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1st and 2nd GENERATION ETHANOL FROM BIOMASS CROPS

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Contents

List of abbreviations

1G: 1st generation biofuels;

2G: 2nd generation biofuels;

AA: acetic acid;

AD: anaerobic digestion;

AIL: acid insoluble lignin;

ANOVA: analysis of variance;

B133: genotype Biomass 133 [*Sorghum bicolor* (L.) Moench];

DAS: days after sowing;

$EtOH_{1G}$: first generation ethanol;

$EtOH_{2G}$: second generation ethanol;

$EtOH_{1G+2G}$: combined first and second generation ethanol;

FA: formic acid;

FPU: filter paper units;

G: genotypes;

GHG; greenhouse gas;

HMF: 5-hydroxymethylfurfural;

HT: harvest time;

Log R_0: severity factor;

LSD: Least Significant Difference;

P: pretreatment;

SB: sugarcane bagasse;

SD: standard deviation;

SSF: simultaneous saccharification and fermentation;

S506: genotype Sucros 506 [*Sorghum bicolor* (L.) Moench];

TDW: total dry weight;

TOC: total organic carbon;

TS: total solids,

VS: volatile solids;

WIS: water insoluble solids;

WL: water level;

WUE: water use efficiency;

W_L: residual lignin;

Y_{ETOH}: overall ethanol yield;

Y_g: glucose yield.

Abstract

Biofuels aim to face the replacement of fossil fuels and mitigate the climate change caused by the use of fossil sources. However, the production of some conventional biofuels has been heavily criticized for causing deforestation (through direct or indirect land-use change (iLUC)), and for competing with food and animal feed production. Food equity and security could be guaranteed by the use of non-edible feedstock for biofuel production (so called second generation biofuels, 2G; while first generation biofuels are 1G), such as lignocellulosic materials, that can be obtained from biomass crops cultivated on marginal land (e.g., perennial biomass crops), or from agricultural residues of food crops (e.g. sugarcane bagasse, wheat straw, corn stover). Among second generation biofuels, 2^G ethanol has a considerable potential to replace oil to some degree, as it is a liquid fuel that can be easily integrated into the existing infrastructure for fuel distribution.

Because of the recalcitrance of lignocellulosic biomass, a pretreatment is needed in order to increase the accessibility of cellulose and hemicellulose to the enzymes that break down the cellulose and hemicellulose polymers into their monomeric units in the subsequent enzymatic hydrolysis step. However, an important aspect to take into account is related to the environmental impact of pretreatment, caused by the use of high quantity of water and chemicals that generate large amount of waste streams, even toxic for the environment. Water used during pretreatment and the whole process can be wasted or not, on the basis of catalyst and the amount of water used. Based on these considerations, calcium hydroxide ($Ca(OH)_2$) is an alkaline catalyst potentially suited for lignocellulosic material. In fact, it can easily be removed from the water used for impregnation of biomass, by carbonating with CO_2. The resulting $CaCO_3$ may be

recovered to be used in several applications, thus the use of calcium hydroxide does not originate waste water.

In chapter 1 calcium hydroxide as impregnation agent before steam explosion of sugarcane bagasse was compared with auto-hydrolysis, assessing the effects on enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) at high solid concentration of pretreated solid fraction. In addition, anaerobic digestion of pretreated liquid fraction was carried out, in order to appraise the effectiveness of calcium hydroxide before steam explosion in a more comprehensive way. In Chapter 2, auto-hydrolysis and steam explosion preceded by either sulphuric acid or calcium hydroxide impregnation were compared in switchgrass, still studying the effects on enzymatic hydrolysis and SSF at high solid concentration of pretreated solid fraction. Anaerobic digestion of the liquid fraction was also carried out, to provide further insight into pretreatment effects on lignocellulosic biomass.

As water is an expensive input in both cultivation of biomass crops and subsequent pretreatment, Chapter 3 addressed the effects of variable soil moisture on biomass growth and composition of biomass sorghum. Moreover, the effect of water stress was related to the characteristics of stem juice for 1^{st} generation ethanol and structural carbohydrates for 2^{nd} generation ethanol.

In the frame of chapter 1, calcium hydroxide was proven to be a suitable catalyst for sugarcane bagasse before steam explosion, in order to enhance fibre deconstruction. After pretreatment and enzymatic hydrolysis, calcium hydroxyde at high concentration (0.7% w w^{-1}) exhibited the best yield of glucose. In turn, this determined the highest ethanol yield from SSF of the solid fraction. Conversely, autohydrolysis was found to be more suitable for methane production.

In chapter 2, effect of calcium hydroxide on switchgrass showed a great potential when ethanol was focused, whereas acid addition produced higher methane yield. Low concentration of lime was shown less aggressive and secured more residual solid after simultaneous saccharification and fermentation, resulting in higher energy output per unit raw biomass.

In chapter 3 it can be observed that during crop cycle the amount of cellulose, hemicellulose and AIL changed causing a decrease of 2G ethanol amount obtained from biomass through SSF. Biomass physical and chemical properties involved a lower glucose yield and concentration at the end of enzymatic hydrolysis and, consequently, a lower 2G ethanol concentration at the end of simultaneous saccharification and fermentation, proving that there is strong relationship between structure, chemical composition, and fermentable sugar yield. Lastly, the increase of dry biomass yield during crop growth was accompanied by a decrease in ethanol concentration and yield at the end of simultaneous saccharification and fermentation, indicating that the best time to harvest both hybrids tested (Sucros 506 and Biomass 133) was at the end of crop cycle. Nevertheless, the significantly higher concentration of ethanol at the early crop stage could be an important incentive to consider biomass sorghum as second crop in the season, to be introduced into some agricultural systems, potentially benefiting farmers and, above all, avoiding the exacerbation of the debate about fuel vs food crops. Moreover, high values of water use efficiency of 2G ethanol and water use efficiency of combined 1G and 2G ethanol, compared to water use efficiency of 1G ethanol, reduce the strife for water use when growing biomass sorghum for advanced biofuel production.

1 General Introduction

Biofuels aim to face the replacement of fossil fuels and mitigate the climate change caused by the use of fossil sources (Fargione et al., 2008).

During the last fifty years the world's population has doubled and further increases are foreseen, hence looking ahead we face major challenges in satisfying needs of both energy and food. As a consequence of population growth, fossil based economies are causing the global warming due to the increase of CO_2 emissions generated from oil, coal and natural gas combustion (IPCC, 2014). Nowadays, it is generally accepted that the use of renewable and alternative energy sources, for example biomass, is necessary not only due to compensate for the progressive depletion of limited fossil stocks, but also to mitigate the damage to the climate caused by the CO_2 generated from fossil fuels combustion and other GHG emissions (mainly methane and nitrous oxide) from human activities (IPCC, 2014).

Climate change has been intensively debated during the past 20 years, and several international and national agreements have been signed to reduce its environmental impact. The first agreement, which legally established binding obligations for developed countries to mitigate their GHG emissions, was the Kyoto protocol. It was adopted in Kyoto (Japan) in 1997, and implemented in 2005. One of the targets was to achieve an average 5% reduction in GHG emissions during the period 2008-2012, compared to the level registered in 1990. In 2009, the European Union pledged a 20% unilateral reduction target for 2020, compared to 1990 levels. It is hoped that this target may increase to 30% through the cooperation of other developed countries.

Moreover, as today's economies are highly dependent on fossil sources, the demand for personal mobility and the transport sector in emerging economies will require greater oil supplies (Banse et al., 2008).

Before the concerns raised by climate change in recent decades, since the 1970s many oil importing countries experienced economic recessions due to the cartel on oil prices adopted by the Organization of the Petroleum Exporting Countries (OPEC). In response to the crisis, many non-OPEC countries highly prioritized strategies for the development of alternative sources of energy and the possibility of fuel substitutions in their economic development plans. In particular, the International Energy Agency (IEA) was established in 1974 mainly to ensure energy security, by lessening dependence on oil. IEA aims to achieve energy security promoting efficiency, diversity and flexibility in the energy sector of its member states.

Hence, the need for alternative and more carbon-neutral energy sources has raised interest in renewable fuels produced from biomass, which have the potential to reduce GHG emissions while overcoming the dependence on fossil fuel supply (Walker, 2010; Fairley, 2011). Vegetables, namely upper plants are considered carbon sinks as they use CO_2 and water for photosynthesis (Scurlock and Hall, 1998). This CO_2 fixed during plant growth will be released by the combustion of plant-derived biofuels (Cherubini et al., 2011). In concept, this carbon cycle will be neutral if the same amount of CO_2 is sequestered as is released during the combustion of biofuel products.

In the report from the Intergovernmental Panel on Climate Change (IPCC) addressing the transportation sector, second generation biofuels, together with electric and hybrid vehicles, have been identified as key mitigation technologies for commercialization before 2030 (IPCC, 2007). However, all the environmental effects of producing and using biofuels require careful consideration: in fact, the production of some

conventional biofuels has been heavily criticized for causing deforestation (Gawel and Ludwig, 2011) (through direct or indirect land-use change (iLUC)), and for competing with food and animal feed production (IEA, 2013). Owing to the fact that expanding current biofuel production from sugar- and starch-based crops (so called first generation, 1G) has raised concerns about competition with crops cultivated for food and natural resources, such as water and productive land (Mohr and Ramam, 2013), it is of crucial importance to investigate which biofuels have positive environmental and social impacts. Life cycle assessment (LCA) has become an important methodology to evaluate the environmental benefits of biofuels (de Vries et al., 2010; Fazio and Monti, 2011). Many studies indicate that this can be generally true, but the extent of these benefits will depend on species, crop management, land allocation, scale level and environmental characteristics (Fazio and Monti, 2011). Moreover, the variation in the results obtained in LCA studies depends on the quality of the input data (Borjesson and Tufvesson, 2011). Biogas, ethanol, butanol and biogasoline are the major transportation biofuels that can be obtained by processing the sugar, starch, lipid and present in biomass. A certain biofuel can be a good or poor alternative in terms of GHG emission, depending on the raw material used, and the production process and location (Borjesson, 2009; Kendall and Chang, 2009).

However, food equity and security could be guaranteed by the use of non-edible feedstock for biofuel production (Naik et al., 2010) (so called second generation biofuels, 2G), such as lignocellulosic materials, that can be obtained from biomass crops cultivated on marginal land (e.g., perennial biomass crops), or from agricultural residues of food crops (e.g. sugarcane bagasse, wheat straw, corn stover). Adding to this, 2G biofuels are recognized to have greater GHG mitigation potential than 1G biofuels produced from sugar-, lipid- and starch-based crops (Directive 2009/28/EC).

2nd generation ethanol ($EtOH_{2G}$) has a considerable potential to replace oil to some degree, as it is a liquid fuel that can be easily integrated into the existing infrastructure for fuel distribution. The use of ethanol as a fuel have been introduced about 100 years ago, with the famous Model T Ford, the world's first mass-produced car, designed by Henry Ford (Solomon et al., 2007). He furthermore stated in the New York Times (1925) that: "*The fuel of the future is going to come from apples, weeds, sawdust – almost anything. There is fuel in every bit of vegetable matter that can be fermented.*" During the World Wars in the 20th century, ethanol was used to supplement gasoline in Europe, the US and Brazil. Post-war military demobilization and the expansion of new fields in the 1940s brought cheap oil to the market again and eliminated ethanol.

1.1 Bioethanol today

Today's flexi-fuel vehicles developed in ethanol rich countries as Brazil can use ethanol blends up to 95% depending on the climate. However, ethanol is mostly used as an additive to gasoline up to a maximum of 20%, and can be used in most modern spark-ignition engines without any need of modifications (Wyman, 1994).

The commercial production of fuel ethanol relies mainly on the fermentation of sugar and starch (1st generation ethanol, $EtOH_{1G}$) while lignocellulosic ethanol (2nd generation ethanol, $EtOH_{2G}$) entered the market only recently (2013) (Balan et al., 2013). The USA and Brazil have been the leading countries in the production of 1st generation ethanol ($EtOH_{1G}$) from corn starch and sugarcane sugar, respectively (RFA, 2012). In both countries, programmes for large-scale production of alcohol were initiated during the early 1970s, in response to the oil embargo of 1973 conducted by OPEC countries. Some years after, in the early 1980s, several countries in Europe decided to start similar programmes for large-scale ethanol production.

The amount of ethanol produced by USA and Brazil together in 2013 was 74 billion litres, accounting for 84% of the world production, while the amount of ethanol produced in Europe was 6.7 billion litres (RFA, 2014). The feedstocks used in Europe are maize (47%) and wheat (31%) grain, and sugar beet (14%). The three largest European ethanol producers are France, Germany, and Spain, followed by Austria and Sweden (ePure, 2014).

However these raw materials are also used for food and feed production, exacerbating the debate on iLUC (Tait, 2011). Furthermore, $EtOH_{1G}$ ethanol usually results in higher GHG emissions than $EtOH_{2G}$, which is produced from lignocellulosic biomass such as wood, agricultural residues such as wheat straw, maize stover and biomass crops (Directive 2009/28/EC).

Currently, only negligible amounts of second generation bioethanol are produced in several demonstrative plants around the world that work at industrial scale (Lennartsson et al., 2014). At the moment, among the companies that started to produce $EtOH_{2G}$, Beta Renewables and Novozymes in Crescentino (Italy) use wheat straw, rice straw and *Arundo donax* as feedstocks in Crescentino (Italy); while in Emmetesburg (Iowa, USA) POET-DSM opened last year a plant where corn cobs, leaves, husk and stalk are converted in ethanol; in Nevada (Iowa, US) DuPont Biofuels is near to complete a commercial cellulosic ethanol plant.

1.2 Ethanol from lignocellulosic biomass

Lignocellulosic biomass can be converted to ethanol through two different ways (Hamelinck et al., 2005): 1) the thermochemical route, in which biomass is gasified (or liquefied) followed by catalytic or microbial conversion of the syngas (or bio-oil) to other fuels; and 2) the biochemical route, where the polymeric sugars constituting

cellulose and hemicellulose are hydrolysed into monomeric units and then converted to ethanol by fermentation with organisms such as yeasts.

In this thesis, the biochemical route was followed, which appears more promising in view of the recent industrial move towards cellulosic ethanol.

The biochemical pathway of the production process mainly consists of four steps: pretreatment, hydrolysis, fermentation and product recovery. Depending on whether hydrolysis and fermentation are carried out in the same vessel at the same time or not, the so called simultaneous saccharification and fermentation (SSF) or the separate hydrolysis and fermentation (SHF) process is performed (Wingren et al., 2003).

Before pretreatment, biomass is mechanically milled to make it easier to handle and process (Hendricks and Zeeman, 2009).

1.2.1 Pretreatment

Because of the recalcitrance of lignocellulosic biomass, a pretreatment is needed in order to increase the accessibility of cellulose and hemicellulose to the enzymes that break down the cellulose and hemicellulose polymers into their monomeric units in the subsequent enzymatic hydrolysis step (Mosier et al., 2005).

In particular, lignocellulosic biomass is composed of cellulose, hemicellulose, lignin, extractives, and several inorganic materials (Sjöström, 1993). Cellulose is a polysaccharide, presents crystalline and amorphous regions, and consists of a linear chain of several hundred to many thousands of β (1→4) linked D-glucose units (Morohoshi, 1991; Dellmer and Amor, 1995). The cellulose chains are packed by hydrogen bonds in so-called microfibrils (Ha et al., 1998). These fibrils are attached to each other by hemicellulose, which is an amorphous polymer of different sugars as well as other polymers such as pectin, and covered by lignin. The microfibrils are often

associated in the form of bundles or macrofibrils (Delmer and Amor, 1995). This structure makes cellulose resistant to both biological and chemical treatments. In softwood, hemicellulose is mainly constituted by mannose, while in hardwoods and agricultural residues, xylose is the dominant sugar in hemicellulose. Furthermore, hemicellulose contains galactose, glucose, arabinose, and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid, and galacturonic acid. While cellulose is mostly crystalline and strong, hemicelluloses have a random, amorphous, and branched structure with little resistance to hydrolysis, so that it is more easily hydrolyzed by acids into its monomer components (Sjöström, 1993; Delmer and Amor, 1995). Lignin is a very complex molecule constructed of phenylpropane units linked in a three-dimensional structure which is particularly difficult to degrade. Lignin is the most recalcitrant component of the plant cell wall, and the higher the proportion of lignin, the higher the resistance to chemical and enzymatic degradation. Generally, softwoods contain more lignin than hardwoods and most of the agriculture residues. There are chemical bonds between lignin and hemicellulose and even cellulose (Palmqvist and Hahn-Hägerdal, 2000). Lignin constitutes one of the drawbacks of using lignocellulosic materials in fermentation, increasing fibre resitance to chemical and biological degradation.

One of the main objectives of pretreatment step is to increase the available surface for enzymatic attack (Chandra et al., 2007; Alvira et al., 2010).

To be most efficient, a pretreatment should secure a series of outcomes (Galbe and Zacchi, 2012):

- result in high recovery of all carbohydrates;
- result in high digestibility of the cellulose in the subsequent enzymatic hydrolysis;
- produce no or very limited amounts of sugar and lignin-degradation products;

- pretreatment liquid should be subjected to fermentation without any need of detoxification;

- result in high concentration of solids as well as liberated sugars in the liquid fraction;

- involve a low energy demand or be performed in such a way that the energy can be re-used in other process steps as secondary heat;

- require low capital and operational costs;

- require as few water as possible.

The best method and conditions of pretreatment depend greatly on the type of lignocelluloses.

Pretreatment methods can be classified into four main groups: biological, physical, chemical and physico-chemical.

Biological pretreatments have low environmental impact, since they consist in the use of microorganisms such as brown, white and soft-rot fungi. These microorganisms have the ability to degrade lignin and hemicellulose, but very little part of cellulose (Taherzadeh et al., 2008). However, pretreatment time is very long to reach appreciable hydrolysis rate.

Physical pretreatments aim to break down the lignocellulosic biomass into smaller particles (Brodeur et al., 2011). These methods (chipping, milling or grinding) increase the surface area available for enzymatic attack and reduce cellulose crystallinity. The main drawback of physical agents is the high amount of energy required, often resulting in financial unfeasibility feasible (Kumar et al., 2009).

Chemical pretreatments aim to enhance enzymatic hydrolysis separating hemicellulose and/or lignin from the cellulose. In particular, alkaline catalysts remove lignin and have a small direct effect on cellulose and hemicellulose, but cause fibre swelling which increases the internal surface area (Kassim et al., 1986). Alkali pretreatment was

considered to be more effective on agricultural residues than wood materials at higher lignification (Chandra et al., 2007). Acid catalysts are also used in order to hydrolyze the hemicellulose (Taherzadeh et al., 2008). However acid are corrosive for the equipment used. Organosolv catalysts, such as methanol, ethanol, acetone and ethylene glycol can be used to solubilize lignin and, consequently, increase the enzymatic digestibility of lignocellulose (Itoh et al., 2003). Ozone is an oxidant able to decompose lignocellulose (Alvira et al., 2010). Ionic liquids have received much attention recently for their ability to solubilise cellulose (Weerachanchai and Lee, 2013).

Physico-chemical pretreatments are a combination of physical and chemical means.

In the steam pretreatment, also called steam explosion, high-pressure saturated steam is applied to the material for a few minutes (5-20), and it can be carried out with or without the addition of chemical catalyst. The pressure is then rapidly decreased by discharging the material into a flash vessel. It is usually run at temperatures around 160-240 °C. In steam pretreatment with no catalyst, also called autohydrolysis, hemicellulose is hydrolysed by acids released from acetyl groups in the hemicellulose, and water acts as an acid at high temperatures (Varga et al., 2004; Ruiz et al., 2006). Adding an acid catalyst during steam pretreatment increases the recovery of hemicellulose sugars and improves the enzymatic hydrolysis of the solid fraction (Stenberg et al., 1998; Galbe and Zacchi, 2007).

The effectiveness of steam pretreatment is determined by the temperature and residence time, and the severity is defined using the so-called severity factor R_0 (Overend and Chornet, 1987).

$$\text{Log } R_0 = Log\left(t \cdot exp^{\left(\frac{T-Tref}{14.75}\right)}\right)$$

Where t is the residence time in minutes and T is the treatment temperature in °C.

When a catalyst is added, the combined severity factor (CS) is used to define the severity of pretreatment:

$$\mathrm{CS} = \mathrm{Log}\, R_0 - pH$$

Liquid hot water (LHW) pretreatment is run at 160-240 °C for about 15 minutes. Most of hemicellulose, as well as half of the lignin and part of cellulose, is dissolved. During LHW pretreatment, organic acids are generated through the cleaving of acetyl and uronic acid groups from hemicellulose, and these acids favour catalysis (Alvira et al., 2010).

Wet oxidation consists in treating biomass at 170-200 °C and 1-1.2 MPa for 10-15 minutes with the addition of air or oxygen. Lignin and hemicellulose are solubilized resulting in increased digestibility of the remaining cellulose (Alvira et al., 2010).

Ammonia fibre explosion (AFEX) consists in treating biomass with liquid ammonia at 60-100 °C, and the combination of ammonia and high pressure causes swelling and physical disruption of the biomass fibres (Alvira et al., 2010).

1.2.2 Hydrolysis

Hydrolysis can be carried out using acids or enzymes. Dilute acid hydrolysis is carried out with an acid concentration in the range of 1-5% and requires high temperature (160-230 °C) and pressure (1 MPa), with retention times of seconds to minutes. It is efficient in hydrolyzing the hemicellulose fraction, but not cellulose. To solubilize cellulose, an acid concentration of about 10-30 % is needed. Concentrated acid hydrolysis can be

performed at moderate temperature (below 50 °C) and atmospheric pressure, but requires longer retention times (Hamelinck et al., 2005).

Compared to acid hydrolysis, enzymatic hydrolysis is highly specific and is carried out under milder conditions (about 50 °C and pH 5). Differently from acid hydrolysis, enzymatic hydrolysis is environmentally friendly and it does not lead to by-products that inhibit enzyme or yeast activity (Wyman, 1994; Hamelinck et al., 2005). In the experiments presented in this thesis, hydrolysis was carried out by enzymes. Enzymes used in hydrolysis are excreted by fungi such as the *Trichoderma*, *Penicillum*, *Aspergillus* and *Phanerochaete* genuses. In particular, the cellulase systems are represented by three major activities: endoglucanases (EGs), exoglucanases, including glucohydrolases and cellobiohydrolases (CBHs), and β-glucosidases. Specifically, endoglucanases act randomly on the amorphous region of the cellulose, attacking the β-1,4 - glycosidic bonds, liberating glucose oligomers of various lengths and exposing new terminal ends of the cellulose chain and act in a progressive manner, releasing either glucose units (glucohydrolases) or cellobiose units (cellobiohydrolases). Cellobiohydrolases cleave off from reducing the exoglucanases bind to end (Labudova and Farkas, 1983). Also hemicellulases are required for the hydrolysis of hemicellulose. Endo-1,4-β-D-xylanase and endo-1,4-β-D-mannase act by depolymerizing the hemicellulose backbone, while β-D-xylosidase, β-D-mannosidase and β-D-glucosidase hydrolyse small oligosaccharides into xylose, mannose or glucose, respectively, by cleaving the oligomer's β-1,4 bonds (Hrmovà et al., 1989).

1.2.3 Fermentation

Monomeric sugars resulting from saccharification are fermented into ethanol, together with the formation of carbon dioxide. A wide variety of bacteria, yeast and filamentous

fungi have the ability to ferment sugars into ethanol (Olsson et al., 1996). The requirements for optimum fermentation are high ethanol productivity, high yield from all types of sugars (pentose and hexose) and tolerance to high ethanol and inhibitor concentrations in the fermentation broth (Mielenz, 2001). *Escherichia coli, Zymomonas mobilis*, *Saccharomyces cerevisiae*, *Pichia stipitis* and *Candida Shehatae* are the most relevant yeasts for ethanol production (Palmqvist and Hahn-Hägerdal, 2000). *S.cerevisae* and *Z.mobilis* are able to ferment glucose to ethanol but not pentose, while *E.coli*, *P. stipitis* and *C.sheatae* are naturally xylose-fermenting strains, but they have low ethanol and inhibitor tolerance.

Baker's yeast, *S. cerevisiae*, is the most commonly used microorganism in traditional $EtOH_{1G}$ production (Hahn-Hägerdal et al., 2006; Wu et al., 2010). The wild type *S. cerevisiae* can efficiently ferment hexose with high yields and it has been shown to have a relatively good tolerance to lignocellulose-derived inhibitors (Klinke et al., 2004). Nowadays, metabolic engineering has achieved substantial progress in building strains able to ferment both pentoses and hexoses (Olson et al., 2012; Erdei et al., 2013). In this work, wild type of *S.cerevisiae* was used.

1.3 Agricultural and biomass sources

There is a wide range of lignocellulosic feedstocks that are differentiated by their origin, composition and structure (Bonin and Lal, 2012). In this work three different materials were used. Sucarcane bagasse was used (*Saccharum officinarum* L.) as agricultural residues, one crop grown specifically for non-food use that can be cropped in marginal lands as Switchgrass (*Panicum virgatum* L.), and two hybrids of sweet sorghum (*Sorghum bicolor* L. Moench) as crop to use as 1^{st} and 2^{nd} generation ethanol production.

1.3.1 Sugarcane bagasse

Sugarcane bagasse is the lignocellulosic residue of the sugarcane-based sugar and ethanol industries. Sugarcane is a perennial true grasses of the genus *Saccharum*, tribe Andropogoneae, native to the warm temperate to tropical regions of South Asia and used for sugar production. It has fibrous stalks that are rich in sugar and measure two to six metres tall. Brazil is the largest producer of sugar cane in the world.

Sugarcane is an important food and bioenergy source and a significant component of the economy in many countries in the tropics and subtropics (Waclawovsky et al., 2010).

After processing for sugar extraction and ethanol industries, about 280 kg of bagasse remains per ton of sugarcane, and that means 70 tons per hectare (Macrelli et al., 2012). Bagasse is commonly discarded as agricultural waste or burned for energy supply in sugar and ethanol mills. However, both alternatives are considering polluting and inefficient from an energy point of view (Furlan et al., 2013). Sugarcane bagasse is primarily composed of cellulose (40-45%), hemicellulose (30-35%), and lignin (20-30%). So it provides a valuable inexpensive feedstock that could be utilized for the biological production of fuels, such as bioethanol, offering economic, environmental and energetic advantages.

1.3.2 Switchgrass

Switchgrass (*Panicum virgatum* L.) is a perennial C_4warm season bunchgrass native to North America, and it has demonstrated high productivity in a wide geographical range, suitability for marginal land, low water and nutrient requirement, and environmental benefits (McLaughin et al., 2002; Heaton et al., 2004; Wright and Turhollow, 2010). Switchgrass is propagated by seed and once established is both a perennial and self-seeding crop, which means farmers do not have to plant and reseed after annual

harvesting (Lewandoski et al., 2003). Switchgrass stand can survive for ten years or longer. For these characteristics switchgrass combines more of the attributes desirable for bioenergy feedstock production than other grasses.

1.3.3 Biomass sorghum

Sorghum is a fast growing C4 plant native to tropical zones but with a wide adaptability to different environmental conditions and sweet sorghum (*Sorghum bicolor* L. Moench) is any of the many varieties of the sorghum grass whose stalks have a high sugar content (FAO 2014). The growing interest in bioenergy and particularly in bioethanol is a great challenge for this relatively new crop that could be used for both thermo-electrical energy and biofuel. Nonetheless, the quantitative and qualitative production of sweet sorghum strongly depends on the use of appropriate and improved agronomic management techniques which is, in some aspects, still largely unknown (Zegada and Monti, 2012).

The production of bioethanol from soluble sugars contained in the juice is likely more economical than from maize starch, the latter needing an additional pretreatment to convert starch into fermentable substrate (Smith et al., 1987; Prasad et al., 2007). It is considered a ‘camel’ for its feature to produce appreciable dry biomass yield in water stress conditions, thus becoming during the last years object of several studies as dedicated bioenergy crops (Mastrorilli et al., 1999). Moreover, it has also high N use efficiency which may limit the fertiliser apply and reduce the environmental releases without compromising biomass yield (Barbanti et al., 2006).

In this respect, sweet sorghum may produce bioethanol from the soluble sugars contained in the stems and also contribute to the production of bioethanol from the

lignocellulosic biomass, which residue after stem sugars extraction, as a second generation biofuel (Ballesteros et al., 2004).

1.4 Aims and arrangement of this thesis

The experimental work carried out in the frame of this thesis addressed pretreatments in the production of ethanol from lignocellulosic materials. As described previously, pretreatments enhance the enzymatic digestibility of biomass before fermentation, thus playing an important role in achieving high sugar and ethanol yields in the process. However, pretreatment step is often associated with a large amount of waste water to manage. Water used during pretreatment and the whole process can be wasted or not, on the basis of catalyst and the amount of water used. Based on these considerations, calcium hydroxide ($Ca(OH)_2$) is an alkaline catalyst potentially suited for lignocellulosic material, which can easily be removed from the water used for impregnation, by carbonating with CO_2. The resulting $CaCO_3$ may be recovered to be used in several applications (Carvalho et al., 1997; Patanè et al., 2012), thus the use of calcium hydroxide does not originate waste water.

In Chapter 1, calcium hydroxide as impregnation agent before steam explosion of sugarcane bagasse was compared with auto-hydrolysis, assessing the effects on enzymatic hydrolysis and SSF at high solid concentration of pretreated solid fraction. In addition, anaerobic digestion of pretreated liquid fraction was carried out, in order to appraise the effectiveness of calcium hydroxide before steam explosion in a more comprehensive way. In Chapter 2, auto-hydrolysis and steam explosion preceded by either sulphuric acid or calcium hydroxide impregnation were compared in switchgrass, still studying the effects on enzymatic hydrolysis and SSF at high solid concentration of

pretreated solid fraction. Anaerobic digestion of the liquid fraction was also carried out, to provide further insight into pretreatment effects on lignocellulosic biomass.

As water is an expensive input in both cultivation of biomass crops and subsequent pretreatment, especially in view of the savings necessitated to face climate change, Chapter 3 addressed the effects of variable soil moisture on biomass growth and composition of biomass sorghum. Moreover, the effect of water stress was related to the characteristics of stem juice for 1st ethanol and structural carbohydrates for 2nd ethanol. Nowadays it is widely known that biomass suitability for 2nd ethanol depends on cellulose content, but also on biomass physical and chemical properties (Corredor et al., 2009). Therefore, is not sufficient to assess the quantity of fibres in biomass, but also their convertibility to ethanol. Given these premises, juice fermentation, enzymatic hydrolysis and SSF were conducted on two hybrids of biomass sorghum subjected to two water levels and harvested at three different dates.

CHAPTER 1

Combined ethanol and methane production using steam pretreated sugarcane bagasse

Abstract

Efficient energy production relies on complementary use of crop residues, to enhance the amount of energy obtained per unit biomass. In this frame, sugarcane bagasse (SB) was pretreated and the resulting solid and fraction served, respectively, for simultaneous saccharification and fermentation (SSF) at high solid concentration (15%), and anaerobic digestion (AD). More specifically, SB was subjected to twelve pretreatments to enhance fibre deconstruction and subsequent energy output: steam explosion alone (195 °C for 5, 10 and 15 minutes), after impregnation with 0.4% and 0.7% $Ca(OH)_2$, and at 205 °C for the same three times after 0.7% $Ca(OH)_2$ addition. Enzymatic hydrolysis was carried out on pretreated solid fraction (slurry), and glucose and xylose analysis were performed on solid and liquid fraction. On this latter, inhibitors (acetic and formic acid, furfural and 5-hydroxymethylfurfural) were also determined. Based on high glucose yield in the slurry, three pretreatments were selected for SSF of the solid fraction. The same pretreatments underwent AD of the liquid fraction. Inhibitors increased at increasing time and temperature, although never achieved critical levels. Lignin removal (range, 17-38%) was enhanced by lime addition, whereas increasing temperature and time did not contribute to delignification. Glucose yield in solid fraction varied accordingly. SSF exhibited the highest ethanol yield with mild lime addition (60% of theoretical) vs. steam alone (53%). However, modest yields were generally evidenced (average, 55%), as a result of high viscosity especially in the case of high lime dose in SSF at high solid concentration. Combined energy yield (ethanol, methane and solid residue) proved lime effectiveness as catalyst in steam explosion of SB, beside two intrinsic advantages consisting in low water consumption in SSF at high solid concentration, and the possibility of lime removal from downstream effluents through carbonation.

1.1 Introduction

Many studies have been conducted on the use of sugarcane bagasse (SB) as a source for second generation bioethanol ($EtOH_{2G}$). A wide consensus supports the use of SB for $EtOH_{2G}$ production in terms of environmental benefits (Dias et al., 2011; Furlan et al., 2013), compared to burning it in order to power a first generation ethanol plant. However, a few studies demonstrate the economic advantages of integrating first and second generation ethanol process (Dias et al., 2011; Furlan et al., 2013). Owing to this, a large number of experiments have been carried out to investigate the best route to obtain $EtOH_{2G}$ from SB, as reported by Macrelli et al. (2012).

In this frame, pretreating biomass is a prerequisite to maximize the enzymatic convertibility of SB cellulose and hemicellulose into fermentable sugars (Galbe et al., 2007). Steam pretreatment, also known as steam explosion, is one of the most studied and promising methods (Toor et al., 2013). It is often preceded by impregnation with a catalyst, mainly consisting of acidic gases or liquids (e.g., sulphur dioxide, sulphuric acid) (Martin et al., 2002; Sassner et al., 2007; Carrasco et al., 2010). Catalyst addition determines a higher pretreatment efficiency than steam explosion alone, this latter also called auto-hydrolysis process (Bondesson et al., 2013). However, the use of a catalyst impacts on the environment because of water and chemical supply that generate large amounts of downstream waste to be disposed. Compared to this, it would be much more advisable to use a catalyst securing high yields of $EtOH_{2G}$, no adverse impact on the environment and whose by-products have a market value.

In general, chemical catalysts involve issues such as equipment corrosion and the need of processing downstream effluents, resulting in high water consumption (Ramos, 2003). Among chemical catalysts, acids do not remove lignin, a fraction of biomass that is not converted into ethanol, hindering enzymatic hydrolysis (Jorgensen et al., 2007).

Lignin may block enzymes activity by restricting access to cellulose and hemicellulose, resulting in a rate limited enzymatic hydrolysis and subsequent curb of potential ethanol yield (Chang et al., 2000; Rabelo et al., 2011). Compared to acids, alkaline pretreatments can effectively abate lignin in agricultural residues as SB (Fuentes et al., 2011), although they did not prove satisfactory in processing recalcitrant substrates as softwood (Chandra et al., 2007).

Lime, i.e. calcium hydroxide ($Ca(OH)_2$), is an alkaline catalyst suited to enhance degradation of lignocellulosic biomass of agricultural origin. Adding to this, it can be removed by carbonating the waste water with CO_2 (Chang et al., 2001). The resulting $CaCO_3$ may have several applications as mitigating the drought stress in tomato (Patanè et al., 2012), thus becoming a valuable by-product instead of a downstream waste.

The yeast strain *Saccharomyces cerevisiae* is well suited for simultaneous saccharification and fermentation (SSF) of hydrolysed lignocellulosic material. However, *S. cerevisiae* ferments hexoses but not pentoses; hence, adopting a pathway to convert pentose sugars into additional energy is crucial to improve the energy output of the whole process. SSF of both hexoses and pentoses with engineered yeast strains is seen a promising option in the near future (Oloffson et al., 2008). At present, methane production through anaerobic digestion (AD) of pretreated liquid appears the most reliable practice (Kaparaju et al., 2009; Dererie et al., 2011) to complement $EtOH_{2G}$ production through SSF of the solid fraction.

Given these premises, the aims of the present study were to compare the effects of lime used as catalyst in SB impregnation before steam explosion, on sugar yields after pretreatment and enzymatic hydrolysis. Time, temperature and catalyst concentration during pretreatment were varied. Pretreatments that in the enzymatic hydrolysis had showed top glucose yields underwent separation of the solid (slurry) from the liquid

fraction. The former was subjected to SSF at high solid concentration (15%); the latter to AD. Finally, combined energy yield was calculated as the sum of the energy contained in ethanol, methane and the solid residue after SSF.

1.2 Material and Methods

1.2.1 Process configuration

SB was impregnated with/without lime, prior to being subjected to steam explosion under different conditions of time and temperature, making up a total of 12 combinations (Table 1.1). Pretreated samples were separated into a solid (slurry) and liquid fraction. The former was repeatedly washed with distilled water and subjected to enzymatic hydrolysis. Both fractions were analysed for glucose and xylose. Enzymatic hydrolysis and the subsequent analysis served to identify pretreatment conditions resulting in top glucose yields, to be selected for SSF. The corresponding liquids underwent AD. Figure 1.1 describes process configuration from raw biomass to final energy products.

Table 1.1 - Experimental conditions and relative Severity Factor (Log R_0) in sugarcane bagasse pretreatment.

Pretreatment	**$Ca(OH)_2$** % (w/w)	**Time** (min)	**Temperature** (°C)	**Log R_0**
1	-	5	195	3,5
2	-	10	195	3,8
3	-	15	195	4,0
4	0.4	5	195	3,5
5	0.4	10	195	3,8
6	0.4	15	195	4,0
7	0.7	5	195	3,5
8	0.7	10	195	3,8
9	0.7	15	195	4,0
10	0.7	5	205	3,8
11	0.7	10	205	4,1
12	0.7	15	205	4,3

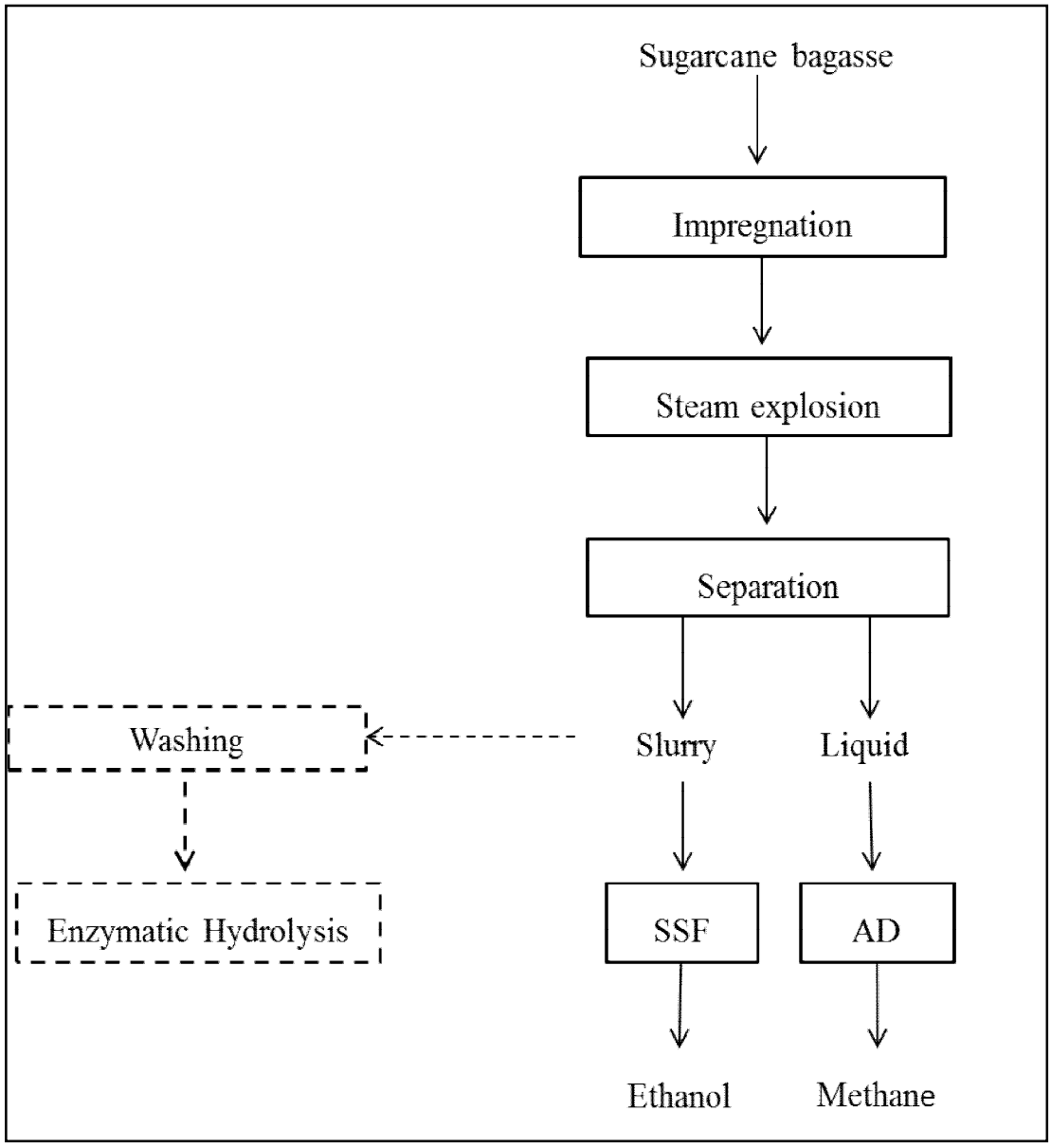

Figure 1.1 - Process configuration from the raw material to final ethanol and methane. Dashed graphics indicate the procedure followed to select pretreatment solids (slurries) for simultaneous saccharification and fermentation (SSF), and the corresponding liquids for anaerobic digestion (AD).

1.2.2 Raw material

SB was air dried (total solids, 93%) and chopped into pieces of approximately 0.5 mm size for the analysis of structural carbohydrates, lignin (Klason lignin and acid soluble lignin), ash and extractives (Sluiter et al., 2005; Sluiter et al., 2008a).

1.2.3 Pretreatments

The 12 pretreatments assessed in the experiment (Table 1.1) can be divided into two main groups: autohydrolysis, consisting of steam alone at increasing time (P1-P3); alkaline pretreatment, consisting of nine combinations of $Ca(OH)_2$ concentration, temperature and time (P4-P12).

The raw material (20-50 mm size) was immersed for one hour either in water at a liquid to solid ratio of 20:1 (w/w), or in an aqueous solution containing $Ca(OH)_2$ at 0.4% or 0.7% (w/w), and stored in a sealed bucket for 1 hour. Thereafter, the wet SB was dewatered in a 3 L press (Tinkturenpressen HP5M, Fischer Maschinenfabrik GMBH, Germany), to reach a dry matter content of 45-50%.

The steam pretreatment was then performed in a 10 L reactor, loaded with an amount of wet SB corresponding to 400 g dry matter. More in detail, the steam unit was composed of a 10 L reactor connected to a controlling computer and the flash chamber.

Pretreated material was then divided into two fractions: liquid, resulting from filtration through a 2.5 μm sieve, and residual solid. The solid fraction was washed and then analysed for structural carbohydrates and lignin, while the liquid fraction was analysed for the content of total sugars, monomers and inhibitors (acetic acid (AA), formic acid (FA), furfural and 5-hydroxymethylfurfural (HMF)), according to a method from the US National Renewable Energy Laboratory (Sluiter et al., 2008b). The content of water-insoluble solids (WIS) was determined using the method developed by Weiss et al. (2010). All the chemical and physical traits were analysed in duplicates.

1.2.4 Enzymatic hydrolysis

Pretreated slurries were repeatedly washed with distilled water to completely remove the liquid fraction, and were subjected to enzymatic hydrolysis (Figure 1.1) at a loading

of 5% WIS for 48 h at 50 °C. The hydrolysis was performed in 50 mL plastic tubes containing two steel balls in a rotating incubator at 100 rpm. The enzyme used, CelliCTec3 (Novozymes, Bagsvaerd, Denmark), was added at an amount corresponding to 10 FPU g^{-1} WIS. Enzyme activity was measured according to Adney and Baker (2008). Sodium acetate was used as buffer. During the hydrolysis, the pH was maintained at 5 with 10% NaOH. All tests were conducted in duplicates.

1.2.5 Simultaneous saccharification and fermentation

Slurries resulting in top glucose yields in enzymatic hydrolysis were further investigated to determine their potentials for producing $EtOH_{2G}$ through SSF at high solids concentration (15% WIS). This procedure was performed in a 2 L fermenter (Infors AG, Bottmingen, Switzerland), previously sterilized at 121 °C for 20 minutes, using 650 g of unwashed material at 15% WIS. The pH was adjusted at 5 with 10% NaOH. The Cellic CTec3 (Novozymes, Bagsvaerd, Denmark) enzyme was added at 10 FPU g^{-1} WIS, while temperature was maintained at 45 °C for 2 hours as a pre-hydrolysis step. Thereafter, the mixture was cooled to 35 °C and supplied with 3 g L^{-1} of *S. cerevisiae* yeast (Ethanol Red, Lesaffre, Marcq-en-Baroeul, Roubaix, France), and 0.5 g L^{-1} NH_4PO_4 as nutrient source. SSF was performed at 35 °C for 96 hours. Samples were taken after 2, 4, 7, 10, 12, 24, 48, 72 and 96 hours, and analysed by HPLC for ethanol, monomeric sugars, acetic acid, formic acid, and sugar degradation products. All SSFs and analyses were performed in duplicates.

1.2.6 Anaerobic digestion

AD was performed to determine methane yield in pretreated liquids corresponding to the slurries chosen for SSF. Prior to AD, total organic carbon (TOC), total solids (TS) and volatile solids (VS) were determined. TOC content was determined by a total

carbon analyser (TOC-5050A) with an auto-sampler (ASI-5000A). The carrier gas flow was set at 150 mL min^{-1} at a working temperature of 680 °C. TS were determined at 105 °C for 24 hours. Finally, VS were determined by ashing the dried sample at 550 °C for 2 hours. All analyses were conducted in duplicates.

Inoculum was collected from a municipal water-treatment plant (Källbyverket, Lund, Sweden), and maintained in mesophilic conditions until the end of biogas emission. TS and VS content of the starved inoculum were determined as in pretreated liquid. Thereafter, inoculum and pretreated liquid were mixed in a 2:1 (VS/VS) ratio, to give a total 500 g broth in 1 L bottles, kept in an incubator at 37 °C for only 10 days, in view of the highly degradable carbohydrates contained in the liquid fraction. Anaerobic digestion was monitored using the Yieldmaster (BlueSens ®, Herten, Germany) system: biogas volume was measured continuously with precision mass flow meters (Ritter MilliGascounter®, Bochum, Germany); methane concentration was gauged with an infrared (IR) sensor, and the data were collected via BACCom units to BACVis software (BlueSens ®, Herten, Germany). Data of methane yield (NmL g^{-1} VS at standard temperature and pressure) were corrected deducting the amount of CH_4 produced from blank samples containing inoculum alone.

1.2.7 Analytical determinations

Monomeric sugars from analysis of the raw material were measured using high-performance anion-exchange chromatography coupled with pulsed amperometric detection. The chromatographic system (ICS-3000, Dionex Corp., Sunnyvale, California, USA) was equipped with a Carbo Pac PA1 analytical column (Dionex Corp., Sunnyvale, California, USA). Deionized water was used as eluent at a flow rate of 1 mL

min^{-1}, and the column was cleaned with a solution of 200 mM NaOH dissolved in 170 mM sodium acetate. The sample injection volume was 10 µL.

The amounts of monomeric sugars, by-products and ethanol in the liquids after enzymatic hydrolysis and SSF were determined by HPLC with a refractive index detector. Glucose, xylose, arabinose, galactose and mannose were separated using an Aminex HPX-87P column (Bio-Rad, Hercules, California, USA) at 85 °C with a flow rate of 0.5 mL min^{-1} using water as eluent. Ethanol, AA, FA, furfural and HMF were separated using an Aminex HPX-87H column (Bio-Rad, Hercules, California, USA) at 50 °C with a flow rate of 0.5 mL min^{-1}, using 5 mmol L^{-1} sulphuric acid as eluent. All samples had been passed through a 0.2 µm filter before analysis.

1.2.8 Calculations and statistical analysis

Lignin removal was calculated as proposed by Kim et al. (2006):

$$Lignin\,removal = 1 - W_{\mathrm{L}} \qquad (1.1)$$

where W_L is the fraction of residual lignin expressed as follows:

$$W_{\mathrm{L}} = \frac{L \cdot Y_T}{L_0} \qquad (1.2)$$

where L is Klason lignin in pretreated biomass, Y_T is pretreatment yield of total solids, and L_0 is Klason lignin in raw material.

Sugar yield was calculated by dividing the total amount of glucose and xylose determined in pretreatment liquid and washed slurry after enzymatic hydrolysis, by the

total amount contained in the raw material. For each sugar, the former proportion represents pretreatment yield, while the latter is enzymatic hydrolysis yield.

Ethanol yield was calculated using the measured amounts of glucose and ethanol in the fermentation broth at the end of SSF, by the following formula:

$$Y_{\mathrm{EtOH}} = \frac{C_{EtOH}(1-WIS_{end})\cdot\frac{M}{1000}}{0.51\cdot\left[WIS\cdot M\cdot\sigma_{glc}+V_{hyd}\cdot c_{glc}\right]} \qquad (1.3)$$

where Y_{ETOH} is the overall ethanol yield resulting from SSF (% of theoretical value); C_{ETOH} is the final concentration of ethanol (g L^{-1}); M is the total mass (g); WIS and WIS_{end} are the fractions of water insoluble solids (%) calculated at the beginning and the end of SSF, respectively; σ_{glc} is the mass fraction of glucose in pretreated fibres (g g^{-1}); V_{hyd} is the starting volume in the reactor (L); c_{glc} is the concentration of glucose at the start of SSF (g L^{-1}).

To better evaluate lime effectiveness as catalyst, a response surface analysis was carried out with the SigmaPlot 10 software (Systat Software Inc.,Chicago, Illinois, USA), using $Ca(OH)_2$ concentration and a severity factor (Log R_0) that combines residence time and temperature, to identify optimum conditions for lignin removal, within the range tested in this experiment. The severity factor was calculated as follows:

$$\mathrm{Log}\ R_0 = Log\left(t\cdot\varepsilon^{\left(\frac{T-Tref}{14.75}\right)}\right) \qquad (1.4)$$

where t is residence time (min), T, pretreatment temperature (°C), and $Tref$ the reference temperature (100 °C).

The combined energy yield, i.e. ethanol from SSF, methane from AD and the amount of energy in the residual solid after SSF, was calculated per unit dry weight of the raw material, assuming 27.1, 50 and 17.8 kJ g^{-1} as respective energy content for ethanol, methane and solid residue (Bondesson et al., 2013; Furlan et al., 2013).

In all traits, normal distribution and equal variance of data were controlled through the Kolmogorov–Smirnov and Bartlett tests, respectively. Data were then submitted to one way analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, California, USA). The lowest significant difference (LSD) test at $P \leq 0.05$ was used to separate means of significant traits.

1.3 Results and discussion

1.3.1 Characteristics of the raw material

Table 1.2 reports the composition of the raw material. SB consisted of ca. 47% glucan and 25% xylan. These amounts were in the same range as SB analyses performed in other studies (Carrasco et al., 2010; Rabelo et al., 2011), indicating a good intrinsic suitability for SSF and AD. Conversely, extractives showed a higher amount compared to the cited sources.

Table 1.2 - Composition of sugarcane bagasse expressed a s percentage of dry matter. In brackets, the standard deviation of mean values.

Glucan	Xylan	Arabinan	Galactan	Lignin[a]	Ash	Extractives
46.6 (±0.66)	24.6 (±0.29)	1.5 (±0.01)	0.4 (±0.01)	26.6 (±0.05)	0.2 (±0.01)	5.3 (±0.13)

[a] Acid-solble lignin plus Klason lignin

1.3.2 Pretreatment evaluation

1.3.2.1 Sugars and inhibitors in pretreatment liquid

Glucose and xylose concentrations as monomeric and oligomeric forms (Table 1.3) released into pretreatment liquid following auto-hydrolysis (P1-3) were very close to data obtained in another study on SB (Carrasco et al., 2010). Monomeric glucose was very low in the auto-hydrolysis, and still below detection limit with lime addition (P4-12). The same pattern with somewhat higher data was shown for oligomeric glucose that averaged 2 g L^{-1} in P1-3; only 0.6 and 0.8 g L^{-1} in P4-9 and P10-12, respectively. Xylose always exhibited higher concentrations than glucose, and the auto-hydrolysis proved still more effective than lime addition in releasing both forms of the sugar: monomeric xylose did not pass the detection limit with lime addition, while oligomeric xylose averaged 18 g L^{-1} in P1-3; only 7 g L^{-1} in P4-12. Thus, the same proportion of ca. 2.5:1 was evidenced between the auto-hydrolysis and lime addition in the two sugars' concentrations.

Inhibitors produced during pretreatment include pentose-degradation products as furfural and FA, hexose-degradation products as HMF, beside AA that is formed when side chains of acetyl groups are released during hemicellulose solubilisation. Inhibitors did not reach concentrations hampering yeast activity, although differences were found between steam alone and after lime impregnation (Figure 1.2). More in detail, the concentration of AA varied but was always quite lower than 5 g L^{-1}, the threshold at which the inhibition of *Saccharomyces cerevisiae* becomes critical, curbing fermentation activity (Taherzadeh et al., 1997). Also FA varied but always remained below 3.7 g L^{-1}, the threshold acknowledged for strong inhibitory effects (Maiorella et al., 1983). Furfural and HMF, already at 1-5 g L^{-1} exert a negative role on fermentation,

although final ethanol yield is barely affected at such concentration (Palmqvist et al., 1996). However, only furfural reached 2 g L^{-1} in P3, while HMF always remained below 1 g L^{-1}.

Despite the low level generally evidenced for all inhibitors, pretreatment time and temperature influenced their concentrations, i.e. longer time and higher temperature were often associated with higher concentrations. This was especially true in the case of time with FA, furfural and AA. Conversely, increase in lime concentration did not determine consistently higher levels of any of the four inhibitors.

Table 1.2 - Sugars in pretreated liquid.

Pretreatment	**Glucose** (g L^{-1})		**Xylose** (g L^{-1})	
	monomer	oligomer	monomer	oligomer
1	0.5	3.1	1.1	27.3
2	0.4	1.4	3.9	15.9
3	0.8	1.4	5.1	10.9
4	*b.d.l.*	0.6	*b.d.l.*	6.8
5	*b.d.l.*	0.6	*b.d.l*	7.7
6	*b.d.l.*	0.6	*b.d.l.*	7.6
7	*b.d.l.*	0.9	*b.d.l.*	5.5
8	*b.d.l.*	0.7	*b.d.l.*	5.8
9	*b.d.l.*	0.8	*b.d.l.*	6.7
10	*b.d.l.*	1.0	*b.d.l.*	7.3
11	*b.d.l.*	0.8	*b.d.l.*	7.1
12	*b.d.l.*	0.8	*b.d.l.*	7.6
$LSD_{0.05}$	0.01	0.14	0.03	0.4

b.d.l. means below detection limit. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

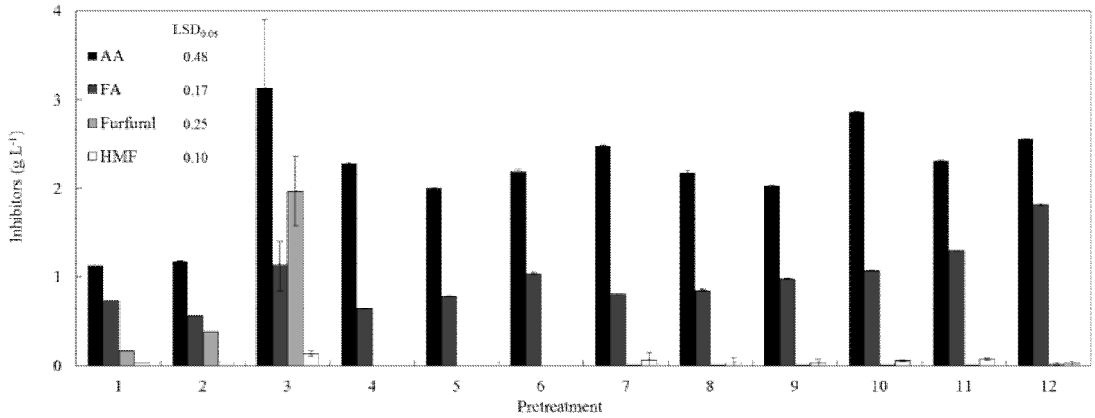

Figure 1.2 - Concentration of inhibitors in pretreatment liquid. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

1.3.2.2 Lignin removal

The amount of lignin removed from the raw material after pretreatment ranged between 17% (P3) and 38% (P7) (data not shown). Without lime (P1-3), a lignin removal of 21% was observed in the average, while low (P4-6) and high (P7-9) lime concentration increased lignin removal up to 25 and 33%, respectively. This is consistent with the effect expected from a stronger impregnation of the raw material with the catalyst. Conversely, increasing temperature during steam explosion did not enhance lignin removal, as the data of 26% obtained with 205 °C (P10-12) demonstrates (data not shown).

The overall effect of lime concentration, time and temperature, the latter two combined in the severity factor (eq. 1.4), is best depicted by the plot of lignin removal in response to concentration of $Ca(OH)_2$ and Log R_0 (Figure 1.3). The two factors concurred to delignification of the raw material in an opposite way. Hence, lignin removal as low as 10% is envisaged at no lime addition in combination with high severity, whereas a removal up to almost 40% is predicted with the opposite combination. This pattern is

consistent with the findings of Kim et al. (2006), showing that high temperature, leading to high severity, had no effect on lignin removal.

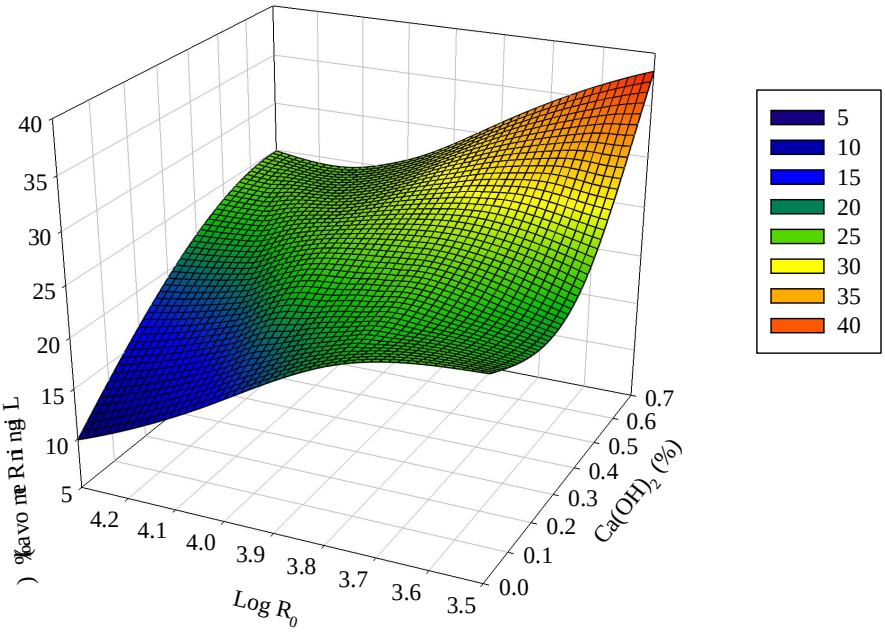

Figure 1.3 - Lignin removal in response to severity factor (Log R_0) and $Ca(OH)_2$ concentration during pretreatment.

1.3.2.3 Glucose and xylose yield

In all pretreatments, glucose from the cellulose fraction was mostly released after enzymatic hydrolysis (Figure 1.4.a). The highest yield of this sugar resulting from enzymatic hydrolysis, 82%, was obtained in pretreatment 7, which is consistent with the highest lignin removal shown by the same pretreatment: since lignin is a major hindrance to cellulose hydrolysis, its removal promotes the release of glucose during the enzymatic hydrolysis step (Chang et al., 2000; Fuentes et al., 2011). Lime substantially contributed to improving cellulose conversion into glucose during enzymatic hydrolysis: as a result, yields of 78 and 75% were evidenced for P3-6 and P7-9,

respectively, compared to 68% for P1-3. In contrast to lime, pretreatment time decreased glucose yield after enzymatic hydrolysis, although to a modest extent: in fact, a glucose yield of 72, 73 and 69% was evidenced with a respective 5, 10 and 15 minutes of residence time. Lastly, increasing temperature curbed the amount of glucose released in enzymatic hydrolysis: average glucose yields of 75 and 65% were shown by P7-9 and P10-12, respectively, which is in accordance with the higher concentration of inhibitors observed at 205 °C vs. 195 °C (Figure 1.2).

Concerning xylose from hemicellulose (Figure 1.4.b), the mildest pretreatment (P1) resulted in the highest yield of this sugar in the combined pretreatment and enzymatic hydrolysis (94%). Lime addition did not improve the overall yield, but the role of enzymatic hydrolysis and pretreatment diverged, reciprocally: in fact, decreasing pretreatment yields of P1-3, P4-6 and P7-9, (54, 21 and 16%, respectively) were counterbalanced by increasing enzymatic yields (23, 64 and 63% in the same respective cases). Conversely, pretreatment time affected enzymatic hydrolysis, decreasing the concentration of xylose detected in the broth to a greater extent than in glucose: 57, 50 and 43% xylose yield with 5, 10 and 15 minutes, respectively. This drop is consistent with the amount of degradation products from hemicellulose (i.e., furfural and FA) rising in time. Likewise, the increase of temperature determined a decrease in xylose yield after enzymatic hydrolysis as much as in xylose (63 and 53% for P7-9 and P10-12, respectively), which is still in good agreement with the high amount of degradation products found at higher temperature (Figure 1.2).

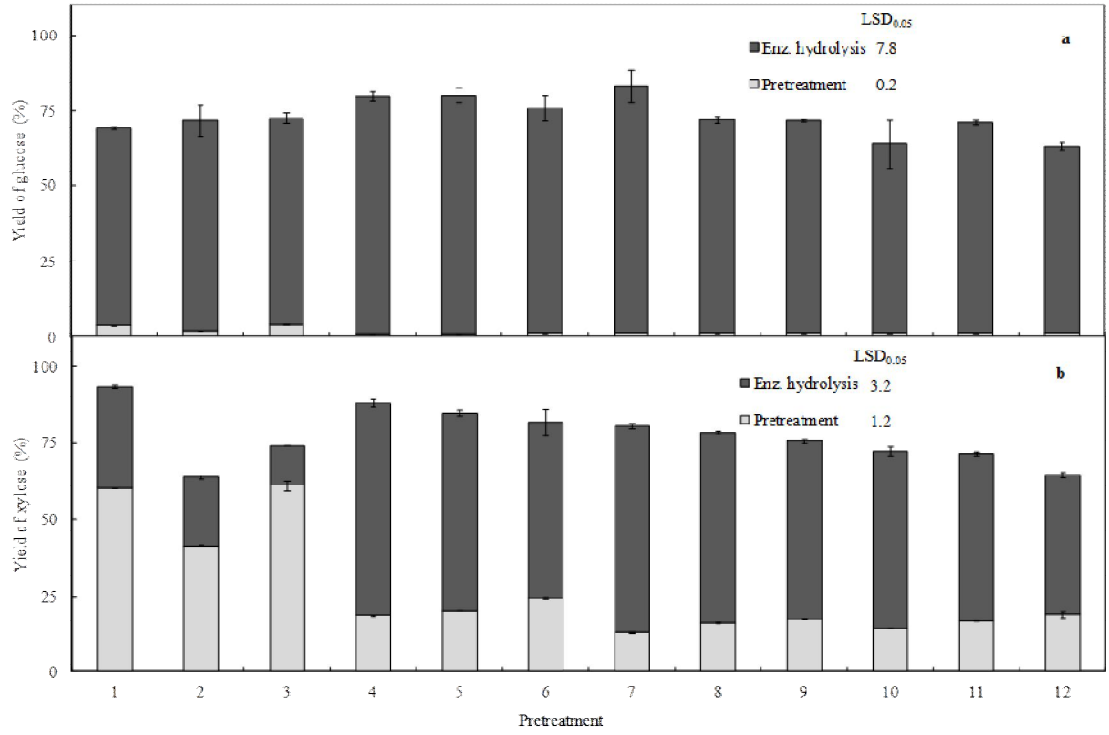

Figure 1.4.ab - Glucose (1.4.a) and xylose (1.4.b) yield in pretreated solid and liquid fraction following enzymatic hydrolysis, as respective percentage of total glucan and xylan content in the raw material. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

1.3.3 Simultaneous saccharification and fermentation

Figure 1.5 reports the results from the SSF runs on the solid material obtained from the three pretreatments resulting in top glucose yields with and without lime addition (P3, P5 and P7).

Differences among ethanol concentration produced at the end of SSF were relatively small, and their ANOVA was not significant: P3, P5 and P7 attained 29, 27.2 and 26.3 g L^{-1} of ethanol, respectively. The corresponding ethanol yields (eq. 1.3) were 60% for SSF performed on P5, while P3 and P7 achieved a lower, almost identical yield (53 and 52%, respectively).

However, these three values rank in the low range for this trait, which appears to be mainly due to a low yield in the fermentation phase, since no problem was noted in the

enzymatic hydrolysis. In fact, several parameters may negatively affect ethanol yield in SSF run at high WIS concentration (15%), as reported by Hoyer et al. (2013). In particular, the lack of improvement in ethanol yield between low (P5) and high (P7) lime addition may be due, beside high WIS concentration, to the high viscosity caused by $Ca(OH)_2$ supply passing the threshold for solubilisation in P7. This, in turn, hampered any yield increase (Palmqvist et al., 2012). Viscosity of the material used for SSF is a subject of growing concern (Palmqvist et al., 2012), especially in SSF conducted at high WIS concentration. Intrinsically, this is critical condition, as several parameters contribute to increase viscosity in the fermenter and consequently decrease ethanol yield. Washing the material prior to SSF could be used to secure high ethanol yield (Lu et al., 2010), although this involves a higher use of water, reflecting on the amount of downstream waste to handle. A more feasible way to achieve this goal could be extending pre-hydrolysis time, as shown by Hoyer et al. (2013).

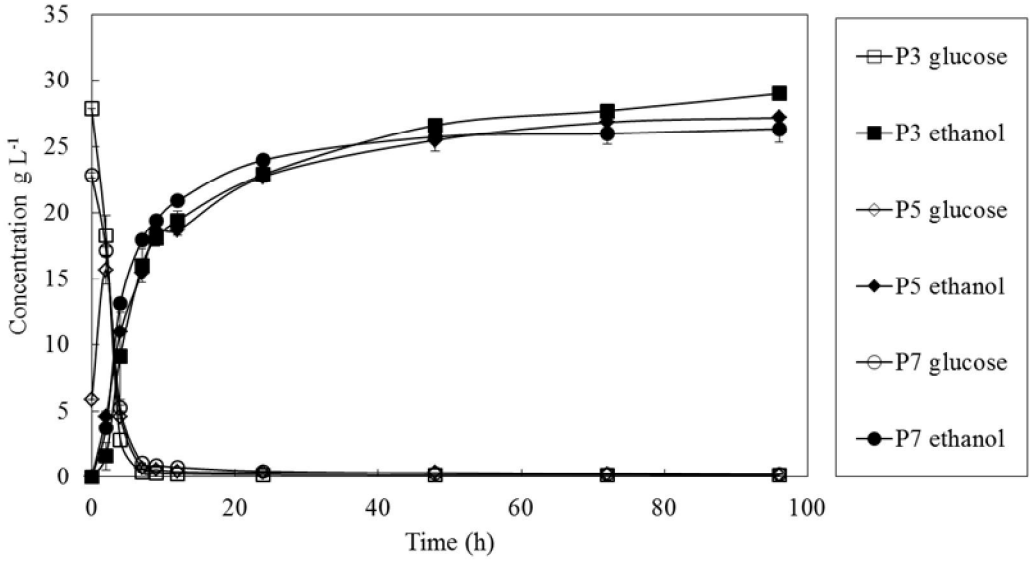

Figure 1.5 - Concentration of glucose (empty symbols) and ethanol (filled symbols) during the simultaneous saccharification and fermentation carried out on the slurries of three selected pretreatments. Error bars show ±SD.

1.3.4 Anaerobic digestion

Table 1.4 reports TOC, TS and VS contents of the three pretreatment liquids before AD, and methane yield at the end of AD. Liquid from P3 yielded the highest methane potential (276 NmL g^{-1} VS), followed by P5 (237 NmL g^{-1} VS) and, finally, P7 (169 NmL g^{-1} VS). Differences in the amount of methane produced by the three pretreatments are consistent with the TOC content of each liquid. In turn, this is consistent with the analysis of sugars in pretreatment liquid, showing a higher amount of glucose and xylose in P3 compared to P5 and P7 (Table 1.3). Moreover, the lower methane production of P5 and P7 compared to P3 may also be due to the higher amount of lignin degradation products in P5 and P7, hampering anaerobic digestion (Klinke et al., 2004).

Table 1.4 - Characteristics of selected pretreatment liquids, and methane yield after anaerobic digestion.

Pretreatment	**TOC** (g L^{-1})	**TS** (%)	**VS** (% TS)	**CH_4** (NmL g^{-1} VS)
3	16.4 a	2.6 a	84.4 a	276 a
5	9.5 b	2.1 b	70.6 b	237 ab
7	9.3 b	2.7 a	82.4 a	169 b

TOC, total organic carbon; TS, total solids; VS, volatile solids. Letters (a, b, etc.) indicate significantly different means according to the LSD test ($P \leq 0.05$).

1.3.5 Combined energy yield

Figure 1.6 summarises the energy yield as ethanol and methane, plus the energy content of residual solid after SSF. The highest yield was obtained by P7 (9.3 kJ g^{-1} TS), followed by P3 and P5 almost at par (8.9 and 8.8 kJ g^{-1} TS, respectively). This overall

energy output was in the range of other studies conducted on agricultural lignocellulosic residues (i.e., corn stover and oat straw) (Dererie et al., 2011; Bondesson et al., 2013). More in detail, lime addition enhanced the ethanol yield by a respective 8 and 18% for 0.4 and 0.7 % $Ca(OH)_2$ (P5 and P7), compared to the autohydrolysis (P3) (3.7, 4.0 and 4.4 kJ g^{-1} TS in P3, P5 and P7, respectively). Conversely, in steam alone (P3) methane from the liquid was 22 and 33% higher, respectively, than in P5 and P7 (1.4, 1.2 and 1.1 kJ g^{-1} TS in the three respective pretreatments). Lastly, at the end of the process the solid residue contained almost the same amount of energy (non-significant ANOVA), ranging between 3.6 kJ g^{-1} TS (P5) and 3.8 kJ g^{-1} TS (P3 and P7). Therefore, ethanol was shown to be the main component of the total energy output from SB (average, 45%), while methane was the minor component (average, 14%).

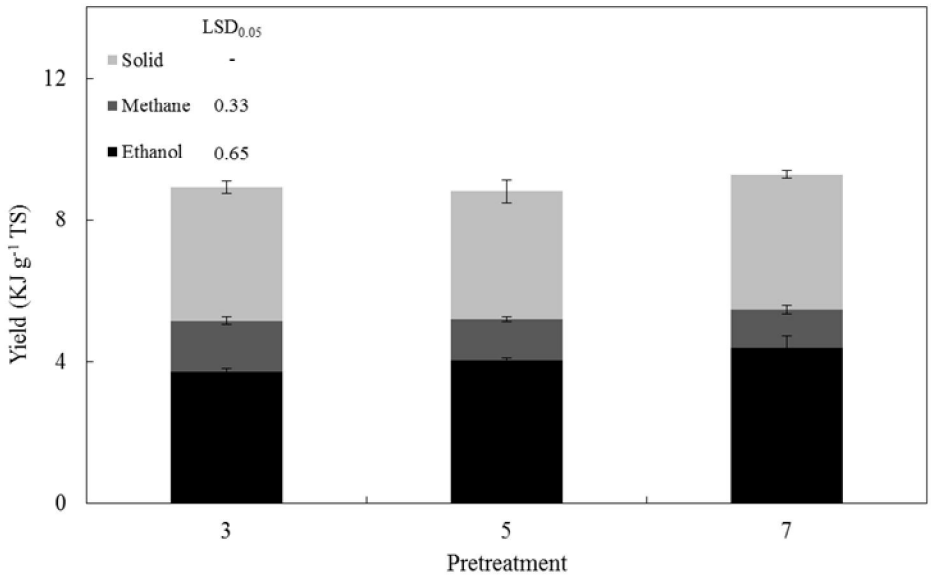

Figure 1.6 - Combined energy yield per unit dry weight of the raw material, in terms of ethanol from simultaneous saccharification and fermentation (SSF), methane from anaerobic digestion (AD), and energy content of the residual solid. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

1.4 Conclusions

Lime was proven to be a suitable catalyst for sugarcane bagasse before steam explosion, in order to enhance fibre deconstruction. After pretreatment and enzymatic hydrolysis, lime at high concentration (P7) exhibited the best yield of glucose. In turn, this determined the highest ethanol yield from SSF of the solid fraction. Conversely, steam explosion with no lime addition (P3) was found to be more suitable for methane production. When the total amount of energy produced under various forms was focused (ethanol, methane and the energy content of solid residue), P7 attained top level, followed by P3 and P5 (lime at low concentration). Beside its efficacy in improving energy output, lime owns a major advantage consisting in the possibility to be removed from the downstream effluent through carbonation. Conversely, the high viscosity of the fermentation broth, determined by catalyst mass added to SSF at high solid concentration, remains a point of concern for future research work. On concluding, in a biorefinery prospect lime represents a favourable option to improve ethanol yield from sugarcane bagasse.

CHAPTER 2

Combined ethanol and methane production from switchgrass (*Panicum virgatum* L.) impregnated with lime prior to steam explosion

Abstract

Pretreatments are crucial to achieve efficient conversion of lignocellulosic biomass to soluble sugars. In this light, switchgrass was subjected to 13 pretreatments including steam explosion alone (195 °C for 5, 10 and 15 minutes) and after impregnation with the following catalysts: $Ca(OH)_2$ at low (0.4%) and high (0.7%) concentration; $Ca(OH)_2$ at high concentration and higher temperature (205 °C for 5, 10 and 15 minutes); H_2SO_4 (0.2% at 195 °C for 10 minutes) as reference acid catalyst before steam explosion. Enzymatic hydrolysis was carried out to assess pretreatment efficiency in both solid and liquid fraction. Thereafter, in selected pretreatments the solid fraction was subjected to simultaneous saccharification and fermentation (SSF), while the liquid fraction underwent anaerobic digestion (AD) to produce additional energy as methane. Lignin removal was lowest (12%) and highest (35%) with steam alone and 0.7% lime impregnation, respectively. In general, higher cellulose degradation and lower hemicellulose hydrolysis were observed in this study compared to others, depending on lower biomass hydration during steam explosion. Mild lime addition (0.4% at 195 °C) enhanced ethanol in SSF (+28% than steam alone), while H_2SO_4 boosted methane in AD (+110%). However, methane represented a lesser component in combined energy yield. Mild lime addition was also shown less aggressive and secured more residual solid after SSF, resulting in higher energy yield per unit raw biomass. Decreased water consumption, avoidance of toxic compounds in downstream effluents, and post process recovery of $Ca(OH)_2$ as $CaCO_3$ represent further advantages of pretreatments involving mild lime addition before steam explosion.

2.1 Introduction

The depletion of oil reserves and the effects of fossil energy on the global climate provide a strong incentive to search for alternative energy sources. Especially the transport sector relies on oil derived products: to alleviate this dependence, bioethanol from lignocellulosic biomass could represent a valuable substitute for gasoline (Hahn-Gerdal et al., 2006).

Among grasses for energy uses, perennial species are preferred over annual ones for their ability to combine high biomass yields with low energy and financial costs (Boehenel et al., 2008; Fazio and Barbanti, 2014). Moreover, perennial grasses deploy a vast range of positive externalities from the environmental viewpoint: increased soil carbon sequestration and reduced nitrate leaching (Boehemel et al., 2008; Tilman et al., 2009; Smith et al., 2013; Cattaneo et al., 2014a); improved soil biological quality, and establishment of beneficial interactions with soil organisms (Cattaneo et al., 2014b). Among perennial species, switchgrass (*Panicum virgatum* L.) is a C_4 grass that has demonstrated high productivity in a wide geographical range, suitability for marginal land (Varvel et al., 2008), low water and nutrient requirement, beside environmental benefits (McLaughin et al., 1998; Monti et al., 2012). Switchgrass is propagated by seed, thus the cost of establishment is lower than other perennial species as Miscanthus (*Miscanthus* × *giganteus*) and Giant Reed (*Arundo donax*), which are sterile and need to be propagated through vegetative organs.

Switchgrass is a promising feedstock for the production of second generation bioethanol (Keshwani et al., 2009), which is considered a more sustainable form of energy, as it does not directly affect the food commodity market (Naik et al., 2010). However, second generation bioethanol involves that lignocellulosic biomass be subjected to various kinds of pretreatments for efficient fermentation (Galbe and Zacchi, 2007).

Among them, steam pretreatment is one of the most frequently used (Toor et al., 2013), often in combination with an acid catalyst (Martin et al., 2002; Sassner et al., 2008; Carrasco et al., 2010). Biomass impregnation with acid catalyst prior to steam explosion has often demonstrated higher pretreatment efficiency than steam explosion alone (also called autohydrolysis) (Bondesson et al., 2013). Sulphuric acid and sulphur dioxide have been tested as acid catalysts, using variable concentrations, temperatures and residence times. However, sulphuric compounds involve serious drawbacks such as acid corrosion of equipment and the need to implement extensive processing of downstream effluents, resulting in high water consumption (Ramos et al., 2003). Thus, pretreatments without sulphur would be preferable, if they can bridge the yield gap with sulphur-based processing.

In a biorefinery concept, the choice of catalyst is not only important for its ability to increase the yield in final product, but also for catalyst fate (Thomsen et al., 2005). In this sense, the use of a chemical that increases the yield in second-generation bioethanol, and whose by-products have a market value, is preferable. Compared to acids, alkalis remove lignin, the only fraction of biomass that is not converted into bioethanol (Fuentes et al., 2011), blocking enzymes activity by restricting access to the cellulose fraction (Kim et al., 2006). This, in turn, reflects in a lower ethanol yield (Chang et al., 2001). Alkaline catalysts as lime ($Ca(OH)_2$) have actually been shown to reduce the lignin content of herbaceous biomass as switchgrass (Chang et al., 2004). Lime can be easily removed from the water used for impregnation before steam explosion, by carbonating with CO_2. The resulting $CaCO_3$ may be recovered to be used in several applications, such as the mitigation of drought stress in tomato cultivation (Patanè et al., 2012). Hence, it can be considered a secondary product showing no

negative impact. Moreover, calcium hydroxide, compared to sodium hydroxide, is safer to handle and has a lower cost (Wyman et al., 2005).

Saccharomyces cerevisiae is the microorganism most commonly used for the fermentation of hexoses, although it cannot ferment pentoses that are also contained in switchgrass. A process configuration aimed for pentose utilization is of paramount importance to increase the overall energy yield and maximize the economic value of biomass. Among several alternatives, methane production through anaerobic digestion (AD) appears the most feasible method for utilizing residual energy content in a raw material (Bondesson et al., 2014). This is based on the fact that none of the microorganisms assayed so far on pentoses (Ahring et al., 1999; Nigam et al., 2001; Jin et al., 2004; Ruohonen et al., 2006; Chu et al., 2007; Georgieva et al., 2007; Bondesson et al., 2014) has proved as efficient as *S. cerevisiae* in fermenting hexoses, although improvements are envisaged (Bondesson et al., 2014). Often, pentose fermenting microorganisms (e.g. *Escherichia coli*, *Pichia stipitis* and *Candida shehatae*) exhibit low ethanol yield and tolerance to increasing alcohol concentration and high sensitivity to inhibitors in the hydrolisate after the pretreatment step (Torry-Smith et al., 2003).

Given these premises, the aim of this work was to investigate the influence of lime impregnation before steam explosion, on ethanol and methane production from switchgrass. As reference practice, steam explosion after impregnation with sulphuric acid was also included. The time, temperature and lime concentration during pretreatment were varied and the sugar yield determined in each case. Pretreated solid fraction was subjected to simultaneous saccharification and fermentation (SSF) at high solids loading for ethanol production. Pretreatment liquid was subjected to AD for methane production.

2.2 Material and Methods

2.2.1 Origin of the raw material

Switchgrass (*Panicum virgatum* L.) was used as substrate in this study. The lowland cultivar ”Alamo” had been seeded in 2002 at the experimental farm, University of Bologna, Cadriano (BO), Italy (44° 33’ N, 11° 21’ E, 32 m above sea level), on a deep alluvial soil with a clayey-loamy texture. The area features a mean annual temperature of 13.3 °C and precipitation of 700 mm, which are typical of the Mediterranean North environmental zone (Metzger et al., 2005). This is a zone with mild winter and long growing season, although precipitation is mostly concentrated in the cold semester. Switchgrass was still in full production in 2011. In that year crop management consisted of nitrogen fertilization in the spring time (120 kg N ha^{-1} as urea), no irrigation and no need of weed, pest and disease control. At the end of the growing season (October 5, 2011), switchgrass was harvested as a whole plant at seed-ripening stage and chopped in ca. 20 mm pieces. Biomass samples were oven dried (40 °C) and ground to a particle size of 0.5 mm for the analysis of structural carbohydrates, lignin (Klason lignin and acid soluble lignin), extractives, and ash (Sluiter et al., 2005; Sluiter et al., 2008a).

2.2.2 Process configuration

After 1 hour of impregnation with water alone, or with alkaline ($Ca(OH)_2$) or acid solution (H_2SO_4), switchgrass was subjected to steam explosion under different conditions of time and temperature, making up a total of 13 combinations (Table 2.1). Pretreated samples were separated into solid (slurry) and liquid fraction. The former was repeatedly washed with distilled water and subjected to enzymatic hydrolysis. Both fractions were analysed for glucose and xylose. Enzymatic hydrolysis and the subsequent analysis served to identify pretreatments that resulted in the highest glucose

yields, to be selected for SSF. The corresponding liquid fractions were selected for AD. Figure 2.1 describes process configuration from raw biomass to final energy products, including the implementation of enzymatic hydrolysis to test pretreatment efficiency.

Table 2.1 - Experimental conditions and associated severity factor (Log R_0) in switchgrass pretreatment.

Pretreatment	**$Ca(OH)_2$** (% w/w)	**H_2SO_4** (% w/w)	**Time** (min)	**Temperature** (°C)	**Log R_0**
1	-	-	5	195	3.5
2	-	-	10	195	3.8
3	-	-	15	195	4.0
4	0.4	-	5	195	3.5
5	0.4	-	10	195	3.8
6	0.4	-	15	195	4.0
7	0.7	-	5	195	3.5
8	0.7	-	10	195	3.8
9	0.7	-	15	195	4.0
10	0.7	-	5	205	3.8
11	0.7	-	10	205	4.1
12	0.7	-	15	205	4.3
13	-	0.2	10	195	3.8

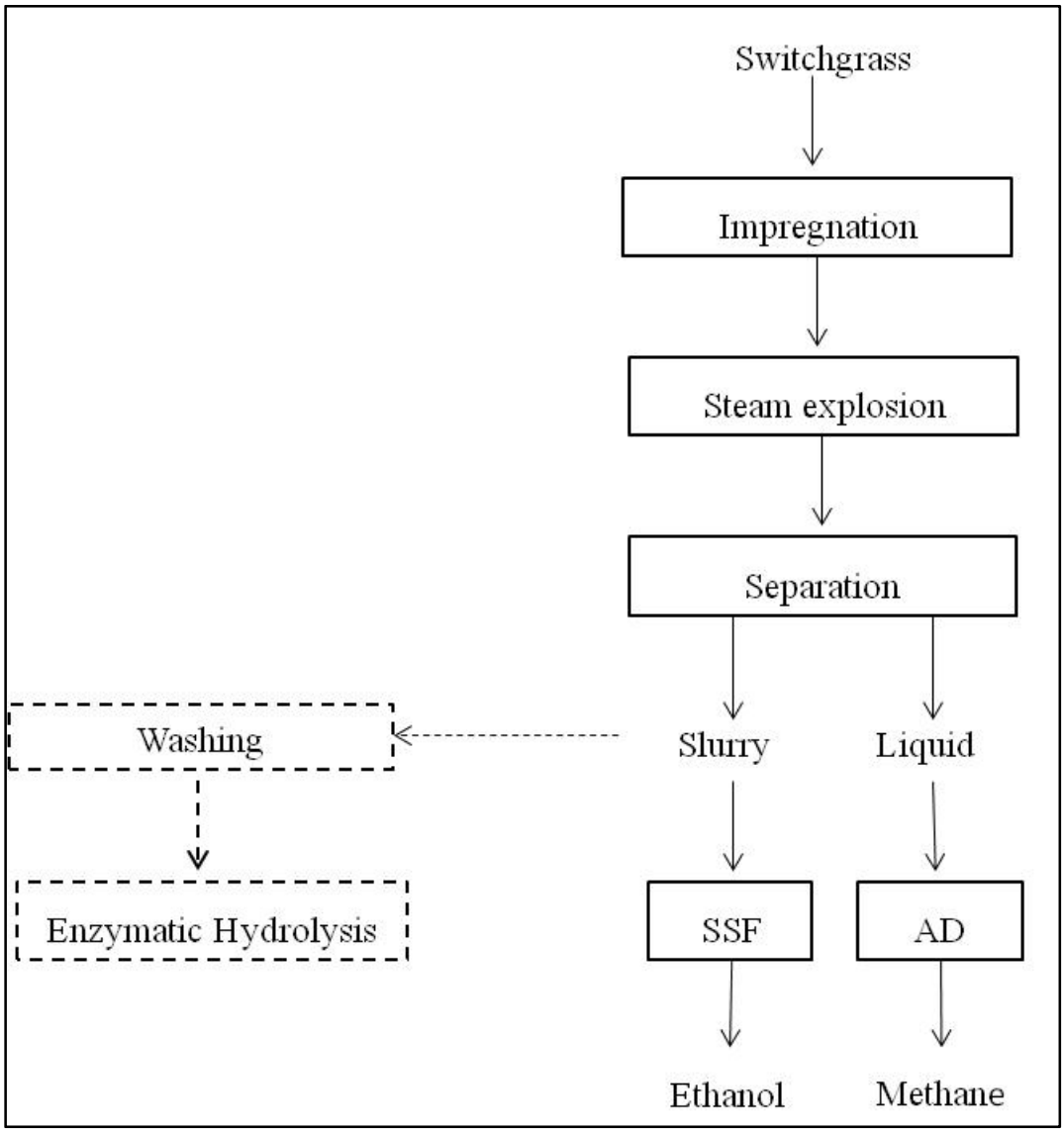

Figure 2.1 - Process configuration from the raw material to final ethanol and methane. Dashed graphics indicate the assessments carried out to select pretreated slurries for simultaneous saccharification and fermentation (SSF), and the corresponding liquids for anaerobic digestion (AD).

2.2.3 Pretreatments

The 13 pretreatments assessed in the experiment (Table 2.1) can be divided into three main groups: autohydrolysis, consisting of steam alone at increasing time (P1-P3); alkaline pretreatment, consisting of nine combinations of steam (195 and 205 °C) and lime ($Ca(OH)_2$ at 0.4 or 0.7% w/w) for 5, 10 and 15 min (P4-P12); acid pretreatment

(P13) using sulphuric acid (0.2% w/w) as a reference, since H_2SO_4 is the catalyst most frequently used in steam pretreatment.

Using steam alone, the raw material (20 mm air-dried samples) was previously immersed in water for 1 hour at a 20:1 water to dry weight ratio. When adding calcium hydroxide, the raw material was impregnated in an aqueous solution containing 0.4% or 0.7% $Ca(OH)_2$ at a 20:1 water to dry weight ratio, and stored in a sealed bucket for 1 hour. For sulphuric acid, the same procedure was followed, using a 0.2% concentration of H_2SO_4 with the same 20:1 water to dry weight ratio. In all pretreatments, after 1 hour of impregnation, switchgrass was dewatered in order to remove the excess solution using a 3 L capacity press (Tinkturenpressen HP5M, Fischer Maschinenfabrik GMBH, Germany), reaching a dry matter content between 50 and 60%.

Following this step, steam explosion was performed in a reactor of 10 L capacity, loaded with an amount of impregnated switchgrass corresponding to 400 g dry matter. Steam temperature and residence time were set according to each specific pretreatment (Table 2.1). The reactor was connected to a computer controlling process parameters and the discharge of pretreated material into a downstream vessel.

Discharged material was then divided into two fractions: pretreatment liquid resulting from filtration through a 2.5 μm sieve, and a residual solid (slurry). The slurry was analysed for structural carbohydrates and lignin using the aforementioned methods, while pretreatment liquid was analysed for the content of total sugars (glucose, xylose and arabinose), their monomeric fractions and, by difference, the oligomeric fractions, and some inhibitors (acetic acid, formic acid, furfural and 5-hydroxymethylfurfural (HMF)), according to a U.S. National Renewable Energy Laboratory (NREL) procedure (Sluiter et al., 2008b). In the slurry, the content of water-insoluble solids (WIS) was also

determined using the method developed by Weiss et al. (2010). All the chemical and physical traits were analysed in duplicates.

2.2.4 Enzymatic hydrolysis

The slurries from the 13 pretreatments were repeatedly washed with distilled water to remove pretreatment liquid, and were subjected to enzymatic hydrolysis (Figure 2.1) at a loading of 5% WIS. Hydrolysis was carried out in plastic tubes containing two 50 mL steel balls to improve mixing in a rotating incubator at 100 rpm. The enzyme, CelliCTec3 (Novozymes, Bagsvaerd, Denmark), was added at an amount corresponding to 10 FPU g^{-1} WIS. Enzyme activity was measured according to Adney and Baker (Adney and Baker, 2008). Sodium acetate was used as buffer adjusted at pH 5. Hydrolysis was allowed to continue for 48 h at 50 °C. The pH was set manually at 5 with 10% sodium hydroxide. Following enzymatic hydrolysis, the concentrations of glucose and xylose were determined in the slurry. All tests were conducted in duplicates.

2.2.5 Simultaneous saccharification and fermentation

Slurries showing the highest glucose yields during enzymatic hydrolysis, were chosen for SSF. Slurries were pressed to reach a 15% WIS content with the same procedure described previously. SSF was performed in 2 L fermenters (Infors AG, Bottmingen, Switzerland) previously sterilized at 121 °C for 20 minutes, using 650 g of unwashed material at 15% WIS. The pH was adjusted at 5 with 10% NaOH. Temperature in the fermenter was set at 45 °C, Cellic CTec3 (Novozymes, Bagsvaerd, Denmark) enzyme was added at 10 FPU g^{-1} WIS, and temperature was maintained at 45 °C for 20 hours as a pre-hydrolysis step. Thereafter, the mixture was cooled to 35 °C and added with 3 g L^{-1} of *S. cerevisiae* Ethanol Red (Lesaffre, Marq-en-Barceul, Roubaix, France) yeast, and

0.5 g L^{-1} NH_4PO_4 as nutrient source. SSF was performed at 35 °C for 96 hours. Samples were taken after 2, 4, 7, 10, 12, 24, 48, 72 and 96 hours, and analysed by HPLC for ethanol, monomeric sugars, acetic acid, formic acid, and sugar degradation products. All SSFs and analyses were performed in duplicates.

2.2.6 Anaerobic digestion

AD was performed using the method described by Hansen et al. (2004), to determine potential methane yield in the four pretreatment liquids corresponding to the slurries chosen for SSF. Prior to AD, the total organic carbon (TOC) content was determined in pretreatment liquids by a total carbon analyser (Shimadzu, TOC-5050A) with an auto-sampler (ASI-5000A). The carrier gas flow was set to 150 ml min^{-1} and the working temperature was 680 °C. In parallel to this, total solids (TS) were determined drying the samples at 105 °C for 24 hours, and volatile solids (VS) were determined by ashing the dried samples at 550 °C for 2 hours. All analyses were conducted in duplicates.

Inoculum (active sludge) from an anaerobic digester was collected from a municipal water-treatment plant (Källbyverket, Lund, Sweden), and was maintained in mesophilic conditions (35 °C in the dark with repeated manual stirring) until the end of biogas emission. TS and VS content of the starved inoculum was determined with the same procedure used for pretreatment liquid. Thereafter, inoculum and pretreatment liquid were mixed in a 2:1 (VS/VS) ratio, to give a total 500 g broth in bottles of 1 L volume, kept in an incubator at 37 °C for 10 days. Anaerobic digestion was monitored using the system Yieldmaster (BlueSens ®, Herten, Germany): biogas volume was measured with precision mass flow meters (Ritter MilliGascounter®, Bochum, Germany); methane concentration with an infrared (IR) sensor, and the data were collected via BACCom units to BACVis software (BlueSens ®, Herten, Germany).

2.2.7 Analytical determinations

Sugars from structural carbohydrates in the raw material, and from slurry and pretreatment liquid were determined by HPLC equipped with a refractive index detector. Glucose, xylose, arabinose, galactose and mannose were separated using an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) at 85 °C with a flow rate 0.5 ml min^{-1} using water as eluent.

Ethanol, acetic acid, formic acid, furfural and HMF in pretreatment liquid were determined by HPLC with a refractive index detector, using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operating at 50 °C with a flow rate of 0.5 ml min^{-1}, using 5 mmol l^{-1} sulphuric acid as eluent. All samples had been filtered through a filter of pore diameter 0.2 µm before analysis.

2.2.8 Calculations and statistical analysis

Lignin removal was calculated as proposed by Kim et al. (2006):

$$Lignin\,removal = 1 - W_L \qquad (2.1)$$

where W_L is the fraction of residual lignin expressed as follows:

$$W_L = \frac{L \cdot Y_T}{L_0} \qquad (2.2)$$

where L is the amount of Klason lignin in the pretreated material (g), Y_T the yield of total solids (%) determined after pretreatment, and L_0 the amount of Klason lignin in the raw material (g).

Sugar yields were calculated as percent sugar recovered after pretreatment, on the raw material basis. Specifically, glucose and xylose yields were calculated by dividing the total of each sugar, determined in pretreatment liquid and washed slurry after enzymatic hydrolysis, by the total amount contained in the raw material. For each sugar, the former proportion represents pretreatment yield, while the latter is enzymatic hydrolysis yield.

Ethanol yield was calculated using the measured amounts of glucose and ethanol in the fermentation broth at the end of SSF, by the following formula:

$$Y_{\mathrm{EtOH}} = \frac{C_{EtOH}(1-WIS_{end})\cdot\frac{M}{1000}}{0.51\cdot\left[WIS\cdot M\cdot\sigma_{glc}+V_{hyd}\cdot c_{glc}\right]} \qquad (2.3)$$

where Y_{ETOH} is the overall ethanol yield resulting from SSF (% of theoretical value); C_{ETOH} is the final concentration of ethanol (g L^{-1}); M is the total mass (g); WIS and WIS_{end} are the fractions of water insoluble solids (%) calculated at the beginning and the end of SSF, respectively; σ_{glc} is the mass fraction of glucose in pretreated fibres (g g^{-1}); V_{hyd} is the starting volume in the reactor (L); c_{glc} is the concentration of glucose at the start of SSF (g L^{-1}).

To better evaluate the effectiveness of $Ca(OH)_2$ as catalyst, a response surface analysis was carried out with the SigmaPlot 10 software (Systat Software Inc.,Chicago, IL, USA), using $Ca(OH)_2$ concentration and a severity factor (Log R_0) that combines residence time and temperature, to identify optimal conditions for lignin removal. The severity factor (Overend et al., 1987) was calculated as follows:

$$\mathrm{Log}\, R_0 = Log\left(t\cdot exp^{\left(\frac{T-Tref}{14.75}\right)}\right) \qquad (2.4)$$

where t is the residence time (min), T pretreatment temperature (°C), and $Tref$ the reference temperature (100 °C).

The combined energy yield, i.e. ethanol from SSF, methane from AD and the amount of energy in the residual solid after SSF, was calculated per unit dry weight of the raw material, assuming 27.1, 50 and 17.4 kJ g^{-1} energy content for ethanol, methane and solid residue, respectively (Mc Laughin et al., 1996; Bondesson et al., 2013).

In all traits, normal distribution and equal variance of data were controlled through the Kolmogorov–Smirnov and Bartlett tests, respectively. Data were then submitted to one way analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, CA, USA). The lowest significant difference (LSD) test at $P \leq 0.05$ was used to separate means of significant traits.

2.3 Results and Discussion

2.3.1 Raw material composition

On a dry weight basis, switchgrass biomass consisted of 16.0 ± 0.1% extractives, 30.3 ± 0.3% glucan, 29.0 ± 0.7% xylan, 4.2 ± 0.3% arabinan, 16.4 ± 1.3% lignin, and 2.5 ± 0.5% ash. These data are in the range of other analysis carried out on switchgrass (Suryawati et al., 2009; Isic et al., 2008). However, a wide analytical range was also observed in other works, concerning extractives and ashes (Ramos et al., 2003; Alizadeh et al., 2005; Keshwani et al., 2009).

2.3.2 Pretreatment evaluation

2.3.2.1 Sugars and inhibitors in pretreatment liquid

Figure 2.2 shows the concentration of inhibitors in pretreatment liquid: acetic acid and furfural are pentose degradation products, while formic acid and HMF are hexose degradation products. Formic acid exerts a stronger inhibition on *S. cerevisiae* (Jönsson

et al., 2013) than acetic acid. Both acids were below the thresholds of cell death of the yeast, although concentrations above the thresholds of inhibition (6 and 4.6 g L^{-1} for acetic and formic acid, respectively) (Larsson et al., 1999) were detected in the two pretreatments conducted at high temperature (205 °C) and lime concentration (0.7%) for 10 and 15 min (P11-12). In general, even a low addition of $Ca(OH)_2$ (0.4%) to steam explosion enhanced the content of acetic and formic acid: 5.2 and 1.5 g L^{-1} (average of P4-6) vs. 2.4 and 0.6 g L^{-1} (average of P1-3), respectively. Lime concentration and temperature further augmented the level of the two respective compounds: 5.6 and 2.5 g L^{-1} (average of P7-9); 6.2 and 4.0 g L^{-1} (average of P10-12). Lastly, residence time only enhanced formic acid when passing from 5-10 min (average, 1.5 g L^{-1}) to 15 min (3.1 g L^{-1}).

In contrast to this, furfural and HMF contents were significantly increased only by acid addition to steam explosion (H_2SO_4 at 0.2%). Even so, HMF remained quite low (0.7 g L^{-1}), whereas furfural attained a level of concern (2.3 g L^{-1}), given the fact that concentrations so low as 1-5 g L^{-1} are acknowledged to affect fermentation, although final ethanol yield is generally uninfluenced (Boyer et al., 1992).

Glucose and xylose concentration in the liquid varied with pretreatment conditions (Table 2). Glucose in its monomeric form was above detection limit only in the autohydrolysis (P1-3), and with H_2SO_4 addition (P13). This is in accordance with Balan et al. (2012). Glucose oligomers were always found in amounts higher than the corresponding monomer (Table 2.2).

Monomeric glucose concentrations with lime addition correspond to pretreatment yields (ca. 0.1%) very similar to that calculated from data of Wyman et al. (Wyman et al., 2011) with the same catalyst. Conversely, our data with sulphuric acid corresponds to

half the yield observed in the cited work (Wyman et al., 2011) (3.8 vs 7.4%) with the same catalyst.

Monomeric xylose depicted a similar behaviour as glucose (Table 2.2): detectable amounts were only shown using autohydrolysis, or especially, after acid addition. Regarding oligomeric xylose, the highest values were found using autohydrolysis (average, 19.6 g L^{-1}). Conversely, low levels were noticed with alkaline pretreatment under mild conditions (195 °C for 5 minutes) (P4 and P7), and in acid addition (P13). This effect of autohydrolysis and lime in enhancing the oligomeric vs monomeric fraction of xylose is consistent with the findings of Wyman et al. (2011).

Similar pretreatment effects were observed by Kim et al. (2011): high hemicellulose removal with autohydrolysis and mild acid catalysis, and strong retention (≥ 85%) of initial cellulose in the solid phase. However, in the cited study (Kim et al., 2011) a higher concentration of lime was used (1 g g^{-1} of biomass), in association with lower temperature (120 °C), longer retention time (4 hours), and higher water to solid ratio. Especially this last condition is detrimental in a perspective of full scale operation, hampering lime recovery at the end of the process. Lime recovery is an important step to reduce process environmental impact, thus this study tries to track a pretreatment route complying with this issue.

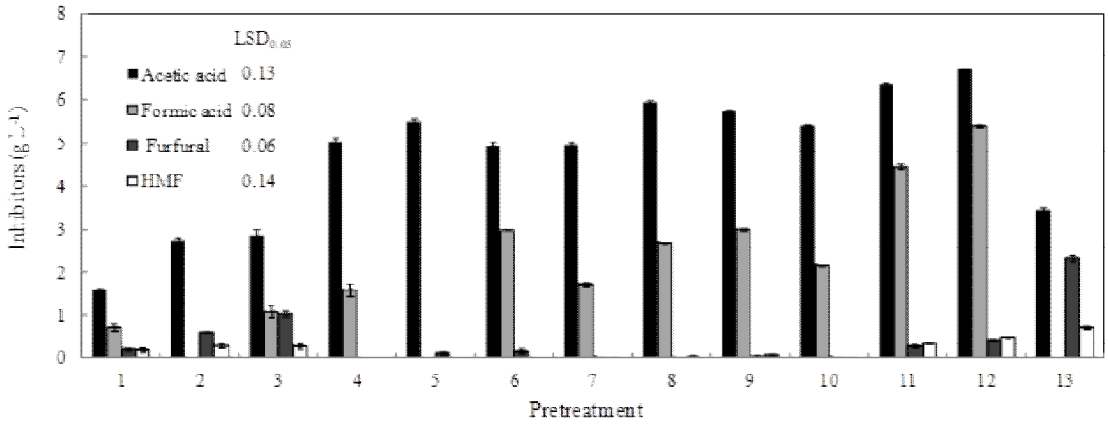

Figure 2.2 - Concentration of inhibitors in pretreatment liquid. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

Table 2.2 - Sugars in pretreatment liquid.

Pretreatment	Glucose ($g\ L^{-1}$)		Xylose ($g\ L^{-1}$)	
	monomer	oligomer	monomer	oligomer
1	1.9	3.2	1.1	17.1
2	1.8	3.2	3.9	25.4
3	1.5	2.1	5.1	17.7
4	*b.d.l.*	2.3	0.2	6.7
5	*b.d.l.*	3.9	0.3	16.7
6	*b.d.l.*	3.7	*b.d.l.*	15.6
7	*b.d.l.*	3.1	*b.d.l.*	12.4
8	*b.d.l.*	4.6	*b.d.l.*	20.1
9	*b.d.l.*	4.3	*b.d.l.*	19.1
10	*b.d.l.*	3.9	*b.d.l.*	18.8
11	*b.d.l.*	4.4	*b.d.l.*	18.2
12	*b.d.l.*	4.2	*b.d.l.*	14.0
13	4.3	2.5	16.0	13.8
$LSD_{0.05}$	0.02	0.16	0.09	0.81

b.d.l. means below detection limit. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

2.3.2.2 Lignin removal

The amount of lignin removed from the raw material after pretreatment ranged between 9 and 38% (data not shown). Steam alone was least effective in removing lignin (average of P1-3, 11.5%). Supplying $Ca(OH)_2$ at low concentration (0.4%) and standard temperature (195 °C), lignin removal increased to an average 18.1%. At high

concentration (0.7%), the best delignification was achieved (34.6%). At high concentration and temperature (205 °C), almost the same result was obtained (30.9%). Compared to this, supplying H_2SO_4 determined a modest lignin removal (14.6%).

The overall effect of lime concentration, time and temperature, the latter two combined in the severity factor (eq. 2.4), is best depicted by the plot of lignin removal in response to $Ca(OH)_2$ and Log R_0 (Figure 2.3). It is perceived that the alkaline catalyst played a stronger role in lignin removal, than the increase in severity. Based on this, the highest delignification occurred at high lime concentration, in combination with a moderate severity. Garlock et al. (2011) observed a similar pattern of lignin removal in switchgrass upon the effect of multiple pretreatments. However, they obtained a stronger lignin removal (50% vs 33% in this study) with higher lime addition (1 g $Ca(OH)_2$ g^{-1} vs 0.125 g g^{-1} in this study) and water to solid ratio (16:1 vs 1:1), although less severe conditions were adopted (Log R_0 3.0 vs 3.9). Thus, it appears that the two former factors played a major role in enhancing switchgrass delignification. However, the cost and the burden associated with higher catalyst dosage and more diluted pretreatment should be accounted for, in the perspective of full scale operation.

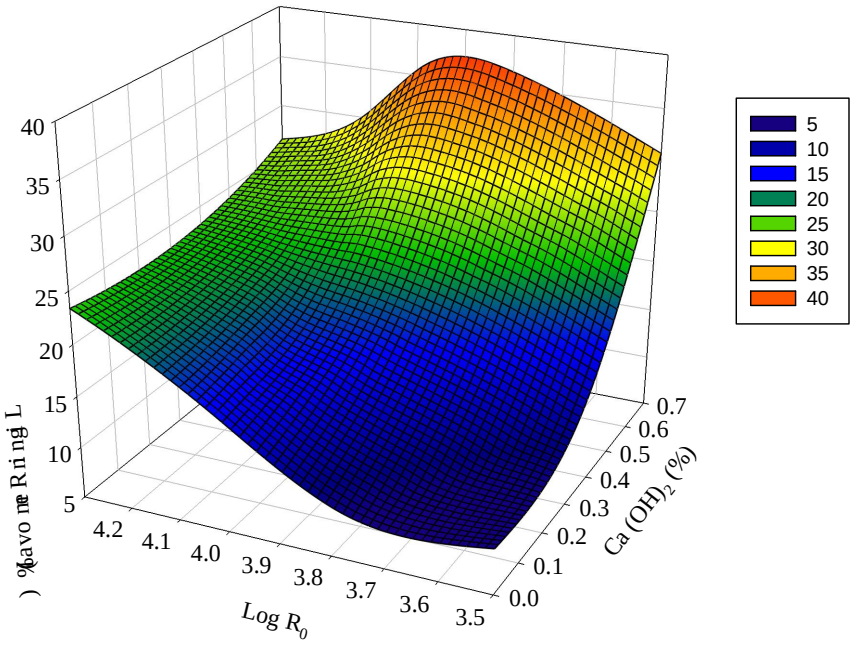

Figure 2.3 - Lignin removal in response to severity factor (Log R_0) and $Ca(OH)_2$ concentration during pretreatment.

2.3.2.3 Glucose and xylose yield

Glucose and xylose yields in the slurry after enzymatic hydrolysis and in pretreatment liquid exhibit a contrasting picture between the two sugars (Figures 2.4 and 2.5), as observed in other studies on switchgrass (Larsson et al., 1999; Alizadeh et al., 2005). In general, glucose featured a much higher recovery in the slurry following enzymatic hydrolysis (on average 61%), than in pretreatment liquid (on average 7%). Hence, this sugar partitioned more to the solid fraction (slurry) aimed for SSF, in accordance with its intended use. Xylose showed a more balanced yield between slurry (on average 27%) and pretreatment liquid (on average 33%). The overall yield of glucose and xylose in the two combined fractions represented a similar share (68 and 60%) of the respective amounts of glucan and xylan contained in the raw material.

Large differences were observed among pretreatment conditions (Figure 2.4). In general, the addition of alkaline catalyst did not increase the two sugars' yield, whereas the acid catalyst improved glucose recovery (in both fractions), and also xylose recovery (only in pretreatment liquid). With strong lime addition, Wyman et al. (2011) obtained a 35% xylose release in pretreatment liquid vs. 27% in this study, in exchange for a lower solubilisation of cellulose (1.5% vs 6.5% in this study). Concerning the effect of acid catalyst, Wyman et al. (2011) report a higher xylose yield in pretreatment liquid (74% vs 53% in this study) in exchange for a lower glucose yield (7% vs. 12%), obtained with higher acid concentration (2.5 vs 0.2%), lower severity (Log R_0, 2.8 vs 3.8), and higher water to solid ratio (9:1 vs 1:1) than in this study. Dien et al. (2006) obtained the same glucose release in pretreatment liquid as in this study (11%), operating on switchgrass at an earlier stage (anthesis), i.e. potentially easier to be degraded. It appears, therefore, that in the cited work a lower severity (Log R_0, 2.8 vs 3.8 in this study) compensated for a much higher acid concentration (2.5 vs 0.2%) and water to solid ratio (9:1 vs 1:1).

Besides, increased residence time enhanced glucose enzymatic yield employing autohydrolysis (P1-3), or using lime at low concentration and temperature (P4-6), but not at higher concentration and temperature (P7-12). A positive effect of time was also observed in xylose pretreatment yield, including high $Ca(OH)_2$ concentration (P7-9). However, for this sugar increases in pretreatment yield tended to be compensated by decreases in enzymatic hydrolysis yield.

Lime did not improve enzymatic hydrolysis of the slurry, in contrast to other studies (Garlock et al., 2011; Wyman et al., 2011), where higher yields were evidenced for both hemicellulose and cellulose. This difference could be due to lower solid loading (1% vs 5%) associated to longer residence time (168 vs 48 hours) than in this study. The two

factors combined contribute to enhance the solubilisation of the solid fraction, as demonstrated by Pallapolu et al. (2011).

In general, a higher degradation of cellulose was obtained in exchange for a lower hydrolysis of hemicellulose, with respect to other experiments on switchgrass (Li et al., 2010; Garlock et al., 2011; Wyman et al., 2011). This contrasting effect on the two fibre components is likely due to the lower moisture of switchgrass in steam explosion in this study (ca. 45% vs. 90% in the cited cases). In fact, an elevated water to solid ratio as in the cited works (Li et al., 2010; Garlock et al., 2011; Wyman et al., 2011) facilitates biomass degradation during steam explosion. This is based on the assumption that high water availability can better penetrate cell structure, hydrate cellulose, but especially, remove hemicellulose (Chang et al., 2001). This, in turn, may explain the higher amount of hemicellulose hydrolysed during pretreatment with biomass at high moisture content, or under elevated water to solid ratio. However, high use of water involves a proportionally higher amount of energy required for pretreatment, sugar recovery and downstream processes, resulting in a relevant drawback from several viewpoints.

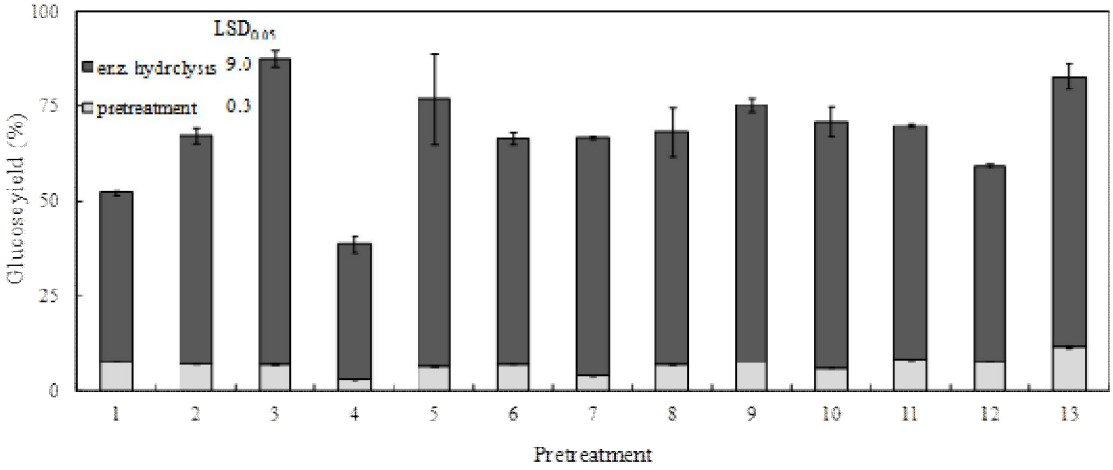

Figure 2.4 - Glucose (monomer and oligomers) yield in pretreatment liquid and slurry following enzymatic hydrolysis, as percentage of total glucan content in the raw material. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

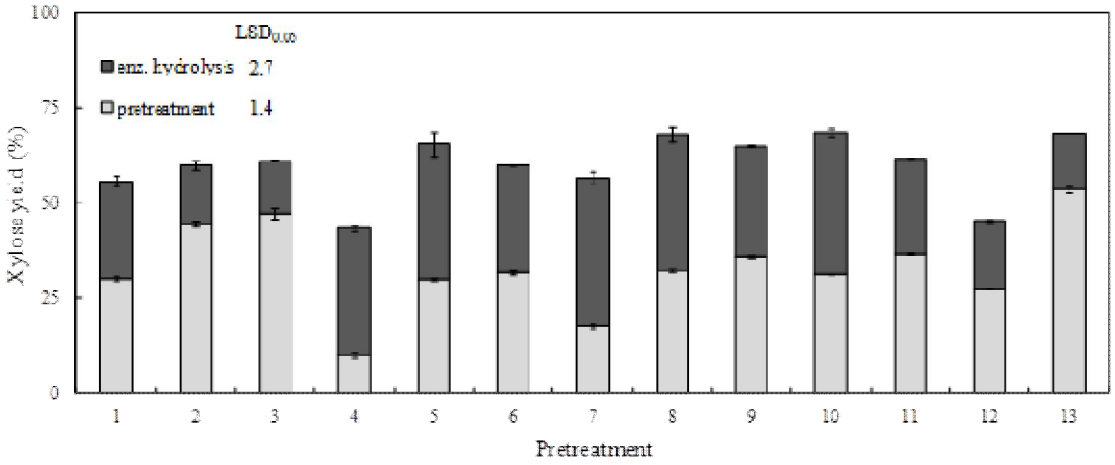

Figure 2.5 - Xylose (monomer and oligomers) yield in pretreatment liquid and slurry following enzymatic hydrolysis, as percentage of total xylan content in the raw material. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

2.3.3 Simultaneous saccharification and fermentation

Based on high glucose yields shown in enzymatic hydrolysis (Figure 2.4), pretreatments 3, 5, 9 and 13 were selected for SSF at high WIS content (15%). The four slurries diverged in the amount of initial glucose (Figure 2.6): 41 g L^{-1} in P3 (steam pretreatment alone) vs. an average of 49 g L^{-1} with alkaline (P5 and P9) or acid catalyst (P13). However, glucose depletion followed the same trend during SSF: steep linear decrease from the aforementioned levels to ca. 5 g L^{-1} in the first 10 hours (average glucose consumption rate, 4.5 g L^{-1} h^{-1}), followed by slow decrease to almost nil at the end of the process (average consumption rate, 0.05 g L^{-1} h^{-1}).

In parallel to this, ethanol concentration increased from zero to ca. 30 g L^{-1} in the first 20 hours, settling around this figure for the rest of time. This pattern corresponds to first-order kinetics: in fact, the four pretreatments fit this curve with very good precision ($R^2 \geq 0.95$**) (function parameters not shown). However, the cumulated amount of ethanol at the end of SSF outlined statistical differences: autohydrolysis (P3) attained a

ca. 10% lower ethanol (29.8 g L^{-1}) than $Ca(OH)_2$ addition at 0.4% (P5) (33.5 g L^{-1}). The other two pretreatments with alkaline catalyst at high dose (P9), and with acid catalyst (P13) featured an intermediate 31.5 g $L^{-1}$of ethanol.

This is in contrast with enzymatic hydrolysis showing a higher glucose yield in P3 than P5 (80 vs. 70%) (Figure 2.4): owing to this, the former pretreatment was expected to yield more ethanol. However, the result we observed may be explained with a sort of alkaline detoxification associated with use of calcium hydroxide in pretreatment, resulting in a better fermentation (Persson et al., 2002).

In general, the ethanol yield obtained with lime addition ranged between 65 and 76% of the theoretical (eq. 3) for P9 and P5, respectively. These data were slightly lower than those obtained on switchgrass by Chang et al. (2001) (70-90%), which may be explained by the slightly higher enzyme loading (25 FPU g^{-1} cellulose) and moisture (WIS, ca. 5%) adopted in the cited work during SSF runs. It has already been observed (Mohagheghi et al., 1992; Varga et al., 2004; Jørgensen et al., 2007) that running SSF experiments at high WIS concentration decreases percent ethanol yield on the theoretical, even though the resulting sugar concentration and consequent ethanol concentration increase. In fact, ethanol concentrations at the end of SSF in this study (Figure 2.6) were higher than those obtained in SSF's conducted at lower WIS: these latter ranged between 14 and 22 g L^{-1} (Chang et al., 2001; Chung et al., 2005; Faga et al., 2010). Final ethanol concentration significantly affects processing costs, in particular distillation (Wingren et al., 2003). This is especially true in light of the fact that an industrial titer threshold of 40 g L^{-1} was indicated as a goal for profitable processing (Zacchi and Axelsson, 1989; Katzen et al., 1999).

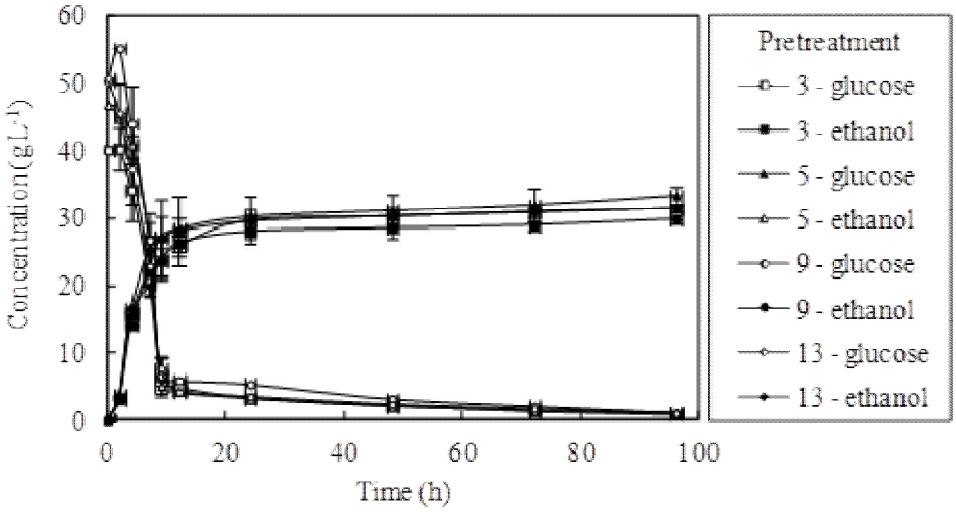

Figure 2.6 - Concentration of glucose (empty symbols) and ethanol (filled symbols) during the simultaneous saccharification and fermentation carried out on the slurries of four selected pretreatments. Error bars show ±SD.

2.3.4 Anaerobic digestion

The four pretreatment liquids selected for AD, consistently varied in TOC, TS and VS (Table 2.3). Acid catalysis (P13) attained the highest levels of TOC and TS, in accordance with a higher recovery of soluble sugars in the liquid fraction (Figure 2.4 and 2.5). Autohydrolysis (P3) exhibited slightly lower TOC and TS, in exchange for higher VS. Lastly, lime (P5 and P9) featured the lowest TOC, TS and VS. This, too, reflects a generally low concentration of xylose in the two pretreatments with lime (Figure 2.4 and 2.5). In fact, utilization of alkaline conditions favours release of polymeric hemicellulose sugars during pretreatment, in comparison with autohydrolysis or acidic conditions. Much of these sugars are transformed into monomeric sugars only after enzymatic hydrolysis, hence they are not completely available for AD in pretreatment liquid.

AD demonstrated a much higher CH_4 output per unit VS, adding alkaline or acid catalyst to steam explosion (Table 2.3). In the case of lime, a dose response is also

perceived. The effect of chemical (acid/base) pretreatment on lignocellulosic biodegradability is already acknowledged in the literature (Taherzadeh et al., 2008; Bruni et al., 2010). However, in our case this effect extends to a liquid with a low level of all inhibitors (Figure 2), thus assumed to be easily degradable.

Table 2.3 - Characteristics of four selected pretreatment liquids, and methane yield after anaerobic digestion.

Pretreatment	**TOC**	**TS**	**VS**	**CH_4**
	(g L^{-1})	(%)	(% TS)	(NmL g^{-1} VS)
3	21.3	2.4	87.6	137.5
5	16.5	2.2	69.3	226.4
9	17.8	1.9	58.9	300.5
13	25.6	2.8	83.4	281.8
$LSD_{0.05}$	2.2	0.6	14.9	139.3

TOC, total organic carbon; TS, total solids; VS, volatile solids. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

2.3.5 Combined energy yield

Combined energy yield (ethanol, methane and residual solid) best shows pretreatment effects referred to unit dry weight of switchgrass biomass (Figure 2.7). Energy from ethanol increased from 3.2 kJ g^{-1} in autohydrolysis (P3) to 4.2 kJ g^{-1} with lime at low concentration (P5), whereas lime at high concentration (P9) and acid addition (P13) did not improve this trait with respect to P3. In contrast to this, energy from methane showed a remarkable increase with acid addition (1.7 kJ g^{-1} in P13 vs 0.8 kJ g^{-1} in P3), while alkaline pretreatments (P5 and P9) did not significantly increase this trait. Lastly, residual energy outlined the same trend as ethanol: significant increase with lime at low

concentration (8.3 kJ g^{-1} in P5 vs 7.2 kJ g^{-1} in P3); no increase with high lime concentration (P9) and sulphuric acid (P13).

Therefore, pretreatments generally enhanced the energy output, as it concerns the two biofuels ethanol and methane. Conversely, strong pretreatments (P9 and P13) had less residual solid than pretreatments with no (P3) or low (P5) catalyst concentration, and this negatively affected the amount of residual energy. As a result, autohydrolysis and the two strong pretreatments were substantially equivalent in terms of combined energy yield (7.2, 7.2 and 7 kJ g^{-1} in P3, P9 and P13, respectively), while low lime concentration (P5) was top ranking (8.3 kJ g^{-1}). Although residual energy may not completely be exploited as it cannot easily be transported as a liquid (ethanol) or gaseous (methane) fuel, the fact remains that residual solid can be used for pellets, or for steam and power generation for internal uses at a power plant. Thus residual energy has to be accounted for, in the overall product yield. Its weight on combined energy yield consistently declined from 44% in autohydrolysis to 33% in acid catalysis, further proving that mild pretreatment conditions as steam alone leave a relevant share of the total energy unexploited in the final residue.

In the literature, higher values of energy outcome from analogous configuration processes are generally reported from similar lignocellulosic sources as corn stover (Bondesson et al., 2013) and oat straw (Dererie et al., 2011). However, the cited studies evidenced a weaker benefit from pretreatments on the combined ethanol and methane: ca. +15% and +3% energy output with acid addition in the two respective sources, compared to +29% with lime addition at low concentration (P5) in this study.

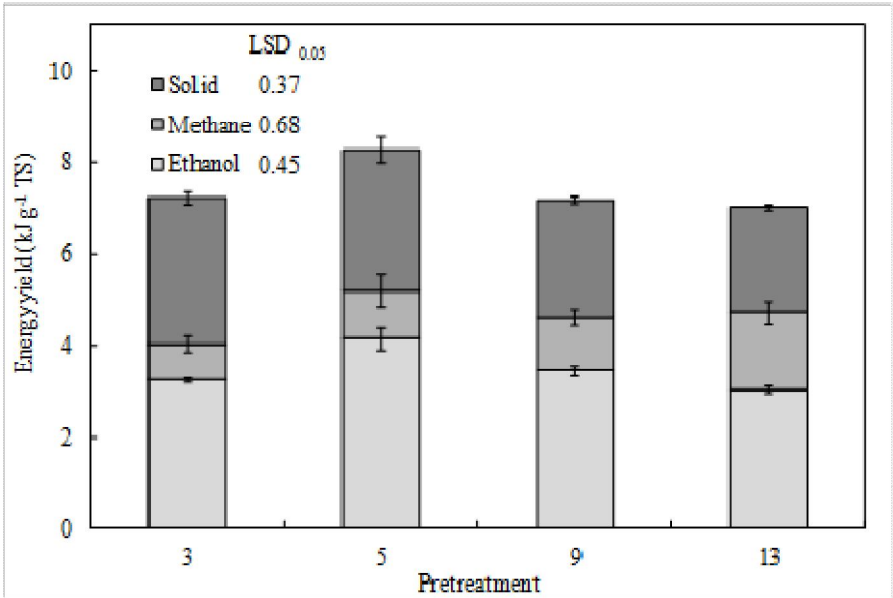

Figure 2.7 - Combined energy yield per unit dry weight of the raw material, in terms of ethanol from SSF, methane from AD, and energy content of the residual solid. Error bars show ±SD. $LSD_{0.05}$ indicates least significant differences at $P \leq 0.05$.

2.2.4 Conclusions

The aim of this study was to compare the effects of steam explosion alone and after impregnation with calcium hydroxide or dilute sulphuric acid on switchgrass, in order to test lime as potential substitute for acid catalyst. Lime showed a great potential when ethanol was focused, whereas acid addition produced higher methane yield. The latter outcome, in association with low concentration of inhibiting compounds in pretreatment liquid, proves that restrained use of sulphuric acid may not be detrimental in steam explosion. However, low concentration of lime was shown less aggressive and secured more residual solid after SSF, resulting in higher energy output per unit raw biomass.

More to this, utilization of lime favours the release of polymeric hemicellulose sugars during pretreatment. Thus, the lime impregnation method could be well suited for applications where hemicellulose sugars will be used for, e.g., production of bioplastics.

This could be an alternative to employing anaerobic digestion as means to add value to final products.

The low water to solid ratio adopted in this study is the premise for reductions in the amount of water consumed during pretreatment, while the use of calcium hydroxide and its final recovery as calcium carbonate avoid to handle effluents containing toxic compounds in downstream processing, and provides a marketable by-product for agricultural applications.

Lastly, simultaneous saccharification and fermentation at high concentration of solids (15% WIS) improved previous records in final ethanol concentration. Further increases may be envisaged through augmented solids concentration (20% WIS). However, this option is responsible for lower ethanol yield on the theoretical maximum, hence potential benefits are at least partially offset.

CHAPTER 3

Effects of water level and harvest time on two hybrids of biomass sorghum [*Sorghum bicolor* (L.) Moench] for first and second generation ethanol production.

Abstract

Among the effects of climate change, the depletion of water resources strongly affects crop yields. As one of the tools to mitigate climate change is the cultivation of dedicated bioenergy crops, the competitiveness between energy and food crops for land and water use could increase in time. In this light, the present study was performed to determine the effect of two water levels (WL, H and L) and three harvest times (HT1, 2 and 3, 90, 118 and 151 days after seeding, respectively) on the growth of two genotypes (G) of biomass sorghum [*Sorghum bicolor* (L.) Moench] (Sucros 506, S506, and Biomass 133, B133) in a greenhouse experiment. Effect of WL and HT were also studied focusing on the characteristics of juice and biomass, in order to devise the most suitable practice to achieve the maximum yield in terms of both 1st ($EtOH_{1G}$) and 2nd ($EtOH_{2G}$) generation bioethanol. Lastly, water use efficiency (WUE) of the juice, total dry weight (TDW) and the amount of $EtOH_{1G}$, $EtOH_{2G}$, and combined 1st plus 2nd generation ethanol ($EtOH_{1G+2G}$) per pot were calculated. S506 produced higher amounts of $EtOH_{1G}$, compared to B133, resulting in higher $EtOH_{1G+2G}$ even if no difference in terms of $EtOH_{2G}$ was evidenced between the two hybrids. Low water availability determined low DBY and juice quantity, thus reflecting in lower amounts of $EtOH_{1G}$ and $EtOH_{2G}$. During crop growth the amount of cellulose, hemicellulose and acid insoluble lignin (AIL) changed causing a decreasing of $EtOH_{2G}$ concentration from HT1 to HT3. Nevertheless, at HT3 the highest amount of $EtOH_{2G}$ was reached, due to the highest DBY. WUE of the juice and TDW resulted to be affected by HT factor, while WUE of $EtOH_{2G}$ was affected by WL. All investigated factors induced statistical differences on WUE of $EtOH_{1G}$.

3.1 Introduction

Biofuels have to face the energy demand due to the depletion of fossil sources, and mitigate the climate change (Monti et al., 2011) caused by the atmospheric release of fossil fuel derived CO_2 (Metz et al., 2007). Among renewable energies, suitable biomasses alternative to fuels should combine low inputs need with high productivity, in order to provide high energy outputs (McKendry, 2002). Among bioenergy crops, biomass sorghum, representing genotypes of *Sorghum bicolor* (L.) Moench featuring high, thick stems with small panicles atop, has been widely studied for its low agricultural inputs, resistance to water stress and high biomass production (Mastrorilli et al., 1995; Guigou et al., 2011; Cosentino et al., 2012). In fact, biomass sorghum has a ratio of energy output to fossil energy input comparatively higher than sugarcane, sugar beet, maize and wheat (Almodares et al., 2009; Wu et al., 2010), and in the same sources its fermentation efficiency has been reported to be higher than 90%

As one of the effects of climate change is the depletion of the water resource (Polley, 2002; Farré and Faci, 2006), water availability is becoming an expensive input in the management of bioenergy crops (Gerbens-Leenes et al., 2009). Moreover, the use of irrigation for energy crops could exacerbate the competition with food crops for the water resource (Dalla Marta et al., 2014). Thus, it is becoming extremely important to evaluate water use efficiency (WUE), i.e. the amount of biomass or deriving biofuels per unit water used for plant growth (Passioura, 1977), in order to assure efficient energy production.

In this frame, many studies focused on the resistance of biomass sorghum to water stress and its biomass yields under drought conditions (Curt et al., 1995; Teetor, 2011; Rocateli et al., 2012), while a few authors have related the water level to the

characteristics of structural carbohydrates of biomass for energy purposes (namely, biogas and 2nd bioethanol production), and who did it (Rocateli et al., 2012; Singh et al., 2012), did not conduct any process transformation of the biomass into ethanol.

Nowadays, it is widely known that biomass suitability for 2nd generation bioethanol not only depends on cellulose content, but also on biomass physical and chemical properties such as the lignin cross-links with cell wall carbohydrates (Corredor et al., 2009). For that reason, it is not sufficient to assess the amount of fibre components in biomass, but also their convertibility into ethanol.

Furthermore, many studies were focused on the maximum accumulation of sugars in stalks (Davila-Gomez et al., 2011), as well as the percent of juice extracted at different crop stages (Teetor et al., 2001). In this framework, many experiments have been conducted in order to appraise the most suitable harvest time when the juice is considered the only source for energy purposes, but no investigation has been planned to identify optimal time and practice when also the residual bagasse is be used for 2nd generation bioethanol production.

Given this background, this study was performed to determine the effect of different water levels and harvest times on the growth and biomass characteristics of two hybrids of biomass sorghum, and to devise the most suitable practice to achieve the maximum yield in terms of both first and second generation bioethanol. Lastly, WUE of total dry weight (TDW), extracted juice, 1st generation ethanol, 2nd generation ethanol and the combined 1st and 2nd generation ethanol, were calculated and discussed to assess the most efficient practice in terms of harvest time, choice of genotype and water level for 1st and 2nd generation bioethanol production.

3.2 Material and methods

3.2.1 Experimental location

The present experiment was conducted during May to October 2014 in a glasshouse at the Department of Agricultural Sciences, University of Bologna, Italy (44° 29' N, 11° 20' E; 32 m above sea level).

3.2.2 Planning and experimental material

Two genotypes of biomass sorghum [*Sorghum bicolor* (L.) Moench] were used. Sucros 506 (S506) and Biomass 133 (B133) were kindly provided by Syngenta Seeds (Casalmorano, CR, Italy). The two hybrids were sown on 20th May 2014 in 54 pots filled with 7 kg of soil on oven dry basis. Six seeds of each sorghum hybrids were sown in each pot. Seedling emergence was recorded 4 days after seeding (DAS), and seedlings were subsequently thinned to two plants per pot. Beginning of differential watering started at 21 DAS, and the experiment went on for 151 DAS.

The soil was brought from the Research Farm of the University of Bologna in Cadriano (Italy) mixed with sand in a 2:1 ratio. Before filling the pots, the soil was air dried and ground to pass a 2 mm sieve. Residual moisture was determined (oven at 105 °C until constant weight) and the following physical-chemical traits were assessed, according to standard procedures (D.M. 13-9, 1999, Italian Ministry of Agricultural and Forest): particle size distribution (sand, silt and clay, 500, 330 and 170 mg g^{-1}, respectively); pH (8.1; soil to water ratio, 1:2.5); total and active limestone (71.2 and 17.5 mg g^{-1}, respectively); cation exchange capacity (17.2 $cmol_c^+$ kg^{-1}); total organic carbon and total kjeldahl nitrogen (6.82 and 0.76 mg g^{-1}, respectively); available P (Olsen) and exchangeable K (14 and 101 mg kg^{-1}, respectively). The volumetric water content of

soil at field capacity and wilting point (Richards' apparatus) were 26.9% and 12.7%, respectively.

3.2.3 Experimental design and treatments

Two watering regimes were applied to the two sorghum genotypes set for three harvest times, in a completely randomized factorial design at four replications, totalling 48 pots.

At 20 DAS, two watering regimes, high (H) and low (L) (70 and 30% of the water holding capacity, respectively), were developed by adding the calculated amount of water determined by the gravimetric method. In H, water was added almost every day after the 20 days from the seeding, while in L watering was carried out three times during the week. Soil moisture was monitored using the gravimetric method weekly, in order to maintain the required amount of water. Extra pots were set up and plants were harvested during the experiment to account for the increase of pot weight due to plant mass. In addition, N fertilizer was applied after thinning, considering that the soil was sufficiently provided in the rest of nutrients.

The three harvest times (HT) were: HT1 at 90 DAS, HT2 at 118 DAS and HT3 at 151 DAS. At each HT, 4 pots for each combination of hybrid (S506, B133) and water regime (H, L) were harvested.

At each HT, stems were manually defoliated prior to juice extraction. Immediately after, they were chipped to a particle size of about 20 mm, and pressed (40 MPa) with a hydraulic press for about 15–20 min. The extracted juice was quantified by weight and then stored at -20 °C into plastic bottles. After juice extraction, bagasse and leaves were fresh weighed and then oven dried at 40 °C, in order to conduct

compositional analysis. A sample of few grams was oven dried at 105 °C, in order to calculate total dry weight (TDW) (g pot^{-1}).

The four replicates of juice and residual biomass (leaves and bagasse) of each hybrid x WL combination were put together for subsequent analysis and fermentation.

3.2.4 Juice fermentation

Fermentation on juice was conducted in duplicates at 30 °C, using glass bottles of 250 mL capacity on a juice volume of 40 mL. Bottles were placed in an orbital shaker maintained at 100 rpm during fermentation. Before fermentation, pH was set at 5 with the appropriate quantity of buffer, and subsequently adjusted at that level with further amounts. The yeast *Saccharomyces cerevisiae*, Ethanol Red, was kindly provided by Lesaffre (Marq-en-Barceul, Roubaix, France), and added at a concentration of 1 g L^{-1}. Each fermentation went on for 72 hours and samples of 1 mL were collected at 2, 4, 7, 9, 12, 24, 48 and 72 hours. Each sample was analysed for sucrose, glucose, fructose and ethanol.

3.2.5 Enzymatic Hydrolysis

After juice extraction, leaves and bagasse were dried in a ventilated oven at 40 °C, chopped at 2 mm and stored in air ventilated conditions before enzymatic hydrolysis. Enzymatic hydrolysis was carried out in duplicates at a solid loading of 5% WIS (water insoluble solid) (Weiss et al., 2010), at a working volume of 50 mL in glass bottles of 250 mL capacity, previously sterilized at 121 °C for 20 minutes.

During the process, the bottles were placed in an orbital shaker kept at 100 rpm. The enzyme, CelliCTec2 (kindly provided by Novozymes A/S, Bagsvaerd, Denmark), was added at a loading of 0.1 g g^{-1} WIS. Sodium acetate at pH 5.0 was used as buffer. Hydrolysis went on for 48 h at 45 °C, during which time the pH was manually adjusted.

Samples of 1 ml were taken at 3, 6, 9, 24 and 48 hours, and analysed for glucose concentration.

3.2.6 Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) was performed in duplicates in 250 mL glass bottles previously sterilized at 121 °C for 20 minutes, at a solid loading of 5% WIS with a working volume of 50 ml. During SSF, the pH was maintained at 5 using sodium acetate buffer, and temperature was set at 35 °C. Cellic CTec2 (Novozymes A/S, Bagsvaerd, Denmark) enzyme was added at 0.1 g g^{-1} WIS, while 1 g L^{-1} of *S. cerevisiae* Ethanol Red (Lesaffre, Marq-en-Barceul, Roubaix, France) was used as yeast.

SSF went on for 96 hours. Samples of 1 mL of broth were taken after 2, 4, 7, 10, 12, 24, 48, 72 and 96 hours, and analysed by HPLC for ethanol concentration.

3.2.7 Analytical determinations

Extractives in biomass were determined following the procedure described by Di Girolamo et al. 2014. Structural carbohydrates (cellulose and hemicellulose) and lignin were determined following the National Renewable Energy Laboratory method (Sluiter et al., 2008a). Briefly, biomass samples were hydrolysed in a water bath (150 mg with 1.5 ml of 72% w/w of H_2SO_4 at 30 °C for 60 min), then diluted with 42 mL of deionized water to reach a final H_2SO_4 concentration of 4%, and autoclaved (121 °C for 60 min). The insoluble residue was separated from the supernatant by vacuum filtration (glass micro-fibre filter Ø 47 mm), washed with about 35 mL deionized water and placed in a crucible. The crucible and glass micro-fibre filter were dried at 105 °C for 12 h to determine the amount of acid insoluble residue (AIR), thereafter they were placed in a muffle furnace at 550 °C for 24 h to determine acid insoluble lignin (AIL).

Monomeric sugars (glucose, xylose and arabinose) in the supernatant after acid hydrolysis were determined by means of HPLC (Waters 1525 Binary HPLC Pump) equipped with a Biorad Aminex HPX-87H column (300 × 7.8 mm) and a refractive index detector (Waters 2414). H_2SO_4 5 mM at a flow rate of 0.5 mL min^{-1} was used as mobile phase; the temperature of the column and detector were maintained at 63 and 50 °C, respectively.

3.2.8 Calculations and statistical analysis

Glucose yield was calculated according to equation 1, where only the measured sugar concentration is accounted for. This approximation is rather accurate for hydrolysis of diluted fiber suspensions (< 5-10% WIS) (Palmqvist and Liden, 2012).

$$Yg = \frac{Cg}{\varphi g \times Cis0 \times Xg0} \quad (3.1)$$

Where Y_g is the theoretical maximum yield of glucose (%, g g^{-1}); φ_g is the molecular ratio of glucose to glucan (1.11); X_{g0} is the initial mass fraction of insoluble solids (g). Water use efficiency (WUE) of TDW (g L^{-1}), juice (g L^{-1}), $EtOH_{1G}$ (g L^{-1}), $EtOH_{2G}$ (g L^{-1}) and $EtOH_{1G+2G}$ (g L^{-1}) was assessed following the respective following equations:

$$WUE_{TDW} = \frac{DBY\ (g)}{W\ (L)} \quad (3.\ 2)$$

$$WUE_{juice} = \frac{Juice\ (g)}{W\ (L)} \quad (3.3)$$

$$WUE_{1G} = \frac{EtOH_{1G}\ (g)}{W\ (L)} \quad (3.4)$$

$$WUE_{2G} = \frac{EtOH_{2G}\ (g)}{W\ (L)} \quad (3.5)$$

$$WUE_{1G+2G} = \frac{EtOH_{1G+2G}\ (g)}{W\ (L)} \quad (3.6)$$

Where WUE_{TDW}, WUE_{juice}, WUE_{1G}, WUE_{2G} and WUE_{1G+2G} represent the respective amounts of TDW, juice, and ethanol obtained through juice fermentation ($EtOH_{1G}$), SSF ($EtOH_{2G}$) and their sum ($EtOH_{1G+2G}$), expressed in grams per litre of water (W) supplied from seeding to harvest.

In all traits, normal distribution and equal variance of data were controlled through the Kolmogorov-Smirnov and Bartlett test, respectively. All chemical traits and parameters calculated were submitted to a three-way completely randomized ANOVA for hybrids, water levels, harvest times and their interactions, through the CoStat 6.3 software (CoHort Software, Monterey, CA, USA). Fisher's Least Significant Difference (LSD) test at $P < 0.05$ was adopted to separate means of statistically significant ANOVA sources.

3.3 Results and discussion

3.3.1 Dry biomass yield and composition

In terms of TDW (Table 3.1), no statistical differences were induced by G, while HT and WL were the factors determining significant differences, as observed in other studies (Zhao et al., 2009; Dalla Marta et al., 2014). Specifically, TDW benefited from water availability, as H level increased DBY by 42% compared to L level. HT also affected TDW, which is consistent with a longer growth period: values steeply

increased from HT1 (51.5 g) to HT2 (+ 42% in 38 days), then slowing down up to HT3 (+ 62% in 61 days from HT1).

Biomass composition (Table 3.1) showed values of extractives, cellulose, hemicellulose and AIL in the range of those observed in other studies conducted on biomass sorghum (Li et al., 2010; McIntosh and Vancov, 2010; Wu et al., 2011). Statistical differences for each trait were observed between the two genotypes, thus confirming the variability within this species (Zhao et al., 2009; Wu et al., 2010).

WL influenced cellulose and lignin: more specifically, high water availability appeared to increase cellulose and AIL content in the biomass. Among sources of variations, HT induced significant differences in all analytical traits, with cellulose and AIL increasing along crop cycle, while extractives decreased from HT1 to HT3. Hemicellulose exhibited a fluctuating trend along crop cycle.

Table 3.1 - Juice, total dry weight and chemical composition of sorghums.

Source	Juice (g)	TDW (g)	Extractives (mg g^{-1})	Cellulose (mg g^{-1})	Hemicellulose (mg g^{-1})	AIL (mg g^{-1})
Genotype (G)						
S506	62.6	83.0	0.30 a	274.3 b	152.8 b	166.9 b
B133	63.3	82.1	0.27 b	287.2 a	163.9 a	178.3 a
P	n.s.	n.s.	**	**	**	**
Water level (WL)						
H	86.3 a	104.5 a	0.27 a	295.7 a	160.2	177.1 a
L	39.6 b	60.6 b	0.29 a	265.8 b	156.4	168.1 b
P	**	**	n.s.	**	n.s.	*
Harvest (HT)						
HT1	26.0 c	51.5 c	0.30 a	273.9 b	153.8 b	163.5 b
HT2	62.4 b	89.4 b	0.28 ab	280.2 ab	162.8 a	175.6 a
HT3	100.5 a	106.7 a	0.27 b	288.2 a	158.4 ab	178.7 a
P	**	**	*	*	*	**
P (G × WL)	n.s.	**	n.s.	**	**	n.s.
P (G × HT)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P (WL × HT)	**	**	n.s.	**	**	n.s.
P (G ×WL × HT)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates.
Extractives represent the water- and ethanol-soluble fraction of VS, containing soluble sugars, chlorophyll, waxes, etc.
TDW, total dry weight.
AIL, acid insoluble lignin.
n.s, not statistically different.

3.3.2 Juice fermentation for $EtOH_{1G}$

Concentration (Figs. 3.1, 3.2, 3.3) and amount (Table 3.2) of ethanol produced from 1st generation process ($EtOH_{1G}$) was affected by all investigated factors and most of their interactions.

In general, concentrations observed were in the lower limit of the range found by other authors (Davila-Gomez et al., 2011), while fermentations conducted on S506 juice reached concentrations similar to those obtained by Zegada-Lizarazu and Monti (2014).

In particular, S506 resulted to be a genotype more adapted than B133 for $EtOH_{1G}$ production , as the 24% increase of ethanol produced from the total amount of juice per pot demonstrates, although the amount of juice extracted was almost the same as in B133 (62.6 vs 63.3 mL in the former vs the latter genotype). This relevant difference in the amount of $EtOH_{1G}$ was apparently due to the higher suitability of S506 juice to be fermented into ethanol, compared to B133 juice: in fact, S506 surpassed by 9% B133 in ethanol concentration, as average of the three harvest times (Figs.3.1, 3.2 and 3.3).

WL strongly affected both the amount and the concentration of $EtOH_{1G}$. Low water availability caused an 18% decrease in ethanol concentration, and a wide difference between quantities of juice extracted (86.3 vs 39.6 g pot^{-1} for H and L, respectively). Other authors have already observed the same effect of water availability on juice production in sorghum (Vasilakoglou et al., 2011; Dalla Marta et al., 2014). The combined difference in juice amount and ethanol concentration during fermentation originated a wide gap, more than two fold, between the amount of $EtOH_{1G}$ in H and L.

HT determined significant differences in terms of $EtOH_{1G}$ amount and concentration (Figs. 3.1, 3.2 and 3.3). The increase of juice quantity during crop cycle had already been observed by Zegada-Lizarazu and Monti (2014), and also Dalla Marta et al. (2014) quantified more juice extracted at the end of crop cycle than at earlier stages. Regarding $EtOH_{1G}$ concentration, HT2 provided the best result (26.3 g L^{-1}), while HT3 reached only 21.7 g L^{-1} , compared to the significantly higher 25.6 g L^{-1} of HT1 (Figs. 3.1, 3.2 and 3.3). Moreover, the quite lower $EtOH_{1G}$ concentration observed in B133 at HT3 compared to the same hybrid at HT1 and HT2 (significant G x WL x HT interaction, Table 3.2) could be due to the wide variability in juice quality observed in biomass sorghum genotypes and along crop cycle (Bala Ravi et al., 1997).

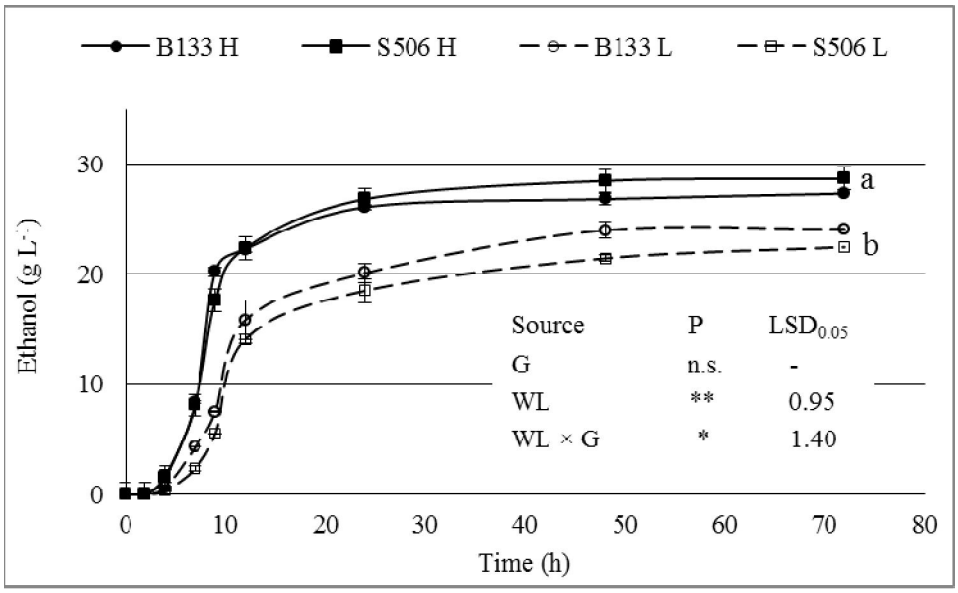

Figure 3.1 – Concentration of $EtOH_{1G}$ at HT1. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

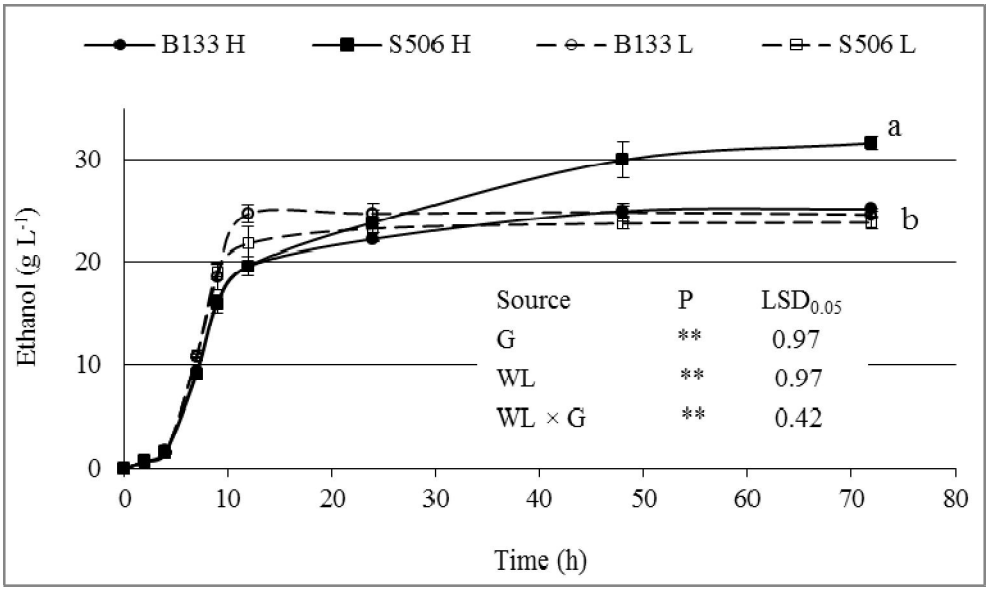

Figure 3.2 - Concentration of $EtOH_{1G}$ at HT2. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

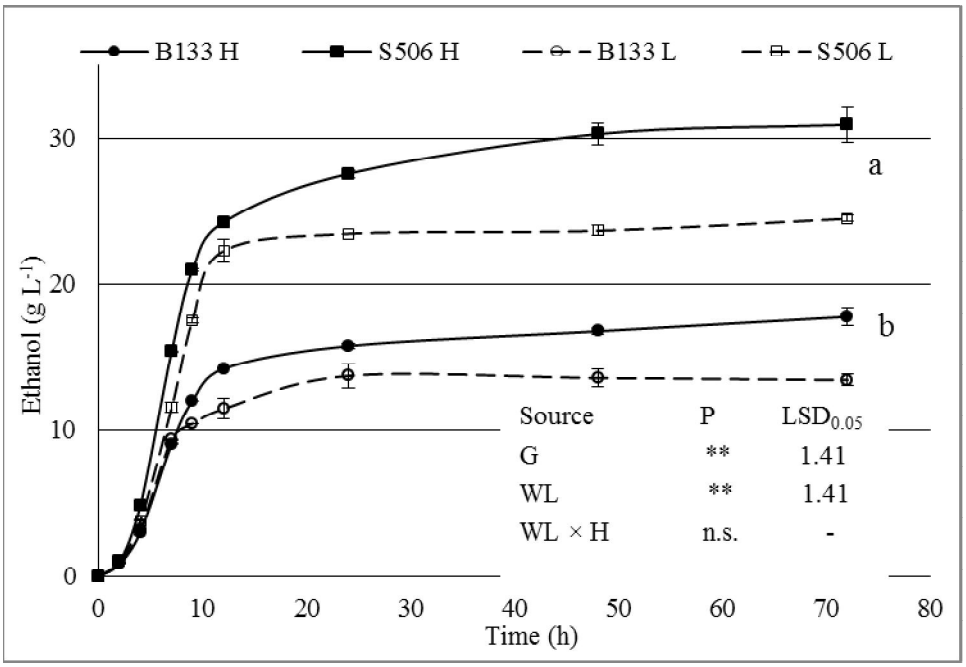

Figure 3.3 - Concentration of $EtOH_{1G}$ at HT3. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively Error bars represent standard deviation.

3.3.3 Enzymatic hydrolysis of bagasse

Figures 3.4 and 3.5 display glucose yield (%) and concentration (g L^{-1}), respectively, as average of the three harvest times observed during the enzymatic hydrolysis.

In general, glucose yields on the theoretical maximum were higher than in the study of Zhang et al. (2011): this difference could be due to the lower WIS concentration adopted in this experiment. In fact, as other studies demonstrated, the WIS concentration strongly affects enzymatic activity, resulting in decreased yields (Palmqvist and Liden, 2012; Hoyer et al., 2013). The study carried out by Goshadrou et al. (2011) appears to corroborate this hypothesis, because during enzymatic hydrolysis run at similar WIS concentrations, they obtained a glucose yield of 65% on sweet sorghum bagasse.

Glucose concentration was affected by G and HT, while WL did not show any effect on the suitability of biomass to be hydrolysed. Specifically, S506 proved to be more adapted and efficient in glucose release than B133. In fact, S506 produced 9.4 g L^{-1} glucose, while B133 stopped at a significantly lower concentration (8.9 g L^{-1}).

During crop cycle, the biomass suitability for hydrolysation of biomass appeared to worsen, as the negative trend of glucose concentration along the three harvest times demonstrated. In particular, concentration of glucose obtained by enzymatic hydrolysis decreased by 7% from HT1 to HT2, and 4% from HT2 to HT3.

In terms of glucose yield, no difference was observed between the two genotypes as well as the two water levels, while harvest time was still shown a factor inducing difference. In particular, glucose yield decreased from HT1 to HT3, starting from 73% with HT1, through 67% of HT2 and reaching the minimum value of 60% in HT3.

These results prove that the suitability of biomass in view of enzymatic hydrolysis for glucose release, decreases along crop cycle. The increase of AIL (Table 3.1) during crop cycle supports the assumption that lignin directly acts as a physical barrier, restricting cellulase access to cellulose, thus reducing this enzyme's activity through non-productive binding (Jeoh et al., 2007). As Corredor et al. (2009) have already observed, biomass suitability for 2nd generation bioethanol not only depends on cellulose content, but also on biomass physical and chemical properties such as lignin cross-links with cell wall carbohydrates. More in detail, secondary cell walls, i.e. those deposited once cell elongation ceases approximately before crop maturity, are usually thicker than primary walls and may be deposited in a number of layers (Pauli and Keegstra, 2008). Above all, in secondary cell walls water is largely replaced by lignin, making them nearly impenetrable to solutes and enzymes (Pauli and Keegstra, 2008). The progressive

decrease of glucose yield from HT1 to HT3 (ca. -10% at each successive harvest) is consistent with this strengthening of cell wall structure.

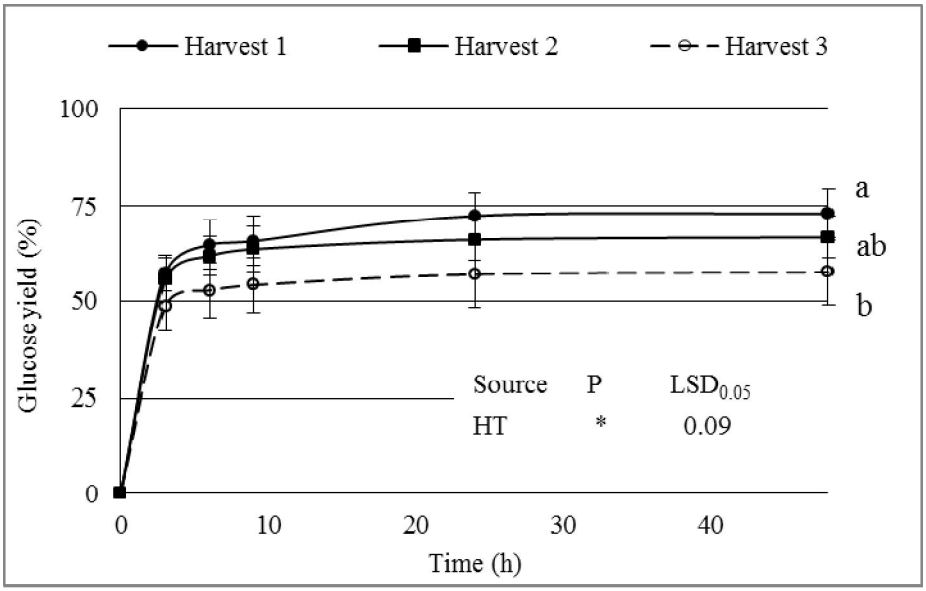

Figure 3.4 – Glucose yield (%) during enzymatic hydrolysis. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

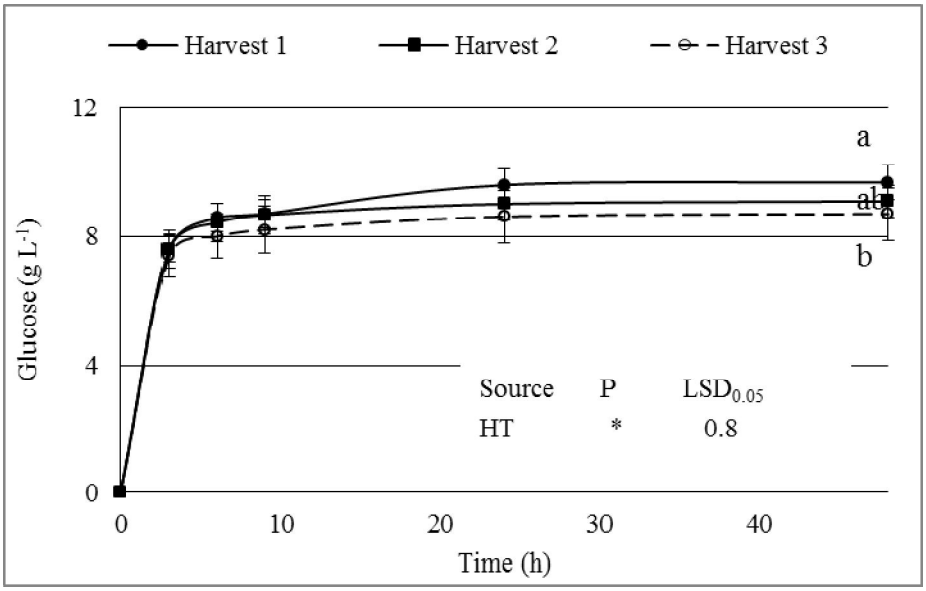

Figure 3.5 - Glucose concentration (g L^{-1}) during enzymatic hydrolysis. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

3.3.4 Simultaneous saccharification and fermentation for $EtOH_{2G}$

Figures 3.6, 3.7 and 3.8 show the ethanol concentration produced during SSF conducted on HT1, HT2 and HT3, respectively. Concentration of ethanol in the three SSFs resulted affected by water level and harvest time, while the genotype did not cause significant variation.

More in detail, ANOVA showed that the average concentration observed in the H level was significant higher than in L, although a difference of only 6% was observed (3.6 and 3.4 g L^{-1} for H and L, respectively).

Concerning the effect of harvest time, HT1 produced more ethanol than HT2 and HT3: in fact, ethanol concentration in HT1 was significantly higher than HT2 and HT3 (3.9, 3.3 and 3.4 g L^{-1} in the three respective cases). Results of $EtOH_{2G}$ concentration observed at each harvest were consistent with values of glucose concentration and yield obtained through enzymatic hydrolysis.

In terms of $EtOH_{2G}$ amount (Table 3.2), no difference was observed between the two hybrids tested, as consequence of almost the same TDW values (Table 3.1) and ethanol concentration produced during SSFs.

$EtOH_{2G}$ amount increased in time: in fact, it augmented by 33 and 46% in HT2 and HT3, respectively, compared to 4.0 g pot^{-1} of $EtOH_{2G}$ registered in HT1, reversing the results observed for $EtOH_{2G}$ concentration. These opposite patterns between $EtOH_{2G}$ concentrations and amounts are due to the significantly higher TDW produced by sorghum during crop cycle with increasing production at later harvests.

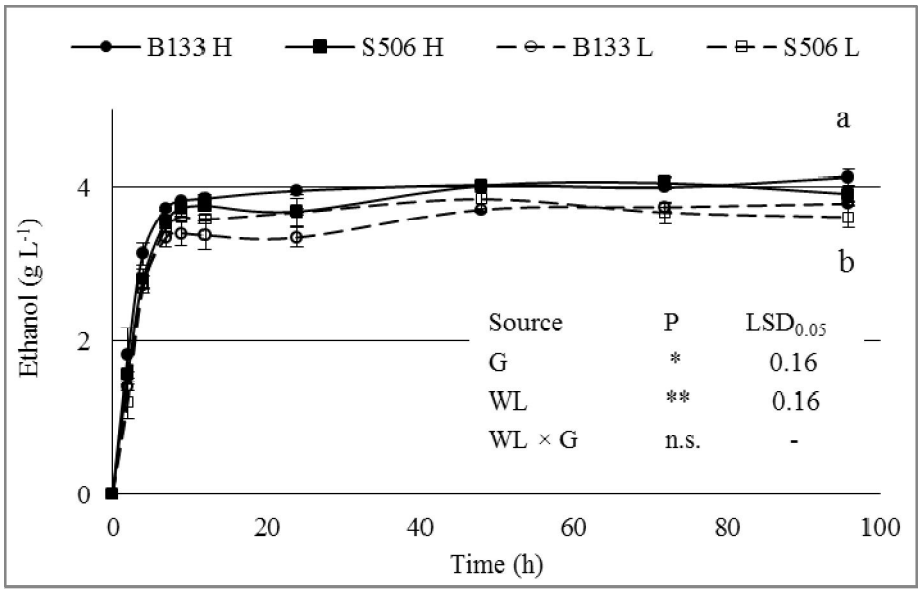

Figure 3.6 – Concentration of $EtOH_{2G}$ at HT1. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

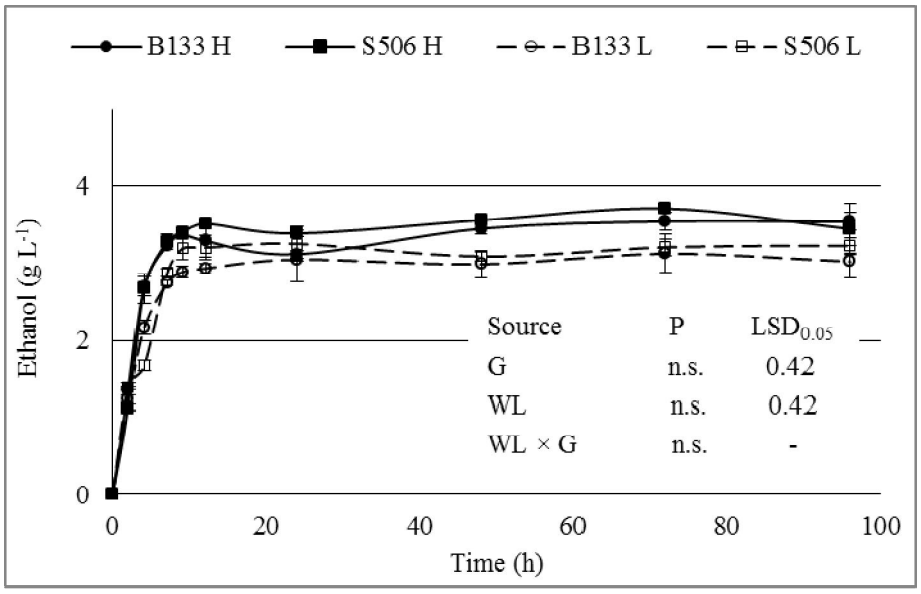

Figure 3.7 - Concentration of $EtOH_{2G}$ at HT2. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

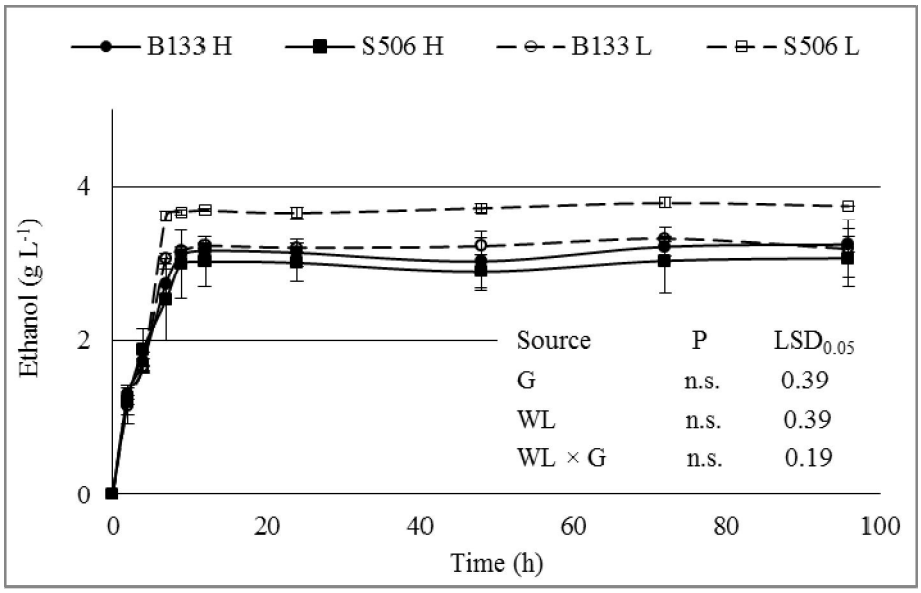

Figure 3.8 - Concentration of $EtOH_{2G}$ at HT3. In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates. n.s., * and ** mean not significant, significant at $P < 0.05$ and 0.01, respectively. Error bars represent standard deviation.

3.5 Combined $EtOH_{1G}$ and $EtOH_{2G}$

In terms of overall $EtOH_{1G+2G}$ production (Table 3.2), S506 produced 17% more than B133, proving to be a hybrid more suitable for ethanol purpose. This difference was due to the higher suitability of S506 juice resulting from higher concentration of $EtOH_{1G}$, while no difference was observed in terms of $EtOH_{2G}$ amount, as already described in the previous paragraphs.

During crop cycle, $EtOH_{1G+2G}$ followed the same trend of juice amount, TDW, $EtOH_{1G}$ and $EtOH_{2G}$, with values increasing by a respective 132 and 212% for HT2 and HT3, compared to HT1.

Also water availability strongly affected the overall amount of $EtOH_{1G+2G}$, the H level resulting more than twice as high as the L level. This was mainly due to the higher amounts of both juice and TDW produced under better water availability.

Table 3.2 – Amounts of 1st, 2nd and combined 1st and 2nd generation ethanol ($EtOH_{1G}$, $EtOH_{2G}$ and $EtOH_{1G+2G}$, respectively).

Source	$EtOH_{1G}$ (g pot^{-1})	$EtOH_{2G}$ (g pot^{-1})	$EtOH_{1G+2G}$ (g pot^{-1})
Genotype (G)			
S506	53.6 a	5.9	59.6 a
B133	40.6 b	5.7	46.3 b
P	**	n.s.	**
Water level (WL)			
H	69.5 a	7.5 a	77.0 a
L	24.7 b	4.1 b	28.9 b
P	**	**	**
Harvest (HT)			
HT1	20.6 c	4.0 c	24.6 c
HT2	51.3 b	6.0 b	57.3 b
HT3	69.4 a	7.4 a	76.8 a
P	**	**	**
P (G×WL)	**	n.s.	**
P (G×HT)	**	**	**
P (WL×HT)	**	**	**
P (G×WL× HT)	**	n.s.	**

In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates.

3.3.6 Water use efficiency

Water use efficiencies of biomass production and deriving ethanol (Table 3.3) were calculated, in order to better understand the impacts on the water resource of crop management and the two bioethanol producing technologies deployed in this experiment.

Regarding juice and TDW, no difference was observed in terms of WUE between the two genotypes, while harvest time was shown a factor of strong influence.

In particular, WUE_{juice} increased remarkably during crop cycle, while WUE_{TDW} reached its highest value at 118 DAS (HT2), and resulted 5 and 18% higher than at HT3 and

HT1, respectively. Water regime was shown to influence WUE_{juice}, as widely acknowledged in the literature (Miller and Ottman, 2010; Vasilakoglou et al., 2011; Dalla Marta et al., 2014). Conversely, no statistical difference was evidenced in WUE_{TDW}: this last finding corroborates the good adaptability of biomass sorghum to low water availability, observed in other studies (Foti et al., 2004; Darcas and Liakatas, 2007). In general, WUE_{TDW} ranged between 3.4 and 4.1 g L^{-1}, which is consistent with values obtained by other experiments (Cosentino et al., 2012; Zegada-Lizarazu et al., 2012).

WUE_{2G} resulted to be significantly affected by water level, while genotypes and harvest time did not determine any statistical difference. H level increased WUE_{2G} by almost 15% with respect to L level.

Compared to this, WUE_{1G} resulted to be significantly affected by all the investigated factors. S506 was shown a genotype more efficient in the use of water with respect to B133, performing 9% higher than this latter. This was due to the higher suitability of S506 juice to be fermented, as explained in the previous paragraph. During crop cycle, WUE_{1G} did not follow the same pattern of WUE_{juice}, reflecting in augmented use efficiency of the water resource: in fact, WUE_{1G} significantly rose from 1.3 g L^{-1} of HT1 to 2.2 g L^{-1} of HT2, in turn passed by 2.4 g L^{-1} of HT3. WUE_{1G} was strongly affected by water level, too: specifically, the H level almost doubled the L level (2.6 vs 1.4 g L^{-1} for H and L, respectively).

Concerning the WUE of overall 1st and 2nd ethanol production (WUE_{1G+2G}), all the investigated factors resulted significant. In particular, S506 performed almost 20% higher than B133, while high water availability yielded 30% more than low availability. The overall efficiency of ethanol achieved through 1st and 2nd generation process, weighed on the amount of water consumed, increased during crop cycle, reaching the

highest value at 151 DAS (HT3) with a WUE_{1G+2G} of 2.7 g L^{-1}. This was statistically indifferentated from HT2 (2.5 g L^{-1}), which in turn was significantly higher than HT1 (1.6 g L^{-1}).

In general, all WUE calculations described biomass sorghum as a water efficient crop, especially when plant growth extended until 151 DAS. Besides, the higher WUE's observed in H vs L level demonstrates the good capacity to convert abundant water into biomass, juice and, ultimately, ethanol.

Table 3.3 - Water use efficiency of juice (WUE_{juice}), dry biomass yield (WUE_{TDW}), 1st generation ethanol (WUE_{1G}), 2nd generation ethanol (WUE_{2G}), combined 1st and 2nd generation ethanol (WUE).

Source	WUE_{juice} (g L^{-1})	WUE_{TDW} (g L^{-1})	WUE_{1G} (g L^{-1})	WUE_{2G} (g L^{-1})	WUE_{1G+2G} (g L^{-1})
Genotype (G)					
S506	2.7	3.8	2.2 a	0.27	2.5 a
B133	2.7	3.8	1.8 b	0.27	2.0 b
P	n.s.	n.s.	**	n.s.	**
Water level (WL)					
H	3.2 a	4.0	2.6 a	0.29 a	2.9 a
L	2.2 b	3.6	1.4 b	0.25 b	1.7 b
P	**	n.s.	**	**	**
Harvest (HT)					
HT1	1.7 a	3.4 c	1.3 c	0.27	1.6 c
HT2	2.7 b	4.1 a	2.2 b	0.28	2.5 b
HT3	3.7 c	3.9 b	2.4 a	0.27	2.7 a
P	**	**	**	n.s.	**
P (G×WL)	n.s.	n.s.	n.s.	n.s.	n.s.
P (G×HT)	n.s.	n.s.	**	**	**
P (WL×HT)	**	**	**	**	**
P (G×WL× HT)	n.s.	n.s.	**	n.s.	**

In significant traits, different letters indicate statistically different data according to the LSD test ($P < 0.05$). Each data is the average of two replicates.

3.4 Conclusions

The two genotypes of biomass sorghum did not produce different TDW, thus resulting in similar $EtOH_{2G}$ amount. However, S506 demonstrated to have a juice better suited for $EtOH_{1G}$, producing higher concentration of ethanol per volume of fermented juice. The overall quantity of ethanol produced, indicated as $EtOH_{1G+2G}$, was still higher in S506, which is due to the difference between $EtOH_{1G}$ amounts observed in the two hybrids. Low water availability decreased TDW, cellulose and AIL, thus resulting in slightly lower $EtOH_{2G}$ concentration compared to high water availability, but in extremely lower $EtOH_{2G}$ amount, due to the strong difference in TDW between H and L water levels.

During crop cycle, the amount of cellulose, hemicellulose and AIL changed causing a decrease of $EtOH_{2G}$ amount obtained from biomass through SSF. Biomass physical and chemical properties involved a lower glucose yield and concentration at the end of enzymatic hydrolysis and, consequently, a lower $EtOH_{2G}$ concentration at the end of SSF, hence proving that there is strong relationship between biomass structure, chemical composition, and fermentable sugar yield.

The increase of TDW during crop growth was accompanied by a decrease in ethanol concentration and yield at the end of SSF, indicating that the best time to harvest both hybrids was at the end of crop cycle. Nevertheless, the significantly higher concentration of ethanol at the early crop stage could be an important advantage to consider biomass sorghum as second crop in the season: its introduction into some agricultural systems could benefit farmers and, above all, avoid the exacerbation of the debate about fuel vs food crops. Moreover, high values of WUE_{2G} and WUE_{1G+2G}, compared to WUE1G, reduce the strife for water use when growing biomass sorghum for advanced biofuel production.

General conclusion

The aim of this dissertation was to gain the suitability of calcium hydroxide as impregnation catalyst before steam explosion for second generation bioethanol production. Adding to this, effects of variable soil moisture and harvest time on biomass growth and composition of two genotypes of biomass sorghum were addressed.

Calcium hydroxide used before steam explosion on sugarcane bagasse, demonstrated to produce a low concentration of inhibiting compounds and remove rather well lignin. Compared to autohydrolysis process, an increase in lignin removal of 25 and 33% for low and high calcium hydroxide concentration, respectively, was observed. This reflected in easier cellulose degradation during enzymatic hydrolysis of solid pretreated material. In fact, calcium hydroxyde substantially contributed to improving cellulose conversion into glucose during enzymatic hydrolysis: as a result, yields of 78 and 75% were evidenced for low and high lime concentration, respectively, compared to 68% for pretreatments with no lime addition. However, differences among ethanol concentration produced at the end of simultaneous saccharification and fermentation were relatively small. The methane produced through anaerobic digestion of pretreament liquids showed that calcium hydroxide is more suitable than the autohydrolysis process. When the total amount of energy produced under various forms was focused (ethanol, methane and the energy content of solid residue), high calcium hydroxide concentration attained top level, followed by no and low calcium hydroxide addition prior to steam explosion. Thus, in a biorefinery prospect, lime represents a favourable option to improve ethanol yield from sugarcane bagasse.

On switchgrass, as described in chapter 2, the effect of calcium hydroxide was compared with autohydrolysis process and sulphuric acid, too. Calcium hydroxide

demonstrated to better remove lignin than no lime addition and sulphuric acid catalyst, and a maximum value of 34.6% was reached in lignin removal at high lime concentration. However, enzymatic hydrolysis on pretreated solid showed that calcium hydroxyde did not improve glucose release. In simultaneous saccharification and fermentation, autohydrolysis attained a ca. 10% lower ethanol concentration (29.8 g L^{-1}) than $Ca(OH)_2$ addition at 0.4% (33.5 g L^{-1}), while high calcium hydroxide concentration and acid catalyst featured an intermediate 31.5 g L^{-1}. Concerning the anaerobic digestion on pretreatment liquid, high calcium hydroxide concentration produced the highest value of CH_4 g^{-1} VS, yielding 7% more than acid catalyst, in turn 20% more productive than low calcium hydroxide concentration. Combined energy yield (ethanol, methane and residual solid) showed pretreatment effects referred to unit dry weight of raw switchgrass biomass: energy from ethanol increased from 3.2 kJ g^{-1} in autohydrolysis to 4.2 kJ g^{-1} with low calcium hydroxide concentration, whereas calcium hydroxyde at high concentration and acid addition did not improve this trait with respect to autohydrolysis. So calcium hydroxyde showed a great potential when ethanol was focused, whereas acid addition produced higher methane yield. In terms of combined energy yield, autohydrolysis and the two strong pretreatments were substantially equivalent (7.2, 7.2 and 7 kJ g^{-1} in autohydrolysis, high calcium hydroxide concentration and acid addition, respectively), while low lime concentration was top ranking (8.3 kJ g^{-1}).

Among the effects of climate change, the water resource depletion strongly affects crop yields. As one of the tools to mitigate climate change is the cultivation of dedicated bioenergy crops, the competitiveness between energy and food crops for land and water use is expected to increase. Given these premises, the study described in chapter 3 was performed to determine the effect of different water levels and harvest times on the

growth of two genotypes of biomass sorghum, on the characteristics of their biomass, and to devise the most suitable practice to achieve the maximum yield in terms of both first and second generation bioethanol. In this experiment it was observed that water availability increased dry biomass yield and juice amount, and the highest yields of both juice and biomass were obtained at the end of crop cycle. Low water availability caused an 18% decrease in 1^{st} generation ethanol concentration. The combined differences in juice amount and 1^{st} ethanol concentration originated a strong difference, more than two fold, between the amounts of 1^{st} generation ethanol with high and low water availability.

Water level did not show any effect on the suitability of biomass to be hydrolysed, while glucose release during enzymatic hydrolysis decreased along crop cycle. Even ethanol concentration in simultaneous saccharification and fermentation resulted affected by harvest time. However, due to the increase of dry biomass yield during time, 2^{nd} generation ethanol amount increased in time: in fact, it augmented by 33 and 46% in the second and the last harvest, respectively, compared to 4.0 g pot^{-1} registered in the first harvest. Likewise, the effect of high water availability on the 2^{nd} generation ethanol amount caused an increase of 45% between low and water level. Nevertheless, the significantly higher concentration of ethanol at the early crop stage could be an important incentive to consider biomass sorghum as second crop in the season, to be introduced into some agricultural systems, to the benefit of farmers and, above all, to avoid the exacerbation of the debate about fuel vs food crops.

Experimental results showed that calcium hydroxide before steam explosion is a favourable catalyst in 2^{nd} generation bioethanol process to achieve high energy yields. So in view to the commercialization of 2^{nd} generation bioethanol plant, this catalyst can reduce the water consumed in the pretreatment step, assuring high energy yields. In the

last experiment described, biomass composition and glucose release thorough enzymatic hydrolysis decreased during crop cycle, while water availability did not have any effect. This work demonstrated that water consumption in the 2nd generation bioethanol process can be reduced. However, in order to promote a low water consumption and increase the efficiency of its use in both field and process step, still careful evaluations and studies under the energetic, environmental and economic viewpoint are needed.

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