilizing cellulose fraction during the pretreatment (12% and 20% for sorghum and wheat straw, respectively), and to the effect of the enzymes naturally secreted by the microorganisms present in the MIX inoculum in presence of sorghum and wheat straw, respectively.

By combining NaOH and enzymatic pretreatment, different behaviors for sorghum and wheat straw were observed. As for sorghum, a further increase in methane production (22%), compared to enzymatic pretreatment alone, was observed. This is mainly due to the effect of alkaline pretreatment which led to a solubilisation of lignin, thus permitting a further solubilisation of cellulose and hemicelluloses fractions. Nevertheless, considering that the sole alkaline pretreatment increased methane production of sorghum by 29%, the use of a combined enzymatic and alkaline pretreatment is not justified, if considered the high cost of commercial enzymes and NaOH. On the contrary, the pretreatment combination did not further improve methane production of wheat straw, with respect to enzymatic pretreatment, and the observed increase is comparable to that obtained by performing an alkaline pretreatment alone (63%). This is probably due to a reduction of enzymatic activity in presence of phenolic compounds produced by the solubilisation of lignin after alkaline treatment, as observed by other authors (Rezaei et al., 2011). Thus, also in this case the combination of the two pretreatment is not justified.

IV.3. Impact of fungal pretreatment on methane production from ensiled sorghum forage and wheat straw

IV.3.1. Selection of fungal strain and culture conditions

In order to choose the most proper fungal strain to perform enzymatic pretreatment, sorghum or cellulose powder were comparatively employed as carbon and energy source in liquid media inoculated with the five fungal strains, according to the protocol described in Chapter II. Aliquots of culture broth were taken after 5 and 10 days of incubation, and culture filtrates separated by filtration were first characterized for their endoglucanase (CMCase), exoglucanase (avicelase) and laccase activities (Table IV.5). CMCase was generally found for all strains at higher levels after 10 days of incubation, with the best results being obtained with *Phanaerochete chrisosporium* and *Polyporus tulipiferus* on cellulose as substrate (4.95 and 5.16 IU mL^{-1}, respectively). On the contrary, avicelase activity was always found at low levels (0.01 – 0.05 IU mL^{-1}) independently from the substrate and the incubation time applied. Laccase activity was only found in trace in culture filtrates of *Irpex lacteus* and *Daedalea quercina* in the range 0.01 – 0.05 IU mL^{-1}. This behavior could be explained by the fact that this class of enzymes is not physiologically present (constitutive) and secreted by the fungi employed, but it has an inducible nature, whose synthesis was not stimulated by the substrates employed. It is also to be considered as a deeper investigation related to the detection of these enzymatic activities; several authors in fact report assay conditions (in terms of reaction pH, temperature and time of incubation, as well as reagents used) significantly different among each others (Vares et al,. 1995; Ander and Messner, 1998; Lei et al., 2011).

Table IV.5. Endoglucanase (CMCase), exoglucanase (avicelase) and laccase enzymatic activities (IU mL^{-1}) present in culture filtrate samples of five fungal strains after 5 and 10 days incubation (CV in the range 7-13%).

		Incubation time 5 days			Incubation time 10 days		
Fungal strain	**Sample**	**CMCase**	**Avicelase**	**Laccase**	**CMCase**	**Avicelase**	**Laccase**
		IU mL^{-1}					
***Irpex lacteus* MIM 100**	**Sorghum**	0.97	0.05	-	3.34	0.03	-
	Cellulose	0.69	0.02	0.05	0.13	//	-
Phanaerochete chrysosporium MIM166	**Sorghum**	0.19	0.00	-	1.67	0.02	-
	Cellulose	0.55	0.00	-	4.95	0.03	-
***Irpex lacteus* MIM 257**	**Sorghum**	1.37	0.03	-	2.58	0.03	-
	Cellulose	0.72	0.01	-	4.24	0.03	-
Polyporus tulipiferus MIM 259	**Sorghum**	2.52	0.05	-	2.99	0.03	-
	Cellulose	0.92	0.01	-	5.16	0.04	-
Daedalea quercina MIM76	**Sorghum**	0.27	0.01	0.03	1.18	0.01	0.02
	Cellulose	0.75	0.01	0.03	2.12	0.01	0.01

According to results, *Polyporus tulipiferus* highlighted the highest CMCase activity at 5 days, both in presence of cellulose and sorghum as substrate. At 10 days of fungal growth, this fungal strain presents the highest CMCase activity in presence of cellulose and similar activity to *Irpex Lacteus* strain in presence of sorghum. Therefore, this strain was chosen for the prosecution of the research, aimed at determining the time course of several enzymatic activities during culture growth in presence of sorghum as inducer, as follows: endoglucanase (CMCase), exoglucanase (avicelase), laccase, β-glucosidase activity and lignin peroxidase (Figure IV.7). Laccase and lignin peroxidase were not evidenced in culture filtrate samples, thus they are not showed in Figure.

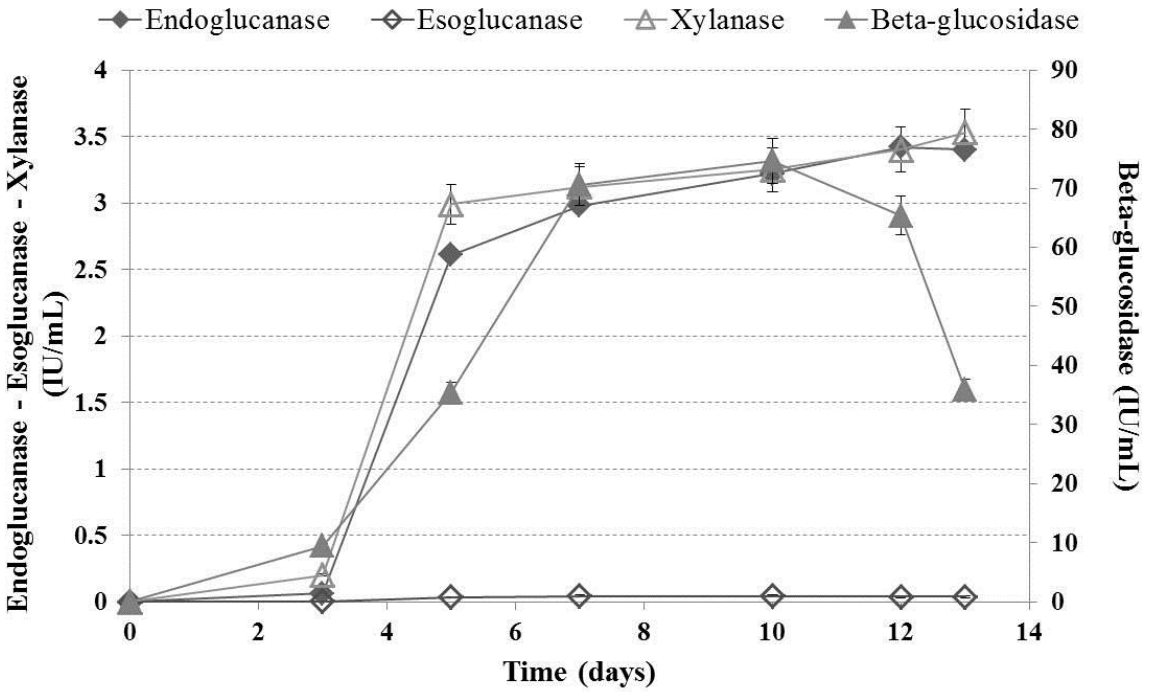

Figure IV.7. Time course of several enzymatic activity (expressed as IU mL^{-1} culture filtrate) present in samples of culture filtrates of the strain Polyporus tulipiferus employing sorghum as carbon and energy source.

β-glucosidase was found higher at 7-10 days (near 70-74 IU mL^{-1}), while endoglucanase increased up to near 3.5 IU mL^{-1} at the end of incubation. Taking into account the enzymatic activities evidenced in culture filtrates as well as their productivity, culture filtrates of *Polyporus tulipiferus* obtained after 7 days incubation in presence of sorghum as inducer was chosen to perform pretreatments.

In order to compare the specific enzymatic activity of the MIX inoculum (Figure IV.1) and the commercial enzymatic preparations, with those added to perform enzymatic pretreatment with fungal filtrate prior to BMP tests, xylanase, endoglucanase and exoglucanase activities present in samples of culture filtrates were expressed in terms of IU g^{-1}VS of substrate in the conditions found for saccharification trials.

As for pretreatment with commercial enzymatic preparations, pretreatments were performed with an initial total solid substrate concentration of 70 gTS L^{-1}, corresponding to an initial volatile solids concentration of 61 and 65 gVS L^{-1} for sorghum and wheat straw, respectively. As shown in Figure IV.7, at day 7, *Polyporus tulipiferus* filtrate was found to contain 3.12 IU mL^{-1} xylanase, 2.98 IU mL^{-1} endoglucanase and 41E-3 IU mL^{-1} exoglucanase activity.

Thus, the corresponding specific enzymatic activities used for sorghum and wheat straw pretreatment were reported in Table IV.6. All the activities resulted hundred times lower than those found in commercial enzymatic preparations and reported in Table IV.4. However, xylanase and endoglucanase activities appeared ten times higher than those naturally found in the MIX inoculum during the course of BMP test (Figure IV.1). Exoglucanase activity was found in trace both in the inoculum (max. 0.04-0.08 IU g^{-1}VS) and in the fungal filtrate, but ten times higher in the latter. Moreover, β-glucosidase activity not present inside the commercial preparations was also found in the fungal filtrate and the corresponding dosages applied to perform the pretreatment on sorghum and wheat straw were 1.15 E+3 and 1.07 E+3 IU g^{-1}VS, respectively.

Table IV.6. Enzymatic activities (IU g^{-1}VS) of fungal filtrate, in the pretreatment of sorghum and wheat straw.

Ensiled sorghum forage			**Wheat straw**		
Xylanase	**Endoglucanase**	**Exoglucanase**	**Xylanase**	**Endoglucanase**	**Exoglucanase**
IU g^{-1}VS					
51.0	48.7	0.7	47.0	45.0	0.6

IV.3.2. Enzymatic pretreatment with culture filtrate

Trials were conducted employing culture filtrates under anaerobic condition to avoid any mycelial growth, both on sorghum and wheat straw. A temperature slightly higher (37 °C) than the optimum value for fungal growth (see Chapter II) was chosen in order to speed up the kinetics of enzyme activity during saccharification trials, while pH (4.5 – 5.5) was maintained in physiological conditions for myceliar microorganisms.

In order to choose the most appropriate pretreatment time, tests were performed by prolonging the contact time between filtrate and substrate up to 48 h. Then, samples at 24 h and 48 h were analyzed in terms of total and reducing sugars (Table IV.7).

Table IV.7. Total and reducing sugars (g L^{-1}) released at 24 and 48 h in samples of untreated and alkaline-pretreated sorghum added with fungal (Polyporus tulipiferus) enzymatic preparation. Values correspond to mean ± standard deviation of measurement performed in duplicate.

		Ensiled sorghum forage		**Wheat straw**	
Condition	**Time**	**Total sugars**	**Reducing sugars**	**Total sugars**	**Reducing sugars**
	h	**g L^{-1}**			
+ Filtrate	24	11.06 ± 0.06	7.12 ± 0.49	10.85 ± 0.86	7.54 ± 0.32
	48	14.83 ± 0.70	12.54 ± 0.87	10.26 ± 1.29	7.42 ± 0.41
+ NaOH+Filtrate	24	9.15 ± 0.51	4.90 ± 0.47	7.04 ± 0.81	1.22 ± 0.08
	48	9.70 ± 0.30	6.20 ± 0.57	7.16 ± 0.65	1.37 ± 0.05

Pretreatments performed on sorghum evidenced the highest release of reducing sugars employing culture filtrate on untreated substrate, with 12.54 g L^{-1} obtained after 48 h incubation. On the contrary, alkaline pretreated sorghum did not show to be a good substrate for *Polyporus* enzymatic filtrate.

As for wheat straw, pretreatments at 24 and 48 h incubation showed similar results, and again alkaline pretreated samples evidenced lower sugars released. The low amount of sugars released evidenced in both alkaline pretreated samples, may be due to the formation of molecules (i.e. phenolic compounds) inhibiting enzymatic hydrolysis, as observed by other authors (Rezaei et al., 2011). An option is to perform fungal pretreatment prior to the addition of the sodium hydroxide reagent. This aspect of the research will be the focus of further future investigation.

However, according to these results, 48 h was chosen as contact time to perform fungal pretreatment.

IV.3.3. Effect of pretreatment on the fibrous composition

Fibre composition changes induced by fungal and combined alkaline-fungal pretreatments on ensiled sorghum forage (Figures IV.8A) and wheat straw (Figures IV. 8B) were investigated, by analysing the solid fraction separated after pretreatment. Results about sole alkaline pretreatment (see Chapter III) were reported for comparative purposes.

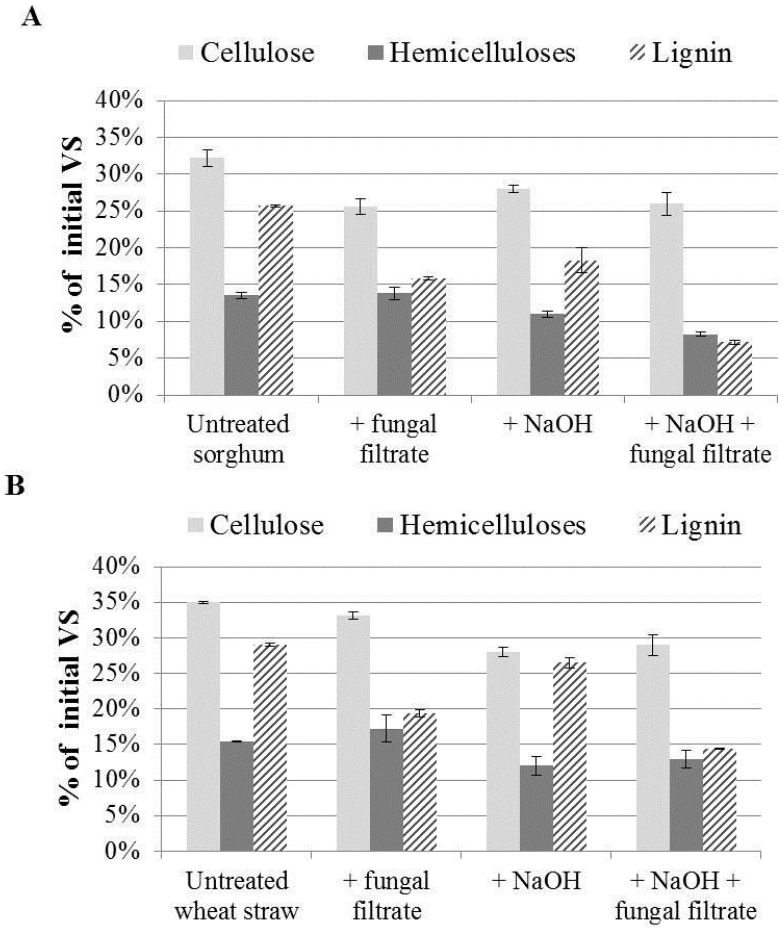

Figure IV.8. Fibrous composition of untreated and pretreated ensiled sorghum forage (A) and wheat straw (B) (results are expressed in terms of % initial VS). Values correspond to mean ± standard deviation of measurement performed in duplicate.

As shown in Figure IV.8, enzymatic pretreatment with fungal filtrate allowed a slight solubilisation of cellulose (21% and 5%) in sorghum an wheat straw samples, due to the low β-glucosidase and endoglucanase (CMCase) enzymatic activities found in fungal filtrate separated by mycelial at day 7 (Figure IV.7 and Table IV.6). Hemicelluloses fraction was not solubilized, probably due both to its more recalcitrant structure (composed of xylose, arabinose, uronic acids and acetyl groups) than that of cellulose, and to a low xylanase activity found in fungal filtrate.

Despite both laccase and lignin peroxidase enzymes were not detected in fungal filtrate, a high solubilisation of lignin was observed in both samples (38% and 33% for sorghum and wheat straw, respectively). Interestingly, lignin solubilisation after fungal pretreatment appeared higher (9% and 24% for sorghum and wheat straw, respectively) than that observed after alkaline pretreatment. This probably due to the action of other lignin degrading enzymes, such as Manganese peroxidase (MnP),

which were not measured in fungal filtrate. Vares and Hatakka (1997) showed that in fungal strains belonging to the family *Polyporaceae*, some of them apparently had a Lignin peroxidase (LiP) ligninolytic system, while others a manganese-peroxidase (MnP) dominating system for lignin degradation. Nevertheless, as stated above, a deeper revision needs to be undertaken, as results reported in terms of lignin degradation may indicate that assays for ligninolytic degradation may not reflect the true ligninolytic activity of the tested fungal strains. As example, the most widely accepted assay for detecting lignin peroxidase, based on the oxidation of veratryl alcohol to veratraldehyde, suffers from some drawbacks; at 310 nm, the wavelength at which the assay is performed, some other materials such as quinonic compounds and aromatic molecules also exhibit strong absorbance thus interfering with the estimation when present in the reaction mixture (Arora and Gill, 2001).

As expected, the addition of sodium hydroxide pretreatment, prior to fungal pretreatment, allowed a further solubilisation of lignin higher for sorghum (up to 72%) than for wheat straw (up to 50%). Interestingly, alkaline pretreatment seemed to have an additive effect, if combined with fungal pretreatment, on lignin solubilisation. The solubilisation of lignin after the sole alkaline pretreatment permitted a consequently hemicelluloses removal in sorghum (19%) and wheat straw (22%) samples, compared to untreated ones. By combining the two pretreatments, a further hemicelluloses solubilisation (20%) was observed only for sorghum, while cellulose was not further solubilized, probably due to a reduction in endoglucanase and β-glucosidase activity presumably due to the presence of phenolic compounds.

IV.3.4. Biochemical methane production tests on ensiled sorghum forage and wheat straw

BMP tests were performed on untreated pretreated samples, as shown in Figure IV.9.

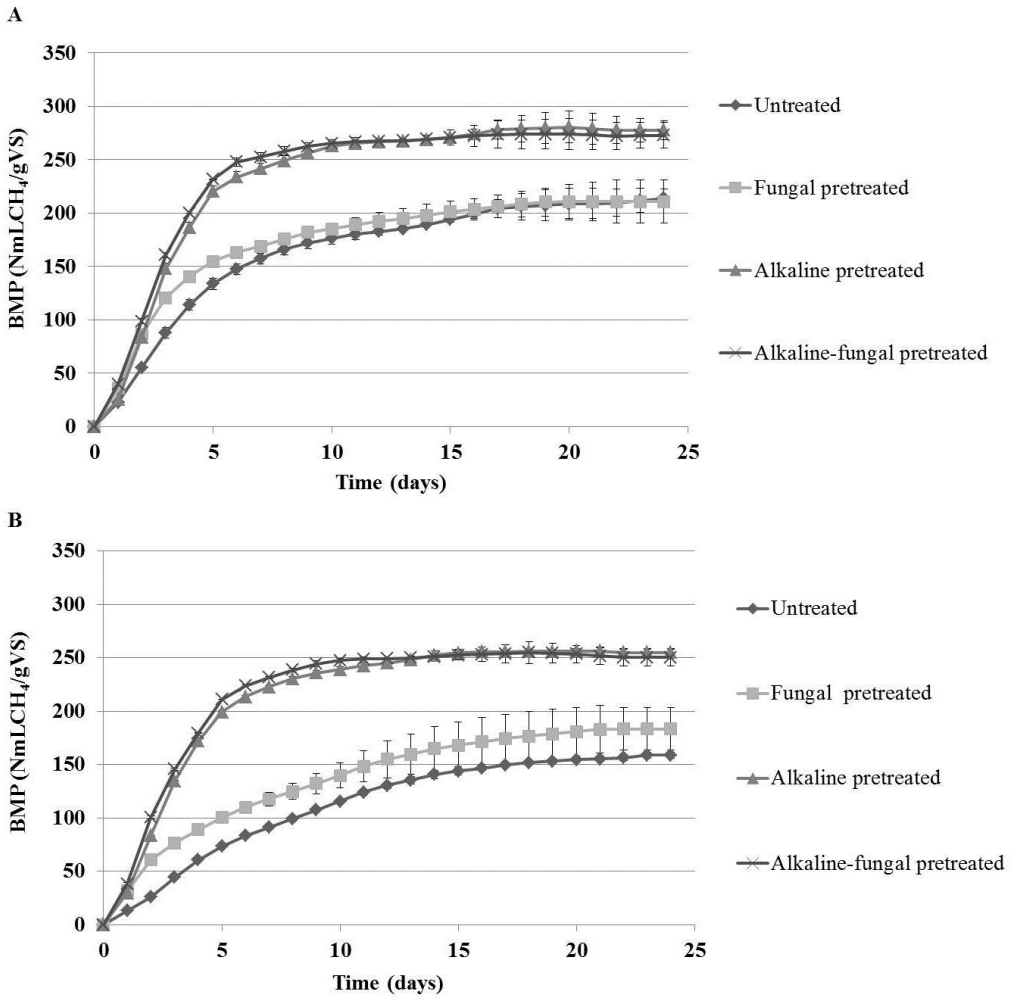

Figure IV.9. Methane yields (BMP, $NmLCH_4\ g^{-1}VS$) trends, at normal temperature and pressure conditions, of untreated, fungal, alkaline and combined alkaline-fungal pretreated ensiled sorghum forage (A) and wheat straw (B). Values correspond to mean± standard deviation of measurement performed in duplicate.

Methane yields of 214±9 and 159±3 $NmLCH_4\ g^{-1}VS$, were observed for untreated sorghum and wheat straw, respectively. These amounts were found lower but in the same range of values than previous ones. Despite, the high lignin solubilisation obtained, enzymatic pretreatment performed with the fungal filtrate did not allow enhancing methane production or anaerobic digestion kinetics of both substrates. This is probably due to different reasons: firstly, the absence of hemicelluloses (i.e. uronic acids, acetyl groups) solubilisation which can limit the consequent enzymatic hydrolysis of available carbohydrates (Kumar et al., 2009a; Pakarinen et al., 2012); secondly, the very low enzymatic activities (endoglucanase, exoglucanase, xylanase and beta-glucosidase) measured; furthermore, the possible inhibition of enzymes of fungal filtrate in presence of phenolic compounds released by the solubilisation of lignin and finally, the enzymatic pretreatment with fungal filtrate which not allow to a

physical redistribution of the substrate structure, as in the case of alkaline pretreatment, such as the accessible surface area and the pore volumes which probably were not enough to permit the entry of enzymes (Fan et al., 1981). A combination of alkaline and fungal pretreatment led to an increase of methane yield (28% for sorghum and of 58% for wheat straw) but similar to that obtained with the sole alkaline pretreatment (30% and 61% for sorghum and wheat straw), probably due to the presence of phenolic compounds which reduce the action of enzymes found in fungal filtrates and did not permit a further methane enhancement. However these aspects will be object of further investigations.

IV.4. Partial conclusions

The characterization of four types of anaerobic sludge inocula showed differences in both enzymatic (xylanase, endoglucanase, exoglucanase and laccase) and metabolic (hydrolytic, acidogenic, acetoclastic and methanogenic) activities. Xylanase activity was the prevailing one for all sludge inocula. Agricultural inoculum (AGR) showed always the highest xylanase and exoglucanase activity, while granular sludge (GR) showed the lowest values, with municipal (WW) and mixed (MIX) inocula in-between. As for metabolic activities, the municipal inoculum (WW) had the highest metabolic activities (except for acetogenic ones). Moreover, results of BMP tests suggest that the inoculum type may influence both the results of methane yield, although slightly, and the methane production rates during BMP tests. In particular, methane yields seemed related with the enzymatic activity trends of the corresponding inocula, while methane production rate appeared related to metabolic activities.

Enzymatic pretreatments performed by using commercial enzymes (xylanase, endoglucanase and exoglucanase) showed a solubilisation of cellulose, which led to an increase of methane yield for both substrates, even in absence of lignin degrading enzymes. By applying a sodium hydroxide pretreatment prior to the enzymatic one, a solubilisation of lignin and hemicelluloses fractions was also observed. This led to a further methane yield increase only for sorghum, but in a similar amount of the sole alkaline pretreatment. As for wheat straw, the combination of the two pretreatments did not have

benefit effects in further increasing methane production compared to the sole enzymatic pretreatment, probably due to an inhibition of enzymatic activity in presence of phenolic compounds released after alkaline pretreatment.

The preliminary results on fungal pretreatment have shown that the use of the enzymatic filtrate, obtained from Polyporus Tulypiferus strain, led to a higher solubilisation of lignin than that observed with the sole alkaline pretreatment, probably due to the presence of lignin degrading enzymes not yet properly analysed. However, the sole fungal pretreatment did not allow enhancing methane yields of both substrates, probably due to different reasons, such as the absence of hemicelluloses solubilisation due to low hemicellulases enzymatic activities, the possible inhibition of fungal filtrate enzymes in presence of phenolic compounds released by the solubilisation of lignin, or because the pretreatment with fungal filtrate did not allow to a physical redistribution of the substrate structure, as in the case of alkaline pretreatment. The application of an alkaline pretreatment prior to the fungal pretreatment led to an increase of methane production similar to that obtained applying the sole alkaline pretreatment, for both substrates. This is probably due to an inhibition of enzymatic activities of the fungal filtrate in presence of phenolic compounds released after alkaline pretreatment.

Chapter V. Influence of sorghum varieties on alkaline pretreatment performances

Influence of sorghum varieties on alkaline pretreatment performances

In this chapter the influence of alkaline pretreatment performances, in terms of chemical composition, physical structure changes and methane production enhancement, was evaluated on six sorghum varieties (one variety of biomass sorghum, two varieties of forage sorghum and three varieties of sweet sorghum). Alkaline pretreatments were performed at different alkaline dosages (4 and 10 gNaOH $100g^{-1}$TS) at 55°C for 12 h, as detailed in Chapter II.

V.1. Characterization of untreated sorghum samples

Results of the chemical composition of the six sorghum varieties (S1: Biomass sorghum, *Biomass* 133; S2: sweet sorghum, *hybrid BMR Sisco; S3:* forage sorghum, *Trudent Headless*; S4: sweet sorghum, *sorghum 405*; S5: sweet sorghum, *sorghum 506*; S6: ensiled sorghum forage, *sudanense hybrid Trudan 8*), summarized in Table V.1, suggest that sorghum composition can vary according to its variety.

Table V.1. Chemical composition of untreated sorghum varieties (S1, S2, S3, S4, S5, S6).

	S1	S2	S3	S4	S5	S6
TS (% wet weight)	91.5 (± 0.0)	91.3 (± 0.1)	94.0 (± 0.2)	91.8 (± 0.2)	87.8 (± 1.2)	92.7 (± 0.2)
VS (%TS)	91.8 (± 1.0)	82.7 (± 4.0)	84.7 (± 0.5)	83.9 (± 0.7)	83.2 (± 1.8)	78.6 (± 3.6)
CEL [a] (%VS)	31.1 (± 1.5)	34.2 (± 1.7)	31.2 (± 1.5)	35.8 (± 0.2)	33.9 (± 0.1)	38.5 (± 1.8)
H-CEL [a] (%VS)	18.0 (± 1.2)	19.4 (± 1.5)	20.1 (± 0.2)	18.6 (± 1.1)	17.2 (± 0.8)	28.3 (± 1.1)
K-LIG [a] (%VS)	24.1 (± 1.6)	22.3 (± 1.1)	24.5 (± 3.6)	23.7 (± 1.5)	25.7 (± 0.1)	32.8 (± 4.5)
GA [a] (%VS)	0.7 (± 0.0)	1.2 (± 0.0)	0.7 (± 0.0)	0.7 (± 0.0)	0.7 (± 0.0)	2.2 (± 0.2)
NTK (%VS)	1.1 (± 0.0)	1.6 (± 0.0)	1.5 (± 1.0)	1.2 (± 0.4)	1.3 (± 0.3)	1.8 (± 0.1)
Fats (%VS)	2.1[b]	2.6[b]	2.9[b]	2.3[b]	2.5[b]	1.8[c]
TOC (%VS)	54.7 (± 0.1)	57.1 (± 0.4)	55.5 (±1.5)	63.6 (± 2.8)	61.5 (± 0.5)	52.7 (± 0.2)
C/N	48(± 0)	37(± 0)	36(± 5)	52(± 3)	48(± 4)	29(± 0)

[a] *CEL = Cellulose; H-CEL = Hemicelluloses; K-LIG = Klason Lignin; GA = Galacturonic acid*
[c] *Fats content was determined by using an automated extraction system for accelerated solvent extraction (Model ASE 200 Dionex, Germany)*
[d] *Fats content was determined with a NIRSystem (5000 monochromator, Foss)*

The VS/TS content was similar for all sorghum varieties with the only exception of sorghum S1 (biomass sorghum), which had a highest VS/TS content. Sorghum varieties S1, S2, S3, S4, and S5 had similar fiber composition, while sorghum S6 presents higher cellulose, hemicelluloses and klason lignin content than other sorghum varieties. The TOC content is quite similar for sorghum varieties S1, S2, and S3, lowest for sorghum S6 and highest for the two sweet sorghum varieties (S4 and S5). Total nitrogen (NTK) content was lowest for sweet sorghum varieties S4 and S5 and for sorghum S1 and highest for sorghum S6. Except for sorghum S6, which as a C/N ratio of 29, for all the other varieties of sorghum the C/N ratio was higher than the desirable level, ranging from 20 to 30, which is considered as optimum for anaerobic digestion (Chandra et al., 2012a).

V.2. Chemical composition of pretreated substrates

Figure V.1 shows the changes in the fibrous composition on the solid fractions separated after alkaline pretreatment, expressed in terms of % initial VS.

As observed, sodium hydroxide pretreatment led to a solubilisation of fibrous fractions (cellulose, hemicelluloses and lignin) for all sorghum varieties, but in different amounts according to their initial chemical composition. By dosing 4 gNaOH $100g^{-1}$TS the highest cellulose solubilisation (42 and 45%) was observed for sweet sorghum samples S2 and S4, respectively. Similar cellulose solubilisations were observed at the same alkaline dosage (4 gNaOH $100g^{-1}$TS) for biomass sorghum (S1) and sweet sorghum (S5), as 30% and 29%, respectively. Forage sorghum varieties (S3 and S6) had the lowest cellulose solubilisation (18% and 4%, respectively). By increasing the sodium hydroxide dosage (up to 10 gNaOH $100g^{-1}$TS) no further cellulose solubilisation was observed for all sorghum varieties.

Hemicelluloses fraction was solubilised in similar amount both at 4 and 10 gNaOH $100g^{-1}$TS for five sorghum varieties (22-25% for S1, 30-32% for S2, 26-25% for S3, 32-35% for S4, 14-18% for S5). As for sorghum S6, a low hemicelluloses solubilisation (10%) was observed at 4 gNaOH $100g^{-1}$TS and a further solubilisation (14%) was noticed by increasing the alkaline dosage up to 10 gNaOH $100g^{-1}$TS.

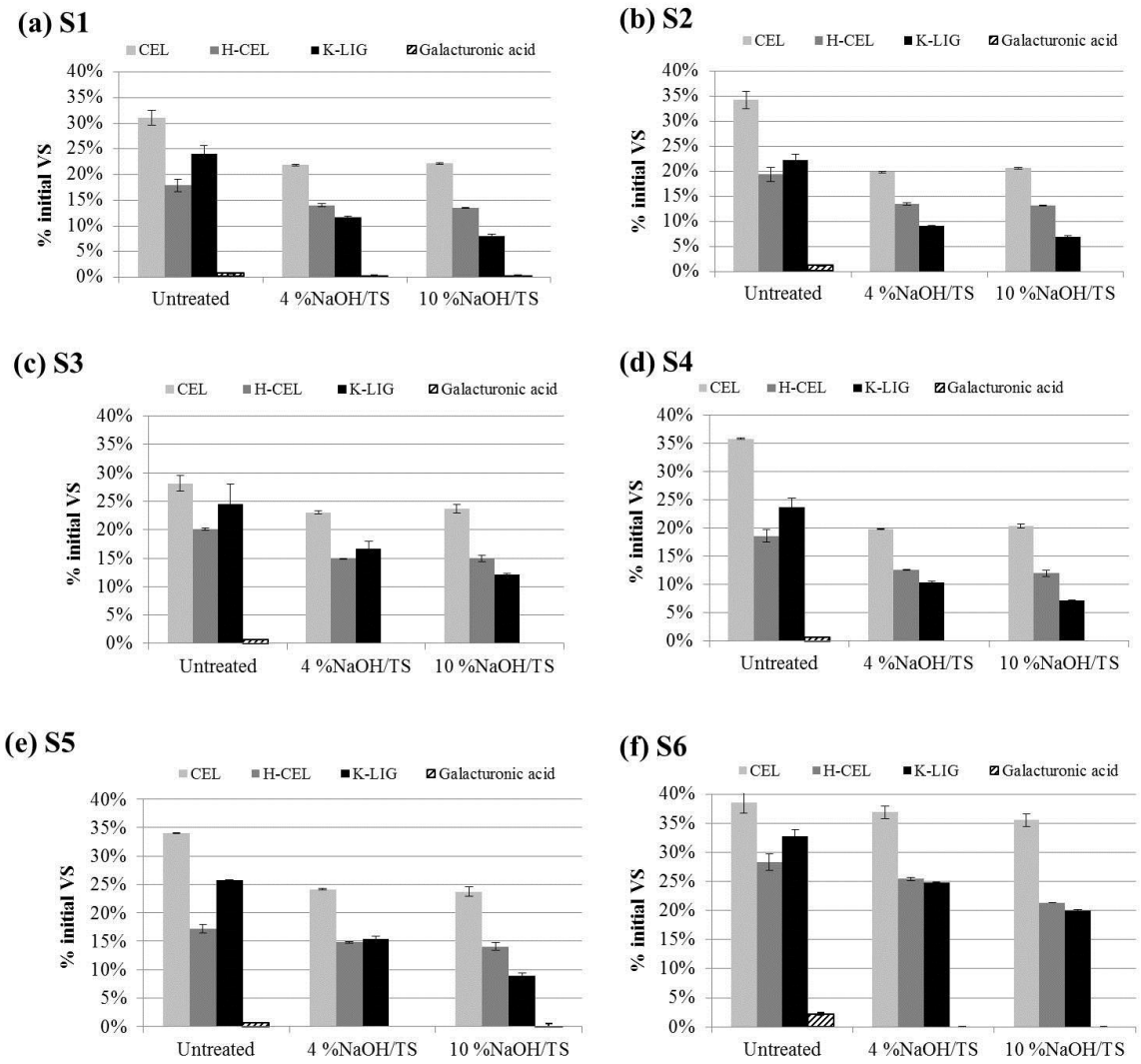

Figure V.1.Chemical composition of untreated and pretreated sorghum varieties: a) S1; b) S2: c) S3; d) S4; e) S5; f) S6. Results of Cellulose (CEL), Hemicelluloses (H-CEL), Klason Lignin (K-LIG) and Galacturonic acid are expressed in terms of % initial VS. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Contrarily to cellulose and hemicelluloses fractions, lignin was solubilised better at high alkaline dosage (10 gNaOH $100g^{-1}$TS) for all sorghum varieties. The highest lignin solubilisation was observed for sorghum varieties S1, S2, S4 and S5 (up to 67%, 68%, 70% and 65%, respectively), while the lowest for forage sorghum varieties S3 and S6 (up to 50 and 39%, respectively).

As reported in Chapter III, results about the effect of thermo-alkaline pretreatments on cellulose, hemicelluloses and lignin solubilisation on different substrates was confirmed by many studies found in literature, while up to date only one study (Kim et al., 2011b) was found about the effect of different type of pretreatment on chemical composition of switchgrass varieties. In their study, Kim et al.

(2011b) confirmed that the pretreatment effect on fibrous solubilisation varied among substrate varieties but it is also high dependent on the harvest season of the switchgrass. A high solubilisation of galacturonic acids was observed for all sorghum samples and higher for ensiled sorghum forage (S6) than for the others, probably due to the initial highest concentration. This result is in agreement with literature data that suggest the efficacy of alkaline pretreatment in uronic acids removal, originated from hemicelluloses and pectins (Monlau et al., 2012c). According to Chandel et al. (2011), hemicelluloses and pectins are bound to cellulose to form a network of cross-linked fibres. Thus, removing uronic acids can increase the accessibility of enzymes to hemicelluloses and cellulose (Pakarinen et al., 2012b).

V.3. Structural characteristics of untreated and pretreated substrates

Results obtained from chemical composition of untreated and pretreated substrates were compared with structural characteristics observed by infra-red spectroscopy analysis. Figure V.2 shows FTIR spectra of untreated samples in 3800 to 800 cm^{-1} region.

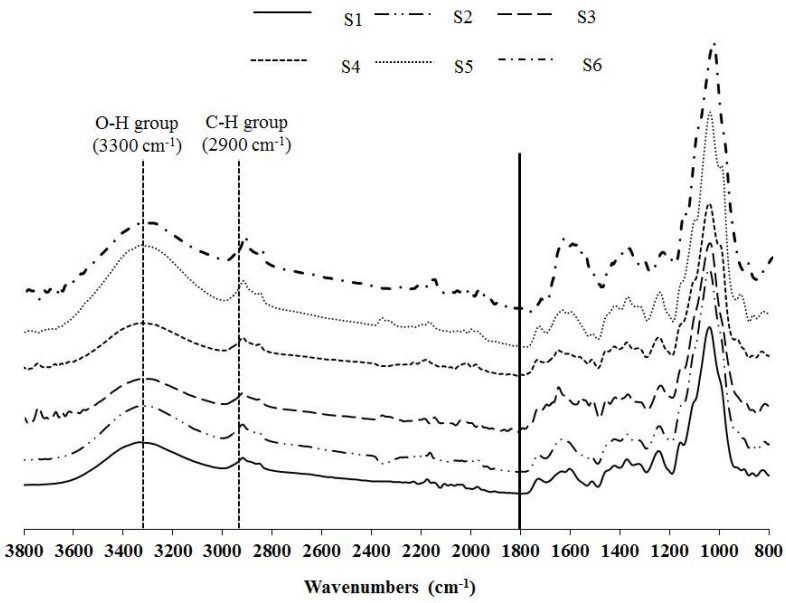

Figure V.2. Fingerprint region (3800-800 cm^{-1}) of the FTIR spectra of untreated sorghum varieties. Values correspond to mean of measurement performed in triplicate.

As shown in Figure V.2, the 3800 cm^{-1} to 1800 cm^{-1} region of the absorbance spectra has only a few bands, which are attributed to the O-H group (at around 3300 cm^{-1}) and the C-H group (at around 2900 cm^{-1}). On the contrary, in the fingerprint region (1800 cm^{-1} to 800 cm cm^{-1}) there are many bands, related to various vibration modes in carbohydrates and lignin. Therefore, the investigation of the spectra of untreated and pretreated sorghum varieties focused on the fingerprint region in 800 to 1800 cm^{-1}, in which each sample shows different pattern of absorbance, as shown in Table V.2.

Table V.2. Absorbances related to bands found in the fingerprint region (1800 cm^{-1} to 800 cm^{-1}), both for untreated and alkaline pretreated (4 and 10 gNaOH $100g^{-1}$TS) sorghum varieties (S1, S2, S3, S4, S5, S6).

Samples	Dosage	Wavenumbers (cm^{-1})						
	gNaOH $100g^{-1}$TS	898	1157	1230	1375	1430	1511	1733
S1	0	0.30	0.43	0.36	0.34	0.31	0.24	0.25
	4	0.27	0.38	0.31	0.29	0.29	0.19	0.14
	10	0.26	0.32	0.14	0.22	0.23	0.13	0.12
S2	0	0.30	0.43	0.34	0.32	0.29	0.23	0.23
	4	0.28	0.43	0.30	0.27	0.24	0.18	0.19
	10	0.24	0.35	0.19	0.25	0.21	0.15	0.18
S3	0	0.29	0.44	0.35	0.32	0.30	0.29	0.26
	4	0.22	0.42	0.29	0.24	0.23	0.22	0.17
	10	0.21	0.38	0.22	0.30	0.21	0.21	0.20
S4	0	0.32	0.44	0.36	0.35	0.33	0.27	0.27
	4	0.29	0.43	0.33	0.31	0.30	0.26	0.23
	10	0.27	0.43	0.32	0.34	0.28	0.26	0.21
S5	0	0.32	0.41	0.35	0.36	0.32	0.23	0.25
	4	0.25	0.41	0.28	0.25	0.22	0.14	0.14
	10	0.24	0.41	0.26	0.33	0.20	0.13	0.17
S6	0	0.32	0.47	0.34	0.36	0.33	0.28	0.21
	4	0.27	0.39	0.23	0.30	0.26	0.19	0.15
	10	0.23	0.40	0.29	0.25	0.23	0.19	0.17

Cellulose related bands in FTIR spectra are seen around 898, 1157, 1375, 1430 cm^{-1}. The peak at 898 cm^{-1} is associated to the C-H deformation of amorphous cellulose (Stewart et al., 1995; Pandey and Pitman, 2003; Kumar et al., 2009b; Corredor et al., 2009), while the bands around 1430 cm^{-1} are related to C-H deformation (asymmetric) of crystalline cellulose (Pandey and Pitman 2003; Gastaldi et al., 1998; Corredor et al., 2009). A decrease of both amorphous and crystalline bands was observed after pretreatment for all samples, due to the cellulose solubilisation. The peak at 1157 cm^{-1} is related to C-O-C vibration in holocelluloses (Pandey and Pitman, 2003; Yang et al., 2009; Shafiei et al., 2010), while the peak at 1375 cm^{-1} related to the C-H deformation (symmetric) in cellulose and

hemicelluloses (Pandey and Pitman 2003; Yang et al., 2009). After pretreatment these peaks decrease in intensity in all samples, suggesting that both cellulose and hemicelluloses are solubilised.

Solubilisation of pectins was observed by changes in the 1230 cm^{-1} peak, related to C-C-O stretching of esters (Sene et al., 1994; Corredor et al., 2009). This band is evident in untreated samples and decreases after sodium hydroxide pretreatment, as confirmed by chemical analyses (Figure V.1).

Lignin related bands in the FTIR spectra are seen around 1511 cm^{-1}, they are attributed to C-O absorption of guayacyl rings in lignin (Pandey and Pitman 2003; Corredor et al., 2009) and tend to decrease after the pretreatment, due to lignin solubilisation.

Hemicelluloses band appeared at 1733 cm^{-1} for all original samples (Pandey and Pitman 2003; Sun and Tomkinson, 2005; Kumar et al., 2009b). A decrease of hemicelluloses band was observed after alkaline pretreatment, indicating that hemicelluloses fraction was solubilised during the pretreatment. The chemical composition analysis (Figure V.1) supports the FTIR observations.

V.4. Methane yields of untreated and pretreated substrates

In Figure V.3 methane yield trends ($NmLCH_4$ $g^{-1}VS$, at normal temperature and pressure conditions) of untreated and pretreated sorghum varieties are showed as a function of the digestion time. Corresponding BMP values obtained at the end of each BMP test (at day 42 for sorghum varieties S1, S2, S3, S4 and S5; at day 30 for sorghum S6) are summarised in Table V.3.

Untreated sweet sorghum varieties (S2, S4, and S5) showed the higher methane yields (335±11, 327±9 and 303±24 $NmLCH_4$ $g^{-1}VS$, respectively) than biomass and forage sorghum varieties (270±13 $NmLCH_4$ $g^{-1}VS$ for S1, 294±1 $NmLCH_4$ $g^{-1}VS$ for S3 and 274±7 $NmLCH_4$ $g^{-1}VS$ for S6). Despite the high variability of methane yield values found in literature, these results are in accordance with literature data, as reported in Chapter I (Table I.2).

Alkaline pretreatment has shown to have a positive effect on lignin solubilisation, as evidenced by analytical measurements. However, it did not have benefit on the enhancement of methane yield for

five varieties of sorghum (S1, S2, S3, S4, S5) (as shown in Figure V.3 and in Table V.3). As only for ensiled sorghum forage (S6) a slight increase (up to 15%) in methane production after the alkaline pretreatment was noticed. To explain these results, the theoretical methane potential (BMP_{theo} $mLCH_4$ $g^{-1}VS$) of all sorghum varieties was calculated by knowing the chemical composition of untreated substrates (Table V.1), according to Symons and Buswell (1933) formula (equation I.1, Chapter I). Thus, by considering only compounds which are degradable during anaerobic digestion (cellulose, hemicelluloses, proteins, and lipids), a theoretical methane potential (BMP_{theo}) of 258±11 $mLCH_4$ $g^{-1}VS$ (S1), 295±13 $mLCH_4$ $g^{-1}VS$ (S2), 274±11 $mLCH_4$ $g^{-1}VS$ (S3), 284±7 $mLCH_4$ $g^{-1}VS$ (S4), 276±5 $mLCH_4$ $g^{-1}VS$ (S5), 363±13 $mLCH_4$ $g^{-1}VS$ (S6) can be expected for all sorghum varieties. Except for sorghum S6, no differences between the experimental methane potential values (BMP) reported in Table V.3 and the theoretical ones (BMP_{theo}) were observed for the other five types of sorghum, confirming that the maximum biodegradability (equation I.2) was obtained without any pretreatment.

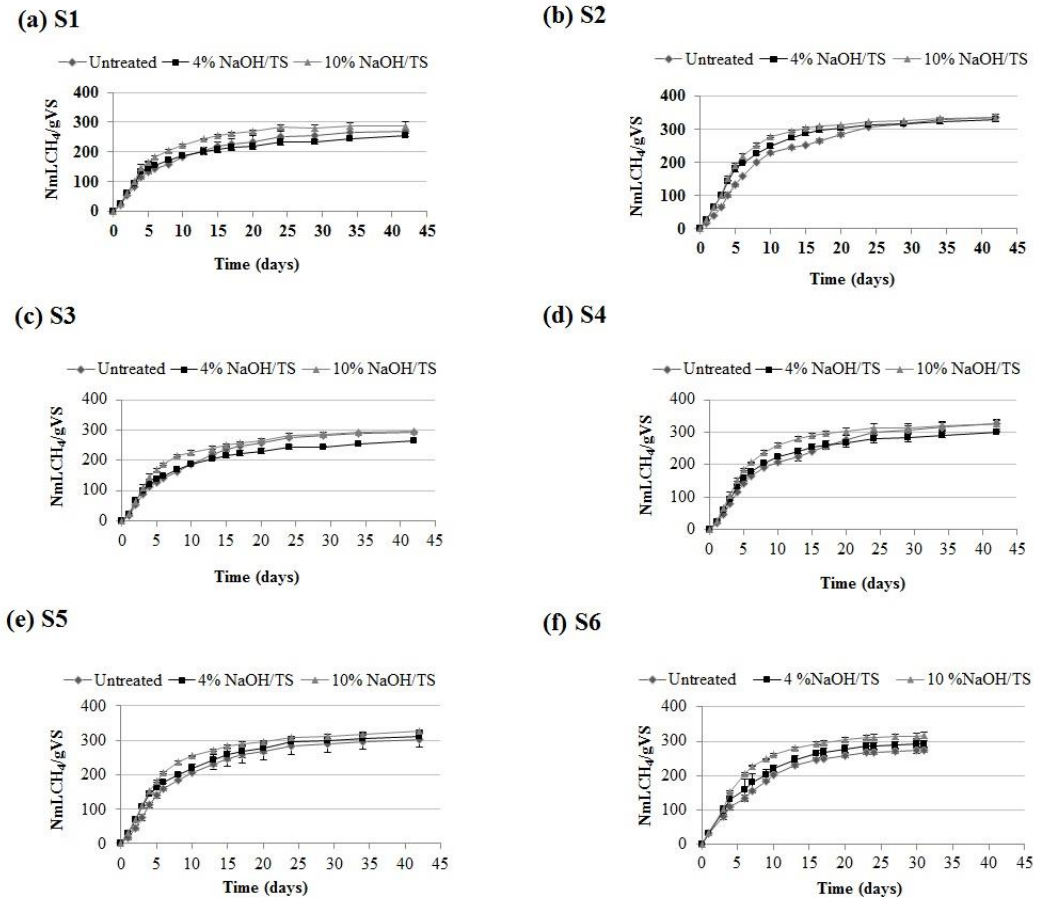

Figure V.3. Methane yields (BMP, $NmLCH_4$ $g^{-1}VS$, at normal temperature and pressure conditions) of untreated and alkaline pretreated sorghum samples: a) S1; b) S2: c) S3; d) S4; e) S5; f) S6. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Experimental results (Table V.3) make it evident the positive effect of alkaline pretreatment on the anaerobic degradation of all sorghum varieties, in terms of anaerobic digestion kinetics. In order to estimate the kinetic constant k_h (d^{-1}), anaerobic digestion process was assumed to follow a first order kinetic model (equation II.3, Chapter II), as it is the case of substrate where hydrolysis is the limiting steps, such as lignocellulosic residues (Angelidaki et al., 2009).

As previously observed in Chapter III, the first order kinetic model was successful in interpreting the experimental production trend, as demonstrated by the high R^2 values. For all varieties, an increase in the first order kinetic constant was observed for both alkaline dosages, due to the solubilisation of cellulose and hemicelluloses and lignin, as evidenced by analytical measurements, which accelerate the hydrolysis process, limiting steps for the anaerobic digestion of lignocellulosic substrates. The increase in the kinetic constant was higher at the highest alkaline dosage, probably due to a highest solubilisation of lignin, as observed with compositional analyses, thus facilitating the accessibility of the other fractions. As stated in Chapter III, similar results about the benefit effect of thermo-chemical pretreatment on anaerobic digestion kinetics were observed by other authors on different substrates (Fernandes et al., 2009; Monlau et al., 2012c), but no studies were found on sorghum varieties. Another aspect that is worth discussing is the difference between kinetic constants obtained on the same substrate (ensiled sorghum forage S6) by performing BMP tests with two different inocula (mix inoculum in Chapter III and granular sludge in this study). Indeed in Chapter III, a higher kinetic constant (0.19 d^{-1}) than that obtained in this study constant (0.11 d^{-1}) was found. As stated in Chapter IV, this is due to different enzymatic and metabolic behaviors which characterized different inocula. In particular granular sludge, characterized by lower enzymatic and metabolic activities than that observed in mix inoculum, had the lowest methanization rate, confirming the results obtained in this study.

Through the first order kinetic model (Chapter II, equation II.3) it is also possible to estimate the ultimate methane yield ($BMP_{t\to\infty}$) of a substrate. Interestingly, in the case of untreated sorghum varieties (S1, S2, S3, S4 and S5) no significant differences were observed between the experimental methane potential values (BMP) and the ultimate methane yields $BMP_{t\to\infty}$,confirming the previous

hypothesis, namely that the maximum biodegradability of these varieties was reached without any pretreatment.

Table V.3. BMP ($NmLCH_4\ g^{-1}VS$), $BMP_{t\to\infty}$ ($NmLCH_4\ g^{-1}VS$) and k_h (d^{-1}) values and its relative increase $I\,k_h$ (%) with respect to the untreated samples, with 95% confidential limits. Values correspond to mean ± standard deviation of measurement performed in duplicate.

Samples	NaOH dosage $gNaOH\ 100g^{-1}TS$	BMP	$BMP_{t\to\infty}$ $NmLCH_4\ g^{-1}VS$	k_h d^{-1}	$I\,k_h$ %	R^2
S1	0	270±13	265±4	0.12±0.01		0.99
	4	255±1	237±3	0.16±0.01	36	0.98
	10	288±14	285±3	0.17±0.00	40	0.99
S2	0	335±11	339±6	0.10±0.00		0.99
	4	328±4	324±3	0.14±0.00	48	0.99
	10	336±2	334±4	0.16±0.01	61	0.99
S3	0	294±1	297±3	0.11±0.00		1.00
	4	264±11	250±3	0.14±0.01	37	0.99
	10	298±11	279±5	0.17±0.01	64	0.99
S4	0	327±9	324±5	0.10±0.00		0.99
	4	301±2	291±3	0.14±0.01	40	0.99
	10	325±15	321±4	0.16±0.01	54	0.99
S5	0	303±24	303±6	0.11±0.01		0.98
	4	312±10	303±4	0.14±0.01	23	0.99
	10	325±6	317±3	0.16±0.00	40	0.99
S6	0	274±7	290±2	0.11±0.00		0.99
	4	293±10	299±4	0.13±0.01	16	0.99
	10	316±9	328±5	0.16±0.01	41	0.98

V.5. Partial conclusions

Results confirmed the influence of the sorghum variety on methane yield of untreated substrates, which varied between 270 and 335 $NmLCH_4\ g^{-1}VS$. Sweet sorghum varieties (S2, S4, S5) had higher methane yields than biomass and forage sorghum varieties (S1, S3, S6). As expected, alkaline pretreatment evidenced positive effects on fibrous solubilisation both at 4 and 10 $gNaOH\ 100g^{-1}TS$, for all substrates, but in different amount according to the variety of sorghum. However, the solubilisation of lignin has not brought benefits in terms of methane increase for five variety of sorghum (biomass sorghum, sweet sorghum varieties and forage sorghum S3), because of their initial anaerobic biodegradability which reached the maximum value without the application of the pretreatment. On the contrary alkaline pretreatment improved methane yield of ensiled sorghum

forage (S6), due to its lower initial anaerobic biodegradability and its higher lignin content if compared to that of the other varieties. The effect of sodium hydroxide pretreatment was observed in terms of anaerobic digestion kinetics increase for all types of sorghum, due to the solubilisation of fibrous fractions, whose amount can vary according to the composition of each untreated sorghum variety.

These results would suggest that the substrate variety may influence not only the methane production of the untreated substrate, but also the alkaline pretreatment performance, evaluated both in terms of chemical composition changes and in terms of methane production and anaerobic digestion kinetics increase.

Chapter VI. General remarks about results of batch tests

General remarks about results of batch tests

In this Chapter, a comparison between the different pretreatment strategies, previously investigated under batch mode, is presented. The comparison takes into consideration their effects both on chemical composition and methane production of ensiled sorghum forage and wheat straw, in order to define the best pretreatment strategy for these substrates with a view to scale-up. Then, information about the parameters which can affect both methane production and anaerobic digestion kinetics were drawn by correlating them with analytical data determinations (soluble COD, cellulose, hemicelluloses and lignin) performed both on untreated and pretreated sorghum and wheat straw.

VI.1. Comparison between pretreatment effects

As observed in previous Chapters, the effect of the pretreatment on chemical composition and methane production of lignocellulosic substrates varied among different pretreatment categories. Thus, Table VI.1 and Figure VI.1 summarize the effects of the various pretreatments (mechanical, thermal, alkaline, biological and their combinations) investigated under batch mode on chemical composition changes and methane production of ensiled sorghum forage (Trudan 8) and wheat straw, in order to define the best pretreatment strategy for a future scale-up of the technology.

Table VI.1. Comparison about pretreatment effects on chemical composition of ensiled sorghum forage and wheat straw ("no": no effect; "+" positive effect).

Pretreatment category	Pretreatment method	Cellulose	Hemicelluloses	Lignin
Physical pretreatment	Mechanical (for sorghum)	no	no	no
	Thermal	+	+	no
Chemical	Alkaline	+	+	+
Physical-chemical-chemical	Thermo-alkaline	+	+	+
	Mechanical-alkaline	+	+	+
Biological pretreatment	Enzymes	+	no	no
	Fungal	+	no	+
Chemical-biological	Alkaline-Enzymes	++	++	+
	Alkaline-Fungal	+	++	++

As summarised in Table VI.1, chemical structure of ensiled sorghum forage was not influenced by the particle size reduction. Thermal pretreatment led to a solubilisation of cellulose and hemicelluloses

fractions, but not of lignin, thus influencing positively only the anaerobic digestion kinetics, as reported in Chapter III. On the contrary, alkaline, thermo-alkaline and mechanical-alkaline pretretaments, led to a solubilisation of cellulose, hemicelluloses and lignin for both ensiled sorghum forage and wheat straw, essentially due to the presence of alkaline reagent. Among biological pretreatments a solubilisation of lignin was only observed after the fungal pretreatment, as expected. By combining biological and alkaline pretreatment, a further solubilisation of lignin was observed, thus improving the solubilisation of the other fractions.

Figure VI.1 summarizes the qualitative effects of the various pretreatments (mechanical, thermal, alkaline, biological and their combinations…) on methane production increase of ensiled sorghum forage (Trudan 8) and wheat straw, compared to untreated substrates. As for alkaline and thermo-alkaline pretreatments only conditions which gave the best results were considered. Null increases have to be considered as not significatives, according to the errors bar values of results reported in previous chapters.

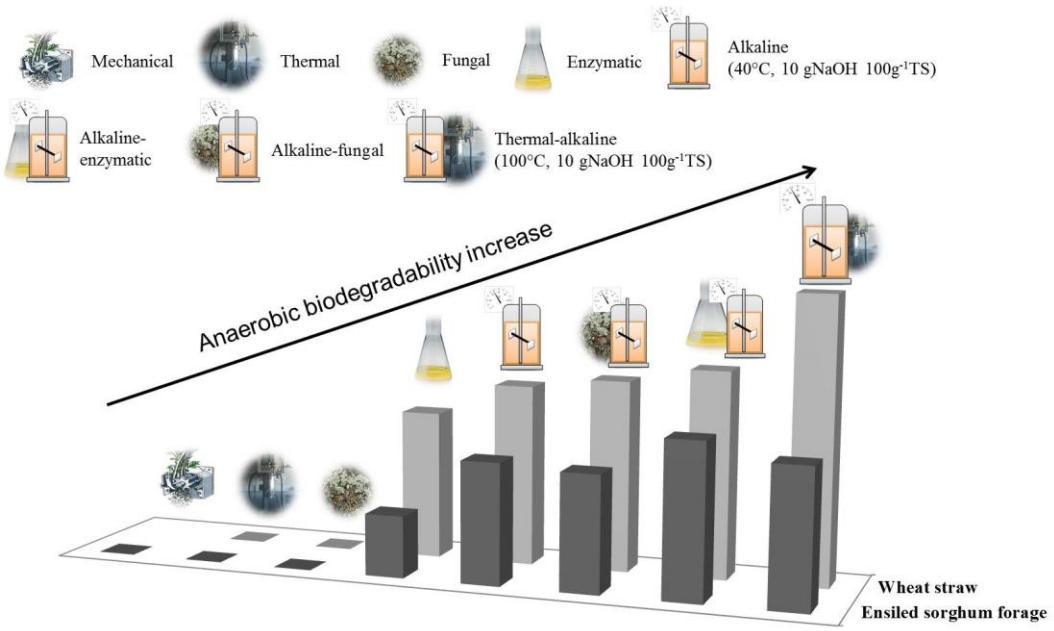

Figure VI. 1. Comparison about pretreatment effects on the increase of anaerobic biodegradability from ensiled sorghum forage and wheat straw, compared to untreated substrates.

According to qualitative (Figure VI.1) and quantitative results reported in previous chapters about anaerobic biodegradability of ensiled sorghum forage and wheat straw, thermo-alkaline pretreatment appeared the most promising pretreatment technology which led to the highest increase in methane

production of wheat straw. As for sorghum, similar results were observed between alkaline, thermo-alkaline and biological-alkaline pretreatments. However, the sole alkaline pretreatment was preferred to treat ensiled sorghum forage for both energetic and economic perspectives. Nevertheless, as detailed in Chapter V, the alkaline pretreatment performances can vary according to substrate varieties. Indeed, in the case of five sorghum varieties, different from Trudan 8, alkaline pretreatment had a positive effect on anaerobic digestion kinetics, but it did not affect methane production as compared to untreated substrates. For this reason, alkaline pretreatment and ensiled sorghum forage (Trudan 8) were chosen in order to evaluate the applicability of such pretreatment in a semi-continuous anaerobic reactor, as reported in Chapter VII.

VI.2. Correlation between chemical composition and specific methane yields

Another aspect that is worth discussing is the information that can be obtained by analytical determinations (cellulose, hemicelluloses, Klason lignin and soluble COD) performed both on untreated and pretreated substrates.

Recently, some authors tried to predict the anaerobic biodegradability and the biochemical methane potential of untreated organic substrates, by measuring the content of relevant organic components, such as soluble carbohydrates, cellulose, hemicelluloses, lignin, proteins, lipids, nitrogen, ash and acid detergent fiber (ADF) (Chandler et al., 1980; Eleazer et al., 1997; Gunaseelan, 2007; Buffiere et al., 2006; Gunaseelan, 2009; Triolo et al., 2011; Monlau et al., 2012c; Monlau et al., 2012b). Gunaseelan (2007) tried to predict methane potentials from five main chemical constituents (total soluble carbohydrates, acid detergent fibers (ADF), lignin/ADF, nitrogen, and ash), which accounted for 90% of the total variation in methane potentials (R^2 = 0.90). Triolo et al. (2011) found a high negative correlation between lignin (ADL) contents and biochemical methane potentials for untreated manure and energy crops (R^2 = 0.88). Similarly, Buffiere et al. (2006) showed a negative correlation between anaerobic biodegradability and the sum of cellulose and lignin contents. Moreover, Eleazer et al. (1997) reported that methane potentials from several untreated municipal solid wastes correlated

positively to the sum of cellulose and hemicelluloses contents. In all of these literature studies, lignin seemed to be the main restrictive factor for methane production, likely by limiting the microbial accessibility to holocelluloses during the fermentative process.

In this paragraph a tentative was made in order to correlate the methane production and/or methane production rate with soluble COD, cellulose, hemicelluloses and klason lignin data which have been drawn from results obtained in batch mode from both untreated and pretreated substrates.

Contrasting indications on the effectiveness of pretreatments on methane production have been drawn by analytical data on COD solubilisation obtained before and after alkaline, thermal and thermo-alkaline pretreatments on both ensiled sorghum forage (Trudan 8) and wheat straw. Indeed, as shown in Figure VI.2, no satisfactory linear correlation was found between the percentages of soluble COD (CODs), with respect to that of untreated substrates (COD), and methane yield values (BMP, $NmLCH_4$ $g^{-1}VS$).

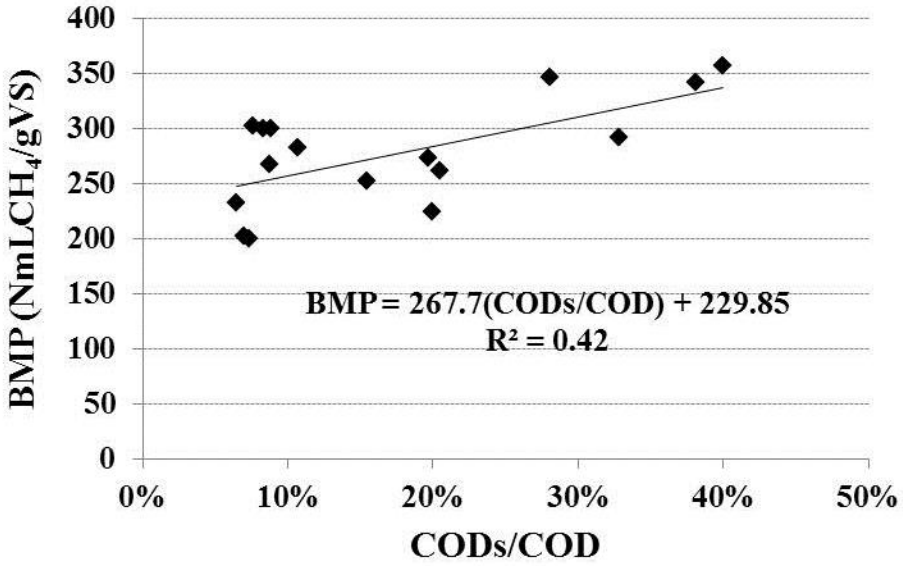

Figure VI.2. Correlations between methane potentials and soluble COD released as CODs/COD (%), of alkaline and thermo-alkaline pretreated ensiled sorghum forage (Trudan 8) and wheat straw.

As a matter of fact, the most interesting increase in the methane yield was obtained by pre-treating wheat straw with 10 gNaOH $100g^{-1}TS$ and at 40 or 100°C, quite in agreement with COD solubilisation data, while increases in methane yield values (up to 12%) with 1 gNaOH $100g^{-1}TS$ at 40°C and 100°C for sorghum and with 1 gNaOH $100g^{-1}TS$ at 100°C for wheat straw (48%) were not expected from the

poor COD solubilisation (8-9% and 8% for sorghum and wheat straw, respectively) observed at these same pretreatment conditions. This is probably to the fact that the sole CODs data are insufficient to quantify the improved biodegradability since it does not include any information on the improved accessibility of the fibrous material. Similarly, COD solubilisation observed at 160°C (20% for both sorghum and wheat straw) did not lead to a higher BMP value than 273 and 224 for sorghum and wheat straw, respectively. This is probably due to the fact that soluble COD released from lignocellulosic substrates is composed of various organic molecules, of which some are easily anaerobically degraded (essentially those form cellulose and hemicelluloses) while others are less (i.e. soluble phenolic compounds from lignin solubilization).

By considering all results from batch tests obtained before and after the pretreatment application both on wheat straw and six sorghum varieties, no satisfactory linear correlations were found between fibrous fractions content and the specific methane production (BMP, $NmLCH_4\ g^{-1}VS$) values. Indeed linear correlation coefficients (R^2) of 0.19 for cellulose, 0.04 for hemicelluloses and 0.23 for lignin, were found and they suggest that the information on fibrous fractions cannot be used to predict in general the methane production from a pretreated substrate.

However, by considering categories of pretreatment separately, some interesting information can be derived from the analysis of fibrous fractions of ensiled sorghum forage (Trudan 8) and wheat straw. By considering data from thermal, alkaline and thermo-alkaline pretreatments, only a good negative correlation between lignin and BMP values was found (R^2=0.76), while no correlations were found by considering both cellulose (R^2=0.24) and hemicelluloses (R^2=0.21) fractions separately and in combination (R^2=0.24). Interestingly, the previously found good correlation with lignin was improved (R^2=0.78) by considering only data from alkaline and thermo-alkaline pretreatments, which affect lignin solubilisation (Figure VI.3). By considering all fibrous fractions, a less significant correlation with BMP values was found (R^2 = 0.59). Thus, for these pretreatment categories, the lignin content seems to be the major parameter affecting methane potentials of untreated and pretreated ensiled sorghum forage and wheat straw. These results were also observed by other authors (Kobayashi et al., 2004; Take et al., 2006; Monlau et al., 2012c). Kobayashi et al. (2004) found a strong negative

correlation (R^2 = 0.95) between the amount of methane produced and the amount of lignin after the steam-explosion of bamboo (243°C, 5 min). Similar strong negative correlation (R^2 = 0.98) was also observed between the amount of Klason lignin in steam-exploded wood chips (258°C, 4.51 MPa) and the amount of methane gas produced (Take et al., 2006). Monlau et al., 2012c observed a strong negative correlation (R^2 = 0.92) between methane yield values and the amount of Klason lignin in thermo-chemical pretreated sunflower stalks (24 h, 55°C; 24 h, 55°C, 4%w/w NaOH; 24 h, 55°C, 4%w/w H_2O_2; 24 h, 55°C, 4%w/w $Ca(OH)_2$; 1h, 170°C; 1h, 170°C, 10%w/w $FeCl_3$; 1h, 170°C, 4%w/w HCl).

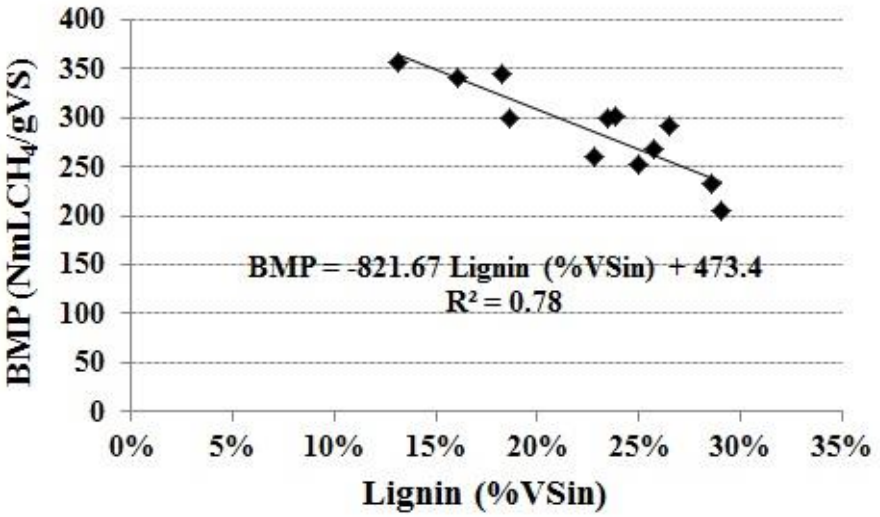

Figure VI.3. Correlations between methane potentials and lignin content (%VSin) of alkaline and thermo-alkaline pretreated ensiled sorghum forage (Trudan 8) and wheat straw.

By considering all results obtained after biological pretreatments (commercial enzymes and fungal filtrates), poor correlations with cellulose (R^2 = 0.41), hemicelluloses (R^2 = 0.17) and lignin (R^2 = 0.03) were found.

However, information can be derived from the correlations found by considering enzymatic and fungal pretreatment separately, in combination with alkaline pretreatment. Indeed, fungal pretreatment in combination with alkaline pretreatment showed the best correlations (Figures VI.4A and VI.4B) between BMP values and hemicelluloses (R^2 = 0.85) and lignin (R^2 = 0.72). By combining hemicelluloses and lignin values, correlations were not improved (R^2 = 0.69). Thus, in the case of

such pretreatment both lignin and hemicelluloses seems to be useful parameters which affect methane production.

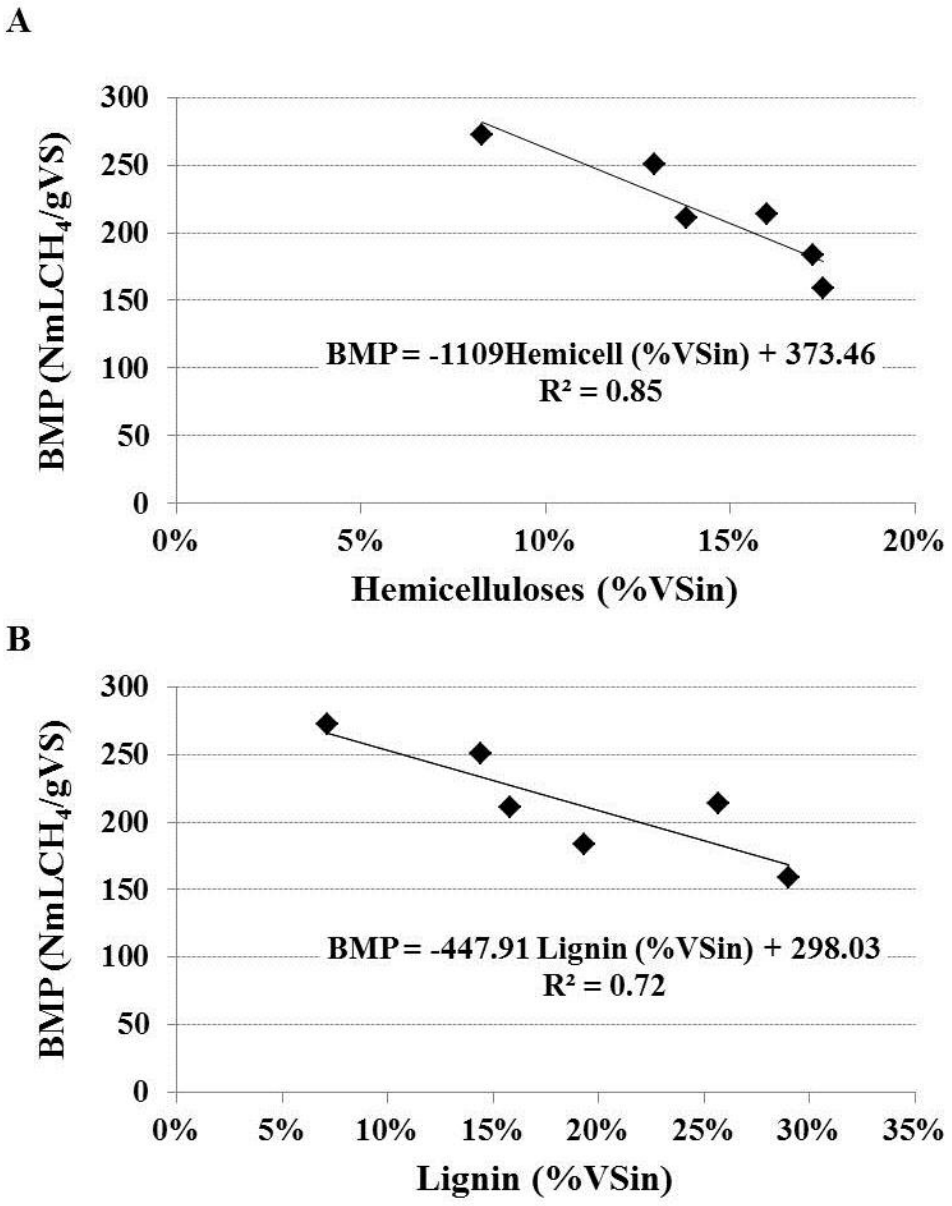

Figure VI.4. Correlations between methane potentials and hemicelluloses (A) and lignin (B) contents (%VSin) of fungal and alkaline-fungal pretreated ensiled sorghum forage (Trudan 8) and wheat straw.

On the contrary, after enzymatic and combined alkaline-enzymatic pretreatment, performed with the use of cellulose and hemicellulose enzymatic preparations, the best correlation (Figure VI.5) was found between cellulose and BMP values (R2=0.81), while not satisfactory correlations were found between BMP values and both hemicelluloses (R2=0.57) and lignin (R2=0.58) values. In this case, cellulose solubilisation plays the major role in affecting methane production from sorghum and wheat straw.

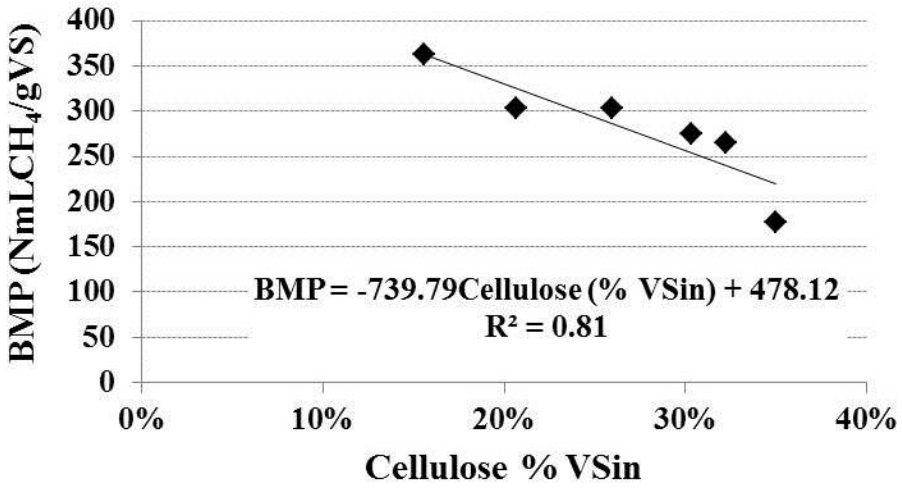

Figure VI.5. Correlations between methane potentials and cellulose content (%VSin) of enzymatic and alkaline-enzymatic pretreated ensiled sorghum forage (Trudan 8) and wheat straw.

These results would suggest that lignin can be considered the most important parameter affecting methane production of lignocellulosic substrates in the case that pretreatments have an effect on lignin solubilisation (i.e. alkaline, thermo-alkaline, fungal and alkaline-fungal pretreatments). On the contrary, as for enzymatic pretreatment with commercial cellulase and hemicellulase enzymes, the key factor, which can explaine methane production is cellulose.

Positive but not satisfactory correlations were observed between anaerobic digestion kinetics and the soluble COD (R^2 = 0.58) or with soluble cellulose and hemicelluloses (R^2 = 0.55) before and after thermal, alkaline and thermo-alkaline pretreatments on both sorghum and wheat straw. As matter of fact, it is known (Angelidaki et al., 2009) that the origin of the inoculum used in BMP tests and its storage time may significantly affect the hydrolysis rate, increasing the uncertainty of k_h estimate and possibly masking the affect of other factors. This aspect was also discussed in Chapter IV.

However, the above presented correlations were built without considering the presence of other compositional or structural characteristics, such as the presence of pectins (polymer of uronic acids), the cellulose crystallinity, the accessible surface area and the pore volumes which can affect the methane production of lignocellulosic substrates. Indeed, cellulose has got both crystalline and amorphous parts and the crystalline one prevents plant cell penetration by micro-organisms or extracellular enzymes. Recently, Pakarinen et al. (2012b) showed that pectins removal can

significantly increase enzymatic hydrolysis of lignocellulosic substrates. Monlau et al. (2012b) found that anaerobic biodegradation of lignocellulosic materials into methane is not only related to the lignin content, but crystalline cellulose had also a negative impact on methane production but in a lower extent than lignin. Zhu et al. (2010) showed that the lignin content and cellulose crystallinity are the two dominant parameters affecting negatively the digestibility of lignocellulosic substrates. Moreover, they suggested that cellulose crystallinity could have a higher influence on short time hydrolysis, whereas the lignin content could have a higher impact on long-time hydrolysis. Additionally, a significant positive correlation was found between methane potential and the content in soluble sugars, proteins and amorphous hemicelluloses in their study. According to Hayashi et al. (2005), the readily accessible regions (amorphous regions) of the lignocellulosic biomass are more efficiently hydrolyzed during enzymatic hydrolysis, resulting in the accumulation of crystalline cellulose. Similarly, Scherer et al. (2000) showed that the most degradable part of spent grains corresponded to their soluble and hemicelluloses fractions, whereas cellulose and lignin were slightly degraded.

Chapter VII. Comparative performance evaluation of semi-continuous anaerobic reactors fed with untreated and alkaline pretreated ensiled sorghum forage

Comparative performance evaluation of semi-continuous anaerobic reactors fed with untreated and alkaline pretreated ensiled sorghum forage

The aim of this Chapter was to validate in two semi-continuous anaerobic reactors the results obtained in batch mode on the effect of alkaline pretreatment (10 g NaOH $100g^{-1}$TS, 24 h, 40°C) on anaerobic digestion of ensiled sorghum forage (Trudan 8). As resulted from batch tests, the methane yields of untreated and alkaline pretreated ensiled sorghum forage were 269±22 and 346±9 $NmLCH_4$ g^{-1}VS respectively, as previously described in Chapter III.

VII.1. Results

Figure VII.1 reports the trend of average weekly Organic Loading Rates (OLR, kgVS m^{-3} d^{-1}), during the course of the experimentation.

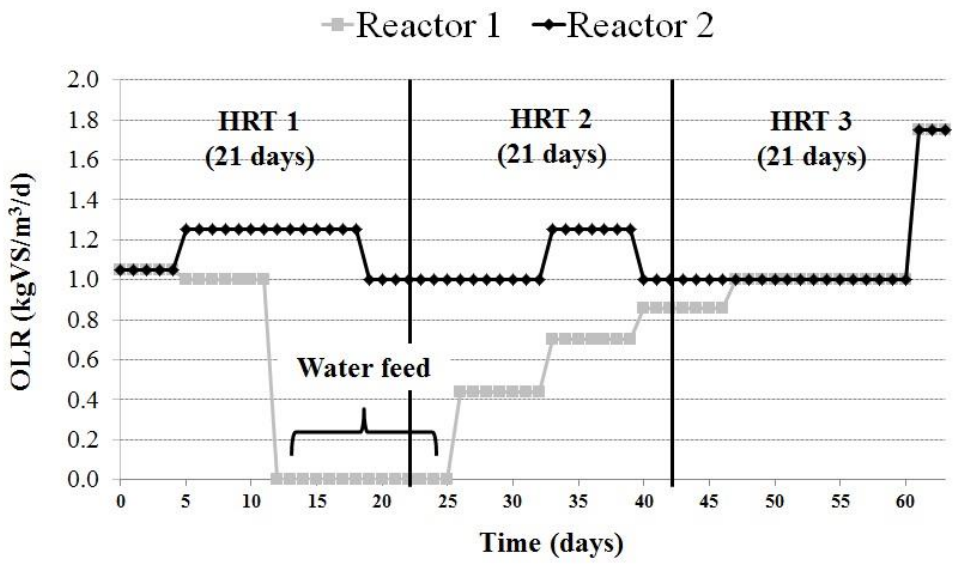

Figure VII.1. Trend of average weekly organic loading rates (OLR, kgVS m^{-3} d^{-1}) during the time of experimentation.

Temporary OLR decreases were operated in response to the build-up of VFA and the consequent decrease in biogas production. The OLR was decreased by reducing the influent sorghum

concentration, while keeping the HRT constant (21 days). Null values of the OLR mean the addition of tap water instead of the sorghum suspension.

Figures VII.2, VII.3, VII.4 and VII.5 report the observed trends for total and single Volatile Fatty Acids (VFA), ammoniacal nitrogen ($N\text{-}NH4^{+}$), pH and alkalinity concentrations in both reactors.

As for total VFA concentrations in reactor 1 (Figure VII.2), they remained high and increased up to 2.2 g L^{-1} during the first HRT, leading to an inhibition of the methanogenesis step and a consequent decrease of methane production. This was due to a higher hydrolysis rate than the methanogenic one, as shown below. As stated before, the OLR was decreased by reducing the influent VS concentration and replacing the feed with tap water. On the contrary, in reactor 2 total VFA concentration remained lower than 0.8 g L^{-1} for all the experimental campaign. However, some VFA peaks were also periodically observed during the last two HRT. Thus, to avoid inhibition, tap water was fed in reactor 2 once a week with a decrease of the weekly average OLR (Figure VII.1) which led to an instantaneous decrease of VFA.

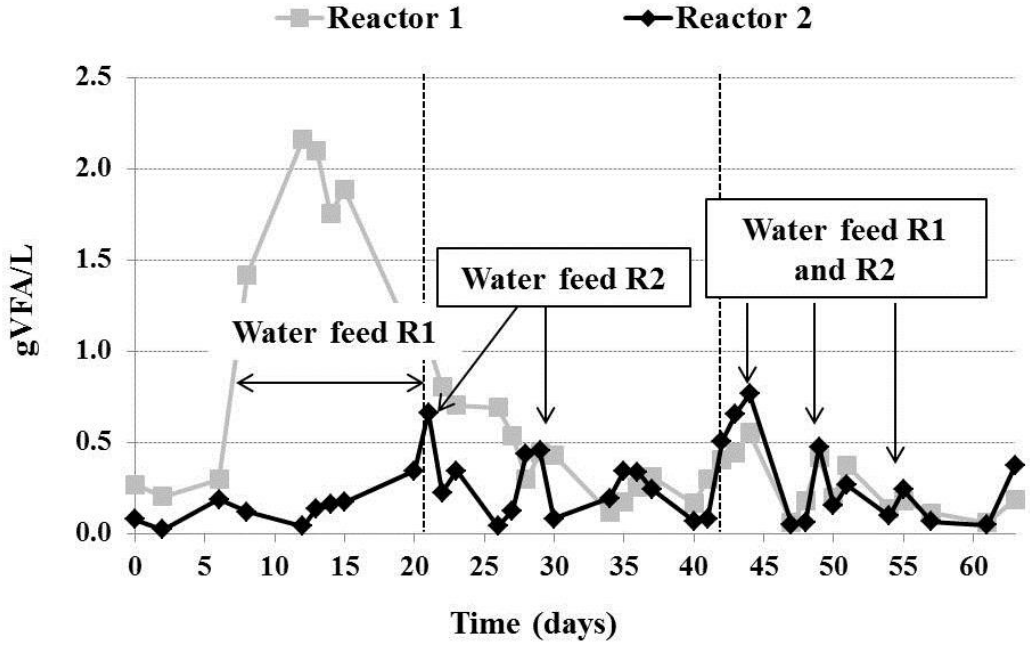

Figure VII.2. Total VFA concentrations (gVFA L^{-1}) trend in reactor 1 and 2.

As observed in Figure VII.3A, the high concentration of total VFA in reactor 1, during the first HRT, was mainly due to the high acetic acid concentration (up to 1.47 g L^{-1}), which is normally converted

into methane. However, also propionic acid and isovaleric acid were increased (up to 0.45 g L^{-1} and 0.19 g L^{-1}), leading to an inhibition of the system with a consequent reduction of the methane production.

After day 8, when the VFA concentration started to grow in reactor 1, the latter was fed with tap water only to favor VFA reduction and this resulted in a slight decrease of VFA concentration up to day 27, when the OLR started to be increased gradually. During the last part of the experimentation, VFA levels remained lower than 0.4 g L^{-1}, and decreased to a satisfactory concentration around 0.20 g L^{-1} during the last two weeks. However, some peaks of acetic and propionic acids were also periodically observed during the last HRT (from day 44 to day 56). Thus, to avoid another methanogenic inhibition, tap water was periodically fed to the reactor, as shown in Figure VII.2.

As for reactor 2, both acetic and propionic acid concentrations remained lower than 0.6 g L^{-1} suggesting the higher stability of the process compared to reactor 1. However, as stated before, some peaks, mainly of acetic acid, were also periodically observed during the last two HRT. Thus, to avoid methanogenic inhibition, tap water was also fed periodically to reactor 2 to avoid the risk of process failure, as shown in Figure VII.2.

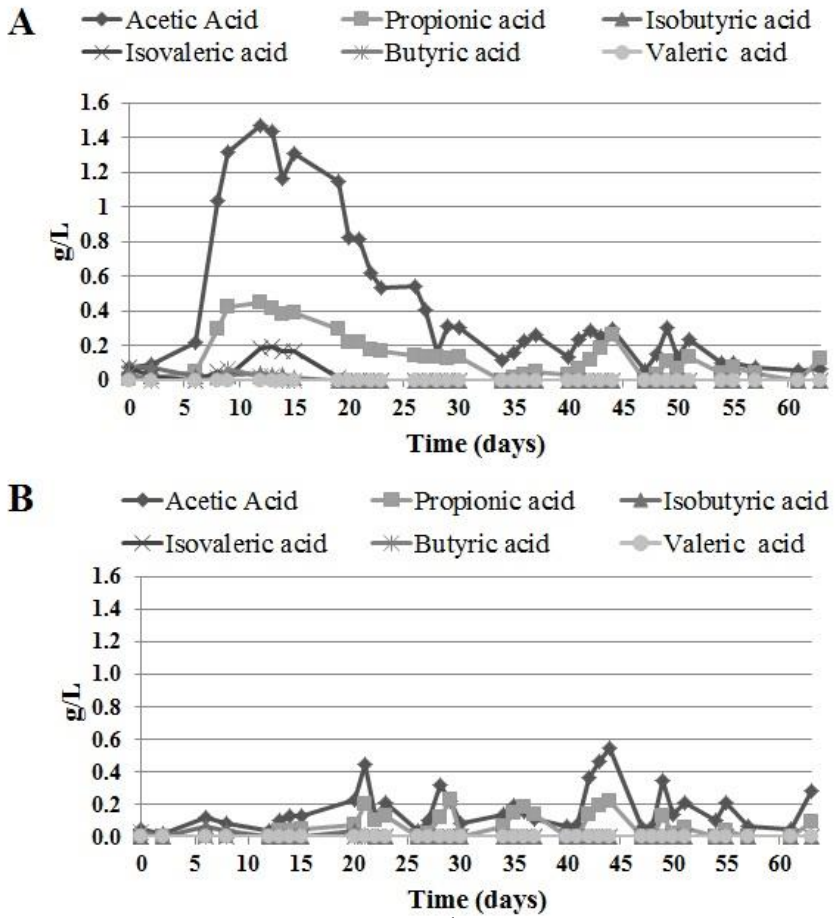

Figure VII.3. Single VFA concentration (gVFA L^{-1}) trends in: (A) reactor 1 and (B) reactor 2.

During the first HRT, an increase of ammoniacal nitrogen concentration was observed in both reactors (Figure VII.4). Then, from day 22 a rapid decrease was observed in both reactors. This trend suggests that the increase in ammoniacal nitrogen during the first HRT was mainly due to the proteins hydrolysis derived from the inoculum and the N decrease was due to a dilution effect which occurred in both reactors and, in a lesser extent, due to the uptake of ammoniacal nitrogen by anaerobic microorganisms. This phenomenon caused a lack of ammonium in the last 21 days and a consequent reduction of the buffer capacity of both reactors. Therefore, the addition of external ammonium source (0.08 gN-NH_4Cl $g^{-1}VS_{in}$) was necessary as both microorganisms N-source and source of alkalinity.

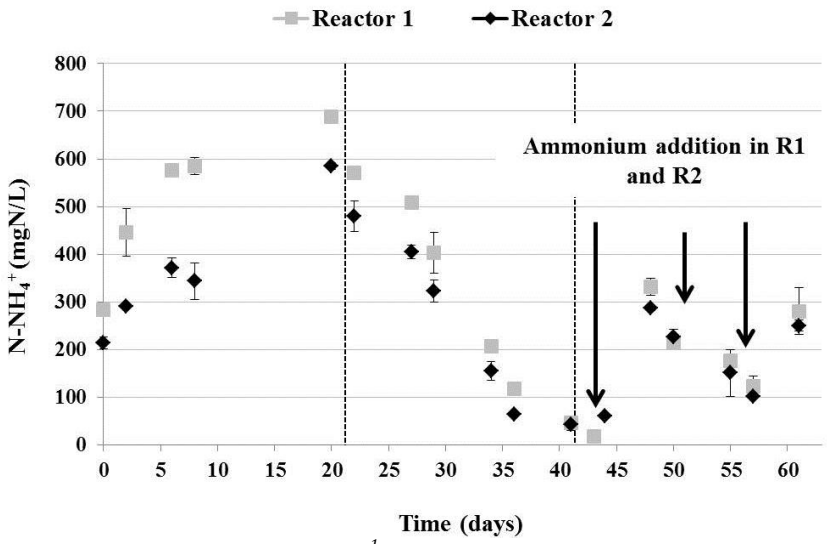

Figure VII.4. Ammonium concentrations (mgN L^{-1}) trend in reactor 1 and 2. Values correspond to mean ± standard deviation of measurement performed in duplicate.

In reactor 1, a decrease of pH (around 7) was observed up to day 9 (Figure VII.5), due to the VFA accumulation and the consequent alkalinity consumption. Indeed, the alkalinity measured at day 9 was 4 $gCaCO_3$ L^{-1}, leading to an increase of the VFA/alkalinity ratio up to 0.4 gVFA $g^{-1}CaCO_3$, higher than the safety threshold value of 0.3 gVFA $g^{-1}CaCO_3$. Therefore, bicarbonate alkalinity (6 $gCaCO_3$ $g^{-1}VS_{in}$) was added at day 9, with a consequent increase of the pH value up to 8. Then, in reactor 1, the pH and the alkalinity slightly decreased during the course of the experimentation.

In reactor 2, pH remained constant (around 7.5) during the whole course of the experimentation, while alkalinity dropped (up to 4.5 $gCaCO_3$ L^{-1}) at the end of the last HRT, leading to an increase of the VFA/alkalinity ratio up to 0.6 gVFA $g^{-1}CaCO_3$, higher than the safety threshold value. However, no bicarbonate alkalinity was added to the system which appeared more stable in terms of pH.

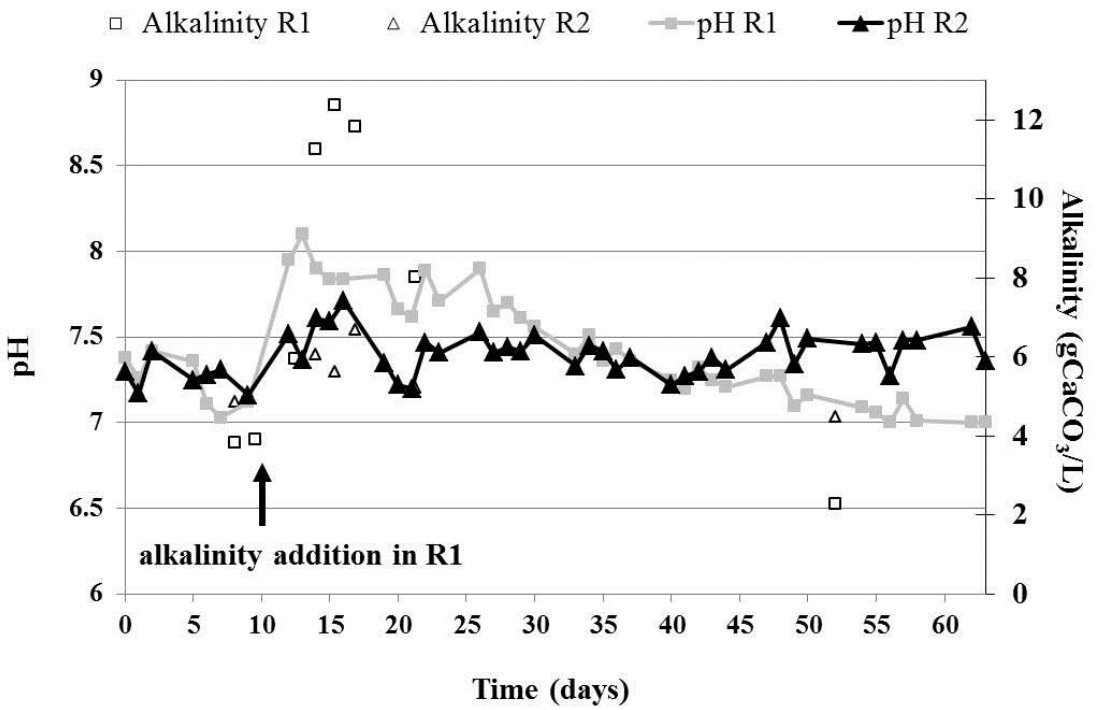

Figure VII.5. pH and alkalinity trends in reactors R1 and R2.

The alkalinity made available in reactor 1 came from the ammonium released from sorghum hydrolysis and externally added (0.08 gN-NH_4Cl $g^{-1}VS_{in}$) to avoid nitrogen shortage (as shown in Figure VII.4). During the last 21 days, the mean concentration of N-NH_4^+ (Figure VII.4) was 0.19 gN L^{-1}, corresponding to an alkalinity concentration of 14 mmol L^{-1}, in addition to the residual alkalinity 3 mmol L^{-1}, remained at day 42 in both reactors. Thus, the available alkalinity in the reactor was 17 mmol L^{-1}, corresponding to 0.8 $gCaCO_3$ L^{-1}, which is similar to that observed (Figure VII.5). To avoid pH drop, the requested alkalinity was computed as the one needed to neutralize the two main acidifying components in the reactor (i.e. the VFA accumulation and CO_2 dissolution). During the last HRT, the mean concentration of VFA in reactor 1 was 254 mg L^{-1} (composed of 69% acetic acid and 31% butyric acid) and corresponding to 63.34 mgVFA $mmol^{-1}$, the mean pH value was 7.2 and the mean value of the CO_2 partial pressure was 43%, corresponding to an alkalinity request of 88 mmol L^{-1} (higher than the available alkalinity in reactor 1 of 17 mmol L^{-1}, corresponding to 0.8 $gCaCO_3$ L^{-1}). These results can explain the observed alkalinity and pH drop in the last 21 days.

As for reactor 2, during the last 21 days, the mean concentration of N-NH_4^+ (Figure VII.4) was 0.18 gN L^{-1}, corresponding to alkalinity concentration of 13 mmol L^{-1}, in addition to the residual alkalinity

3 mmol L^{-1}, remained at day 42 in both reactors. Thus, the available alkalinity came from the ammonium released from sorghum hydrolysis and externally added (0.08 $gN\text{-}NH_4Cl\ g^{-1}VS_{in}$) to avoid nitrogen shortage in reactor 2 was 16 mmol L^{-1}, corresponding to 0.8 $gCaCO_3\ L^{-1}$. The addition of sodium hydroxide with the feed (3.5 $gNaOH\ L^{-1}$) allows to a further alkalinity contribution as 88 mmol L^{-1}, corresponding to 4.4 $gCaCO_3\ L^{-1}$. Therefore, the available alkalinity in the reactor 2 was 103 mmol L^{-1}, corresponding to 5.2 $gCaCO_3\ L^{-1}$, which is similar to that observed (Figure VII.5). The requested alkalinity of reactor 2, in the last 21 days, was computed by considering: i) the mean concentration of VFA as 289 mg L^{-1} (composed of 86% acetic acid and 14% butyric acid) and corresponding to 61.96 $mgVFA\ mmol^{-1}$; ii) the pH value around 7; iii) the mean value of the CO_2 partial pressure as 38%. In this case, thanks to all the alkalinity contributes (ammonium nitrogen and NaOH) the alkalinity requested (51.4 mmol L^{-1}, corresponding to 2.6 $gCaCO_3\ L^{-1}$) remains lower than that available (as 103 mmol L^{-1}, corresponding to 5.2 $gCaCO_3\ L^{-1}$). These results suggest a higher stability of reactor 2 than 1.

The increase of soluble COD (CODs) observed in both reactors during the first month (Figure VII.6) was due to the hydrolysis of the particulate organic matter presents both in the inoculum and in the feed as also suggested by the trend in the Volatile Solids (VS) concentration in both reactors (Figure VII.7). As expected, CODs (outlet) increased more rapidly in reactor 1 than in reactor 2, due to the accumulation of VFA during the first month of experimentation, as previously observed in Figures VII.2 and VII.3A. This indicates an unbalance between the hydrolysis/acidogenesis rate and that of methanogensis, as discussed below.

On the contrary CODs (outlet) in reactor 2 remained lower than that added to the system, according to a lower VFA concentration than that observed in reactor 1 and, after an initial increase, it decreased slowly in the last two HRT.

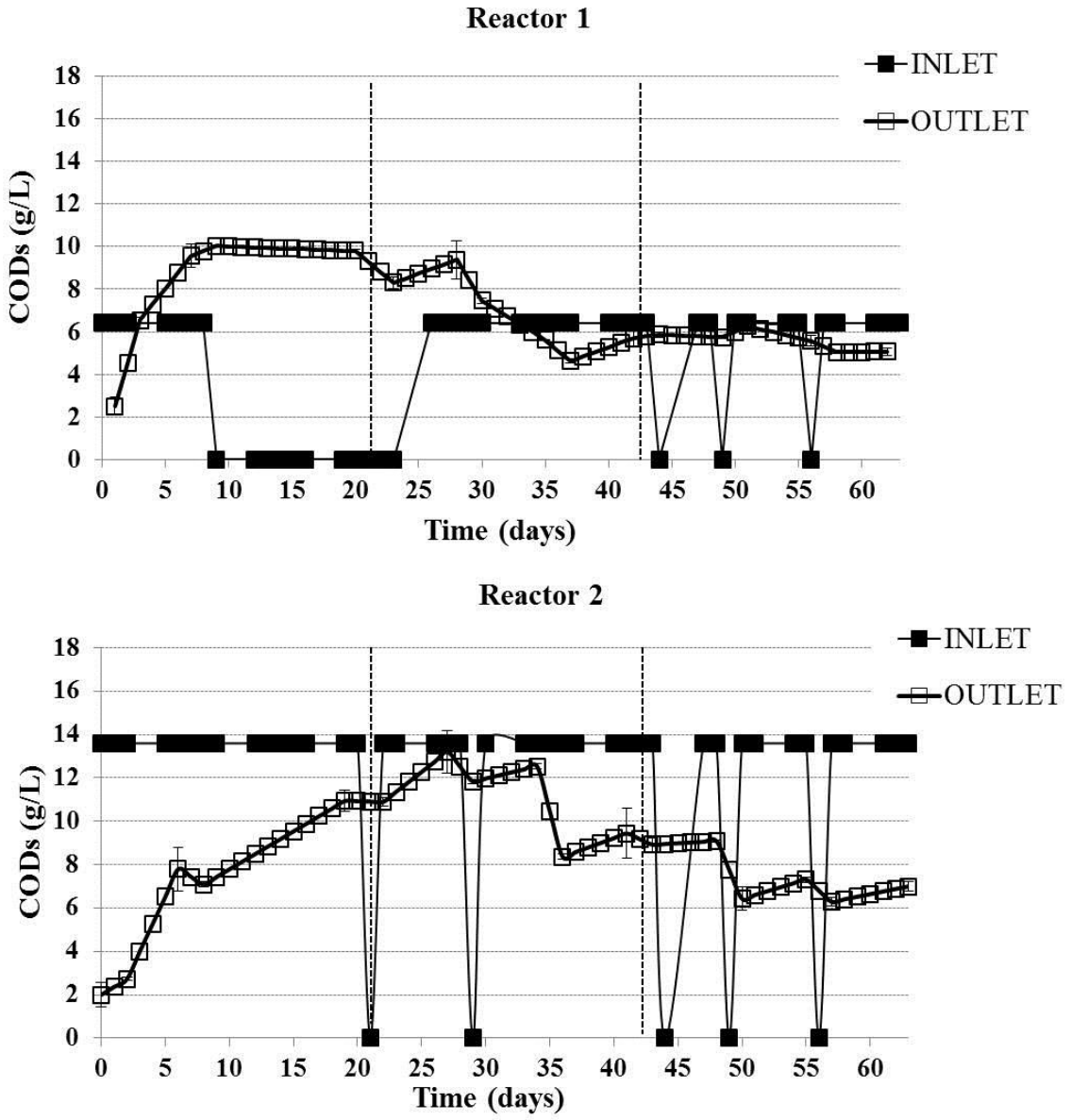

Figure VII.6. Influent and effluent soluble COD concentration trends.

However, the amount of CODs (outlet) observed in the last HRT in both reactors (5.6 and 7.4 gCODs L^{-1}in reactor 1 and 2, respectively), appeared higher than that associated to residual VFA (acetic and propionic acid) concentration in the same period (estimated as 0.22 gCOD L^{-1} for both reactors). This amount of organic matter was not acidified.

The initial VS concentration in both reactors was higher than that added with the feed and decreased significantly during the first month of experimentation (Figure VII.7).

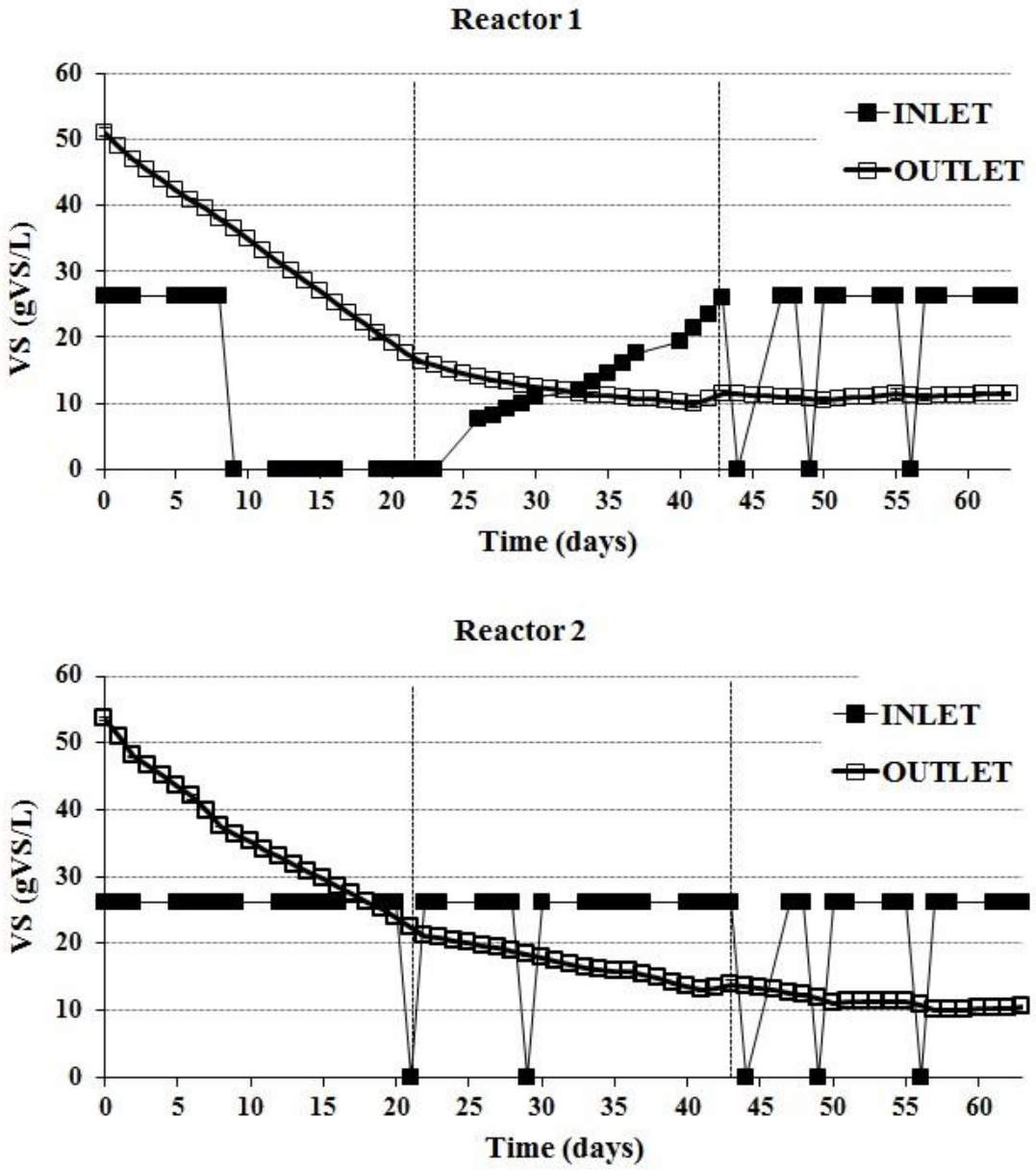

Figure VII.7. VS concentrations (gVS L^{-1}) trend.

This VS (outlet) reduction was more rapid in reactor 1 than in reactor 2, due both to VS degradation and to a dilution effect, due to tap water addition instead of sorghum during the first HRT (Figure VII.1). During the last 16 days, when both reactors were fed with a mean VS concentration of 22 gVS L^{-1}, VS (outlet) concentrations stabilized around a similar mean value for both reactors (11 gVS L^{-1}), suggesting a similar hydrolysis rate, as shown below.

The methane production rate trend (NmLCH_4 d^{-1}, at normal temperature and pressure conditions) for the two reactors, are represented in the Figure VII.8.

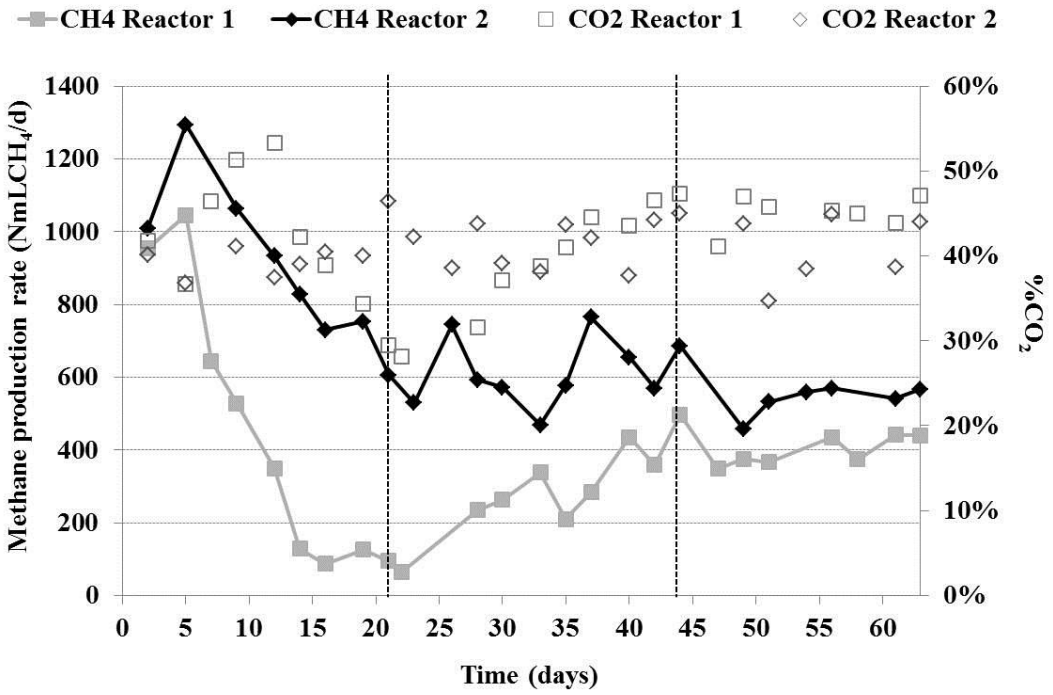

Figure VII.8. Methane production rate ($NmLCH_4\ d^{-1}$) and $\%CO_2$ trend in reactor 1 and 2.

The high initial methane production observed for both reactors was due to the endogenous contribution of the inoculum. Then, the reduction of methane production rate of reactor 1 up to day 24 was concomitant to the increase of VFA concentration and it was therefore due to the inhibition of the methanogenic biomass by VFA accumulation. After day 26, the OLR of reactor 1 started to be increased gradually (Figure VII.1) and the methane production rate kept increasing, well in accordance with the constant decrease in the VFA level. As for reactor 2, periodical decreases in methane production rate can be explained by the increase of VFA concentrations during the course of the last two HRTs, as previously observed. Methane production appeared quite stable only during the last 16 days when both reactors were fed with a mean VS concentration of 22 gVS L^{-1}.

Reactor digestates were also characterized in terms of Total Kjeldahl nitrogen (TKN) and Sodium (Na^+) concentration. The TKN content was 0.87±0.02 gN L^{-1} and 1.03±0.03 gN L^{-1} for reactor 1 and 2, respectively. As for sodium ion, the initial concentration in the inoculum of both reactors was 1.8 g L^{-1}. During the last HRT, Na^+ concentration in the digestate was found to be 0.8±0.1 g L^{-1} and 2±0.1 g L^{-1} for reactor 1 and 2, respectively. As expected, for reactor 1, Na^+ concentration decreased, if compared to the initial concentration in the inoculum due to a negligible addition of Na^+ with the untreated

sorghum. On the contrary, in reactor 2 an amount of 1.5 g Na^+ L^{-1}, similar to that found in the inoculum, was added with the feeding, and thereafter found in the digestate. Nevertheless, despite the use of sodium hydroxide during the pretreatment step, the anaerobic process was not inhibited, because the amount of sodium remains lower than 5.6–53 g L^{-1}, which has been reported to be the range of sodium causing inhibition, depending on the adaptation period, antagonistic/synergistic effects, substrate, and reactor configuration (Chen et al., 2008). When a NaOH pretreatment is performed, sodium concentration in the reactor increases with both the sodium hydroxide dosage used for the pretreatment and the VS concentration of the feed. In this case, by considering 10 gNaOH $100g^{-1}$TS as alkaline dosage, the maximum admissible VS concentration of the feed has to be maintained around 95 gVS_{in} L^{-1}, to keep the sodium (Na^+) concentration lower than the threshold value of 5.6 gNa^+ L^{-1}. By considering that the VS/TS ratio for ensiled sorghum forage is around 87%, this means that the TS concentration of the feed has to be maintained around 110 gTS L^{-1}, which is in accordance with the typical value used for a wet digestion system, which has to be maintained below 20% of TS (Angelidaki et al., 2003). Another issue associated to the sodium concentration in the digestate concerns the possibility to use the digestate as a soil fertilizer and this aspect has to be taken into account for a possible scale-up of this technology. Indeed, a high sodium concentration negatively affects crops cultivation, causing direct toxicity or restraint of root elongation by impeding moisture movement and aeration in soil due to the deterioration of soil physical properties by dispersion effect of sodium ion in soil (Shannon, 1997). Thus, also this aspect has to be taken into account for a possible reuse of digestate as a fertilizer.

VII.2. Discussion

In order to assess the effect of sorghum pretreatment, the average specific methane yield was computed as the slope of the trend line fitting data of the cumulative methane production versus the cumulative VS fed to each reactor. Data related to the last HRT (21 days) were only considered in order to assess the quasi-steady state operating condition. As observed in Figure VII.9, final specific

methane productions were 237 and 297 $mLCH_4$ $g^{-1}VS$ for reactor 1 and 2, corresponding to 88% and 86% of BMP results for the two feeds, respectively. Results evidence that alkaline pretreatment led to an increase of methane production yield of 25%, compared to the specific methane production of the anaerobic digester fed with untreated sorghum.

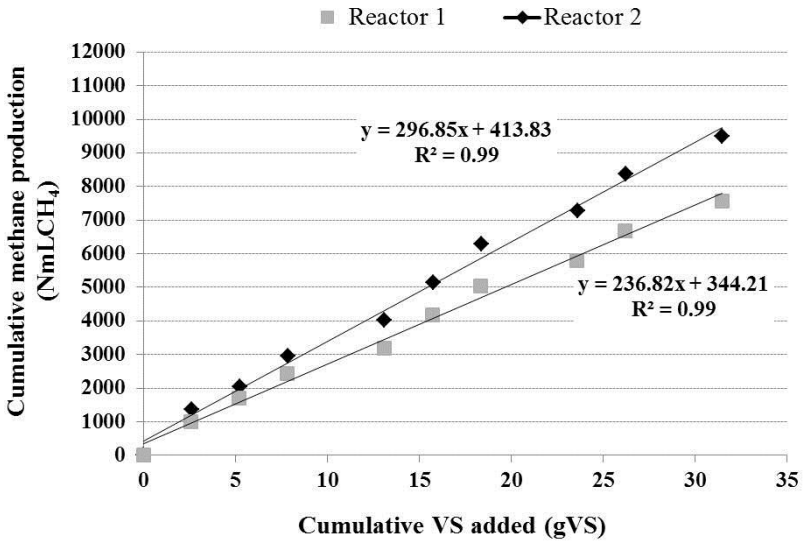

Figure VII.9. Specific methane production for the last HRT.

This result is slightly lower than that observed after batch tests (29%), as reported in Chapter III, and apparently in discordance with the results about VS removal during the last HRT. This can be explaining by estimating and comparing the mean value of COD to VS ratio of the digestate of both reactors in the last 21 days. By knowing the mean COD in the feed (3.1 and 3.2 gCOD d^{-1} for reactors 1 and 2, respectively) and the corresponding mean value of COD associated to the methane produced during the last HRT (2.3 and 2.7 gCOD d^{-1} for reactors 1 and 2, respectively), the mean COD in the digestate can be estimated from the COD mass balance to be 0.8 and 0.4 gCOD d^{-1} for reactors 1 and 2, respectively. The mass of VS in the digestate (1.1 gVS d^{-1}) was equal for both reactors. Thus, the COD to VS ratio in the digestate was higher for reactor 1 than for reactor 2, suggesting that in the latter a higher amount of COD than that of reactor 1 was converted into methane. The increase of COD removal was 31% between the two reactors, confirming the increase of methane production.

A prolongation of time of experimentation may be necessary to confirm the methane increased observed.

The available analytical results allowed for the calculation of hydrolysis, acidogenesis and methanogenesis rates for the two anaerobic reactors which was made, according to equations II.4, II.5 and II.6 (Figure VII.10).

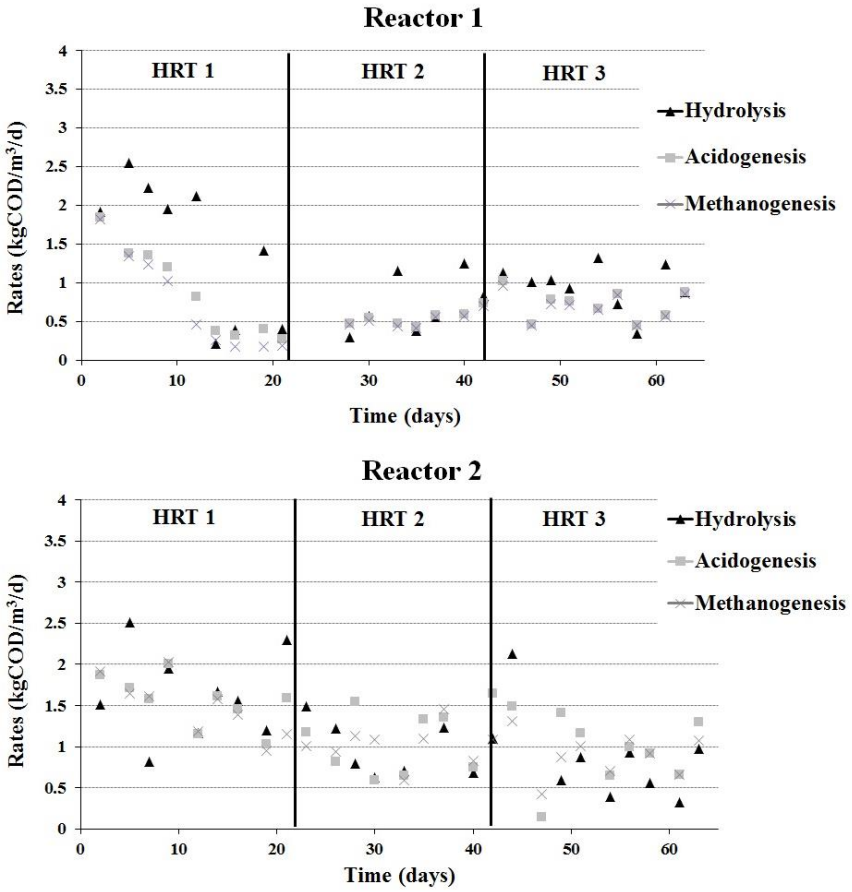

Figure VII.10. Hydrolysis, acidogenesis and methanogenesis rates.

As mentioned before, an endogenous biogas production occurred in both reactors during the first 20 days of experimentation, causing a slight reduction of the methanogenic rate, which was observed for both reactors.

In reactor 1, during the first HRT (21 days) the hydrolysis rate was higher than the methanogenic one. This caused an accumulation of VFA, as observed in Figures VII.2 and VII.3 and a consequent destabilization of the degradation chain, which affected the methane production. During the last period, reactor 1 appeared to stabilize with a hydrolysis rate (0.9±0.3 kgCOD m^{-3} d^{-1}) similar to the methanogenic one (0.7±0.2 kgCOD m^{-3} d^{-1}). As expected, acidogenesis appeared in accordance with

the methanogenesis values, and during the last HRT the mean value of acidogenic rate was identical to that of methanogenesis.

As for reactor 2, all the trends appear similar and more constant during the course of the experimentation, suggesting a well-balanced degradation process. Similarly to reactor 1, mean values of hydrolysis, acidogenesis and methanogenesis were similar during the last HRT (0.8±0.6, 1.0±0.5 and 0.9±0.3 for hydrolysis, acidogenesis and methanogenesis rate, respectively). As suggested by VS concentrations, similar hydrolysis rates were observed for both reactors during the last period of experimentation.

VII.3. Preliminary evaluation of energetic and economic balances

A preliminary energetic and economic analysis was computed by comparing the extra operational costs for the substrate pretreatment with the extra gains due to the improved methane production resulting from the pretreatment (see Chapter II). As for thermal pretreatment (Table VII.1), the heat production from a CHP system, made available by the anaerobic digestion of the pretreated samples, is theoretically sufficient to treat the substrate at 40°C, as confirmed by the heat energy requirement values for pretreatment (197 kWh t^{-1}TS). Therefore, according to our results, the thermal pretreatment's cost was considered null.

Table VII.1. Preliminary economic analysis under three scenarios (S1, France; S2, Germany; S3, Italy).

		Reactor 1	Reactor 2
Specific methane production (m^3 t^{-1}TS)		206 [a]	258 [a]
Heat and electricity from CHP	**Heat (kWh t^{-1}TS)**	841	1054
	Electricity (kWh t^{-1}TS)	830	1040
Electric energy gain (€ t^{-1}TS)	**S1 (0.17 € kWh^{-1})**	141	177
	S2 (0.25 € kWh^{-1})	207	260
	S3 (0.28 € kWh^{-1})	232	291
NaOH cost (€ t^{-1}TS)			41
Heat requirements for pretreatment (kWh t^{-1}TS)			197
Extra net Gain (€ t^{-1}TS)	**S1 (0.17 € kWh^{-1})**		-6
	S2 (0.25 € kWh^{-1})		11
	S3 (0.28 € kWh^{-1})		18

[a] *Obtained by considering the VS content (87 %TS) of ensiled sorghum forage (see Chapter III).*

On the other hand, as mentioned before, a CHP system can produce electric energy. Normally, such energy is sold to the public grid at a fixed rate. Three related economic scenarios are presented, namely S1, S2 and S3, corresponding to the incentive policy of France, Germany and Italy, respectively. The extra net gain was calculated for each scenario, considering the NaOH cost and the extra electric gain obtained through an extra electric production from a CHP system, resulting from the anaerobic digestion of the pretreated samples, compared to those of untreated. According to the data shown in Table VII.1, the extra net gains obtained are positive only for Germany and Italy, varying between -6 and 18 € kWh^{-1}, showing that the net gain is mainly dependent on the call price, which fluctuates from one country to another. Another important aspect to take into account is related to the high cost of the sodium hydroxide that can negatively affects the extra net gains obtained. In this sense, an option could be to reduce the alkaline dosage, while remaining on reasonable levels of methane increase, or to replace NaOH with other cheapest chemicals, such as lime (70 € t^{-1}). However, these economic results have to be considered as preliminary and a more rigorous economic study should be realized taking into account the investment of infrastructure, and additional operating costs.

VII.4. Partial conclusions

In this Chapter, performances of two semi-continuous anaerobic reactors fed, respectively, with untreated and alkaline pretreated ensiled sorghum forage, were compared. Three positive effects of the alkaline pretreatment were observed on the anaerobic digestion performance. Firstly, the addition of sodium hydroxide allowed maintaining a high alkalinity in the system, limiting pH drops and avoiding the destabilization of the anaerobic digestion process, as it occurred in reactor 1. Secondly, under the tested conditions, the pretreatment step did not cause an inhibition of the anaerobic process because the amount of sodium remained lower than the range of sodium causing inhibition during the whole course of the experimentation. Finally, the alkaline pretreatment led to an increase in methane yield of 25%, compared to reactor 1 fed with untreated sorghum. Nevertheless, longer experimental period under steady state operation would have been necessary to confirm the methane increased observed.

As for the preliminary evaluations of economic and energetic balances, the extra net gains the extra net gains obtained are positive only for Germany and Italy, being mainly dependent on the call price, which fluctuates from one country to another. However, a more rigorous economic study should be realized taking into account the investment of infrastructure, and additional operational costs due to alkaline pretreatment.

Chapter VIII. General conclusions and perspectives

The production of methane from lignocellulosic materials (i.e. agricultural residues and energy crops), appear to be one of the most promising alternatives to fossil fuels with no "food versus fuel" dilemma.

However, the main challenge in using lignocellulosic substrates, such as agricultural residues and energy crops, for biogas production, is their recalcitrant structure and composition. Thus, various methods of pretreatment, originally investigated for the production of second generation bioethanol, have been suggested and tested in order to alter the structure of lignocellulosic substrates, facilitating their enzymatic hydrolysis and consequently enhancing their methane production. Nevertheless, the high variability of pretreatment conditions, methods and results, even when similar substrates were compared, suggest that no definite consensus on their effectiveness for the improvement of the anaerobic biodegradability of agricultural residues and energy crops has yet been attained. Moreover, results about the effect of biological pretreatments on methane production of lignocellulosic substrates are still limited and available data are yet insufficient to draw conclusions about the efficacy of these pretreatment technologies for their future scale-up. Thus, a comparison between many different types of pretreatment applied on the same substrate can be useful in order to define the best pretreatment strategy. Moreover, due to the high variability of methane potential and pretreatment results, depending not only on substrate type but also on crop variety, the same pretreatment has to be tested on various varieties of the same lignocellulosic material with different chemical and structural composition. Finally, pilot scale or full scale pretreatment applications are still limited or inexistent mainly due to economic reasons and because operational pretreatment parameters are not well defined yet. Thus for a future scale-up of the technology, pretreatments should be also tested in continuous reactors, in order to gather data not only regarding the methane enhancement but also on the energetic, economic and environmental aspects.

The **first objective** of the thesis was to evaluate the effect of different pretreatment strategies on chemical composition, physical structure and methane production of lignocellulosic substrates. For this purpose, physical (mechanical and thermal), chemical (alkaline with NaOH), biological (with commercial enzymes and fungal enzymatic filtrates) and their combinations were tested on two agricultural substrates (ensiled sorghum forage and wheat straw). The most relevant conclusions are hereafter summarized.

- Both the chemical and the physical structure of ensiled sorghum forage were not influenced by the

particle size reduction (between 2 and 0.25 mm). On the contrary, alkaline, thermal, thermo-alkaline, mechanical-alkaline, biological (i.e. enzymatic and fungal) and alkaline-biological pretreatments led to a solubilisation of cellulose, hemicelluloses and lignin for both ensiled sorghum forage and wheat straw, with more or less success.

- As for anaerobic digestion performances, mechanical pretreatment did not enhance methane potentials nor anaerobic digestion kinetics between 2 and 0.25 mm.
- By combining mechanical and alkaline pretreatment, an increase in both methane yield (20%) and kinetic constants (by 31%) was observed, due to the effect of the alkaline agent (10 gNaOH $100g^{-1}$TS), but these results were not significantly influenced by the particle size reduction.
- Thermal pretreatment performed at 100°C and 160°C did not have a benefit effect in the increasing of methane yield both for sorghum and wheat straw, but led to an increase of anaerobic digestion kinetics (up to 13% and 107% for sorghum and wheat straw, respectively), mainly due to the solubilisation of cellulose and hemicelluloses fractions. Among alkaline and thermo-alkaline pretreatments, the best results in terms of methane production increase were observed by treating wheat straw at 40 and 100°C with 10 gNaOH $100g^{-1}$TS for 24 h (43% and 67%, respectively) and ensiled sorghum forage with the same conditions (up to 32%). An increase in anaerobic digestion kinetics was also observed (up to 65% and 161% for sorghum and wheat straw, respectively).
- Biological pretreatments, performed with commercial enzymatic preparations (i.e. xylanase, endo and eso-glucanase), led to an increase of methane production of both substrates (15% and 55%, for sorghum and wheat straw, respectively). By combining alkaline (10 gNaOH $100g^{-1}$TS, 24 h, 40°C) and enzymatic pretreatment a further increase in methane production was observed only for sorghum (up to 37%). However this value is not justified if compared to that obtained after the alkaline pretreatment alone (29%), taking into account the high cost of commercial enzymes and NaOH. On the contrary, biological pretreatments performed with an enzymatic extract of a fungal strain, did not improve methane production. The combination with alkaline (10 gNaOH $100g^{-1}$TS, 24 h, 40°C), led to an increase of methane production (28% and 58% for sorghum and wheat straw, respectively), similar to that obtained by applying the sole alkaline pretreatment.
- According to results obtained from all pretreatment categories, thermo-alkaline pretreatment (10

gNaOH 100g^{-1}TS, 24 h, 100°C) led to the best results in terms of methane production increase for wheat straw. As for sorghum, similar results were observed between alkaline (10 gNaOH 100g^{-1}TS, 24 h, 40°C), thermo-alkaline (10 gNaOH 100g^{-1}TS, 24 h, 100°C) and biological-alkaline pretreatments. However, the sole alkaline pretreatment was preferred to treat ensiled sorghum forage for both energetic and economic perspectives. Moreover, among the two substrates, wheat straw showed the highest increase in methane production, due to a lower initial anaerobic degradability if compared to untreated sorghum.

The **second objective** was to evaluate the influence of substrate varieties on alkaline pretreatment efficiency, evaluated in terms of chemical composition, structural structure and anaerobic digestion performances. Sodium hydroxide pretreatment (10 gNaOH 100g^{-1}TS, 40°C, 24 h), which was found as the best pretreatment strategy to treat ensiled sorghum forage, was chosen in order to evaluate its influence on six sorghum varieties. In the case of five varieties sorghum, different from ensiled sorghum forage, alkaline pretreatment had a positive effect in increasing anaerobic digestion kinetics (by 31%), but it did not affect methane production of untreated substrates.

Finally, the **last objective** was to evaluate the applicability and implementation of the sodium hydroxide pretreatment (10 gNaOH 100g^{-1}TS, 40°C, 24 h) on ensiled sorghum forage prior to a semi-continuous anaerobic reactor. Interesting results were obtained, suggesting that the addition of sodium hydroxide allowed maintaining a high alkalinity in the system, limiting pH drops and avoiding the destabilization of the anaerobic digestion process, as it occurred in the reactor fed with untreated sorghum. Alkaline pretreatment permitted also an increase in methane yield (25 %) compared to that obtained from the reactor fed with untreated sorghum, without causing any inhibition of the anaerobic process by the accumulation of sodium ions. However, longer experimentation would have been necessary to confirm the results observed. As for the preliminary economic evaluations, the extra net gains obtained by selling the extra electric production from a CHP system, resulting from the anaerobic digestion of the pretreated samples, compared to those of untreated, are positive for all scenarios (from 3 to 18 € kWh^{-1}), suggesting the economic feasibility of the pretreatment step. Nevertheless, these results have to be considered as preliminary. Thus, a more rigorous

economic study should be realized taking into account the investment of infrastructure, and additional operating costs.

This work has contributed to define the best pretreatment strategy at lab scale on two agricultural substrates (sorghum and wheat straw) commonly available at agricultural farms and to confirm the feasibility and benefits of the sodium hydroxide pretreatment of ensiled sorghum forage prior to the anaerobic digestion process. It also contributes to verify that the pretreatment performances can vary according not only to the plant origin but also to its variety, suggesting that a case to case approach as to be applied when assessing the applicability and favourability of a pretreatment.

However, **future works** about the optimization of pretreatments in terms of operational, economical and environmental aspects and the valorization of the anaerobic residue have to be considered to make the entire process more economically-viable.

In this sense, several perspectives and future works can be suggested:

1) Physical and chemical pretreatments have to be optimized in order to maximize methane production and to render the entire process chain economically viable. Optimization of parameters such as solid loading, time, temperature and concentration of chemical reagent has to be considered. Firstly, it has been demonstrated by other authors that in the case of thermal or thermo-chemical pretreatments, the heat requirement depends on the solid loading used to perform the pretreatment step (Zhang et al., 2009; Monlau et al., 2012c). To reduce the heat requirement it is necessary to work with high solid loading during the pretreatment. Thus, it could be interesting to evaluate the impact of the solid loading on pretreatment performances, especially in terms of methane production, thus rendering the process economically viable. Then, the optimization of other operational parameters (residence time, temperature, concentration of chemical reagent) to maximize methane potential is also necessary. The experimental design can represent an interesting option to simplify this optimization step, permitting to optimize one response (i.e. methane potential) by taking simultaneously into account various parameters. As for the combination of chemical and mechanical pretreatment, it should be interesting to consider the mechanical step after a chemical pretreatment and to compare the results in terms of methane production obtained by the two configurations

(mechanical-chemical and chemical-mechanical). Indeed, Zhu et al., 2010b has stated that a particle size reduction step after the chemical pretreatment can decrease significantly the energetic consumption compared to the traditional two stage mechanical-chemical pretreatment.

2) Several bottlenecks remain on the application of biological pretreatments with commercial enzymes and fungi. As for enzymatic pretreatment, the need of a sterilization step to avoid loss of carbohydrates and the high cost of enzymes remain two limitations for the development of this technology at industrial scale. To avoid the sterilization step, anaerobic conditions with nitrogen gas were tested in this work and gave positive results. However, at industrial scale, the use of carbon dioxide, which composes biogas, to generate anaerobic conditions and to avoid proliferation of aerobic microorganisms that consume free sugars, released during enzymatic pretreatment, can be a more viable and economical solution. Another option is the simultaneous addition of enzyme mixture directly in the anaerobic digestion reactor for the concomitant release and degradation of the sugars as previously suggested by Romano et al. (2009) and Quémeneur et al. (2012). Recent findings had shown the benefits of producing biohythane (a mixture of methane and biohydrogen) in a two stage biohydrogen-methane anaerobic digester, compared to one stage anaerobic digester for methane production (Pakarinen et al., 2009; Pakarinen et al., 2011; Monlau et al., 2012a). As biohydrogen production step is generally performed at pH around 5, which is the optimal working pH of enzymes, it could be interesting to test enzymatic pretreatment by injecting enzymes, directly in the first step of a combined hydrogen/methane reactor. Then, to solve the high cost of industrial enzymes, the use of biological pretreatment such as fungi, both in solid state and liquid state fermentation, can be a promising alternative. To solve the problem of long treatment times required for a solid state fermentation of fungal strains, an option could be to favor fungal growth directly during substrate storage which is normally found at agricultural farms. Another option, which was also applied in this thesis, could be to use the enzymatic extracts from a liquid state cultivation of the fungal strains. However, in order to obtain high enzymatic concentrations in the fungal filtrate, cultivation parameters (solid to liquid ratio, time, temperature, composition of cultural medium...), on which depend the concentration of enzymes induced by fungi, have to be optimized. Another option, but probably less economically viable could be to concentrate enzymes with existing technologies (i.e. membrane technologies).

3) As for the digestate, it would be interesting to separate it into solid and liquid fractions and to treat the

solid fraction with thermo-chemical technologies in order to degrade the residual recalcitrant components (i.e. lignin) still present and to allow the accessibility of remainging biodegradable components for their further reinjection in the anaerobic digester.

4) Another important aspect is related to the energetic and economic assessments, which have to be defined for a future scale up of the pretreatment technologies, taking into accounts additional investment and operational cost of the pretreatment step and to evaluate the return of investment. Moreover it should be interesting to take into account the environmental aspects linked to pretreatment of lignocellulosic residues. Indeed, up to date, only one author (Carballa et al., 2010) made a Life Cycle Assessment of solid waste (i.e. kitchen waste and sewage sludge) pretreatment prior to the anaerobic digestion step.

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Appendices

I. List of pubblications

Peer-review journals

- C. Sambusiti, E. Ficara, F. Malpei, J.P. Steyer, H.Carrere (2013). Chemical and structural changes after alkaline pre-treatment to enhance methane production of five varieties of sorghum. Energy, Available online, DOI 10.1016/j.energy.2013.04.025.
- C. Sambusiti, E. Ficara, F. Malpei, J.P. Steyer, H.Carrere (2013). Effect of particle size on methane production of raw and alkaline pre-treated ensiled sorghum forage. Waste and Biomass Valorization, Available Online, DOI: 10.1007/s12649-013-9199-x.
- C. Sambusiti, F. Monlau, E. Ficara, H. Carrere, F. Malpei (2013). A comparison of different pre-treatments to increase methane production from two agricultural substrates. Applied Energy, 104, pp. 62-70.
- F. Monlau, C.Sambusiti, A. Barakat, X.M. Guo, E. Latrille, E. Trably, J.P. Steyer, H. Carrere (2012). Predictive models of biohydrogen and biomethane production based on the compositional and structural features of lignocellulosic materials. Environmental Science and Technology, 46, pp. 12217-12225.
- C. Sambusiti, E. Ficara, F. Malpei, J.P. Steyer, H. Carrere (2012). Influence of alkaline pre-treatment conditions on structural features and methane production from ensiled sorghum forage. Chemical Engineering Journal, 211-212, pp. 488-492.
- C. Sambusiti, E. Ficara, M. Rollini, M. Manzoni, F. Malpei (2012). Sodium hydroxide pretreatment of ensiled sorghum forage and wheat straw to increase methane production. Water Science and Technology, 66(11), pp. 2447-2452.

Conference proceedings

- C. Sambusiti, E. Ficara, F. Malpei, H. Carrere, J.P. Steyer (2012). Effect of particle size on alkaline pretreatment and methane production of ensiled sorghum forage. In: Proceedings of International WasteEng 2012 Conference, Porto, Portogallo, 10-13 September 2012, paper 215, p. 615 ISBN : 979-10-91526-00-5.
- C. Sambusiti, E. Ficara, M. Rollini, M. Manzoni, H. Carrere, F. Malpei (2012). Comparative study of different pretreatments to enchance methane production of sorghum forage. In: Proceedings of SIDISA 2012

International Symposium of sanitary and environmental engineering 9th edition, Milano, Italia, 26-29 June 2012, Paper SESSION WATER - Energy and WWTP 926, pp. 1-8, on CD-ROM.

- M. Rollini, C. Sambusiti, A. Musatti, M. Manzoni, E. Ficara, F. Malpei (2012). Combination of alkaline and enzymatic pre-treatment to increase bio-methane production potential of sorghum and wheat straw. In: Proceedings of 20th European Biomass Conference and Exhibition, Milano, Italia 18 June – 21 June 2012 Paper 2DV.3.5, pp. 1406 – 1410, on CD-ROM. ISBN: 978-88-89407-54-7, doi: 10.5071/20thEUBCE2012-2DV.3.5.
- C. Sambusiti, E. Ficara, M. Rollini, M. Manzoni, F. Malpei (2011). Alkaline pretreatment of sorghum and wheat straw for increasing methane production. In: Proceedings of International Symposium on Anaerobic Digestion of Solid Waste and Anaergy Crops, Wien, 28 August – 1 September 2011, Austria, Paper IWA-7817R1, pp. 1-8, on CD-ROM.

II. List of tables

III. List of figures