INSTITUTO SUPERIOR TÉCNICO
Universidade Técnica de Lisboa

Dilute acid and enzymatic hydrolysis of sugarcane bagasse for biogas production

Rui Nuno Leitão de Carvalho

Dissertação para obtenção do Grau de Mestre em

Engenharia Biológica

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Outubro de 2009

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Finally, my sincere gratitude to my closest family – my mother and my cats Lancelote and Carolina – for all their love and support, which were two vital ingredients in the elaboration of this work.

Cover image – scintillation vials containing sugarcane bagasse being enzymatically digested.

Abstract

The depleting fossil fuel resources and the environmental problems with greenhouse gases have sprung forth the awareness of the importance of renewable and cleaner sources of energy, such as biogas produced from lignocellulosic biomass. Sugarcane bagasse is a cheap and abundant raw material which can be used for this purpose. However, this biomass is resistant to microbial attack and must be previously pretreated.

In the present work, sugarcane bagasse was subjected to dilute sulphuric acid treatment and enzymatic hydrolysis. The acid treatment was performed in an autoclave, at 121ºC with 0.95% (w/w) of acid. The parameters varied were residence time, bagasse particle size and the addition of a supplemental post-treatment incubation period. In the enzymatic reaction were tested the role of enzyme loadings and the effect of some parameters of the acid treatment in the bagasse's enzymatic digestibility. The most relevant products resulting from these treatments were analyzed for their biochemical methane potential.

The obtained saccharifications were low, possibly due to a combination of bagasse recalcitrance and weak acid hydrolysis. Carbohydrate recovery in the acid hydrolysate was almost 40%, mostly in the form of xylose. Less than 14% of the pulp's total carbohydrates were hydrolyzed by enzymatic action. However, this hydrolysate possessed a good biochemical methane potential, seemingly promising for biogas production.

Some relevant features limiting the overall treatment effectiveness were identified, paving way for future studies to significantly improve this process.

Keywords: Biogas; Sugarcane bagasse; Pretreatment; Dilute Acid; Enzymatic Hydrolysis; Biochemical methane potential.

Resumo

A depleção das reservas de combustíveis fósseis e os problemas ambientais com os gases de efeito de estufa impulsionaram a sensibilização para energias renováveis e mais limpas, tais como o biogás produzido a partir de matérias lenhocelulósicas. O bagaço de cana-de-açúcar é uma matéria-prima barata e abundante que pode ser usada para este fim. No entanto, esta biomassa é resistente ao ataque microbiano e tem de ser pré-tratada.

Neste trabalho, bagaço de cana-de-açúcar foi sujeito a tratamento com ácido sulfúrico diluído e a hidrólise enzimática. O tratamento ácido foi efectuado numa autoclave a 121ºC, com 0.95% (p/p) de ácido. Os parâmetros variados foram: tempo de residência, tamanho das partículas de bagaço e a adição de um tempo de incubação suplementar pós-tratamento. Na reacção enzimática foi testado o papel da carga de enzima e o efeito de alguns parâmetros do tratamento ácido na digestibilidade enzimática do bagaço. Os produtos destes tratamentos considerados mais relevantes foram analisados em termos do seu potencial bioquímico de metano.

As sacarificações obtidas foram baixas, possivelmente devido a uma combinação de recalcitrância do bagaço e hidrólise ácida pouco severa. A recuperação de carbohidratos no hidrolisado ácido foi quase 40%, sobretudo sob a forma de xilose. Menos de 14% dos carbohidratos totais da polpa foram hidrolisados por acção enzimática. No entanto, este hidrolisado possuía um bom potencial bioquímico de metano, parecendo prometedor para a produção de biogás.

Foram identificados alguns factores relevantes que limitaram a eficácia dos tratamentos, abrindo caminho para que estudos futuros melhorem significativamente este processo.

Palavras-chave: Biogás; Bagaço de cana-de-açúcar; Pré-tratamento; Ácido Diluído; Hidrólise enzimática; Potencial bioquímico de metano

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1. Biofuels

1.1 General

Fossil fuel resources have been for a long time the main energy source to supply the world's needs. These needs have kept on increasing, but oil reserves are limited and it's expected a great decline in worldwide production of crude oil in the near future (Sun *et al.*, 2002). Furthermore, its use causes a great environmental impact in the emission of greenhouse gases (GHG). Those reasons have aroused interest in the research of alternative and renewable energy sources.

Biomass has arisen as an interesting renewable source of fuel which, even if it cannot completely replace oil, will greatly help to lower our actual dependence on fossil fuels. Biomass can be defined as "*all organic material of vegetal or animal origin, which is produced in natural or managed ecosystems (agriculture, aquaculture, forestry), all or not industrially transformed*" (Vandame, 2009). The most widely known fuels derived from it – biofuels – are ethanol, biodiesel and biogas. Liquid fuels are currently the most popular, with ethanol being produced in large scale in Brazil (UN-Energy, 2007) and biodiesel gaining recognition in Europe, as a replacement and for blending with mineral diesel. So far, biogas production and use has been the smallest out the three, though countries like Germany and Sweden have been actively researching its exploitation and paving way for its purification and use as a vehicle fuel (Gasföreningen, 2008).

The main advantages of biofuels are the fact that are renewable in a sustainable time frame and that they are theoretically carbon neutral, meaning that the carbon dioxide emissions of biofuel burning are captured again by the plants and serve as raw material for its production. Biofuels can be classified as first, second and third generation, according to the origin and processing of their biomass (Figure 1.1).

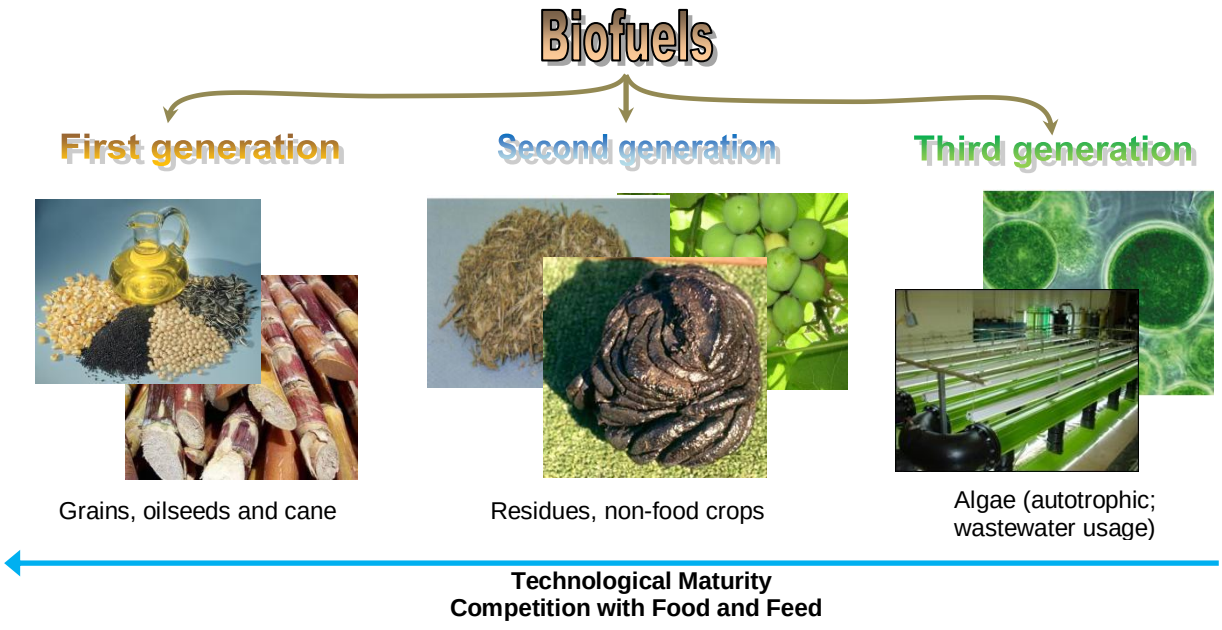

Figure 1.1 – Comparison of first, second and third generation biofuels in terms of raw materials, technological maturity and competition with food crops.

First generation biofuels are made using feedstocks such as seeds or grains from cereals, which yield starch, or sugarcane, from which sugar is extracted. These carbohydrates can be directly fermented into ethanol. Oilseeds can be pressed to yield vegetable oil passible of being transformed into biodiesel. Since these feedstocks could instead enter the animal or human food chain, there has been a growing debate on whether the usage of these fuels could compete with food, increasing prices and lowering availability (UN-Energy, 2007). Also, with the exception of cultivating sugarcane in warm climates (like Brazil's), production of first generation biofuels is far from an ideal closed carbon cycle, since there is a significant petroleum usage during the whole process (to make fertilizers, power farm equipment, transport feedstocks), which make greenhouse gas reductions in the order of 20% to 50% (IEA, 2004) (Figure 1.2).

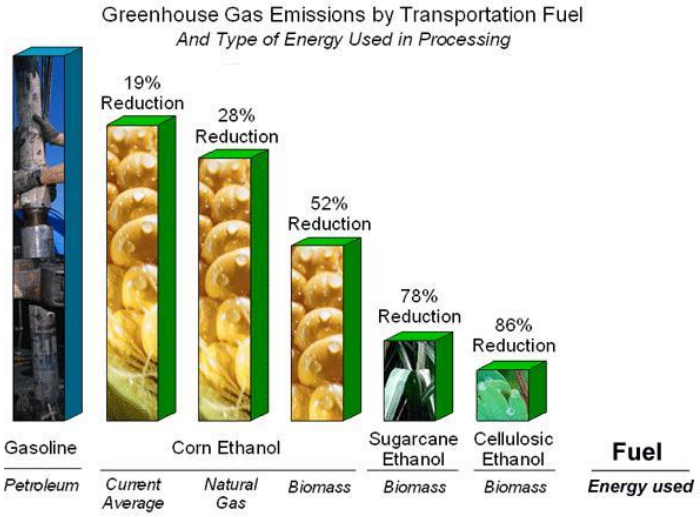

Figure 1.2 – Reductions of GHG emissions by first generation (American corn and Brazilian sugarcane) ethanol and second generation (cellulosic) ethanol (adapted from Wang *et al.*, 2007)

The appearance of second and third generation biofuels came as a possible solution to avoid direct competition for commodities, while benefiting from increased GHG reductions. Second generation biofuels are produced from non-food crops or waste materials, such as food wastes, manure and agricultural residues. Third generation biofuels use algae to produce carbohydrates and lipids, which can be used for producing bioethanol and biodiesel, respectively. This technology is still not very mature, but has potentially very high yields per terrain usage, while not displacing terrain for food production (Brigs, 2004).

Some of the raw materials used by second generation biofuels are crops that are not used for food purposes, such as switch grass, jatropha and cereals that bear little grain. However, if farmers can make more money by cultivating crops for biofuel, most likely they will partially abandon food crops, thus raising the 'food vs fuel' dilemma again (Brown, 2006). The use of residues doesn't suffer from this problem. Used frying oils can be converted to biodiesel, while manure, food wastes and wastewater can be used for biogas production. The non-food parts of current crops, such as stems, leaves and husks (lignocellulosic parts) are largely underused, being simply burnt or used for the paper and pulp industry. However, they have also a great potential for being converted into biofuels like ethanol and biogas.

1.2 Biogas

Biogas is a gaseous mixture of methane (45-85%) and carbon dioxide, with smaller amounts of other gases, such as nitrogen, ammonia and hydrogen sulphide. Its production has always been linked to residue utilization, as its first widespread production was from the anaerobic digestion of domestic sewage. Ever since the first oil shocks, there was an increased interest in producing this fuel from other sources of organic material, such as manure and industrial wastewaters from sugar and 'pulp and paper' factories. A great deal of biogas plants were built in Sweden in the 1980s and 1990s. Its main usage has been burning for generating heat and electricity, but more recent technological developments have allowed the upgrading of biogas (removal of CO_2 and minor gases), to yield a 95% CH_4 gas called bio-methane, suitable for use as vehicle fuel, substituting natural gas (Figure 1.3).

Figure 1.3 – Swedish vehicles powered by biogas. Left - Car in front of the biogas plant at Linköping; center – Bus in Stockholm; right – Biogas train "Amanda", operating on the line between Linköping and Västervik (adapted from Gasföreningen, 2008).

The countries which have invested the most on biogas have already a significant number of plants, most of which are sewage treatment plants, and several landfills from which biogas is recovered. Biogas from landfills has usually less methane content than gas produced in digesters, as the conditions for methane production cannot be optimized in the same way they are inside the controlled environment of a digester. Therefore, digester gas had usually more than 55% methane, while landfill gas has 45-55%. The amount of gas produced from agricultural and industrial wastes is still small, but has seen a steady increase in the last years. Digester technology also allows a local production, with farmers being able to have their own small digesters, coupled to combined heat and power generators. Anaerobic digestion produces a solid residue (bio-manure) usable as a fertilizer, which represents an additional saving in petroleum use.

Like other countries with very active biogas research, the current trend in Sweden is an increasing biogas demand, which has been superior to supply. It is currently the country with the highest amount of biogas upgrading plants, with a growing network of distribution lines and filling stations (the later reached 100 on August 2009) (Gasföreningen, 2008).

This demand can only be satisfied by an increase in production supported by biomass from crops. Several studies have concluded that the potential production in Sweden is 10 terawatts hours) (Linné and Jönsson, 2004). Large scale plants are already being planned, with capacities of 50 gigawatt hours per year.

Biogas produced from crops and wood will play an important role in this production expansion, thus the importance of investing and researching the methanization of these lignocellulosic feedstocks.

2. From Lignocellulose to Biogas

Biogas production directly from lignocellulosic materials is slow and has a low yield, so many types of pretreatments are being studied and compared in order to improve this factor. This includes research on the characterization of lignocelluloses and the effect of several of their structural properties in pretreatment and anaerobic digestion effectiveness.

2.1 Lignocellulose Structural Features

2.1.1 General

All plant cells are surrounded by a polysaccharide-rich wall, which provides support, strength and shape to the plant. This structural material in the cell wall is known as lignocellulose. It is composed mainly of cellulose, hemicellulose and lignin, possessing smaller amounts of pectin, proteins, extractives and ash. These components are present in different proportions at different parts of the wall, with the outer wall (primary wall) being composed mainly of lignin and the inner secondary wall containing the majority of the carbohydrates in biomass (Figure 2.1) (Carpita *et al.*, 2001; CCRC, 2007). This distribution varies with the plant species, age, growth conditions and with certain parts of the plant (Jørgensen *et al.*, 2007).

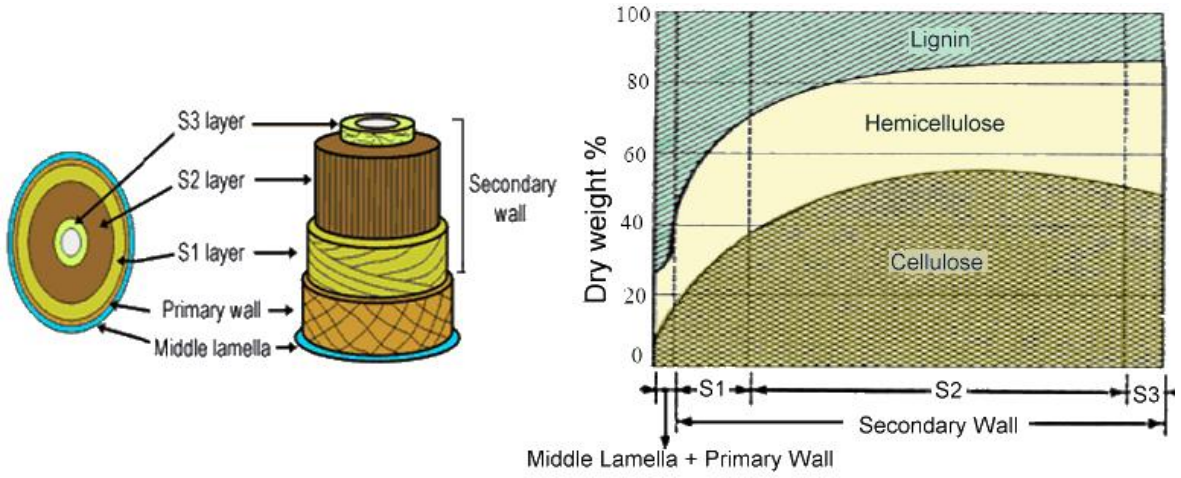

Figure 2.1 – Cell wall layers and distribution of cellulose, hemicellulose and lignin in a typical plant cell wall (adapted from CCRC (2007) and Panshin *et al.* (1980)).

Lignocellulosic materials can be grouped in three groups, according to the type of plant of origin: from softwoods (conifers), from hardwoods (angiosperm trees) and from grasses (Poaceae family). The latter includes grain crops, lawn grasses and leaf and stem crops such as the sugarcane.

Lignin content of grasses is usually lower than either softwoods or hardwoods. The cellulose and hemicellulose contents can vary considerably, and the differences often lay more in the simple sugars that compose hemicellulose than the macro component percentage itself.

2.1.2 Cellulose

Cellulose is a linear polymer of D-glucose units connected by β-1,4 glycosidic bonds, which accounts for about 40-50% of biomass. The degree of polymerization can go from 500 to 15000 (Holtzapple, 1993). The orientation of these bonds allows the formation of intra- and intermolecular

hydrogen bonds, resulting in the aggregation of 36 chains into elementary crystalline microfibrils. The hydrophobic nature of the cellulose surface results in the formation of a dense layer of water that may hinder diffusion of enzymes and degradation products in its surroundings (Matthews *et al.*, 2006).

The microfibrils are imbedded in a matrix of mostly hemicellulose and pectin, and are covered with lignin. This results in the association of several microfibrils into macrofibrils, which are arranged into bigger cellulose fibers (Figure 2.2) (Delmer *et al.*, 1995). This highly structured configuration gives cellulose a high tensile strength, insolubility in most organic solvents and resistance to enzymatic (and microbial) attack (Ward *et al.*, 1989).

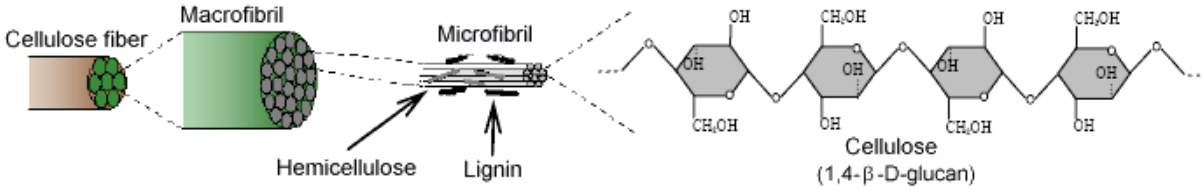

Figure 2.2 – Structure of cellulose (adapted from Taherzadeh *et al.* (2008)).

Although the major part (about 2/3) of cellulose is in its crystalline form (Chum *et al.*, 1985), microfibrils also have amorphous areas, where the several chains lose their parallel orderly fashion and acquire a much more disorganized orientation. The chain molecules pass successively through crystalline and amorphous areas, usually beginning and ending with the first configuration (Figure 2.3) (Panshin *et al.*, 1980).

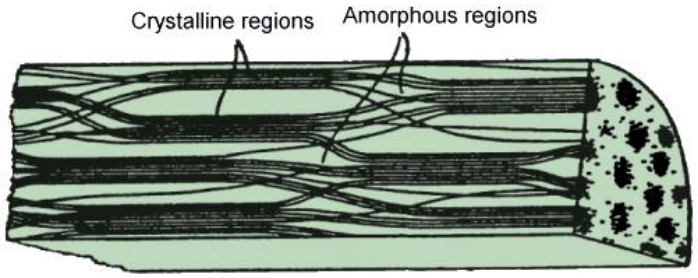

Figure 2.3 – Cross view of a macrofibril, showing microfibrils in crystalline and amorphous conformations (adapted from Panshin *et al.* (1980)).

It is widely accepted that the higher the crystalline content of cellulose is, the more difficult is the enzymatic attack to hydrolyze this polymer. The amorphous regions are more accessible to enzymes and are therefore more easily hydrolyzed, while in the crystalline areas enzyme contact efficiency is decreased (Chang *et al.*, 2000). Acid hydrolysis of cellulose has also been reported to be more effective when the substrate crystallinity was decreased (Han *et al.,* 1983). Consequently, one strategy to improve the digestibility of cellulose is to reduce its degree of crystallinity.

However, some studies contradicted these results, reporting that crystallinity had no influence of digestibility, and that it was the increased internal surface area (pore volume) that accompanied the decrease in crystallinity that was responsible for the observed increased in cellulose digestion (Puri, 1984; Sinitsyn *et al.*, 1991). This apparent conflict in results comes from the fact that one treatment acts on more than one feature of cellulose: particle size (external area), pore size volume (internal area), crystallinity, degree of polymerization are often connected, making it difficult to analyze one factor separately. In any case, crystallinity may be an important factor in cellulose digestibility, but due

to the heterogeneous nature of this material, it is only one of the possible factors that affect it (Taherzadeh, 2008).

Surface area is almost always linked with crystallinity, but most researchers consider it a crucial factor for effective cellulose degradation. The accessible surface area available can be limiting in the hydrolysis step, since cellulase activity requires direct physical adsorption onto the target molecules surface (Sun *et al.*, 2002). There are two types of surface area: external surface area is related to the size and shape of particles, while the internal surface area is dependant of the pore size and the capillary structure of the fibers. While dry fibers have usually large external specific surface areas, their specific internal surface area is often much smaller. That area can be increased by swelling the fibers, by wetting them with water or other polar solvents. However, water is known to cause some re-crystallization of highly amorphous cellulose. Drying the fiber can result on the irreversible collapse and shrinking of the capillary structure of the fibers, reducing the surface area (Fan *et al.*, 1980).

2.1.3 Hemicellulose

Hemicellulose is a complex, highly branched polysaccharide, which is found associated with cellulose and it's commonly about 30% of biomass weight. Unlike cellulose, hemicellulose polymers are chemically heterogeneous, have lower degrees of polymerization (under 200) and are mostly amorphous. The basic monomeric residues present are xylose, arabinose, mannose, glucose, galactose, glucuronic acid and its methyl ether. Hemicelluloses are classified according to the sugar that composes the backbone. In softwoods mannan (main sugar is mannose) is present, and in hardwoods and grasses it's xylan (Jørgensen *et al.*, 2007; CCRC, 2007).

Xylan has a backbone of 1,4-β-linked xylose residues, several of which are acetylated (Kuhad *et al.*, 1997). Despite homoxylans (linear and unsubstituted) having been reported, the most common is to find heteroxylans, which possess small ramifications containing other sugars or acids. According to the abundance of the other residues, xylans are further classified into subgroups. Grasses are composed mainly of glucuronoarabinoxylans, having arabinose and glucuronic acid as the most abundant substituents (Carpita, 1996; Saha, 2003).

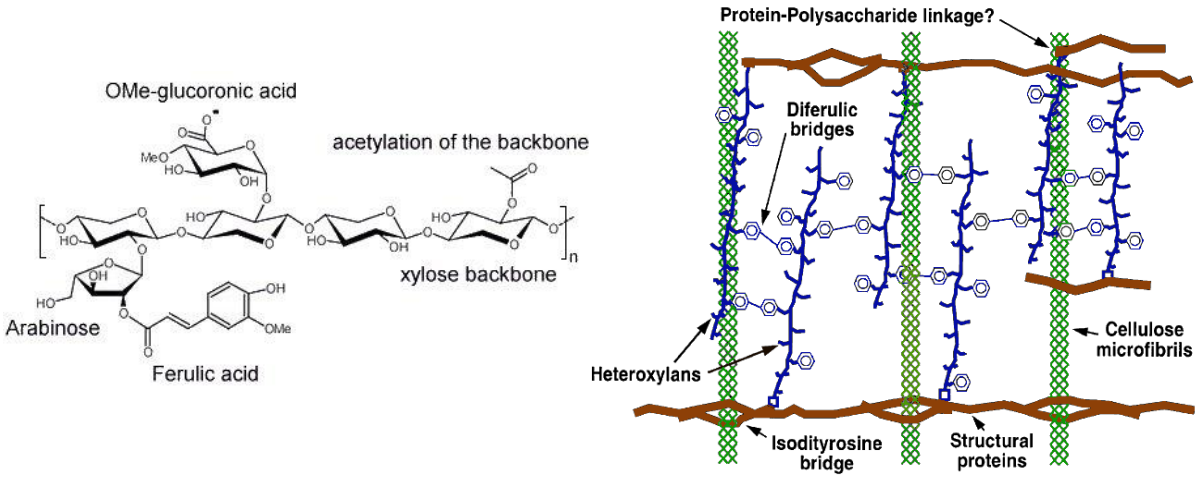

Figure 2.4 – Basic structural composition of a glucuronoarabinoxylan (left) and model of corn fiber cell walls, depicting the binding effect played by xylans on cellulose microfibrils (adapted from Hövel *et al.* (2003) and Saha (2003)).

Heteroxylans can crosslink with each other, adsorb to cellulose, bind to structural proteins and lignin, forming part of the matrix in which cellulose is embedded. It forms a physical barrier to microbial and enzymatic attack on cellulose (Figure 2.4). Its acetyl content has the added effect of sterically hindering enzyme activity, interfering with substrate recognition. That applies not only to cellulose, but also to the enzymatic hydrolysis of hemicellulose itself (Chang *et al.,* 2000).

Hemicellulose has weaker bonds than cellulose, which can be extensively broken using suitable kinds of pretreatments, such as dilute acid hydrolysis. Removing hemicellulose by these methods greatly facilitates subsequent cellulose digestion (Saha *et al.*, 2005).

2.1.4 Lignin

Lignin is not a well defined and uniform compound, but a complex network of chemically similar substances with very different molecular weights. Lignins are highly branched polymers comprised of coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol, each of which has an aromatic ring with different substituents (Brown, 2003). Linking these monomers is a variety of bonds, such as alkyl-alkyl, alkyl-aryl and aryl-aryl ether bonds (Figure 2.5).

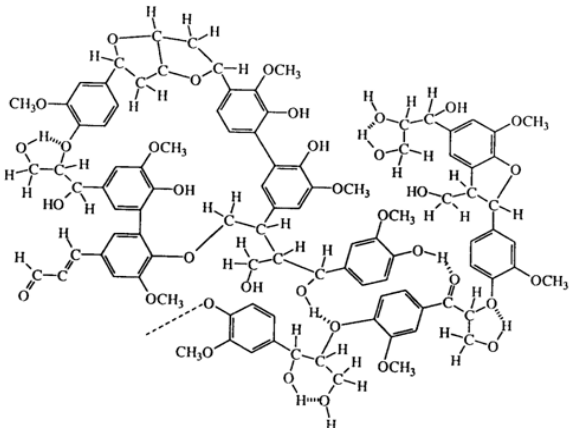

Figure 2.5 – Structure of a section of a lignin polymer (from Larson *et al.* (1994)).

Lignin is able to form covalent bonds with residues from hemicellulose. This way, this component provides integrity, structural rigidity and prevention of swelling of lignocelluloses. It is commonly accepted as one of the major factors responsible for biomass recalcitrance. In fact, there are various ways in which lignin can difficult digestion, such as enzymatic sterical hindrance and prevention of fiber swelling, the later being an important factor to increase internal surface area (Mooney et *al.,* 1998). This effect can vary with lignin composition, since softwoods are frequently more recalcitrant than hardwoods. It is thought that this is caused by the greater amounts of guaiacyl subunits present in softwoods, as opposed to the mixture of guaiacyl and syringyl subunits of hardwoods (Ramos *et al*., 1992).

Furthermore, dissolved lignin is known to inhibit cellulases and xylanases, which further complicates digestion (Berlin *et al*, 2006).

To summarize, there are several structural features that can influence digestion. They can be grouped into physical and chemical features. There is still some disagreement of the effect of some of the features, but the common reported results are presented on Table 2.1.

Table 2.1 - Summary of relationships between structural features and digestibility (adapted from Zhu, 2005).

Structural features		Reported relationships with digestibility
Physical	Surface area	Favorable
	Crystallinity	Unfavorable / No Correlation
	Degree of polymerization	Unfavorable / No Correlation
	Pore volume	Favorable
	Particle size	No Correlation
Chemical	Lignin	Unfavorable
	Hemicellulose	Unfavorable
	Acetyl groups	Unfavorable

2.2 Sugarcane Bagasse

Out of the many agricultural residues available, sugarcane bagasse has a great biotechnological potential, which is partially due to its abundance, since it is one of the largest agro-industrial by-products, being produced in over 200 countries. Every year, more than 400 million tons of sugarcane bagasse are produced around the world (UNSD, 2005). There are currently several approaches to deal with this waste. Some of them involve using it for the pulp industry, while others incinerate it for obtaining electricity. More recent is the use of this biomass for biofuel production, namely ethanol and biogas. A study on the lifecycle analysis of four scenarios to manage this waste (landfilling with methane recovery, incineration, anaerobic digestion, pulp production) has considered anaerobic digestion of bagasse as one the methods that allowed a better energy recovery per ton of biomass, while having the best environmental performance of the three energy recovering scenarios analyzed (Kiatkittipong et al., 2009).

As far as terrain usage goes, bagasse also has high yields, rounding 80 tonnes of material per hectare (against 1,2 and 20 tonnes of wheat, other grasses and trees, respectively), and good annual regeneration capacity. Composition-wise, the average ash contents of 2.4% are attractive when employing bioconversion methods using microbial intervention (Pandey *et al*., 2000). Cellulose content is typically 39-42% and lignin 20-25%. Hemicellulose has been commonly observed in the 25% to 27% range (Gámez *et al.*, 2006; Bustos *et al.*, 2003, Yu *et al.*, 2008), although higher values like 36% have also been reported (Sasaki *et al.*, 2003; Cordova *et al.*, 1998).

Among other biotechnological applications, it's particularly interesting to note that bagasse has been used for the production of industrially important enzymes, such as cellulases and xylanases, the same enzymes that are responsible for an effective degradation of bagasse and other lignocellulosic materials (Parameswaran, 2009; Adsul *et al.*, 2004).

2.3 Biomass Pretreatments

2.3.1 Overview

Whether it's ethanol or biogas production, biomass is degraded by action of microorganisms, which in turn use enzymes in their metabolism. Polymer hydrolysis is the first phase in bio-digestion, and it's almost always the limiting step of the whole process. Consequently, to attain good productivities of biofuels is necessary to supply substrates which are easy targets for hydrolytic

enzymes' action. Lignocellulosic materials in their raw form, due to the structural features mentioned, are highly resistant to fermentation or anaerobic digestion. Therefore, there is a need to apply pretreatments which will act on these obstacles and pave way for a successful and extensive microbial degradation into the products of interest (Figure 2.6).

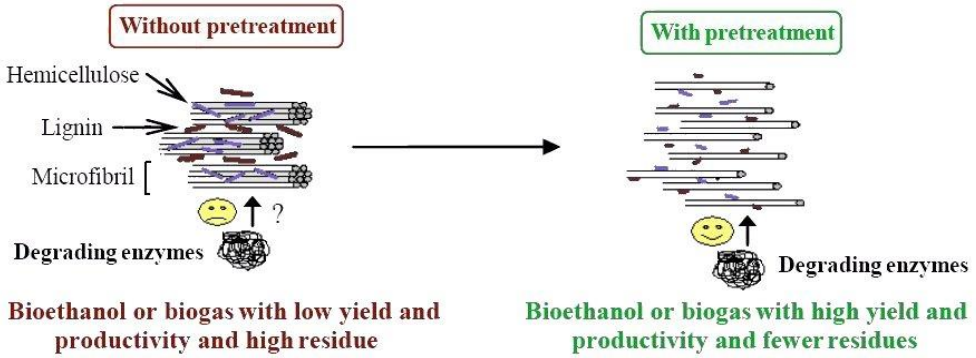

Figure 2.6 – Effect of pretreatment on degrading enzymes accessibility (adapted from Taherzadeh *et al.,* 2008).

The effects of pretreatments are usually focused on lignin and hemicellulose removal, cellulose crystallinity reduction and accessible surface area increase. There are some widely accepted requirements that pretreatments should meet (Sun *et al.*, 2002):

- Improve the formation of sugars or create a reactive cellulose fiber for enzymatic attack;
- Minimize the degradation or loss of carbohydrates;
- Avoid formation of byproducts that are inhibitory to enzymatic/microbial activity;
- Minimize the use of energy, chemicals and capital equipment to produce a cost-effective process that is scalable to industrial size.

Over the last years many different pretreatment methods have been researched. They can be grouped into physical, physico-chemical, chemical and biological methods (Figure 2.7).

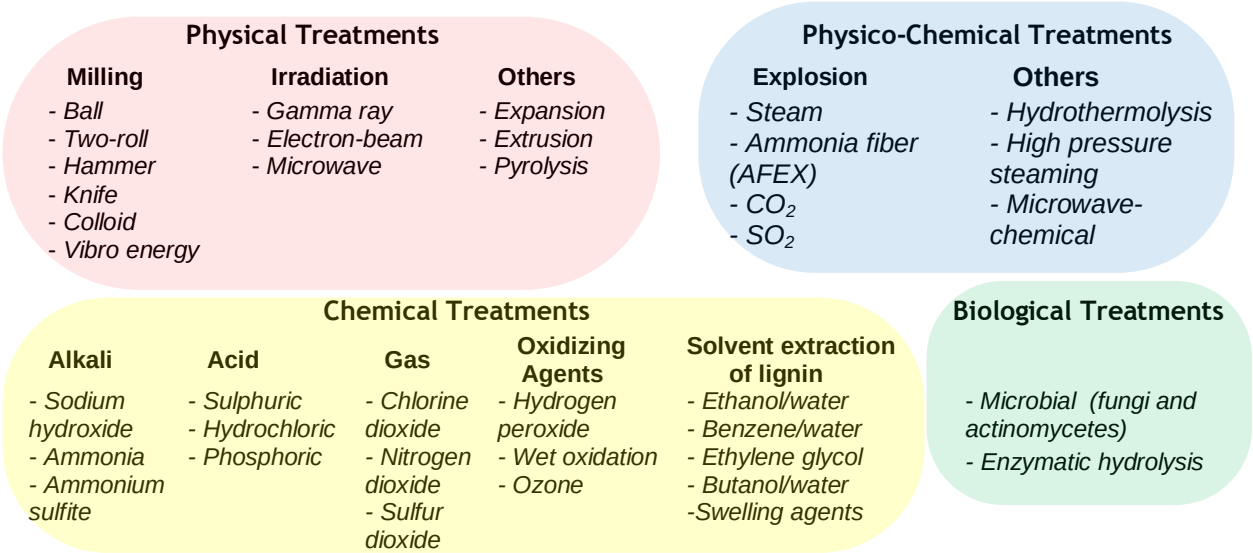

Figure 2.7 – Most common pretreatment processes of lignocellulosic materials.

The extent to which individual technology is studied varies greatly, with some processes being well-researched and already in applied in pilot scale, while others still haven't got past the laboratorial stage. It's also important to remark that there is a great diversity of lignocellulosic materials. Technologies and conditions that work well with one certain type don't necessarily generate the same

kind of results in a different type of material. Still, it is possible to collect individual data and get a profile on the strong and weak points of a treatment.

2.3.2 Physical Pretreatments

Physical pretreatments affect the physical structural features of biomass, meaning they act on reducing crystallinity, increasing pore size and reducing degrees of polymerization. They can involve mechanical methods (milling), radiation or pyrolysis. One of the advantages of physical pretreatment is that it is relatively insensitive to the physical and chemical characteristics of the biomass employed.

2.3.2.1 Milling

When milling, the final target size of the materials is usually from 2 to 0.2 mm. This size reduction is accompanied by a sharp decrease in cellulose crystallinity, making it more accessible to cellulases. It is also thought that it disrupts lignin-carbohydrate complexes, which further aids the enzymatic hydrolysis (Mais *et al.*, 2002). There are several types of milling, such as ball-milling, knife-milling and hammer-milling. The choice of what method to use lays in the properties of the starting material. Processes like colloid-milling are only suitable for wet materials, while hammer-milling can be more favorable when treating dry solids such as waste paper (Walpot, 1986). This often comes down to the energetic cost of the operations. Overall, energy consumption increases exponentially with the decrease in the final particle size. Still, some types of milling are high energy consumers with some biomass types, but behave much better with others (Table 2.2). Still, milling is an energy-intensive process, which is one of the most important limitations for wide-scale industrial application.

Table 2.2 – Comparison of energy requirements of knife and hammer-milling of lignocellulosic materials, with different size reductions (adapted from Sun *et al.*, 2002)

Lignocellulosic Materials	Final Particle Size (mm)	Energy Consumption (kWh/ton)	
		Knife mill	Hammer mill
Hardwood	1.60	130	130
	2.54	80	120
	3.20	50	115
	6.35	25	95
Straw	1.60	7.5	42
	2.64	6.4	29
Corn Stover	1.60	*n/a*	14
	3.20	20	9.6
	6.35	15	*n/a*
	9.50	3.2	*n/a*

n/a – not available

Smaller particles are better digested for biogas production, resulting in greater solids reduction and biogas yields. However milling is much more effective when combined with other pretreatments (e.g. ammonia, enzymatic hydrolysis) before being used in a digester (Zhang *et al.*, 1998). Processes consisting of milling followed by enzymatic hydrolysis or simultaneous milling and hydrolysis have been studied. In either case, it was observed increased saccharification of lignocellulose, even at relatively low enzyme concentrations (Sidira *et al*, 1989; Zeng *et al.*, 2007; Mais *et al*, 2002).

One limitation of milling is the fact that it is unable to remove lignin. This restricts the access of enzymes and inhibits their activity, preventing the hydrolysis of cellulose from reaching close to the maximum theoretical values.

2.3.2.2 Irradiation

Irradiation is a pretreatment which has been used alone and combined with other kinds of pretreatments, with an ultimate goal of accelerating enzymatic hydrolysis (Mammar *et al*., 1990).

In the presence of lignin, radiation affects directly the cellulose component, breaking up glucoside bonds, thus creating fragile fibers and low molecular weight oligosaccharides. Excess of radiation (above 100MR), however, can lead to the decomposition of glucose's ring structure. In studies involving irradiation pretreatments, subsequent enzymatic hydrolysis didn't improve if the biomass had no lignin. When using bagasse and following irradiation with acid or enzymatic hydrolysis, the saccharification yields doubled and quadrupled, respectively, when compared with non-irradiated bagasse (Kumakura *et al.*, 1983).

Studies with microwave radiation have been pursued due to their high heating efficiency and ease of operation (Zhu *et al.*, 2005b). Studies at elevated pressure applied on rice straw also resulted in a material much more susceptible to enzymatic hydrolysis. The key factor in this process is that in the presence of water and at temperatures above 175ºC, the acetic acid in hemicellulose is hydrolyzed and catalyzes the hydrolysis of hemicellulose and lignin. However, cellulose crystallinity was apparently unaltered (Azuma *et al.*, 1984). Later studies using sugarcane bagasse showed that microwave pretreatment could also be made at atmospheric temperature, by using a mixture of water/glycerine as the immersion medium for the lignocellulosic material. In both works, enzymatic hydrolysis of the treated material resulted in the recovery of about 50% of the carbohydrates as reducing sugars (Kitchaiya *et al.*, 2003).

The drawback to irradiation methods is they are expensive and difficult to apply on an industrial scale.

2.3.2.3 Pyrolysis

In pyrolysis, biomass is submitted to temperatures above 300ºC. This causes cellulose to rapidly decompose, resulting in gaseous products and residual char. At lower temperature, the reaction speed is lower, but so are the volatile products formed. Acid hydrolysis in mild conditions of the pyrolysis residues results in reducing sugars, amounting to 80-85% conversion of cellulose (Fan *et al.*, 1987). However, it is more common to use products of pyrolysis as fuels and not as a pretreatment step for ethanol or biogas production.

2.3.3 Chemical Pretreatments

2.3.3.1 Acid Hydrolysis

Acid hydrolysis of lignocellulosic materials is one the most investigated and well-documented pretreatment methods. A great variety of biomass has been treated with acids such as sulphuric, nitric, hydrochloric and phosphoric, with sulphuric being the most common.

There are two types of acid hydrolysis: one which uses concentrated acids and another which uses diluted acids. Concentrated acid hydrolysis takes place at low temperatures (e.g. 40ºC), using concentrated acids (30-72%). This method provides high sugar yields (nearly 100% of theoretical hexose yields) and has the low-temperature advantage. However, it requires large volumes of acid

(which must be recovered), which are toxic and corrosive, thus needing reactors highly resistant to corrosion. Furthermore, the acid stream produced must be neutralized, a process which originates a lot of gypsum. For these reasons, its commercial potential is small and little work has been made in this field (Wyman, 2003; Brown, 2003).

On the other hand, dilute acid hydrolysis has been extensively reviewed and is considered to be one of the treatment methods with greater potential for wide-scale application. It employs diluted acids (0.3-2%) and high temperatures (120-220ºC), with variable treatment times (some minutes up to an hour). It can be used as a pretreatment to increase cellulose accessibility to enzymes, or a method for direct saccharification. The latter is usually carried out at high temperatures, since cellulose hydrolysis is favored (McMillan, 1994).

Acid treatment works primarily on hemicellulose, hydrolyzing it to monomeric sugars to a great extent. Complete hemicellulose removal from the solids is even possible. Lignin is not significantly removed. Although some part of it is solubilized, it recondenses forming an altered lignin polymer (Torget et al., 1991). Still, this disruption and redistribution of lignin weakens the carbohydrate-lignin matrix, increasing cellulose accessible surface area (Yang *et al.*, 2004; Wyman, 2003). Effects on crystallinity vary. That's because, on one hand, chemical treatments remove amorphous lignin and hemicellulose components, increasing biomass crystallinity. On the other hand, they loosen the highly packed crystalline structure through swelling, and so decrease crystallinity (Gharpuray *et al.,* 1983). As such, the change in biomass crystallinity is dependent on which of the two effects is predominant.

Dilute-acid pretreatment can be performed either in short retention time (e.g. 5 min) at high temperatures (e.g. 180 °C) or in a relatively long retention time (e.g. 30-90 min) at lower temperatures (e.g. 120 °C) (Taherzadeh *et al.*, 2008). For a given material, the best conditions for hemicellulose removal and recovery in the hydrolysate don't always translate into the best enzymatic digestibility. Studies with olive tree biomass found 170 °C and 1% sulfuric acid to be the optimal conditions for hemicellulose recovery (83%), but the enzyme accessibility of the corresponding pretreated solid was not very high (enzymatic yield of 47.8% at 72h of reaction time). It was only at 210 °C with 1.4% acid concentration that the maximum enzymatic hydrolysis yield (76.5%) was obtained, but the hemicellulose recovery was minimal (<5%) (Cara *et al.*, 2008).

Different types of reactors have been applied to this treatment. Continuous processes usually employ lower solids loadings (5-10% w/w) and temperatures above 160ºC, while batch processes commonly have 10-40% solids loadings and temperatures below 160ºC Grohmann *et al.,* 1985).

Among the studies of dilute acid hydrolysis of sugarcane bagasse, Aguilar *et al.* (2002) studied the kinetics of this process and observed the variation with time of xylose, glucose, acetic acid and furfural. The experimental conditions tested were three different temperatures (100ºC, 122ºC, 128ºC) and sulfuric acid concentrations (2%, 4%, 6% (w/w)), at a solids loading of 10% (w/w). At temperatures of 122ºC and higher, xylose concentration showed a clear increase in the beginning, as the xylan degraded. It reached a maximum (at 20 min of treatment time), after which it started to decrease, due to degradation rates becoming significant. This was accompanied by an increase in furfural concentration. Both glucose and acetic acid quickly reached stable values, after which no further change was visible (Figure 2.8). Glucose degradation only happened at 128ºC, at very long

treatment times. This shows that to achieve the optimal conditions for maximum hemicellulose recovery, special care should be taken with reaction time, since there is only a relatively small time interval in which hemicellulose degradation has occurred to a very significant extent and sugar destruction is still small. The optimal hemicellulose recovery conditions in this work were 2% acid, 122ºC and 20 min of treatment, with a 92% recovery of xylan as xylose. At 100ºC and 128ºC, the highest values of xylose concentration were 19 and 16 g/L, respectively.

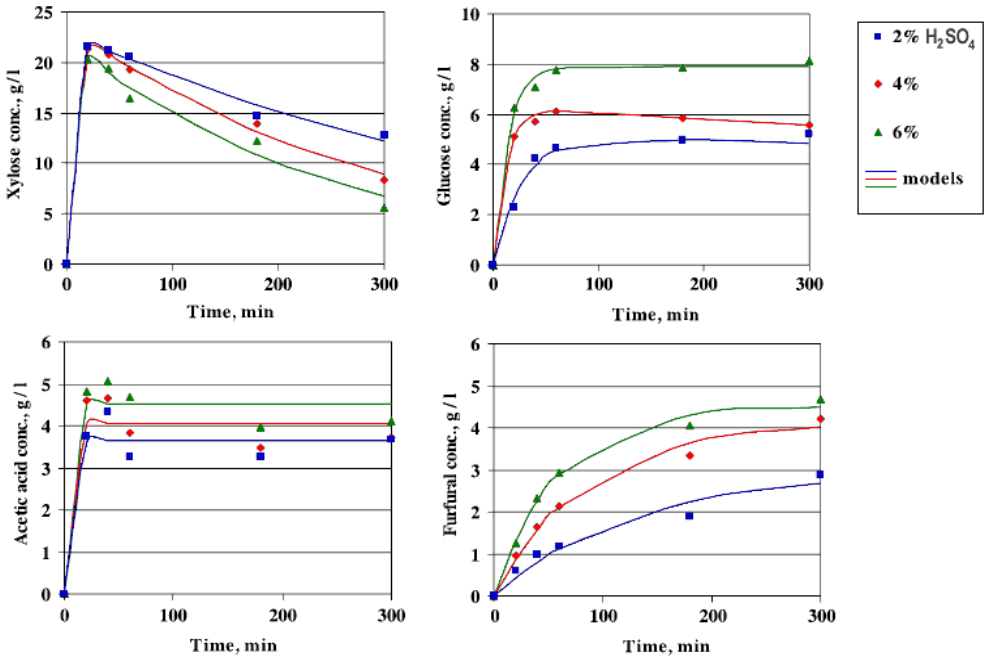

Figure 2.8 - Experimental and predicted dependence of the xylose, glucose, acetic acid and furfural concentrations on time, during dilute acid hydrolysis of sugarcane bagasse, at 122ºC and at several H_2SO_4 concentrations (adapted from Aguilar *et al.*, 2002).

Other acids have also been used to treat bagasse. Out of sulphuric, hydrochloric (Bustos *et al.*, 2003), nitric acid (Rodriguéz-Chong *et al.*, 2004) and phosphoric acid (Gaméz *et al.*, 2006), which were tested in the same range of temperatures and acid concentrations, treatment with phosphoric acid was the one which yielded the greatest ratio of sugars/inhibitors in the hydrolysates, even though the maximum hemicellulose conversions were observed with sulphuric acid.

Lavarack *et al.* (2002) also worked with sugarcane bagasse and sulphuric acid. Treatment parameters such as acid concentration, residence times and temperature were varied. The maximum xylose yields went up to 80% of theoretical values. In Martin *et al.* (2007a), sugarcane bagasse was hydrolyzed with 2% sulphuric acid, for 20, 40 and 60 min, achieving the highest (80%) xylose recoveries at longer times. The pretreated solid was subjected to enzymatic hydrolysis and 66% of the cellulose was converted to sugars.

In all the aforementioned works, xylose was always the most abundant sugar in the hydrolysates, with glucose present in smaller amounts. This showed cellulose in bagasse is little affected by dilute acid hydrolysis, while good removals of hemicellulose are obtained. Presence of significant concentrations of microbial growth inhibitors have been consistently observed, with acetic acid and furfural being the most relevant. Their formation is the major drawback of dilute acid hydrolysis. Even though they don't affect enzymatic hydrolysis, they are unwanted compounds in fermentations or digestions.

2.3.3.2 Alkaline hydrolysis

Lignocellulosic materials can also be treated with alkaline solutions, such as sodium hydroxide, lime or ammonia. Alkaline methods are primarily delignification methods, although there is also a significant solubilization of hemicellulose. (Millett *et al*., 1976). The mechanism of alkaline hydrolysis is the saponification of intermolecular ester bonds crosslinking hemicellulose and lignin. The removal of these linkages substantially increases biomass porosity and causes cellulose swelling (Tarkow *et al.,* 1969). Dilute NaOH hydrolysis also causes a decrease in polymerization degree and crystallinity (Fan et al., 1987). It is a treatment that can greatly increase enzymatic saccharification of biomass, and its effectiveness depends on the lignin content of the materials, having been shown to be more effective on agricultural residues than on wood materials (Xu *et al.*, 2007). Out of several treatments applied to cotton stalks, NaOH treatment (2% NaOH in 90 min at 121°C) returned the highest delignification (65%) and enzymatic cellulose conversion (60.8%) (Silverstein *et al.*, 2007).

The high cost of caustic soda and the need for its recovery are the main obstacles to its large-scale implementation. Therefore, pretreatments using aqueous ammonia and lime (calcium hydroxide) have been developed to try to reduce the process cost. These two chemicals are effective in removing both lignin and acetyl groups in biomass and have promising futures (Chang *et al.*, 1997).

Treatment with aqueous ammonia (25-28%) on sugarcane bagasse (20 min in autoclave at 120ºC) improved its enzymatic digestion by cellulases and xylanases, resulting in carbohydrate yields of 72.9% for cellulose and 82.4% for hemicellulose (Kurakake *et al.*, 2001).

Another process using aqueous ammonia, termed ammonia recycled percolation (ARP), was studied by Kim *et al.* (2003) for the treatment of corn stover. Ammonia at 15% contacted with biomass in a reactor operated in flow-through mode, at an operational temperature of 170ºC, for reaction times up to 90 min. It was observed 70–85% removal of the total lignin and 40–60% solubilization of hemicellulose, while cellulose was retained at 95%. The enzymatic digestibility of the treated solids was very high, reaching 92.7% glucan conversion after 72h (though 80% of this value was achieved at 24h). However, despite these promising results, the process is expensive and thus economically unfeasible.

2.3.3.3 Alkaline Peroxide (Oxidative delignification)

Alkaline peroxide, also known as oxidative delignification, like its names imply, is a pretreatment which removes lignin under alkaline conditions in the presence of hydrogen peroxide (H_2O_2). In this method, biomass is immersed in a pH-adjusted water (e.g. to pH 11-12 using NaOH) containing H_2O_2 at room temperatures for long periods of time (e.g. 6-24 h) (Taherzadeh *et al.*, 2008). This method detaches and solubilizes lignin, loosening the lignocellulosic matrix and causing a more open three-dimensional relationship between lignin, cellulose, and hemicellulose at the molecular level, thus increasing the amount of cellulose available for hydrolysis by enzymes (Martel *et al.*, 1990). However, when treating oak, it was also observed some degradation of soluble sugars (Kim *et al.*, 2001). But in the studies of Saha *et al.* (2007), no measurable furfural and hydroxymethylfurfural were detected in the process, having obtained good fermentable/digestible hydrolysates.

The pretreatment of sugarcane cane bagasse with 2% hydrogen peroxide, at 30ºC for 8h enhanced its susceptibility to enzymatic hydrolysis. About 50% of lignin and most of hemicellulose content of bagasse was solubilized, which translated into an increase of the cellulose content from 42% in the original cane bagasse to 75% in the oxidized pulp. Saccharification of this pulp residue with cellulase for 24 h yielded glucose with 95% efficiency (Azzam, 1989). Amjed *et al.* (1992) also applied alkaline peroxide treatment to bagasse, recovering 72.5% of cellulose and 83.2% of xylose after 24h of enzymatic hydrolysis.

2.3.3.4 Ozonolysis

Treatment of lignocellulosic materials with ozone is referred as ozonolysis. Ozone degrades lignin by direct ring cleavage, effectively removing a great portion of lignin from biomass (Yosef *et al.*, 1994). Hemicellulose is also partially removed, cellulose is mostly unaffected and no toxic compounds exist in the treated material (Neely, 1984). The pretreatment is usually carried out at room temperature and pressure, with process variables being moisture content of the sample, particle size and ozone concentration. The percentage of water in the feed is the most important factor that influences solubilization, with optimum water content being around 60% for corn stover (Quesada *et al.,* 1999), while being considered to be 30% for other fibers (Taherzadeh *et al.*, 2008).

Studies with wheat straw observed a five-fold increase in its enzymatic digestibility, after ozonolysis removed 60% of lignin. After ozonolysis pretreatment of poplar saw dust, lignin content of the biomass decreased from 29% to 8%, and enzymatic hydrolysis increased from 0% to 57% (Vidal *et al.*, 1988).

The large amounts of ozone needed for this process makes it too expensive to wide-scale application (Sun *et al.*, 2002).

2.3.3.5 Wet oxidation

Wet oxidation (WO) is the process of treating material with water and air or oxygen at temperatures above 120ºC (like 148-200ºC), for a certain period of time (e.g 15-30 min). The main reactions in wet oxidation pretreatment are a low temperature hydrolytic reaction that forms organic acids, and a high-temperature oxidative reaction (McGinnis *et al.*, 1983). This results in hemicelluloses being hydrolyzed, lignin undergoing both cleavage and oxidation and cellulose being partly degraded, causing it to become highly susceptible to enzymatic hydrolysis (Schultz *et al.*, 1984). In the case of hemicellulose, it was observed WO had the tendency of transferring it from the solid to the liquid fraction without totally hydrolyzing it, yielding sugars in oligomeric form (Martin *et al*, 2008). The most important parameters in WO are the temperature, reaction time and oxygen pressure. The presence of oxygen enables operation at comparatively reduced temperatures, as it participates in the oxidative reactions and enhances the generation of organic acids. It is an exothermic process, and once it is initiated, it becomes self-sufficient heat-wise (Schmidt *et al.*, 1998). Temperature control becomes a critical factor because of the fast rates of reaction and heat generation (Garrote *et al.*, 1999).

This method is not well-suited to materials with high lignin contents, as it has been shown the yield decreases with an increased lignin fraction. In addition, since the lignin is solubilized and

oxidized, it cannot be used as a solid fuel, losing value as a byproduct. This is a phenomenon that happens with many other delignification methods (Schmidt *et al*, 1998).

WO can be carried out in neutral conditions or be catalyzed by alkaline or acidic conditions. In the study of Martin *et al.* (2007b), sugarcane bagasse was subjected to wet oxidation at 185ºC and 195ºC, residence times of 5 and 15 minutes, and at acidic, neutral and alkaline pH values. The best results were obtained with alkaline WO at 195ºC for 15 minutes. Not only was lignin and hemicellulose extensively removed (40-50% and 93-94%, respectively), but the enzymatic hydrolysis of the treated material was also the highest, with 74.9% of cellulose conversion after 48h. Furthermore, the production of inhibitory byproducts was reduced under alkaline conditions.

2.3.3.6 Solvent extraction of lignin (Organosolv)

The organosolv process uses an organic or aqueous organic solvent mixture to remove or decompose the network of lignin. Lignocellulose is mixed with organic liquid and water and then heated, dissolving the lignin and part of the hemicellulose, while leaving more reactive cellulose in the pulp. (Itoh *et al.*, 2003). A large number of organic or aqueous/organic solvents can be used at temperatures of 150-200°C without addition of catalysts. Some of them are oxalic, salicylic and acetylsalicylic acids. But the use of inorganic acid or alkaline catalysts can either help reducing the operating temperature or enhancing the delignification process (Chum *et al.*, 1985).

Overall, esters, ketones, glycols, organic acids, phenols, and ethers have been used for this treatment. When choosing solvents for a process, it should be kept in mind that after the process the solvents must be removed and preferentially recycled, as they are often inhibitory to downstream biological processes (Sun *et al.*, 2002). Therefore, not only should the solvent price be considered, but also its simplicity of recovery. The use of low-molecular-weight alcohols such as ethanol and methanol has been preferred, since it is relatively easy to separate them by boiling and condensing.

The major advantage of solvent extraction of lignin it that, unlike chemical delignification processes, the liquid fraction contains low molecular weight lignin with a high purity, which can have several uses, such as fuel or raw material for lignin-based adhesives (Pan *et al.*, 2005).

Mesa *et al.* (2009) submitted sugarcane bagasse to an organosolv process using a water-ethanol mixture (1:1), using either an acid (H_2SO_4) or alkaline (NaOH) catalyst, in concentrations 1.25-1.5% (w/w), for 60-90 minutes. It was observed that alkaline treatments resulted in a higher delignification (up to 44%), while acid treatment removed more hemicellulose (up to 60%). After enzymatic hydrolysis of the treated materials, the highest glucose conversions (~30%) were obtained with bagasse treated with 1.25% H_2SO_4 for 60 minutes. They concluded that lignin content was less important than hemicellulose for the enzymatic degradation of organosolv treated bagasse.

2.3.4 Physico-Chemical Pretreatments

This type of methods includes treatments that combine both chemical and physical methods.

2.3.4.1 Explosion

Explosion treatments involve contact of biomass with a suitable substance (steam, ammonia, CO_2) at high temperature and pressure for a certain amount of time, after which there is a quick drop

of pressure, making the materials undergo an explosive decompression. The most studied and applied method uses steam, and it is thus called steam explosion. Other methods are ammonia fiber explosion (AFEX) and CO_2 explosion.

Steam Explosion

The principle behind steam explosion is somewhat similar to microwave treatment: hemicellulose hydrolysis is catalyzed by the acids (acetic) that are released from it, only in this case steam is the agent responsible for the acetic acid cleavage. However, this hydrolysis is not always complete, resulting in oligosaccharides instead of monomeric sugars (Galbe *et al.*, 2007). The high pressure and temperature conditions also induce lignin transformation, improving cellulose accessibility.

The process is initiated at temperatures between 160 and 260ºC, and the residence time until the pressure drop varies widely, from 30 seconds to 20 minutes (Sun *et al.*, 2002). Therefore, there is a great range for experimentation, but optimal conditions have been found to be either high temperature and short residence time (e.g. 270 ºC, 1 min) or lower temperature and longer residence time (e.g. 190 ºC, 10 min). Very severe conditions result in considerable sugar loss (Duff *et al.*, 1996, Ruiz *et al.*, 2008). Other relevant parameters have been studied in Cullis *et al.* (2004), where it was shown that higher particle size and humidity of the raw material were beneficial to the process.

This process is well documented for many materials and was tested in lab- and pilot processes by several research groups and companies, and has been appreciated as being cost-efficient. It has already been demonstrated at commercial scale (Chum *et al.,* 1985). Its limitations are a weaker effect on lignin, incompletely disrupting the lignin-carbohydrate matrix, and the destruction of a fraction of xylan, consequently forming degradation products inhibitory to microbial activity (Mackie *et al.*, 1985).

A variation to this method is the added use of impregnating agents (H_2SO_4, SO_2 or CO_2) to improve downstream enzymatic hydrolysis, increase hemicellulose removal and reduce formation of inhibitors. The highest sugar recovery from sugarcane bagasse was reported at 220ºC, 30 seconds, 2 water-to-solids ratio and 1% H_2SO_4, obtaining 65.1g of sugar /100 g bagasse (Morjanoff *et al.*, 1987). However, in the work of Martin *et al.*, (2002), it was compared steam explosion without any additions, with SO_2 and with H_2SO_4. It was shown that despite yielding more sugars, usage of H_2SO_4 also resulted in greater inhibitor concentrations than when SO_2 was used.

Ammonia Fiber Explosion (AFEX)

AFEX has a concept similar to steam explosion, but uses liquid ammonia, takes place at lower temperatures (90-100ºC) and has residence times in the range 10-60 minutes (Teymouri *et al.*, 2005). Common ammonia loadings are 1-2 kg per kg of biomass. Being an alkaline treatment, it works by modifying the lignin fraction of biomass. However, little matter is solubilized in this treatment, be it hemicellulose or lignin. The small solubilization of hemicellulose is probably due to its limited degradation to oligomer sugars and its deacetylation (Gollapalli *et al.*, 2002). AFEX appears to act on changing the structure of the material, increasing the reactivity of cellulose and accessible surface area. These changes result in increased water holding capacity and higher digestibility (McMillan, 1994). This treatment has the added benefits of not needing small particles and of not producing inhibitory compounds to biological treatments, fermentation and digestion processes (Sun *et al.*, 2002). However, its effectiveness decreases with increasing lignin content, so while with bermuda

straw (5% lignin) and sugarcane bagasse (15% lignin), downstream enzymatic hydrolysis achieved 90% conversion of cellulose and hemicellulose, with newspaper (18-30% lignin) and aspen chips (25% lignin) the conversions didn't rise above 40 and 50%, respectively (Holtzapple *et al.*, 1991; McMillan, 1994).

Another disadvantage of AFEX is that ammonia must be recycled to be an economically feasible and environmental friendly process (Eggeman *et al.*, 2005).

CO_2 Explosion

Gases can also be employed in an explosion treatment. In the work of Zheng *et al.* (1998), out of nitrogen, helium and supercritical carbon dioxide, the later has demonstrated better results on treatment of lignocellulosic materials such as a recycled paper mix, sugarcane bagasse and the repulping waste of recycled paper. The usage of supercritical CO_2 offers several benefits, since it is non-toxic, nonflammable, easy to recover after extraction and environmental friendly. Furthermore, its mass transfer characteristics are similar to a gas and it possesses a solvating power akin to a liquid (Zheng *et al*., 1996; Larson *et al.*, 1986). CO_2 molecules are small, so they can penetrate small pores, just like water and ammonia. Its great diffusivity allows it to actively affect biomass, acting on delignification and increasing the accessible surface area for enzymatic attack. This can be enhanced by using co-solvents such as ethanol–water or acetic acid–water (Pasquini *et al.*, 2005). This is partly due to CO_2 forming carbonic acid when dissolved in water, which will help hydrolyze hemicellulose. Since it works at low temperatures (supercritical temperatures like 35ºC), the decomposition of monosaccharides by the acid is minimized, generating a product mostly free of inhibitory by-products.

Increasing the pressure results in a material that yields more sugars after hydrolysis. This is due to faster penetration of CO_2 inside the pores, before it is exploded. In works with sugarcane bagasse at 35ºC and 3000 psi, it was obtained almost 45% of theoretical glucose conversion after 25h enzymatic hydrolysis. While these results are lower than AFEX's, CO_2 explosion was found more cost-effective than the former (Zheng *et al.*, 1998).

It has also been achieved simultaneous CO_2 explosion and enzymatic treatment (Park *et al.*, 2001). The cellulases used were sustained at pressures of up to 160 bar for 90 min at 50 °C and were significantly stable. The hydrolysis under these conditions was fast and achieved 100% conversion of commercial cellulose (Avicel). Zheng *et al.* (1998), who separated explosion and enzymatic treatment, didn't achieve more 70% Avicel conversions, even at higher pressures.

Unfortunately, CO_2 explosion in overall may still be too expensive for commercial application.

2.3.4.2 Hydrothermolysis

Hydrothermolysis or liquid hot water (LHW) consists of cooking the lignocellulosic materials in hot water and is one of the pretreatment methods that has been applied for many years in the pulp industry. It's used liquid hot water under pressure, with temperatures between 180ºC and 270ºC, which penetrates the fibers and hydrates cellulose, also removing part of hemicellulose and lignin. This creates cellulose fibers with an enlarged accessible and susceptible surface, which are more accessible to enzymatic attack (Figure 2.9). Subsequent enzymatic hydrolysis of corn stover converted 90% of cellulose (van Walsum *et al.*, 1996; Zeng *et al.*, 2007). The generated hydrolysates

are rich in hemicellulosic oligosaccharides (80-90% xylan recovery). These conversion values are on par with results obtained with some dilute acid treatments or steam explosions, but they have the added advantages of not employing chemicals and of having no need for special non-corrosive reactor materials. Furthermore, preliminary size reduction of the biomass is unnecessary and the final hydrolysates possess a small amount of inhibitors of microbial growth (van Walsum *et al.*, 1996).

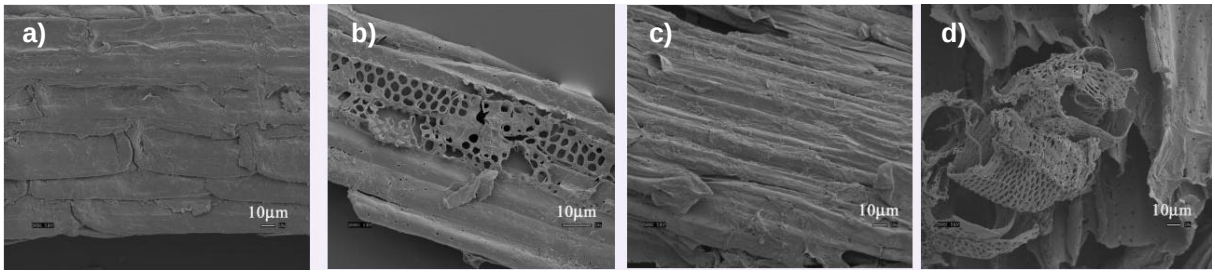

Figure 2.9 – SEM images (at 500x) of corn stover a) Native; b) Enzymatically treated; c) Pretreated by LHW; d) Pretreated by LHW and enzymatic hydrolysis. (adapted from Zeng, *et al.*, 2007).

LHW treatment of de-starched corn fiber at 160ºC for 20 min dissolved 75% of the xylan (Dien *et al.*, 2006), while treatment of alfalfa fiber at 220ºC for 2 min resulted in nearly total hemicellulose dissolution (Sreenath *et al.*, 1999). As for sugarcane bagasse, Laser *et al.* (2001) tried temperatures in the range 170-230ºC for 1-46 min, with solids loadings of 1 to 8%. The best results were achieved at high temperatures (220ºC), short residence times (2 min) and low solids concentration <5%, resulting in 90% of xylan recovery and producing a fiber which was 90% converted to ethanol in a simultaneous saccharification and fermentation process. Higher solids loading resulted into the release of inhibitory compounds and the decrease of ethanol producing rate.

The main limitation of LHW treatment is when dealing with high lignin substrates. While hemicellulose removal is great, the extent to which lignin is removed is smaller, which, as already mentioned, limits enzymatic hydrolysis. For that reason it has been suggested that this treatment could be adapted into a two-stage process, adding a treatment which is effective in lignin removal (e.g ammonia) (Kim *et al.*, 2006).

2.3.4.3 Microwave-chemical

Common microwave treatment uses high temperatures, which may degrade some useful components of biomass. However, microwave radiation can be combined with chemical treatments, accelerating the chemical reaction rates (Caddick, 1995). In the works of Zhu *et al*. (2005a; 2006), three combinations of chemical/microwave treatments were tested on rice straw: microwave/alkali, microwave/acid/alkali and microwave/acid/alkali/H_2O_2. These treatments, particularly the later, were more effective than simple chemical treatments, significantly removing hemicellulose and lignin, and creating hydrolysates rich in glucose and poorer in xylose. Xylose could only be recovered from the hydrolysates in microwave/acid/alkali/H_2O_2 treatment. It was also with this treatment that the highest enzymatic saccharification of the treated fiber was obtained.

2.3.5 Biological Pretreatments

Microorganisms can also be used to treat the lignocelluloses and enhance enzymatic hydrolysis. Brown-, white- and soft-rot fungi can degrade lignin and solubilize hemicellulose (Schurz, 1978). Brow-

rot fungi mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocellulosic materials, an oxidative process which is possibilitated by the action of lignin peroxidases (LiP) and manganese peroxidases (MnP) that these organisms produce (Fan et al., 1987; Malherbe *et al.*, 2002).

It is possible to obtain good results through careful species selection. *Pleurotus ostreatus* selectively degraded more of the lignin fraction rather than the holocellulose (cellulose + hemicellulose), and increased the susceptibility of rice straw to enzymatic hydrolysis (Taniguchi *et al.*, 2005). In the work of Keller *et al.* (2003), preliminary tests showed a three- to five-fold improvement in enzymatic cellulose digestibility of corn stover after pretreatment with *Cyathus stercoreus*.

Some bacterial strains have also been reported to biologically pretreat lignocellulosic biomass. Kurakake *et al.* (2007) used two bacterial strains, *Sphingomonas paucimobilis* and *Bacillus circulans*, for treating office paper from municipal wastes. Pretreatment with the combined strains enabled sugar recovery of 94% in subsequent enzymatic hydrolysis.

Patel *et al.* (2007) pretreated sugarcane bagasse with a variety of fungal species. Treatment with *Phenerochaete chrysosporium* resulted in the highest amount of sugars in the liquid fraction, but it was with a combination of *Aspergillus awamori* and *Pleurotus sajor-caju* that the highest fermentability to produce ethanol was achieved.

Biological treatments occur at mild environmental conditions, using low temperatures and no chemicals, which is good advantage over chemical treatments, which involve much energy and money spent on heating and chemicals. The downside of this process is the slow rate of hydrolysis, which decreases its economic feasibility. For that reason biological treatments have not been developed as intensively as physical and chemical pretreatments (Chambel, 2008). Still, they could be used as a first step before applying other treatments (e.g acid), potentially lowering their requirements of chemicals, temperature and time, resulting in less biomass degradation and lower inhibitory compunds concentrations (Keller *et al.*, 2003).

Another variation to biological pretreatment is using it not only for lignin removal, but also for biological removal of specific components such as antimicrobial substances. In Yu *et al.* (2008), hydrolysates from dilute acid treatment of sugarcane bagasse were treated with the bacterium *Ralstonia eutropha*, with the goal of removing organic inhibitors while producing polyhydroxyalkanoates (PHAs). It was observed that the major organic inhibitors (formic acid, acetic acid, furfural and acid soluble lignin) were effectively utilized and removed to concentrations below 100 ppm, while at the same time, PHA biopolyesters were synthesized and accumulated to 57 wt% of cell mass. Also, since the strain did not use xylose, arabinose and oligosaccharides, which were the predominant sugars in the hydrolysate, the process water could possibly be used for fermentation/digestion.

Although interesting results have been achieved with many kinds of pretreatments, all of them still have some challenges to overcome before reaching widespread application. When selecting a process to treat lignocellulosic biomass, one should take into consideration the properties of the raw

material and the characteristics, advantages and disadvantages of the various methods. Some of them are summarized in Table 2.3.

Table 2.3 – Change in biomass compositional features for various pretreatment techniques (adapted from Zhu, 2005).

Pretreatments	Compositional Features			Advantages	Disadvantages
	Cellulose	Hemicellulose	Lignin		
Ball-Milling	Intensive decrystallization	No Removal	No Removal	Intensive decrystallization	Energy-intensive
Microwave Irradiation	Some depolymerization	Significant solubilization	Significant solubilization	High heating efficiency, easy operation	Expensive
Dilute Acid	Some depolymerization	80-100% solubilization	Little solubilization, more redistribution	Mild condition, high xylose yields	Acid recovery, byproduct inhibition
Sodium hydroxide	Substantial swelling	Substantial solubilization	Substantial solubilization (>50%)	Effective removal of lignin's ester bonds	Expensive reagent, alkali recovery
Lime	Little depolymerization	Significant solub-ilization (30%)	Partial solubilization (-40%)	Effective lignin & acetyl removal, inexpensive	Less effective due to poor solubility of lime
ARP	Less than 5% depolymerization	~50% solubilization	~70% solubilization	Effective delignification	Alkali recovery, relatively expensive
Alkaline Peroxide	Significant swelling	~50% solubilization	~50% solubilization	Effective delignification	Some decomposition of soluble sugars
Ozonolysis	Almost no depolymerization	Little solubilization	Up to 70% solubilization	Effective delignification, mild conditions	Expensive, high needs of ozone
Wet Oxidation	Some depolymerization	>90% solubilization	40-50% solubilization	Effective removal of hemicellulose and lignin	Not effective for high lignin
Organosolv	Significant swelling	Substantial solubilization	Substantial solubilization	High xylose yields, effective delignification	Solvent recovery expensive
Steam Explosion	Some depolymerization	80-100% solubilization	Little solubilization, more redistribution	Energy efficient, no recycling cost	Xylan degradation, byproduct inhibition
AFEX	Decrystallization	Up to 60% solubilization	10-20% solubilization	Less xylan loss, no inhibitor formation	Ammonia recovery, not effective for high lignin
CO_2 explosion	Significant swelling	Substantial solubilization	Substantial solubilization	No inhibitory byproducts, low temperature	Expensive process
Hydro-thermolysis	Significant swelling	Nearly total solubilization	Substantial solubilization	No chemicals, not corrosive,	Not very effective for high lignin
Biological	20–30% depolymerization	Up to 80% depolymerization	40% delignification	Low energy requirement, effective delignification	Cellulose loss, slow hydrolysis rate

2.4 Enzymatic Hydrolysis

2.4.1 General

While enzymatic hydrolysis can still be considered part of the biological pretreatments, its role in converting lignocellulosic materials to biogas is slightly different that with other pretreatments. First of all, it's not a treatment that has success alone, since the lignocellulose is resistant to enzymatic attack. Therefore, it is used between a first pretreatment and the anaerobic digestion, in order to convert cellulose and hemicellulose to simple sugars, obtaining an easily-metabolizable sugar-rich liquid fraction.

Unlike utility costs, which are low due the mild conditions employed and the lack of corrosion, the enzymes themselves – cellulases and xylanases – are costly (Duff *et al*., 1996). While this has limited their application in commercial scale, much effort has been made in order to reduce enzyme production costs, with companies like Novozymes and Genencor having already achieved great cost reductions and efficiency increases, particularly on cellulases (Zhang *et al.*, 2006). Still, enzyme loadings should be minimized in order to reduce production costs, but that will have the adverse effect

of increasing the time needed to achieve a greater extension of hydrolysis. Here the pretreatment step also comes into play, since the rate and extent of hydrolysis depends significantly on the structural features resulting from pretreatment. While within some limitations, a poor pretreatment can be offset by using a higher enzyme loading, a great pretreatment can reduce the required amounts of enzyme needed to achieve the same effect. Therefore, the extent of pretreatments should be weighed against the effects of enzymatic saccharification, in order to find an optimal condition which will minimize the cost the both steps combined (Figure 2.10).

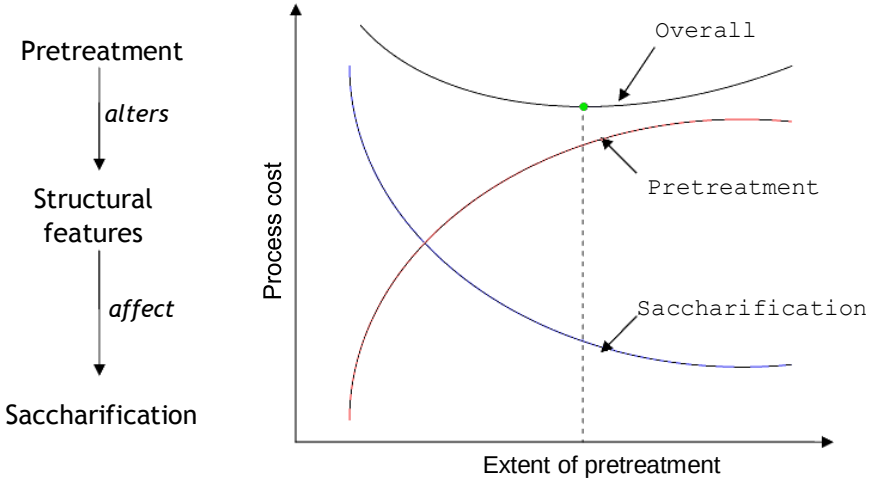

Figure 2.10 - Relationship between pretreatment extent and process costs (adapted from Zhu, 2005).

2.4.2 The enzymes and the hydrolysis reaction

The term cellulase actually corresponds to a family of enzymes, which can be subdivided into three groups, which exert a collaborative effort to break down cellulose. They are endoglucanases (1,4-β-D-glucan glucanohydrolases), exoglucanases (1,4-β-D-glucan cellobiohydrolases) and β-glucosidases (β-D-glucoside glucohydrolases) (Philippidis, 1994).

Cellulases are synthesized by fungi, bacteria and plants, but most research is focused on microbial cellulases, particularly fungal. That's because even though cellulotic bacteria, particularly the cellulolytic anaerobes, produce cellulases with high specific activity, they have very low growth rates and require anaerobic growth conditions. On the other hand, the aerobic mesophilic filamentous fungus *Trichoderma reesei* has shown good results, with some strains releasing up to 30g/l of extracellular enzymes, mostly cellulases (Chambel, 2008). It also produces enzymes from the three cellulase groups. They are more resistant to chemical inhibitors and exhibit better stability at 50ºC than other fungal cellulases. Their downside is they are sensitive to product inhibition and have a slow activation, even at their optimum temperature (Philippidis, 1994).

The basic hydrolysis mechanism involves three steps: adsorption of the biocatalyst to the cellulose surface, biodegradation of cellulose, ending with enzyme desorption. However, the way enzymes from the each group act on cellulose is different, and they complement each other.

The first phase of the process, involving mostly the solid cellulose, constitutes what's called primary hydrolysis. It's the rate-limiting step for the whole cellulose hydrolysis process. Endoglucanases hydrolyze internal bonds in the cellulose chain and break intermolecular bonds between adjacent cellulose chains. They act mainly on the amorphous parts and cleave glucosidic

bonds randomly, generating soluble carbohydrate chains with low degrees of polymerization (cellodextrins) and creating new chain ends on the crystalline solid fraction. Exoglucanases start acting only from the chain ends, and then they go along the chain, being capable of degrading crystalline cellulose. They release mainly cellobiose, but also glucose and small cellodextrins (e.g. cellotriose). One exoglucanase can either act on the reducing or the non-reducing ends or the chains, but microorganisms often produce more than one type of exoglucanase, degrading cellulose chains from both directions (Zhang *et al.*, 2004). This difference in binding capacity between endo- and exo-glucanases comes from the enzyme structure. Both have a small cellulose binding module and a larger catalytic domain, which are connected by a flexible linker section. This grants some freedom to the catalytic domain to move around the binding module that is fixed to the substrate surface. However, endoglucanases have a more open active site, which enables action in the middle of the glucan chain, while exoglucanases have a tunnel-shaped active site which can only hydrolyze chain ends (Teeri, 1997).

Secondary hydrolysis is a process that occurs in the liquid phase, involving the soluble sugars released in the previous step. Endo- and exoglucanases beak down larger oligosaccharides into cellobiose, but the most important step is the action of β-glucosidases, which converts most cellobiose (or in some cases, bigger cellodextrins) into monomeric glucose (Figure 2.11).

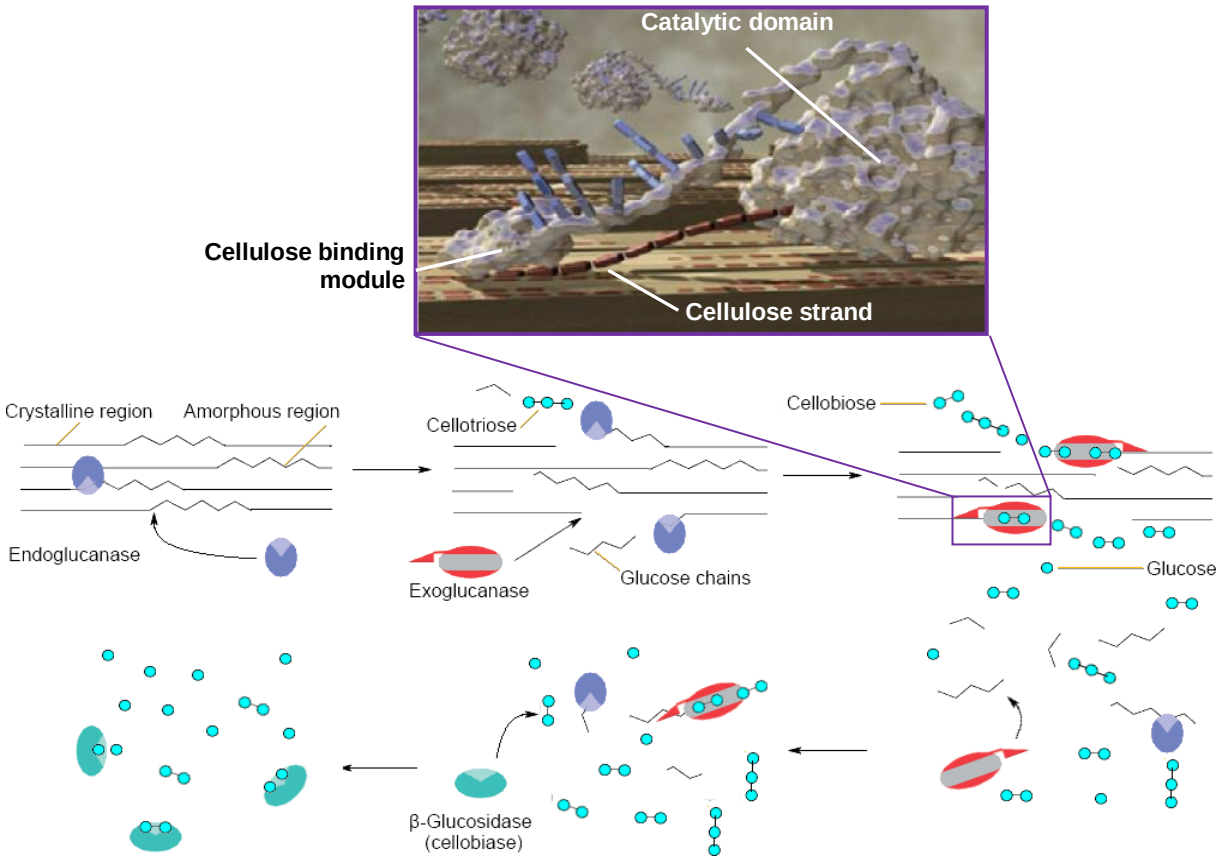

Figure 2.11 – Mode of cellulolytic enzyme action and computer model of the structure of the exoglucanase CBH I (adapted from Zhu, 2005; Jørgensen, 2007 and NREL, 2007).

The degradation of hemicellulose, particularly the glucuronoarabinoxylans present in grasses, involves the joint action of many enzymes. Most of these enzymes are also produced by fungal species like *T. reseei.* Similar to cellulases, there are endo-xylanases, exo-xylanases and β-

xylosidases, where the first two attack the large xylan backbone from the inside and from the chain ends, respectively, and the β-xylosidases hydrolyze oligmomeric sugars to xylose (Saha, 2003). However, hemicellulose structure is more complex than cellulose, and many xylanases are unable to cleave the glycosidic bonds between xylose units that are substituted. Therefore accessory enzymes must be present to remove the sugars and acids in the side chains (Table 2.4 and Figure 2.12 – A hypothetical plant heteroarabinoxylan structure showing different substituent groups with sites of attack by microbial xylanases (adapted from Beg *et al.*, 2001) (Lee *et al.*, 1987). However, many accessory enzymes only remove side chains from xylose oligosaccharides, thus requiring a partial hydrolysis of xylan before the side chains can be cleaved. This creates a continuous cooperative effort between all the enzymes involved (Poutanen *et al*., 1991).

Table 2.4 – Enzymes involved in the hydrolysis of heteroarabinoxylans (adapted from Saha *et al.*, 1999).

Enzymes	Mode of action
Endo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone.
Exo-xylanase	Hydrolyzes β-1,4-xylose linkages releasing xylobiose.
β-Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides.
α-Arabino-furanosidase	Hydrolyzes terminal nonreducing a-arabinofuranose from arabinoxylans
α-Glucuronidase	Releases glucuronic acid from glucuronoxylans.
Ferulic acid esterase	Hydrolyzes feruloylester bonds in xylans.
ρ-Coumaric acid esterase	Hydrolyzes *ρ* -coumaryl ester bonds in xylans.
Acetylxylan esterase	Hydrolyzes acetylester bonds in acetyl xylans.

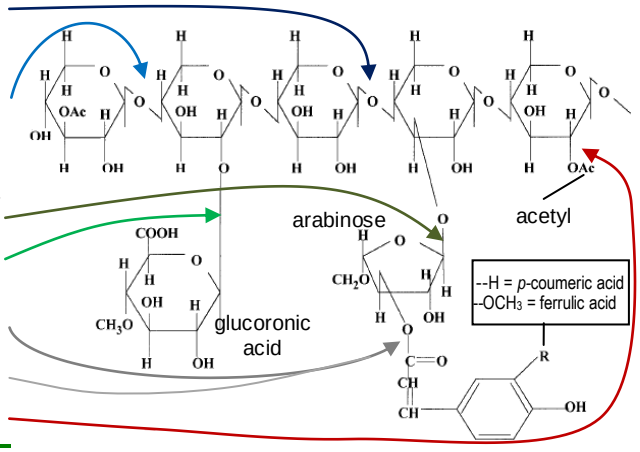

Figure 2.12 – A hypothetical plant heteroarabinoxylan structure showing different substituent groups with sites of attack by microbial xylanases (adapted from Beg *et al.*, 2001)

2.4.3 Factors affecting enzymatic hydrolysis

There are various factors that can hinder enzymatic activity and lower digestibility. Besides the structural features already mentioned (lignin, acetyl, hemicellulose, surface area, crystallinity), the experimental conditions (temperature, pH, substrate and enzyme loadings) in which the reaction takes place also have a big effect in overall glucose yield and release kinetics (Figure 2.13).

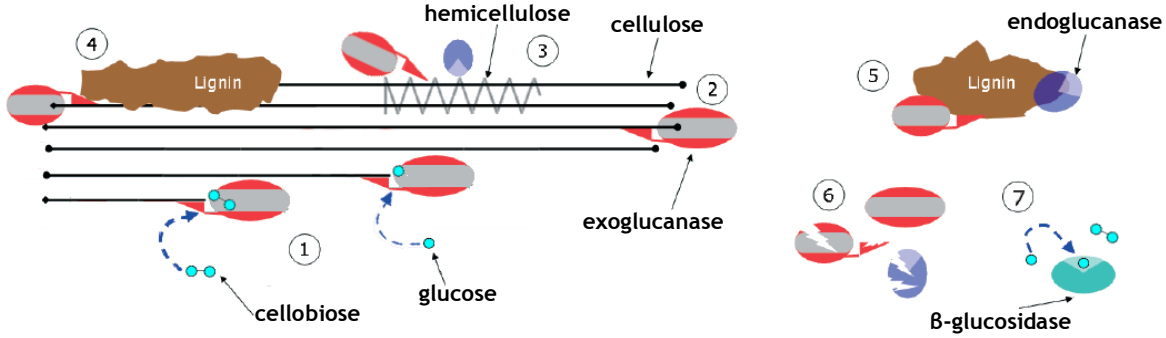

Figure 2.13 - Simplistic overview of some factors limiting efficient hydrolysis of cellulose: 1 - Product inhibition of cellobiohydrolases by cellobiose (majorly) and glucose, respectively; 2 - Unproductive binding of exoglucanases onto a cellulose chain. 3 and 4 - Hemicelluloses and lignin associated with or covering the microfibrils prevent the cellulases from accessing the cellulose surface; 5 - Enzymes (both cellulases and hemicellulases) can be unspecifically adsorbed onto soluble lignin particles or surfaces; 6 - Denaturation or loss of enzyme activity due to mechanical shear, proteolytic activity or low thermostability; 7- Proudct inhibition of β–glucosidases by glucose (adapted from Jørgensen *et al*., 2007).

Structural features

It has already been mentioned that lignin, acetyl content and crystallinity (measured as crystallinity index – CrI) are all obstacles to enzyme activity. Their relative importance to this process has been studied by Chang *et al.* (2000) and Zhu (2005). For each factor that was individually altered, digestibility at short times (1h) and long times (3 days) was recorded (Table 2.5)

Table 2.5 – Effects of Lignin Content, Acetyl Content, and CrI on Digestibility (taken from Chang *et al.*, 2000)

Lignin content		CrI		Acetyl content		3-d Digestibility		1-h Digestibility	
High	Low	High	Low	High	Low	High	Low	High	Low
✔		✔		✔			×		×
✔		✔			✔		×		×
✔			✔	✔		×		×	
✔			✔		✔	×		×	
	✔	✔		✔		×			×
	✔	✔			✔	×			×
	✔		✔	✔		×		×	
	✔		✔		✔	×		×	

Based on these results, it was concluded that lignin and crystallinity have more important roles than acetyl content, meaning it's the values from those two factors that mostly determines if digestibility is high or low. Lignin in its solid form blocks the access to cellulose, while in soluble form may cause enzyme adsorption. In either form, it's an agent which hinders enzymes from reaching their targets. High cellulose crystallinity makes it harder for enzymes that get there to actually find binding places to start degradation, but low crystallinity, even with high lignin, resulted in high digestibility. Chang *et al.* (2000) represented this in a schematic model, in which enzymes had to flow through pipes with valves called lignin and acetyl, which were never completely closed, but could limit enzymatic access to a substrate (cellulose) pool. These valves were placed in parallel, meaning that opening either one (removing that component) could increase the flow of enzymes to the substrate pool. However, the pipe for acetyl was small, so removing acetyl had small effects on the amount of enzymes that passed through. Lignin, on the other hand, had a much larger pipe, which could potentially help many enzymes to reach their destination. After the enzymes arrived at the substrate tank, the speed they worked with (effectiveness) depended on crystallinity. So even if the valves were closed, low crystallinity enabled the few enzymes that reached the pool to work quickly and hydrolyze much substrate (Figure 2.14).

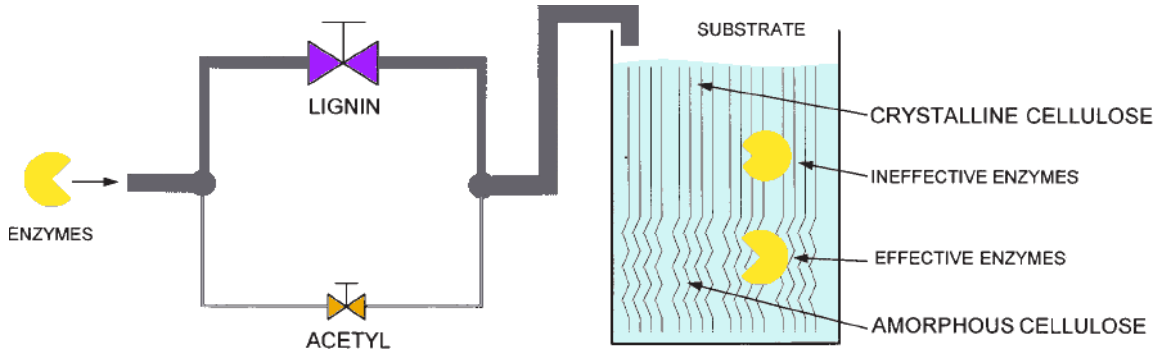

Figure 2.14 – A schematic model for the effects of lignin, acetyl groups, and crystallinity on enzymatic hydrolysis. (adapted from Chang *et al.*, 2000).

In practical terms, this means that low crystallinity is the major factor benefiting shorter reaction times and initial rates of hydrolysis, but also enhancing the final extent of the reaction. In the case of lignin, its removal allows the extent of the reaction to reach near theoretical values on the long run, but does not significantly increase the initial reaction rate. In terms of the model, if there are many enzymes in the substrate tank, they will finish their job, even if it takes a long time due to their slow work speed. Acetyl content has a similar role to lignin, but its effect in smaller on cellulose and greater on hemicellulose (Zhu, 2005) (Figure 2.15).

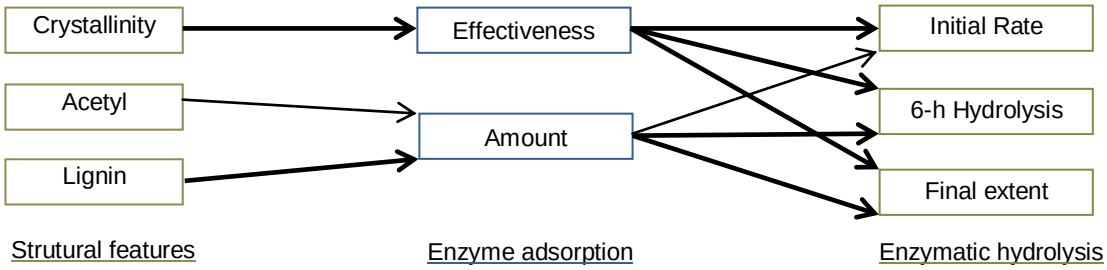

Figure 2.15 – Diagram for the effects structural features on enzyme adsorption and enzymatic hydrolysis of biomass. Thicker lines indicate a more significant effect (adapted from Zhu, 2005).

Reaction conditions

Two of the most important conditions to be planned are temperature and pH. Increasing temperature augments the frequency of collision between substrates and active sites, resulting in higher reaction speeds. However, too high temperatures cause enzyme denaturation, a phenomenon in which changes in the biocatalyst's internal structure cause (mostly) irreversible activity loss. There is an optimal temperature at which reaction rates are the fastest and enzymes retain activity for a long period of time. pH can also affect activity, since at certain pH values the shape of the active site can get changed, possibly denaturing the enzyme (Ortega *et al.*, 2001).

Most commercial cellulases and xylanases, including the ones used in the present work, have optimal conditions at 50-60ºC and pH 4.0-5.0 (Figure 2.16).

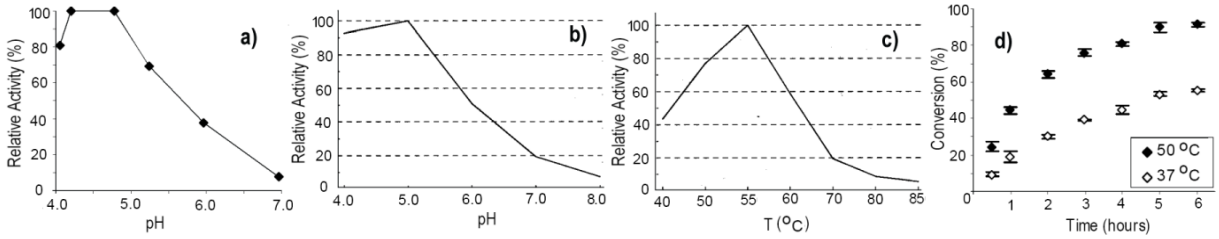

Figure 2.16 – a) and b) - Effect of pH on the activity of Accellerase 1000 and GC 140 xylanase (both from Genencor), respectively; c) Effect of temperature on GC 140 xylanase activity; d) % Cellulose digestibility at different temperatures, using Accellerase 1000 at 0.2 mL product/g cellulose on Phosophoric Acid Swollen Cellulose, at 1.2% cellulose loading and pH 5.0 (taken from respective product specification sheets).

Agitat ion can also influence the hydrolysis. High agitation speeds are beneficial to provide sufficient contact between the substrate and enzymes and to promote heat and mass transfer within the reaction vessel. Works have verified that using 340 instead of 170 rpm results in a small gain in conversion at longer reaction times (Temborg *et al.*, 2001). Excessive agitation may however cause a great mechanical shear and lead to enzyme deactivation (Ganesh *et al.*, 2000).

High substrate loadings usually result in higher glucose concentrations in the final liquor. While this is desirable from an economical stand point, as it will provide product stream more concentrated

for the digestion, it can negatively affect the hydrolysis rate and yield. Until a certain threshold, higher substrate concentration will increase contact between the substrate and enzymes, but past that point inhibition by end-product will become quite significant and reaction kinetics suffer (Cheung *et al.*, 1997). It is well known that cellobiose is a potent inhibitor of exoglucanases, with glucose and hemicellulosic sugars causing lesser inhibition (Holtzapple *et al.*, 1990). This inhibition is more marked in short reaction times, where there is the greatest rate of formation of cellobiose (Zhu, 2005). Among the strategies used to alleviate these inhibitory effects is the supplementation of β-glucosidase. Currently, cellulase producers already add extra β-glucosidase to their commercial products to aid the process. Still, as this enzyme is also inhibited by its product, glucose, the improvement only goes so far. For that reason, some researchers have focused on glucose removal. When dealing with ethanol, there's the simultaneous saccharification and fermentation, but other than that there's the use of membrane reactors using ultrafiltration membranes with a cut off of 50 KDa or less. This way, product inhibition from glucose and cellobiose can be reduced and the lifetime of the enzymes increased (Gan *et al*, 2002).

But even with those technologies, operating at a high substrate concentration presents some technical difficulties. In pilot scale plants, the maximum dry matter that can be handled has often been reported to be 15–20%. Otherwise, the initial viscosity of the material gets too high, making mixing difficult and inadequate and rising the power consumption in stirred tank reactors (Tolan, 2002).

Using higher enzyme loadings can increase the rate and yield of hydrolysis. This is partially because the extent of substrate inhibition depends on the ratio of total substrate to total enzyme (Huang *et al.*, 1991). The drawback, as previously mentioned, is a more costly operation. Moreover, the influence of increasing enzyme loading on biomass digestibility highly depends on structural features resulting from pretreatment. This is related to the limiting enzyme concentration after which there is no significant effect in the product formation. Substrates that have had a good pretreatment usually have lower limiting enzyme concentrations than those with poorer pretreatments (Chambel, 2008; Zhu, 2005).

Cellulase dosages of 10 FPU/g (filter paper unit, defined as a micromole of reducing sugar as glucose produced by 1ml of enzyme per minute) are often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield within acceptable reaction times (48-72h) at a reasonable enzyme cost (Gregg *et al.* 1996). However cellulase producers often use their own units for demonstrating enzymatic performance on certain substrates (Figure 2.17), requiring laboratory analyses to determine the equivalent activity in FPUs.

One of the limitative factors evidenced in Figure 2.13 is the unproductive absorption of cellulases onto a cellulose fiber. Exoglucanases bind strongly to the cellulose chain with their catalytic core. Obstacles can make the enzymes halt and become unproductively bound. This irreversible adsorption is partially responsible for the fact that cellulase activity decreases during the hydrolysis (Xiao *et al.*, 2004). The addition of surfactants is capable of modifying the cellulose surface properties, minimizing this effect. Non-ionic surfactants (e.g Tween 20, 80) are believed to be more suitable for enhancing the cellulose hydrolysis. The reaction rate was improved by 33% using Tween 80 as a surfactant in the hydrolysis of newspaper (Sun *et al.,* 2002; Castanon *et al.*, 1981).

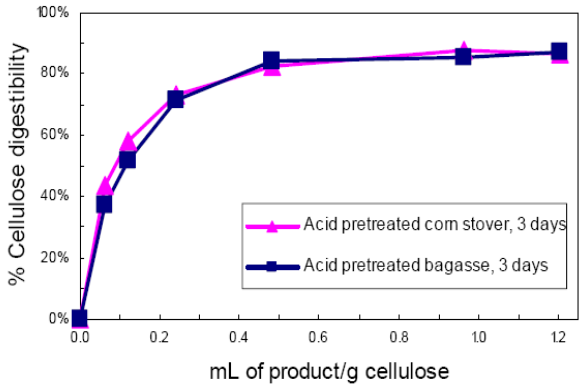

Figure 2.17 – Effect of Accellerase 1000 loading on cellulose digestibility for washed acid-pretreated corn stover and sugar cane bagasse. Reaction conditions were 7% cellulose loading, 50°C, pH 5.0, 3 days of reaction time. Acid pretreatment was carried out at a solids concentration of 20% (w/w), temperature of 165°C, 1.44% (w/w) acid concentration and an approximate residence time of 8 minutes (taken from product specifications datasheet).

2.5 Anaerobic digestion

2.5.1 Microbial diversity and the degradative steps

Unlike ethanolic fermentation, which is commonly carried out by a selected yeast strain capable of metabolizing a limited number of substrates (mostly glucose), anaerobic digestion is a much more complex process, requiring the syntrophic and cooperative interaction between several types of microorganisms. It's a process divided in four key stages, with different trophic groups intervening in each one of them.

In the first, the hydrolysis stage, organic macromolecules are broken down into monomers like sugars, fatty acids and amino acids. In the second, the acidogenesis stage, these components are further broken down into VFAs (volatile fatty acids: short-chained fatty acids like acetate, butyrate or propionate), organic acids and alcohols, along with small amounts of hydrogen. The largest fraction of H_2 and acetate comes from the third step, the acetogenesis stage, in which bigger VFAs and other organic acids from the previous stage are converted into the two aforementioned substances. After the final stage, the methanogenesis, methane and carbon dioxide are formed as the main final products (Miyamoto, 1997) (Figure 2.18).

Most acidogens cannot effectively break down organic polymers. However, hydrolytic bacteria carry out depolymerization and release monomers that are metabolized by acidogens. As previously mentioned, hydrolysis is the rate limiting step of the whole process (Chynoweth *et al.*, 1989). Adding monomeric substrates bypasses the first stage and makes the process quicker. Methanogenesis takes place thanks to archaeons known as methanogens. In this food chain they can be divided into two trophic groups: the methylotrophs (or acetoclastic), which metabolize acetate and other methylated compounds such as methylamines and methanol; the hydrogenotrophs, archaeons with a chemolitotrophic metabolism which use carbon dioxide and hydrogen. Both groups produce methane, thus ending this complex metabolic chain (Sam-Soon *et al*., 1990; Gerardi, 2003).

Other species of *Archaea* and *Bacteria* relevant to these reactions are the commonly called sulfur-reducing bacteria (SRB). They reduce oxidized sulfur compounds like sulfate to either elemental sulfur or sulfides like H_2S. While some of them can be acetogens, others couple hydrogen sulfide formation

to the oxidation of acetate, succinate or other organic acids. They compete with the autotrophic acetogens and methanogens for hydrogen and/or acetate, and with heterothrophic acetogens for VFAs (Stefanie *et al.*, 1994; Colleran *et al.*, 1995; Stams *et al.*, 2005). H_2S formation is undesirable, not only for being a corrosive gas that can damage the pipes and other equipment (Gerardi, 2003), but also for its toxic effects on microorganisms. Cellulose-degrading organisms and acetoclastic methanogens are some of the most affected (Tursmand *et al.*, 1989; Hilton *et al.*, 1988). Therefore, optimization of reactional conditions often involves various strategies to minimize its production, precipitate it or shift its solubilization equilibrium, converting it into its less toxic ionized form (HS^-). H_2S is of increased concern when the substrate has appreciable ratios of sulfate/COD, which changes the equilibrium between methanogenic and sulfate-reducing genera, favoring SRB (Raskin *et al.*, 1996).

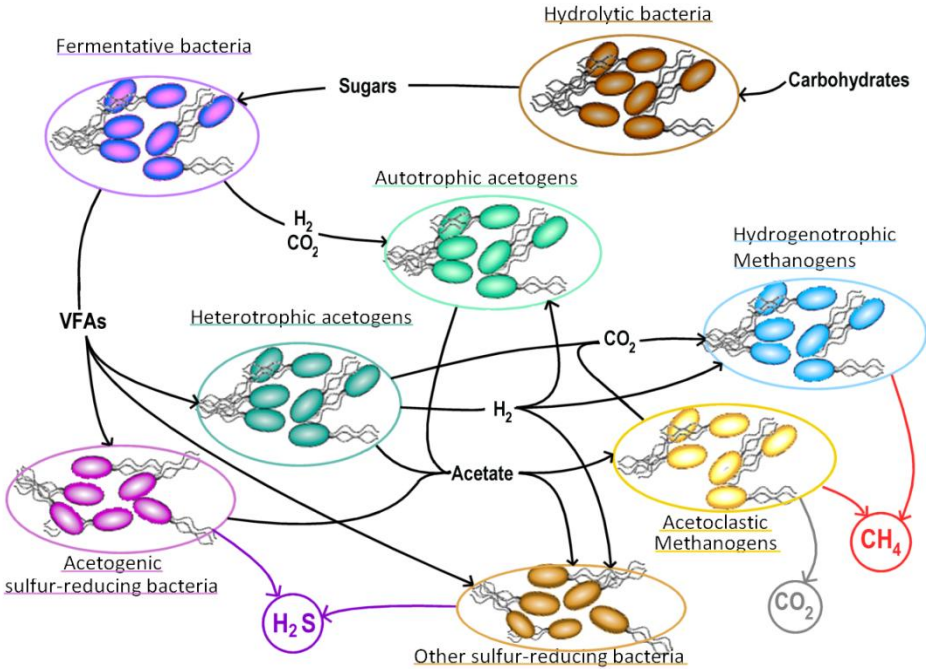

Figure 2.18 – Main metabolic fluxes between trophic groups during anaerobic digestion of lignocellulosic materials.

2.5.2 Biochemical Methane Potential

The methane production of various substrates in anaerobic digesters is subject to a multitude of operational variables. Extensive optimizations can be made with respect to loading rates, reactor configurations, temperature regimes, alkalinity supplements, among many others. Extensive research has been made in this field, especially when applied to anaerobic treatment of wastewaters and municipal solid waste (MSW). (Speece, 2008).

Analogously to the Biochemical Oxygen Demand (BOD), which represents the amount of organic matter in wastewaters passive of being aerobically degraded by microorganisms, the Biochemical Methane Potential (BMP) informs about the amount of organic matter passive of being biologically converted to methane in anaerobic conditions. Since most organic matter can be degraded under both anaerobic and aerobic conditions, both will usually measure similar amounts. However, BOD measures the depletion of an oxidized product (dissolved oxygen), being commonly used as an indicator of water pollution; BMP measures the formation of a reduced product (methane) and it was

originally developed to measure the ultimate biodegradability and associated methane yield of various feedstocks during anaerobic digestion. BMP assay is a valuable, quick and inexpensive tool for measuring potentials of biomass to biogas conversions, thus giving preliminary estimates of methane production from the assayed substrates, before research is moved on to pilot scale experimentation. (Chynoweth *et al.*, 1993; Speece, 2008).

This assay was originally devolved by Owen *et al.* (1979), and has since then seen many adaptations and optimizations, giving birth to minor and larger variations (ASTM, 1992).

The test involves a batch incubation of a substrate in ideal conditions for anaerobic decomposition. Some of these conditions include excess of inoculum and nutrients, substrate concentration below inhibitory levels, moderate temperatures, excess buffering capacity of the inoculum and strict anaerobic conditions.

A typical profile of methane production during BMP usually follows first order kinetics, characterized by an ultimate methane potential (B_∞) and a conversion kinetic constant k_h (equation (2.1), Figure 2.19). Duration of BMP tests is commonly 30 days. Less time will not yield trustworthy results, since even simple substrates like sugars and starch may take up to 30 days in the reactor until biogas production halts. This value can extend up to 120 days when dealing with very recalcitrant lignocellulosic materials (SUCCEED, 1999). A typical curve registers a sharp increase in production in the beginning, followed by an asymptotic stabilization (Figure 2.19 a). Deviations to this profile can occur when a biomass sample has both fast and slow digestible components (e.g. soluble and structural carbohydrates).

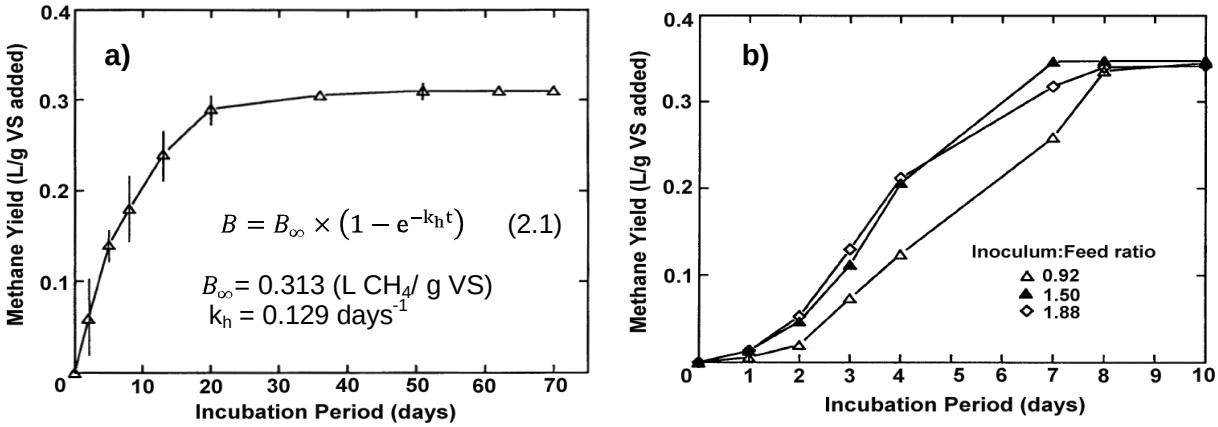

Figure 2.19 – a) A typical biochemical methane potential profile (from napiergrass). In the equation, *B* is the methane production at a certain time *t* ; b) Effect of inoculum:feed ratio on the biochemical methane potential assay of cellulose. (Adapted from Chynoweth *et al.*, 1993).

It is important that the inoculum is acclimatized with the substrate. Microorganisms, when faced with unfamiliar substrates or toxic compounds, do not respond very quickly to them, usually needing some days before significant microbial activity is restored, accompanied by a rise in methane production. If an unacclimated inoculum is used, the BMP test results might not be very conclusive, and effects like lag phases and altered kinetics might be observed (Speece, 2008).

With respect to the inoculum quantity, the inoculum-to-substrate ratio, on a volatile solids (VS) basis, is often 1. However, studies aiming to determine the limiting inoculum size concluded that for

some substrates, higher ratios translated into higher rates of reaction (Figure 2.19 b). Therefore, the ratio 2 became also of common use (Chynoweth *et al.,* 1993; Tong *et al.,* 1990).

Buffering capacity refers to the inoculum's ability to neutralize acids and maintain pH. This is particularly important since there's a tendency for the pH to decrease due to the release of VFAs and other acids during the acidogenesis stage. Methanogenic activity is optimal in the range 6.0-8.0 and declines sharply otherwise (Figure 2.20) (Clark *et al.*, 1970). This capacity is measured in terms of partial (or bicarbonate) alkalinity.

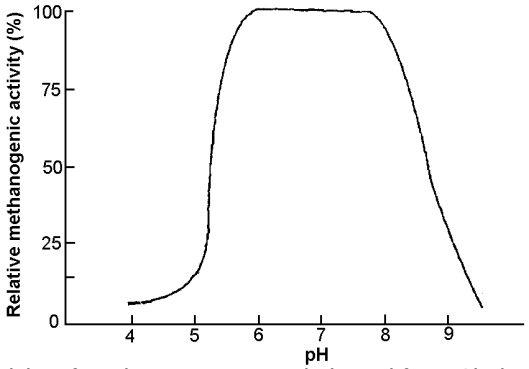

Figure 2.20 – The relative activity of methanogens to pH (adapted from Clark *et al.*, 1970)

As for substrate characteristics, particle size on the range of centimeters and millimeters seems to have no effect on digestibility. As for the structural components, no correlations were found, with the exception of lignin (Chynoweth *et al.*, 1993). It has been demonstrated that materials with high lignin contents have lower digestibilities, as lignin-carbohydrate complexes are of difficult accessibility for microorganisms (Stinson *et al.*, 1995; Pueboobpaphan)

The methane content of the biogas produced can be estimated based on the substrates chemical formula. So when dealing with a substrate purely lipidic, methane content is usually in the 70% range. With carbohydrates, on the other hand, methane content accounts for about 50% of the gas produced (Table 2.6) (Baez-Smith, 2008).

Table 2.6 – Theoretical Methane Content of Biogas (adapted from Baez-Smith, 2008).

Substrate	Chemical Composition	Methane % of Total Gas
Fats	$C_{15}H_{31}OCOOH$	72
Proteins	C_4H_6ON	63
Carbohydrates	$C_6H_{12}O_6$	50

Several important waste materials and feedstocks have been analyzed for BMP. Some of those results are present in Table 2.7.

However, while studies with sugarcane have been made, no literature work was found assaying the BMP of sugarcane bagasse. Since bagasse is one of most abundant residues produced during sugarcane refining, it is very interesting to investigate its potential for biogas production.

Table 2.7 – Range of BMP data for biomass and waste feedstocks (adapted from Chynoweth *et al.*, 2001).

Substrate	B_∞ (L CH_4 /g VS)	k_h ($days^{-1}$)
Napiergrass	0.19-0.34	0.05-0.16
Water hyacinth	0.19-0.32	0.09-0.11
Sugarcane	0.23-0.30	0.05-0.16
Willow	0.13-0.30	0.01-0.04
MSW	0.20-0.22	0.13-0.16
Avicel Cellulose	0.37	0.14

3. Materials and Methods

3.1 Biomass

Sugarcane bagasse from two different origins was used during this work. The first one originated from India. Its date of harvest was unknown. It had been previously stored at the laboratory, at room temperature, for some months. It was used for most of the first part of this work, involving the study of some variables in acidic and enzymatic hydrolysis. The second source of bagasse was from Pakistan. It was collected directly from the producer during late June 2009 and sent to Lund's Faculty of Engineering. This bagasse was used for the latter part, the integrated hydrolysis and anaerobic digestion.

During the experimental period of this work, both bagasses were stored inside plastic bags and were placed inside a cold room, at 4ºC.

Figure 3.1 - Aspect of the Indian bagasse (left) and Pakistanese bagasse (right).

3.2 Primary Characterization

During various steps of the bagasse treatment, it was necessary to determine some simple parameters of the resulting solid and/or liquid fractions. That included contents of total solids, moisture, volatile solids and ash. The two original bagasses were also characterized based on their particle size distribution.

Comparison of sample characteristics and of experimental data from the reactions described further ahead is often reported as relative percent difference (RPD), calculated by equation (3.1).

$$RPD = \left(\frac{X_1 - X_2}{\overline{X}}\right) \times 100 \quad (3.1)$$

Where: X_1 and X_2 = measured/read values
$\overline{X}$ = arithmetic mean of X_1 and X_2

3.2.1 Determination of Total Solids

The total solids (TS) content was determined by adapting National Renewable Energy Laboratory's (NREL) Laboratory Analytical Procedure (LAP) "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples" (Sluiter *et al.*, 2008a). Since this procedure was followed up by volatile solids determination, the crucibles used were incinerated for over two

hours at 550ºC in a muffle furnace, and then left to cool inside a desiccator. A certain amount of biomass sample was placed in the crucibles and their weight with and without biomass was recorded. The crucibles were placed inside a convective oven at 105 ºC overnight for about 24 hours, or longer if constant weight had not yet been achieved (± 1% change in sample weight after two hours of reheating). The TS was calculated using equation (3.2), with moisture being the remaining percentage:

$$TS(\%) = \left(\frac{Weight_{dried\ 105ºC\ crucible+sample} - Weight\ incinerated_{crucible}}{Weight_{initial\ sample}} \right) \times 100 \quad (3.2)$$

All TS (and VS) determinations were made in quadruplicate. The reported results were averages of those values.

3.2.2 Determination of Volatile Solids

The volatile solids (VS) content was determined by placing the crucibles containing the dry solids obtained from TS determination in muffle furnace at 550ºC, for 2.5 hours. The crucibles were left to cool at room temperature inside a desiccator and then their weight was recorded. The VS content was calculated with equation (3.3), and the ash percentage was the difference to 100%.

$$VS(dry\ basis\ \%) = \left(\frac{Weight_{dried\ 105ºC\ crucible+sample} - Weight\ incinerated_{crucible+ash}}{Weight_{dried\ 105ºC\ crucible+sample} - Weight\ incinerated_{\ crucible}} \right) \times 100 \quad (3.3)$$

3.2.3 Bagasse Particle Size Distribution

A sample of each initial bagasse was passed through a set of sieves with mesh pore sizes of 10; 4; 1.6; 0.85 and 0.18 mm. Every fraction obtained was weighted, creating particle size distribution profiles.

3.3 Fiber Analysis

The following group of procedures, labeled fiber analysis, was executed in order to get a more in-depth knowledge of the chemical composition of biomass. This includes extractives and structural components like carbohydrates and lignin. These methods were only applied to the original Indian bagasse and one of its acid-treated derivatives, the one mentioned in section 3.5.1.

3.3.1 Milling

As a requirement for the following protocols, all biomass samples were milled. For this purpose, the biomass samples were left in a convection oven at 45ºC for about a week. The milling was carried out in an AEG laboratory cutting mill, model AM 80 MX2, rotating at 50 Hz, equipped with a sieve with an equivalent pore size of 0.5mm (Figure 3.2).

The milled samples were stored inside sealed plastic bags and placed inside a cold room, at 4ºC.

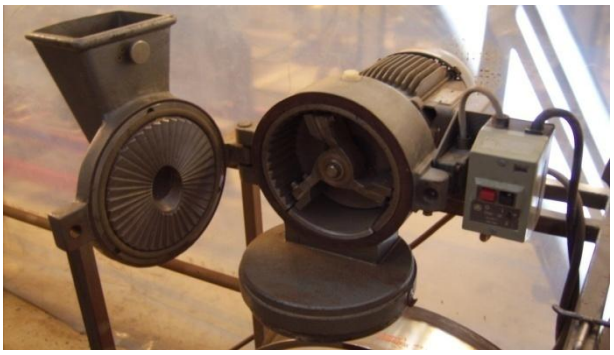

Figure 3.2 – Equipment used for biomass milling.

3.3.2 Determination of Extractives

The samples were analyzed for water extractives and ethanol extractives, according to the LAP "Determination of Extractives in Biomass" (Sluiter *et al.,* 2005).

The process of removing extractives present in the biomass is particularly important when performing analysis of structural components, because these chemicals can interfere and lower the precision of that analysis. A Soxhlet extraction procedure was applied to 7.2968 and 7.0029 g (dry weight) of original and acid-treated bagasse, respectively. The samples had been previously put into thimbles and dried for 24 hours at 60ºC. The thimbles were inserted into glass Soxhlet extraction tubes, which were connected to receiving flasks in heating mantels below, and condensers above, as shown in Figure 3.3.

Figure 3.3 – Soxhlet extraction setup used for water and for ethanol extractives determination

The water extraction was carried out first, by adding 190 mL of HPLC-grade water into the receiving flask. The extraction was run for about 8 hours, adjusting the heating mantles to assure that the liquid inside the Soxhlet tube refluxed 4 times per hour. Before stopping the extraction, it was verified the water inside the Soxhlet tube looked completely clear. The receiving flasks containing the water residue were then removed and replaced by similar flasks containing 190 mL of ethanol. This extraction was carried out in a similar fashion to the previous one, lasting around 10 hours, also having 4 refluxes per hour. Likewise, the extraction was only stopped after the liquid present in the Soxhlet tube looked clear.

The water and ethanol extraction residues had their respective solvents evaporated by placing them in rotary vacuum evaporators equipped with water baths at 60ºC, in case of water, and 40ºC, in case of ethanol. The solvent-free solids were then weighted and the extractives content (in a dry-weight basis) was calculated according to equation (3.4).

$$\%\ Extractives = \left(\frac{Weight_{flask+residue\ after\ extraction} - Weight_{receiving\ flask}}{Weight_{initial\ dry\ sample}}\right) \times 100 \quad (3.4)$$

Where: *residue after extraction* = water extractives or ethanol extractives.

The total extractives percentage (*%Extractives*$_{total}$) was calculated by the sum of ethanol extractives and water extractives.

3.3.3 Sample preparation and hydrolysis

Before the extractives-free samples were analyzed for structural carbohydrates and lignin, they were prepared and acid-hydrolyzed. This process and the remaining fiber analysis steps were all made based on LAP "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter *et al.*, 2008b). Duplicates were made for each sample and the reported results were averages of the replicates.

Approximately 300 mg of sample were added into tared pressure tubes and their weight was recorded. It was then added 3 mL of 72% sulfuric acid to each pressure tube. After a brief mixing, the tubes were put into water baths at 30ºC, and the mixture was incubated for an hour, begin stirred every 5 minutes. After this reaction, the acid concentration inside was diluted to 4% by adding 84 mL of deionized water. The tubes were then autoclaved for one hour at 121ºC, using the liquids setting. To measure the background sugar degradation during this step, a set of sugar recovery standards (SRS) was prepared. It contained known concentrations of sugars (1.3 g/L glucose; 0.39 g/L xylose; 0.06 g/L arabinose, 0.12 g/L mannose) and acid was added, so that it was also 4% sulfuric acid. The SRS was also used in duplicate and was autoclaved along with the samples.

3.3.4 Determination of acid insoluble and acid soluble lignin

The portion of acid insoluble lignin was determined using the sample resultant from the last step, which had undergone concentrated and diluted acid hydrolysis. The autoclaved hydrolysates were vacuum filtered through filtering crucibles. The filtrates were captured and saved for acid soluble lignin and carbohydrate determination. The acid insoluble residues (AIR) remaining in the crucibles were rinsed with deionized water and then dried at 105ºC for 19 hours, until constant weight was achieved. Their dry weight was recorded and then the crucibles were put inside a muffle furnace at 575ºC for 24 hours. After cooling inside a desiccator, the crucibles containing the ash were weighted.

Aliquots of the filtrates from the hydrolysate liquors were analyzed for acid soluble lignin by UV-Vis Spectrophotometry, within six hours of the hydrolysis, as indicated in the protocol. A blank of deionized water was run on an Ultraspec 1000 UV-Vis Spectrophotometer (Pharmacia Biotech), at 240nm and using a 1cm light path quartz cuvette. The samples were diluted 8 times to have an absorbance in the range 0.6-1.0.

The percentages of acid insoluble (AIL) and acid soluble lignin (ASL) were determined using equations (3.5) and (3.6), respectively. The total lignin was the sum of these two parcels. These results came referred in an extractives-free basis.

$$\%\ AIL_{extr.free} = \left(\frac{Weight_{dried\ 105^{o}C\ crucible+AIR} - Weight\ incinerated_{crucible+ash}}{Weight_{extr.free\ sample}}\right) \times 100 \quad (3.5)$$

$$\%\ ASL_{extr.free} = \left(\frac{Absorbance \times Volume_{filtrate} \times Dilution\ factor}{\varepsilon \times Weight_{extr.free\ sample}}\right) \times 100 \quad (3.6)$$

Where: $Volume_{filtrate}$ =0,087 L
ε = Absortivity of soluble lignin from bagasse biomass at 240 nm = $L.g^{-1}.cm^{-1}$
Dilution factor = 8
(Weights are in g)

3.3.5 Structural Carbohydrate Determination

The carbohydrate content of the samples was determined based on the HPLC analysis of the monomeric sugars glucose (for cellulose), xylose, arabinose and mannose (for hemicellulose).

It was used a HPLC system with automatic sampling, composed of a Jasco AS-950-10 injector, a Jasco PU-980 pump, a Shimadzu CTO-6A oven and a ERC-7515A refractive index (RI) detector (Figure 3.4). It was used an Aminex HPX-87P column and a 125-0119 precolumn (both from Biorad).

Figure 3.4 – Jasco HPLC system used for fiber analysis.

Twenty mililiters of the filtrates saved in the last step were first neutralized to pH 6 by adding calcium carbonate while stirring. After reaching the desired pH, the supernatants were decanted and then filtered through 0.2 µm polyether sulfone filters into the autosampler vials.

It was also prepared a calibration verification standard (CVS), containing 2,5g/L of each of the sugars to be analyzed plus galactose and cellobiose. This standard was used to check that the calibration curves used were valid.

The samples and the CVS were analyzed on the HPLC system using 0.2 µm filtered and degassed HPLC grade water as the mobile phase, a flowrate of 0.6 mL/min, column temperature of 80ºC, RI detector set on range 256, at 30ºC, and a sample running time of 35 minutes.

The sugar concentrations were calculated based on the peak areas measured and calibration curves already available for the column and settings used (Annex 7.1 – Figure 7.1 to Figure 7.4), using equations (3.7) – (3.10).

$$Glucose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{glucose}(mV.\min) + 0.0317}{15.097} \quad (3.7)$$

$R^2 = 0.9998$

$$Xylose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{xylose}(mV.\min) + 0.0317}{15.097} \quad (3.8)$$

$R^2 = 0.9998$

$$Arabinose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{arabinose}(mV.\min) - 0.0416}{14.426} \quad (3.9)$$

$R^2 = 0.9998$

$$Mannose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{mannose}(mV.\min) - 0.0197}{15.147} \quad (3.10)$$

$R^2 = 0.9995$

The sugar recovery standards (SRS) come into play to calculate the percentage of sugar recovered after the 4% acid hydrolysis. The amount of each sugar recovered was calculated with equation (3.11).

$$\%R_{sugar,x} = \left(\frac{Concentration\ detected\ by\ HPLC}{Known\ concentration\ of\ sugar\ before\ hydrolysis}\right)_{SRS} \times 100 \quad (3.11)$$

Where $\%R_{sugar,\,x}$ =Recovery percentage of a sugar after 4% acid hydrolysis

The sugar concentrations were then corrected with this factor, according to equation (3.12).

$$C_x = \frac{Concentration\ detected\ by\ HPLC \times Dilution\ factor}{\%R_{sugar,x}/100} \quad (3.12)$$

Where C_x = concentration of a sugar in the hydrolyzed sample after correction for loss on 4% acid hydrolysis.
Dilution Factor = 1

To calculate the correspondent concentration of polymeric sugars, it was necessary to multiply the calculated concentrations with an anhydro factor, to compensate for the water molecules incorporated during polymeric sugar hydrolysis. Therefore, for hexoses (glucose, galactose and mannose) that value was 162/180, and for pentoses (xylose and arabinose) it was 132/150. The percentage of each sugar on an extractives free basis was then calculated with equation (3.13).

$$\%\ Polymeric\ Sugar_{extr.free} = \left(\frac{C_x \times Anhydro\ factor \times Volume_{filtrate}}{Weight_{extr.free\ sample}}\right) \times 100 \quad (3.13)$$

Where $Volume_{filtrate}$ =0,087 L
Concentrations are in g/L
Weights are in g

The percentage of hemicellulose was considered to be the sum the percentages of xylan, mannan, arabinan, galactan while cellulose equal to glucan alone.

To correct all the above mentioned percentages (lignin, sugars) from an extractives free basis to an as-received basis it was necessary to use the percentage of total extractives (from water plus ethanol):

$$\%Component_{as\ received} = \%Component_{extr.free} \times \left(\frac{100 - \%Extractives_{total}}{100}\right) \quad (3.14)$$

3.4 Other Analytical Protocols

3.4.1 HPLC determination of sugars and organic acids

The same HPLC system indicated above was used for the determination of the concentrations of cellobiose, glucose, xylose and acetic acid, using an Aminex HPX-87H column and a 125-0129 precolumn (Biorad). However, the column and method used were also capable of detecting lactose, n-propanol, i-propanol, ethanol, propanediol and formic, lactic, succinic, propionic and butyric acids.

The pH of samples was adjusted to around 1 using 20% H_2SO_4 (20 microliters of sulfuric acid for each 1 ml sample). The analysis was made using 5mM H_2SO_4 as the mobile phase, a flowrate of 0.6 mL/min, column temperature of 55ºC, and a sample running time of 40 minutes.

The concentrations of interest were calculated based on the peak areas measured and calibration curves already available for the column and settings used (Annex 7.1 – Figure 7.5 to Figure 7.8), using equations (3.15) –(3.18).

$$Glucose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{glucose}(mV.\mathrm{s}) + 3.21}{1810.1} \quad (3.15)$$

$R^2 = 1$

$$Xylose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{xylose}(mV.\mathrm{s}) - 8.23}{2001.2} \quad (3.16)$$

$R^2 = 1$

$$Cellobiose\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{cellobiose}(mV.\mathrm{s}) - 178.61}{2265.8} \quad (3.17)$$

$R^2 = 0.997$

$$Acetic\ acid\ concentration\ \left(\frac{g}{L}\right) = \frac{Peak\ area_{acetic\ acid}(mV.\mathrm{s}) - 0.70}{1042.9} \quad (3.18)$$

$R^2 = 1$

3.4.2 Reducing sugars determination – DNS method

The DNS method is a simple, relatively quick method to quantify reducing sugars in liquid samples using spectrophotometry. It was used intensively throughout this work, as many reactions were followed by analyzing their reducing sugar content.

3.4.2.1 Preparation of reagents

The DNS reagent, which is central to the method, was prepared by mixing the following quantities of chemicals into 1L of deionized water (all chemicals were analytical grade): 10 g of dinitrosalicylic acid; 0.2 g of phenol; 0.5 g of sodium sulphite; 10g of sodium hydroxide; 200g of potassium sodium tartrate.

The sodium hydroxide was dissolved in 200 mL of water. Then it was added more 100 mL of water and the sodium sulphite. This was followed by the addition of phenol and more water, until the total volume was 500 mL. The potassium sodium tartrate and the dinitrosalicylic acid were then added and dissolved and the resulting solution left to rest overnight, cooled in a recipient covered with aluminium foil. Finally the remaining water was added, until the final volume was 1L.

The prepared reagent was stored in a cold room at 4ºC, and was protected from the action of light being placed in recipients covered with aluminium foil or in dark tinted glass bottles.

It was also used a pH 4.8 phosphate buffer, composed of 0.2 mM di-sodium hydrogen phosphate (Na_2HPO_4) and 45 mM sodium di-hydrogen phosphate (NaH_2PO_4) in deionized water.

3.4.2.2 Analysis method

The samples and blank were prepared for analysis by mixing them with phosphate buffer and DNS reagents in microcentrifuge (Eppendorf) tubes, in the quantities indicated in Table 3.1

Table 3.1 – Component quantities (μL) used in the DNS method sample preparation

Component	Blank	Tests
Phosphate Buffer	500	450
Sample	-	50
DNS reagent	750	750

The resulting solutions were then put in boiling water for 5 minutes. After cooling, their absorbance at 540 nm was read in an Ultraspec 1000 UV-Vis Spectrophotometer (Pharmacia Biotech), using a 1 cm light path plastic cuvette. Appropriate pre-dilutions were made so that the absorbance values didn't exceed 0.8.

A calibration curve for this method was made using analytical grade D-glucose solutions made in MiliQ water. Solutions with concentrations of 0 (blank), 0.5, 0.7, 1, 1.3, 1.5, 1.7 and 2 g/L were prepared and were analyzed in triplicate, following the same method described for the reaction samples (Annex 7.2.1 – Figure 7.9).

Sugar concentrations were calculated by means of equation (3.19), obtained from trendline of the calibration curve and by multiplying the absorbance by the dilution factor used.

$$Reducing\ sugar\ concentration\ \left(\frac{g}{L}\right) = \frac{Absorbance_{540\ nm} \times Dilution\ factor + 0.2283}{0.4443} \quad (3.19)$$

$R^2 = 0.9961$

3.4.3 Determination of partial and total alkalinity

The samples for alkalinity measurement were centrifuged in a Labofuge 200 lab centrifuge (Thermo Scientific) at 3000 G for 5 minutes and the supernatant was collected. 6 g of the sample were evaluated as partial alkalinity (PA) by titration to pH 5.75, and the total alkalinity (TA) by titration to pH 4.3 with standardized 0.1 N HCl, using a TitraLab TIM800 titration manager pH meter and a ABU901 Autoburete (both from Radiometer Copenhagen). PA and TA were calculated by equations (3.20) and (3.21).

$$PA\ (mg\ CaCO_3/L) = \frac{volume\ 1 \times F\ value(CaCO_3)}{Weight_{sample}} \quad (3.20)$$

$$TA\ (mg\ CaCO_3/L) = \frac{(volume\ 1 + volume\ 2)\ \times F\ value\ (CaCO_3)}{Weight_{sample}} \quad (3.21)$$

Where: *volume 1* = volume of 0.1 N HCl used to reach pH 5.75 (mL)
volume 2 = volume of 0.1 N HCl used to go from pH 5.75 to pH 4.3 (mL)
F value($CaCO_3$) (factor returned by the titrator to convert the volume of HCl spent into mg $CaCO_3$/L) = 5000.52

3.5 Dilute Acid Pretreatment

The main pretreatment applied to the bagasse was a dilute acid hydrolysis with sulphuric acid (H_2SO_4). All the pretreatment methods described here were only applied to the Indian bagasse. Besides the main pretreatment process, variations to that protocol were tested and its results were compared to the original one.

All solid samples after the pretreatment were stored inside sealed plastic bags. The liquid samples were placed inside glass bottles. Both types of samples were stored in a cold room, at 4ºC.

3.5.1 Main pretreatment process

10g of bagasse (dry weight) were added to a 500 mL Erlenmeyer flask and mixed with 189mL of 0.1M H_2SO_4, to have a final working volume of 200 mL and a loading of 5% TS. Final acid concentration was therefore ~0.93% (w/w). It was also prepared a control with the same amount of bagasse and with a volume of deionized water equal to the volume of acid solution (189mL).

The pair of vessels was autoclaved at 121°C for 15 minutes, using the liquids setting, for a total autoclave program running time of about 3 hours. After autoclaving, the solution in each vessel was adjusted to pH 7, by addition a 5N solution of NaOH and, if necessary, a 0.1N HCl solution.

The liquid part (hydrolysate) was separated by vacuum filtration, first by using a Munktell analytical filter paper grade 3 (pore size >10 µm), followed by a refiltering through a Whatman glass microfibre filter grade GF/A (pore size 1.6 µm).

The solids (acid-treated bagasse) were recovered as much as possible and then placed inside a convective oven at 105ºC for 6 hours. After drying they were stored for posterior use.

The hydrolysate was analyzed in triplicate for reducing sugar concentration by the DNS method. The reported results were averages. It was also analyzed for some sugar and organic acids concentration by HPLC method.

The biomass saccharification percentages (on a total solids basis) were calculated using the concentrations of reducing sugars dissolved in the hydrolysates and according to equation (3.22). The saccharification (on a total carbohydrates basis) was calculated by equation (3.23). HPLC results for the hydrolysate indicated that the majority of the sugars present in the hydrolysates was xylose. Therefore, the anhydro factor used for calculations was 132/150 (0.88).

$$\%Saccharification_{TS\ basis} = \frac{(C_T - C_C) \times Anhydro\ factor \times Working\ Volume}{Weight_{dry\ bagasse\ sample}} \times 100 \quad (3.22)$$

$$\%Saccharification_{Carbohydrate\ basis} = \frac{\%Saccharification_{TS\ basis}}{Bagasse_{carbohydrates}} \quad (3.23)$$

Where: C_T = reducing sugars concentration in the acid-treated hydrolysate (g/L)
C_C = reducing sugars concentration in the respective control hydrolysate (g/L)
$Weight_{dry\ bagasse\ sample}$ = bagasse dry weight placed in the reactor (g)
$Bagasse_{carbohydrates}$ = total carbohydrate content of the dry bagasse, as determined by fiber analysis (%)

3.5.2 Pretreatment variations

3.5.2.1 Post-autoclaving incubation: three sets of acid/control Erlenmeyer pairs were prepared as described above and were autoclaved using the same conditions. After autoclaving, the vessels were placed in a shaking water bath at 30ºC and with an agitation of 70 rpm and left there for different periods of time. One pair was removed at 6 hours of incubation time, another at 12 hours and the final one at 24 hours. Their downstream processing (neutralization, filtration, DNS analysis and storage) was the same as described in the main pretreatment process.

3.5.2.2 Residence times in the autoclave: 2.5 g of bagasse (dry weight) were added to a 200 mL Erlenmeyer flask and mixed with 47.5 mL of 0.1M H_2SO_4, to have a final working volume of 50 mL and a loading of 5% TS. Three sets of acid/control pairs were prepared. One was autoclaved 15 minutes at 121ºC, another one was set to 30 minutes at 121ºC, while the last one was 60 minutes at 121ºC. Their post-processing was in everything identical to main pretreatment process.

3.5.2.3 Combined milling + acid treatment: this process was identical to the main pretreatment process, with the exception that the bagasse used was first milled, using the same equipment and conditions as described in section 3.3.1.

3.6 Enzymatic Hydrolysis

All work carried out in this section was made using Indian bagasse, except for the last section. All tests were batch reactions and all reactors were run in triplicates, and the reported reducing sugar concentration results were their averages.

3.6.1 Enzymes

The enzymes (GC 140 xylanase, Y5 xylanase and Accellerase™ 1000) being used in these studies were kindly provided by Genencor, a division of Danisco A/S Denmark. Accellerase™ 1000 is a mixture of cellulase enzymes mainly with exoglucanase, endoglucanase, hemi-cellulase and beta-glucosidase activities. It has an optimal activity in the temperature range 50-65ºC and pH range 4.0-5.0. It has a reported endoglucanase activity of 2500 CMC U/g and a beta-glucosidase activity of 400 pNPG U/g. One CMC U unit of activity liberates 1 µmol of reducing sugars (expressed as glucose equivalents) in one minute under assay conditions of 50ºC and pH 4.8. One pNPG unit denotes 1 µmol of Nitrophenol liberated from para-nitrophenyl-B-D-glucopyranoside in 10 minutes at 50ºC and pH 4.8.

GC 140 xylanase is derived from a genetically modified strain of *Trichodema longibrachiatum*, and possesses an activity of 7250 GXU/g (Genecor Xylanase Units). The manufacturer reported that these units were determined using 0.25% aqueous solution of Remazol Brilliant Blue-dyed birchwood xylan as substrate, at 30 °C and pH 4.5. The optimum condition ranges were temperatures of 50-60ºC and pH 4.5-5.0.

Y5 xylanase is also derived from *Trichodema longibrachiatum*, with a reported 50000 U/g of endo-1,4-β-xylanase. In the specification sheet was indicated that its formulation was a powdery solid, which

didn't match the amber-colored solution available in the laboratory. No optimal temperature and pH conditions were given. Due to practical constraints, no enzymatic activity verification was performed.

Most of the enzymatic treatments carried out involved using two of these enzyme preparations simultaneously (usually Accellerase plus one xylanase), in volumetric ratios of 1:1.

3.6.2 Effect of enzyme loading on hydrolysis of untreated bagasse

0.5 g (dry weight) of bagasse were added to 20 mL scintillation vials. These were sterilized in an autoclave at 121°C for 15 minutes, using the liquids setting. Afterwards, different volumes of GC 140 xylanase and Accellerase 1000 were added to the vials. Final working volumes of 10 mL and 5% of TS were obtained by adding pH 4.8 phosphate buffer.

Eight different enzyme loadings were tested. It was used a 1:1 volumetric ratio between GC 140 xylanase and Accellerase 1000, as shown in Table 3.2. The control for these reactions was a vial with just bagasse and buffer.

The vials were incubated for 72h, in a shaking water bath, at 50°C and 70 rpm.

Table 3.2 – Experimental setup for studying the effect of different AccelleraseTM 1000 and GC 140 xylanase loadings in the hydrolysis of sugarcane bagasse.

Reactor label	Control	0.95%	1.9%	2.85%	3.8%	4.75%	5.7%	6.65%	7.6%
Working volume (mL)	10								
TS (%)	5 (0.5 g bagasse (dry weight))								
Accellerase 1000									
(% working volume)	0	0.95	1.9	2.85	3.8	4.75	5.7	6.65	7.6
mL product /g cellulose	0	0.48	0.96	1.44	1.91	2.39	2.87	3.35	3.83
GC 140 Xylanase									
(% working volume)	0	0.95	1.9	2.85	3.8	4.75	5.7	6.65	7.6

Due to practicality issues, these reactions were carried out in two batches, the first one covered the four lower enzyme concentrations and the second run covered the remaining concentrations.

The reaction was monitored by following the amount of reducing sugars released into the liquid phase. Samples of 100 µL were taken every 2 hours until 10h of reaction time, and then at 13, 16, 20, 26, 32, 36, 38, 48, 60 (only in the second batch) and 72h of reaction time. The samples were analyzed by the DNS method.

To calculate the saccharification of the enzymatic reaction, it was necessary to deduct two background sugar concentrations. One was the level of reducing sugars already present in the bagasse, which was measured by the control reactors run simultaneously with the samples, containing only bagasse and buffer. The other one was the background sugar concentration of the enzymes. Since most enzymatic preparations are stabilized in polysaccharides, this value is often significant. To that end, enzymatic reactors containing buffer and enzyme concentrations used in the experiments were prepared and ran separately for 72h. Although the ideal was to have one of these controls for each concentration of Accellerase and GC 140 xylanase used, in the end it was only possible to run two concentrations, 7.6% and 5.7% (v/v). For the remaining concentrations, the background enzyme sugars were calculated by a three point linear regression, with the two abovementioned concentrations plus the 0% concentration (with 0g/L of sugars). The saccharifications were then calculated by equations (3.24) and (3.25).

$$\%Saccharification_{TS\ basis} = \frac{(C_S - C_{CB} - C_{CE}) \times Anhydro\ factor \times Working\ Volume}{Weight_{dry\ bagasse\ sample}} \times 100 \quad (3.24)$$

$$\%Saccharification_{Carbohydrate\ basis} = \frac{\%Saccharification_{TS\ basis}}{Bagasse_{carbohydrates}} \times 100 \quad (3.25)$$

Where: C_S = reducing sugars concentration in the hydrolyzed samples (g/L)
C_{CB} = reducing sugars concentration in the control with bagasse and buffer (g/L)
C_{CE} = reducing sugars concentration in the control with enzyme and buffer, with the same enzyme concentration as the one used in the sample. (g/L)
$Weight_{dry\ bagasse\ sample}$ = bagasse dry weight placed in the reactor (g)
$Bagasse_{carbohydrates}$ = total carbohydrate content of the dry bagasse (untreated or acid treated), as determined by fiber analysis (%)

3.6.3 Effect of acid pretreatment with post-autoclaving incubation

A similar reaction to the one described above was made, but using different types of bagasse: the acid-treated bagasse from the main process, that wasn't incubated after autoclaving (thus called 0h); the ones incubated for 6, 12 and 24 hours (addressed as 6h, 12h and 24h, respectively). For each of the four types of bagasse, four reactors were made, involving the acid treated pulp and its respective control bagasse (labeled untreated), as shown in Table 3.3.Two different controls were prepared. One with untreated bagasse and buffer (Control 1) and the other with acid treated bagasse and buffer (Control 2), to measure the background sugar concentration after acid hydrolysis.

Table 3.3 – Experimental setup used for each type of bagasse (0h, 6h, 12h and 24h), for studying the effect of dilute H_2SO_4 pre-treatment on the enzymatic hydrolysis of sugarcane bagasse.

Reactor label	Control 1	Control 2	Rx 1	Rx 2
Working volume / TS	10 mL / 5%			
Untreated bagasse dry weight (g)	0.5		0.5	
Acid-treated bagasse dry weight (g)		0.5		0.5
Accellerase 1000 (% working volume)	0	0	5.7	5.7
GC 140 Xylanase (% working volume)	0	0	5.7	5.7

The reaction was followed as described in the step above, quantifying reducing sugars present with the DNS method.

The saccharification to reducing sugars (TS basis) was calculated similarly to what's described in equation (3.22), with Control 1 being the control for Rx 1, and Control 2 for Rx 2. It was assumed that the major sugar present in the liquid fraction was glucose. Therefore, for all calculations involving enzymatic reactors, the anhydro factor used was 162/180 (0.9).

3.6.4 Effect of combined milling and acid pretreatment

The setup used for the milled bagasse enzymatic hydrolysis can be taken from 3.6.3 and Table 3.3, with the difference it was used the milled acid treated and control bagasses prepared in 3.5.2.3. The rest of the method was also very similar, although the reaction was only carried out for 48h.

3.6.5 Effect of changing the enzyme combinations

In this experiment, while Accellerase 1000 was always used, it was studied how the absence of xylanase affected the hydrolysis and how was the performance of Y5 xylanase in this reaction. To this

end was used acid treated bagasse prepared in 3.5.1 and its control (untreated bagasse), in a setup similar to the previous one (Table 3.4).

The reaction was followed for 48h, with samples taken every 2 hours until 10h of reaction time, and then at 13, 16, 20, 26, 32, 36, 38 and 48 hours.

Biomass conversion to sugars (TS basis) was calculated using Control 1 as the control for all reactors containing untreated bagasse and Control 2 for all reactors containing acid treated bagasse.

Table 3.4 – Experimental setup for studying the effect of the presence of xylanases on the enzymatic hydrolysis of sugarcane bagasse.

Reactor label	Control 1	Control 2	Rx 3	Rx 4	Rx 5	Rx 6
Working volume / TS	10 mL / 5%					
Untreated bagasse dry weight (g)	0.5		0.5		0.5	
Acid-treated bagasse dry weight (g)		0.5		0.5		0.5
Accellerase 1000 (% working volume)	0	0	5.7	5.7	5.7	5.7
Y5 Xylanase (% working volume)	0	0	0	0	5.7	5.7

3.6.6 Enzymatic hydrolysis of Pakistanese bagasse

It was run an experiment to study the behavior of Pakistanese bagasse upon acid and enzymatic treatments, before proceeding to select an enzyme loading to use in the integrated process with anaerobic digestion. The bagasse used was taken from the larger scale acid hydrolysis treatment described in section 3.7.2.

Table 3.5 – Experimental setup for studying enzymatic hydrolysis of Pakistanese bagasse

Reactor label	Working volume/ TS	Untreated bagasse (g)	Acid treated bagasse (g)	Accellerase 1000 (% working volume)	GC 140 xylanase (% working volume)
Control 1	10 mL 5%	0.5		0	0
Control 2			0.5	0	0
1.9% NT		0.5		1.9	1.9
1.9% T			0.5	1.9	1.9
3% NT		0.5		3	3
3% T			0.5	3	3
3.8% NT		0.5		3.8	3.8
3.8% T			0.5	3.8	3.8
5.7% NT		0.5		5.7	5.7
5.7% T			0.5	5.7	5.7
7.6 % NT		0.5		7.6	7.6
7.6% T			0.5	7.6	7.6

The protocol followed the one in section 3.6.2, with a couple of differences. First, it was used both untreated and acid treated bagasses. Second, the enzyme loadings tested were partially different.

The experiment was carried out in multiple batches. The reaction in the batch involving the 1.9% and 3.8% was followed for 72h. The remaining reactors were only followed for 48h.

3.7 Integrated Pretreatment and Anaerobic Digestion

The biogas production potential of several fractions of bagasse was evaluated, from the original to the acid and enzyme treated, using both solid and liquid fractions. It was used Pakistanese bagasse in a batch anaerobic digestion process, preceded by none, one or two pretreatments. The following

scheme illustrates how the pretreatment steps were connected to each other and which fractions were tested for biogas production (Figure 3.5).

This method adapted the protocol suggested by Angelidaki *et al.* (2009), to determine the bio methane potential of the substrates.

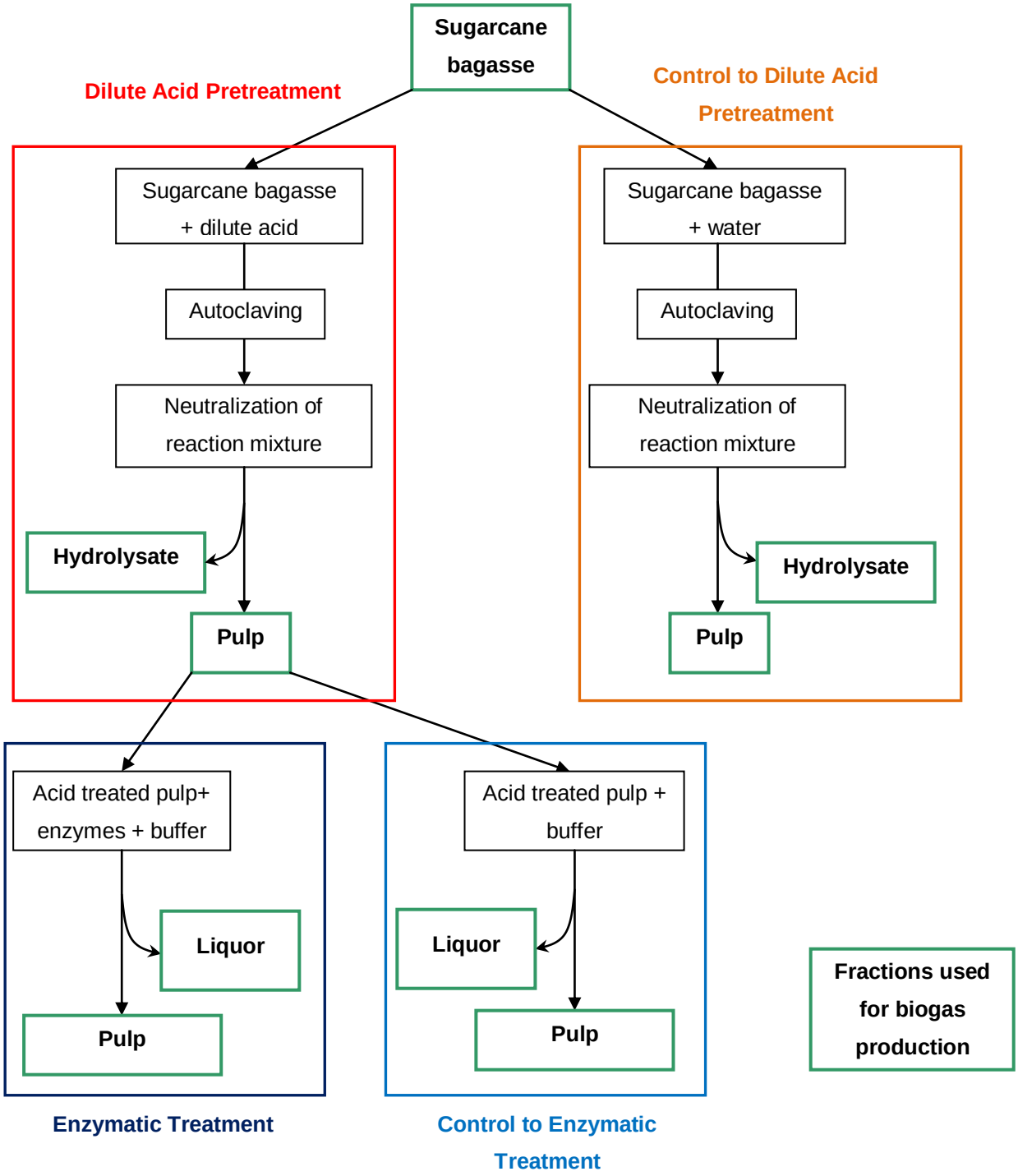

Figure 3.5 – Flow sheet presentation the pretreatment steps employed and the fractions used in the batch anaerobic digestion.

3.7.1 Inoculum

The inoculum used was an anaerobic sludge from the biogas plant at Wrams Gunnarstorp castle (Bjuv, Sweden). This plant treated pig manure, slaughterhouse wastes and waste products from the Findus food processing factory. The sludge was received July 2009.

It was degassed, that is, pre-incubated in order to deplete the residual biodegradable organic material present in it. That incubation was made at 37ºC (process temperature) in a shaking water bath, for about a week. After that, it was stored at room temperature. Before use, it was analyzed for TS, VS, partial and total alkalinity.

3.7.2 Dilute Acid Pretreatment

A hundred grams of bagasse (dry weight) were added to two 5 L Erlenmeyer flask each and mixed with 1878 mL of 0.1M H_2SO_4, to have final working volumes of 2 L and loadings of 5% TS. A control using water was also prepared. The autoclaving was made at 121ºC for 30 minutes and the neutralization and filtration occurred in similar fashion to what's described in 3.5.1.

Samples of the pulps were taken to analyze for TS and VS. The hydrolysates were analyzed for TS, VS and reducing sugars concentration.

The pulps were not dried, and instead were stored inside sealed plastic bags and placed inside a cold room at 4ºC, to keep moisture content. The hydrolysates were placed in a glass bottle and stored in a freezer.

3.7.3 Enzymatic Treatment

Fifty grams (TS) of bagasse from the acid-treated pulp prepared in the previous step were added to a 3 L Erlenmeyer flask and mixed with pH 4.8 phosphate buffer to have a final working volume of 1 L and a loading of 5% TS. It was added 3% (working volume) of Accellerase 1000 and also 3% of GC 140 xylanase. An appropriate control was also prepared, using only bagasse and buffer.

The flasks were put into a shaking water bath at 50ºC and 70 rpm for 13 hours. Samples were taken at the beginning and end of the reaction and analyzed in triplicate with the DNS method. After the reaction, the solid fraction was separated by vacuum filtration (refer to section 3.5.1).

Both fractions were analyzed for TS and VS.

3.7.4 Anaerobic digestion

3.7.4.1 Experimental Setup

The batch anaerobic digestion was carried out in 500 mL Erlenmeyers, using triplicates for every type of substrate used (refer to Figure 3.5 – 9 substrates, 27 flasks).

The inoculum and substrates were mixed based on their VS content, and in order to have final working volumes of 300 mL. The original bagasse, the acid treated pulp and hydrolysate, as well as the enzymatic treated pulp and liquor were mixed with inoculum using an inoculum/substrate ratio of 2 (VS basis). The masses of inoculum and substrate added for each reactor were calculated based on equations (3.26) and (3.27). The liquid relative densities were approximately equal to 1 and the pulps

were also considered to have a density of 1, either because they were used in small quantities (original bagasse) and/or because they were saturated with liquid.

$$Mass_{substrate} = \frac{Working\ volume \times \%VS_{inoculum} \times \%TS_{inoculum}}{\%VS_{inoculum} \times \%TS_{inoculum} + ratio \times \%VS_{substrate} \times \%TS_{substrate}} \quad (3.26)$$

$$Mass_{inoculum} = ratio \times Mass_{substrate} \times \frac{\%VS_{substrate} \times \%TS_{substrate}}{\%VS_{inoculum} \times \%TS_{inoculum}} \quad (3.27)$$

Where: *ratio* = 2
masses are in g and volume in mL

The remaining substrates, which were controls, were used in the same quantities calculated for their respective counterparts.

The control for the digestion reaction was composed of reactors containing only inoculum and water, to measure the background inoculum activity. Since the mass of inoculum mixed with each substrate was not the same, and considering that the amount of gas produced was expected to vary slightly with the amount of inoculum used, two different reactor compositions were used. One 'mimicked' the composition of the reactors containing the original bagasse, which employed the greatest amount of inoculum. The other one covered the other end of the spectrum, the reactors containing acid treated hydrolysate, which used the least amount of inoculum. The effect of enzymes alone on the anaerobic digestion was disregarded, as the amount of enzyme present in the reactors was negligible. Therefore, control reactors using enzymes, inoculum but no substrate were not prepared.

A reference was prepared, that is, a set of triplicates of reactors containing inoculum and analytical grade cellulose (Avicel), using the same 1:2 VS ratio used with the bagasse samples. This reference assay was used to give an idea of the inoculum response towards more simple and "standard" substrates (Angelidaki *et al.*, 2009).

Reactor composition is summarized in Table 3.6.

Table 3.6 – Composition of each reactor for anaerobic digestion.

Type of substrate	Mass of Inoculum (g)	Mass of Substrate (g)
Original Bagasse	296.62	3.38
Acid treated pulp	276.09	23.91
Control to acid treated pulp	276.09	23.91
Acid treated hydrolysate	157.54	142.46
Control to acid-treated hydrolysate	157.54	142.46
Enzyme treated pulp	282.22	17.78
Control to enzyme treated pulp	282.22	17.78
Enzyme treated liquor	214.74	85.26
Control to enzyme treated liquor	214.74	85.26
None (water)	296.62	3.38
None (water)	157.54	142.46
Reference (Avicel - cellulose)	297.31	2.69

The substrate and inoculum were added to the vessels and the pH values of the mixtures were recorded. They were then flushed with nitrogen for about three minutes to remove most of the oxygen from the liquid and the headspace, to create and anaerobic environment. After each flask was flushed, it was immediately clogged and linked to an empty sealed bag, to collect gas. The reactors were placed inside shaking water baths at 37ºC and 70 rpm (Figure 3.6). The start of the digestion was July 22, 2009, around 19:00.

The reaction was followed by analyzing the volume of gas produced and its composition, particularly in carbon dioxide and methane. Whenever an analysis was made, the temperatures in all the regions of the room where the gas bags stood were also recorded. For the first five days, these analyses were made every day. Afterwards, they started to be less frequent, as the daily volume of gas produced was not enough to justify that frequency. The flasks were taken out of the baths and manually shaken on every analysis session.

Figure 3.6 – Experimental setup for the anaerobic digestion

3.7.4.2 Determination of gas composition and total gas volume

300 µL gas samples were taken from each reactor using a 2 mL gas-tight plastic syringe. The gas composition was analyzed by gas chromatography (GC), injecting the samples in a 6890 Network GC system (Agilent Technologies), equipped with a Haysep N 80/100, 9 ft, 1/8 mesh column, a molecular sieve (Molesieve 5 A 60/80, 6 ft, 1/8), a stainless-steel column and a thermal conductivity detector.

For all the reactors, except the ones with cellulose, it was used a method using helium as the carrier gas, with a flowrate of 28.8 mL/min, column temperature at 60ºC and detector temperature at 150ºC (main method). The method used for the Avicel-containing reactors was identical in everything except the column temperature, which was set at 80ºC (Avicel method).

The software used had already implemented a program that determined approximate gas compositional percentages of the samples, based on the peaks areas detected for nitrogen, oxygen, carbon dioxide and methane. But for a more precise calculation of the percentage of methane, the

integrated area of the methane peaks was converted to volumes of gas using two calibration curves – one for each of the methods used (Annex 7.2.2, Figure 7.10 and Figure 7.11). The calibration curves were made by injecting known volumes of methane using a gas mixture composed of methane and carbon dioxide (60% CH_4, 40% CO_2 – supplied by Air Liquide) into the GC system. The volume of methane injected was then plotted against the peak area for methane, obtaining equations (3.28) and (3.29). The methane percentage was then calculated by equation (3.30).

$$Volume_{methane,main\ method}\ (\mu L) = \frac{Peak\ area\ (mV.S) - 785.49}{156.39} \quad (3.28)$$

$R^2 = 0.9992$

$$Volume_{methane,Avicel\ method}\ (\mu L) = \frac{Peak\ area\ (mV.S) - 132.69}{156.87} \quad (3.29)$$

$R^2 = 0.9991$

$$\%CH_4 = \frac{Volume\ CH_4\ detected\ by\ GC}{Volume\ of\ GC\ injected\ sample} \times 100 \quad (3.30)$$

Where: *Volume of GC injected sample* = 0.3 mL

The amount of total gas produced was determined by emptying the gas bags using a gas measuring syringe. Since the temperature inside the room was not homogeneous, and also to standardize the results, all volumes were converted to standard temperature and pressure (STP) conditions, that is, 0ºC and 1 atm, considering it was an ideal gas.

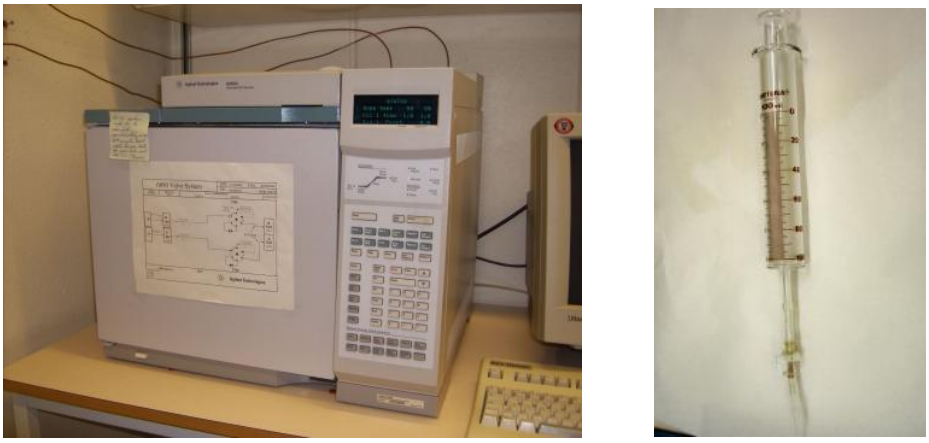

Figure 3.7 – GC system used for gas composition analysis (left) and gas measuring syringe used for determination of gas volume produced (right).

The digestion was run for 52 days. After stopping the reaction, the headspace of the reactors was measured. That measurement was made by weighting the amount of water (admitting density =1) needed to fill the reactors from the surface of the reaction sludge to the stopper level.

The reaction sludge was analyzed for TS, VS, pH and alkalinity.

Whenever the gas was sampled and the gas bags emptied, methane production was calculated by multiplying the methane content of the analyzed sample by the total gas volume present in the bags. The cumulative methane production was followed by adding the individual methane productions recorded in each analysis session (equation (3.31)).

However, these values didn't represent the total methane production, as the headspaces of the reactors had appreciable volumes of methane, but which could only be accounted for at the end of the digestion (equation (3.32)).

$$Cumulative\ CH_{4,\ i}(mL) = \sum_{i} \left(Volume_{gas,i} \times \%CH_{4,i}\right) \quad (3.31)$$

Where: *Cumulative* $CH_{4,\ i}$ = methane accumulated in the gas bags at the analysis number *i* (mL)
$Volume_{gas,i}$ =volume of gas measured on the analysis number *i* (mL)
$\%CH_{4,i}$ = percentage of methane present in the gas on the analysis number *i*

$$Final\ CH_4\ (mL) = Cumulative\ CH_{4,\ n} + Volume_{headspace} \times \%CH_{4,n} \quad (3.32)$$

Where: *Cumulative* $CH_{4,\ n}$ = methane accumulated in the gas bags at the end of the reaction
$\%CH_{4,n}$ = percentage of methane present in the gas on the analysis number *n* (last one)

3.7.4.3 Determination of biochemical methane potential

The result of a biochemical methane potential (BMP) test, as defined in Angelidaki *et al.* (2009), is the total quantity of methane produced from a given weight of a certain substrate. This specific methane production of one sample can be reported as volume of methane produced per gram of VS.

However, since the inoculum alone also produced some methane, it was necessary to subtract that background methane production. Yet, the controls with inoculum and water only covered two concentrations. So, it was assumed that the amount of methane produced varied linearly with the mass of inoculum added. It was then established a trendline with the background methane production values as a function of the initial mass of inoculum added to the reactors. Those corrected CH_4 volumes were then used to determine the final BMP (equation (3.33).

$$BMP\left(\frac{mL\ CH_4}{g\ VS}\right) = \frac{Final\ CH_{4(sample)} - Final\ CH_{4(inoculum)}}{Weight_{substrate} \times \%VS_{substrate} \times \%TS_{substrate}} \quad (3.33)$$

Where: *Final* $CH_{4(sample)}$ = final methane production of a reactor
Final $CH_{4(inoculum)}$ = respective background methane production due to the inoculum
(Weights in g and volumes in mL)

BMP evolution with digestion time was followed by determining a 'pseudo BMP', based on the cumulative methane production from the gas bags alone. When the curves exhibited typical BMP profiles, first order kinetic models were adjusted to them, in order to determine their kinetic constant k_h. That adjustment was made using the least squares method and the *Solver* tool from Microsoft™ Excel.

To calculate the reduction of VS, a similar calculation method was used, since a part of the final VS of each reactor was due to inoculum. Therefore, it was established a linear trendline of final inoculum VS as a function of the starting inoculum VS. The VS reduction of each reactor was then calculated using that correction (equation (3.34)).

$$VS\ reduction\ (\%) = \frac{Initial\ VS_{(substrate)} - \left(Final\ VS_{(sample)} - Final\ VS_{(inoculum)}\right)}{Initial\ VS_{(substrate)}} \times 100 \quad (3.34)$$

Where: *Final* $VS_{sample)}$ = final VS of a reactor (g)
Final $VS_{(inoculum)}$ = respective amount of VS due to the inoculum (g)
Initial $VS_{(substrate)}$ = amount of substrate VS added to the reactor (g)

4. Results and Discussion

4.1 Bagasse primary characterization

From Figure 3.1 it was possible to observe that the aspect of the bagasse varied with its source, as the Indian bagasse had a lighter color than its Pakistanese counterpart. In Table 4.1 are summarized the some of the characteristics of the two.

Table 4.1 – Characteristics of the sugarcane bagasses used in this work. (Values in percentages).

Bagasse origin	India	Pakistan
TS	91.6	82.4
Moisture	8.4	17.6
VS (dry basis)	98.1	96.6
Ash (dry basis)	1.9	3.4
Particle size distribution (mm)		
≥ 10	1.0	4.6
[4;10[	12.7	18.2
[1.6;4[	41.8	20.6
[0.85;1.6[	25.0	13.4
[0.18;0.85[	19.2	34.4
< 0.18	0.3	3.7

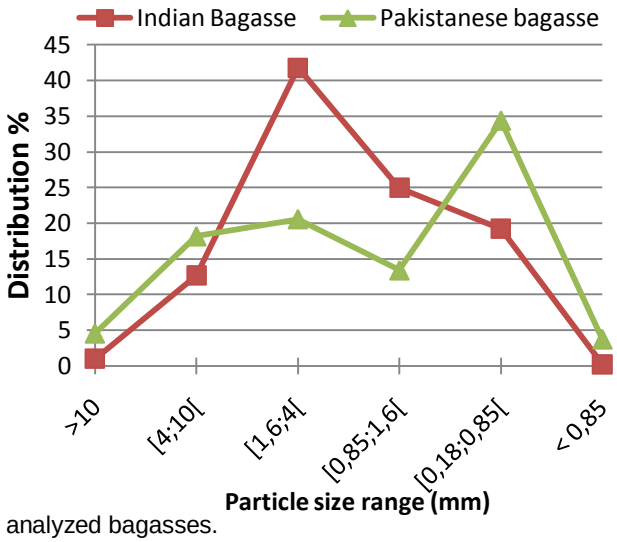

Figure 4.1 – Distribution profile of the particle sizes of the analyzed bagasses.

The moisture content of the Pakistanese bagasse was significantly higher, and so was the inorganic matter. As for the particle size, the Pakistanese bagasse had a greater fraction of both very big and very small particles, particularly the latter. When working with this type of bagasse, there were always many fines floating around, while such thing didn't happen when working with Indian bagasse. While the greater amount of small particles may benefit hydrolysis by having greater surface area available, the larger amount of inorganic matter may be worse for biogas yield on a dry substrate basis.

4.2 Indian bagasse fiber analysis

Table 4.2 – Chemical composition of untreated and acid treated sugarcane bagasse (Values in percentages).

Component	Original (Untreated) bagasse		Acid treated bagasse	
Total Carbohydrates:	**56.3**		**52.1**	
Cellulose (glucan)	39.7		45.3	
Total Hemicellulose:	16.6		6.8	
Xylan		*14.6*		*6.1*
Arabinan		*1.8*		*0.7*
Mannan		*0.1*		*0.0*
Total Lignin:	**30.4**		**25.7**	
Acid Insoluble Lignin	22.2		21.4	
Acid Soluble Lignin	8.2		4.3	
Total Extractives:	**4.9**		**21.8**	
Water Extractives	3.1		18.2	
Ethanol Extractives	1.8		3.6	
Others*	**8.3**		**0.4**	

* includes inorganic non-extracted residues and the remaining unaccounted biomass percentage.

The chemical composition of the original Indian bagasse and the acid treated bagasse prepared by the main pretreatment process are summarized in Table 4.2.

Independently of the bagasse type, glucan showed to be the most abundant component. The total carbohydrate content was a little above 50%. For biogas production, all of the carbohydrates are valuable components, since many organisms are present, and thus many kinds of sugars can be metabolized. The main component of hemicellulose was xylan. The other sugars existed in smaller quantities and galactose wasn't detected.

Composed 16.6% of hemicellulose, this bagasse had a significantly lower content than other values found in literature (Sasaki *et al.*, 2003; Rodríguez-Chong *et al.*, 2003, Pessoa *et al.*, 1997; Cordova *et al.*, 1998). Still, the main component of hemicellulose was still xylose, with arabinose being a distant second. Glucan content was within the expected range, but lignin was higher than observed on any of the mentioned works. This means this bagasse was not expected to be particularly good for saccharification, since it had a relatively low carbohydrate content and a high lignin content, which would difficult the former's breakdown.

Comparing both types of bagasse, it could be observed that, during acid hydrolysis, carbohydrates, specifically hemicellulose, were solubilized to a certain degree, but lignin was less removed. Even if the acid didn't completely break the structural binding and dissolved some components, it may have left a significant part of the material very "loose", more prone to be removed by the water and ethanol extraction. This would explain the much higher amount of extractives on the acid treated bagasse.

There wasn't a great fluctuation in the total carbohydrate and lignin fractions, but the individual components of each fraction suffered some significant changes.

Within the carbohydrates, the increase in the total percentage of glucan comes as no surprise, seeing as cellulose is little affected by the acid treatment. Counterbalancing is the hemicellulose, which is very sensitive to degradation by acid. Its content didn't go, however, below 6%. Even though it was reported to be possible almost 100% hemicellulose removal by dilute acid hydrolysis (Taherzadeh *et al.*, 2008; Silverstein, 2004), the conditions and equipment used, or maybe the biomass itself, didn't allow such to happen. In any case, these results hinted that the use of a xylanase even after acid treatment could benefit the enzymatic reaction.

As expected, lignin did not suffer a great degree of solubilization, except the acid soluble lignin, but its percentage was already small to begin with. Whether lignin disruption happened and cellulose's susceptibility to enzymatic degradation increased was evaluated during the enzymatic hydrolysis experiments.

4.3 Dilute acid pretreatment

After autoclaving, the aspect of the two treated and untreated bagasses was significantly different. The untreated bagasse appeared unchanged while the acid treated had acquired a reddish color. The pH of solution with acid treated bagasse was 0.8-0.9 while in the untreated was around 4.6-4.7. For the working volume used (200mL), it was needed approximately 8.3 mL of 0.5 NaOH solution to bring the acid treated bagasse solution to pH 7, while it was a matter of very few microliters with the

untreated bagasse. After neutralization, while the untreated bagasse still looked relatively unchanged, the acid treated had acquired a much darker color. While Figure 4.2 refers to the milled bagasse, the general appearance is valid for all the acid treatments made with Indian bagasse, albeit with larger particles. After filtration and drying, the acid treated pulp had a different texture from that of its untreated counterpart. It had a rougher feel and it was much more brittle. A lot of smaller particles were present and some had agglomerated into coarser particle aggregates.

Figure 4.2 – Appearance of the milled bagasses during two stages of the dilute acid pretreatment: after autoclaving (left picture) and after neutralization (right picture). In both pictures, the flask of the left has acid treated bagasse and the one on the right has untreated bagasse.

The reducing sugar concentrations of the hydrolysates were obtained by following the protocols described in 3.5.1 and 3.5.2.1. The main pretreated hydrolysate (0h) and the ones which were placed in the water bath for 6, 12 and 24 h, are presented in Figure 4.3

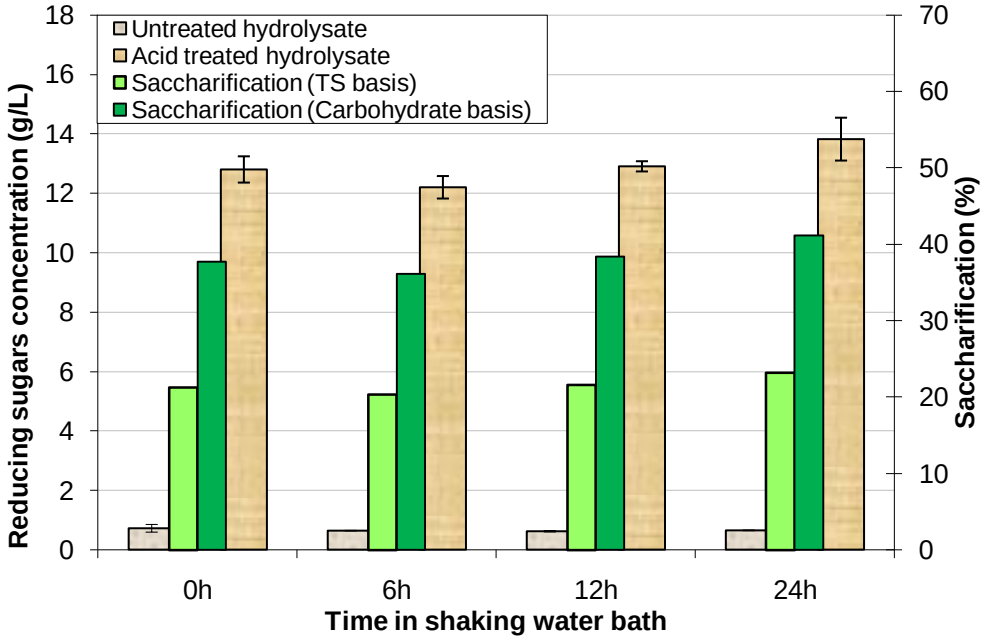

Figure 4.3 – Effect of dilute sulphuric acid treatment and time in shaking water bath after autoclaving in the sugar concentrations present in the hydrolysate. The bars are an average of the values obtained from the triplicate analysis. Their standard deviation is represented by the vertical error bars. Hydrolysates' sugar concentration is read on the left axis, while saccharifications are read on the right axis.

There was a very large increase in the amount of reducing sugars released into the hydrolysate when acid treatment was applied, comparing to the untreated (control) bagasse. Overall, after this acid pre-treatment step, the hydrolysates had about 13 g/L of solubilized reducing sugars, which corresponded to 22% of the TS used. Since the origin of these sugars was the biomass carbohydrates, the maximum theoretical conversion would be the amount of carbohydrates present. Using that perspective, it was achieved around 38% of carbohydrate saccharification.

The time in the shaking water bath seemed to have no significant effect on sugar release. Although it was observed a slightly higher sugar concentration in the 24h hydrolysate (RPD of 7.7% vs. 0h), its standard deviation was also greater.

The HPLC analysis of the main pretreated hydrolysate returned 10.31g/L of xylose, 0.62g/L of both cellobiose and glucose, and 2.07 g/L of acetic acid. No other organic acids were found within the ones tested. These results were within expectations, since the main component solubilized during acid treatment is hemicellulose, which is mostly made of xylose. The acetic acid present was mostly from the acetylated residues of xylan.

Recovery of xylan as xylose and glucan as glucose and cellobiose was estimated using equation (3.23). No HPLC data was available for the untreated (control) hydrolysate, but it was assumed that all reducing sugars detected were xylose, since xylan is the most fragile molecule. The anhydro factor of cellobiose was (324/342). Those calculations returned 2.8% glucan recovered as glucose and a further 2.7% recovered as cellobiose. Xylose recovery, however, was 115%. The reason for this value laid in a conflict between the xylose concentration detected on the hydrolysate and the xylose content of the raw bagasse. Since the values from HPLC seemed to be in accordance with the values from DNS analysis, it seems more probable that the quantification mistake belonged to the fibre analysis, which, as discussed earlier, returned a significantly lower xylose content than expected.

To suport this hypothesis, the sugar and acetic acid concentrations obtained in this treatment were compared to the ones observed in Aguilar *et al.* (2002) (Figure 2.8). Their treatment was operated at a simillar temperature, but with double the solids loading. The concentrations observed in this work were nearly half of Aguilar's. Since linearity between solids loading and product concentrations is usually observed in the 5-10% range, this would mean that treatments in both works solubilized similar amounts of sugars. However, in their work that amount corresponded to nearly 90% of xylan removal, while here the total xylan content went from 16% to 6%, which would correspond to a much smaller removal. In order to get conclusive results the fiber analysis should be repeated.

But if the xylan removal was actually lower in this work, then a probable cause would lay in the sulphuric acid concentration, since the one used here was less than half of Aguilar's work. In the work of Lavarack *et al.* (2002), the optimum treatment conditions were also achieved at 2% (w/w) sulphuric acid concentration.

The pretreatment variation described in 3.5.2.2 was applied to study the effect of the residence time in the autoclave at maximum temperature (121ºC). Increasing the residence time from 15 to 30 minutes resulted in higher sugar concentrations (RPD 12.3%). However, further increasing the time to 60 min resulted in a minimal increase. Therefore, residence times of 30 min seemed more attractive in

terms of the hydrolysate's sugar concentration, when weighting sugar amount against time spent in the treatment (Figure 4.4).

Due to limitations in the quantity of raw material available, this experiment was scaled down from the main pretreatment process, using total volumes equal to ¼ of the first one. This scale down seemed to slightly affect negatively the sugar release, as shown by the results for 15 minutes of this experiment and by the 0h from Figure 4.3, since the same residence time was used in both cases. This could be partially attributed to internal liquid circulation in smaller vessels being more limited.

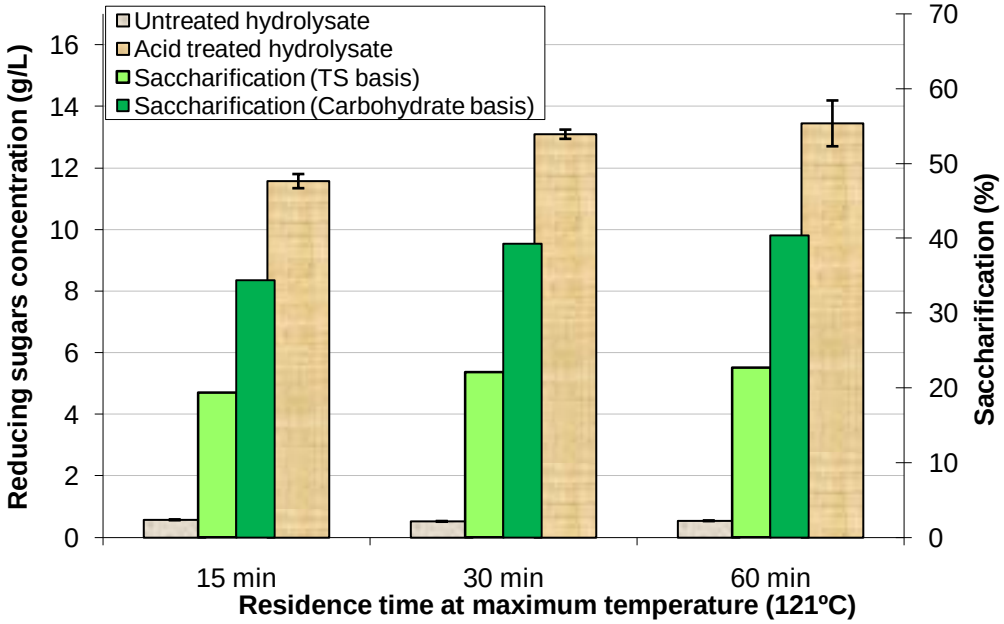

Figure 4.4 - Effect of dilute sulphuric acid treatment and autoclave residence time sugar concentrations present in the hydrolysate. The bars are an average of the values obtained from the triplicate analysis. Their standard deviation is represented by the vertical error bars. Hydrolysates' sugar concentration is read on the left axis, while conversions are read on the right axis.

Results from the DNS analysis of the hydrolysate obtained with combined milling and acid pretreatment are illustrated in Figure 4.5, in which they are compared to the hydrolysate from the main pretreatment process (non-milled). The reducing sugar concentration of milled acid treated bagasse was 20.8% (in RPD) inferior to its respective non-milled bagasse. These results were slightly unexpected, since particle size reduction should increase surface area and acid diffusion through the fiber and should thus not hinder the reaction. In the work of Lavarack *et al.* (2002), dilute acid hydrolysis of bagasse and bagacillo (the finer size fraction of bagasse, separated during later stages of sugarcane refining) was carried out, from which was observed that particle size had a negligibly effect on the rate of hydrolysis. Despite this, a possible reason for this difference would be that, since no agitation was applied during treatment, particles may have deposited in the bottom of the flask, forming a wet cake. Due to their small size, acid penetration and diffusion on that layer of biomass might have been hindered.

Alternatively, it's possible that the use of smaller particles resulted in greater sugar degradation. Considering a typical profile of xylose with process time (Figure 2.8) and the remaining results from Aguilar et al. (2002), xylan recovery is a very sensitive process, as xylan's monomeric sugar degradation is much more accelerated than glucan's. The increased contact between xylan and acid

due to size reduction might have had the effect on generating more sugar degradation byproducts like furfural. Assuming that the xylose profile would be similar to the one Figure 2.8, it would mean that the pretreatment time used was already past the maximum recovery peak, into the section where degradation becomes more significant than monomeric sugar release. But since in the mentioned work it were used small particles, this hypothesis is less probable. Still, an HPLC analysis targeting furfural and/or acetic acid detection in the hydrolysates would have been able to clear up that fact.

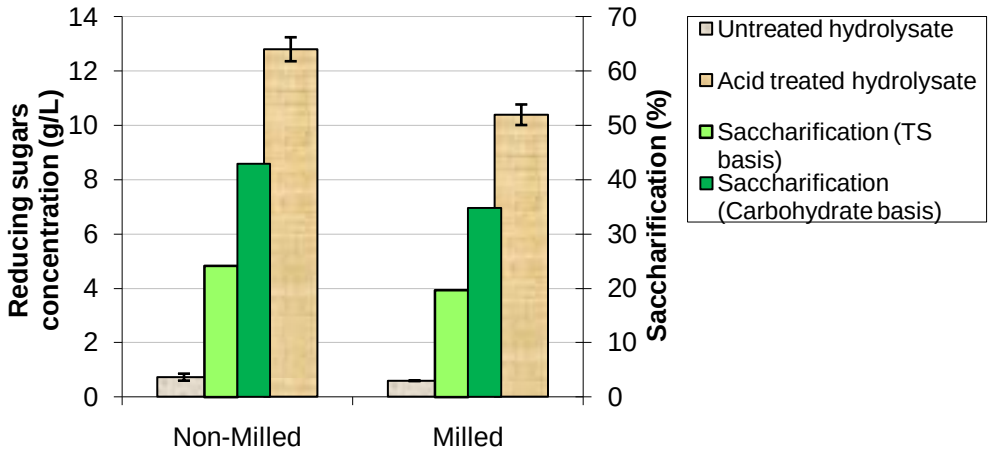

Figure 4.5 - Effect of milling bagasse on acid pretreatment. The bars are an average of the values obtained from the triplicate analysis. Their standard deviation is represented by the vertical error bars. Hydrolysates' sugar concentration is read on the left axis, while conversions are read on the right axis.

This brings us to the main limitation of using the DNS method for analyzing this kind of pretreatments. While it can give a good estimate of the amount of readily metabolizable substrates available, it does not give insight into the other components present in solution, out of which the most relevant are possible inhibitors of microorganism development, such as carboxylic acids, furans and phenolic compounds.

4.4 Enzymatic Hydrolysis

4.4.1 Effect of enzyme loading on the hydrolysis of untreated bagasse

The enzymatic reaction setup described in 3.6.2 was carried out to test the influence of enzyme loadings in the enzymatic conversion of bagasse. The temperature and pH (50º and 4.8, respectively) were chosen in order to be within both enzymes' intervals of optimal activity. In Figure 4.6 is shown the evolution of the saccharification during enzymatic hydrolysis, at different enzyme loadings. The reaction was carried out for 72h to give enough time for the curves to stabilize.

The results showed a great dispersion of data, making it difficult to extract information about the effect of changing the enzyme loading on biomass saccharification. The curves for the lower concentrations returned the most dissonant results, with 2.85% and 3.80% showing some unexpectedly high values in the 30-48h mark, exhibiting higher saccharification yields than with higher enzyme loadings. However, by the end of the 72h reaction, their values fell back into lower levels. Experimental error may have been the main cause for this, as there still wasn't any familiarity of the operator with the analysis method, missing some of its nuances. The one most likely to affect more

severely the results was when taking out the 50 µL aliquot for DNS analysis from the samples taken from the reactors. While the samples taken in the first hours of the reaction were usually a clear liquid, later samples had solid particles deposited in the bottom of the Eppendorf. If taking out the 50 µL wasn't careful enough, some particles could be also dragged along. These suspended solids would then interfere with the spectrophotometric analysis, causing higher readings than expected. The 4.75% curve, on the other hand, achieved lower sugar concentrations than expected, but no explanation could be found for this case.

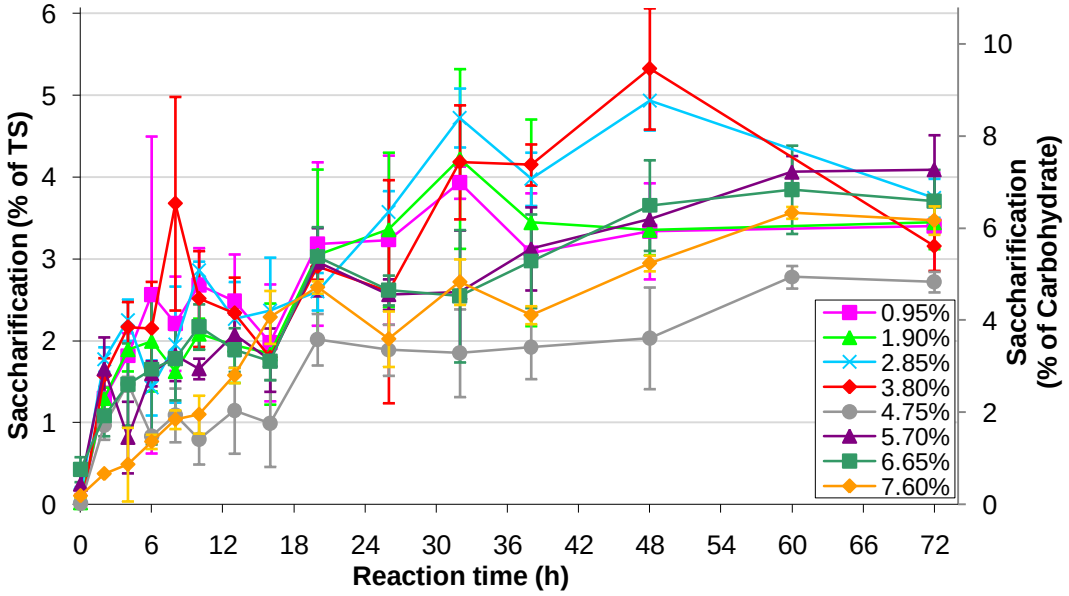

Figure 4.6 – Saccharification yields during enzymatic hydrolysis of untreated sugarcane bagasse at different enzyme loadings. The points are an average of the values obtained from the triplicate reactors. Their standard deviation is represented by the vertical error bars. (Legend: x % = x% (v/v) of Accellerase 1000 + x% (v/v) GC 140 xylanase).

Still, some important general considerations could be taken from this data. First, as expected, enzymatic hydrolysis alone was not a good method to release sugars from bagasse. The saccharification was roughly 3-4% on a TS basis (or 6-7% on carbohydrate basis), even when employing high Accellerase and xylanase loadings (reducing sugar concentrations detected in the liquid fraction were inferior to 4 g/L – Annex 7.3, Figure 7.13). The curves for 0.95%, 1.9%, 5.7%, 6.75% and 7.6% exhibited similar profiles, which could indicate that the loading range examined (corresponding to 0.48 – 3.83 mL Accellerase/g cellulose) was already past the limiting enzyme concentration. This would match with the Accellerase loading profile shown in Figure 2.17.

A certain stabilization of the hydrolysis of sugars could be observed after 48h of reaction time. This meant that using longer reaction times would bring no significant benefit to the final sugar yield.

4.4.2 Effect of acid pretreatment with post-autoclaving incubation

The enzymatic digestibility of the acid treated bagasses prepared during the main pretreatment process and the post-autoclaving incubation were tested with the setup described in 3.6.3, using enzyme loadings of 5.7% of Accellerase 1000 and 5.7% of GC 140 xylanase. This high loading was used to ensure that the reaction rate and extent were not limited by enzyme activity.

The evolution of reducing sugars concentrations present in the liquid fractions is represented in Figure 4.7.

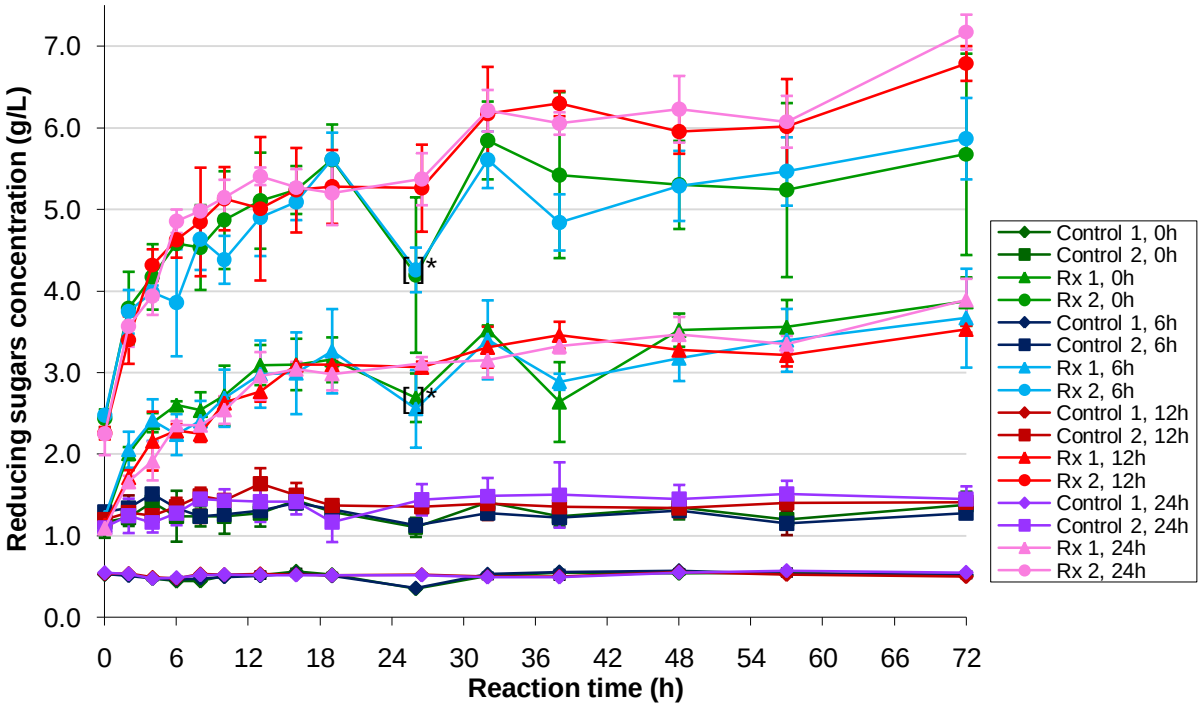

Figure 4.7 - Effect of dilute sulphuric acid treatment and time in shaking water bath in the amount of sugar released during the enzymatic hydrolysis of sugarcane bagasse, using a 1:1 mixture of Accellerase 1000 and GC 140 xylanase and a loading of 5.7% of each enzyme. The points are averages of the values obtained from the triplicate reactors. Their standard deviation is represented by the vertical error bars. (Legend: Control 1 – untreated bagasse without enzymes; Control 2 – acid treated bagasse without enzymes; Rx 1 – untreated bagasse with enzymes; Rx 2 – acid treated bagasse with enzymes; 0h, 6h, 12h and 24h – time the reactors were incubated post autoclaving.)
* the DNS reagent used was changed, but the blank was still made with the old one.

After an initial rapid increase of sugar concentration, a slowdown on the sugar release rate seemed to occur after 13h of reaction time, though greater stability only happened later, after 32h. This was possibly related to the crystallinity of cellulose, as the major part of the more amorphous cellulose is the first to be extensively converted, leaving behind the harder-to-digest crystalline cellulose.

The reactors with untreated bagasses (Rx 1) had a curve very similar to what was obtained in the earlier experiment, at a 5.7% loading (Figure 4.6). The sugar concentrations were quite low, never quite reaching 4 g/L. Even though no acid was used, these bagasses underwent autoclaving, neutralization, filtration and drying, just like the acid treated bagasse; therefore they are not identical to the original bagasse. The fact that the same sugar concentration profiles are obtained means that air drying of the untreated bagasse didn't cause irreversible structure collapse, or at least it didn't have a relevant effect on its enzymatic digestibility. However, it is possible that the same wouldn't happen with acid treated bagasse (see section 4.5.1).

With the acid treated bagasses (Rx 2), the sugar concentrations reached higher levels, between 5.7 and 7.2 g/L. But there was an obvious larger background sugar concentration when acid treated bagasse was used, as shown by all the Control 2 reactors. This could be attributed to the formation of reducing sugars during the pretreatment step and, since the solids weren't washed, remained

associated to the solid fraction. The maximum sugar concentration for 0h acid treated bagasse was at 32 hours, after which it suffered a slight decrease accompanied by a growing standard deviation. This was due to some of the triplicates starting to exhibit different behaviours, with one of them showing more or less constant sugar concentrations, another one where they continued to increase and another which suffered a decrease. This could be explained by possible bacterial contamination in some of the reactors, consuming the sugars released by hydrolysis.

Although in this experiment sugar concentrations didn't reach very high values, it should be kept in mind that end product inhibition by glucose is one of the main reasons which could limit enzymatic activity. It is therefore desirable that the initial sugar concentrations are as low as possible. While in this case, the difference between Control 1 and 2 was around 0.6 g/L, it may prove advantageous to wash the solids after acid treatment and join that washing stream with the hydrolysate.

In order to better compare the results of acid treated and untreated bagasses, these results were expressed as saccharification yields, with 'Control 1' and 'Control 2' reactors being the 'bagasse+buffer' controls correspondent to 'Rx 1' and 'Rx 2' reactors. That way, the different background sugar concentrations of untreated and acid treated bagasses were eliminated (Figure 4.8).

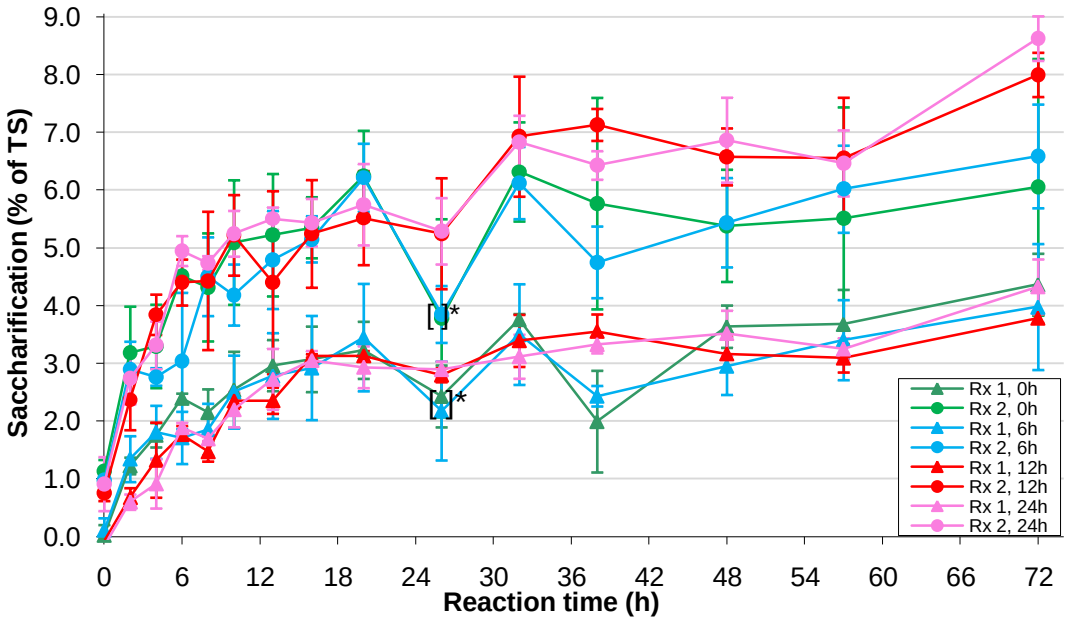

Figure 4.8 – Saccharification yields (TS basis) of untreated and acid treated sugarcane bagasse, using a 1:1 mixture of Accellerase 1000 and GC 140 xylanase and a loading of 5.7% of each enzyme. The points are averages of the values obtained from the triplicate reactors, subtracting the values of the respective control reactors. Their standard deviation is represented by the vertical error bars. (Legend: Rx 1 – untreated bagasse with enzymes; Rx 2 – acid treated bagasse with enzymes; 0h, 6h, 12h and 24h – time the reactors were incubated post autoclaving.)
* the DNS reagent used was changed, but the blank was still made with the old one.

Enzymatic treatment of acid treated bagasse obtained greater yields than when untreated bagasse was used. The maximum yields on a TS basis were 6.0-8.6% for acid treated bagasse, while untreated bagasse reached only 3.8-4.3% (or 11.6-16.5% against 6.7-7.7% on a carbohydrate basis - Annex 7.3, Figure 7.14). This means that the acid treatment rendered structural carbohydrates more accessible to enzymatic hydrolysis.

The effect of time in the shaking water bath was significant for longer reaction times with the acid treated bagasse. Both 12h and 24h bagasses exhibited a biphasic curve. At 72h of reaction time, the saccharification of the 24h bagasse was 35% higher than the 0h bagasse. However, there was a greater variability with the bagasses from 0h and 6h, which shed some uncertainty over whether the incubation time actually improved digestion. In the very least, for smaller reaction times, the differences in conversion were much smaller. Furthermore, when planning a large scale application for this process, it is unlikely that this reaction would be run for 72 hours, as the sugar release speed is much lower after just 13 hours, making longer reaction times economically disadvantageous. For that reason, using this pretreatment variation is probably not a good approach and wasn't further considered in this work.

It is relevant to note that not all curves have their starting point at 0% saccharification, especially with acid treated bagasse. This is likely due to the fact that preparing all the reactors and taking the first samples took some time (~15 minutes), over which the enzymes had already started the reaction. While the room temperature was inferior to their optimum 50ºC, their activity may still have been significant (Figure 2.16 d), which combined with the high initial rates of reaction of the treated bagasse resulted in an apparent 'head start' of the conversion.

4.4.3 Effect of combined milling and acid pretreatment

The milled bagasse pulps obtained from the acid pretreatment (Figure 4.9) were used the enzymatic setup mentioned in 3.6.4.

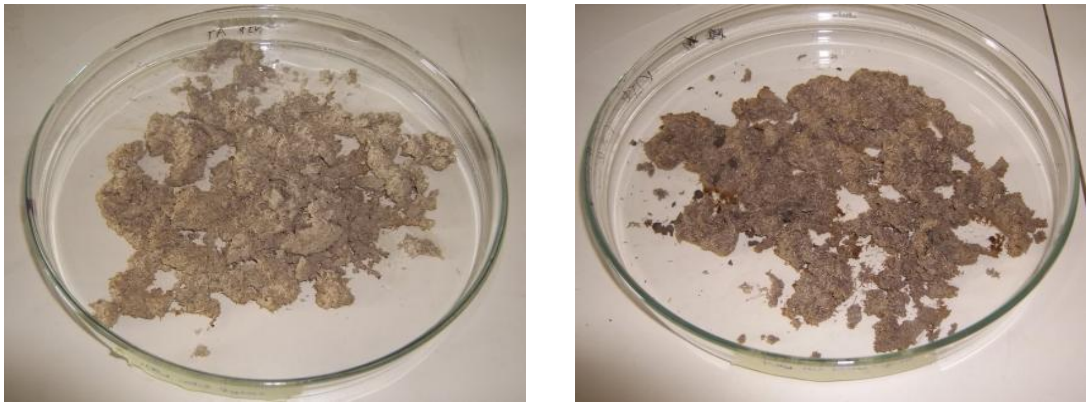

Figure 4.9 – Appearance of the dry milled bagasses used for enzymatic hydrolysis: left – untreated (control); right – acid treated

The saccharification of the milled pulps was compared against the non-milled pulps (Figure 4.10).

The use of untreated milled bagasse resulted in higher saccharification extents than when using non-milled bagasse, quite likely due to a great crystallinity decrease. After 16h of reaction time, saccharification of untreated milled bagasse was much higher than its non-milled counterpart and was on par with non-milled acid treated bagasse. Longer reaction times further enhanced this difference. Even at 48 h, it did not look that the yield had stopped increasing. It would have been interesting to let this reaction run for a longer time to be able to observe a stabilization of the sugar release, thus drawing some more conclusions. However, the initial reaction rate was lower. From 0 to 13h, the sugar concentrations were significantly below the non-milled acid treated bagasse, only catching up at 16h.

Acid treated milled bagasse didn't return such good results as it was expected. The initial reaction rate was on par with the non-milled acid treated bagasse, but other than that it did not seem that the final reaction extents were superior to milled untreated bagasse. In fact, apart from the initial hours of the reaction, it seemed that the acid treatment did not significantly improve the enzymatic hydrolysis of milled bagasse. Milling is an energy intensive process, while dilute acid treatment generates chemical residues that must be dealt with. Using both of them in an industrial process comes at a great cost, and should only be used if there is a significant advantage in doing so. Therefore, for the treatment conditions employed, milling combined with dilute acid pretreatment did not appear to be a good choice for bagasse treatment.

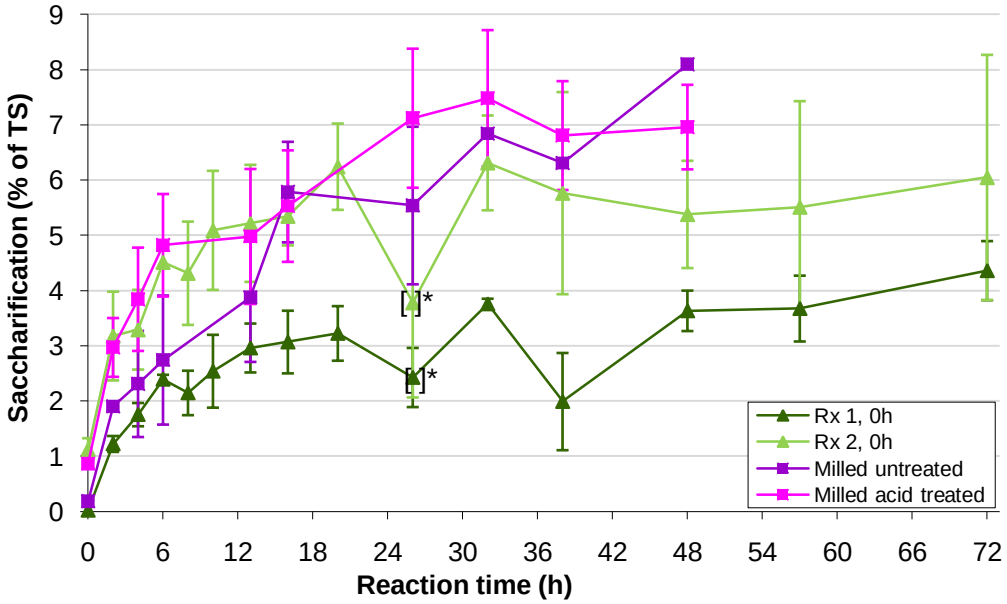

Figure 4.10 – Enzymatic saccharification of acid treated and untreated milled and non-milled bagasse, using a 1:1 mixture of Accellerase 1000 and GC 140 xylanase and a loading of 5.7% of each enzyme. The points are averages of the values obtained from the triplicate reactors, subtracting the values of the respective control reactors. Their standard deviation is represented by the vertical error bars. (Legend: Rx 1, 0h – untreated non-milled bagasse (0h in water bath); Rx 2, 0h – acid treated non-milled bagasse (0h in water bath).
* the DNS reagent used was changed, but the blank was still made with the old one.

4.4.4 Effect of changing enzyme combinations

The enzymatic reaction setup described in 3.6.5 was used to study the enzymatic reaction in the absence of a xylanase, and with a different xylanase that had a reported higher specific activity. The results from these reactions are presented in Figure 4.11, and compared with bagasse from the main acid pretreatment process (no incubation, 15 min residence time).

The reactions were run only for 48h since the previous experiments with non-milled bagasses had already shown that by that time the reaction had already slowed down considerably, and that it was enough to get a clear profile of the reaction.

It was observed that reactors without any xylanase (Rx 3 and Rx 4) reached lower levels of sugar concentration than when using either GC 140 or Y5 xylanase. This happened with untreated (Rx 1, Rx 3 and Rx 5) or acid treated bagasses (Rx 2, Rx 4 and Rx 6). This meant that there was still an appreciable amount of xylan in the acid treated bagasse. Without any xylanase, not only is the xylan not converted to reducing sugars, but also, like previously mentioned, it lowers the area accessible for enzymatic attack. On one hand, this was in agreement with the results from the fiber analysis, which

indicated the presence 6.1% of xylan in the solids after acid treatment. On the other hand, it conflicted with the nearly total xylan removal suggested by the sugar concentrations present in the acid treated hydrolysates. The reactors without xylanase allowed determining how much cellulose was degraded, since the sugars in the liquid fraction would be mostly glucose (apart from some residual xylanase activity displayed by Accellerase). At the 48h mark, it was approximately 10.5%.

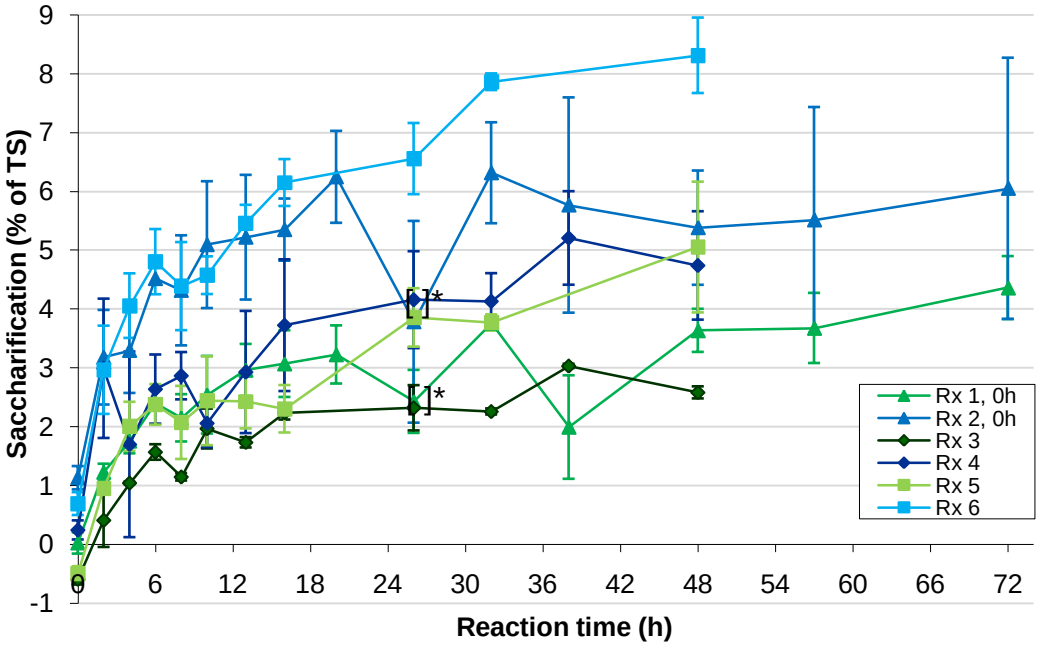

Figure 4.11 – Enzymatic hydrolysis of acid treated and untreated bagasse when using Accellerase 1000 and not employing any xylanase and when using Accellerase 1000 plus Y5 xylanase. Each enzyme was employed in a 5.7% working volume loading. The points are averages of the values obtained from the triplicate reactors, subtracting the values of the respective control reactors. Their standard deviation is represented by the vertical error bars. (Legend: Rx 1, 0h – untreated bagasse with GC 140 xylanase (0h in water bath); Rx 2, 0h – acid treated bagasse with GC 140 xylanase (0h in water bath); Rx 3 – untreated bagasse withou any xylanase; Rx 4 – acid treated bagasse withouth any xylanase; Rx 5 – untreated bagasse with Y5 xylanase; Rx 6 – acid treated bagasse with Y5 xylanase).
* the DNS reagent used was changed, but the blank was still made with the old one.

Interestingly enough, even though the untreated bagasse had a much higher xylan content, the usage of a xylanase didn't improve the effectiveness of the enzymatic treatment much more than it did with the acid treated bagasse. It's likely that the strong lignin-hemicellulose bonds hindered the enzymatic hydrolysis of xylan. To understand this behaviour better, detection of individual glucose and xylose contents in these liquid fractions is needed.

When using Y5 xylanase, the saccharification profiles up to the 20h mark were equivalent to when it was used GC 140 xylanase. After that, higher saccharifications were obtained with Y5, whether the bagasse was acid treated or not. The real improvement over GC 140 may be overestimated due to the possible contamination of the reactors containing the later. However, since Y5 xylanase did not seem advantageous for lower reaction times and little reliable product information was available, this enzyme wasn't used in other parts of this work.

The saccharification values of untreated bagasse from at 0h were negative. A possible explanation for this occurrence may be linked to the way saccharification is calculated. It involves a control with substrates but without enzymes, and a control with enzymes but no substrate. Both the substrate and enzymes have a certain amount of reducing sugars, which are released into the liquid

fraction. However, then sum of those values may be superior to the amount of sugars initially solubilized when enzymes and substrate are used together, due to an alteration of solubilization equilibrium with the solid fractions. A way to confirm this hypothesis would be to prepare a reactor with enzymes and substrate plus a reaction inhibitor and compare it with the controls used. This effect is most likely dependent on the characteristics of the substrate used and may become "hidden" by high initial rates of reaction, which would explain why it wasn't observed with the untreated bagasse. Also, the sugar concentrations measured were often close to the detection limit of this DNS method (0.5 g/L – Annex 7.2.1), which may have brought some more uncertainty to the values registered.

13h and 48h saccharification yields (on a carbohydrate basis) from all the above mentioned enzymatic hydrolysis experiments are summarized in Figure 4.12.

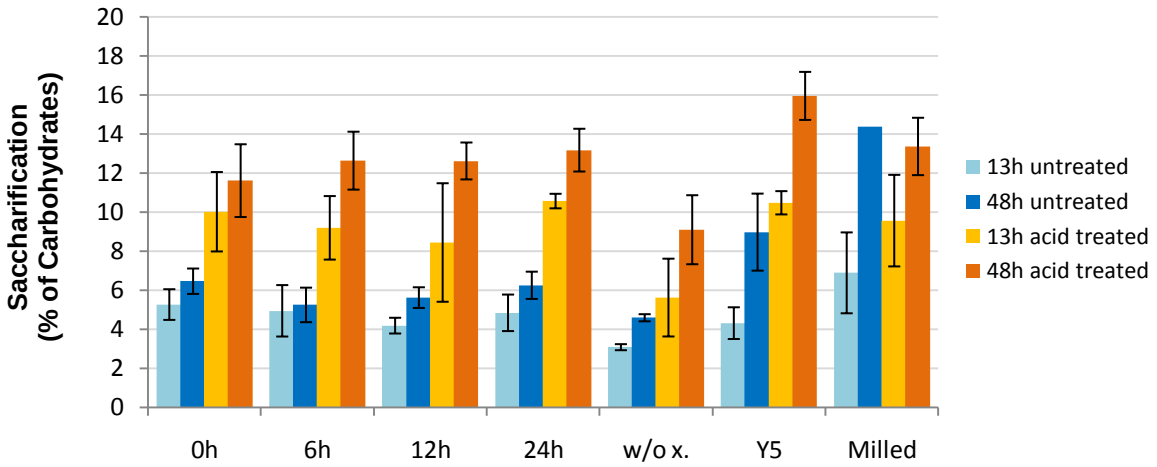

Figure 4.12 - Enzymatic saccharification of untreated and acid treated bagasse to reducing sugars (carbohydrate basis), for the various reactions carried out, at 13h and 48h. Bars are averages from the triplicate reactors and their standard deviation is represented by the vertical error bars. Legend: 0h, 6h, 12h, 24h – used bagasse incubated after autoclaving for 0h, 6h, 12 and 24h, respectively; w/o x. – reaction carried out without any xylanase; Y5 – used Y5 xylanase; Milled – bagasse was milled before acid treatment and enzymatic hydrolysis.

Overall, in all reactions carried out, carbohydrate conversions never reached very high values, with the highest being 16%, which was achieved with Y5 xylanase.

These saccharification values were far inferior to all the literature works consulted with either dilute acid or other pretreatments, especially when taking into consideration that the sugars obtained in this work were hydrolyzed from both cellulose and hemicellulose. The most direct comparisons can be made with the results presented in Figure 2.17 or in Accellerase 1000 technical bulletin #1 (Annex 7.5). In the later, with an Accellerase loading inferior to the ones used in this work (0.24 mL/g cellulose), it was obtained 60% of cellulose digestibility after 24 hours of reaction, which went up to 70% after 72h. However, the bagasse had been treated with 1.44% of acid at 165ºC. This means that the causes for the low enzymatic hydrolysis obtained here were not in the enzymes and reactional conditions employed, but in the original material and in the acid pretreatment.

As for the acid pretreatment, it was already mentioned that the conditions used in this work were milder than those observed in other works. The results for the percentage of hemicellulose removal were not conclusive, but it's likely that the treatment was not severe enough to ensure very reactive fibers. This would result in the weaker enzymatic hydrolysis observed.

The raw bagasse itself can also be of importance. In the work of Martin *et al.* (2007b), enzymatic hydrolysis of untreated bagasse returned a cellulose conversion of 5% after 48h, which is somewhat comparable to what was obtained in this work. However, Kuo *et al.* (2009) obtained ~20% of untreated bagasse conversion (TS basis) into reducing sugars. As it was suggested by the high lignin content, the bagasse used here is most likely more recalcitrant to degradation than the average bagasse, which further emphasizes the need for harsher conditions in the acid treatment.

4.4.5 Enzymatic hydrolysis of Pakistanese bagasse

The Pakistanese bagasse was submitted to the setup explained in 3.6.6 in order to study its response to enzymatic hydrolysis and to find a suitable enzyme loading for use in the anaerobic digestion. The acid treated and untreated bagasses used are shown in Figure 4.13. Appearance wise, unlike the stark contrast observed with the Indian bagasse, the acid treated bagasse was not very different from the untreated one. However, the first was still more brittle than the former and had a greater amount of smaller particles.

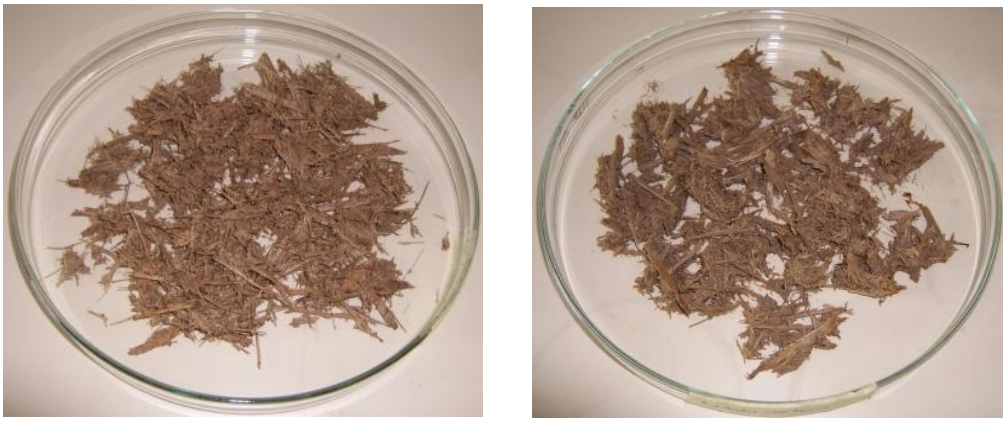

Figure 4.13 – Appearance of the dry Pakistanese bagasses untreated (left) and acid treated (right).

The second batch of reactors (loadings 5.7% and 7.6%) was run for 72h. This longer reaction time was merely to confirm that sugar release had stopped, similarly to what was observed with Indian bagasse. And in fact, after 48h there was a significant stabilization of the sugar conversion (Figure 4.14).

Like with the Indian bagasse, there was no clear increase in reaction rate and yield when enzyme loadings were increased. The 3.8% loading with acid treated bagasse was the only one which escaped that trend. For unknown reasons, it exhibited conversions higher than the remaining enzyme loadings used.

It is interesting to note that the curves for untreated Pakistanese bagasse achieved lower saccharifications than with untreated Indian bagasse (Figure 4.3). Like it was mentioned above, raw material can have a significant effect on its enzymatic digestibility, and the plant strain, harvest methods and sugarcane processing used to obtain the Pakistanese bagasse resulted in biomass more recalcitrant to enzymatic hydrolysis.

Only one enzyme loading (5.7%) was tested for acid treated Indian bagasse, and its acid pretreatment consisted of 15 min at 121ºC, as opposed to the 30 min of the Pakistanese bagasse pretreatment. Still, while a direct comparison can't be made, the curves obtained for both acid treated

bagasses at 5.7% enzyme loading (5.7% T - Figure 4.14 vs. Rx 1, 0h - Figure 4.10) were relatively on par with each other, though the initial reaction rate was greater with the Indian bagasse. This opens up the possibility of acid treatment mitigating the difference in digestibility between these two bagasses.

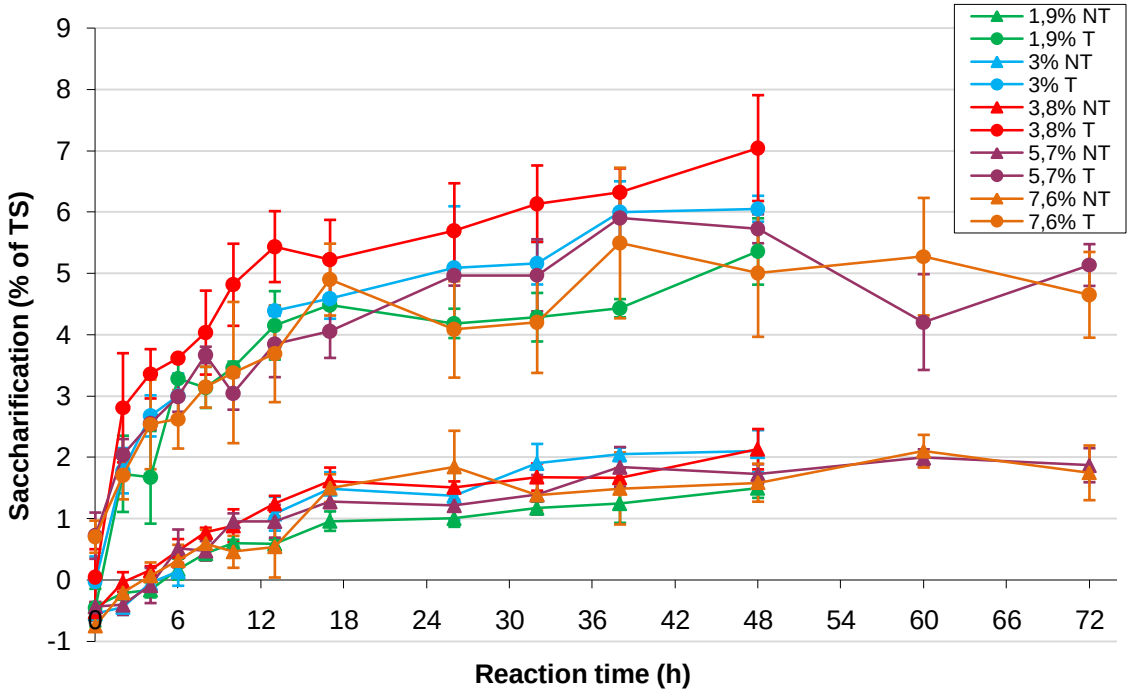

Figure 4.14 – Enzymatic hydrolysis of untreated and acid treated Pakistanese bagasse, at various loadings of a 1:1 mixture of Accellerase 1000 and GC 140 xylanase. The points are averages of the values obtained from the triplicate reactors, subtracting the values of the respective control reactors. Their standard deviation is represented by the vertical error bars. (Legend: x % = x% (working vol.) of Accellerase 1000 + x% (working vol.) GC 140 xylanase; NT – untreated bagasse; T – acid treated bagasse).

Unlike the acid pretreatment, no inhibitory compounds were expected to be formed during enzymatic hydrolysis. Therefore, the only factors weighted when choosing a loading to use in the anaerobic digestion were reducing sugar concentrations and amount of enzymes spent. But from the results obtained, no limiting enzyme concentration was found. The higher values with 3.8% loading were not considered very reliable, since they were probably derived from an experimental error. The 3% loading was chosen, while keeping in mind that more loading optimization studies using lesser amounts of enzymes should be made in future.

4.5 Integrated Pretreatment and Anaerobic Digestion

4.5.1 Dilute acid pretreatment

The dilute sulphuric acid treatment for this section was carried out in a scale four times larger than the main pretreatment process used with the Indian bagasse, since larger quantities of acid treated bagasse were needed for both the downstream enzymatic hydrolysis and for the anaerobic digestion.

The sugar concentrations achieved in the acid treated hydrolysate were about 11 g/L, which were lower than the values observed with Indian bagasse. On Figure 4.15 is presented a comparison between the Pakistanese bagasse hydrolysate and Indian bagasse treated for 30 min in the autoclave (from section 4.3). There was a big difference in the scale of the reaction (50mL vs 2L), but during this work was shown that reactors on a 50mL scale had actually lower sugar concentrations than the main

pretreatment process (volume=200mL). So either increasing the scale is also disadvantageous to the sugar release or the cause lies in the bagasse itself. It was already shown that the Pakistanese bagasse was more recalcitrant to enzymatic hydrolysis. Taking into consideration that the Pakistanese bagasse also has higher inorganic (ash) content, it's likely that its behavior during acid hydrolysis was comparatively inferior to the Indian bagasse.

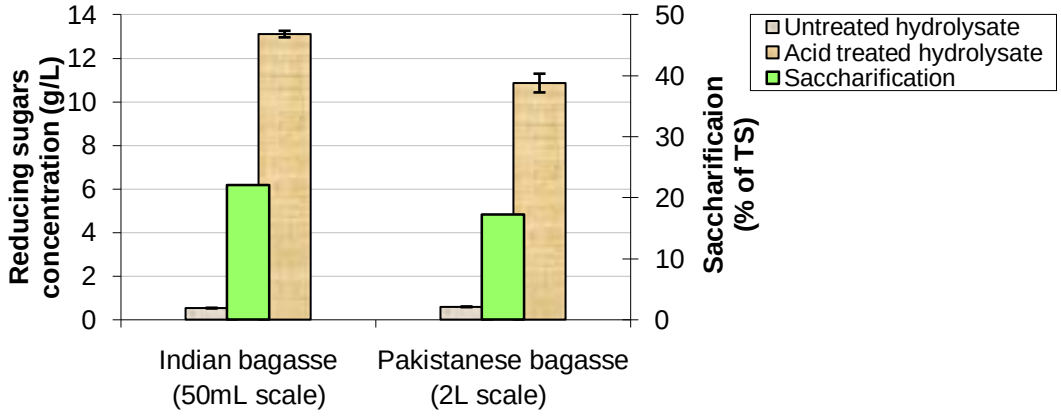

Figure 4.15 – Comparison of reducing sugar concentrations of hydrolysates from Indian and Pakistanese bagasse treatments. Samples were incubated in the autoclave for 30 minutes at 121ºC. The bars are an average of the values obtained from the triplicate analysis. Their standard deviation is represented by the vertical error bars.

4.5.2 Enzymatic treatment

The results from the large scale enzymatic reaction showed much higher reducing sugar concentrations than expected. The reaction was stopped at 13h, which according to Figure 7.17 (Annex 7.3), there should exist around 4 g/L of reducing sugars. However, in Figure 4.15 can be observed higher sugar concentrations after the enzymatic reaction, reaching over 9.5 g/L at 13h. The background sugar concentration was also greater than observed on the smaller scale experiments control reactors, where it ranged from 1 to 1.5 g/L. Still, even after deducting the control reactors and calculating the conversion yield, it was more than doubled in the 1L reaction (over 10%, against ~4% in the small scale reactions).

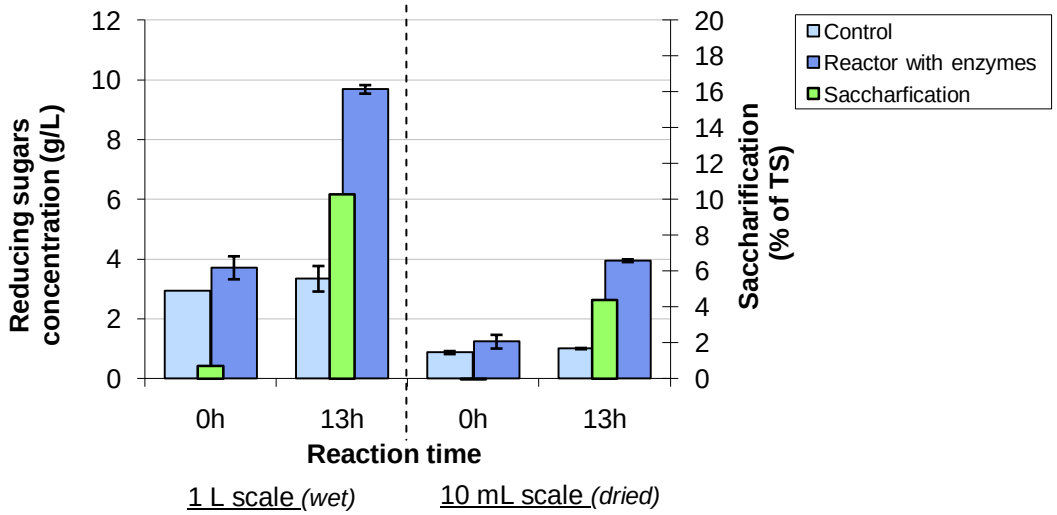

Figure 4.16 – Reducing sugar concentrations of the liquid fractions during enzymatic hydrolysis of acid treated Pakistanese bagasse, carried out with a 1L scale and in a 10mL (scintillation vial) scale.

Since most of the processing was similar to the small scale reactions, this great difference could originate from two different factors, or maybe a combination of the two. One was the upscaling. Even though the vessels in the two experiments were submitted to the same shaking speed (50rpm), it could have happened that the shaking was more effective in the large Erlenmeyer than in the scintillation vials, which favored the reaction. The second difference in the processing lay in the fact the acid treated bagasse was not dried after acid treatment, and instead was used wet for the enzymatic reaction. It has already been discussed that the act of drying the bagasse could cause structural damage and decrease its accessible surface area. While that wasn't observed with untreated Indian bagasse, it is quite plausible that acid treated bagasse, whose structure is in a more fragile state, might have been more prone to structural collapse upon drying, limiting not only the enzymatic hydrolysis of cellulose, but also imprisoning the simple sugars which were formed in during acid treatment and that remained associated with the solids. It could prove interesting to execute future treatments without drying the bagasse.

4.5.3 Anaerobic digestion

4.5.3.1 Reactor composition, gas production and methane content

Some of the characteristics of the sludge used as inoculum are summarized in Table 4.3.

Table 4.3 – Characteristics of the inoculum.

TS (%)	VS(%)	pH	PA (ppm $CaCO_3$)	TA (ppm $CaCO_3$)
2.9	61.9	8.11	12368	15408

The inoculum had a very high alkalinity, so it was expected that it would have more than enough buffering capacity for the acids released during acidogenesis.

The TS and VS contents of the substrates used are shown in Table 4.4, as well as the amount of VS loaded into the reactors.

Table 4.4 – TS, VS and amount of VS loaded into the reactors, for the various substrates.

Type of substrate	TS (%)	VS (%)	VS loaded into reactor (g)
Original Bagasse	82.4	96.6	2.69
Acid treated pulp	12.2	85.5	2.50
Control to acid treated pulp	12.2	96.5	2.82
Acid treated hydrolysate	2.6	38.5	1.43
Control to acid-treated hydrolysate	0.2	75.1	0.25
Enzyme treated pulp	15.7	91.5	2.56
Control to enzyme treated pulp	12.6	91.2	2.04
Enzyme treated liquor	3.2	71.1	1.95
Control to Enzyme treated liquor	1.3	37.3	0.42
Reference (Avicel - cellulose)*	100	100	2.69

* No actual VS analysis was carried out.

The VS content of the acid treated hydrolysate was quite low when compared to other substrates. That was due to the sodium sulphate content (which should be present in 14.2g/L), resultant from the neutralization process. Since the acid treated pulp was 'wet' with the hydrolysate, its VS content also came affected. This is reinforced by the fact that 'Control to enzyme treated pulp' is technically the same acid treated bagasse, only it's diluted in more water, having 91% VS. For this reason it is difficult

to accurately judge if the acid treatment process altered the organic (VS) content of the pulp, but with these considerations in mind, those changes were most likely inferior to 5%.

The enzymatic treatment had a negligible effect on the VS content of the pulp, even though its carbohydrates were partially solubilized. The VS content of the respective liquors was not very high, with the control liquor having 37% of VS. Since the acid treated bagasse was used 'wet' for the enzymatic hydrolysis, some sodium sulphate was still carried on to this reaction, thus having a relatively high amount of this inorganic salt (estimated to be about 5 g/L).

All reactors' initial pH fell in the range 8.0-8.3 and wasn't significantly altered after digestion, due to the good buffering capacity of the inoculum (Annex 7.4, Table 7.4).

The total volume of biogas produced by each type substrate is presented in Figure 4.17.

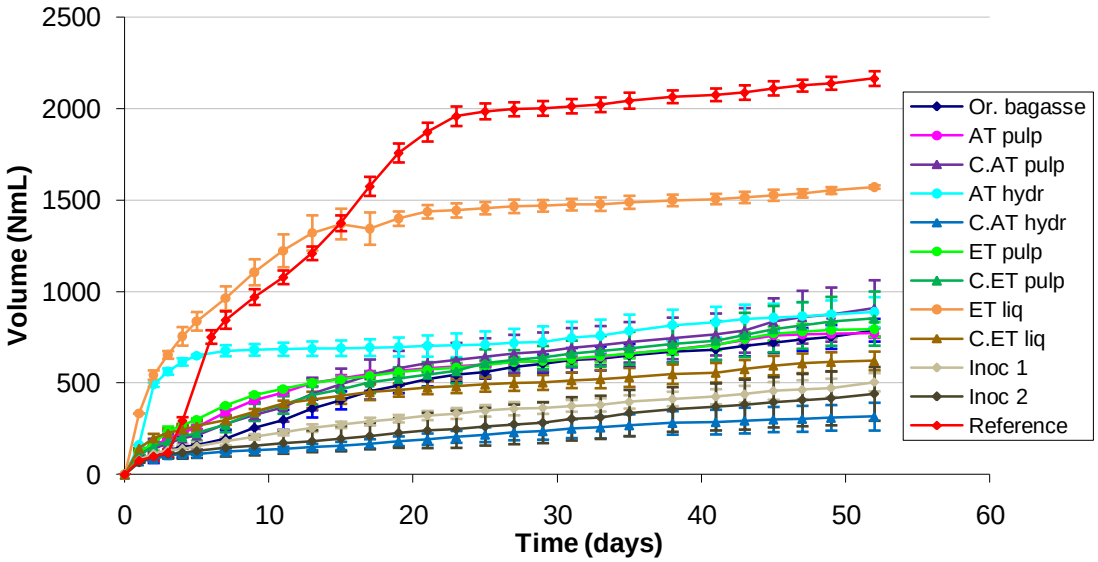

Figure 4.17 – Volume of biogas produced during the anaerobic digestion process. Points are averages from the triplicate reactors. Their standard deviations are represented by the vertical error bars. Legend: Or. bagasse – Original pulp (bagasse); hydr – hydrolysate; liq - liquor; AT – acid treated; C.AT – control to acid treated; ET – enzyme treated; C. ET – control to enzyme treated; Inoc 1 and Inoc 2– Inoculum with water only.

The total amount of gas produced regardless of its methane content is an indication of microbial activity. The substrates with greater amounts of monomeric sugars (the hydrolysate from the acid pretreatment and liquor from the enzymatic treatment) originated quick increases in gas production. The reference substrate (Avicel) had a slow start, but then quickly started producing more gas and eventually overcame the production of the other reactors. Since it was pure cellulose, it could be more easily metabolized in its entirety than more complex lignocellulosic substrates, but since it had a low initial availability of easily metabolizable sugars, there was a first phase where microorganisms first had to adapt in order brake down the cellulose. Similarly, the pulps and the controls to the other substrates, as they didn't possess large quantities of simple sugars, didn't register a very quick increase in gas production, albeit still greater than cellulose alone. However, the production rate didn't increase afterwards, so the gas volumes produced never got very high. In the case of the pulps, this showed that they were a more complex substrate, which the microorganisms had greater difficulty metabolizing. For the controls to the liquid fractions, it means that the already low sugar content had been used up and there was no other source of carbon available.

The gas produced with acid treated hydrolysate had a high initial production rate, but it quickly slowed down and achieved final gas volumes similar to the ones obtained with pulps. Its methane content was also lower, taking more time to stabilize above 50% than the other reactors (inoculum only excluded) (Figure 4.18). Since it was the substrate with the highest reducing sugar concentration, this behavior supports there was some microbial activity inhibition, which could partially have been caused by sugar degradation products resulting from the acid treatment. Moreover, there have been several studies about sodium inhibition of anaerobic digesters (Feijoo *et al.*, 1995). In the acid treated hydrolysate was present sodium sulphate, resultant from the neutralization of sulphuric acid. But with sodium concentrations of approximately 3 g/L, it wasn't expected great inhibition. Nevertheless, in the aforementioned studies it was said that inocula not used to sodium could feel its toxicity at lower concentrations. Since the sludge used was from a biogas plant fed on food processing residues and manure, it may not have been used to moderate-to-high salinity. Also, since more sulphate was present, the sulfur-reducing population might have been more stimulated and hydrogen sulfide production increased, which would decrease methane production.

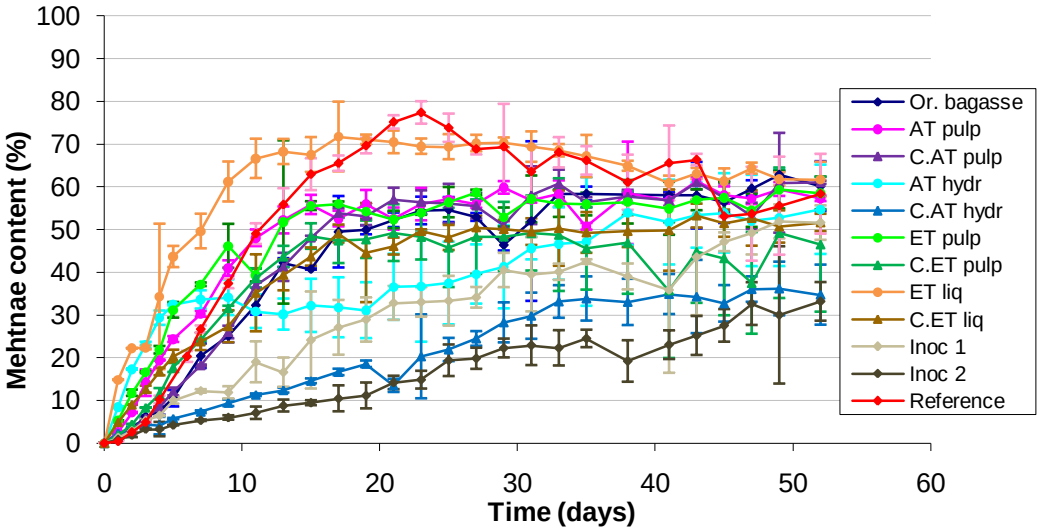

Figure 4.18 – Evolution of biogas composition during the anaerobic digestion process. Points are averages from the triplicate reactors. Their standard deviations are represented by the vertical error bars. Legend: Or. bagasse – Original pulp (bagasse); hydr – hydrolysate; liq - liquor; AT – acid treated; C.AT – control to acid treated; ET –enzyme treated; C. ET – control to enzyme treated; Inoc 1 and Inoc 2– Inoculum with water only.

However, its control reactor exhibited lower gas production than the reactors with inoculum and water, which was a clear indication that there were some components in the substrate solution that decreased the microbial activity of the inoculum. Since no acid was employed in that control, it's unlikely that sugar degradation products were the cause. Also, little sodium sulphate was present, excluding the possibility of sodium inhibition. It's possible that there could be compounds present in the bagasse, which could be washed out in hot water and were toxic to microbial activity.

Overall, the methane content present in most of the reactors initially increased until it reached a stable level, around 50-60%. This percentage was within the expected values, since carbohydrate--based substrates were used (Table 2.6). With Avicel and enzymatic treated liquor, the increase in methane content was quicker and it registered values around 70%. After that, it decreased back to the 50-60% range. When not using any substrate (lowest inoculum concentration) and with the control to the acid treated hydrolysate, the methane content never rose above 36%.

Since the gas composition was not very different between different samples, the overall profiles for cumulative methane production were similar to the ones presented in the total gas production (Annex 7.4, Figure 7.18). This means that the highest methane production was achieved with Avicel, followed by the enzyme treated liquor. All the other substrates resulted in far lower productions.

4.5.3.2 Biochemical methane potential

Since the substrate VS and inoculum mass employed were slightly different for every substrate, a more accurate comparison can be made by analyzing their biochemical methane potential by unit of mass of VS, subtracting the background production of the inoculum.

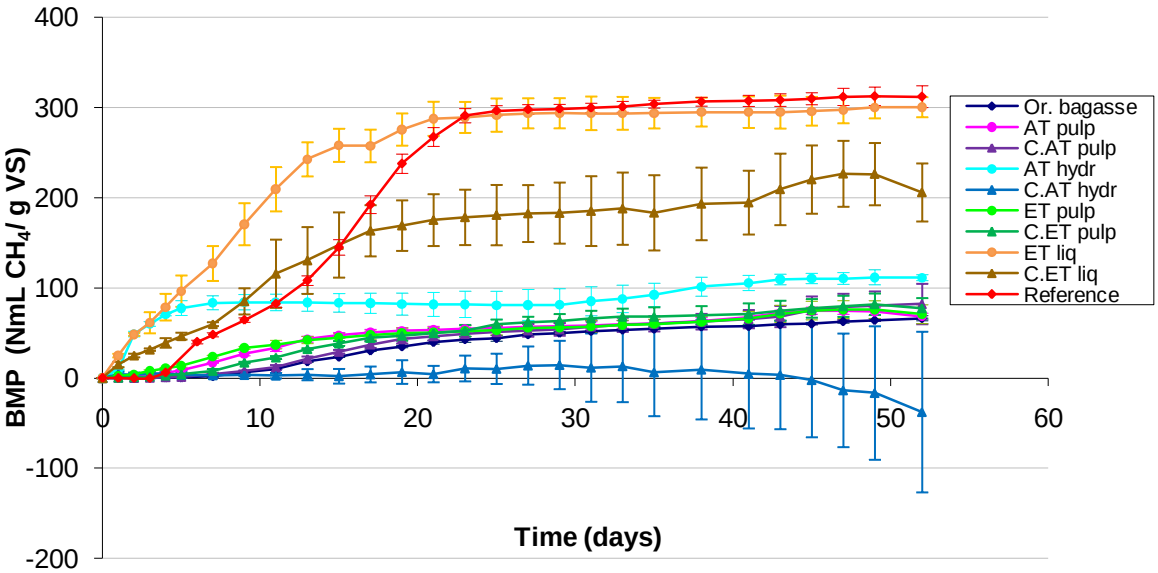

Figure 4.19 – Evolution of the bio-methane potential of the various substrates during anaerobic digestion. Legend: Or. bagasse – Original pulp (bagasse); hydr – hydrolysate; liq - liquor; AT – acid treated; C.AT – control to acid treated; ET –enzyme treated; C. ET – control to enzyme treated.

From the analysis of the BMP profiles it's possible to observe more clearly the existence of lag phases for the cellulose-rich substrates (pulps and Avicel), whereas substrates based on simple sugars began methane production immediately. This was an indication that the inoculum was not well adapted to digestion of lignocellulosic materials. The anaerobic digester from which the inoculum was taken was fed partially with animal manure and mostly with food industry wastes. Therefore cellulose wouldn't be a significant component present in the wastes. This means that this BMP test wasn't executed in the optimal conditions for the methanization of bagasse, and that the inoculum should have been acclimatized with these types of carbohydrates, by means of a pre-incubation with a cellulosic substrate for about one month.

Avicel was the substrate with the second highest final BMP (Figure 4.22). The value for Avicel obtained here (0.318 L/g VS) was a little lower than what was found in literature works (Chynoweth *et al.,* 2001). This was most likely related to the lack of inoculum adaptation to cellulose, which did not allow extracting the full potential of Avicel. Its BMP profile didn't follow the first order kinetics, so it wasn't possible to obtain a kinetic constant and compare it with the literature.

Similarly, the atypical nature of the curves belonging to the pulps didn't supply reliable data to extract kinetic information. Therefore, the kinetic constant was only estimated for the acid treated hydrolysate and for the liquors from enzymatic treatment.

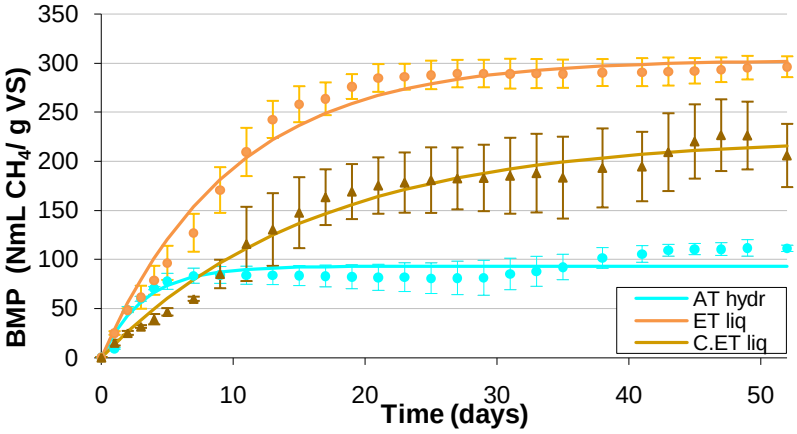

Figure 4.20 – First order kinetic constants obtained for some substrates

Substrates	k_h ($days^{-1}$)
Acid treated hydrolysate	0.31
Enzyme treated liquor	0.10
Control to enzyme treated liquor	0.06

Figure 4.21 – First order kinetic model adjustment for the BMP evolution of acid treated hydrolysate (AT hydr), enzyme treated liquor (ET liq) and its control (C. ET liq). Dots are experimental data and the lines are the models.

While the final BMP gives an idea of the maximum methane production possible from a substrate, the k_h value is also a characteristic of a given substrate and gives information about the time required to generate a certain ratio of its ultimate methane potential (Mace *et al*, 2003). Like the previous charts suggested, the acid treated hydrolysate was the one that reached its full potential earlier (higher k_h), even though its potential was low, while on the other end of the spectrum there's the control to the enzyme treated liquor, which despite having a high BMP takes a longer time to reach it, most likely due to being very diluted in the reactional mixture.

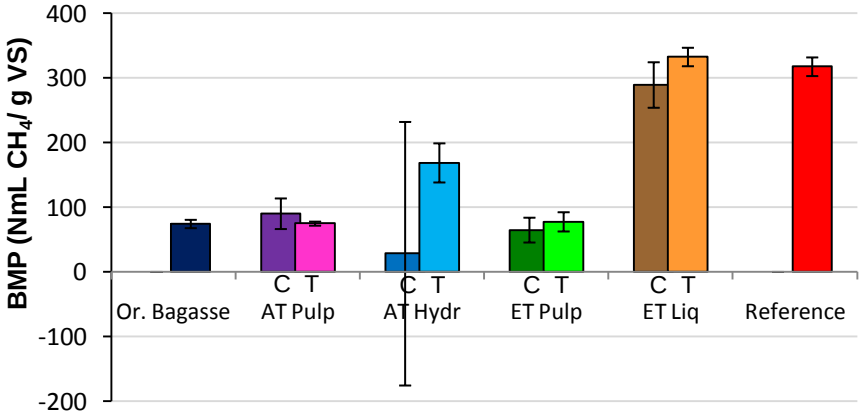

Figure 4.22 – Final bio-methane potential of the various substrates. Legend: Or. Bagasse – Original pulp (bagasse); AT Pulp – acid treated pulp; AT Hydr – acid treated hydrolysate; ET Pulp – enzyme treated pulp; ET Liq – enzyme treated liquor; C – control; T – treated. Their standard deviation is represented by the vertical error bars.

The enzyme treated liquor and its control had BMPs similar to Avicel. This was because they possessed mostly simple (reducing) sugars, which were easy to metabolize. Furthermore, no microbial inhibitory products were formed. The main difference between the treated liquor and its control was that the former had a greater concentration of sugars derived from enzymatic hydrolysis,

producing higher volumes of methane more quickly (Annex 7.5, Figure 7.18 Figure 7.19). However, their maximum digestibilities were nearly identical.

The behavior of the acid treated hydrolysate and its control confirmed what was previously suggested. Even though the reducing sugar concentration of the acid treated hydrolysate was the highest, its BMP was not. This seemed to be a clear indication of the presence of toxic compounds that hindered biomass digestion. On average, its control reactor had a very low BMP, but the individual reactors behaved quite differently, originating a very high standard deviation. The experiment with this substrate should be repeated in order to get more reliable information

There was little difference in performance between all the pulps used. This was not entirely unexpected, as both acid and enzymatic treatments didn't hydrolyze cellulose to a great extent, therefore not causing great alterations to the bagasse chemical composition. As already mentioned, the main factor limiting the accessibility of cellulose to microorganisms during digestion is the lignin content. Since the treatments applied are not particularly effective at removing lignin, bagasse digestibility was not greatly affected.

As for VS reductions, the pulps were less than 40% degraded, which can be attributed to the low accessibility of cellulose and the presence of lignin, which is a hardly digestible organic compound. As expected, substrates like Avicel and the enzymatic liquors had VS reductions close to 100%. VS reduction was only 56% in the acid treated hydrolysate, even though its organic content was mostly simple sugars. This is in agreement with the small methane productions caused by the inhibition.

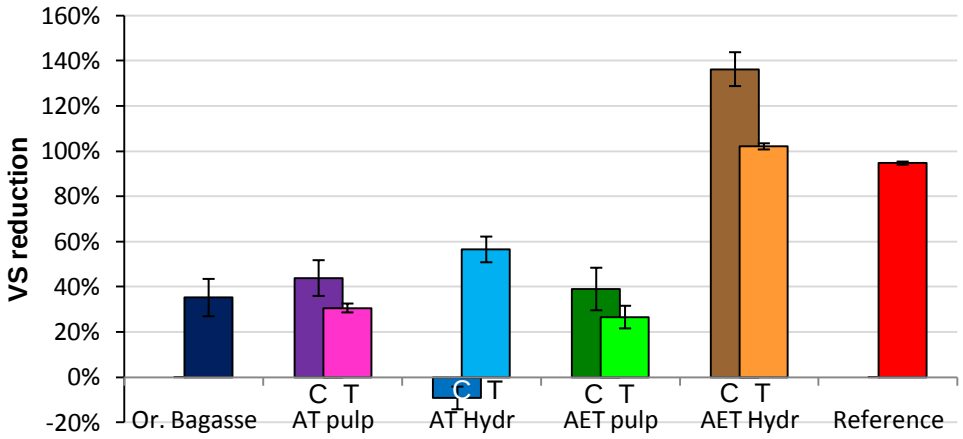

Figure 4.23 – VS reduction of the various substrates after digestion. Legend: Or. Bagasse – Original pulp (bagasse); AT Pulp – acid treated pulp; AT Hydr – acid treated hydrolysate; ET Pulp – enzyme treated pulp; ET Liq – enzyme treated liquor; C – control; T – treated.

The control to the acid treated hydrolysate and to the enzyme treated liquor registered VS reductions lower than 0% and higher than 100%, respectively. These values were most likely a numerical instability problem caused by the small amounts of substrate VS employed (less than 0.5 g) in comparison with inoculum VS. So, if the actual inoculum VS reduction had shifted slightly from what was predicted by the controls, it would be enough to have a big effect of the calculated substrate VS reduction. Taking into consideration the BMP of these substrates, the real VS reduction of the control acid treated hydrolysate is probably close to 0%. The control to acid and enzymatic treated hydrolysate, on the other hand, was most likely consumed to 100%, just like its treated counterpart.

5. Conclusions and future work

This work consisted in the chemical characterization of sugarcane bagasse from two different origins (India and Pakistan) and its treatment by dilute sulphuric acid hydrolysis and enzymatic hydrolysis. Some liquid and solid fractions resulting from these treatments were tested for their biochemical methane potential.

The dilute acid treatment caused the solubilization of mainly xylan. Still, it's suspected that the extent was relatively low. These results were possibly due to the temperature and acid concentration not being high enough, resulting in xylan being only partially hydrolyzed. Incubating the hydrolysate mixture after the autoclave treatment did little to improve this. The optimum residence time in the autoclave was 30 minutes, but the gain achieved from the initial 15 minutes was not very substantial. Acid treatment of milled bagasse resulted in lower conversions than the non-milled bagasse, though it was possibly caused by diffusional problems and not the chemical characteristics of the material.

Enzymatic hydrolysis was performed on untreated bagasse to observe the effect of enzyme loading, but due to a great data dispersion it was not possible to conclusively identify the limiting enzyme loading, though the results suggested that all loadings were above it. The pulps from the main acid treatment, with and without posterior incubation, were subjected to enzymatic hydrolysis. Although there was a significant improvement over untreated bagasse, the overall saccharification yields were below 14% of the carbohydrate content after 48h. When only milling was applied to the bagasse, the final were yields on par with the acid treatment, though the reaction rate was lower. Acid treatment of milled bagasse was only able to increase the rate, but not the final yield, suggesting that the present milling step does not hold much promise. When altering the enzyme combinations, it was found that xylan hindered the reaction, even with the acid treatment. A second enzyme loading optimization study, this time for Pakistanese bagasse, both untreated and acid treated, allowed to conclude: 1) bagasse origin can have a very significant effect on rate and final yield of the reaction; 2) the range of enzyme loadings used was clearly above the limiting enzyme loading.

The overall saccharifications in both types of treatments were much lower than expected, but this work allowed identifying several important parameters and other minor protocol alterations which can easily be improved upon. Their detection and discussion has provided valuable lessons: 1) The greatest limitation to the whole process was the mild conditions used in acid pretreatment. The bagasse used here was more recalcitrant than average, demanding the use of higher temperatures and/or acid concentrations. This alone is expected to produce significant improvements on the subsequent treatment. Alternatively, it could mean the dilute acid hydrolysis may have not been the most adequate treatment for this biomass. 2) The enzyme loadings could be drastically cut, especially if a good pretreatment was made. 3) Not drying the pulp after acid hydrolysis may also bring great benefits to the enzymatic reaction.

As for the biochemical methane potential, the liquors from the enzymatic treatment returned the most positive results, having obtained high specific methane productions and total reduction of volatile solid. The hydrolysate from the acid treatment suffered from a comparatively lower methane production and incomplete volatile solids consumption, most likely due to the formation of inhibitory

and toxic compounds to microorganism growth. This means that the former is suitable for biogas production, while the later needs some detoxification treatment before its full potential can be unleashed.

The pretreatments didn't greatly affect the digestion of pulps, but that could be attributed to their weak effect on the chemical composition of the substrates. The reduction of their lignin content may be a possible strategy to improve digestibility.

Dilute acid followed by enzymatic hydrolysis could have potential to be a good method for saccharifying bagasse, yielding sugary liquors that are easily and extensively converted to biogas. However, much optimization is still required, particularly on the acid treatment, in order to maximize the amount of sugars released from biomass.

The work described here was the beginning of a research project at the Department of Biotechnology of Lund's Faculty of Engineering, aiming to convert bagasse into biogas. Therefore, this was a preliminary work which partially aimed to bring up topics of research related to bagasse degradation. Some works which would help answer some of the most preeminent questions raised here would be:

- Repeating the dilute acid hydrolysis pretreatment using more severe conditions. This should be accompanied by a more extensive characterization of the components present in the hydrolysates, in an attempt to monitor concentrations of inhibitory and toxic compounds to microbial life.
- Testing alternative pretreatments which focus on delignification and studying their effect not only on a downstream enzymatic hydrolysis but also on the anaerobic digestibility of the treated solids.
- Setting up enzymatic hydrolysis experiments at lower enzyme loadings and different ratios of cellulases: xylanases, or if the pretreatment so permits, eliminate altogether the usage of a xylanase. To that end, liquid fractions should be analyzed for individual sugar concentrations. Furthermore, enzymatic activity of the commercial enzymes should be analyzed with standard conditions, in order to have a good basis of comparison with other scientific works.
- Evaluating the effect of residence time in autoclave in the enzymatic digestibility. Also, confirming the effect of air drying treated bagasses on their structure and enzymatic digestibility.
- Researching possible pretreatments to be used in the acid treated hydrolysates, in order to detoxify them and enable good performances in anaerobic digestion.
- Performing tests of biochemical methane potential using an appropriate acclimatized inoculum, using substrates not only from an optimized acid and enzymatic treatment process, but also from other pretreatment technologies which have reasonable chances of being viable in the near future.

However, these works would only represent the first phase of the much larger ambition of taking this to a large scale. So it is also important that further research works never forget the aim of creating an environmental friendly and economically viable process that may become one of the solid foundations of a future with sustainable energy.

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Zhu, S., Wu, Y., Yu, Z., Wang, C., Yu, F., Jin, S., Ding, Y., Chi, R., Liao, J., Zhang, Y. (2006), *Comparison of three microwave/chemical pretreatment processes for enzymatic hydrolysis of rice straw.* Biosystems Engineering, 93: 279-283.

7. Annexes

7.1 HPLC analysis

7.1.1 Fibre analysis

Present below on tables are portions of the output data given by the HPLC analysis software, evidencing various peaks detected in the samples analyzed (in duplicates), their retention times and peak areas.

Table 7.1 – Software output for the samples for fiber analysis analyzed by HPLC

Peak name	Retention time (min)	Peak area (mV.min)	Peak name	Retention time (min)	Peak area (mV.min)
Untreated bagasse					
Unknown	4.242	0.1	*Unknown*	4.15	≈0
Unknown	7.933	0.2	*Unknown*	7.917	0.1
Glucose	12.6	22.1	Glucose	12.6	22.6
Xylose	13.675	6.8	Xylose	13.675	6.8
Arabinose	15.608	1.0	Arabinose	15.608	1
Mannose	16.417	0.1	Mannose	16.408	0.1
Unknown	18.25	1.0	*Unknown*	18.242	2.1
Acid treated bagasse					
Unknown	7.833	≈0	*Unknown*	4.125	≈0
Unknown	10.767	0.1	*Unknown*	7.942	0.2
Glucose	12.6	34	Glucose	10.758	0.1
Xylose	13.675	3.9	Xylose	12.6	30.1
Arabinose	15.6	0.4	Arabinose	13.675	3.3
Unknown	18.233	2	*Unknown*	15.592	0.5
Sugar Recovery Standard (SRS)					
Unknown	4.225	0.1	*Unknown*	4.217	0.1
Unknown	7.908	0.2	*Unknown*	7.817	≈0
Glucose	12.6	51.4	Glucose	12.592	21.3
Xylose	13.667	10.3	Xylose	13.667	4.4
Galactose	14.4	2.7	Galactose	14.392	1.2
Arabinose	15.608	1.9	Arabinose	15.608	0.8
Mannose	16.358	3.1	Mannose	16.35	1.7
Unknown	18.258	1.1	*Unknown*	18.233	0.6
Calibration Verification Standard (CVS)					
Unknown	8.792	≈0			
Cellobiose	10.292	39.2			
Glucose	12.608	38.1			
Xylose	13.683	35.4			
Galactose	14.417	31.2			
Arabinose	15.617	35.9			
Mannose	16.358	37.4			

Using the calibration curves below, these peak areas were converted to concentrations. Their accuracy was verified by the CVS, by means of the CVS Recovery %, which was calculated by equation (7.1), for each of the sugars involved.

$$\%\ CVS\ Recovery = \left(\frac{Sugar\ concentration\ detected\ by\ HPLC}{Known\ concentration\ of\ sugar}\right) \times 100 \quad (7.1)$$

Where: *Known concentration of sugar* = 2.5g/L for all sugars used.

Table 7.2 – Calibration verification standards sugar concentration and recovery

Sugars	Concentration (g/L)	CVS Recovery (%)
Glucose	2.510	100.41
Xylose	2.511	100.43
Arabinose	2.488	99.52
Mannose	2.457	98.28

From which it can be concluded that the following calibration curves were acceptable.

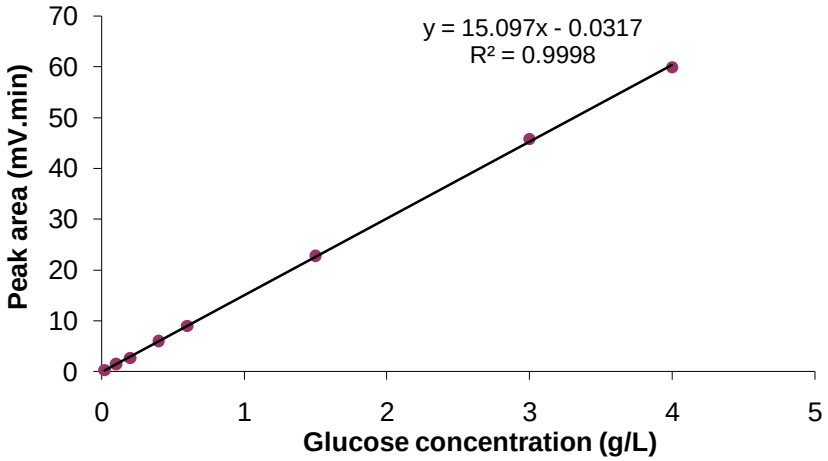

Figure 7.1 – Calibration curve for fiber analysis HPLC glucose determination.

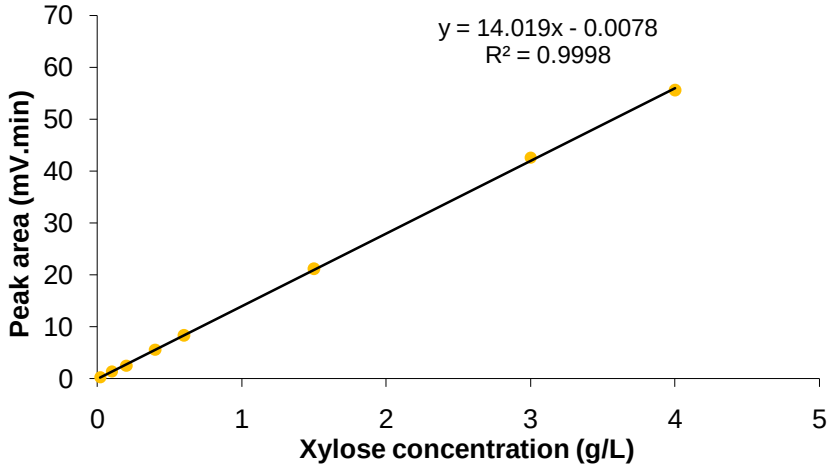

Figure 7.2 – Calibration curve for fiber analysis HPLC xylose determination.

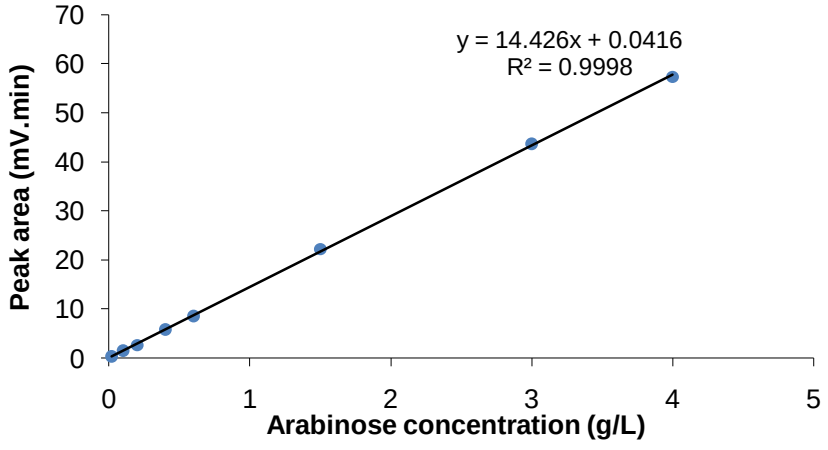

Figure 7.3 – Calibration curve for fiber analysis HPLC arabinose determination

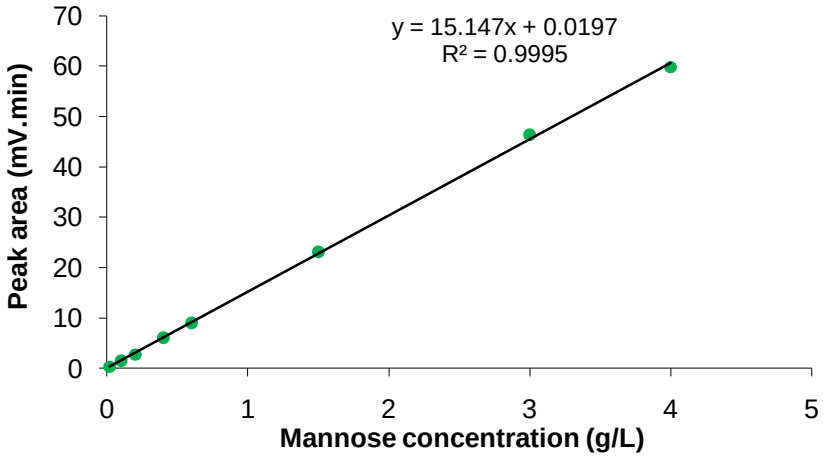

Figure 7.4 – Calibration curve for fiber analysis HPLC mannose determination.

7.1.2 Sugar and organic acid determination

Table 7.3 – Software output for main acid pretreated hydrolysate when analyzed by HPLC

Peak name	Retention time (min)	Peak area (mV.sec)
Sulphuric acid	6.133	7552.9
Unknown	6.617	38.8
Cellobiose	7.208	101.8
Unknown	7.692	146.4
Glucose	8.908	227.1
Xylose	9.55	4116.8
Unknown	10.425	311.4
Glycerol	13.225	5.5
Acetic acid	14.783	430.3

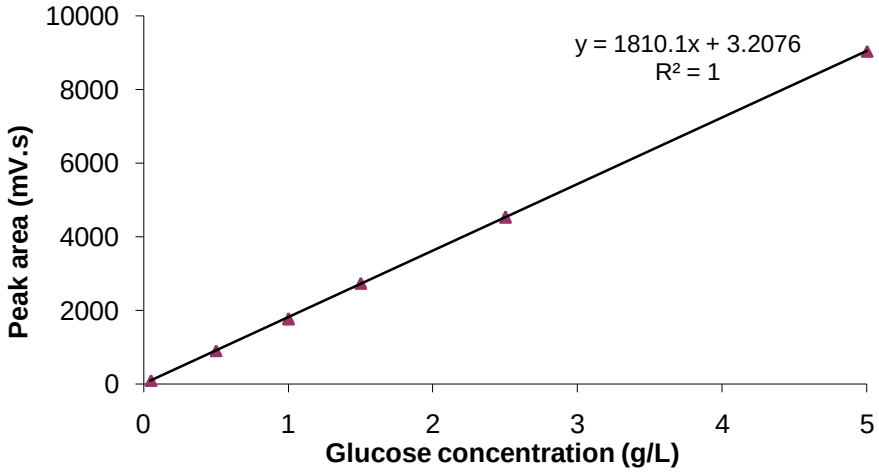

Figure 7.5 – Calibration curve for '*sugars and acids*' HPLC glucose determination.

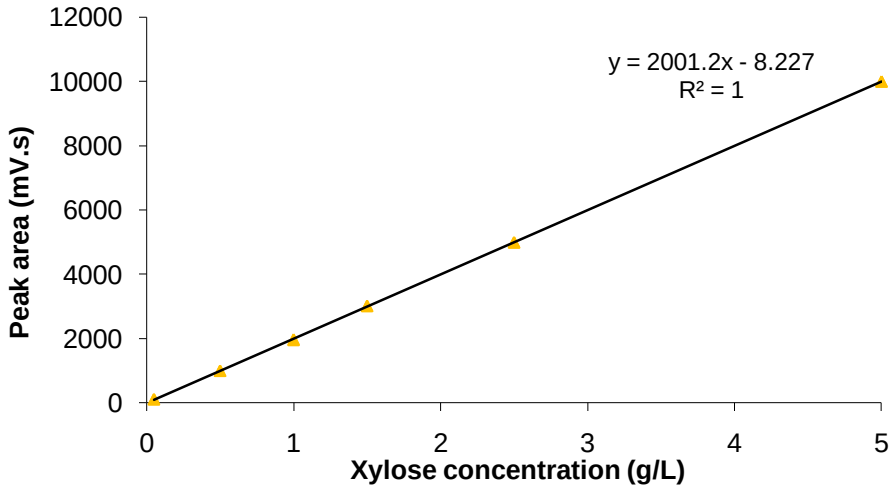

Figure 7.6 – Calibration curve for 'sugars and acids' HPLC xylose determination.

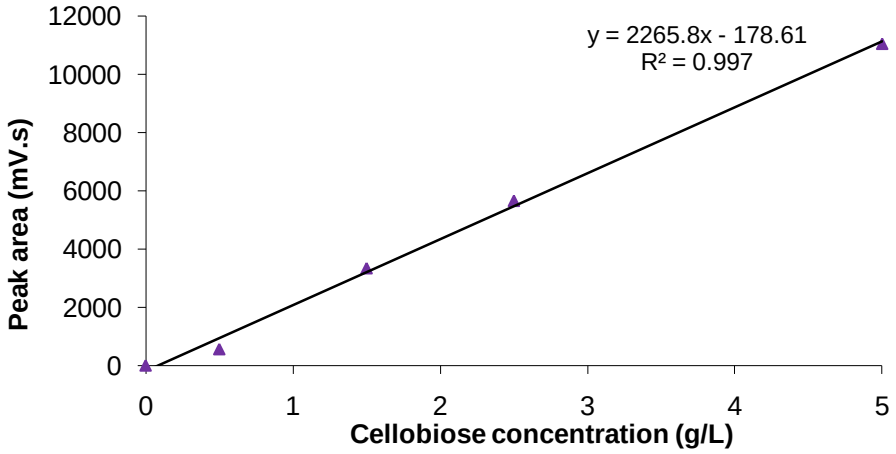

Figure 7.7 – Calibration curve for 'sugars and acids' HPLC cellobiose determination.

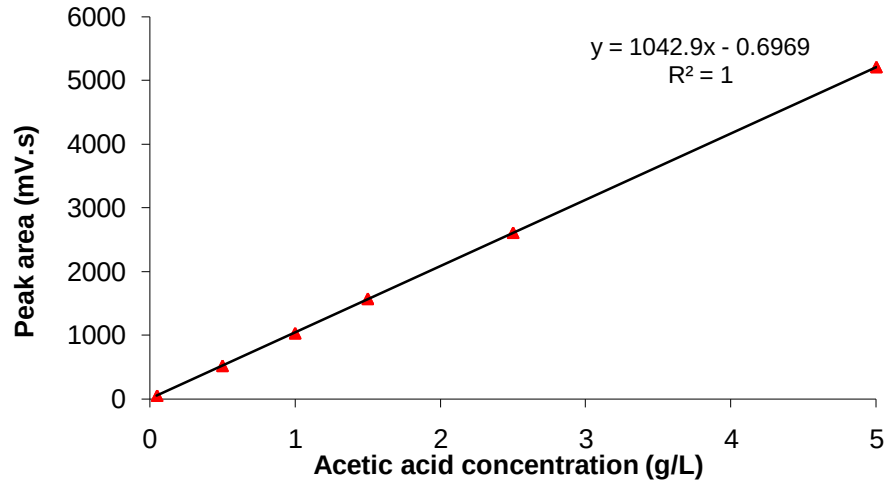

Figure 7.8 – Calibration curve for 'sugars and acids' HPLC acetic acid determination.

7.2 Other calibration curves

7.2.1 DNS method

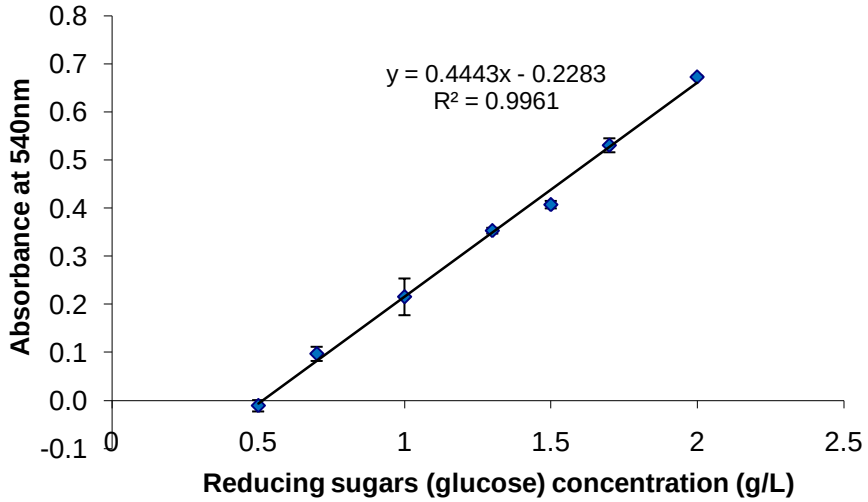

Figure 7.9 – Calibration curve for DNS reducing sugar determination, using D-glucose. The values are averages of the triplicate readings and their standard deviation is represented by the vertical error bars.

7.2.2 Gas chromatography

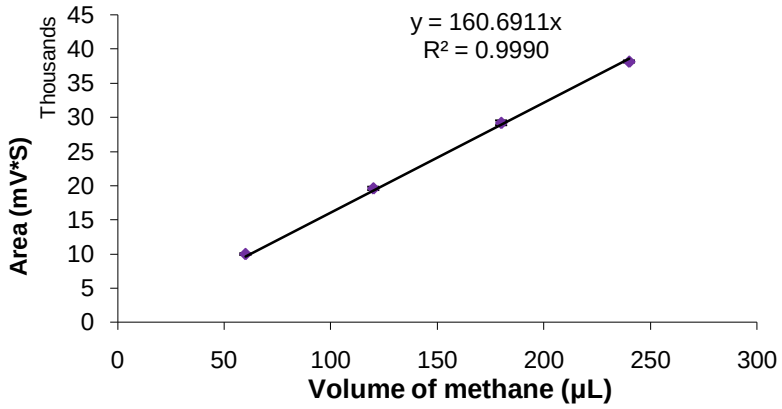

Figure 7.10 – Calibration curve for methane quantification of mostly all the gas samples (main method).

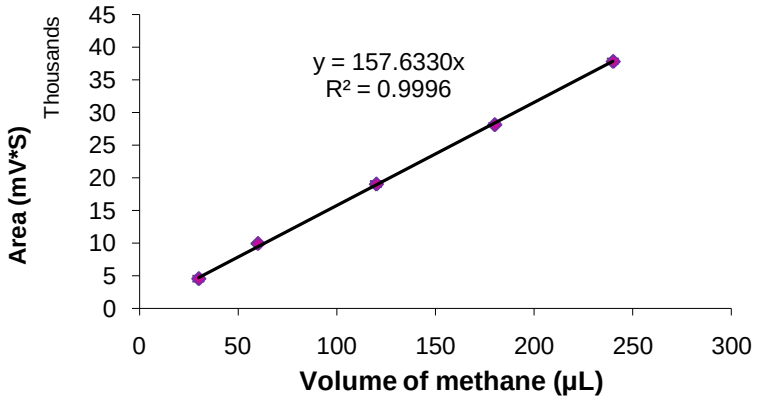

Figure 7.11 – Calibration curve for methane quantification of the samples from Avicel-containing reactors (Avicel method).

7.3 Enzymatic reactions complimentary charts

In this section are present charts that complement the information presented on section 3.6. Aside from the charts depicting the background sugar concentrations of the enzymes used in this study, it is also presented the data for Figure 4.6 through Figure 4.14, expressed as reducing sugar concentrations and as saccharification yield on a carbohydrate basis.

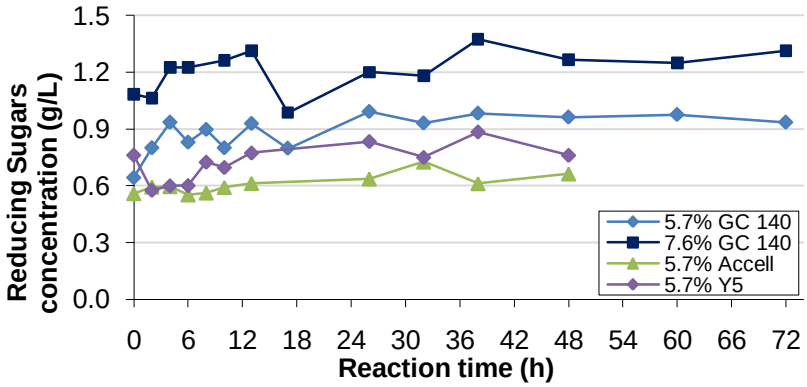

Figure 7.12 – Reducing sugars concentration detected in the reactors with enzymes and no substrate. Legend: 5.7% GC 140 – 5.7% of GC 140 xylanase and 5.7% of Accellerase 1000; 7.6% GC 140 – 7.6% of GC 140 xylanase and 7.6% of Accellerase 1000; 5.7% Accell – 5.7% of Accellerase 1000; 5.7% Y5 – 5.7% of Y5 xylanase and 5.7% of Accellerase 1000 (% in v/v).

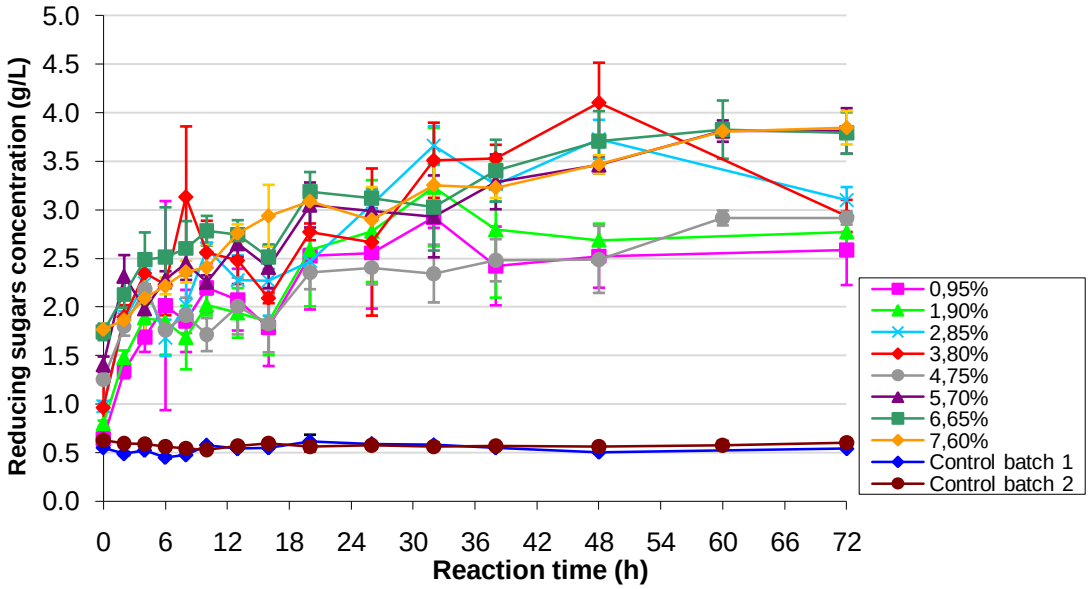

Figure 7.13 – Reducing sugars concentration in the liquid fraction during hydrolysis of the untreated sugarcane bagasse at different enzyme loadings. The points are an average of the values obtained from the triplicate reactors. Their standard deviation is represented by the vertical error bars. (Legend: *x* % = *x*% (working vol.) of Accellerase 1000 + *x*% (working vol.) GC 140 xylanase).

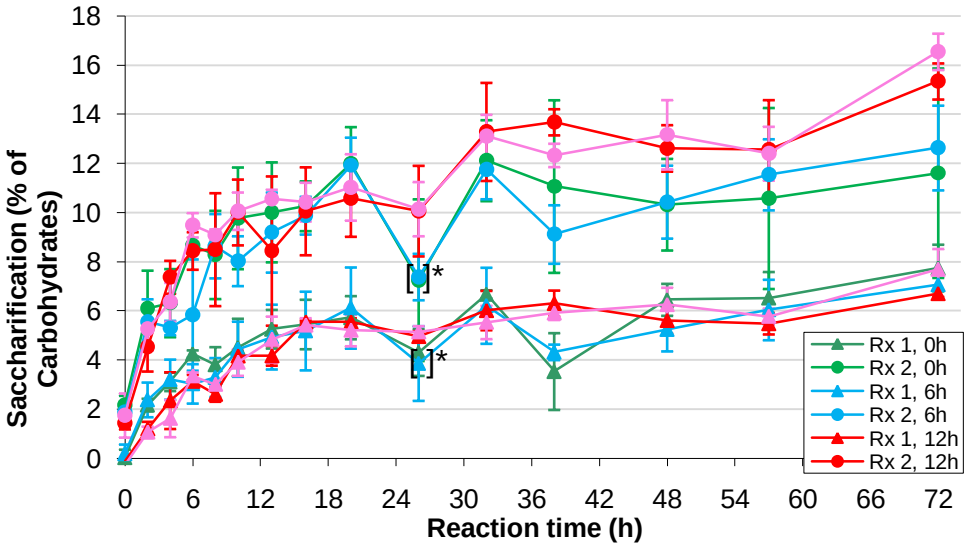

Figure 7.14 – Saccharification yields (Carbohydrate basis) of untreated and acid treated sugarcane bagasse, using a 1:1 mixture of Accellerase 1000 and GC 140 xylanase and a loading of 5.7% of each enzyme. The points are averages of the values obtained from the triplicate reactors, subtracting the values of the respective control reactors. Their standard deviation is represented by the vertical error bars. The biomass conversion percentage (based on total solids) can be read on the right axis. (Legend: Rx 1 – untreated bagasse with enzymes; Rx 2 – acid treated bagasse with enzymes; 0h, 6h, 12h and 24h – time the reactors were incubated post autoclaving.)
* the DNS reagent used was changed, but the blank was still made with the old one.

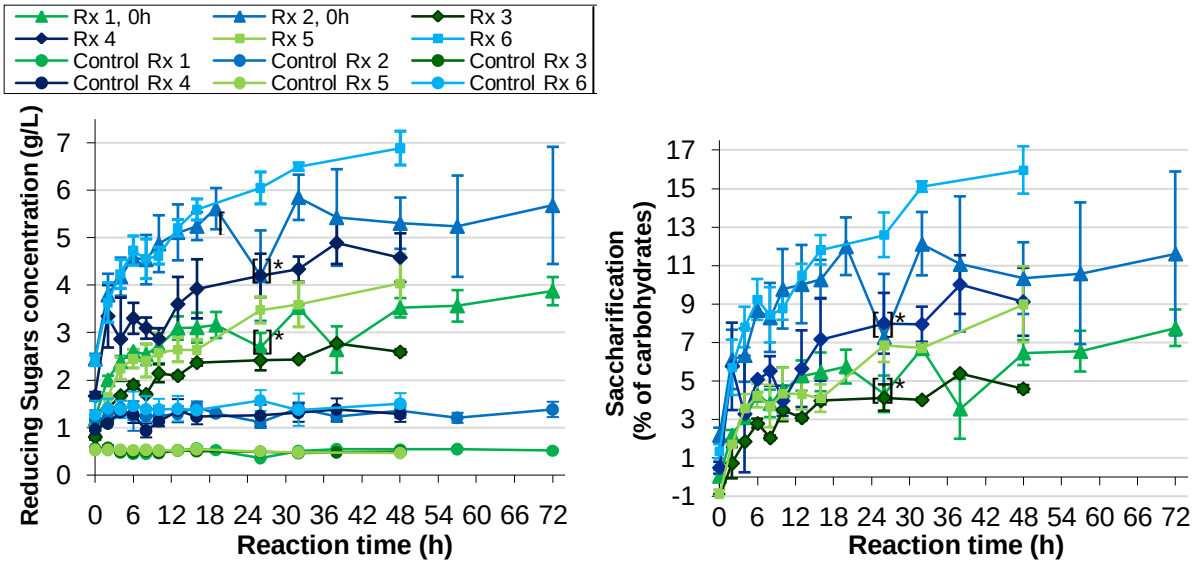

Figure 7.15 – Enzymatic hydrolysis of acid treated and untreated bagasse when using Accellerase 1000 and not employing any xylanase and when using Accellerase 1000 plus Y5 xylanase, expressed in reducing sugar concentrations (left) and saccharification on a carbohydrate basis (right). Each enzyme was employed in a 5.7% working volume loading. The points are averages of the values obtained from the triplicate reactors. Their standard deviation is represented by the vertical error bars. (Legend: Rx 1, 0h – untreated bagasse with GC 140 xylanase (0h in water bath); Rx 2, 0h – acid treated bagasse with GC 140 xylanase (0h in water bath); Rx 3 – untreated bagasse withou any xylanase; Rx 4 – acid treated bagasse withouth any xylanase; Rx 5 – untreated bagasse with Y5 xylanase; Rx 6 – acid treated bagasse with Y5 xylanase; Control – Same as the respective reactors (Rx 1 to Rx 6), but without enzymes).

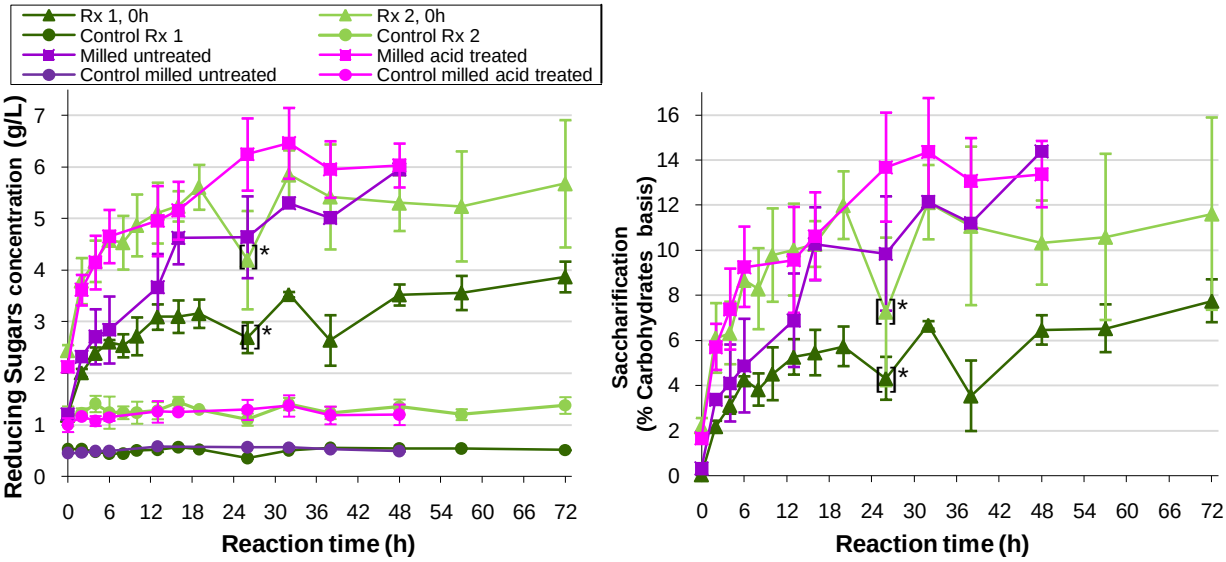

Figure 7.16 – Enzymatic hydrolysis of acid treated and untreated milled and non-milled bagasse, expressed in reducing sugar concentrations (left) and saccharification on a carbohydrate basis (right). The points are averages of the values obtained from the triplicate reactors. Their standard deviation is represented by the vertical error bars. (Legend: Rx 1, 0h – untreated non-milled bagasse (0h in water bath); Rx 2, 0h – acid treated non-milled bagasse (0h in water bath); Control – Same as the respetive reactors, but without enzymes
* the DNS reagent used was changed, but the blank was still made with the old one

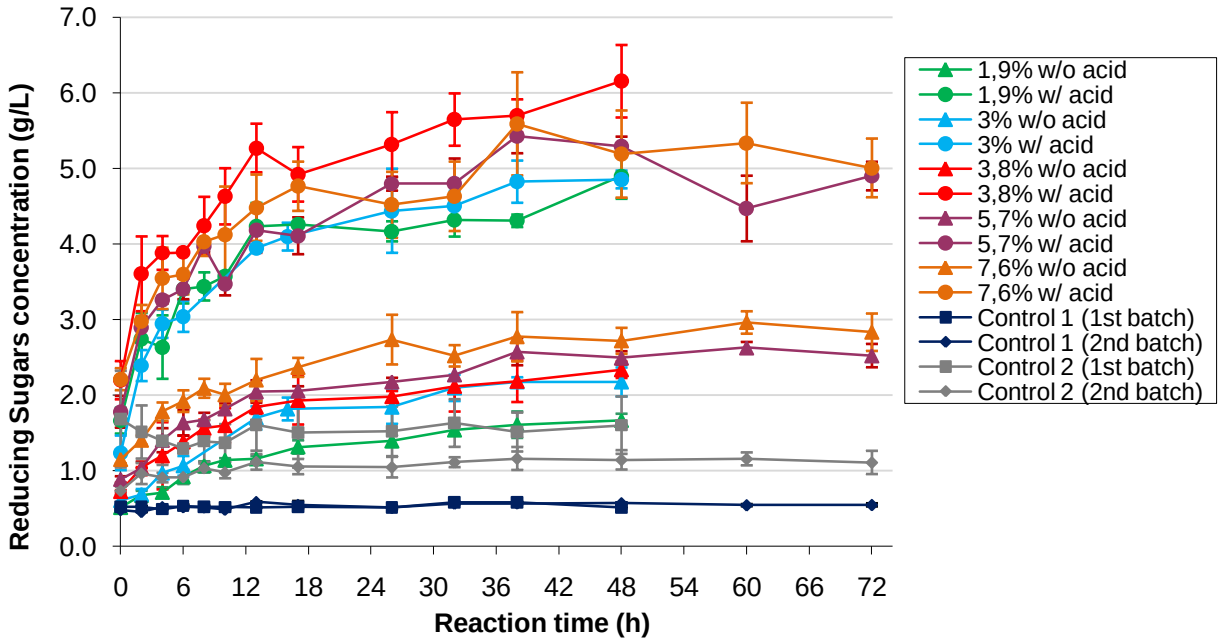

Figure 7.17 – Reducing sugars concentration during enzymatic hydrolysis of untreated and acid treated Pakistanese bagasse, featuring also the control reactors. The points are averages of the values obtained from the triplicate reactors. Their standard deviation is represented by the vertical error bars. (Legend: x % = x% (working vol.) of Accellerase 1000 + x% (working vol.) GC 140 xylanase; NT – untreated bagasse; T – acid treated bagasse; Control 1 – untreated bagasse without enzymes; Control 2 – acid treated bagasse without enzymes.

7.4 Anaerobic digestion additional data

Table 7.4 – pH, VS and partial and total alkalinity data for each of the triplicate reactors used in the anaerobic digestion.

Reactors	Substrate	Initial pH	Final VS (g)	Final pH	PA (ppm CaCO3)	TA (ppm $CaCO_3$)
1	Original Bagasse	8.22	5.75	8.06	12844	14945
2		8.197	6.12	8.15	12787	15393
3		8.188	6.14	8.00	12904	15236
4	Acid-treated pulp	8.202	5.65	8.13	12225	14614
5		8.216	5.74	8.16	12354	14237
6		8.208	5.67	8.14	12353	14518
7	Control to acid-treated pulp	8.213	5.71	8.11	12163	14280
8		8.201	5.28	7.73	11720	14295
9		8.22	5.61	8.06	11903	14481
10	Acid-treated hydrolysate	8.305	2.68	8.02	10923	13114
11		8.3	2.81	8.08	10857	12533
12		8.288	2.83	8.13	10837	13020
13	Control to acid-treated hydrolysate	8.328	2.43	8.15	6479	7889
14		8.34	2.44	8.23	6436	8016
15		8.325	2.41	7.94	6624	8188
16	Acid & Enzyme-treated pulp	8.152	6.05	8.16	12205	14282
17		8.162	5.79	8.14	12364	14680
18		8.131	5.92	8.09	12184	14571
19	Control to Acid & enzyme-treated pulp	8.15	5.16	8.15	12125	14754
20		8.131	5.19	8.22	12360	15187
21		8.126	5.51	8.19	12362	14933
22	Acid & Enzyme-treated hydrolysate	8.086	2.96	7.92	10697	12863
23		8.079	2.97	8.13	10520	12923
24		8.049	3.01	8.15	10617	12811
25	Control to Acid & Enzyme-treated hydrolysate	8.079	2.88	8.18	10185	12601
26		8.065	2.83	8.02	10087	12347
27		8.073	2.89	8.19	10243	12202
28	Innoculum + water	8.152	4.34	8.23	5858	8332
29		8.202	Reactor lost			
30		8.192	4.22	8.09	12816	15282
31		8.123	4.22	8.21	12862	15236
32	Innoculum + water	8.286	2.17	8.09	6880	8497
33		8.309	2.12	8.00	6869	8466
34		8.25	2.16	8.19	6880	8641
35	Reference (Avicel - cellulose)	8.335	Reactor lost			
36		8.222	4.44	8.15	13200	15364
37		8.202	4.41	8.23	13005	15237
38		8.157	4.41	8.09	13000	15033

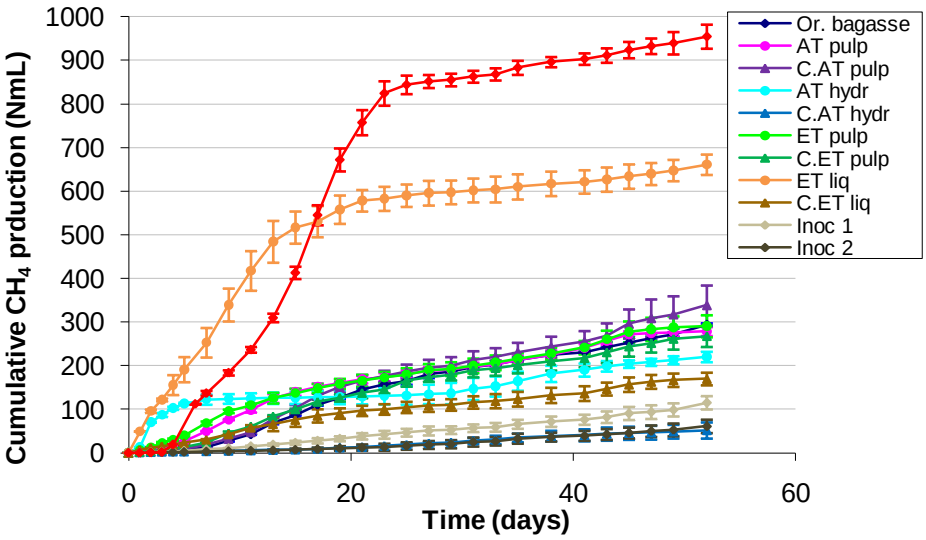

Figure 7.18 – Cumulative methane production during the anaerobic digestion process. Points are averages from the triplicate reactors. Their standard deviations are represented by the vertical error bars. Legend: Or. bagasse – Original pulp (bagasse); hydr – hydrolysate; liq - liquor; AT – acid treated; C.AT – control to acid treated; ET –enzyme treated; C. ET – control to enzyme treated; Inoc 1 and Inoc 2– Inoculum with water only. (1 – higher inoculum loading; 2 – lower inoculum loading).

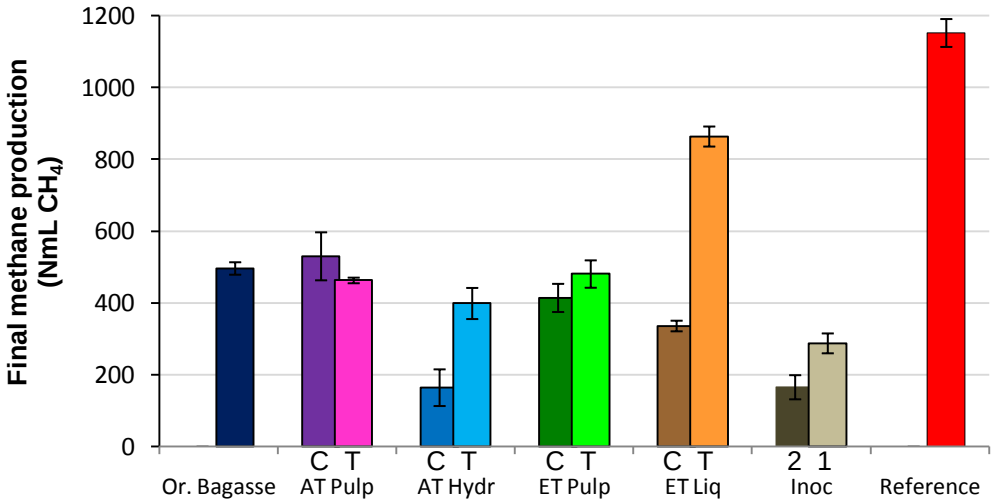

Figure 7.19 – Final methane production from the various substrates after anaerobic digestion. Legend: Or. Bagasse – Original pulp (bagasse); AT Pulp – acid treated pulp; AT Hydr – acid treated hydrolysate; ET Pulp – enzyme treated pulp; ET Liq – enzyme treated liquor; C – control; T – treated. Inoc 1 and Inoc 2– Inoculum with water only (1 – higher inoculum loading; 2 – lower inoculum loading).Their standard deviation is represented by the vertical error bars.

7.5 Enzyme information data sheets and technical bulletins

product description

DANISCO

Y5 Xylanase

Version:01 Issue: 12/02 ROW

A feed enzyme system specifically developed for use in wheat, triticale, rye or corn-based broiler, laying hen, turkey and duck diets.

Rate of use:
0.5 kg/tonne (0.05%) of finished feed, included either directly or via a premix.

Guaranteed minimum activity:
50,000 U/g endo-1,4-beta-xylanase EC 3.2.1.8. Analysis methods detailing assay conditions are available upon request.

Processing stability:
The feed enzyme system is adsorbed onto a specially prepared wheat carrier using a patented process, which ensures stability during pelleting. For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 85°C (185°F).

Storage stability:
Store in dry conditions. The constituent enzyme activities remain stable for at least 12 months when stored in original packaging at <22°C (<72°F) and 4 months when included in a vitamin/mineral premix and stored at <22°C (<72°F).

Ingredients:
Dried *Trichoderma longibrachiatum* fermentation product, wheat flour, calcium propionate (preservative).

Characteristics:
The product is supplied as a light brown, fine granular product as follows:

Particle size based on sieve fineness

	Approx %
> 1400 micrometers	8
<1400 to > 125 micrometers	81
< 125 micrometers	11

Bulk density - 650 kg/m³

Moisture content – Maximum 12%

Particle size distribution studies by Method DIN 53734 indicate absence of particles less than 10 micrometers.

Safety and purity:
The product contains enzymes produced by strains of *Trichoderma*. Enzymes are produced in accordance with FAO/WHO JECFA and FCC recommendations as follows:

Heavy metals	< 40 ppm	Aflatoxins	Negative by test
Lead	< 10 ppm	Antibiotic activity	Negative by test
Arsenic	< 3 ppm	Salmonella/25g	Negative by test
Cadmium	< 0.5 ppm	E. coli/25g	Negative by test
Mercury	< 0.5 ppm		

There are no viable producing micro-organisms in the final product. None of the ingredients is of animal origin.

Package sizes:
The product is available in 25 kg multi-wall paper bags.

Handling:
Provide for good ventilation and avoid dust formation. It is recommended to use protective glasses, respiratory mask and gloves during handling. In case of accidental contact with skin or eyes, the only action needed is copious flushing with water. See the Material Safety Data Sheet for further information.

Danisco Animal Nutrition
PO Box 777, Marlborough, Wiltshire, SN8 1XN, United Kingdom
Tel +44 (0) 1672 517777 Fax +44 (0) 1672 517778

GC 140

Developmental Xylanase Enzyme

Product Information

■ INTRODUCTION

GC 140 xylanase is derived from a genetically modified strain of *Trichoderma longibrachiatum* (formerly *Trichoderma reesei*). GC 140 xylanase is a food-grade product especially designed to modify xylan polymers and can be used as a processing aid in various applications.

■ ADVANTAGES

- Xylanase activity breaks down insoluble fibers to increase digestibility
- Contains virtually no protease, lipase, or amylase side activity
- Contains low amounts of cellulase side activity

■ UNIT DEFINITION

The activity of GC 140 xylanase is expressed in Genencor Xylanase Units (GXU). The substrate is an aqueous solution of Remazol Brilliant Blue-dyed birchwood xylan. Xylanase activity is calculated from the amount of dyed substrate released by the enzyme in a 10 minute incubation at pH 4.5 and temperature 30° C, as measured spectrophotometrically using a xylanase standard.

■ TYPICAL CHARACTERISTICS

Activity:	7250 GXU/g minimum
Appearance:	Amber liquid
Solubility:	Miscible in water
pH:	4.8 - 5.2
Specific gravity:	1.08 - 1.18 g/ml

The GC 140 enzyme is GRAS (Generally Recognized As Safe) and complies with current FAO/WHO and FCC recommended specifications for food-grade enzymes, and also meets additional internal specifications which are available upon request.

■ APPLICATIONS

GC 140 xylanase can be used in most applications which require modification or degradation of non-starch carbohydrates, specifically xylan polymers. It may also be used to treat fibrous materials to make the nutrients more available to animals and/or humans.

Such applications include:

- Animal feed
- Baked goods
- Brewing
- Ready-to-eat cereals
- Treatment of whole grains, grain mashes, or doughs as a processing aid and/or to reduce viscosity

■ DOSAGE GUIDELINES

GC 140 xylanase is a concentrated enzyme product which can be used at low doses for most applications.

For best results:

A. Agitate for a short time to ensure that the undiluted enzyme is well dispersed in liquid formulations, *or*

B. Dilute prior to addition to dry or viscous products, facilitating dispersion of the enzyme (i.e. 10% solution in cool water). Generally, GC 140 Xylanase can be diluted into the water used for normal processing.

Dosage of GC 140 xylanase is dependent upon:

- Type of plant material
- Degree of hydrolysis desired
- Temperature of the process
- pH and other chemical parameters
- Time permitted for enzymatic action

The optimum dosage of GC 140 xylanase can be determined by small-scale experimentation. A starting dose of 25 - 75 ppm (w/w), with the exact amount based upon the material and the enzyme application, is recommended.

■ TEMPERATURE & pH CONDITIONS

GC 140 xylanase is designed for activity over a range of reaction conditions. The optimum temperature range is 50 - 60° C (120 - 140° F) and the optimum reaction pH is 4.5 - 5.0. Small-scale experiments should be conducted to obtain the optimum reaction conditions for each application.

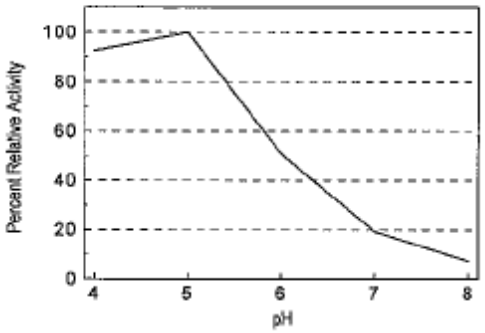

Figure 1: Effect of pH on GC 140 Xylanase Activity
Xylanase assay conditions: 40° C, 10 minutes

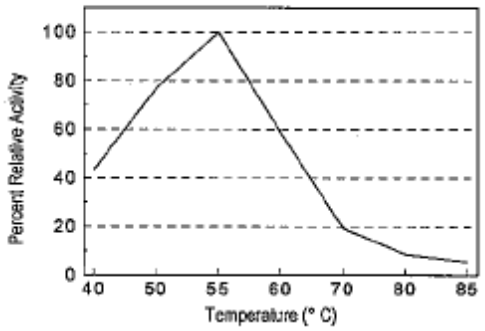

Figure 2: Effect of Temperature on GC 140 Xylanase Activity
Xylanase assay conditions: pH 5.3, 10 minutes

■ STORAGE

As with all liquid enzyme products, it is recommended to hold this product under refrigerated conditions for long-term storage. GC 140 xylanase should not lose significant activity if held at room temperature for up to three months. To ensure maximum stability, do not expose the product to high temperatures during storage.

■ SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the Enzyme Technical Association (ETA) handbook *Working Safely With Enzymes*, and the Association of Manufacturers of Fermentation Enzyme Products (Amfep) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available from Genencor International.

■ TECHNICAL SERVICE

Information covering specific applications of this product is available. Genencor International will work with customers to enhance processes and solve problems. Let us know what you need and we will assist you.

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ACCELLERASE™ 1000

Cellulase Enzyme Complex for Lignocellulosic Biomass Hydrolysis

Product Information

■ DESCRIPTION

ACCELLERASE™ 1000 cellulase is an enzyme complex preparation intended specifically for the lignocellulosic biomass processing industries, including renewable fuels and chemicals. ACCELLERASE™ 1000 will facilitate process development and scale up in this emerging industry. Key features that are expected to be important at commercial scale biorefineries are already built in to this first generation product. Benefits observed with the proprietary enzyme complex and the unique product formulation of ACCELLERASE™ 1000 compared to conventional cellulases include:

- □ Enhanced saccharification performance on a variety of feedstocks.
- □ Ability to operate in simultaneous saccharification and fermentation (SSF) processes, two step sequential hydrolysis and fermentation (SHF) processes or hybrids of the two.
- □ High beta-glucosidase activity to minimize residual cellobiose, which may lead to higher rates of saccharification and ultimately to a faster ethanol fermentation. Yields may also be improved.
- □ Unclarified product. The remaining nutrients from enzyme production are available to the yeast in addition to the fermentable sugars produced by saccharification. This may lead to faster ethanol fermentations, reduce the cost of ethanol fermentation raw materials, and possibly even improve ethanol yields.
- □ Minimal formulation to ensure that enzyme formulation chemicals do not interfere with saccharification carbohydrate profile analysis or subsequent yeast fermentation.

ACCELLERASE™ 1000 enzyme complex contains a potent combination of enzymes which effectively modify and digest non-starch carbohydrates, the structural material of lignocellulosic biomass. Lignocellulosic material is composed mainly of cellulose, hemicellulose, and beta-glucans which are associated with each other and also with lignin, pectins, proteins, starch, and lipids. This product is capable of efficiently hydrolyzing lignocellulosic biomass into fermentable monsaccharides. ACCELLERASE™1000 contains high levels of beta-glucosidase to ensure almost complete conversion of cellobiose to glucose.

ACCELLERASE™ 1000 is produced with a genetically modified strain derived from *Trichoderma reesei.* The production host is inactivated at the end of the controlled fermentation.

■ TYPICAL CHARACTERISTICS

ACCELLERASE™ 1000 enzyme complex contains multiple enzyme activities; mainly exoglucanase, endoglucanase, hemi-cellulase and beta-glucosidase. The endoglucanase activity is standardized on the basis of its activity on carboxymethylcellulose (CMC). Beta-glucosidase activity is standardized on the basis of activity on pNP-glucoside. The biomass hydrolysis performance of this enzyme preparation is a result of the synergistic effect of all the main and accessory activities and cannot be completely evaluated on the basis of the declared activities alone.

Endoglucanase Activity:	2500 CMC U/g (minimum)
Beta-Glucosidase Activity:	400 pNPG U/g (minimum)
Appearance:	Brown liquid
pH:	4.8 - 5.2

The activity of ACCELLERASE™ 1000 enzyme complex is expressed in carboxymethycellulose (CMC U) activity units. One CMC U unit of activity liberates 1 µmol of reducing sugars (expressed as glucose equivalents) in one minute under specific assay conditions of 50°C (122°F) and pH 4.8. Beta-glucosidase is reported in pNPG units. One pNPG unit denotes 1 µmol of Nitrophenol liberated from para-nitrophenyl-B-D-glucopyranoside in 10 minutes at 50°C (122°F) and pH 4.8. Detailed assay methods are available upon request.

■ APPLICATIONS

ACCELLERASE™ 1000 enzyme complex will hydrolyze the non-starch carbohydrates in lignocellulosic biomass into fermentable monosaccharides as well as aid materials handling by liquefaction and viscosity reduction. Feedstocks including paper pulp, corn stover, sugar cane bagasse, wood chips, waste paper and many others can all be hydrolyzed using ACCELLERASE™ 1000. ACCELLERASE™ 1000 can work with a variety of pretreatments including dilute acid, AFEX and steam expansion. This can be done using simultaneous saccharification and fermentation (SSF) or in a two step sequential hydrolysis and fermentation (SHF) configuration. Please be aware of pH and temperature stability optima and limits of the enzyme depending on your process configuration. Saccharification performance may be enhanced by the addition of other Genencor® enzymes depending on the composition of the pretreated feedstock.

■ DOSAGE GUIDELINES

The optimum dosage levels of ACCELLERASE™ 1000 enzyme complex will vary considerably with different substrates and their associated pretreatment technologies and conditions. Operating conditions such as pH, temperature and reaction time may also affect enzyme performance. An ACCELLERASE™ 1000 dosage rate of 0.1 - 0.5 g (0.1 - 0.5 mL) per g cellulose or roughly 0.05 to 0.25 mL per g of biomass (depending on biomass composition) is recommended as a starting point for optimization of enzyme dosage. ACCELLERASE™ 1000 rapidly liquefies and hydrolyzes a variety of substrates within 24 hours, with some additional benefit by extending the time. Small-scale experiments are recommended to determine optimum enzyme dosage in each system. See Figures 1 and 2 for an example of such experiments.

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Genencor® enzymes

Saccharification only:

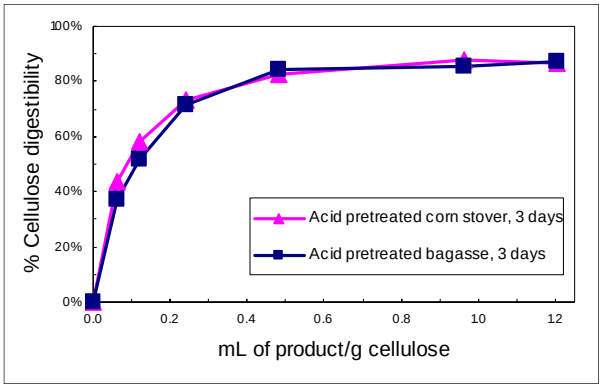

Figure 1: % Cellulose digestibility vs. mL of product per g cellulose for washed acid-pretreated corn stover and sugar cane bagasse using ACCELLERASE™ 1000 at 7% cellulose loading, 50˚C, pH 5.0, and in 3 days.

Simultaneous Saccharification and Fermentation (SSF):

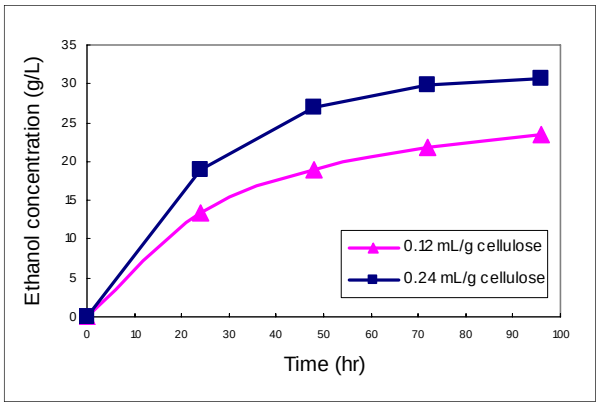

Figure 2: Ethanol concentration vs. time for two doses of enzyme (0.12 mL/g cellulose and 0.24 mL/g cellulose) in an SSF process. SSF of acid pretreated sugar cane bagasse using ACCELLERASE™ 1000 at 7% cellulose solids, pH 5.0, 38°C.

EFFECT OF pH AND TEMPERATURE

ACCELLERASE™ 10000 enzyme complex has the best operational stability in the following ranges:

Temperature: 50 - 65°C (122 - 149°F)

pH: 4.0 - 5.0

ACCELLERASE™ 1000 enzyme complex is easily inactivated at temperatures above 70°C (158°F) or at pH levels above 7.0 or below 4.0 Long term storage should be at 4°C (39°F).

PACKAGING

ACCELLERASE™ 1000 enzyme complex is available in various package sizes. Please consult your Genencor® representative for detailed information.

STORAGE

It is advisable to store ACCELLERASE™ 1000 enzyme complex under refrigerated conditions of 4°C (39°F) and sheltered against direct sunlight. Storage above 20°C (70°F) should be avoided. The product can settle gradually, so large-scale storage with either gentle agitation or occasional pump recirculation is recommended.

SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the Enzyme Technical Association (ETA) handbook *Working Safely With Enzymes*, and the Association of Manufacturers and Formulators of Enzyme Products (Amfep) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available from Genencor®.

TECHNICAL SERVICE

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ACCELLERASE™ 1000

Cellulase Enzyme Complex for Lignocellulosic Biomass Hydrolysis

Technical Bulletin #1: Saccharification

■ SUMMARY

ACCELLERASE™ 1000 cellulase is an enzyme preparation intended specifically for the lignocellulosic biomass processing industries, including renewable fuels and chemicals. ACCELLERASE™ 1000 will facilitate process development and scale up in this emerging industry. Key features that are expected to be important at commercial scale biorefineries are already built in to this first generation product. Benefits observed with the proprietary enzyme complex and the unique product formulation of ACCELLERASE™ 1000 compared to conventional cellulases include:

- □ Enhanced saccharification performance on a variety of feedstocks.
- □ Ability to operate in simultaneous saccharification and fermentation (SSF) processes, two step sequential hydrolysis and fermentation (SHF) processes or hybrids of the two.
- □ High beta-glucosidase activity to minimize residual cellobiose, which may lead to a higher rate of saccharification and ultimately to a faster ethanol fermentation. Yields may also be improved.
- □ Unclarified product. The remaining nutrients from enzyme production are available to the yeast in addition to the fermentable sugars produced by saccharification.
- □ Minimal formulation to ensure that enzyme formulation chemicals do not interfere with saccharification carbohydrate profile analysis or subsequent yeast fermentation.

Technical Bulletin #1 describes saccharification using ACCELLERASE™ 1000 enzyme complex. This bulletin first shows hydrolysis of a model cellulose (Phosophoric Acid Swollen Cellulose or PASC), and then discusses a more real-world application in the biofuel and biochemical industry where ACCELLERASE™ 1000 was used to hydrolyze dilute acid pre-treated biomass such as corn stover and sugar cane bagasse into fermentable glucose and xylose sugars. Enzyme hydrolysis experiments were performed on these two substrates at 7% cellulose loading (~13% solids) and the cellulose digestibility is reported.

ACCELLERASE™ 1000 is produced with a genetically modified strain derived from *Trichoderma reesei.* The production host is inactivated at the end of the controlled fermentation.

■ SUBSTRATE INFORMATION

Substrates used in this work :

1. Phosphoric acid swollen cellulose (PASC) produced by a standard method (Wood, 1988) in which Avicel PH101 (Fluka 11365) was treated with 85% ortho-phosphoric acid, precipitated with acetone, washed and stored as a slurry. Although not an industrial lignocellulosic biomass substrate, this is a very easily digestible "amorphous" cellulose useful in demonstrating intrinsic stability and activity characteristics of cellulase preparations.
2. A dilute sulfuric acid pretreated corn stover (PCS) was produced and provided by NREL, as detailed in Schell et al 2003. The PCS was pretreated at a solids concentration of 20% (w/w), temperature of 165°C, 1.44% (w/w) acid and an approximate residence time of 8 minutes.
3. A dilute sulfuric acid pretreatment sugar cane bagasse (APB) produced in a similar manner to the PCS.

The cellulose composition of the corn stover and bagasse were 55.6% and 53.6% respectively. Compositional analyses were performed using the standard assays detailed in the NREL protocols for Standard Biomass Analytical Procedures.

http://www1.eere.energy.gov/biomass/analytical_procedures.html

■ ENZYME PROPERTIES

ACCELLERASE™ 1000 enzyme complex contains multiple enzyme activities; mainly exoglucanase, endoglucanase, hemi-cellulase and beta-glucosidase. The endoglucanase activity is standardized on the basis of its activity on carboxymethylcellulose (CMC). Beta-glucosidase activity is standardized on the basis of its activity on para-nitrophenyl-B-D-glucopyranoside (pNPG). Detailed assay methods are available upon request. The product data sheet contains additional information on enzyme pH and temperature stability ranges. The biomass hydrolysis performance of this enzyme preparation is a result of the synergistic effect of all the main and accessory activities and cannot be completely evaluated on the basis of the declared activities only.

Endoglucanase Activity:	2500 CMC U/g (minimum)
Beta-Glucosidase Activity:	400 pNPG U/g (minimum)
Appearance:	Brown liquid
pH:	4.8 - 5.2

■ SACCHARIFICATION CONDITIONS

Enzymatic digestion studies were carried out based on NREL standard assay method LAP-009 "Enzymatic Saccharification of Lignocellulosic Biomass" (http://devafdc.nrel.gov/pdfs/9578.pdf), except for modification of enzyme and cellulose loading. The PASC hydrolysis was done at 1.2% cellulose and dosed with 0.2 mL product/g cellulose of ACCELLERASE™ 1000. pH optimum experiments were conducted at 2.5% PASC and dosed with 0.12 mL product/g cellulose. The acid pretreated corn stover and bagasse saccharification experiments were carried out at a higher 7% cellulose loading (~13% solids) at 50 ºC and pH 5.0. The amount of enzyme dosed was 0.24 mL product/g cellulose. Samples of the hydrolysate were taken at various time intervals over 5 days and then analyzed for glucose concentration and cellobiose concentrations. All samples were performed in duplicate. The data reported was the average of the duplicates. The % cellulose digestibility was calculated by dividing the amount of cellulose digested by the amount of cellulose loaded. Glucose and cellobiose concentrations were determined using a Waters HPLC system (Alliance system, Waters Corp., Milford, MA). The HPLC column used for sugar analysis was purchased from BioRad (Aminex HPX-87P, BioRad Inc., Hercules, CA).

■ RESULTS AND DISCUSSION

Figure 1A demonstrates the conversion of phosphoric acid swollen cellulose (PASC), loaded at 1.2% cellulose, over 6 hours. The optimal pH range for ACCELLERASE™ 1000 is shown to be 4.0 to 5.0 in Figure 1B. Figure 2 demonstrates that ACCELLERASE™ 1000 will rapidly hydrolyze acid pretreated sugar cane bagasse or corn stover into fermentable sugars within 24 hours at

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the dosage level used, but that some additional benefit will be seen by extending the time. It is also shown that ACCELLERASE™ 1000 hydrolyses biomass at a significantly faster rate than Spezyme® CP, a common benchmark enzyme. Figure 3 shows the low residual cellobiose levels using ACCELLERASE™ 1000 versus Spezyme® CP. Most fermentation organisms, such as yeast, are unable to metabolize cellobiose. Additionally, the lower cellobiose levels may lead to a much faster hydrolysis during the first few days from reduced inhibition of the cellulases.

The optimum dosage levels of ACCELLERASE™ 1000 will vary considerably with different substrates and their associated pretreatment technologies and conditions. Operating conditions such as pH, temperature, and reaction time should also be optimized to improve cellulose digestibility.

An ACCELLERASE™ 1000 enzyme complex dosage rate of 0.1- 0.5 g (0.1 - 0.5 mL) per g cellulose or roughly 0.05 to 0.25 mL per g of biomass (depending on biomass composition) is recommended as a starting point for the optimization of enzyme dosage. Small-scale experiments are recommended to determine optimum enzyme dosage in each system.

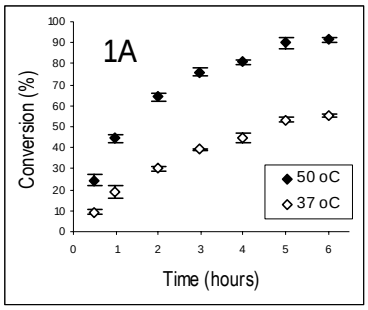

Figure 1A: % Cellulose digestibility using ACCELLERASE™ 1000 at 0.2 mL product/g cellulose on PASC at 1.2% cellulose loading, 37°C and 50°C, and pH 5.0. Note that the

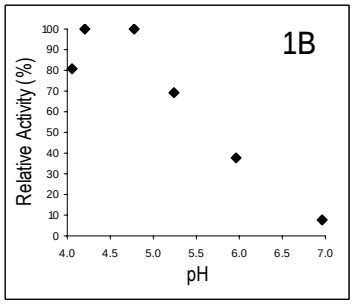

Figure 1B: shows the pH optima to be 4.0 to 5.0 on 2.5% PASC after 7 hours at 25°C. Loading was 0.12 mL ACCELLERASE™ 1000/g cellulose.

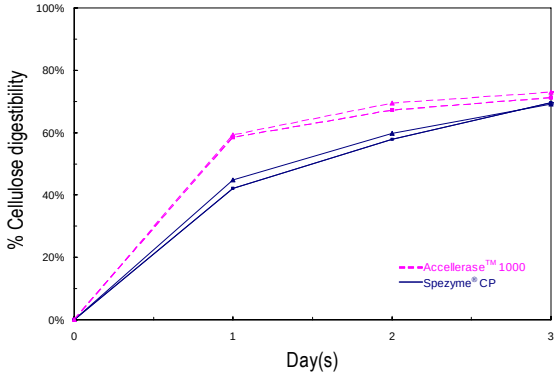

Figure 2: % Cellulose digestibility using ACCELLERASE™ 1000 at 0.24 mL product /g cellulose vs. an equivalent enzyme loading of Spezyme® CP on acid pretreated corn stover(▲) and sugar cane bagasse (■) at 7% cellulose loading, 50°C, and pH 5.0.

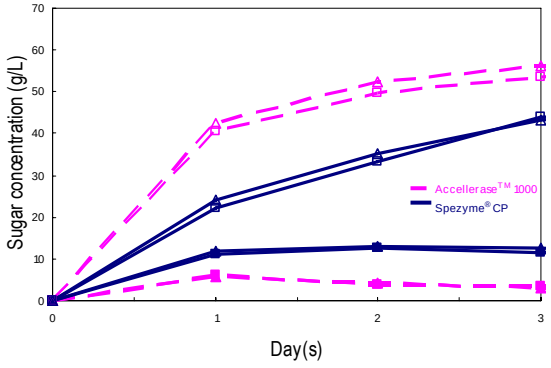

Figure 3: Low residual cellobiose (filled symbols) and higher glucose (open symbols) levels seen using ACCELLERASE™ 1000 at 0.24 mL product /g cellulose vs. the benchmark cellulase product Spezyme® CP on acid pretreated corn stover (▲) and sugar cane bagasse (■), 7% cellulose loading, 50°C, and pH 5.0.

SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the Enzyme Technical Association (ETA) handbook *Working Safely With Enzymes*, and the Association of Manufacturers and Formulators of Enzyme Products (Amfep) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available from Genencor®.

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REFERENCES

1. Wood, T. (1988) in Methods in Enzymology, Vol. 160. Biomass - Part A: Cellulose and Hemicellulose (Wood, W. & Kellog, S. Eds.), pp. 19-25, Academic Press, San Diego, CA, USA

2. Schell, D., Farmer, J. Newman, M., McMillan, J. 2003. Dilute-sulfuric Acid Pretreatment of Corn Stover in Pilot Scale Reactor; Investigation of Yields, Kinetics and Enzymatic Digestibilities of Solids. App. Biochem. Biotechnol. Vol. 105-108, 69-85.

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