Optimization of the conversion of lignocellulosic agricultural by-products to bioethanol using different enzyme cocktails and recombinant yeast strains.

by

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Abstract

The need to mitigate the twin crises of peak oil and climate change has driven a headlong rush to biofuels. This study was aimed at the development of a process to efficiently convert steam explosion pretreated (STEX) sugarcane bagasse into ethanol by using combinations of commercial enzyme cocktails and recombinant *Saccharomyces cerevisiae* strains. Though enzymatic saccharification is promising in obtaining sugars from lignocellulosics, the low enzymatic accessibility of the cellulose and hemicellulose is a key impediment thus necessitating development of an effective pretreatment scheme and optimized enzyme mixtures with essential accessory activities. In this context, the effect of uncatalysed and SO_2 catalysed STEX pretreatment of sugarcane bagasse on the composition of pretreated material, digestibility of the water insoluble solids (WIS) fraction and overall sugar recovery was investigated. STEX pretreatment with water impregnation was found to result in a higher glucose recovery (28.1 g/ 100 bagasse) and produced WIS with a higher enzymatic digestibility, thus was used in the optimization of saccharification and fermentation. Response surface methodology (RSM) based on the 3^3 factorial design was used to optimize the composition of the saccharolytic enzyme mixture so as to maximize glucose and xylose production from steam exploded bagasse. It was established that a combination of 20 FPU cellulase/ g WIS and 30 IU β-glucosidases/ g WIS produced the highest desirability for glucose yield. Subsequently the optimal enzyme mixture was used to supplement enzyme activities of recombinant yeast strains co-expressing several cellulases and xylanases in simultaneous saccharification and fermentations SSFs. In the SSFs, ethanol yield was found to be inversely proportional to substrate concentration with the lowest ethanol yield of 70% being achieved in the SSF at a WIS concentration of 10% (w/v). The ultimate process would however be a one-step "consolidated" bio-processing (CBP) of lignocellulose to ethanol, where hydrolysis and fermentation of polysaccharides would be mediated by a single microorganism or microbial consortium without added saccharolytic enzymes. The cellulolytic yeast strains were able to autonomously multiply on sugarcane bagasse and concomitantly produce ethanol, though at very low titres (0.4 g/L). This study therefore confirms that saccharolytic enzymes exhibit synergism and that bagasse is a potential

substrate for bioethanol production. Furthermore the concept of CBP was proven to be feasible.

Opsomming

Die behoefte om die twee krisisse van piek-olie en klimaatsverandering te versag, het veroorsaak dat mense na biobrandstof as alternatiewe energiebron begin kyk het. Hierdie studie is gemik op die ontwikkeling van 'n proses om stoomontplofde voorafbehandelde (STEX) suikerriet bagasse doeltreffend te omskep in etanol deur die gebruik van kombinasies van kommersiële ensiem mengsels en rekombinante *Saccharomyces cerevisiae* stamme. Alhoewel ensiematiese versuikering belowend is vir die verkryging van suikers vanaf lignosellulose, skep die lae ensiematiese toeganklikheid van die sellulose en hemisellulose 'n hindernis en dus is die ontwikkeling van' n effektiewe behandelingskema en optimiseerde ensiemmengsels met essensiële bykomstige aktiwiteite noodsaaklik. In hierdie konteks, was die effek van ongekataliseerde en SO_2 gekataliseerde stoomontploffing voorafbehandeling van suikerriet bagasse op die samestelling van voorafbehandelde materiaal, die verteerbaarheid van die (WIS) breuk van onoplosbare vastestowwe in water (WIS), en die algehele suikerherstel ondersoek. Daar was bevind dat stoomontploffing behandeling (STEX) met water versadiging lei tot 'n hoër suikerherstel (21.8 g/ 100g bagasse) en dit het WIS met 'n hoër ensimatiese verteerbaarheid vervaardig en was dus gebruik in die optimalisering van versuikering en fermentasie. Reaksie oppervlak metodologie (RSM), gebasseer op die 3^3 faktoriële ontwerp, was gebruik om die samestelling van die 'saccharolytic' ensiemmengsel te optimaliseer om sodoende die maksimering van glukose en 'xylose' produksie van stoomontplofde bagasse te optimaliseer. Daar was bevestig dat 'n kombinasie van 20 FPU sellulase/ g WIS en 30 IU 'β-glucosidases/ g' WIS die hoogste wenslikheid vir glukose-opbrengs produseer het. Daarna was die optimale ensiemmengsel gebruik om ensiemaktiwiteit van rekombinante gisstamme aan te vul, wat gelei het tot die mede-uitdrukking van verskillende 'cellulases' en 'xylanases' in gelyktydige versuikering en fermentasie SSFs. In die SSFs was daar bevind dat die etanol-produksie omgekeerd proporsioneel is tot substraat konsentrasie, met die laagste etanolopbrengs van 70% wat bereik was in die SSF by 'n WIS konsentrasie van 10% (w/v). Die uiteindelike proses sal egter 'n eenmalige "gekonsolideerde" bioprosessering (CBP) van lignosellulose na etanol behels, waar die hidrolise en fermentasie van polisakkariede deur' n enkele mikro-organisme of mikrobiese konsortium sonder bygevoegde 'saccharolytic' ensieme

bemiddel sal word. Die ‘cellulolytic’ gisstamme was in staat om vanself te vermeerder op suikerriet bagasse en gelyktydig alkohol te produseer, al was dit by baie lae titres (0.4 g/L). Hierdie studie bevestig dus dat ‘saccharolytic’ ensieme sinergisme vertoon en dat bagasse 'n potensiële substraat is vir bio-etanol produksie. Daar was ook onder meer bewys dat die konsep van CBP uitvoerbaar is.

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Chapter 1: General Introduction and Project Aims

1.1. Introduction

Global economic development in the 20th century depended strongly on the abundant supply of oil, which used to be, convenient to use and with high energy efficiency. However, the world is fast approaching the point where the depletion of ageing oilfields cannot be covered by decreasing new supply coming on stream, and thus crude oil production will inevitably start lagging behind demand for oil (Tsoskounoglou *et al*., 2008). According to the BP Statistical Review of World Energy (2008), currently global oil reserves stand at 1.2 trillion barrels, whereas current annual production arises to 30 billion barrels. Consequently, current reserves may only cover current global production for about 40 years.

Moreover it has become widely accepted that the combustion of petroleum carbon is the major contributor to the observed increase in atmospheric carbon dioxide, with concomitant global warming effects. To ensure that economic prosperity is not hampered in the 21st century, it is thus paramount that alternative energy sources that are preferably renewable and carbon-free or of low-carbon are developed in time to mitigate the twin crises of Peak Oil and climate change (Tsoskounoglou *et al*., 2008). This convergence of market pressure (supply instability and high oil prices) and concern for the environment (the greenhouse effect) has driven a headlong rush to biofuels.

Biofuels are liquid or gaseous fuels for the transport sector that are predominantly produced from biomass. They are generally considered as offering many priorities, including sustainability, reduction of greenhouse gas emissions, regional development, social structure and agriculture, as well as security of supply (IEA, 2006). There are a variety of biofuels potentially available (bio-oil, biomethanol, biodiesel, bioethanol), but the main biofuels being considered globally are biodiesel and bioethanol. Biodiesel is the fuel that can be produced from straight vegetable oils, edible and non-edible, recycled

waste vegetable oils, and animal fat, while bioethanol can be produced from sucrose containing feedstocks, starchy materials and lignocellulosic biomass (Balat *et al.*, 2008). In recent years, lignocellulosic biomass, such as agricultural and forestry residues, waste paper, and industrial wastes, has been recognized as an ideally inexpensive and abundantly available source of sugar for fermentation into ethanol, a sustainable transportation fuel. Lignocellulose is composed of sugars polymerized to cellulose and hemicellulose and the complex polyphenolic structure lignin. The composition of these materials varies but the major component is cellulose, followed by hemicellulose and lignin respectively.

There are three major steps in the process of converting lignocellulosic materials into ethanol, i.e. thermochemical pretreatment, hydrolysis and subsequent fermentation of the released sugars by specialized organisms (Gray *et al*, 2006). Pretreatment is required to alter the biomass macroscopic and microscopic size and structure, as well as its submicroscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields (Mosier *et al.*, 2005).

Since lignocellulosic materials contain polysaccharides such as cellulose and hemicellulose, that are not readily available for bioconversion, they have to be hydrolysed by means of acids or enzymes to monosaccharides that can then be fermented to ethanol by microorganisms. Although acid-based technologies are more established, enzymatic saccharification is more promising as it offers numerous advantages and there is potential to improve the technology on an industrial scale, making bioethanol competitive with other fuels (Martin *et al.*, 2006). The advantages of enzymatic hydrolysis include better yields, lower utility cost as it is conducted at mild conditions (pH 4.8 and temperature 50℃), and the absence of a corrosion problem. Furthermore, enzyme manufacturers have recently reduced costs substantially using modern biotechnology (Balat *et al.*, 2008).

Currently uneconomically high enzyme loadings are required to achieve high saccharification yields. This has been attributed to non-productive binding of cellulase and hemicellulases with lignin and other portions of the lignocellulose and inhibition by carbohydrate oligomers, the released sugars and their degradation products (Boussaid and Saddler 1999; Palonen *et al*., 2004; Xiao *et al*., 2004; García-Aparicio *et al*., 2006; Kumar and Wyman, 2008). It has, however, been shown that optimizing the composition of the saccharifying enzyme mixture by supplementation with accessory enzymes such as xylanases, ferulic acid esterases and laccases, the concentration of enzyme needed can be reduced, thus reducing the cost and rendering enzymatic saccharification economically feasible. Although the combination of enzymes influences hydrolysis, it is apparent that the efficacy of enzymatic complexes is inextricably linked to the structural characteristics of the substrate, e.g. cellulose crystallinity, degree of cellulose polymerization, surface area, lignin content, and/or the modifications that occur as saccharification proceeds (Mansfield *et al*., 1999). It thus becomes paramount to optimize saccharolytic enzyme complexes for each lignocellulosic substrate.

The final stage of lignocellulose bioconversion to ethanol is the fermentation of sugars released during saccharification. Various microorganisms (bacteria, yeast or fungi) ferment carbohydrates to ethanol under anaerobic conditions. These microbes acquire energy (in the form of adenosine triphosphate) during the fermentation and are therefore dependent upon ethanol production for growth and long-term survival. For lignocellulosic ethanol to be economically viable, microorganisms that can ferment both hexose (glucose, mannose and galactose) and pentose (xylose and arabinose) sugars will be essential. Currently, there is no naturally occurring microorganism that can ferment all these sugars, but this hurdle could be overcome by appropriate metabolic engineering.

The ultimate process would, however, be a one-step “consolidated” bioprocessing (CBP) of lignocellulose to ethanol, where the four biologically mediated transformations, i.e. production of saccharolytic enzymes (cellulases and hemicellulases); hydrolysis of carbohydrate components present in biomass to sugars; fermentation of hexose sugars; and fermentation of pentose sugars (Lynd *et al*., 2005), would be mediated by a single

microorganism or microbial consortium without added saccharolytic enzymes (van Zyl *et al.*, 2007). Microorganisms with the properties required for CBP are not currently available, but efforts are underway for their development. One strategy being applied involves engineering non-saccharolytic organisms that exhibit high product yields and titers so that they express a heterologous saccharolytic system enabling lignocellulose utilization. The yeast *Saccharomyces cerevisiae* is an attractive host organism for this strategy given that it is a proven ethanol-producer, exhibits tolerance to inhibitors commonly found in hydrolyzates resulting from biomass pretreatment, enjoys GRAS (Generally Regarded As Safe) status, and has well-established tools for genetic manipulation (van Zyl *et al.*, 2007).

1.2. Aims and Objectives

The broad aim of this study was to optimize the conversion of sugarcane bagasse to bioethanol by improving the application of lignocellulolytic enzymes and recombinant yeast strains.

1.2.1. Specific Objectives

The specific objectives of the research were to:

- Investigate the efficacy of different steam explosion pretreatment conditions on improving enzymatic digestibility of sugarcane bagasse.

- Screen various enzyme cocktails for those with the best hemicellulase and cellulase activity against steam explosion pretreated sugarcane bagasse.

- Investigate synergism between cellulases, hemicellulases and other accessory enzymes in hydrolysing steam sugarcane bagasse.

- Screen various recombinant *S. cerevisiae* yeast strains for their ability to hydrolyse and ferment sugarcane bagasse cellulose and hemicellulose to bioethanol.

- Investigate synergism between the screened enzyme cocktails and yeast strains for optimum bagasse hydrolysis and fermentation in a Simultaneous Saccharification and Fermentation (SSF) process.

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Chapter 2: Literature review

2.1. Fuel properties of bioethanol

Bioethanol (ethyl alcohol, grain alcohol, C_2H_5OH or EtOH) is by far the most common renewable fuel with an annual global production of about 17,3 million gallons in the year 2008 (RFA, 2009). It is a liquid, which is advantageous in terms of storage, delivery and infrastructural compatability. Bioethanol is an oxygenated fuel (35% oxygen), thus particulate and NO_x emissions from combustion in compression–ignition engines are reduced (Tables 1 and 2).

Table 1: Properties of Ethanol, Gasoline and Diesel (US Department of Energy)

Property	Ethanol	Gasoline	Diesel
Chemical Properties			
Formula	C_2H_5OH	C4 to C12	C3 to C25
Molecular weight	46.07	100–105	≈200
% carbon (by weight)	52.2	85–88	84–87
% hydrogen (by weight)	13.1	12–15	33–16
% oxygen (by weight)	34.7	0	0
C/H ratio (by weight)	4	5.6-7.4	7
Stoichiometric air-to-fuel ratio	9	14.2- 15.1	14.5- 15.1
Physical Properties			
specific gravity	0.796	0.72–0.78	0.81–0.89
Liquid density (lb/gd)	6.61	6.0–6.5	6.7–7.4
Vapor pressure at 100 °F (psi)	2.3	8–15	0.2
Boiling point (°F)	172	80–437	370–650
Solubility in water (ppm)	infinite	240	none
Thermal Properties			
Lower heating value (Btu/gal)	76,000	118,000	131,000
Higher heating value (Btu/gal)	84,400	122,000	139,000
Heat of vaporization (Btu/gal)	2378	900	700
Research octane rating	108	90–100	--
Motor octane rating	92	81–90	--
Flammability limits (% vol. in air)	4.3-19	1.4-7.6	1–6
Specific heat (Btdlb-°F)	0.57	0.48	0.43
Autoignition temperature (°F)	793	495	≈600
Flash point, closed cup, °F	55	-45	165

Other properties of bioethanol include a higher octane number (108), broader flammability limits, higher flame speeds and higher heats of vaporization (Table 1). These properties allow for a higher compression ratio and shorter burn time, which lead to theoretical efficiency advantages over gasoline in an internal combustion (IC)

engine (Balat and Balat, 2009). Octane rating is a measure of auto ignition resistance also referred to as "knock resistance." The right amount of octane ensures that combustion occurs at the right time, delivers the most efficient power and prevents cylinder knocking.

Table 2: Percent change in emissions for E85 vs. Gasoline (Yanowitz and McCormick, 2009)

Emission	Comparison	Average Change (%)	95% Confidence Range (%)		Number Tested	P Value For Paired t test
Total Hydrocarbons	E85 vs. gasoline in same FFV	-8	-19	4	89	0.20
	E85 vs. gasoline in similar non FFV	-18	-28	-7	71	0
Nonmethane Organic Gas	E85 vs. gasoline in same FFV	12	-56	182	6	0.43
	E85 vs. gasoline in similar non FFV	-43	-43	-43	1	NA
Nonmethane Hydrocarbon	E85 vs. gasoline in same FFV	-10	-17	-3	72	0.03
	E85 vs. gasoline in similar non FFV	-27	-37	-16	72	0
Benzene	E85 vs. gasoline in same FFV	-70	-82	-50	6	0.16
	E85 vs. gasoline in similar non FFV	-86	-86	-86	1	NA
1,3-Butadiene	E85 vs. gasoline in same FFV	-62	-83	-13	6	0.01
	E85 vs. gasoline in similar non FFV	-91	-91	-91	1	NA
NOx	E85 vs. gasoline in same FFV	-18	-27	-9	93	0
	E85 vs. gasoline in similar non FFV	-54	-60	-46	73	0
Particulate Matter	E85 vs. gasoline in same FFV	-34	-98	-2395	3	0
	E85 vs. gasoline in similar non FFV					
CO	E85 vs. gasoline in same FFV	-20	-39	4	93	0
	E85 vs. gasoline in similar FFV	-18	-27	8	73	0
Formaldehyde	E85 vs. gasoline in same FFV	63	51	75	92	0
	E85 vs. gasoline in similar non FFV	56	39	76	72	0
Acetaldehyde	E85 vs. gasoline in same FFV	1786	1424	2233	92	0
	E85 vs. gasoline in similar non FFV	2437	2130	2786	72	0
Methane	E85 vs. gasoline in same FFV	92	72	114	86	0
	E85 vs. gasoline in similar non FFV	91	75	108	71	0

Bioethanol can be used as a transportation fuel in several formulae: low-level blends (e.g. ≤ 22% ethanol in gasoline), high-level blends (e.g. ≥ 85% ethanol in gasoline), neat (containing no gasoline but usually containing water in amounts ≤ 20%), and as ethyl tert-butyl ether (ETBE) (Lynd, 1996). It is however most commonly blended with gasoline at a ratio of 10% ethanol to 90% gasoline, known as E10.

2.1.1. Feedstocks for bioethanol production

Balat *et al*. (2008) classified different feedstocks that can be used for the production of bioethanol, including sucrose containing feedstocks (e.g. sugarcane and sweet sorghum), starchy materials (e.g. wheat and corn) and lignocellulosic biomass (e.g. bagasse and straw). Production of ethanol from lignocellulosic biomass has the advantage of an inexpensive, abundant and diverse raw material that has limited market opportunities at present. Moreover, lignocellulosic ethanol does not affect food security and may also assist in alleviating problems with disposal of lignocellulosic material.

2.1.1.1. Lignocellulose - a valuable resource

Lignocellulosic biomass includes agricultural and forestry waste, municipal solid wastes, waste from the pulp and paper industry, wood and herbaceous energy crops. Lignocellulosics are mainly composed of cellulose (insoluble fibers of β-1,4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans), and lignin (a complex polyphenolic structure). The composition of these materials varies (Table 3), but the major component is cellulose (35–50%), followed by hemicellulose (20–35%) and lignin (10–25%), respectively (Saha, 2003).

Apart from holocellulose and lignin, lignocellulosics are also composed of extractive (soluble in water or organic solvent) and non-extractive non-cell wall materials (NCWM). The non-extractives are mainly inorganic ash components such as silica and alkali salts, but also includes pectin, proteins, and starch. The extractives often have protective biological and anti-microbial activities (Cowan, 1999).

Table 3: Composition of different lignocellulosic material

Biomass	Glucan	Xylan	Mannan	Galactan	Arabinan	Lignin	Reference
HARDWOODS							
Black poplar	43.5	15.5	2.5	2.3	1.5	26.2	Negro *et al*., 2003
Maple	44.9	17.3	2.9	N.D	2.8	28.0	Wyman, 1996
Walnut	46.2	16.5	2.6	N.D	1.8	21.9	Wyman, 1996
Salix	41.5	15.0	3.0	2.1	1.8	25.2	Sassner *et al*., 2006
Birch	38.2	18.5	1.2	N.D	N.D	22.8	Hayn *et al*., 1993
Alamo	49.9	17.4	4.7	1.2	1.8	18.1	Wyman, 1996
SOFTWOODS							
Pine	46.4	7.8	10.6	N.D	2.2	29.4	Wyman, 1996
Fir	49.9	5.3	12.3	2.3	1.7	28.7	Soderstrom *et al*, 2003
OTHERS							
Olive tree branch	25.0	9.8	0.7	1.4	2.1	18.8	Cara *et al*., 2007
Maize residues (bagasse)	36.8	22.2	N.D	2.9	5.5	23.1	Ohgren *et al*., 2005
Wheat straw	30.2	18.7	N.D	0.8	2.8	17.0	Ballesteros *et al*., 2006
Barley straw	33.1	20.2	N.D	0.9	3.8	16.1	Garcia-Aparicio *et al*., 2006

N.D-Not detected

2.1.1.2. Sugarcane bagasse

Throughout the work presented in this thesis, the lignocellulosic material, sugarcane bagasse, was hydrolysed and fermented. Sugarcane bagasse is the fibrous residue obtained after extracting the juice from sugarcane (*Saccharum officinarum*) in the sugar production process and it averages 30% by weight of the crushed sugarcane (Mbohwa and Fukuda, 2003). Most of the bagasse produced in the sugar industry is used as a fuel for generating the energy required by the sugar mills. However, with improvements in the thermal efficiency of combustion units, the energetic demands of sugar factories could be satisfied with reduced amounts of bagasse. Therefore, a surplus of bagasse would become available for alternative uses, including ethanol production.

Bagasse is an interesting raw material for industrial bioconversion processes since it is cheap, abundant and rich in carbohydrates (including approximately 40% of the dry matter content as cellulose and 25% as hemicelluloses). Moreover, logistic problems are minimal, as it is available in high amounts at sugar mill sites and its utilisation

helps to solve the disposal problem for sugar mills. Because of its low ash content (~2.4%), bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash contents, respectively, for application in bioconversion processes using microbial cultures (Pandey *et al*., 2000).

2.1.2. Structural features of lignocellulose

2.1.2.1. Cellulose

The molecule of cellulose is described as a β-1,4-D-glucan (Figure 1). It is an unbranched chain of β-D-glucopyranose units, in the chair conformation ($^4C_1$), that are joined by glycosidic linkages between the hemi-acetal hydroxyl (OH) group at C1 on one residue and the OH group at C4 on the next residue with the loss of the elements of water. Free rotation around the C1–O and O–C4′ bonds in the glycosidic linkages is limited by van der Waals repulsions between the glucose units. These are minimal when the torsion angles, ϕ and φ (Figure 1), for the C1–O and O–C4′ bonds are 98° and 143°, respectively. The predicted regular chain conformation of a cellulose molecule, determined by these angles, is helical with a repeat of 1.03nm (Stone, 2005).

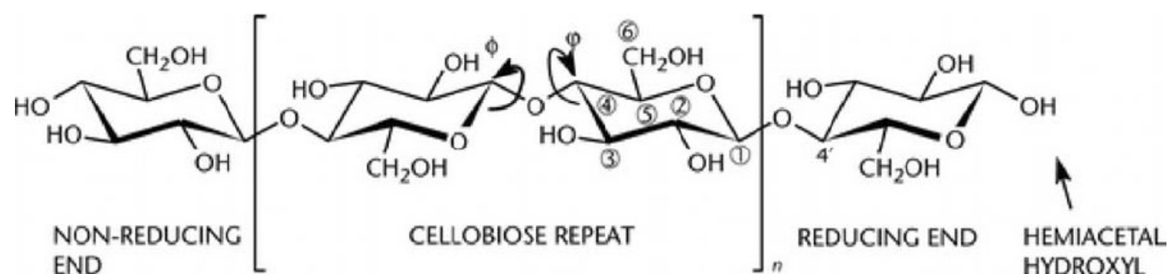

Figure 1: A β-1,4-D-glucan molecule terminated by a 'reducing end' bearing a free (unsubstituted) hemiacetal hydroxyl (on the right) and a 'nonreducing' end (on the left). The hydroxymethyl groups at C6 of alternate glucose residues are on opposite sides of the chain. Each carbon atom of the glucose ring carries an axial hydrogen atom (adapted from Stone, 2005).

Cellulose is thus an extensive, linear-chain polymer with numerous OH groups (3 per anhydroglucose (AGU) unit), in which all the hydroxyl (OH) and the hydroxymethyl (CH_2OH) substituents are equatorial. Every second AGU ring is rotated 180° in the plane to allow the preferred bond angles of the acetal oxygen bridges. Thus, the

molecule may be looked upon as a polymer of the disaccharide cellobiose defined by two adjacent structural units (Figure 1). The cellulose chain consists at one end of a D-glucose unit with an original C4–OH group (the nonreducing end) while the other end is terminated with an original C1–OH group (the reducing end). The average degree of polymerization (DP_{av}) of the cellulose molecules depends on the source and treatment of the raw material, with plant fibres having DP values between 800 and 10000 (Klemm *et al*., 2005).

Overall, the cellulose molecule has a 'ribbon-like' conformation. The ribbon-like polymer chains are packed into sheets that are stabilized by hydrogen bonds within and between adjacent chains. In native cellulose (cellulose I allomorph) intrachain hydrogen bonding, occurs parallel to the chain axis and on successive chain units. Glucose units on adjacent chains in the sheets also hydrogen bond but no intersheet hydrogen bonding occurs. The sheets however, associate to form stacks through van der Waals interactions (Stone, 2005). The extensive interchain (2 per AGU) and intrachain (2~3 per AGU) hydrogen bonds hold chains firmly side-by-side producing straight, stable supramolecular fibers of great tensile strength.

The regular packing of the cellulose molecules in the microfibrils determines the chemical reactivity, physical properties, and biological functions of cellulose. Structural features of cellulose commonly considered to limit rates of enzymatic hydrolysis include crystallinity index, degree of polymerization, and accessible area. Sun *et al*. (2004a) extracted bagasse cellulose and comparatively studied six samples by both degradation methods, hydrolysis and thermal analysis, and non-degradation techniques, FT-IR and CP/MAS ^{13}C-NMR spectroscopy, and reported the highest degree of polymerization to be about 1400.

2.1.2.2. Hemicellulose

Unlike chemically homogeneous cellulose, hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Softwood hemicellulose consists of glucomannan, galactoglucomannan, arabinan, and a small amount of arabino-(4-*O*-methylglucurono)-xylan (Figure 2). Glucomannans are composed of β-(1,4)-linked D-mannose and β-(1,4)-linked D-

glucose residues, presented in a 3:1 ratio. The mannose units are randomly distributed in the chain and have attached various amounts of α-linked galactose end groups. In softwood galactoglucomannans, the α-D-galactopyranosyl units are linked as a single-unit side chain to both D-glucosyl and D-mannosyl units of the main chain by (1,6) bonds. Arabinogalactan consists of (1,3)-linked β-D-galactopyranosyl units, each of them bearing a substituent to the Cα position. The main chain contains (1,3)-linked β-D-galactopyranosyl or 3-*O*-β-arabinopyranosyl-α-L-arabinofuranosyl units (Dumitriu, 2005).

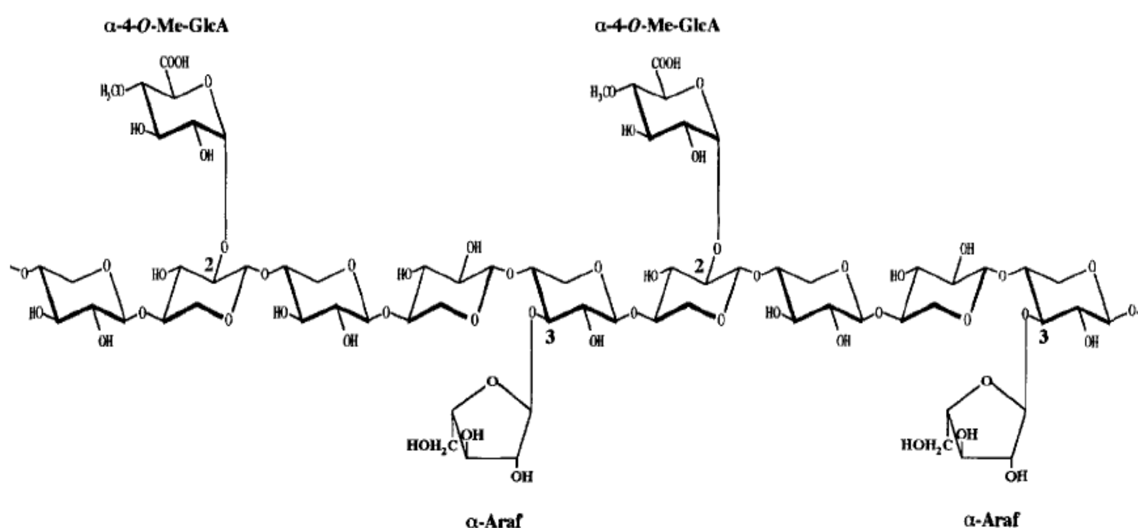

Figure 2: Composition of arabino-4-Omethylglucuronoxylan (softwood xylan). Numbers indicate the carbon atoms at which substitutions take place. α-Araf: α-Arabinofuranose; α-4-OMe-GlcA: α-4-Omethylglucuronic acid (adapted from Sunna and Antranikia, 1997).

Hardwood hemicelluloses by xylans and a small proportion of glucomannans are represented. Hardwood xylans are linear polymers, constituted of (1,4)-linked β-xylanopyranosyl units that constitute the main skeleton (Dumitriu, 2005). Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, and acetic, ferulic, and *p*-coumaric acids. The frequency and composition of branches are dependent on the source of xylan. The backbone consists of *O*-acetyl (Figure 3), α-L-arabinofuranosyl, α-1,2-linked glucuronic or 4-*O*-methylglucuronic acid substituents. About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid linked to *O*-2 and/or *O*-3 of xylose residues, and also

by oligomeric side chains containing arabinose, xylose, and sometimes galactose residues (Saha, 2003).

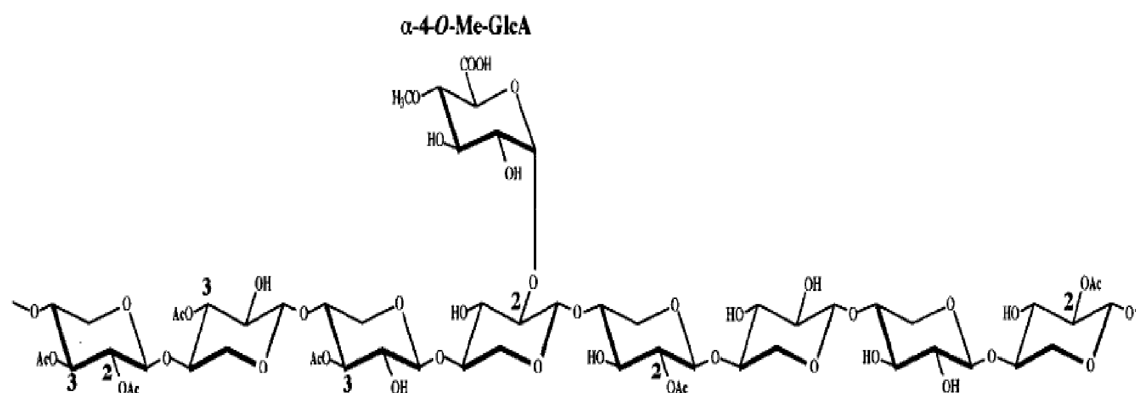

Figure 3: Composition of Oacetyl-4-Omethylglucuronoxylan (hardwood xylan). Numbers indicate the carbon atoms at which substitutions take place. Ac: Acetyl group; α-4-OMe-GlcA: α-4-Omethylglucuronic acid (adapted from Sunna and Antranikia, 1997).

In annual plants, the commonest hemicelluloses included in farm crop consist of (1,4)-β-D-xylopyranosyl units in the main chain, with side chains of one to several α-L-arabinofuranosyl, D-galactopyranosyl, and β-D-glucuronopyranosyl units. Also, L-rhamnosyl, L-galactosyl, and L-fucosyl units, and units of various methylated sugars are presented. These hemicelluloses could be partially acetylated, with the amount of acetyl groups varying up to 12% (Dumitriu, 2005). Sun *et al.* (2004b) used a combination of sugar analysis, nitrobenzene oxidation of bound lignin, molecular determination, Fourier transform infrared, and ^{1}H and ^{13}C NMR spectroscopy and thermal analysis to elucidate the physico-chemical properties, and structure of sugarcane bagasse hemicellulose. They reported that bagasse hemicellulose has a backbone of xylose residues, with β-(1,4) linkages, branched mainly through arabinofuranosyl and 4-*O*-methyl glucopyranosyl units (Figure 4). They also found that ferulic and *p*-coumaric acids were esterified to the hemicellulose.

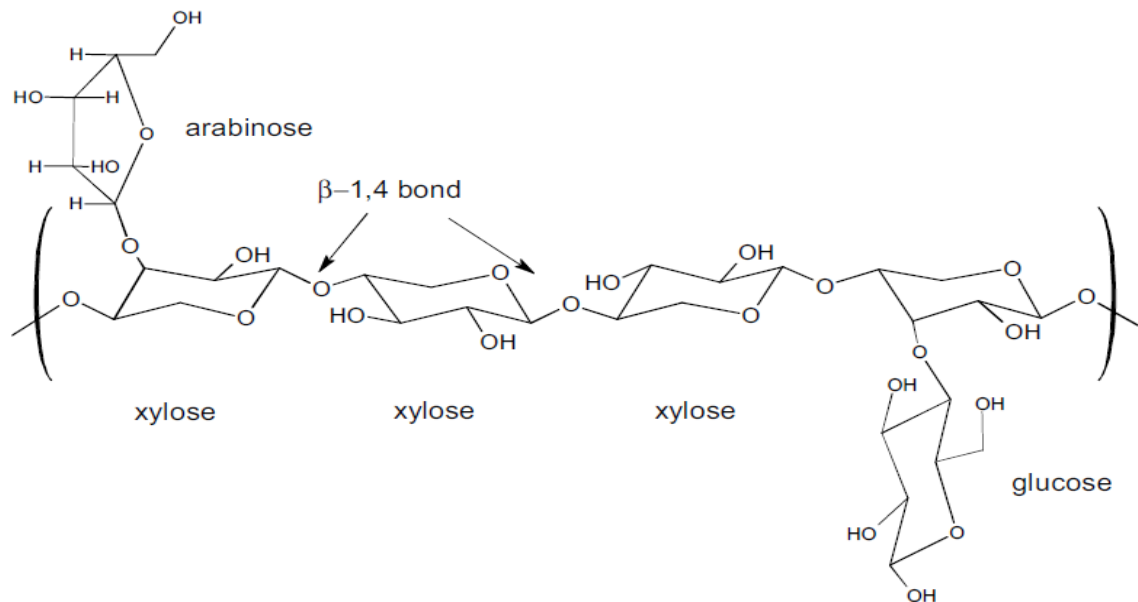

Figure 4: Simplified structure of sugarcane bagasse hemicellulose (adapted from Walford, 2008)

2.1.2.3. Lignin

Lignin is the third most abundant natural polymer present in nature after cellulose and hemicelluloses, respectively. Lignins are amorphous, highly complex, mainly aromatic, polymers of the phenylpropane units: *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 5). These monomers are linked by alkyl-alkyl, alkyl-aryl and aryl-aryl ether bonds (Walford, 2008). Lignins are generally classified into three major groups based on their monomeric units: guaiacyl lignin in softwoods (gymnosperms), guaiacyl–syringyl lignin in hardwoods (angiosperms), and guaiacyl–syringyl–*p*-hydroxyphenyl lignin in grasses (gramineae) (Higuchi, 2006).

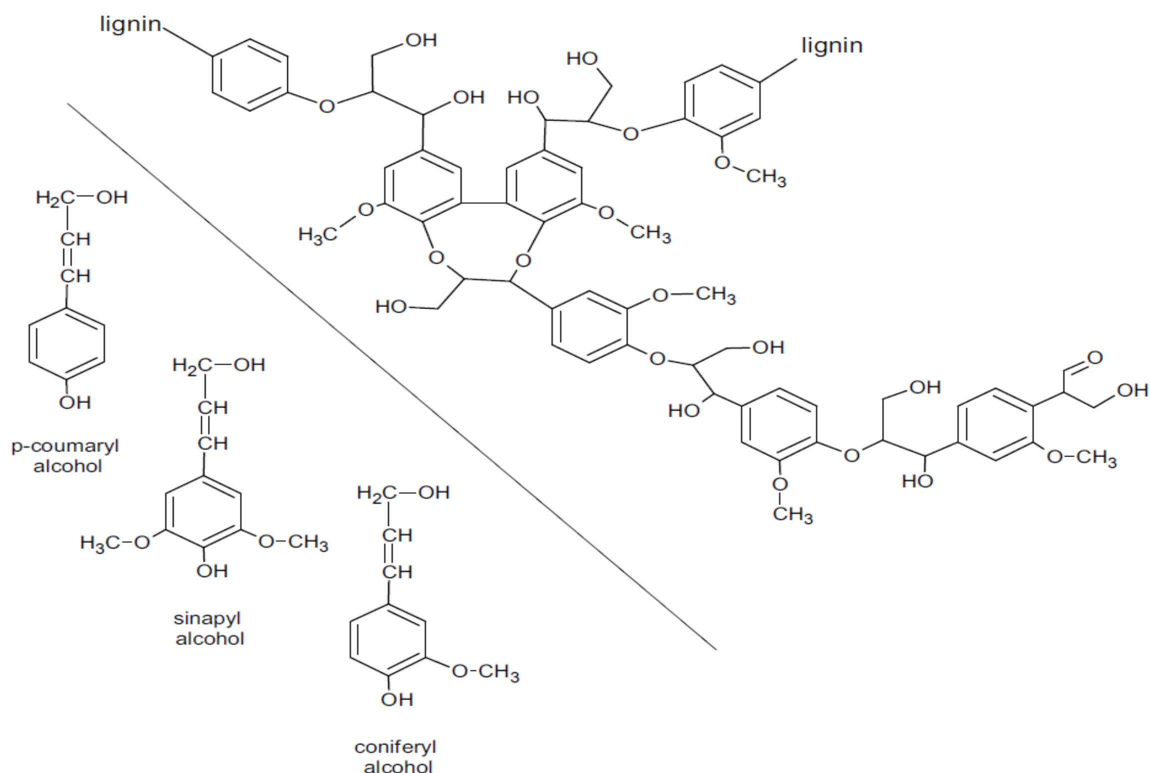

Figure 5: Phenyl-propane precursors (Left) and a model lignin structure (right) (adapted from Walford, 2008). The three different monomeric precursors, monolignols, that can be incorporated into lignin polymers. Hydroxyphenyl or H-type lignins are derived from *p*-coumaryl alcohol and are found in the cell walls of xylem cells in reaction wood, and in grasses. Guaiacyl or G –type lignins are derived from coniferyl alcohol and are found in gymnosperm and angiosperm lignins. Syringyl or S-type lignins incorporate sinapyl alcohol units and are found in angiosperm lignins only (Rogers and Campbell, 2004).

Softwood lignin is a three dimensional heterogeneous polymer in which the monomeric guaiacylpropane units (>90%) are connected by both ether and carbon–carbon linkages: several substructures are involved in the lignin macromolecules, of which guaiacylglycerol-β-aryl ether is the most abundant interphenylpropane linkage (40%–60%), followed by the substructures, phenylcoumaran (10%), dibenzodioxin (10%), diarylpropane (<5%), pinoresinol (<5%), biphenyl (5%–10%), diphenyl ether (5%), etc. The lignin of hardwoods is composed of guaiacylpropane and syringylpropane units connected by linkages similar to those found in conifer lignin; the ratio of the syringyl unit to the guaiacyl unit (1~3) is different among species. Grass lignin is composed of guaiacylpropane, syringylpropane, and *p*-hydroxyphenylpropane units also connected by similar linkages to those found in softwood lignin. *p*-Coumaric acid (5%–10% of lignin) is mostly esterified at the γ-position of the propyl side chains of the lignin. The lignin content of the woody stems

of softwoods, hardwoods, and grasses (bamboo, wheat, etc) ranges from 15% to 36% (Higuchi, 2006).

Eight bagasse lignin fractions were isolated and subjected to a comprehensive structural characterization by UV, FT-IR, and ^{1}H and ^{13}C NMR spectroscopies and thermal analysis by Sun *et al*. (2003). They concluded that bagasse lignins are typical grass lignins composed of syringyl, guaiacyl, and a small amount of *p*-hydroxyphenyl units (SGH-type lignins), with a small amount of esterified *p*-coumaric acid and mainly etherified ferulic acid. β-*O*-4 ether bonds were found to be the major linkages between the lignin structural units together with common carbon-carbon linkages such as β-β, 5-5', and β-5.

Sun *et al*. (2003) however reported heterogeneities in the chemical structure as four of the lignin fractions were rich in syringyl units and contained large amounts of noncondensed ether structures, whereas the other three fractions had a higher degree of condensation and were rich in guaiacyl lignins. This heterogeneity of sugarcane bagasse lignin had been previously reported by He and Terashima (1990), who analysed different morphological regions of sugarcane by microautoradiography and some degradative analyses. Both groups attributed the heterogeneities to differences in the structure of the lignin among fiber, vessel and parenchyma, with the lignin in the secondary wall of fiber and metaxylem being composed of syringyl (S)-, guaiacyl (G)- and *p*-hydroxyphenyl (H)-propane units with accompanying phenolic acid residues, and the proportion of these monolignols being S>G>H while the lignin in vessels of protoxylem contains more G and H units than S units.

2.2. Conversion of Lignocellulose to ethanol

There are three major steps in the process of converting lignocellulosic materials into ethanol (Figure 6): (i) thermochemical pretreatment - a preprocessing step that improves enzyme access to the cellulose; (ii) enzymatic saccharification - the use of cellulases and on some occasions hemicellulases; and (iii) fermentation of the released sugars by specialized organisms (Gray *et al.*, 2006).

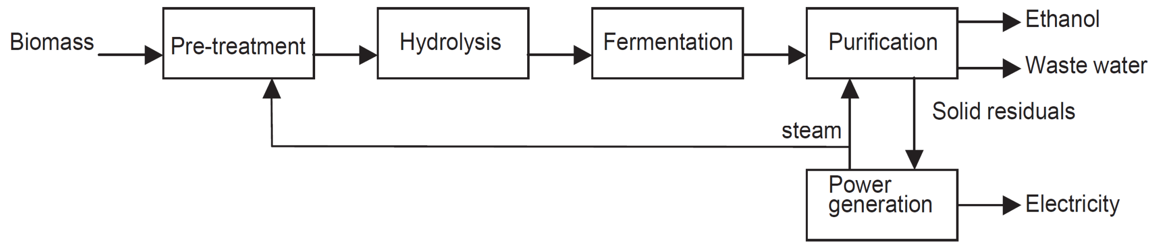

Figure 6: Generalised biomass to ethanol process (adapted from Hamelinck *et al*., 2005).

2.2.1. Pretreatment

The term "pretreatment" refers to a process step that alters the structure of lignocellulosic biomass from its native form, in which it is recalcitrant to cellulase enzyme systems, into a form in which enzymatic hydrolysis is effective (Figure 7). Pretreatment is required to alter the biomass macroscopic and microscopic size and structure, as well as its submicroscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. The objective is to break the lignin seal and disrupt the crystalline structure of cellulose (Mosier *et al*., 2005; Wyman *et al*., 2005; Taherzadeh and Karimi, 2008; Kumar *et al*., 2009; Alvira *et al*., 2010).

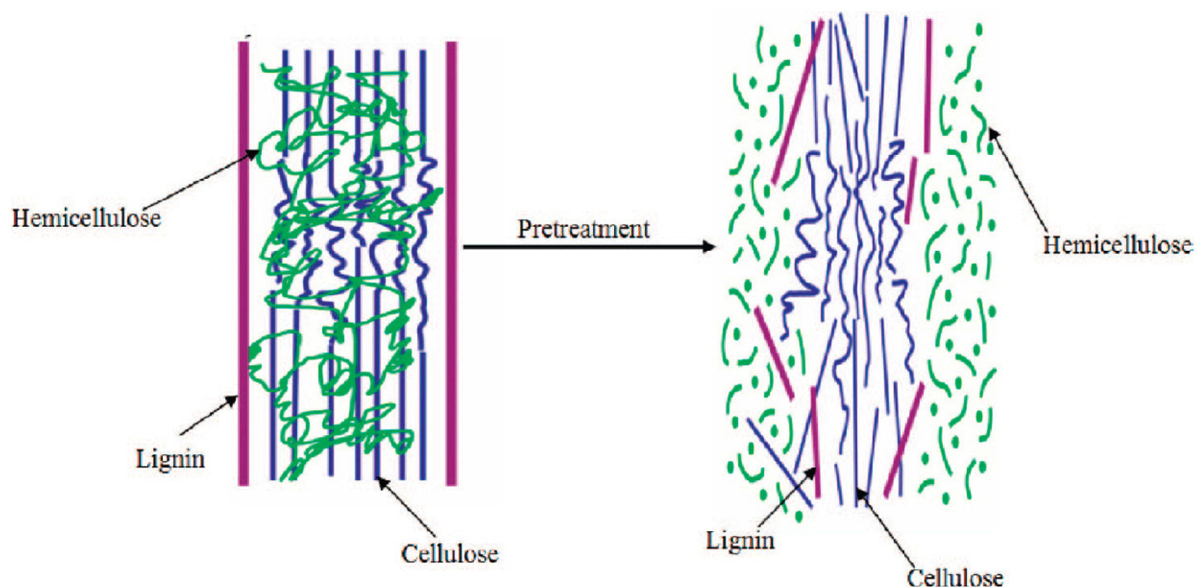

Figure 7: Schematic of goals of pretreatment on lignocellulosic material (adapted from Kumar *et al*., 2009).

To be effective, a pretreatment process must avoid the need for reducing the size of biomass particles, preserve the pentose (hemicellulose) fractions, yield a highly digestible pretreated solid, limit formation of degradation products that inhibit growth of fermentative microorganism, minimize energy demands, avoid production of solid-waste residues, be effective at low moisture content, operate in reasonable size and moderate cost reactors, result in high yields for multiple crops, sites ages, and harvesting times, and limit cost (Alvira *et al*., 2010).

Table 4: Summary of various processes used for the pretreatment of lignocellulosic biomass (modified from Kumar *et al*., 2009)

Pretreatment process	Principle and advantages	Limitations and disadvantages
Mechanical comminution	Reduces cellulose crystallinity	Power consumption usually higher than inherent biomass energy
Steam explosion	Causes hemicellulose degradation and lignin transformation; cost-effective	Destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms; not effective for softwoods without catalyst addition
AFEX	Increases accessible surface area; removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes	Not efficient for biomass with high lignin Content
CO_2 explosion	Increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds	Does not modify lignin or hemicelluloses
Ozonolysis	Reduces lignin content; does not produce toxic residues	Large amount of ozone required; expensive
Acid hydrolysis	Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	High cost; equipment corrosion; formation of toxic substances
Alkaline hydrolysis	Removes hemicelluloses and lignin; Increases accessible surface area	Long residence times required; irrecoverable salts formed and incorporated into biomass
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Pyrolysis	Produces gas and liquid products; pulsed electrical field; ambient conditions; disrupts plant cells; simple equipment	High temperature; ash production process needs more research
Biological	Degrades lignin and hemicelluloses; low energy requirements	Rate of hydrolysis is very low

Categories of pretreatment methods include physical (grinding and milling), physicochemical (hydrothermolysis, steam explosion (STEX), and wet oxidation (WO)), chemical (alkali, dilute acid, organic solvents and oxidizing agents), biological, electrical, or a combination of these (Mosier *et al*., 2005; Wyman *et al*., 2005; Taherzadeh and Karimi, 2008; Kumar *et al*., 2009; Alvira *et al*., 2010) (Table 4). There is no universal pretreatment process due to the diverse nature of different biomass feedstocks hence a pretreatment technology has to be selected based on the characteristics of the feedstock of interest. In the work presented in this thesis, steam explosion was used to pretreat sugarcane bagasse.

2.2.1.1. Steam explosion (STEX) pretreatment

Steam explosion is the most commonly used method for the pretreatment of lignocellulosic materials. It is an attractive option because of its limited use of chemicals, low energy consumption, short reaction time and, depending on the conditions used, high sugar recovery (Chandra *et al*., 2007). This pretreatment technology has been extensively investigated and found to be effective on sugarcane bagasse and several other lignocellulosics (Kaar *et al*., 1998; Martin *et al*., 2002a, 2006).

2.2.1.2. Mode of operation of steam explosion pretreatment

This pretreatment combines thermal, mechanical forces and chemical effects (Alvira *et al*., 2010). Uncatalysed STEX involves heating lignocellulosic material at high temperatures and pressures in a batch reactor, followed by mechanical disruption of the pretreated material by violent discharge into a collecting tank (explosion). It is typically initiated at a temperature of 160 - 260°C (corresponding pressure, 0.69 - 4.83MPa), then the biomass/steam mixture is held for a period of time (several seconds to a few minutes) to promote hemicellulose hydrolysis, and the process is terminated by the explosive decompression. Figures 8 and 9 show the flow sheet and a photo of the pilot plant at CIEMAT-Renewable Energies Department, (Madrid, Spain) were STEX pretreatment of sugarcane bagasse was carried out.

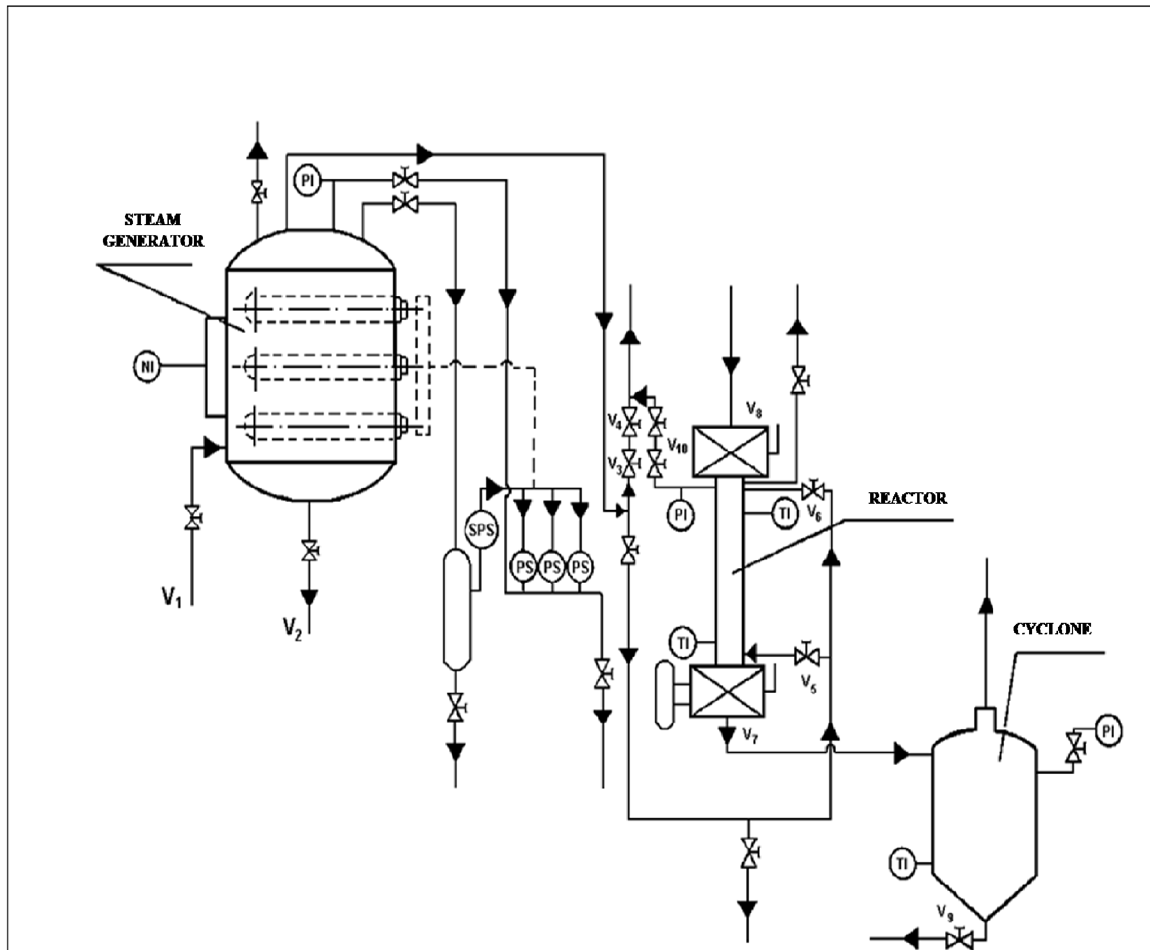

Figure 8: Flow-sheet of the pilot plant at CIEMAT-Renewable Energies Department, Madrid, Spain. This Steam Explosion pilot plant is made up of three units: (1) the steam generator or boiler, that supplies steam at 250 ºC; (2) a 2L reactor made of stainless steel 316, equipped with two valves, the input valve in the top of the reactor and the output valve in the bottom of the reactor; and (3) the cyclone (50 L) where the pretreated material is collected after the pretreatment (adapted from Ballesteros *et al.*, 2004).

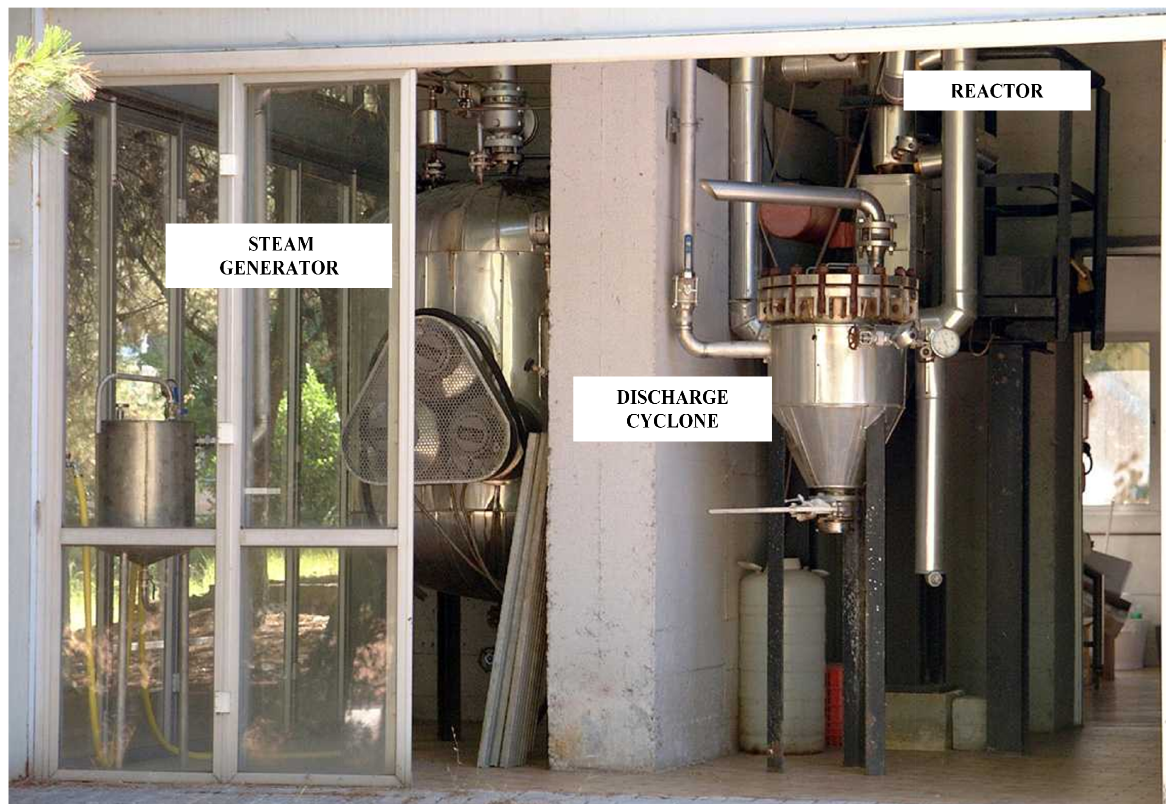

Figure 9: A picture of the Steam Explosion unit located at CIEMAT-Renewable Energies Department, Madrid, Spain. It's made up of three different parts as described in Figure 8.

Acetic acid released from acetylated hemicelluloses has been considered the main acid catalyst in autohydrolysis, though other acids such as formic and levulinic acids (Figure 10) are also produced and may impact on the chemical effect and overall pretreatment efficiency (Ramos, 2003). Moreover, water itself acts as an acid (hydroxic acid) at high temperatures. Depending on the severity of the pretreatment, the acids generated may further catalyze hydrolysis of the cellulose to glucose. Steam explosion pretreatment can be catalysed by the addition of H_2SO_4 (or SO_2) (Carrasco *et al*., 2010) or CO_2 (Ferreira-Leitao *et al*., 2010). The SO_2 and CO_2 are solubilised in water to H_2SO_3 and H_2CO_3, respectively, which enhance acid catalysis of the hemicellulose fraction (Ferreira-Leitao *et al*., 2010).

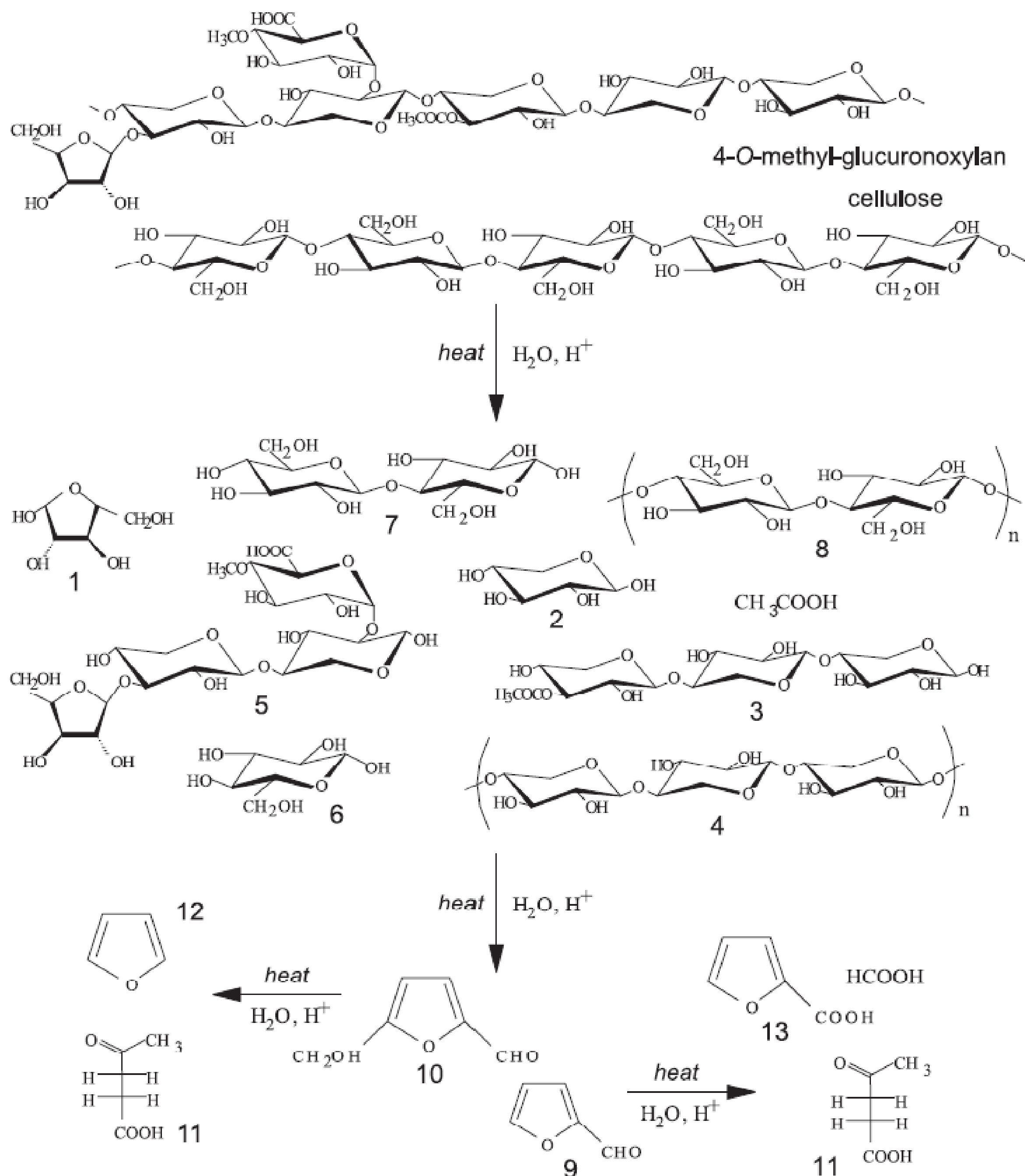

Figure 10: Hydrolysis of 4-O-methylglucuronoxylan and cellulose as a result of the steam explosion of hardwoods. (1) Arabinose; (2) xylose; (3) acetylated xylooligomers (DP of 3); (4) xylooligomers of higher molecular mass; (5) acidic, branched oligosaccharides; (6) glucose; (7) cellobiose; (8) cellooligomers; (9) furfural; (10) hydroxymethylfurfural; (11) levulinic acid; (12) furan; and (13) 2-furoic acid (pyromucic acid). Formation of formic and acetic acid is also indicated (adapted from Ramos, 2003)

The mechanical forces are generated by the rapid decompression termination which causes the expansion of the cellulose fibrils, causing physical disruption and rupturing of the glucose chains and resultantly the fibers are separated (Sun and Cheng 2002).

At high pressure the water is in liquid form and when the reactor is suddenly exposed to atmospheric pressure, the water is vaporised resulting in the mechanical disruption of the lignocellulose structure.

The high-pressure steam radically modifies the plant cell wall structure, yielding a dark brown material (slurry) from which partially hydrolysed hemicelluloses are easily recovered by water-washing, leaving a water-insoluble fraction (WIS) composed of cellulose, residual hemicelluloses and a chemically modified lignin that can be further extracted by mild alkali, dioxane, ethanol, or oxidative agents such as alkaline hydrogen peroxide and sodium chlorite (Ramos, 2003). Most of the lignin is not removed during the pretreatment, but is redistributed on the fiber surfaces as a result of melting and depolymerization/repolymerization reactions (Li *et al*., 2007).

Steam-explosion pretreatment is affected by residence time, temperature, chip size, moisture content and impregnating agent (catalyst) and the combined effect of both temperature (T) and time (t), which is described by the severity factor (R_o). Kaar *et al*. (1998) to identify the optimum conditions for uncatalysed steam explosion of sugarcane bagasse by using a range of operating temperatures (188-243°C) and residence times (0.5-44 min) in a 10 L batch reactor. They, however, found that the total sugar recovery, and thus the ethanol ”potential”, of the process was relatively insensitive to changes in reaction conditions due to the trade-off between xylose recovery and glucose recovery. They also observed that xylose conversion (17 to 85%) and mass recovery (78-99%) was better at low severities whilst glucose conversion (41 to 67%) preferred a higher severity with a temperature optimum of 216°C.

Catalyst addition can allow for decreased time and temperature requirements, effectively improve hydrolysis, decrease production of inhibitory compounds, and lead to complete removal of hemicellulose. Morjanoff and Gray (1987) obtained sugar production of 65.1 g sugar/100 g starting bagasse and reported the optimal conditions for STEX pretreatment of sugarcane bagasse to be as follows: 220°C; 30 s residence time; water-to-solids ratio, 2; and 1% H_2SO_4. Martin *et al*. (2002a) went on to investigate the effects of using different impregnating agents on STEX of bagasse.

After hydrolysis of the pretreated material by cellulolytic enzymes, H_2SO_4-impregnation gave the highest glucose yield (35.9 g/100 g) but the lowest total sugar yield (42.3 g/100 g) whilst the highest yields of xylose (16.2 g/100 g dry bagasse), arabinose (1.5 g/100 g), and total sugar (52.9 g/100 g) were obtained in the SO_2-impregnated bagasse. The low total sugar yield from the H_2SO_4-impregnated bagasse was attributed to the dehydration of xylose to furfural. Sendelius (2005) also investigated the effect of water, 2% SO_2 and 0.25 g H_2SO_4 per 100 g sugarcane bagasse as impregnating agents at various temperatures and residence times and concluded that SO_2-impregnation at 180ºC during 5 minutes, was the most prominent tested pretreatment condition. More recently, Carrasco *et al*. (2010) investigated conditions for SO_2 catalysed STEX for good pentose recovery and concluded that a temperature of 190 °C for 5 min using 2% (of water content) of SO_2 were the best conditions obtained in their study.

The use of CO_2 as an impregnating agent for sugarcane bagasse STEX was investigated by Ferreira-Leitao *et al*. (2010). The highest glucose yield (86.6% of theoretical) was obtained after pretreatment at 205°C for 15 min. Comparable pretreatment performance was obtained with CO_2 as compared to when SO_2 is used, although higher temperature and pressure were needed. Apart from the higher severity required, CO_2 has very attractive characteristics such as high availability, low cost, low toxicity, low corrosivity and low occupational risk (Ferreira-Leitao *et al*. (2010). The steam explosion results, here reviewed, indicate that steam explosion processing optimums are highly feedstock (sugarcane variety) dependent, since different carbohydrates compositions dictate different conditions.

Limitations of STEX include partial xylan degradation, incomplete disruption of the lignin-carbohydrate matrix and generation of compounds inhibitory to microbial growth, enzymatic hydrolysis and fermentation. Washing the pretreated biomass with water removes the inhibitors along with water-soluble hemicellulose. Inevitably, the water wash also removes soluble sugars, such as those generated by hemicellulose hydrolysis, consequently lowering saccharification yield.

2.2.1.3. Inhibitors produced during steam explosion pretreatment

Inhibitory compounds produced during pretreatment can be divided into four categories: sugar degradation products, lignin degradation products, compounds derived from lignocellulose structure (Figure 11) and heavy metal ions (Klinke *et al.*, 2004; Mussato and Roberto, 2004). The degradation products formed by STEX pretreatment of bagasse depend on the temperature, time and addition of catalysts. Generally, the major inhibitory compounds released include furans such as furfural and 5-hydroxymethylfurfural (5-HMF), weak acids such as acetic acid and formic acid, and phenolics such as 4-hydroxybenzaldehyde (4-HB), syringaldehyde and vanillin (Klinke *et al.*, 2004).

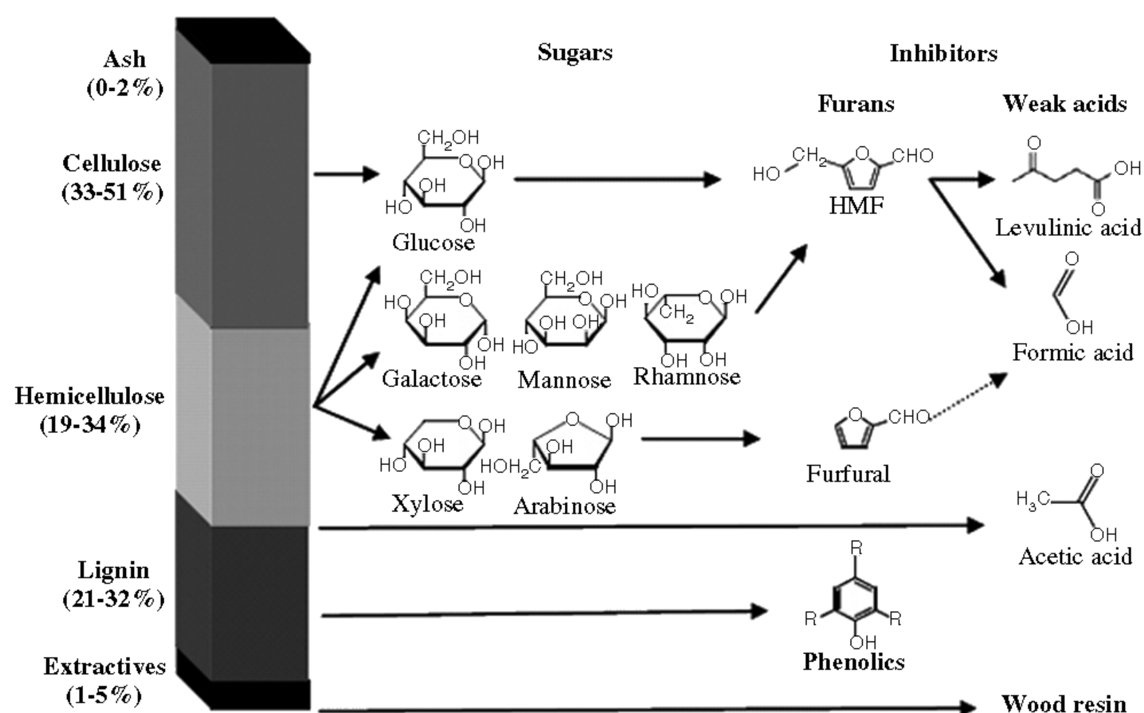

Figure 11: Average composition of lignocellulosic biomass and main derived hydrolysis products (adapted from Almeida *et al.*, 2007).

The furans (furfural and HMF) originate from the dehydration of pentose and hexose sugars, respectively. Acetic acid is formed by de-acetylation of hemicelluloses, while formic and levulinic acids are products of HMF breakdown (Figure 11). Formic acid can additionally be formed from furfural under acidic conditions at elevated temperatures (Almeida *et al.*, 2007). The phenolics syringaldehyde, coumaric acid,

vanillin, 4-hydroxybenzaldehyde, and ferulic acid are formed by solubilisation and hydrolytic or oxidative cleavage of lignin (Klinke *et al*., 2004).

2.2.1.4. Mechanism of inhibition and effects of lignocellulosic inhibitors

The biological activity of lignocellulosic inhibitors poses a problem for the biomass-to-ethanol process as they have adverse effects on the biological processes. These compounds affect yeast metabolism in several ways, including extension of the lag phase, and reduction of the growth rate, ethanol yield and specific ethanol productivity, as well as causing a reduction in viability. The known inhibition mechanisms of furans, weak acids and phenolics are represented in figure 12 and have been extensively reviewed (Palmqvist and Hahn-Hägerdal, 2000; Klinke *et al*., 2004; Almeida *et al*., 2007).

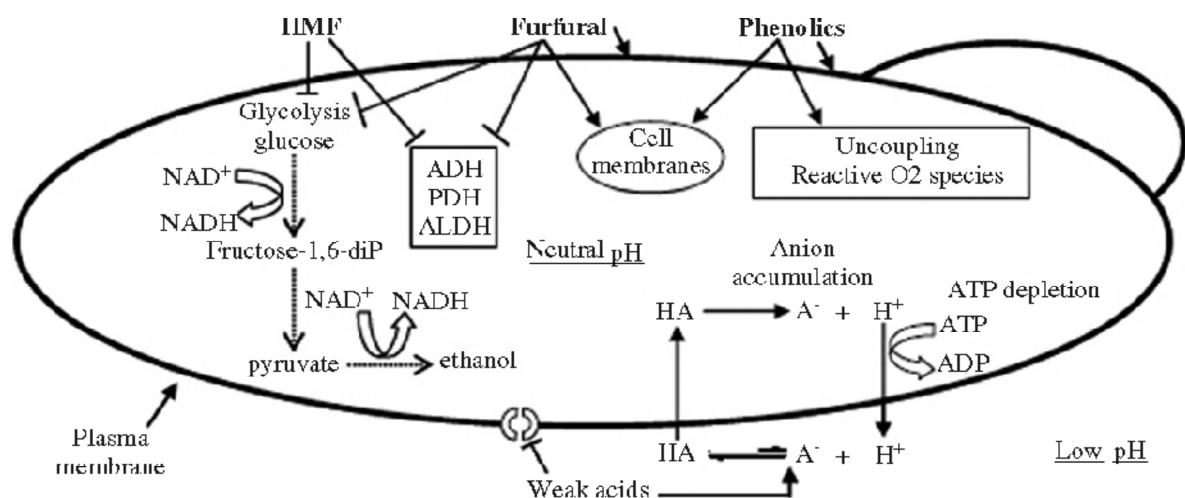

Figure 12: Schematic view of known inhibition mechanisms of furans, weak acids and phenolic compounds in *S. cerevisiae*. HMF: inhibition of ADH (alcohol dehydrogenase), (PDH) pyruvate dehydrogenase and ALDH (aldehyde dehydrogenase), inhibition of glycolysis (either enzyme and/or cofactors). Furfural: same as HMF, plus cell membrane damages. Weak acids: ATP depletion, toxic anion accumulation and inhibition of aromatic amino acids uptake. Phenolic compounds: uncoupling, generation of reactive O_2 species and membrane damage (adapted from Almeida *et al*., 2007).

2.2.1.5. Furans

The furaldehydes, HMF and furfural, decrease the volumetric ethanol yield and productivity, inhibit growth or give rise to a longer lag phase (Almeida *et al*., 2009). Several mechanisms may explain the inhibition effects of ethanol fermentation by furans (Figure 12). It has been shown that furfural and HMF directly inhibited alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and pyruvate

dehydrogenase (PDH) (Modig *et al*., 2002). Decreased activity of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and hexokinase in the presence of furfural has also been reported (Banerjee *et al*., 1981). The reduction of furans, to less toxic alcohols, by yeast may also result in NAD(P)H depletion (Palmqvist *et al*., 1999). Furthermore metabolic flux analyses have shown that furfural affects glycolytic and TCA fluxes, which are involved in energy metabolism (Sarvari *et al*., 2003). In *S. cerevisiae* furfural induces reactive oxygen species accumulation which results in damage to mitochondrial and vacuole membranes, the actin cytoskeleton and nuclear chromatin (Allen *et al*., 2010). Thus in a nutshell the effects of furans can be explained by a re-direction of yeast energy to fixing the damage caused by furans and by reduced intracellular ATP and NAD(P)H levels, either by enzymatic inhibition or consumption/regeneration of cofactors (Almeida *et al*., 2007).

2.2.1.6. Phenolics

As for furans, generally phenolics decrease biomass yield, growth rate and ethanol productivity more than ethanol yields. Inhibition mechanisms of phenolic compounds on *S. cerevisiae* and other eukaryotic microorganisms have not yet been completely elucidated, largely due to the heterogeneity of the group and the lack of accurate qualitative and quantitative analyses. Phenolic compounds may act on biological membranes (Figure 12), causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Heipieper *et al*., 1994). Weakly acidic phenolic compounds may destroy the electrochemical gradient by transporting protons back across the mitochondrial membranes (Terada, 1990).

2.2.1.7. Weak acids

The weak acids, acetic, levulinic and formic acid, commonly formed during hydrolysis inhibit yeast fermentation by reducing biomass formation and ethanol yields (Phowchinda *et al*., 1995). The growth-inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into the cytosol were it dissociates, due to the neutral intracellular pH, decreasing the cytosolic pH (Pampulha and Loureiro-Dias, 1989). The decrease in intracellular pH is compensated by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis (Figure 12) (Verduyn *et al*., 1992). Consequently, less ATP is

available for biomass formation. Weak acids have also been shown to inhibit yeast growth by reducing the uptake of aromatic amino acids from the medium, probably as a consequence of strong inhibition of Tat2p amino acid permease (Bauer *et al.*, 2003).

Carrasco *et al.* (2010) and Ferreira-Leitao *et al.* (2010) clearly showed that under all conditions autohydrolysis of sugarcane bagasse yielded significantly lower, if any, inhibitory compounds compared to catalysed STEX. Martin *et al.* (2002a) identified and compared the inhibitory compounds in hydrolysates of bagasse steam-exploded with H_2SO_4 and SO_2 impregnation, and by autohydrolysis. The yields of most of the fermentation-inhibiting compounds were found to be much higher for the hydrolysate obtained with H_2SO_4-impregnation probably due to dehydration of glucose and xylose to HMF and furfural. Sulphuric acid impregnation led to a threefold increase in the concentration of furfural and HMF, and a two-fold increase in the concentration of aliphatic acids (formic, acetic, and levulinic acids). However, the total content of phenolic compounds was not strongly affected by the different pretreatment methods. As proof of the inhibitory effect of these compounds, the hydrolysate of H_2SO_4-impregnated bagasse was fermented considerably poorer than the other two. In a separate study, Laser *et al.* (2002) observed that inhibition of fermentation rate of STEX pretreated bagasse increased as a function of furfural concentration. It was noted however that STEX yields lesser phenolic compounds than wet oxidation, another promising method for pretreatment (Martin *et al.*, 2007a).

2.2.1.8. Strategies to overcome inhibition

Inhibition of yeast fermentation is clearly a significant hurdle in the development of an economic lignocellulose to ethanol process. Strategies to overcome this inhibition include hydrolysate detoxification (Martin *et al.*, 2002b; Mussato and Roberto, 2004; Chandel *et al.*, 2007; Cheng *et al.*, 2008), improvement of *S. cerevisiae* strain tolerance via directed evolution or adaptative strategies (Martin *et al.*, 2007b), use of a careful fermentation control (process design strategy), and targeted metabolic engineering for improved yeast tolerance towards specific inhibitors (Almeida *et al.*, 2008a; Almeida *et al.*, 2008b; Heer *et al.*, 2009; Alriksson *et al.*, 2010; Guadalupe Medina *et al.*, 2010; Yang *et al.*, 2010). These methods are well reviewed by Almeida *et al.*, (2007).

Several detoxification methods have been developed to improve hydrolysis and fermentation of sugarcane bagasse hydrolysates. These include neutralisation, overliming with calcium hydroxide, activated charcoal, ion exchange resins and enzymatic detoxication using laccase (Mussato and Roberto, 2004; Chandel *et al*., 2007). STEX pretreated bagasse enzymatic hydrolysates were detoxified using the enzyme laccase and by overliming (Martin *et al*., 2002b). Laccase treatment removed almost 80% of the phenolic compounds and an almost two-fold increase of the specific productivity of the *S. cerevisiae* strain TMB 3001 was observed in the detoxified hydrolysates when compared to the undetoxified hydrolysates. Detoxification should however be avoided as it results in an additional process cost (von Sivers *et al*., 1994) and possible loss of fermentable sugars (Rivard *et al*., 1996).

In a different approach, Martin *et al*. (2007b) adaptated a xylose-utilizing genetically engineered strain of *S. cerevisiae* to STEX sugarcane bagasse hydrolysates through cultivation for 353 h on media with increasing concentrations of inhibitors, including phenolic compounds, furaldehydes and aliphatic acids. The ethanol yield after 24 h of fermentation of the bagasse hydrolysate with the adapted strain was double that attained with the non-adapted strain.

Naturally yeast can reduce HMF and furfural to the less toxic compounds, 2,5-bis-hydroxymethylfuran (HMF alcohol) and furfuryl alcohol, respectively. *S. cerevisiae* also has the natural ability to metabolize some phenolic compounds present in lignocellulose hydrolysates. In the process design strategy an improved fermentation process which favours the natural conversion capacity of the yeast is used. An example would be a fed-batch fermentation in which there is gradual addition of substrate at a rate matching the intrinsic bioconversion capacity of the yeast culture. In this fermentation mode, the organism acquires enhanced bioconversion capacity by short-term adaptation.

Lastly, the strain development strategy involves improvement of yeast tolerance to lignocellulosic hydrolysates by overexpressing homologous or heterologous genes encoding enzymes that confer resistance towards specific inhibitors. This has been

achieved for acetic acid (Guadalupe Medina *et al*., 2010; Yang *et al*., 2010), furfural (Almeida *et al*., 2008b; Heer *et al*., 2009), HMF (Almeida *et al*., 2008a; Almeida *et al*., 2008b; Alriksson *et al*., 2010; Yang *et al*., 2010), and the phenolic inhibitors coniferyl aldehyde (Alriksson *et al*., 2010) and vanillin (Yang *et al*., 2010). Due to the complexity of the lignocellulose hydrolysate it might be necessary to apply targeted metabolic engineering, strain adaptation and fermentation control in combination for the development of efficient lignocellulose-based ethanol processes.

2.2.2. Enzymatic hydrolysis of lignocellulosic material

Since lignocellulosic materials contain polysaccharides, such as cellulose and hemicelluloses, which are not readily available for bioconversion, they have to be hydrolysed by means of acids or enzymes to monosaccharides which can then be fermented to ethanol. Although technologies using acids are more established, enzymatic saccharification is more promising as it offers numerous advantages and there is potential to improve the technology on an industrial scale, making bioethanol competitive with other fuels (Martin *et al*., 2006). Advantages of enzymatic hydrolysis include better yields, lower utility cost as it is conducted at mild conditions (pH 4.8 and 45-50℃) and absence of a corrosion problem. Furthermore, enzyme manufacturers have recently reduced costs substantially using modern biotechnology (Balat *et al*., 2008).

Generally glycosidases (*O*-glycoside hydrolases, EC 3.2.1.x) catalyse the hydrolysis of glycosidic bonds in oligo- and polysaccharides (Davies and Henrissat, 1995). Cellulases and hemicellulases are the main glycosidases involved in the hydrolysis of the cellulose and hemicellulose fractions of lignocellulosic material and an updated list of their glycosyl hydrolase families (GHF) is maintained on the CAZy database. However, the low enzymatic accessibility of the native cellulose is a key problem for biomass-to-ethanol processes. As already discussed, cellulose in lignocellulosics is closely associated with hemicelluloses and lignin. The lignin is partly covalently associated with hemicelluloses, thus preventing the access of hydrolytic agents to cellulose. In addition, the crystalline structure of cellulose itself represents an extra obstacle to hydrolysis (Martin *et al*., 2007). Thus, lignocellulose hydrolysis requires synergistic action between several cellulases, xylanases, cinnamoyl esterases and/or

pectinolytic enzymes. Synergism defines a phenomenon where enzyme systems exhibit higher collective activity than the sum of the activities of the individual enzymes.

2.2.2.1. Cellulose hydrolysis

The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by three major types of enzymes (Figure 13): (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (cellodextrinases; EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases; EC 3.2.1.91), and (iii) β-glucosidases (β-glucoside glucohydrolases; EC 3.2.1.21). Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Lynd *et al.*, 2002). β-Glucosidases complete the hydrolysis process by catalyzing the hydrolysis of soluble cellodextrins and cellobiose to glucose (Balat *et al.*, 2008).

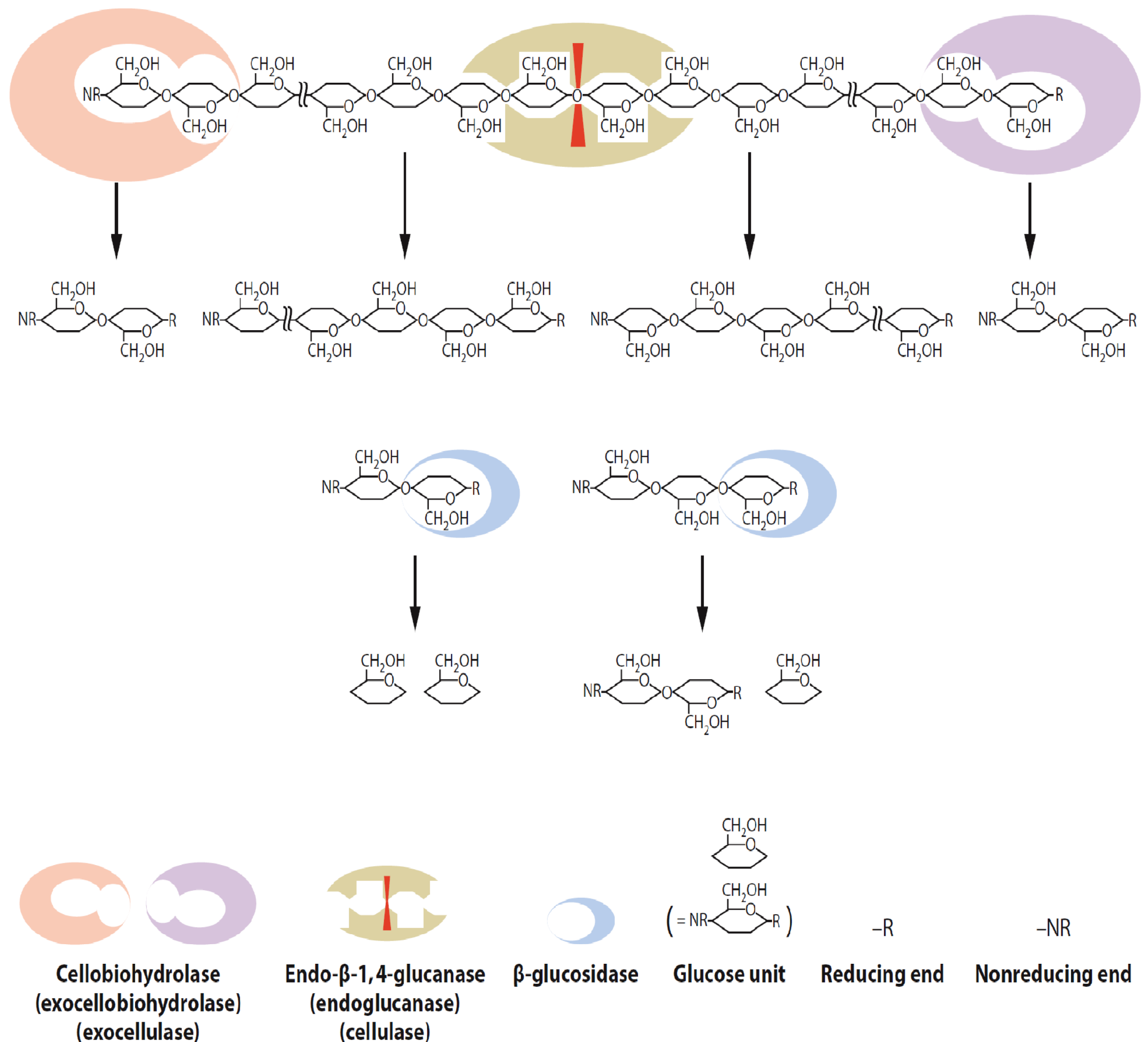

Figure 13: Schematic view of the biodegradation of cellulose. Cellobiohydrolases act on the nonreducing or reducing termini of cellulose fibers to processively release cellobiose. Endo-β-1,4-glucanases randomly cleave cellulose chains. β-glucosidases hydrolyze cellobiose or cello-oligomers to glucose from the nonreducing ends (adapted from Watanabe and Tokuda, 2010).

Cellulase systems exhibit a high degree of synergism. Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases; (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains; (iii) synergy between exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of the first

two enzymes; and (iv) intramolecular synergy between catalytic domains and carbohydrate-binding modules (CBMs) (Lynd *et al*., 2002).

2.2.2.2. Factors affecting cellulose hydrolysis

Enzymatic hydrolysis of lignocellulose is limited by various obstacles that reduce enzyme efficiency (Figure 14). These include end-product inhibition, presence of lignin and hemicellulose which shields cellulose fibrils making them inaccessible to cellulases, adsorption of enzymes to lignin and mechanical shear which may denature/ degrade enzymes (Jørgensen *et al*., 2007). Also, structural characteristics of the substrate, e.g.

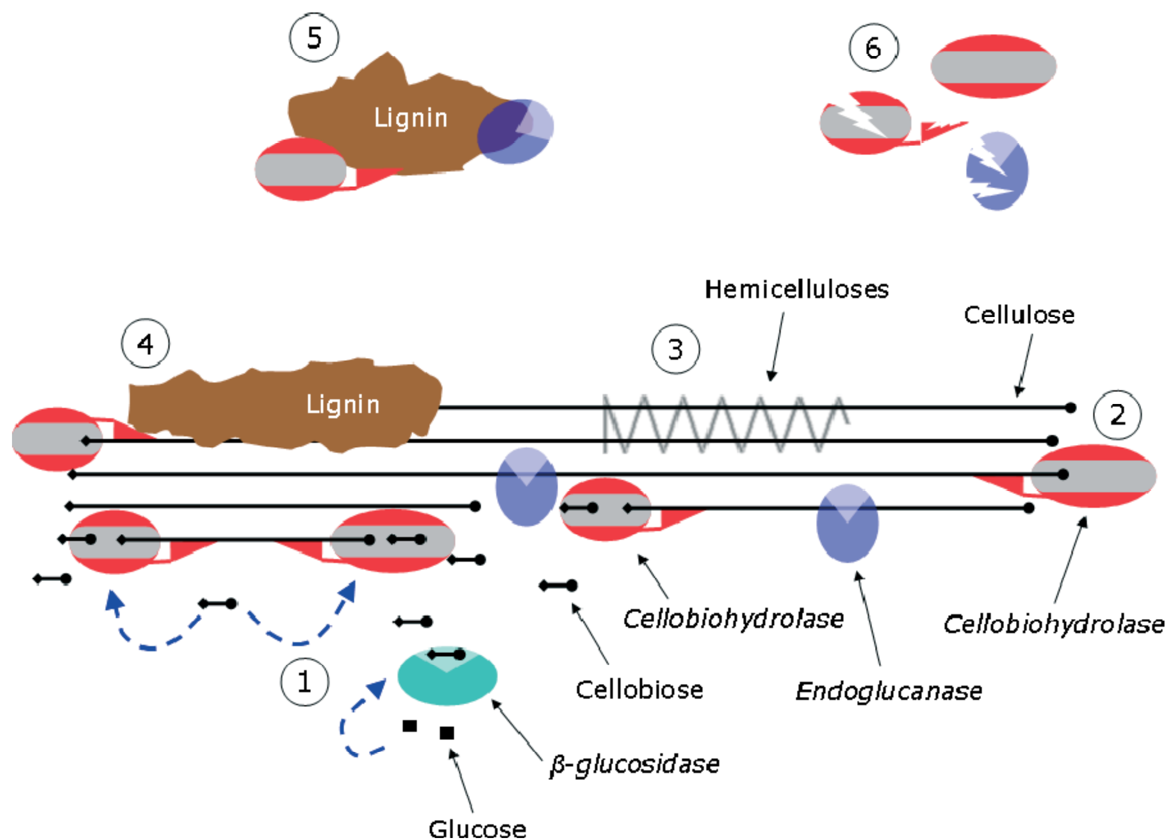

Figure 14: Simplistic overview of factors limiting efficient hydrolysis of cellulose (symbolised by the straight lines). 1: Product inhibition of β–glucosidases and cellobiohydrolases by glucose and cellobiose, respectively. 2: Unproductive binding of cellobiohydrolases onto a cellulose chain. Due to the processivity of cellobiohydrolases and their strong binding of the cellulose chain in their catalytic core, obstacles can make the enzymes halt and become unproductively bound. 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 lignin particles or surfaces. 6: Denaturation or loss of enzyme activity due to mechanical shear, proteolytic activity or low thermostability (adapted from Jørgensen *et al*., 2007).

cellulose crystallinity, degree of cellulose polymerization, surface area, lignin content, and/or the modifications that occur during pretreatment affect the final enzymatic saccharification (Mansfield *et al*., 1999; García-Aparicio *et al*., 2006). These obstacles are compounded when high substrate loadings are used.

2.2.2.3. Hemicellulose hydrolysis

The hemicellulytic system is more complex due to the heterologous nature of hemicellulose, which is composed of various sugar units, with attached side chains and side groups. Hemicellulases include enzymes that break down both β-1,4-xylan (xylanases; EC 3.2.1.8 and β-xylosidases; EC 3.2.1.37) and various side chains (α-l-arabinofuranosidases; EC 3.2.1.55, α-glucuronidases; EC 3.2.1.139, acetyl xylan esterases; EC 3.1.1.72, ferulic acid esterases; EC 3.1.1.73, α-galactosidases; EC 3.2.1.22, endo-1,4-β-D-mannanases; EC 3.2.1.78 and β-mannosidases; EC 3.2.1.25) (Jørgensen *et al*., 2003; Shallom and Shoham, 2003; Polizeli *et al*., 2005; Gray *et al*., 2006). Table 5 lists the enzymes involved in the hydrolysis of hemicellulose and their modes of action while their sites of attack on xylan are depicted in Figure 15.

Table 5: Enzymes involved in the hydrolysis of hemicellulose (adapted from Saha *et al*., 2003).

Enzyme	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-Arabinofuranosidase	Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolyzes feruloylester bonds in xylans
***p*-Coumaric acid esterase**	Hydrolyzes *p*-coumaryl ester bonds in xylans

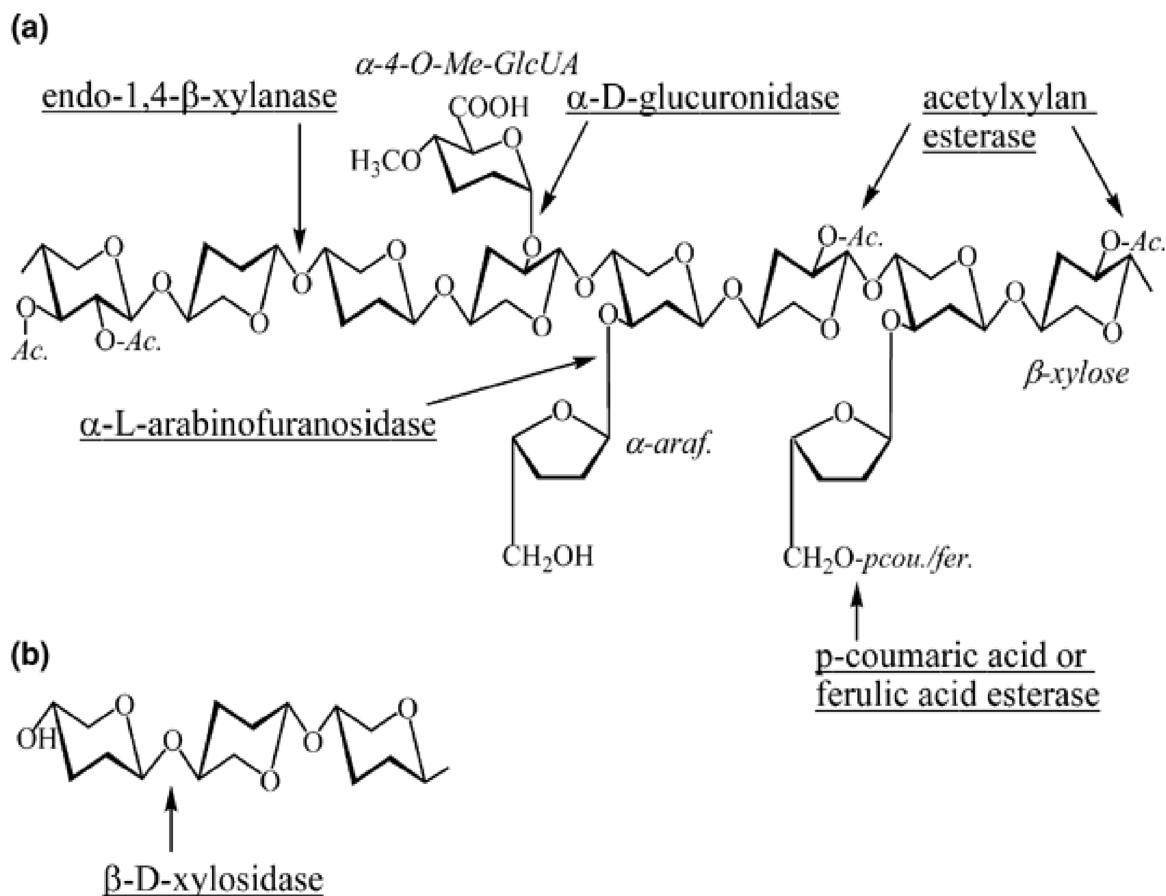

Figure 15: (a) Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4-β-linked xylose residues. Ac., Acetyl group; α-araf., α -arabinofuranose; α-4-O-Me-GlcUA, α-4-O-methylglucuronic acid; pcou., *p*-coumaric acid; fer., ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase (adapted from Collins *et al.*, 2005).

Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase) cleaves the glycosidic bonds in the xylan backbone, reducing the DP of the substrate (Figure 15) as β-D-xylopyranosyl oligomers are initially released, and mono-, di- and trisaccharides of β-D-xylopyranosyl at a later stage. The bonds for hydrolysis are not randomly selected, but depend on the nature of the substrate molecule, i.e. chain length, degree of branching and the presence of substituents. β-D-Xylosidases (1,4-β-D-xylan xylohydrolases) then hydrolyze xylooligosaccharides to D-xylose. Their highest affinity is for xylobiose as their affinity is inversely proportional to the DP of xylooligosaccharides (Polizeli *et al.*, 2005).

The modes of action of the enzymes involved in cleavage of side chains are described in Table 5. These enzymes are important as removal of the side chains affords greater accessibility for endoxylanase hydrolysis. Thus synergistic and cooperative effects among xylan-degrading enzymes are essential for enhanced susceptibility of heteropolymeric xylan to endoxylanases.

2.2.2.4. Lignin degradation

The extracellular enzymes involved in lignin degradation are laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase), lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), versatile peroxidase (VP) and H_2O_2-forming enzymes such as glyoxal oxidase and aryl alcohol oxidase, all secreted by various species of white-rot fungi in different combinations (Hatakka, 1994). The microbial lignin degradation process involves unspecific one-electron oxidation of the benzenic rings in the different lignin substructures by powerful extracellular hemoperoxidases (lignin peroxidases and manganese peroxidases), and in some fungi laccase, acting synergistically with peroxide-generating oxidases (Ruiz-Dueñas and Martínez, 2009). The oxidation generates radical species that undergo further reactions resulting in extensive degradation and fragmentation of the polymer.

2.2.2.5. Optimization of enzymatic saccharification

Despite intensive research, the requirement of uneconomically high enzyme loadings to achieve high saccharification yields coupled with long process times due to rapid decrease of the hydrolysis rate (Gregg and saddler, 1996; Sheehan and Himmel, 1999) still hamper large-scale utilization of lignocellulose for bioethanol production. This has been attributed to nonproductive binding of cellulase and hemicellulases with lignin and other portions of the lignocellulose and inhibition by carbohydrate oligomers, the released sugars and their degradation products (Boussaid and Saddler 1999; Palonen *et al*., 2004; Xiao *et al*., 2004; Kumar and Wyman, 2008). In addition, enzyme recycling is difficult as enzymes adsorb to residual lignocellulosic material (Lu *et al*., 2002). Thus, development of methods to increase enzyme effectiveness is critical in order to make lignocellulose hydrolysis for ethanol production economically feasible.

It is well documented that the conjugated action of cellulases, hemicellulases and accessory enzymes results in a higher final sugar production as compared to cellulases alone (Adsul *et al.*, 2005; Tabka *et al.*, 2006; Berlin *et al.*, 2007; Sorensen *et al.*, 2007; Prior and Day, 2008; Kumar and Wyman, 2009a). It is suggested that the so called ''accessory'' enzymes such as xylanase, FAE and pectinase stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers. Tabka *et al.* (2006) for example proved a synergistic effect between cellulase, FAE and xylanase under a critical enzymatic concentration (10 U/g of cellulases, 3 U/g of xylanase and 10 U/g of FAE) with the best results obtained with a combination of FAE and xylanase treatment. Feruloyl esterases are involved in the liberation of ferulic acid and other cinnamic acids from plant cell wall polysaccharides (Benoit *et al.*, 2008). Ferulic acid is known to crosslink plant cell wall polysaccharides to each other and to lignin (Burr and Fry, 2009), such that its removal could have led to the improved hydrolysis. However, lignocellulosics show significant quantitative and qualitative differences in their non-cellulosic polysaccharide components with further differences in composition introduced according to the pretreatment technology employed. It has thus been suggested that enzyme mixtures could be customized for particular feedstocks and pretreatments in order to optimize hydrolysis (Berlin *et al.*, 2005; Eggeman and Elander, 2005).

More recently, statistically designed experiments have been employed to construct optimized saccharolytic enzyme mixtures for various substrates (Berlin *et al.*, 2007; Kim *et al.*, 2008; Zhou *et al.*, 2009). This method is a powerful tool for optimization and analysis of the effects of each component of the saccharolytic mixture as well as interactions between components, and several authors have used this technique for optimization of bioprocesses (Wen and Chen, 2001; Zheng *et al.*, 2008). Prior and Day (2008) evaluated the hydrolysis of AFEX and NH_4OH pretreated bagasse with combinations of cellulase, β-glucosidase and hemicellulase. They performed two-way analysis of variance (ANOVA) to determine if any significant differences ($p<0.05$) occurred between factors. Their results showed that significant xylanase activity in enzyme cocktails appears to be required for improving hydrolysis of both glucan and xylan fractions of ammonia pretreated sugarcane bagasse. These results indicate that

the optimized cellulase mixture is an available and efficient paradigm for the hydrolysis of lignocellulosic substrate.

Apart from the composition of the saccharolytic enzyme mixture, optimisation of saccharification parameters, e.g. temperature, pH and substrate loading, is also paramount for optimal saccharification (García-Aparicio *et al*., 2004; Vásquez *et al*., 2007). Vásquez *et al*. (2007) optimized cellulose conversion of "cellulignin*",* the solid fraction from acid hydrolysis of sugarcane bagasse, using the response surface methodology with pH, enzyme loading, solid percentage, and temperature as factor variables. Using the "desirability" function, conditions that optimize both, conversion to glucose and glucose concentration were found to be as follows: 47°C, 10% substrate loading, and 25.9 FPU/g of pretreated "cellulignin".

It has been shown that addition of surfactants such as nonionic detergents [e.g. Tween-20, and polyethylene glycol (PEG)] and protein [e.g. Bovine serum albumin (BSA)] significantly increases the enzymatic conversion of cellulose into soluble sugars (Eriksson *et al*., 2002; Alkasrawi *et al*., 2003; Borjesson *et al*., 2007a; Kristensen *et al*., 2007; Kumar and Wyman, 2009b).

Three different explanations to the surfactant effect on cellulose hydrolysis have been postulated: (i) surfactants may increase enzyme stability and prevent denaturation of enzymes during hydrolysis; (ii) surfactants could affect the structure of the substrate and make it more accessible for enzymatic hydrolysis; (iii) and/or surfactants could positively affect enzyme–substrate interactions leading to more effective conversion of cellulose (Helle and Duff, 1993; Kristensen *et al*., 2007; Borjesson *et al*., 2007b). Studies on steam-pretreated spruce (SPS) propose that the dominating mechanism responsible is the influence of surfactants on cellulase interaction with lignin surfaces (Eriksson *et al*., 2002; Borjesson *et al*., 2007b). Surfactant adsorption to lignin is believed to prevent unproductive binding of enzymes to lignin, thereby producing higher yields and better recycling of enzymes. This is in accordance with other results showing less adsorption of enzymes to lignocellulose during hydrolysis in the presence of a surfactant (Park *et al*., 1992; Helle and Duff, 1993). Added protein such

as BSA is also believed to bind to lignin, preventing unproductive binding of cellulases (Kristensen *et al*., 2007).

2.3. Hydrolysis and fermentation strategies (Process configurations)

The final stage of lignocellulose bioconversion to ethanol is the fermentation of sugars released during saccharification. Various microorganisms (bacteria, yeast or fungi) ferment carbohydrates to ethanol under anaerobic conditions. These microbes acquire energy (in the form of adenosine triphosphate) through the fermentations and are therefore dependent upon ethanol production for growth and long-term survival. Among numerous microorganisms that have been exploited for ethanol production, *Saccharomyces cerevisiae* remains the prime species.

The main metabolic pathway involved in ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or EMP pathway), through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced (Figure 16). Since lignocellulose hydrolysates contain not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides, microorganisms that can also efficiently ferment these sugars are required for the successful industrial production of bioethanol.

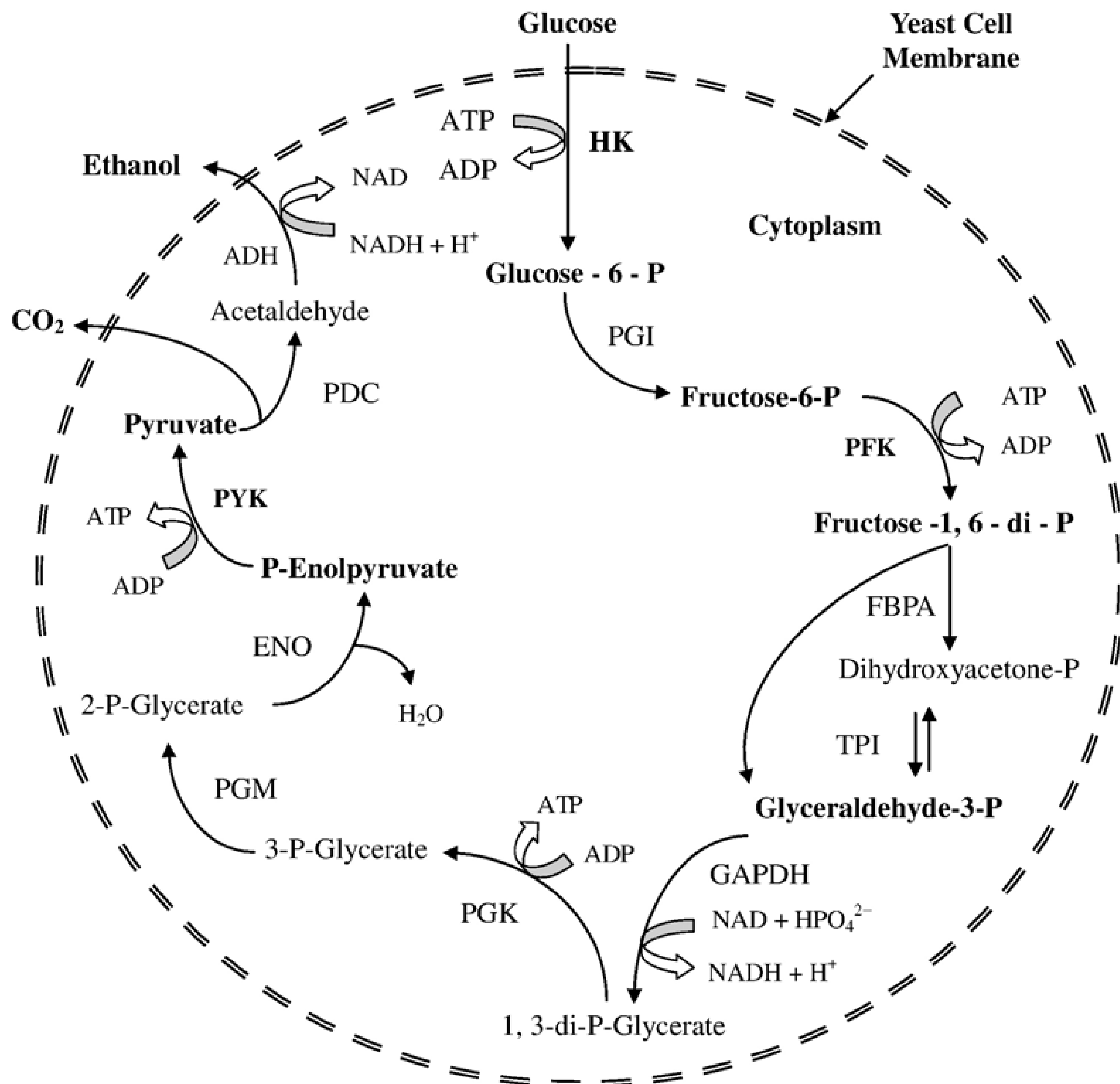

Figure 16: Metabolic pathway of ethanol fermentation in *S. cerevisiae*. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase (adapted from Bai *et al.*, 2007).

Four biologically mediated events occur in the course of producing ethanol from lignocellulosic material using enzymatic hydrolysis: cellulase production, cellulose hydrolysis, hexose fermentation and pentose fermentation. The process configurations that have been proposed for these biological steps are illustrated in Figure 17 and described below. These configurations differ in the degree to which the biological events are integrated.

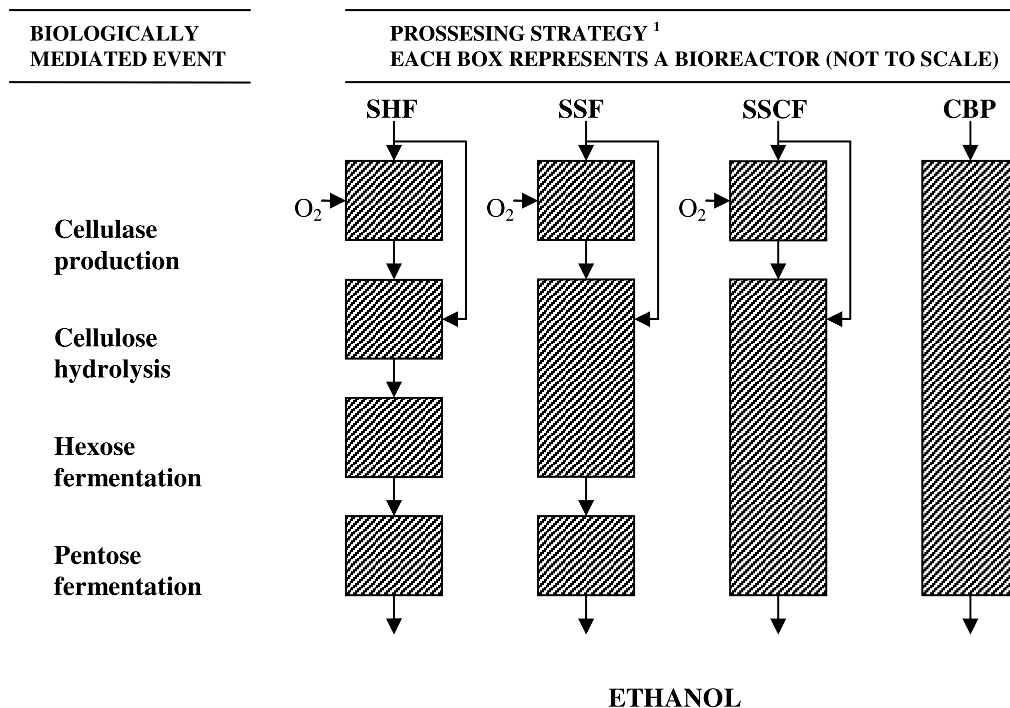

Figure 17: Consolidation of biologically mediated events in cellulosic ethanol production (adapted from Lynd, 1996).

2.3.1. Separate Hydrolysis and Fermentation (SHF)

In this configuration, pretreated lignocellulosic material is enzymatically hydrolysed and subsequently fermented in separate steps. The enzymatic hydrolysate first enters the glucose fermentation reactor. The mixture is then distilled to remove the bioethanol leaving the unconverted xylose behind. In a second reactor, xylose is fermented to bioethanol, and the bioethanol is again distilled. The major advantage of SHF is that hydrolysis and fermentation occur at optimum conditions. The optimum temperature for saccharolytic enzymes is usually between 45°C and 50°C depending on the producing organism, whilst the optimum temperature for most of the fermenting microorganisms is between 30°C and 37°C (Taherzadeh and Karimi, 2007).

The main disadvantage is that saccharolytic enzymes are inhibited by glucose, cellobiose, xylose and other oligosaccharides such that the rate of hydrolysis is progressively reduced as the endproducts accumulate. Although glucose acts as an inhibitor cellobiose is more inhibitory reducing cellulase activity by 60% at a concentration as low as 6 g/L. Glucose is however a major inhibitor of β-glucosidases, reducing β-glucosidase activity by 75% at a concentration of 3 g/L (Philippidis *et al*., 1993). Another drawback in this configuration is possible microbial contamination during the hydrolysis and fermentation steps. The hydrolysis step takes rather long, up to 96 hrs, and the difficulty in sterilizing the enzymes makes them a possible source of contamination.

2.3.2. Simultaneous Saccharification and Fermentation (SSF)

SSF combines enzymatic hydrolysis of lignocellulosic material and fermentation in one bioreactor. In this configuration, the glucose produced during saccharification is fermented simultaneously by microorganisms present in the media. The principal advantage of SSF compared to SHF is that the inhibitory effect of glucose and cellobiose on cellulases is minimized by keeping a low concentration of these sugars in the media thereby increasing the rate of hydrolysis. Apart from this main advantage, there are several other advantages, including (i) lower enzyme requirement, (ii) higher ethanol yields, (iii) lower risk of contamination since glucose is removed immediately and bioethanol is produced, (iv) shorter process time and (v) fewer reactors are required (Olofsson *et al*., 2008)

Inevitably, SSF also has disadvantages relative to SHF. The difference in temperature optima for saccharification and fermentation is the main drawback of SSF. Due to this discrepancy, saccharification becomes the limiting step in SSF. Besides this, the yeast cannot be reused in an SSF process due to impracticalities in separating the yeast from the lignin after fermentation. Cellulase inhibition by the produced ethanol might also be a problem in SSF (Bezerra and Dias, 2005). However, due to problems with mechanical mixing and mass transfer (Hoyer *et al*., 2008), only low substrate concentrations are currently practical for SSF making ethanol inhibition a less important factor.

Regardless of the shortfalls mentioned above, SSF is the preferred configuration in laboratory studies and pilot scale plants. Most of the research done on optimisation of sugarcane bagasse pretreatment and saccharification employed SSF for fermentation of hydrolysates (Martin *et al.*, 2006a; Vásquez *et al.*, 2007). Total glucose consumption was observed by Rudolf *et al.* (2008) when they investigated SSF of STEX sugarcane bagasse by the *S. cerevisiae* strain TMB3400. Hernandez-Salas *et al.* (2009) used SHF on bagasse hydrolysates and reported that the ethanol yields were much lower than those reported for SSFs, showing that SSF is a much more potent configuration.

2.3.3. Nonisothermal Simultaneous Saccharification and Fermentation (NSSF)

Since the saccharification step in SSF is operated at a temperature lower than its optimum level, the enzyme activities are far below potential, and consequently the enzyme requirement is raised. To alleviate this problem, a nonisothermal simultaneous saccharification and fermentation process (NSSF) was suggested by Wu and Lee (1998) and further investigated by Oh *et al.* (2000). In this configuration, saccharification and fermentation occur simultaneously but in two separate reactors that are maintained at different temperatures. Lignocellulosic biomass is retained inside a column reactor and hydrolyzed at 50°C, the optimum temperature for saccharification. The effluent from the column reactor is recirculated through a fermenter, which runs at its optimum temperature (e.g. 30°C).

Cellulase activity is increased 2-3 fold when the saccharification temperature is raised from 30°C to 50°C. According to Wu and Lee (1998), NSSF improved the enzymatic reaction in the SSF to the extent of reducing the overall enzyme requirement by 30-40% while both ethanol yield and productivity were substantially higher than those in the SSF with the terminal yield attainable in 4 days with SSF, reachable in 40 h with NSSF. They observed that the effect of temperature was most significant on β-glucosidase activity.

2.3.4. Simultaneous Saccharification and Co-fermentation (SSCF)

More recently, the SSF technology has been improved to simultaneous saccharification and co-fermentation (SSCF) in which there is simultaneous fermentation of hexoses and pentoses. After pretreatment, the hydrolysed hemicellulose and the solid cellulose fractions are not separated, allowing the hemicellulose sugars and cellulose to be fermented together in a single bioreactor. In SSF, hexoses and pentoses are fermented by different microorganisms in separate bioreactors. With the SSCF configuration, it is suggested to ferment both hexoses and pentoses in one bioreactor using a single microorganism. In SSCF, the enzymatic saccharification continuously releases hexose sugars, which increases the rate of glycolysis such that the pentose sugars are fermented faster and with higher yield (Balat *et al*., 2008).

2.3.5. Consolidated Bioprocessing (CBP)

In all the strategies outlined thus far, the saccharolytic enzymes should be provided externally. The ultimate process would however be a one-step “consolidated” bioprocessing (CBP) of lignocellulose to ethanol, where the four biologically mediated transformations, i.e. production of saccharolytic enzymes (cellulases and hemicellulases), hydrolysis of carbohydrate components present in biomass to sugars, fermentation of hexose sugars, and fermentation of pentose sugars (Lynd *et al*., 2005), would be mediated by a single microorganism or microbial consortium without added saccharolytic enzymes (van Zyl *et al.*, 2007). CBP is also known as direct microbial conversion (DMC).

Microorganisms with the properties required for CBP are not currently available, but efforts are underway for their development. Two strategies can be employed in such developments: (i) engineering naturally occurring saccharolytic microorganisms to improve product-related properties, such as yield and titer (native cellulolytic strategy), and (ii) engineering non-saccharolytic organisms that exhibit high product yields and titers so that they express a heterologous cellulase system enabling lignocellulose utilization (recombinant cellulolytic strategy).

Engineering a microorganism for CBP of pretreated lignocellulosic material is a daunting task. Besides high ethanol yield and productivity, industrial strains need to be able to first effectively hydrolyse both the cellulose and hemicellulose components of lignocellulosic material, with minimal requirement for pre-processing, and then concurrently ferment both hexoses and pentoses under robust industrial conditions that require minimum nutrient requirements and also exhibit high ethanol and inhibitor tolerance.

The yeast *S. cerevisiae* is an attractive platform organism for the recombinant cellulolytic strategy given that it is a proven ethanol-producer, exhibits tolerance to high sugar concentrations, ethanol and inhibitory compounds commonly found in hydrolyzates resulting from biomass pretreatment, has the ability to grow at low oxygen levels, enjoys GRAS (Generally Regarded As Safe) status, and has well-established tools for genetic manipulation (van Zyl *et al.*, 2007). Wildtype *S. cerevisiae*, however, lacks some major attributes of an ideal CBP yeast. Firstly, it falls short of the major requirement of sufficiently expressing and producing extracellular saccharolytic enzymes (cellulases and hemicellulases). Secondly, *S. cerevisiae* can ferment all the hexoses (glucose, fructose, galactose and mannose) (Figure 18) in the lignocellulosic hydrolysate to ethanol, but not the pentoses (xylose and arabinose), which can be a significant portion (25% for sugarcane bagasse). Despite the ability of *S. cerevisiae* to also utilize the disaccharides sucrose and maltose, the yeast cannot utilize cellobiose and cello-oligosaccharides, which are major endproducts of cellulose hydrolysis. Development of *S. cerevisiae* as a CBP yeast therefore rests on the ability to engineer the yeast so that it can exhibit the attributes it lacks naturally.

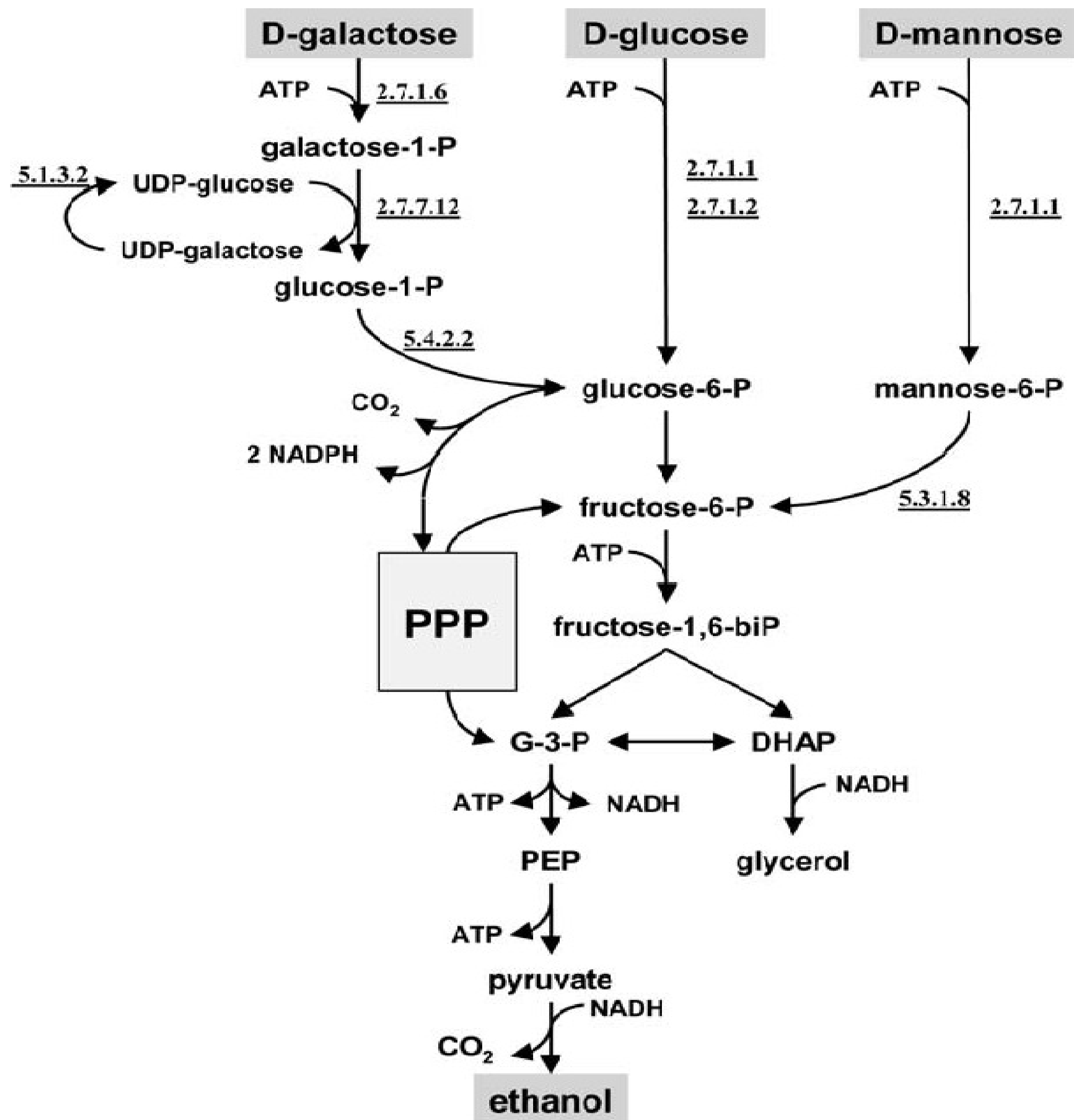

Figure 18: Hexose catabolism of *Saccharomyces cerevisiae*. Underlined EC numbers represent enzymes/steps present in *S. cerevisiae* metabolism. The gene names encoding the various enzymes are given in parentheses in the legend of this figure. Glucose catabolism: 2.7.1.1, hexokinase (*HXK1/HXK2*); 2.7.1.2, glucokinase (*GLK1*); Galactose catabolism: via the Leloir pathway: 2.7.1.6, galactokinase (GAL1); 2.7.7.12, galactose-1-phosphate uridylyltransferase (*GAL7*); 5.1.3.2, UDP-glucose 4-epimerase (*GAL10*); 5.4.2.2 phosphoglucomutase (*GAL5/PGM2*). Mannose catabolism: 2.7.1.1, hexokinase I (*HXK1*); 5.3.1.8, mannose- 6-phosphate isomerase (*PMI40*). G-3-P, Glyceraldehyde- 3-phosphate; DHAP, dihydroxy-acetone-phosphate; PEP, phospho-enol pyruvate; PPP, Pentose phosphate pathway (adapted from van Maris *et al*., 2006).

2.3.6. Progress in the engineering of *S. cerevisiae* for CBP of pretreated lignocellulosic material

2.3.6.1. Expression of cellulases in *S. cerevisiae*

As already discussed in Section 2.2.2.1., complete cellulose hydrolysis requires three major types of enzymatic activity: (i) endoglucanases; (ii) exoglucanases; and (iii) β-glucosidases. In the recent past, there have been several reports of genes coding for cellulases being cloned from various bacteria, filamentous fungi and plants and, expressed in *S. cerevisiae* (van Zyl *et al*., 2007). Cellobiohydrolases (CBHs) have a much lower specific activity compared to the other cellulase system components, endoglucanases and *β*-glucosidases, which makes their expression in *S. cerevisiae* more of a challenge. CBHs are key components for fungal cellulase systems, contributing ~80% of the total mass for the *Trichoderma reesei* system, making their functional secretion indispensable for CBP. CBHs have been successfully cloned and expressed in *S. cerevisiae* showing activity on a variety of substrates (Fujita *et al*., 2004; Den Haan *et al*., 2007a; Voutilainen *et al*., 2007; 2008; 2009), but studies to increase their expression levels are still required.

Fungal and bacterial endoglucanase (EG) expression in *S. cerevisiae* has been much more successful (Fujita *et al*., 2002; Toda *et al*., 2005; Den Haan *et al*., 2007b; Qin *et al*., 2008; Ganiger *et al*., 2008) than CBH expression and this can be attributed to the higher specific activity of endoglucanases compared to cellobiohydrolase. β-Glucosidase was successfully expressed at sufficient levels to sustain growth on cellobiose, indicating that its expression will not be a limiting step in cellulase system reconstruction (van Rooyen *et al*., 2005; Mcbride *et al*., 2005).

Successful co-expression of cellulases has been reported by several researchers (Fujita *et al*., 2002; 2004; Den Haan *et al*., 2007b). Fujita *et al*. (2002) codisplayed the endoglucanase II (EGII) of *T. reesei* and the β-glucosidase 1 of *Aspergillus aculeatus* on the cell surface of *S. cerevisiae*. The resulting yeast cells could grow in synthetic medium containing barley β-glucan as the sole carbon source and directly fermented the β-glucan, yielding 93.3% of the theoretical ethanol yield. Fujita *et al*. (2004) went on to demonstrate synergistic saccharification and fermentation of amorphous

cellulose by codisplaying three enzymes, *T. reesei* endoglucanase II and cellobiohydrolase II and *A. aculeatus* β-glucosidase 1, on the cell surface of *S. cerevisiae*. The resulting yeast strain could directly produce ethanol from amorphous cellulose (which the yeast strain codisplaying only β-glucosidase 1 and endoglucanase II could not). In a report by Den Haan *et al*. (2007b), two cellulase encoding genes, an endoglucanase of *T. reesei* (EGI) and the β-glucosidase of *Saccharomycopsis fibuligera* (BGL1), were co-expressed in *S. cerevisiae*. Through simultaneous production of sufficient extracellular endoglucanase and β-glucosidase activity, the resulting strain was able to grow on medium containing phosphoric acid swollen cellulose (PASC) as sole carbohydrate source with concomitant ethanol production. These results demonstrate that efficient one-step conversion of cellulose to ethanol by recombinant *S. cerevisiae* cells expressing cellulolytic enzymes is practicable, and represent significant progress towards CBP of cellulosic biomass.

2.3.6.2. Expression of hemicellulases in *S. cerevisiae*

β-1,4 xylan, the most abundant component of hemicellulose, is a complex polysaccharide consisting of a backbone of β-1,4 linked xylopyranoside, which is partially substituted with acetyl, glucuronosyl and arabinosyl side chains. Xylan is hydrolyzed to xylo-oligosaccharides by endo-β-xylanase (EC 3.2.1.8), followed by β-D-xylosidase (EC 3.2.1.37) that hydrolyzes xylo-oligosaccharides to D-xylose.

Many researchers have studied the production of xylanolytic enzymes in *S. cerevisiae* through heterologous production of bacterial or fungal xylanases (La Grange *et al*., 1996; Li and Ljungdahl, 1996; Luttig *et al*., 1997; Nuyens *et al*., 2001; Lee *et al*., 2009) and β-xylosidase (Margolles-Clark *et al*., 1996; La Grange *et al*., 1997). Attempts have been made to co-produce xylanase and β-xylosidase in *S. cerevisiae* as a means of converting xylan to cell mass or ethanol through simultaneous saccharification and fermentation (La Grange *et al*., 2000; 2001). La Grange *et al*. (2001) coexpressed the *A. niger* β-xylosidase (*xlnD*) and the *T. reesei* xylanase II (*xyn2*) genes in *S. cerevisiae* and the resulting yeast was able to degrade birchwood xylan to D-xylose through the coproduction of β-xylanase and β–xylosidase. Progress in the cloning and expression of xylanolytic enzymes in *S. cerevisiae* was well reviewed by Van Zyl *et al*. (2007) and Ahmed *et al*. (2009).

2.3.6.3. Engineering *S. cerevisiae* for sugar fermentation

S. cerevisiae is naturally unable to utilise the 5-carbon sugars D-xylose and L-arabinose present in plant biomass, and must thus be engineered to both transport and ferment them. Xylose is the second most abundant carbohydrate in lignocellulosic material (Ohgren *et al*., 2005; Garcia-Aparicio *et al*., 2006; Ballesteros *et al*., 2006) and its fermentation to ethanol is paramount for economical lignocellulosic ethanol production.

In fungi, D-xylose catabolism begins with its conversion by xylose reductase (XR; EC 1.1.1.21) and xylitol dehydrogenase (XDH; EC 1.1.1.9) to D-xylulose, which is then phosphorylated to D-xylulose 5-phosphate by xylulokinase (XK; EC 2.7.1.17) before being assimilated via the non-oxidative pentose phosphate pathway (PPP). On the other hand, in most bacteria (e.g., *Escherichia coli* and *Streptomyces sp*.), D-xylose is directly isomerized to D-xylulose by xylose isomerase (XI; EC 5.3.1.5). These two heterologous pathways have been used to construct recombinant xylose fermenting *S. cerevisiae* strains i.e.: i) the xylose reductase (XR) and xylitol dehydrogenase (XDH) pathway (Karhumaa *et al*. 2007) and ii) the xylose isomerase (XI) pathway (Walfridsson *et al*., 1996; Kuyper *et al*., 2003; Brat *et al*., 2009; Madhavan *et al*., 2009) (Figure 19).

Figure 19: Engineering pentose metabolism in yeasts. The pentose phosphate pathway (PPP) in yeasts consists of the oxidative phase, which consists of glucose 6-phosphate dehydrogenase (*ZWF1*) and 6-phosphogluconate dehydrogenase (*GND1*), and the non-oxidative phase, which is carried out by D-ribulose-5-phosphate 3-epimerase (*RPE1*), ribose-5-phosphate ketol-isomerase (*RKI1*), transketolase (*TKL1*) and transaldolase (*TAL1*). Phosphoglycerate isomerase (*PGI1*) completes the cycle. The PPP has been engineered with the heterologous expression of D-xylose reductase (*XYL1*), xylitol dehydrogenase (*XYL2*), D-xylulokinase (*XYL3*), xylose isomerase (*xylA*), L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-phosphate 4-epimerase (*araD*) for the assimilation of D-xylose and L-arabinose. Phosphoketolase (*xfp*) provides a bypass to the PPP, which incorporates inorganic phosphate into xylulose-5-phosphate, resulting in the production of acetyl phosphate and phosphoenolpyruvate. The former can be converted into acetate and ATP via acetate kinase (*ackA*). Alternatively, it can be converted into ethanol via phosphotransacetylase (*pta*), acetaldehyde dehydrogenase (*adhE*) and alcohol dehydrogenase (*ADH1*). Normal glycolysis also produces acetaldehyde via pyruvate decarboxylase (*PDC1*). Acetaldehyde is oxidized to acetate and NADPH by aldehyde dehydrogenase (*ALD6*). Yeast genes are shown in italic caps; bacterial genes in lower case italics (adapted from Jeffreys, 2006).

Heterologous expression of xylose reductase and xylitol dehydrogenase does enable D-xylose utilisation, but incomplete recycling of redox co-substrates in the catalytic steps of the NADPH-preferring XR and the NAD+-dependent XDH results in the formation of xylitol and concomitantly low ethanol yields are realised on xylose. On

the contrary, expression of xylose isomerase does not have these constraints, but its use has been precluded by several problems associated with functional expression of bacterial and archaeal XI genes in yeast (Moes *et al*. 1996; Gárdonyi and Hahn-Hägerdal, 2003). Functional XIs from *Thermus thermophilus* (Walfridsson *et al*., 1996), *Piromyces* sp. E2, (Kuyper *et al*., 2003), *Orpinomyces* (Madhavan *et al*., 2009) and *Clostridium phytofermentans* (Brat *et al*., 2009) have been expressed in *S. cerevisiae*.

Karhumaa *et al*. (2007) compared xylose fermentation by recombinant *S. cerevisiae* strains with the *Pichia stipitis* XR-XDH and the *Piromyces* XI pathways and concluded that despite by-product formation, the XR-XDH xylose utilization pathway resulted in faster ethanol production than using the reported XI pathways. They also observed that xylitol was formed by the XR-XDH-carrying strains only in mineral medium, but not in spruce hydrolysate.

In addition to the introduction of xylose-to-xylulose conversion pathways in *S. cerevisiae*, a number of strategies for metabolic engineering and genetic modification to enhance the rapid and efficient fermentation of xylose to ethanol have been investigated and found to be effective. These include over-expression of the native XK (Johansson *et al*., 2001), changing the intracellular redox balance (Petschacher and Nidetzky, 2008; Bengtsson *et al*., 2009) engineering the xylose transport (Runquist *et al*., 2009), and enhancing the PPP (Jin *et al*., 2005; Kuyper *et al*., 2005). Apart from targeted metabolic engineering, adaptation has been used to obtain improved utilisation of xylose in lignocellulosic hydrolysates, such as bagasse hydrolysate (Martin *et al*., 2007b).

Research has been conducted to construct a yeast strain that can directly convert xylan into ethanol by conferring both xylan hydrolysis and xylose fermentation abilities on a single *S. cerevisiae* strain. Katahira *et al*. (2004) constructed a xylan-utilizing *S. cerevisiae* strain by cell surface codisplaying xylanase II (XYNII) from *T. reesei* and β-xylosidase (XylA) from *Aspergillus oryzae* and introducing genes for xylose utilization, i.e. the *P. stipitis* XR and XDH and the *S. cerevisiae* XK into the strain.

The resultant strain produced ethanol directly from Birchwood xylan, demonstrating direct conversion of xylan to ethanol.

For lignocellulosic ethanol to be cost-effective and efficient, the fermentation of not only major constituents, such as glucose and xylose, but also less predominant sugars, such as L-arabinose, is required. It has been earlier (Section 2.1.2.2.) mentioned that the bagasse hemicellulose xylose backbone is branched mainly through arabinofuranosyl and 4-*O*-methyl glucopyranosyl units (Sun *et al.*, 2004b) which prognosticates the need for arabinose fermentation. Although several yeasts and fungi can utilize L-arabinose as a carbon and energy source, most of them are unable to ferment it into ethanol.

The rarity of ethanolic arabinose fermentation may be due to a redox imbalance in the fungal arabinose pathway that results in the formation of L-arabinitol. The fungal L-arabinose pathway involves the conversion of L-arabinose to L-arabitol by an aldose reductase (AR, EC 1.1.1.21), followed by the L-arabitol dehydrogenase (LAD, EC 1.1.1.12) catalysed conversion of the L-arabitol to L-xylulose, which is then reduced to xylitol by L-xylulose reductase (ALX, EC 1.1.1.10). Richard *et al*. (2003) showed that overexpression of all the structural genes of the fungal L-arabinose pathway (*XYL1*, *lad1*, *lxr1*, *XYL2*, and *XKS1*) in *S. cerevisiae* does not result in fast and efficient fermentation of L-arabinose into ethanol. An L-arabinose-fermenting *S. cerevisiae* strain can alternatively be constructed through the expression of the bacterial L-arabinose pathway that circumvents the intrinsic redox imbalances associated with the expression of the fungal pathway. Bacteria convert L-arabinose to L-ribulose, L-ribulose-5- phosphate and finally D-xylulose-5-phosphate via L-arabinose isomerase (*araA*, EC 5.3.1.4), L-ribulokinase (*araB*, EC 2.7.1.16) and L-ribulose-5-P 4-epimerase (*araD*, EC 5.1.3.4), respectively.

Wisselink *et al*. (2007) constructed an L-arabinose utilising *S. cerevisiae* strain by combining the expression of the structural genes for the L-arabinose utilization pathway of *Lactobacillus plantarum*, the over-expression of the *S. cerevisiae* genes encoding the enzymes of the non-oxidative pentose phosphate pathway, and extensive evolutionary engineering. The resulting strain exhibited high rates of arabinose consumption and ethanol production and a high ethanol yield during anaerobic growth

on L-arabinose as the sole carbon source. A milestone in pentose fermentation was reached more recently as Bettiga *et al*. (2009) constructed a new *S. cerevisiae* strain expressing an improved fungal pathway for the utilization of both L-arabinose and D-xylose. The new strain grew aerobically on L-arabinose and D-xylose as sole carbon sources, producing both biomass and ethanol.

2.4. Conclusions

The South African sugar industry generates approximately 6 million tonnes of sugarcane bagasse per annum from crushing about 21 million tonnes of sugarcane (Mashoko *et al*., 2008) and only a portion of this bagasse is used to generate electricity and steam for the sugar mills in a low efficiency steam cycle. With improvements in thermal efficiency of combustion units, the energetic demands of sugar factories could be satisfied with reduced amounts of bagasse and a bigger surplus would become available for alternative uses, including ethanol production. Fittingly, with the imminent twin crises of Peak Oil and climate change, it is paramount for South Africa to harness this cheap, abundant and carbohydrate rich raw material for the production of bioethanol.

However, the recalcitrance of this lignocellulosic substrate to enzymatic hydrolysis and fermentation necessitates the development of an energy efficient, high-ethanol yield process for economic feasibility. In this context, this study was undertaken to develop a process to efficiently convert sugarcane bagasse into ethanol by using combinations of commercial enzyme cocktails and recombinant *S. cerevisiae* strains. Though enzymatic saccharification is promising for obtaining sugars from lignocellulosics, the low enzymatic accessibility of the cellulose and hemicellulose is a key impediment, thus necessitating development of optimized enzyme mixtures with essential accessory activities. The ultimate process would, however, be a one-step "consolidated" bioprocessing (CBP) of lignocellulose to ethanol, where hydrolysis and fermentation of polysaccharides would be mediated by a single microorganism or microbial consortium without added saccharolytic enzymes.

In this thesis, the effect of uncatalysed and SO_2 catalysed steam explosion pretreatment of sugarcane bagasse on the composition of pretreated material,

digestibility of the water insoluble solids (WIS) fraction and overall sugar recovery was investigated. Steam explosion pretreatment with water impregnation was found to result in a higher sugar recovery and produced WIS with a higher enzymatic digestibility thus was used in optimization of saccharification and fermentation. Response surface methodology (RSM) based on the 3^3 factorial design was used to optimize the composition of the saccharolytic enzyme mixture so as to maximize glucose and xylose production from steam exploded bagasse. Subsequently, the optimal enzyme mixture was used to supplement enzyme activities of recombinant yeast strains co-expressing several cellulases and xylanases in simultaneous saccharification and fermentations SSFs.

The multi-enzyme mixture allowed for a significantly higher conversion of bagasse at lower enzyme loadings compared to using a single cellulase cocktail. The recombinant yeast strains were able to separately hydrolyse and ferment the substrate though at lower rates than in the SSFs. This study confirms that saccharolytic enzymes exhibit synergism and that bagasse is a potential substrate for bioethanol production. Furthermore, the concept of CBP of sugarcane bagasse was proven to be feasible.

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CHAPTER 3

COMPARATIVE SUGAR RECOVERY AND ENZYMATIC HYDROLYSIS OF SUGARCANE BAGASSE STEAM EXPLODED WITH WATER AND SO_2 IMPREGNATION

Comparative sugar recovery and enzymatic hydrolysis of sugarcane bagasse steam exploded with water and SO_2 impregnation

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3.1. Abstract

Sugarcane bagasse is the fibrous residue obtained after sugarcane is crushed to extract its juice. It is a potential lignocellulosic raw material for fuel ethanol production since it is cheap, abundant and rich in carbohydrates. The main aim of this study was to investigate whether the overall ethanol yield in a sugarcane ethanol plant could be increased by steam pretreatment and enzymatic hydrolysis of bagasse. Two different approaches to perform steam pretreatment of sugarcane bagasse were compared with respect to the composition of pretreated material, digestibility of the WIS fraction and overall sugar recovery. In the first approach, sugarcane bagasse was impregnated in water prior to pretreatment at 210°C for 5 minutes (severity factor of 3.94), whereas in the second pretreatment the biomass was impregnated with sulphur dioxide (SO_2) prior to pretreatment under less severe conditions (188°C for 10 minutes, severity factor of 3.59). Results showed that SO_2 impregnation prior to pretreatment at less severe conditions provided similar cellulose digestibility whilst increasing the xylose recovery (75.1%) compared to the un-catalyzed steam pretreatment (53.3%).

3.2. Introduction

Biomass-derived liquid fuels, such as ethanol, are generally considered as offering many benefits, including mitigation of greenhouse gas emissions, regional development, sustainability and security of supply, rendering them an attractive alternative to fuel oil. In addition, plant biomass represents the basis for future supply of renewable chemicals and materials (Ragauskas *et al*., 2006; Kamm, 2007; Gandini, 2008; Gandini *et al*., 2009). Consequently, South Africa is making efforts to support the production and use of biofuels in the transport sector, with the short-term goal of replacing 2% of the national liquid fuel supply (National Biofuels Task Team, 2006).

At present, large quantities of bioethanol are already produced from feedstock rich in starch or sugar (corn, grain or sugarcane) that are assigned to the food market. However, it is generally accepted that the expansion of the bioethanol production and shifting from a petrol-chemical based industry to a carbohydrate based industry on a sustainable basis requires the utilization of the whole plant, including lignocellulosic residues such as sugarcane bagasse. Sugarcane bagasse is produced as a by-product of the sugar factory after extraction of the juice and it can account for up to 30% by weight of the crushed sugarcane (Mbohwa and Fukuda, 2003; Sánchez and Cardona, 2008). This residue contains a significant amount of sugars that would increase the ethanol yield and thus increase production efficiency.

Efficient and cost effective degradation of the cell wall carbohydrates (mainly cellulose and hemicellulose), which represents up to 75% of lignocellulosic materials, into monosaccharides is an important first step and represents a bottleneck of the process (Himmel *et al*., 2007; Jørgensen *et al*., 2007). Among depolymerization processes, those based on enzymatic hydrolysis show promise due to a high potential for improvements by means of biotechnology.

Current technologies for the conversion of lignocellulose to bioethanol require a pretreatment step prior to enzymatic hydrolysis into fermentable sugars, such as glucose and xylose. Hydrothermal treatment such as steam explosion is an interesting option because of its limited use of chemicals, low energy consumption, short reaction time and, depending on the conditions used, high sugar recovery (Chandra *et al*., 2007). Besides, it has been shown to be an effective method to considerably disrupt the lignocellulosic structure of agricultural residues increasing the rate of hydrolysis (Kaar *et al*., 1998; Martin *et al*., 2002, 2006; Ballesteros *et al*., 2006). Steam

explosion pretreatment can be un-catalysed (autohydrolysis) or catalysed by the addition of H_2SO_4 (or SO_2) (Carrasco *et al*., 2010) or CO_2 (Ferreira-Leitao *et al*., 2010). The SO_2 and CO_2 are solubilised in water to H_2SO_3 and H_2CO_3, respectively, that enhance acid catalysis of the hemicellulose fraction (Ferreira-Leitao *et al*., 2010). The pretreated material or slurry is normally filtered to obtain a water-insoluble solids (WIS) fraction, which contains the majority of cellulose and lignin, and a liquid fraction or prehydrolysate, which contains the hemicellulosic sugars solubilized during the pretreatment, as well as different degradation products (Ramos, 2003).

Pretreatment conditions can modify the substrate composition having a great effect on the subsequent enzymatic hydrolysis and fermentation steps. In this context, two different approaches to perform steam pretreatment of sugarcane bagasse were compared with respect to the composition of pretreated material, digestibility of the WIS fraction and overall sugar recovery. In the first approach, sugarcane bagasse was impregnated in water prior to pretreatment at 210°C for 5 minutes (severity factor = 3.94), whereas the biomass was impregnated with sulphur dioxide (SO_2) prior to pretreatment under less severe conditions (188°C for 10 minutes, severity factor = 3.59) in the second pretreatment.

3.3. Materials and methods

3.3.1. Substrate and chemicals

Raw bagasse was supplied by TSB Sugar, Mpumalanga, South Africa. All chemicals, media components and supplements were of analytical grade and sourced from BDH Chemicals Ltd. (Poole, England), Merck (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, USA).

3.3.2. Pretreatment of sugarcane bagasse

Two steam pretreatment conditions were used in this work (Table 2). Un-catalysed steam explosion (STEX) pretreatment was generously performed at CIEMAT biomass unit (CIEMAT Renewable Energies Department, Madrid, Spain) by applying Masonite technology in a small steam-explosion unit operated in batches and equipped with a 10-L reactor. The reactor was charged with 500 g (dry matter) of feedstock per batch, directly heated with saturated steam to a temperature of 210°C and maintained for 5 min before being suddenly depressurized.

SO_2 catalysed steam exploded bagasse slurry was generously supplied by SEKAB biofuels and chemicals AB (Örnsköldsvik, Sweden). Milled bagasse was impregnated with SO_2 by sealing the wet material (500 g dry matter content) for 30 min in plastic bags to which SO_2 gas was added. The reopened bags were subsequently placed in a well-ventilated area to allow excess SO_2 gas to disperse. The average SO_2 content of the material was calculated as 2% (w/w liquid) based on the difference in mass of the material before and after SO_2 exposure. SO_2 impregnated bagasse was then directly heated with saturated steam to a temperature of 188°C and maintained for 10 minutes at 12 bar before being suddenly depressurized.

The pretreated material (slurry) was recovered, cooled to about 40°C, and vacuum-filtered for liquid and solid recovery determination. The solid fraction was water-washed and the resulting solid, denoted as water insoluble solids (WIS), was used for enzymatic hydrolysis experiments. The liquid fraction or prehydrolyzate, containing the water-soluble solids (WSS), was analyzed for sugar, acetic acid and sugar-degradation compounds as described below.

3.3.4. Severity function

The severity function (R_0) is used to correlate the effects of temperature and the residence time at the specific temperature with the effectiveness of biomass pretreatment. Overend and Chornet (1987) defined function (1) to quantify the severity of a biomass hydrolysis system:

$$\mathbf{R}\boldsymbol{o} = \mathbf{t} \times \mathbf{exp}\left[\frac{(\boldsymbol{T} - \mathbf{100})}{\boldsymbol{14.75}}\right] \quad \textbf{(1)}$$

where t is time in minutes and T is the experimental temperature in °C

The effect of the acidity (as indicated by the pH after pretreatment) generated in the reaction media by the addition of an acid catalyst and the release of organic acids from the raw material can also be included by using the combined severity function developed by Chum *et al*. (1990):

$$\textbf{combined severity} = \mathbf{LogR_0} - \mathbf{pH} \quad \textbf{(2)}$$

3.3.5. Enzymatic hydrolysis

The WIS obtained after pretreatment was enzymatically hydrolyzed to determine the effect of the different pretreatment conditions on the enzyme accessibility of the substrate. The enzymatic hydrolysis was performed in 25 ml McCartney bottles at 2% (w/v) substrate loading in 0.05 M citrate buffer (pH 4.8) and an initial reaction volume of 10 ml at 50°C whilst shaking at 150 rpm for 120 hours. Sodium azide was added to the buffer to a final concentration of 0.02% to avoid bacterial contamination. Celluclast® 1.5L (Genencor-Danisco, Denmarck) with a cellulase activity of 74 FPU/ml and a xylanase activity of 310 U/ml, and Novozyme 188 (Novozymes A/S, Denmark) with a β-glucosidase activity of 740 IU/ml, were used in all experiments. The standard enzyme dosage of 15 FPU cellulase/g WIS (equivalent to 30.6 mg protein/g of dry pretreated substrate) was tested in the presence and absence of 15 IU β-glucosidase/g WIS (equivalent to 6.2 mg protein/g dry pretreated substrate). In addition, a 3-fold reduction of the conventional enzyme mixture (5 FPU cellulase/g WIS supplemented with 5 IU β-glucosidase/g WIS) was tested. Samples were withdrawn from the hydrolysis media after 24, 72 and 120 h and sugar content was analyzed by HPLC as described below.

3.3.6. Analytical methods

Enzyme preparations were subjected to standardized methods to determine protein content and major enzyme activities relevant in the conversion of lignocellulose, namely cellulase and cellobiase. Cellulase and β-glucosidase activities were measured according to methods described by Ghose (1987) and the endo-β-1,4-xylanase activity of Celluclast® 1.5L as described by Bailey *et al.* (1992). Protein concentrations were determined using the Bio-Rad Bradford protein assay (Bio-Rad laboratories, München, Germany) as prescribed for the microtitre plate protocol. A standard curve was compiled using 0.05 to 0.5 mg/mL bovine serum albumin.

The composition of the raw sugarcane bagasse and WIS was determined using the standard Laboratory Analytical Procedures for biomass analysis provided by the National Renewable Energy Laboratory (NREL) (CO, USA). The liquid fractions after steam pretreatment were analysed for monomeric sugars and soluble oligomeric sugars. The oligosaccharides concentration was determined as the difference in

monomeric sugar concentration before and after a hydrolysis process with sulphuric acid (3%, v/v) at 121°C for 30 minutes. Sugars (glucose, xylose and arabinose) and by-products (acetic acid, formic acid, hydroximethylfurfural and furfural) in the case of the liquid fraction from pretreatment were analyzed as described elsewhere (Ballesteros *et al*., 2006; García-Aparicio *et al*., 2010). Likewise, cellobiose, glucose, xylose and arabinose concentrations after completion of enzymatic hydrolysis tests were measured from the EH media by HPLC. All analytical determinations were performed at least in duplicate and the averages are reported here.

3.4. Results and discussion

3.4.1. Raw material composition

The composition of the sugarcane bagasse is provided in Table 1. Milled-processed sugarcane bagasse had a chemical composition of 19.9% lignin, 26.1% hemicellulose and 39.6% cellulose on a dry weight basis, which is in agreement with the data reported in literature for sugarcane bagasse (Geddes *et al*., 2010; Monte *et al*., 2010). The hemicellulose component consisted primarily of xylan (22.9%) and small amounts of arabinan (3.2%). The extractives and inorganic content (ash) were relatively low at 5.3% and 2.4%, respectively.

Table 1: Chemical composition of raw sugarcane bagasse.

Component	Extraction method Composition	Dry matter (%, w/w)*
Extractives	**Water**	2.2 ± 0.3
	Ethanol	3.0 ± 0.1
Polysaccharides	**Glucan**	39.6 ± 2.1
	Xylan	22.9 ± 2.0
	Arabinan	3.2 ± 0.2
Lignin	**Acid soluble**	5.9 ± 0.9
	Acid insoluble	14.0 ± 0.9
Ash		2.4 ± 0.3
Total		93.2

*Data represents mean values and standard deviations of four determinations

The results indicate that up to 65.7% of the dry raw material consisted of glucan and hemicellulose that could be used as substrate for ethanol production. The high carbohydrate content coupled to the relatively low ash, extractive and acid insoluble lignin content makes this feedstock a good candidate for second generation

ethanol production through appropriate pretreatment, enzymatic hydrolysis and fermentation.

3.4.2. Steam explosion pretreatment

3.4.2.1. Effects of pretreatment on composition of sugarcane bagasse

The pretreatment conditions evaluated, namely temperature, residence time and impregnating agent, and the combination of these parameters in terms of severity factor (Equation 1) or combined severity factor (Equation 2) are shown in Table 2. Slurry with total solids of 20.0% and 16.8% (w/v) resulted after steam pretreatment of the sugarcane bagasse without and with an acid catalyst, respectively. The WIS content of the slurry varied from 15.2% (w/v) when the biomass was impregnated in water, to 10.4% (w/v) when SO_2 was used as the impregnating agent prior to steam pretreatment. As a result, the WSS content was 4.8% (w/v) and 6.4% (w/v), respectively. Total solid recovery after pretreatment (expressed as insoluble solids remaining after pretreatment per dry weight of the raw material) was about 64.2% and 60% for un-catalyzed and catalyzed steam pretreatment, respectively.

Table 2: Evaluation of temperature (°C), residence time (minutes) and impregnating agent (water or SO_2) as pretreatment conditions.

Pretreatment conditions					Pretreated material: Slurry		
Temperature (ºC)	Time (min)	Impregnation	Severity Factor	Combined Severity Factor (similar pH~3)	Total Solids (%)	WSS (%)	WIS (%)
210	5	Water	3.94	0.94	20.0	4.8	15.2
188	10	SO_2 (2% (w/w))	3.59	0.59	16.8	6.4	10.4

The composition of the different fractions of the pretreated sugarcane bagasse is summarized in Table 3. As can be observed, the glucan levels in the solid fraction after both pretreatments (~59%) increased relative to untreated material (39.6%) due to solubilization of hemicellulose and extractives. Considering the glucan content in the raw material and the solid recovery, 95% of the glucan remained in the solid fraction after pretreatment when water was used as impregnating agent. In the case of

SO_2-catalyzed steam pretreatment, the glucose recovered in the solid fraction was reduced to 91% of the glucose of the raw material.

The sugar composition and concentrations of byproducts from the liquid fraction or prehydrolysate are also shown in Table 3. Most of the hemicellulose-derived sugars were solubilized during pretreatment mainly in oligomeric form. Xylose, the main hemicellulosic sugar, was found in the prehydrolyzate in a concentration of 8.6 g/L and 15 g/L for water and SO_2-catalyzed pretreatment, respectively. These concentrations increased to 26.1 g/L and 30.7 g/L after acid hydrolysis, indicating that 17.5 and 15.7 g/L of the xylose was in oligomeric form. The lower glucose recovery on the WIS in the SO_2-catalyzed pretreatment indicates greater glucan degradation and this was supported by the higher glucose concentration in the SO_2-catalyzed liquid fraction (5 g/L as monomers and 4.4 g/L in oligomeric form) compared to that obtained in the un-catalyzed steam pretreatment liquid fraction (0.4 g/L as monomers and 1.4 g/L in oligomeric form).

Table 3: Composition of sugarcane bagasse steam-pretreated with and without SO_2 impregnation

Steam Pretreatment	**Water Impregnation**	**SO_2 impregnation 2% (w/w liquid)**
Temperature (°C)	210	188
Time (minutes)	5	10
Water Insoluble Solids (WIS), % dw		
Glucan (glucose)	58.6 (64.5) ± 1.3	60.2 (66.2) ±1.5
Xylan (xylose)	6.2 (7.0) ± 0.2	4.2(4.8) ±0.2
Acid Insoluble Lignin	34. ± 2.3	33.1±0.7
Prehydrolyzate (monomeric sugars), g/L		
Glucose	0.4±0.02	5.0±0.2
Xylose	8.6±0.5	15.0± 0.5
Arabinose	nd	2.3±0.9
Prehydrolyzate (monomeric sugars after acid hydrolysis), g/L		
Glucose	1.4±0.1	9.4 ±0.6
Xylose	26.1±1.4	30.7±2.4
Arabinose	nd	1.6 ±1.1
Prehydrolyzate (byproducts), g/L		
Formic Acid	0.8±0.1	0.6±0.1
Acetic Acid	5.0±0.3	4.9 ±0.2
HMF	0.2±0.02	0.8±0.1
Furfural	1.4±0.4	3.6±0.9

The high acetic acid concentration after both pretreatments (5.0 g/L) is due to considerable breakdown of hemicellulose during pretreatment since it is formed mainly by the hydrolysis of acetylated β-D-xylopyranose residues in lignocellulose. This is in agreement with the fact that acetic acid has been considered the main acid catalyst in uncatalysed steam explosion pretreatment, though other acids such as formic and levulinic acids may also be produced and may impact overall pretreatment efficiency (Ramos, 2003). Furfural and 5-hydroxymethylfurfural (HMF), degradation products from pentoses and hexoses, respectively, were also detected with higher concentration for the SO_2-catalyzed steam pretreatment. This also accounts for the lower glucose recovery in the WIS resulting from SO_2-catalyzed steam pretreatment. In addition, furfural and HMF could be degraded to other inhibitors such as formic acid, which was present in both prehydrolysates (0.6-0.8 g/L). Formic acid can also, however, be produced from lignin degradation (Klinke *et al*. 2004).

The phenolics syringaldehyde (13 mg/L), coumaric acid (78 mg/L), vanillin (35 mg/L), 4-hydroxybenzaldehyde (80 mg/L) and ferulic acid (350 mg/L) formed by solubilisation and hydrolytic or oxidative cleavage of lignin were also detected in the uncatalysed prehydrolysate. The *p*-coumaric and ferulic acids are major non-core lignin monomers that crosslink plant cell wall polysaccharides to each other and to core lignin (Ralph *et al*., 1992). Their significant concentration in the prehydrolysate thus indicates substantial lignocellulosic network disorganization. It is noteworthy that the concentration of vanillin formed by the degradation of guaiacyl propane (G) units of lignin was significantly higher than syringaldehyde produced by degradation of syringylpropane (S) units of lignin. This result is consistent with the G/S ratio in sugarcane bagasse protoxylem (Sun *et al*. 2003). The H (hydroxy) phenolic 4-hydroxybenzaldehyde is thought to be an extractive component rather than a lignin component (Klinke *et al*., 2004). These results are consistent with the reasoning that bagasse lignins are typical grass lignins composed of syringyl, guaiacyl and *p*-hydroxyphenyl units (SGH-type lignins), with a small amount of esterified *p*-coumaric acid and mainly etherified ferulic acid (Sun *et al*. 2003).

The sugar recovery in the pretreatment step was estimated considering the sugar composition of the different fractions and the solid recovery. The recovery of the main sugars, namely glucose and xylose, for both pretreatments are shown in Figure 1. Almost a complete glucose recovery was obtained with both pretreatments.

However, the impregnation with SO_2 prior to steam pretreatment led to a higher sugar recovery of glucose (5.1 g/100 g sugarcane bagasse) and xylose in the liquid fraction (16.6 g/100 g sugarcane bagasse). The addition of SO_2 during steam pretreatment provided the greatest xylose recovery in the liquid fraction (75.1%) compared to water impregnation (53.5%).

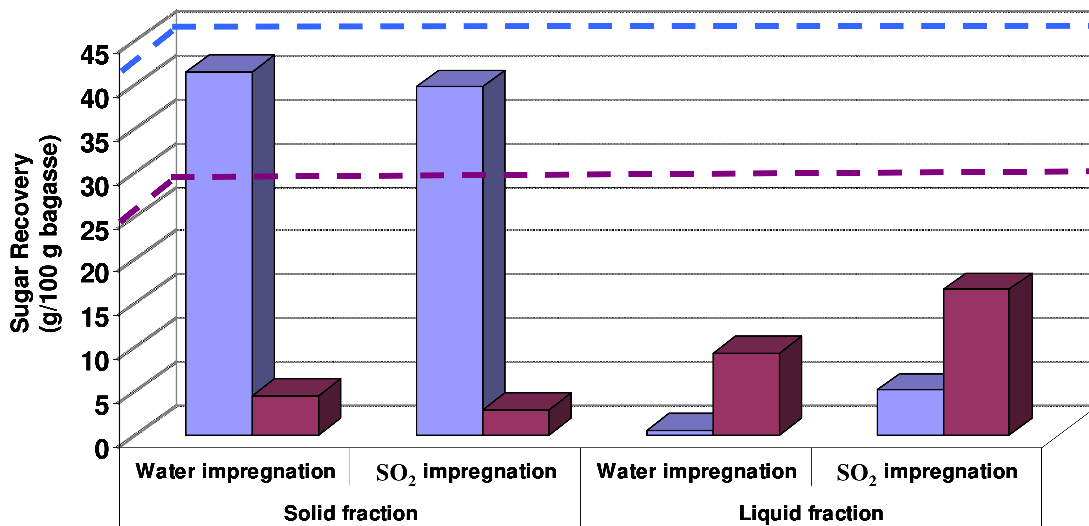

Figure 1: Glucose (■) and xylose (■) recovery, expressed as g/100 g bagasse, on the solid fraction (WIS) and liquid fraction obtained after steam pretreatment with water and SO_2 impregnation. Maximum theoretical yields for glucose (43.6 g/100 g) and xylose (25.9 g/100 g) in unpretreated bagasse. The sugars in the liquid fraction correspond with the monomeric sugars obtained after mild acid hydrolysis of the liquid fraction.

3.4.2.2. Effects of pretreatment on enzymatic hydrolysis of the water insoluble solids

The WIS fraction of each pretreatment was subjected to enzymatic hydrolysis by commercial enzyme preparations at a solid loading of 2% (w/v). Since enzyme cost is one of the main contributors in the global ethanol production process, different enzyme dosages were evaluated. The standard enzyme dosage of 15 FPU cellulase/ g WIS was tested with and without the addition of 15 IU β-glucosidase/g WIS. In addition, a 3-fold lower concentration of the conventional enzyme mixture (5 FPU/g WIS supplemented with 5 IU β-glucosidase/g WIS) was tested.

The cellulose digestibility (expressed as percentage of glucose and cellobiose released in relation to potential cellulose in the WIS) of the different WIS at 24, 72 and 120 hours are represented in Figure 2. After pretreatment, the enzymatic conversion from cellulose to glucose and cellobiose increased between 3.6- and 6.6-fold compared to that of untreated sugarcane bagasse (data not shown). Pretreatment

alters the biomass chemical composition and structure, rendering the resulting WIS less recalcitrant such that enzymatic hydrolysis of the cellulose fraction can be achieved more rapidly and with greater yields. From this preliminary result, it could be concluded that it was essential to pretreat raw sugarcane bagasse prior to enzymatic hydrolysis.

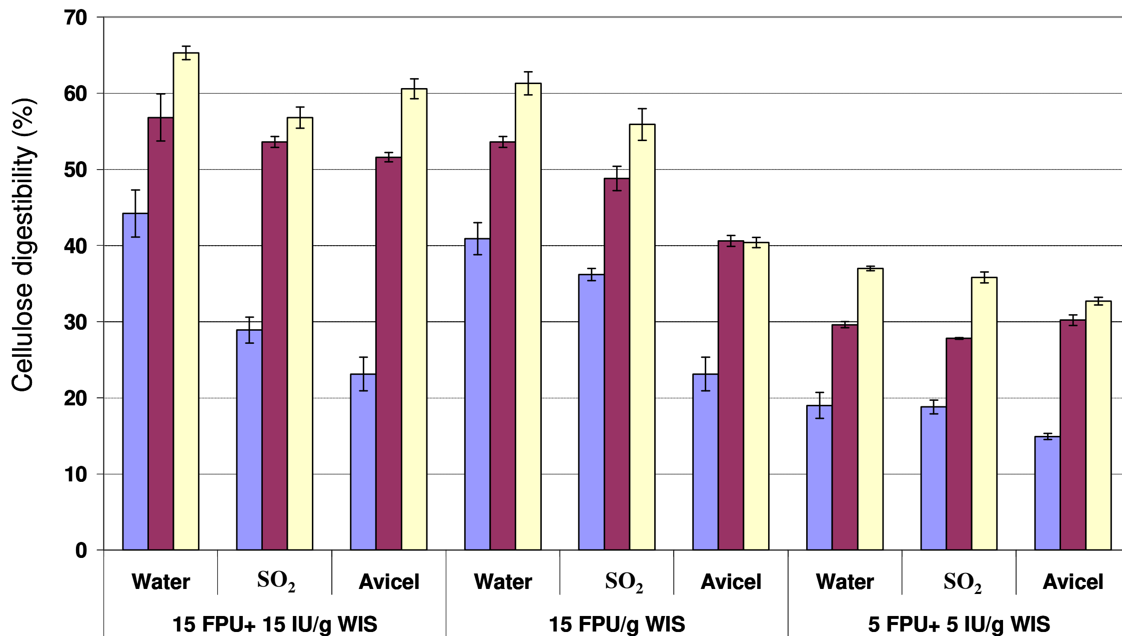

Figure 2: Cellulose digestibility with Celluclast® 1.5L (FPU) and β-glucosidase (IU) of the different WIS expressed as percentage during enzymatic hydrolysis at 24 h (■), 72 h (■) and120 h (■). Commercial cellulose (Avicel) was used as a control. Results represent the mean values from three separate experiments; error bars represent the standard deviation.

The WIS obtained from un-catalyzed steam pretreatment was slightly more digestible than that from SO_2-catalyzed pretreatment, especially at higher cellulase (FPU) dosages. The greatest cellulose digestibility (65.3%) was obtained at 120 hours when 15 FPU Celluclast® 1.5L was supplemented with 15 IU β-glucosidase/ g WIS resulting from un-catalyzed steam pretreatment. At the lower enzyme dosage (5 FPU Celluclast® 1.5L), these differences were not statistically significant. At this dosage, incubation periods up to 120 hours did not equal the yields attained at higher enzyme dosages.

Since both WIS contained residual xylan (Table 3), the xylan conversion during enzymatic hydrolysis was also evaluated. Xylan conversion ranged from 6.6% to 45.0% (data not shown). A similar trend to the cellulose digestibility was observed for xylan conversion. Higher values were obtained when water was used as

impregnating agent prior to pretreatment for all enzyme dosages. The greater xylan conversion (~45%) was obtained when the enzymatic hydrolysis was performed with 15 FPU cellulase supplemented with 15 IU β-glucosidase/g WIS at 120 hours.

The efficacy of enzymatic hydrolysis of lignocellulose is impacted by non-productive binding of cellulases and hemicellulases with lignin, inhibition of enzymes by carbohydrate oligomers, the released sugars and their degradation products (Boussaid and Saddler 1999; Palonen *et al.*, 2004; Xiao *et al.*, 2004; Kumar and Wyman, 2008) and mechanical shear that may denature/ degrade enzymes (Jørgensen *et al.*, 2007). Thus, complete inhibition and/or degradation of enzymes could have resulted in lower enzyme dosages resulting in much lower maximum yields after 120 hours of hydrolysis. The highest yields were attained with β-glucosidase supplementation because in the absence of this activity, cellobiose, the major cellulase inhibitor, will accumulate and cause feedback inhibition.

3.4.2.3. Overall sugar yield

The overall sugar yield was determined taking into account the sugar solubilized during pretreatment and the sugar released during the enzymatic hydrolysis of the solid residue. The results for the main sugars, glucose and xylose, are depicted in Figure 3A. Both pretreatment conditions provided a similar overall sugar yield of 39.7 and 42.2 g/100 g bagasse (dw) when water and SO_2 impregnation were used, respectively. However, the contribution of each sugar was relatively different. A glucose and xylose yield of 28.1 g and 11.5 g/100 g bagasse, respectively, were obtained in the water pretreatment, whereas the xylose yield (17.8 g/100 g bagasse) was closer to the glucose yield (24.7 g/100 g bagasse) in the second pretreatment.

The overall sugar yield obtained in both pretreatments was used to estimate the theoretical ethanol yield (expressed as L/ dry ton of bagasse) that could be produced considering an ethanol fermentation yield of 0.44 g ethanol/g sugar consumed (Tomás-Pejó *et al.*, 2008) (Figure 3B). A potential ethanol yield of 157 and 137 L/ton of bagasse (dw) could be obtained based on the overall glucose yield for the water and SO_2–catalyzed steam pretreatment, respectively. These amounts could be increased up to 221 and 235 L/ton of bagasse (dw), if glucose and xylose are co-fermented.

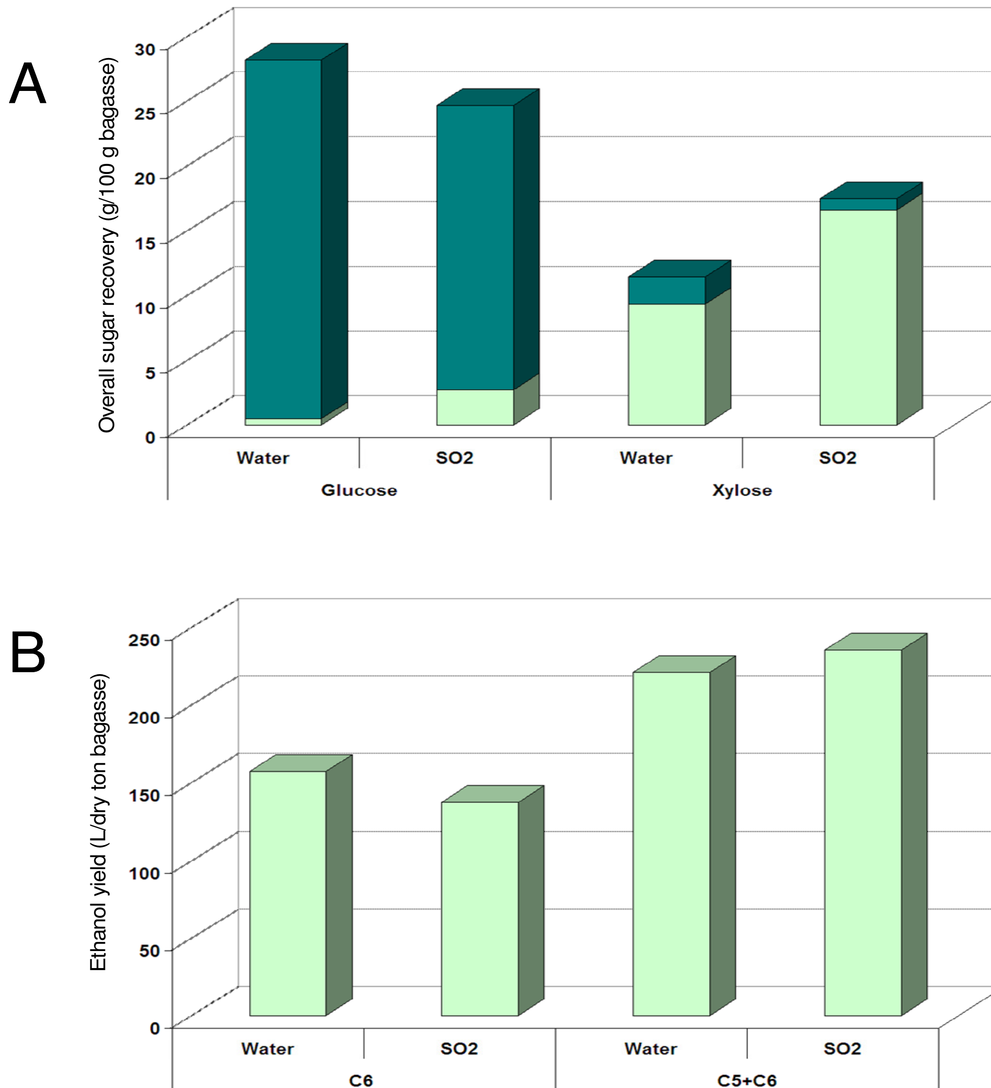

Figure 3: (A) Overall sugar yield, expressed as g/100 g of raw material, of glucose and xylose after pretreatment (■) and enzymatic hydrolysis at 120 hours (■) with 15 FPU Celluclast® 1.5L and 15 IU ß-glucosidase/g WIS for steam pretreatment with water and SO_2 impregnation. (B) Estimation of ethanol yield (L/dry ton of bagasse) based on a fermentation yield of 0.44 g ethanol/ g sugar consumed. The estimation considered the overall glucose recovery (C6) and overall sugar recovery (C5+C6) represented in Figure 3A.

3.5. Conclusions

This study showed that steam pretreatment is an efficient method to increase the enzymatic accessibility of the water-insoluble, cellulose-rich component in sugarcane bagasse. After pretreatment, the enzymatic conversion from cellulose to glucose and cellobiose increased up to 6.6 times, compared to that of untreated bagasse. SO_2 impregnation prior to pretreatment at less severe conditions provided similar cellulose digestibility whilst increasing the xylose recovery (75.1%) compared

to the un-catalyzed steam treatment (53.3%) carried out at a higher severity. However, further optimization of the enzyme cocktail is required to increase the enzymatic hydrolysis yield obtained with the conventional enzyme dosage for both pretreatments.

3.6. Acknowledgements

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CHAPTER 4

OPTIMIZATION OF ENZYMATIC SACCHARIFICATION OF STEAM EXPLODED SUGARCANE BAGASSE USING DIFFERENT COMMERCIAL ENZYME COCKTAILS

Optimization of enzymatic saccharification of steam exploded sugarcane bagasse using different commercial enzyme cocktails

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Keywords: sugarcane bagasse; Steam Explosion Pretreatment (STEX); cellulase; β-glucosidase, xylanase.

4.1. Abstract

Though enzymatic saccharification is promising in obtaining sugars from lignocellulosics, the low enzymatic accessibility of the cellulose and hemicellulose is a key impediment, thus necessitating development of optimized enzyme mixtures with essential accessory activities. Response Surface Methodology (RSM) based on the $3^{(3)}$ factorial design was used to optimize the composition of a saccharolytic enzyme mixture so as to maximize glucose and xylose production from steam exploded sugarcane bagasse. The multi-enzyme mixture allowed for a significantly higher, approximately 2-fold, conversion of bagasse at lower enzyme loadings compared to using a single cellulase cocktail. This study confirms that saccharolytic enzymes exhibit synergism and that bagasse is a potential renewable substrate for the enzymatic production of sugars for fermentation to bioethanol.

4.2. Introduction

Current technologies for the conversion of lignocellulose to bioethanol require a pretreatment step prior to enzymatic hydrolysis into fermentable sugars, such as glucose and xylose. Hydrothermal treatment such as steam explosion is an interesting option because of its limited use of chemicals, low energy consumption, short reaction time and, depending on the conditions used, high sugar recovery (Chandra *et al.*, 2007). Besides, it has been shown to be an effective method to disrupt the lignocellulose structure of agricultural residues (Kaar *et al.*, 1998; Martin *et al.*, 2002, 2006).

Although biological enzyme production cost has recently been reduced considerably (Moreira, 2005; Balat *et al.*, 2008), cellulase production and its use remains one of the most expensive steps during ethanol production from cellulosic biomass, in that it can account for up to 40% of global costs (Sainz, 2009). Thus, significant cost reduction is required in order to enhance the commercial viability of the cellulase production technology. Furthermore, the main obstacle preventing a large-scale utilization of lignocellulose for liquid fuel production is the requirement of uneconomically high enzyme loadings to achieve high saccharification yields due to nonproductive binding of cellulase and hemicellulases with lignin and other portions of the lignocellulose and inhibition by carbohydrate oligomers, the released sugars and their degradation products (Boussaid and Saddler 1999; Palonen *et al.*, 2004; Xiao *et al.*, 2004; Kumar and Wyman, 2008). Thus, development of methods to increase enzyme effectiveness is critical in order to make lignocellulose hydrolysis for ethanol production economically feasible.

Cellulases are a group of enzymes that include endoglucanases that hydrolyze the cellulose polymer exposing reducing and non-reducing ends of the linear polymer of glucose units; exoglucanases and cellobiohydrolases that act on these ends to release cellobiose and cellooligosaccharides, and β-glucosidases (BGL) that cleaves the cellobiose units to liberate glucose – the end product.

All these enzymes work synergistically to hydrolyse cellulose by creating new accessible sites for each other, removing obstacles and relieving product inhibition. It is well documented that the conjugated action of cellulases, hemicellulases and accessory enzymes results in a higher final sugar production as compared to cellulases alone (Adsul *et al.*, 2005; Tabka *et al.*, 2006; Berlin *et al.*, 2007; Sorensen *et al.*,

2007; Prior and Day, 2008; Kumar and Wyman, 2009a). It is suggested that the so called ''accessory'' enzymes such as xylanase, FAE and pectinase stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers. Extensive research is being done for improving the hydrolytic efficiency of such enzymes. One strategy is the optimization of the cellulase mixture by varying components and their ratios. In this context, the addition of accessory enzymes such as xylanases can improve the cellulose accessibility and hence, the cellulose conversion.

However, lignocellulosics show significant quantitative and qualitative differences in their non-cellulosic polysaccharide components with further differences in composition introduced according to the pretreatment technology employed. It has thus been suggested that enzyme mixtures could be customized for particular feedstocks and pretreatments in order to optimize hydrolysis (Berlin *et al.*, 2005; Eggeman and Elander, 2005).

In the present study, enzyme preparations available from Novozymes A/S (Bagsvaerd, Denmark) and Genencor® A Danisco Division US, Inc. (Rochester, NY) were assessed for their efficiency in hydrolysing sugarcane bagasse. Firstly, these enzyme cocktails were analyzed for their cellulase, cellobiase and xylanase activities. Subsequently, the efficiency of different combinations of the cellulase, β-glucosidase and hemicellulase enzyme preparations on the hydrolysis of steam-exploded (210ºC during 5 minutes) sugarcane bagasse was evaluated.

4.3. Materials and methods

4.3.1. Substrate and chemicals

Raw bagasse was supplied by TSB Sugar, Mpumalanga, South Africa. All chemicals, media components and supplements used are of analytical grade standard and were obtained from BDH Chemicals Ltd. (Poole, England), Merck (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, USA).

4.3.2. Pretreatment of sugarcane bagasse

Uncatalysed Steam Explosion (STEX) pretreatment of sugarcane bagasse was generously performed at CIEMAT Biomass Unit (CIEMAT- Renewable Energies Department, Av. Complutense, 22, 28040 Madrid, Spain) as described in Chapter 3

(section 3.3.2.). The solid fraction [Water Insoluble Solids (WIS)] was water-washed and used for enzymatic hydrolysis.

4.3.3. Compositional analysis of Sugar Cane Bagasse and Pretreated Bagasse

The carbohydrate, lignin, ash and extractives composition was determined as previously described (Chapter 3, section 3.3.6.).

4.3.4. Enzymes

Celluclast® 1.5L (74 FPU/ml) and Novozyme 188 (740 IU/ml) were acquired from Novozymes A/S (Bagsvaerd, Denmark), while Optimash™ VR (615 IU/ml) was supplied by Genencor® A Danisco Division US, Inc. (Rochester, NY). Filter paper (FPU) and β-glucosidase activities were determined using the method of Ghose (1987). The xylanase activities were determined by the method of the University of New South Wales, Kensington, Australia Laboratory as described by Ghose and Bisaria (1987). The main characteristics of each enzyme cocktail are summarized and discussed in Chapter 3 (section 3.3.5.).

4.3.5. Protein assays

Protein concentrations were determined using the Bio-Rad Bradford protein assay (Bio-Rad laboratories, München, Germany) as prescribed for the microtiter plate protocol. A standard curve was compiled using (0.05 to 0.5 mg/mL) bovine serum albumin.

4.3.6. Experimental design

A statistically designed experimental approach was used to optimize the composition of the cellulase mixture so as to maximize the amount of sugars produced from the steam-exploded bagasse. Experimental designs were developed and analysed using the STATISTICA 9.0 (Statsoft, Inc., Tulsa, OK, USA) software. A Fractional Factorial Design (FFD) was used to investigate the statistical significances of the factors, i.e. cellulase, β-glucosidase and xylanase loading, on glucose production by enzymatic hydrolysis. High, intermediate and low levels of the factors were considered in the statistical designs and were expressed in coded values for convenience. Low and high levels were coded as –1 and +1, and the midpoint was coded as 0 (Table 1). The experimental design matrix is shown in Tables 2 and 3. The

analysis of the results of this design included the computation of the linear (L), quadratic (Q) and interaction effects, the analyses of the variances (ANOVA) ascribed to them and response surface methodology (RSM). Final glucose concentration and conversion of WIS to glucose were considered as response variables for the process analysis.

Table 1: Factors and code values for enzymatic saccharification fractional factorial designs

Factors	**Level of factors (IU/g substrate)**		
	-1	**0**	**+1**
FPU (Celluclast® 1.5L)	10	15	20
β-glucosidase (Novozyme 188)	20	30	40
Xylanase (Optimash™ VR)	240	320	400

Table 2: $3^{(3)}$ fractional factorial design (1 block, 9 runs) for optimisation of WIS and slurry hydrolysis with different combinations of cellulase (FPU), β-glucosidase and xylanase

Run	**Cellulase (FPU/ g substrate)**	**β-glucosidase (IU/ g substrate)**	**Xylanase (IU/g substrate)**
9	20	40	400
2	10	30	400
6	15	40	240
3	10	40	320
5	15	30	320
1	10	20	240
8	20	30	240
4	15	20	400
7	20	20	320

Table 3: $3^{(3)}$ FFD (1 block, 9 runs) for optimisation of WIS and slurry hydrolysis with different combinations of cellulase (FPU), β-glucosidase and xylanase, coded values

Run	**FPU**	**β-glucosidase**	**Xylanase**
9	1	1	1
2	-1	0	1
6	0	1	-1
3	-1	1	0
5	0	0	0
1	-1	-1	-1
8	1	0	-1
4	0	-1	1
7	1	-1	0

4.3.7. Enzymatic hydrolysis

The enzymatic hydrolysis experiments were carried out at 50°C in 0.05 M citrate buffer at pH 4.8 whilst shaking at 150 rpm. Optimization of the saccharolytic enzyme complex was conducted in 25 ml McCartney bottles at 2% (w/v) substrate loading and an initial reaction volume of 10 ml. Sodium azide was added to the buffer to a final concentration of 0.02% to avoid bacterial growth. Enzyme loadings of 10-20 FPU/g substrate of cellulase (Celluclast® 1.5L) and 15-50 UI/g substrate β-glucosidase (Novozyme 188) were used for hydrolysis. Evaluation of the effect of substrate concentration [4%, 6%, 8% and 10% (w/v)] on enzymatic hydrolysis, using the optimized enzyme cocktail, was conducted in 100 ml Schott bottles with a starting volume of 50 ml. Samples were periodically withdrawn from the reaction from 0 to 120 h, heated at 95°C for 10 min to stop the reaction, centrifuged at 10,000 rpm in Eppendorf tubes to remove residual substrate, and analyzed for glucose, xylose, arabinose, cellobiose, acetic acid and glycerol by HPLC. Samples were stored at -20°C and thawed on ice prior to analysis.

4.3.8. Analytical methods

High performance liquid chromatography was conducted on a Surveyor Plus liquid chromatograph (Thermo Scientific) consisting of a LC pump, autosampler and RI detector. The Rezex RHM polymer-based column (300 x 7.8 mm) was used to separate glucose, xylose, arabinose, cellobiose, acetic acid, glycerol and ethanol concentrations in 0.22 µm filtered samples. The column temperature was maintained at 40°C using a Gecko 2000 column heater. The mobile phase was HPLC grade water at a flow rate of 0.6 ml/min.

Analysis of the inhibitory compounds, formic acid, acetic acid, 5-hydroxymethylfurfural (HMF), furfural, 4-hydroxybenzaldehyde, vanillin, syringaldehyde, ferulic acid and coumaric acid, was done by HPLC while the total phenolic acid composition was determined by the Folin–Ciocalteau method as described by Singleton *et al*. (1999).

4.3.9. Calculations

The glucose yield as % of the theoretical yield (% digestibility) was calculated using the following formula:

$$\textit{Glucose yield (\%)} = \frac{[Glucose] + 1.053\ [Cellobiose]}{1.111 f\ [Biomass]} \times 100\% \quad (1)$$

Where:

[Glucose]	Residual glucose concentration (g/L)
[Cellobiose]	Residual cellobiose concentration (g/L)
[Biomass]	Dry biomass concentration at the beginning of hydrolysis (g/L)
f	Cellulose fraction in dry biomass (g/g)
1.053	Converts cellobiose to equivalent glucose.
1.111	Converts cellulose to equivalent glucose

4.4. Results and Discussion

4.4.1. Chemical composition of substrates

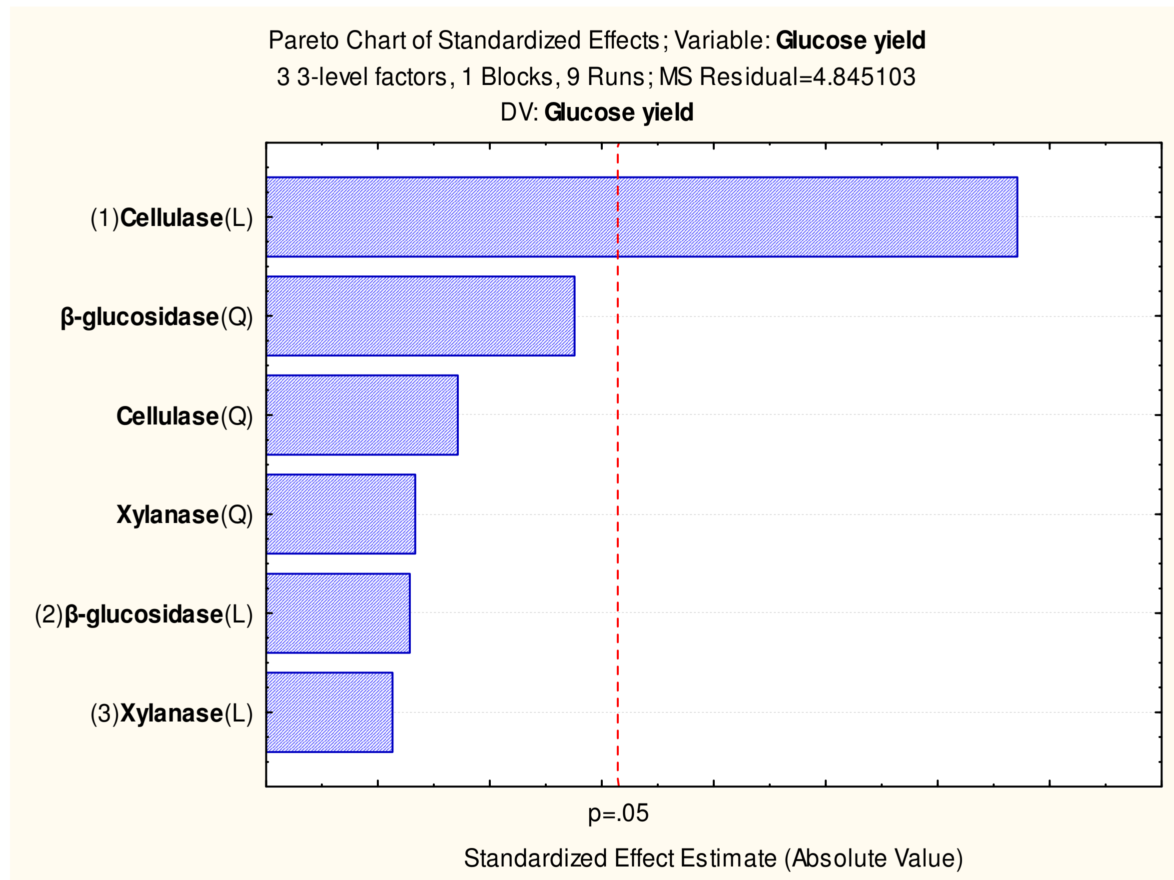
The chemical composition of untreated and STEX pretreated bagasse are discussed in chapter 3. Sugarcane bagasse steam pretreated with water impregnation was used in the optimization of saccharification due to its higher sugar recovery and enzymatic digestibility (chapter 3, 3.4.2).

4.4.2. Optimization of WIS saccharification

The significances of cellulase (FPU), β–glucosidase and xylanase on glucose yield from WIS hydrolysis at 24 hours of hydrolysis are shown in table 4 and depicted in the Pareto chart of standardized effects (Figure 1). ANOVA (STATISTICA 9.0 (Statsoft, Inc., Tulsa, OK, USA) to determine the statistical significance of the effect of each enzyme activity on enzymatic hydrolysis yield revealed that the regression was statistically significant at a 95% confidence level for cellulase (FPU) loading (Figure 1). The determination coefficient for the model was very high (R^2 = 0.98646; R^2 adjusted = 0.94582) (Figure 1) indicating that the model was able to comprehend 98.6% of the variability in the enzymatic saccharification of WIS.

Table 4: Experimental design and results of $3^{(3)}$ fractional factorial design (1 block, 9 runs) for WIS saccharification at 24 hours. Predicted values were computed during the analysis of the experimental design with STATISTICA 9.0

Runs	Experimental factors and coded values			Glucose yield (%)	
	FPU	β-glucosidase	Xylanase	Experimental	Predicted
9	1	1	1	87.64	88.25
2	-1	0	1	74.14	72.68
6	0	1	-1	82.14	80.68
3	-1	1	0	66.07	66.92
5	0	0	0	83.75	84.36
1	-1	-1	-1	66.56	67.17
8	1	0	-1	92.83	93.68
4	0	-1	1	78.34	79.19
7	1	-1	0	87.89	86.43



R^2=0.98646; R^2 Adj=0.94582

Figure 1: Pareto chart of standardized effect estimates for $3^{(3)}$ fractional factorial experimental design for the optimisatiom of WIS glucan enzymatic hydrolysis, to glucose and cellobiose, at 24 hours. Standardized effects are calculated by dividing the effect by its standard error. This Pareto chart shows that Cellulase loading had the most significant positive effect on enzymatic hydrolysis whilst the xylanase had the least effect

Determination of the optimal enzyme mixture for maximum cellulose hydrolysis was performed using the response *desirability* function (Derringer and Suich, 1980) (Figure 2). This method allowed for the definition of values of each component of the saccharolytic mixture that maximize the saccharification of WIS polysaccharides. Celluclast® 1.5 L together with the β–glucosidase preparation Novozyme 188 resulted in a significant increase in glucose yield (expressed as percentage of glucose and cellobiose released in relation to potential cellulose in the WIS, Equation 1). It is well documented that *T. reesei* cellulase complexes are

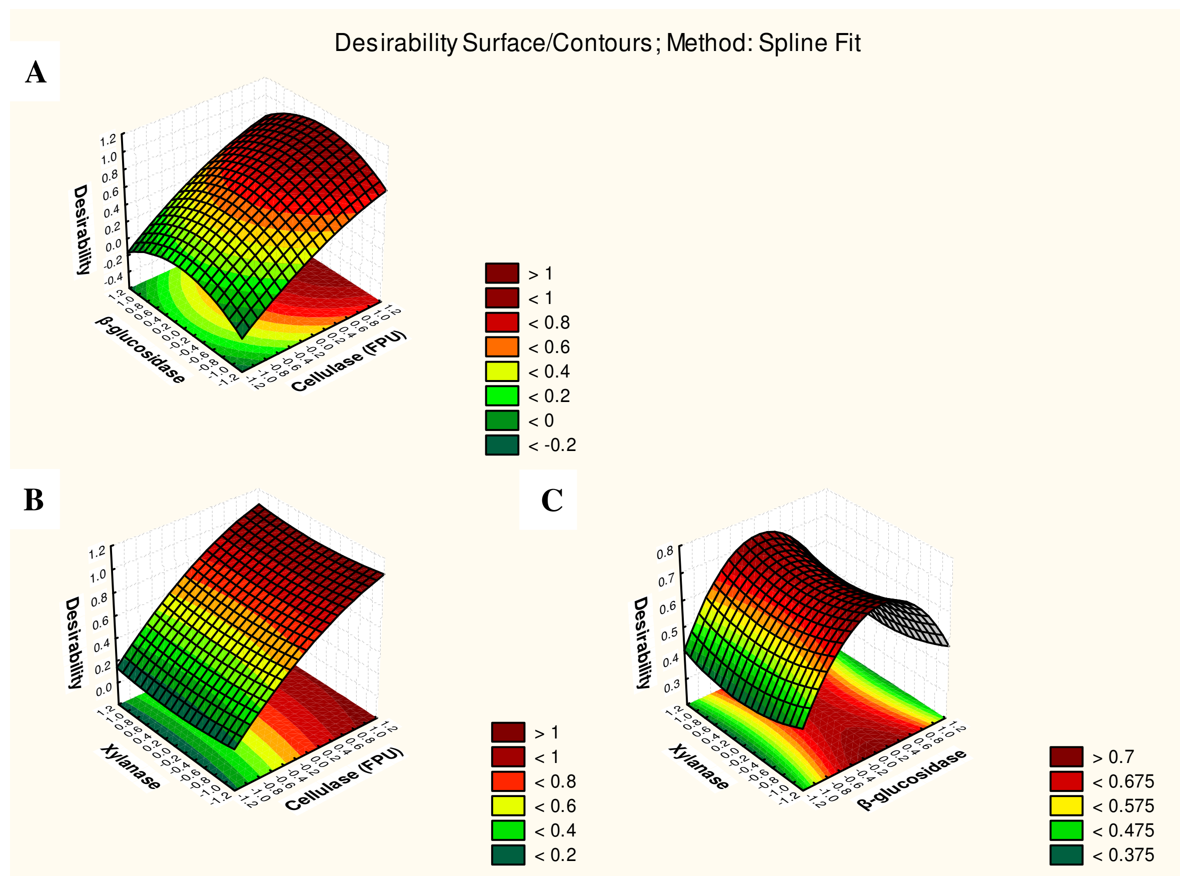


Figure 2: Response surface plots for desirability function of $3^{(3)}$ fractional factorial experimental design for the optimisation of WIS hydrolysis at 24 hours. Displays profiling to inspect the predicted values for glucose yield at different combinations of levels of cellulase, β–glucosidase and xylanase (A. cellulase: β–glucosidase; B. Xylanase: cellulase; C. Xylanase: β–glucosidase), to specify desirability functions for glucose yield, and to specify a search for the levels of cellulase, β–glucosidase and xylanase that produce the most desirable response on glucose yield.

typically low in β–glucosidase activity (Duff and Murray, 1996; Nieves *et al.*, 1998). Thus cellulose hydrolysis by these cellulase complexes results in the accumulation of cellobiose which in turn results in significant cellulase inhibition. As a result, cellobiose (produced by the action of cellobiohydrolases) accumulates resulting in

significant cellulase inhibition during cellulose hydrolysis. Cellobiose is the major inhibitor of cellulases, reducing cellulase activity by 60% at a concentration as low as 6 g/L (Philippidis *et al*., 1993). In accordance with several previous reports on the effect of β-glucosidase supplementation on cellulose hydrolysis (Berlin *et al*., 2007; Prior and Day, 2008; Kumar and Wyman, 2009), the data presented in Figure 2 demonstrates that addition of β-glucosidase to Celluclast® 1.5L improves the overall glucose yield.

However, supplementation of Celluclast® 1.5L with Novozyme 188 (β–glucosidase) showed a parabolic concentration dependence of the glucose yield with a distinct maximum (Figures 2) of about 30 IU/g substrate. The parabolic effect observed with the supplementation of Celluclast® 1.5 L with β–glucosidase and xylanase activities was also observed by Berlin *et al*. (2007) and Zhou *et al*. (2009) among others, when they investigated the optimization of other cellulase mixtures. The mechanism underlying this phenomenon is currently unknown, but these results indicate that a strategy involving the addition of supplementary enzymes in excess is to be avoided, even if their production costs can be ignored. The authors, however, speculated that this negative effect observed at higher levels of supplementation could have resulted from (i) competition for productive binding sites amongst enzymes, and (ii) saturation of the individual enzyme.

It has been determined that synergism is greatest when each cellulase is present at a non-saturating concentration (Woodward *et al*., 1988). Furthermore, Woodward *et al*. (1988) concluded that the greatest degree of synergism was obtained at total enzyme concentrations below that needed to saturate the available binding sites on the substrate. Their findings further proved that a minimum amount of surface area was needed for an individual cellulase to be effective in hydrolyzing cellulose and the hydrolytic efficiency will decrease when the total cellulase concentration exceeds saturation. Furthermore, the possibility that some hydrolysis by-products may influence enzyme accessibility to the cellulose microfibrils and the interaction of individual enzyme could not be excluded. Further experiments should be performed in order to confirm this assumption.

Contrary to previous reports, supplementation with the xylanase Optimash VR did not increase the glucose yield over the values obtained for cellulase and β-glucosidase alone (Figures 1 and 3). Instead, the glucose yield decreased with

increasing xylanase concentration. It is worth noting that the xylan composition of the WIS is very low (6.1%). Thus the negative effect of xylanase supplementation could have been due to competition for binding sites amongst the enzymes. This phenomenon was more pronounced in the early (less than 24 hours) stages of saccharification (Figure 3). Figure 4 shows that the xylanase Optimash VR adsorbs to Avicel, resulting in a drop in protein concentration in the supernatant.

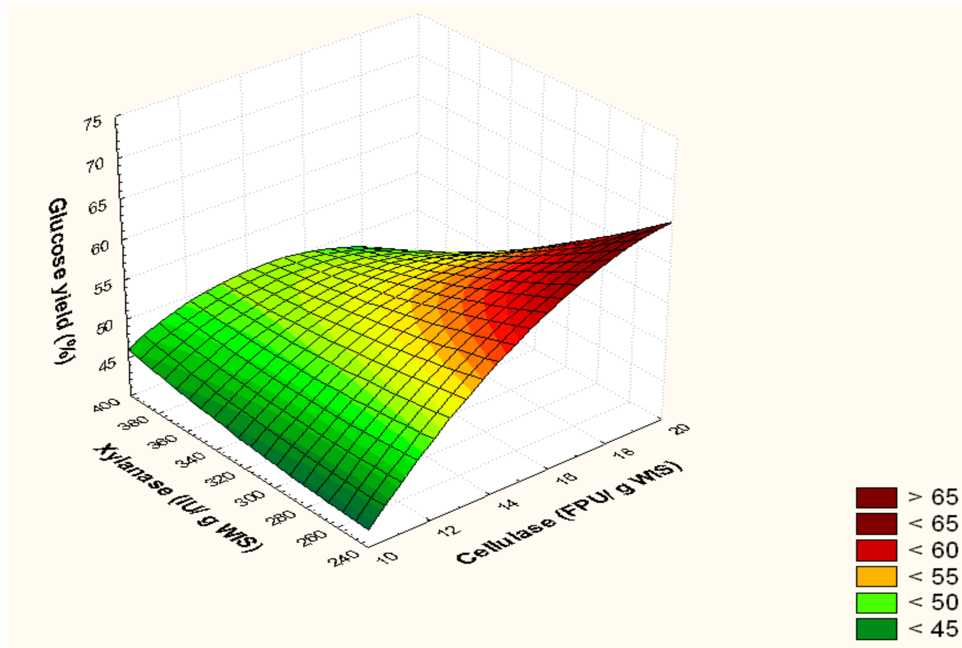


Figure 3: 3D surface plot showing the interactive effect of cellulase (FPU/ g WIS) and xylanase (IU/ g WIS) on glucose yield after 6 hours of WIS (2%, w/v) hydrolysis.

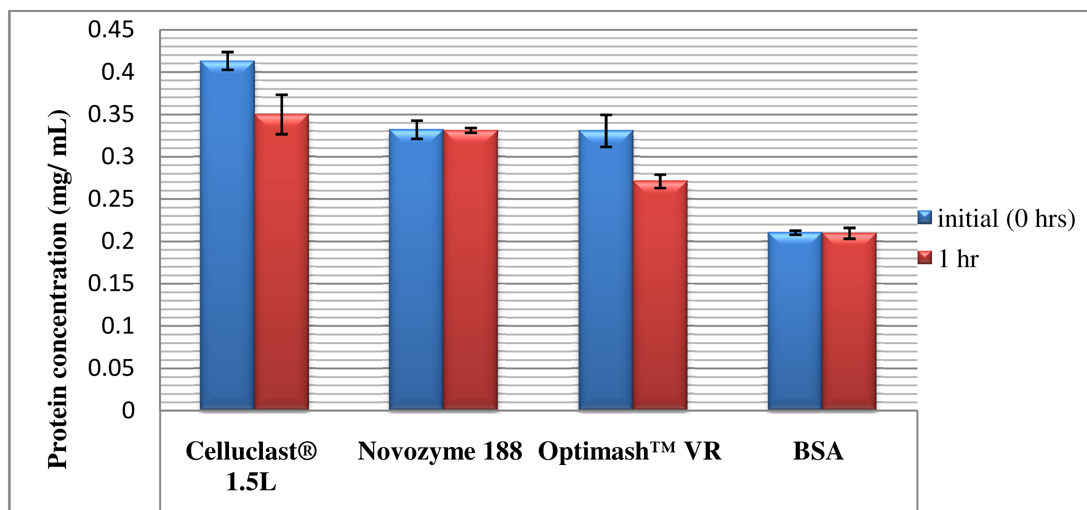
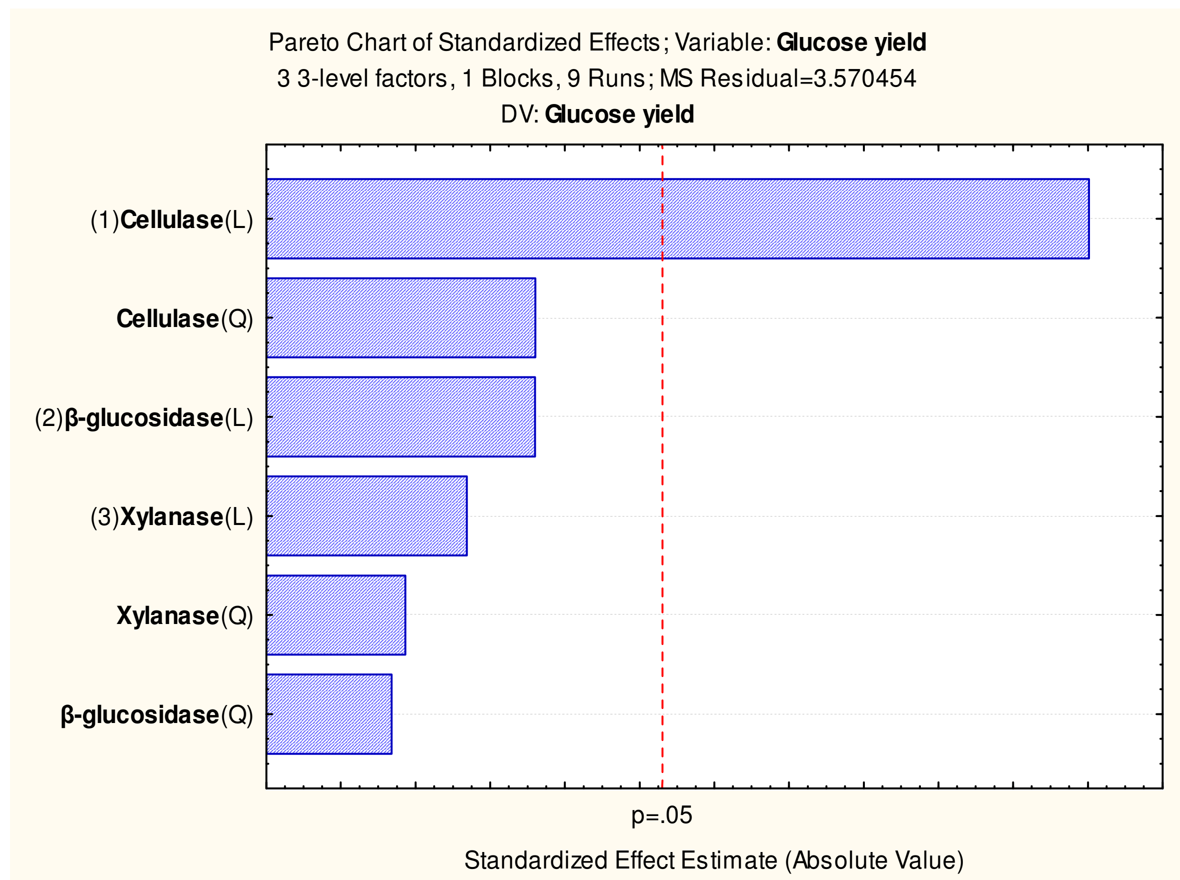


Figure 4: Adsorption of different proteins on avicel (2% w/v) after incubation for 1 hr at room temperature. Initial protein concentration in supernatant (■), final protein concentration in supernatant after 1 hr of incubation (■). Results represent the mean values from three separate experiments; error bars represent the standard deviation.

4.4.3. Optimization of slurry saccharification

As for the WIS, the Pareto chart for glucose yield from the enzymatic saccharification of the slurry (Figure 5) shows that the regression was statistically significant at a 95% confidence level for the cellulase (FPU) loading (Figure 5). Furthermore, the model also presented a high determination coefficient (R^2 = 0.98332; R^2 adjusted = 0.93327) (Figure 5), explaining 98.3% of the variability in the enzymatic saccharification of WIS.



R^2=0.98332; R^2 Adj=0.93327

Figure 5: Pareto chart of standardized effect estimates for $3^{(3)}$ fractional factorial experimental design for the optimisation of slurry enzymatic hydrolysis at 24 hours to glucose. Standardized effects are calculated by dividing the effect by its standard error. This Pareto chart shows that Cellulase loading had the most significant positive effect on enzymatic hydrolysis whilst the xylanase had the least effect.

The parabolic concentration-dependence of the glucose yield observed with the β-glucosidase on WIS was also not observed with the whole slurry (Figures 6) at 24 hours. This could be attributed to the fact that the slurry still contains the water

soluble solids. The water soluble solids consist not only of the hemicellulose fraction, but also solubilised cello-oligosaccharides (Chapter 3, section 3.4.2.1.). Thus the excess β-glucosidase that was not involved in the synergistic hydrolysis of the cellulose fraction of the WIS in the slurry could have been utilized in breaking down the cello-oligosaccharides to glucose. Also, even though the xylanase did not have a significant positive effect, there was neither a negative effect observed. This could be attributed to the fact that the slurry still contains the hemicellulose fraction, hence there was minimum competition for cellulose binding sites between the xylanase and the cellulases. These results confirm the notion that it is critical to customize the saccharolytic enzyme mixture for each specific feedstock in order to optimize hydrolysis.

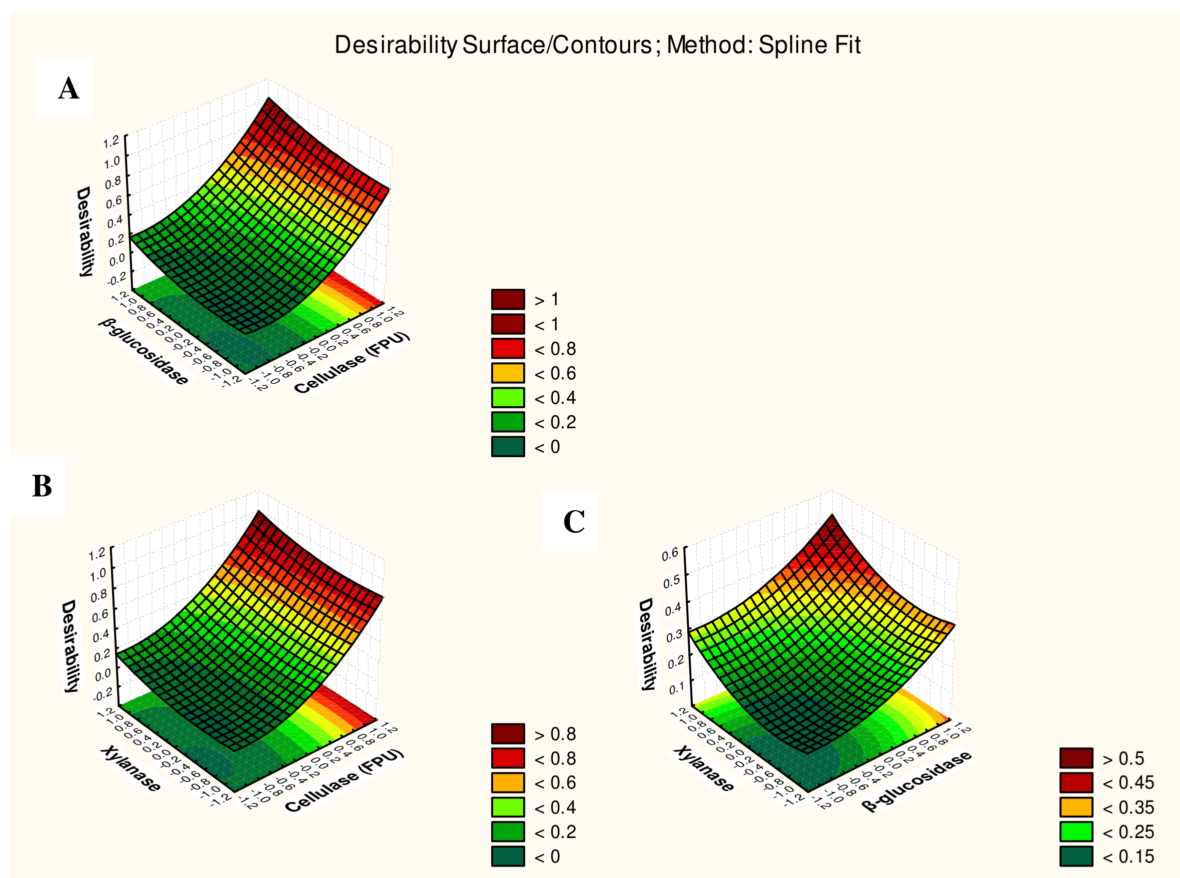


Figure 6: Response surface plots for desirability function of $3^{(3)}$ fractional factorial experimental design for optimisation of slurry enzymatic hydrolysis at 24hours. Displays profiling to inspect the predicted values for glucose yield at different combinations of levels of cellulase, β–glucosidase and xylanase (A. cellulase: β–glucosidase; B. Xylanase: cellulase; C. Xylanase: β–glucosidase), to specify desirability functions for glucose yield, and to specify a search for the levels of cellulase, β–glucosidase and xylanase that produce the most desirable response on glucose yield.

4.4.4. Effect of substrate loading on enzymatic hydrolysis of WIS

The time course of saccharification on 4%, 6%, 8% and 10% (w/v) WIS is illustrated in Figures 7 to10. Saccharification of WIS at all substrate concentrations showed the same kinetics with insignificant yields of cellobiose and xylose. The cellobiose concentration remained low indicating that the β–glucosidase remained active throughout the saccharification. Negligible xylose concentrations were released as the xylan concentration of the WIS was very low and there was no xylanase activity added to the saccharolytic enzyme mixture. The glucose concentration increased exponentially for the first 12 hours and plateaued after that.

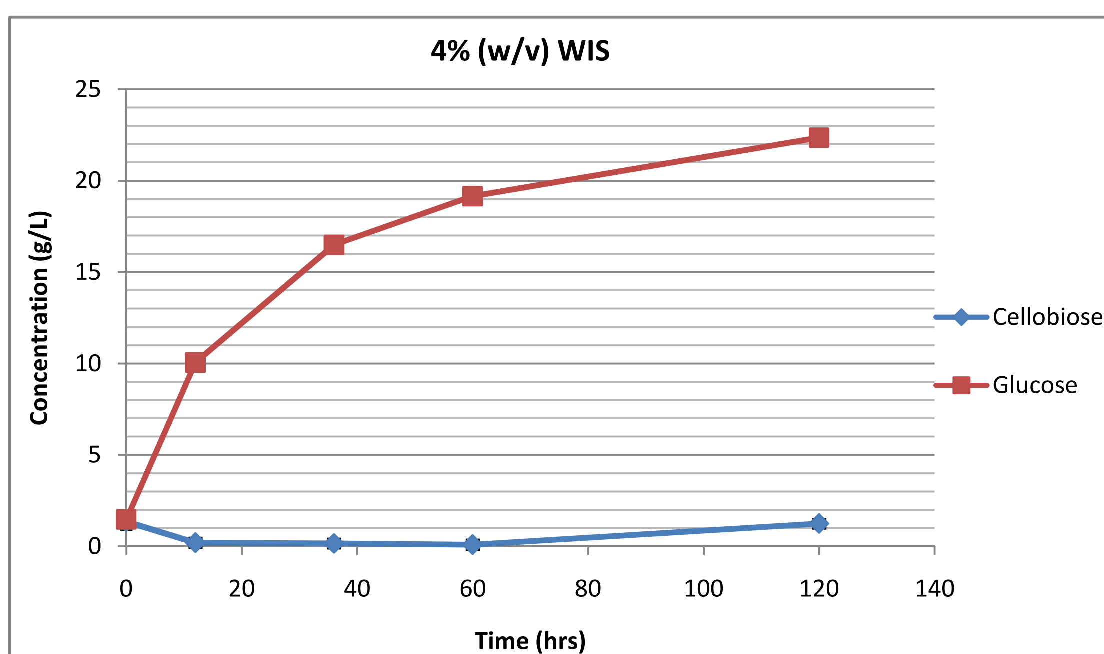


Figure 7: Enzymatic saccharification of WIS (4%, w/v) by the optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS).

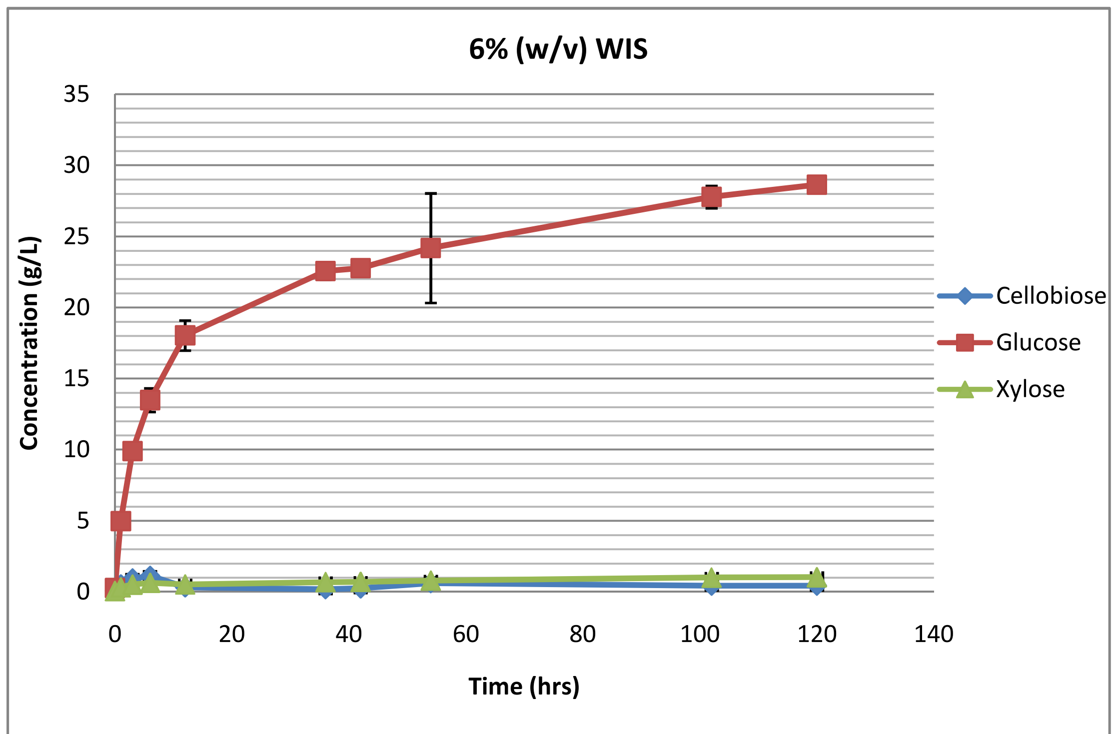


Figure 8: Enzymatic saccharification of WIS (6%, w/v) by the optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS).

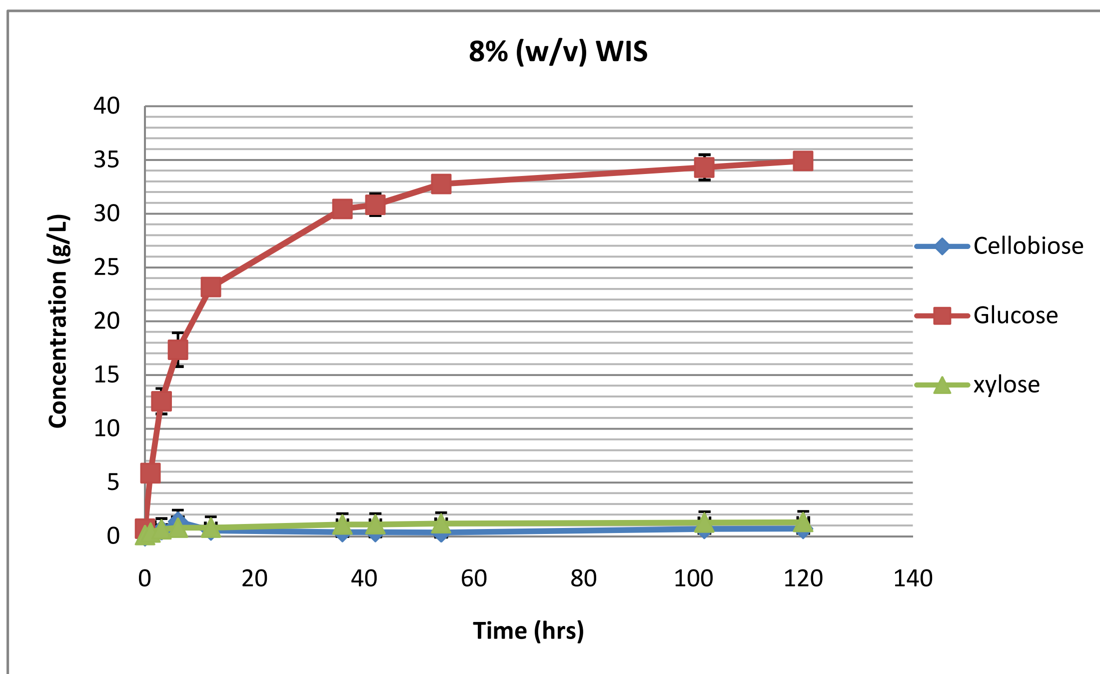


Figure 9: Enzymatic saccharification of WIS (8%, w/v) by the optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS).

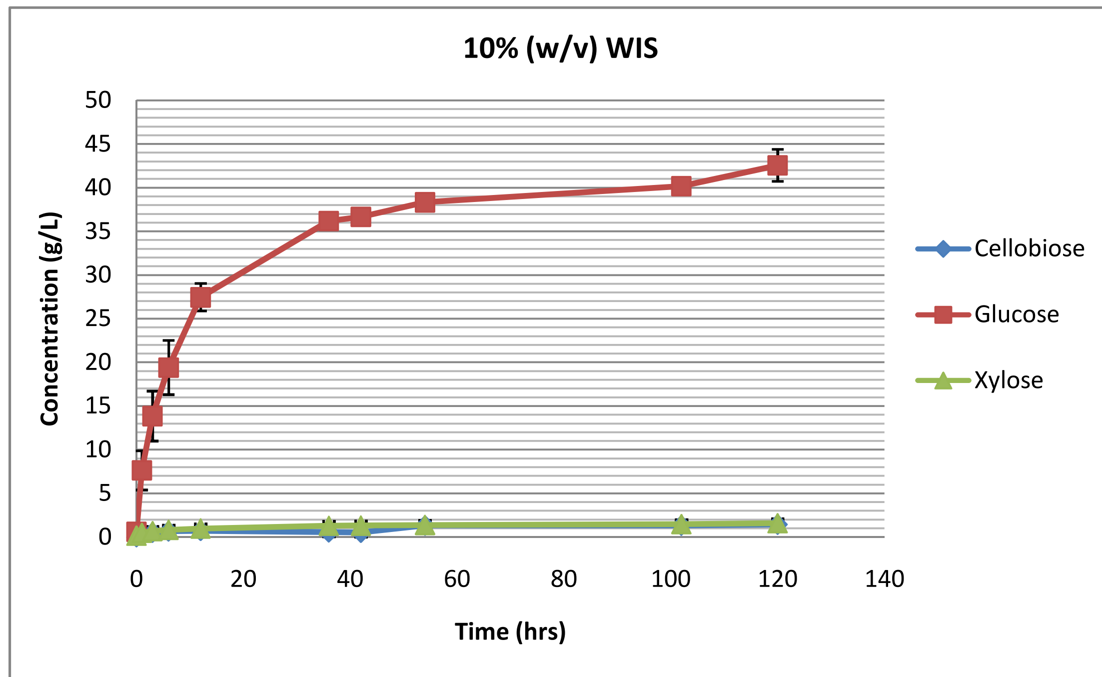


Figure 10: Enzymatic saccharification of WIS (10%, w/v) by the optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS).

As shown in Figure 11, WIS cellulose hydrolysis followed the characteristic profile, with a high initial conversion rate that decreases significantly after 12 hours. It is clear that cellulose conversion (expressed as percentage of glucose and cellobiose released in relation to potential cellulose in the WIS, Equation 1) decreased with increasing solids concentration (constant enzyme to substrate ratio). The highest cellulose conversion (expressed as percentage of glucose and cellobiose released in relation to potential cellulose in the WIS, Equation 1) of 92% was achieved at a substrate loading of 4% (w/v) with the lowest of 67.5% achieved at 10% (w/v) substrate loading. This phenomenon has been previously observed and appears to be an intrinsic or generic effect of enzymatic hydrolysis at increasing solid loadings (Cara *et al*., 2007; Jørgensen *et al*., 2007; Kristensen *et al*., 2009).

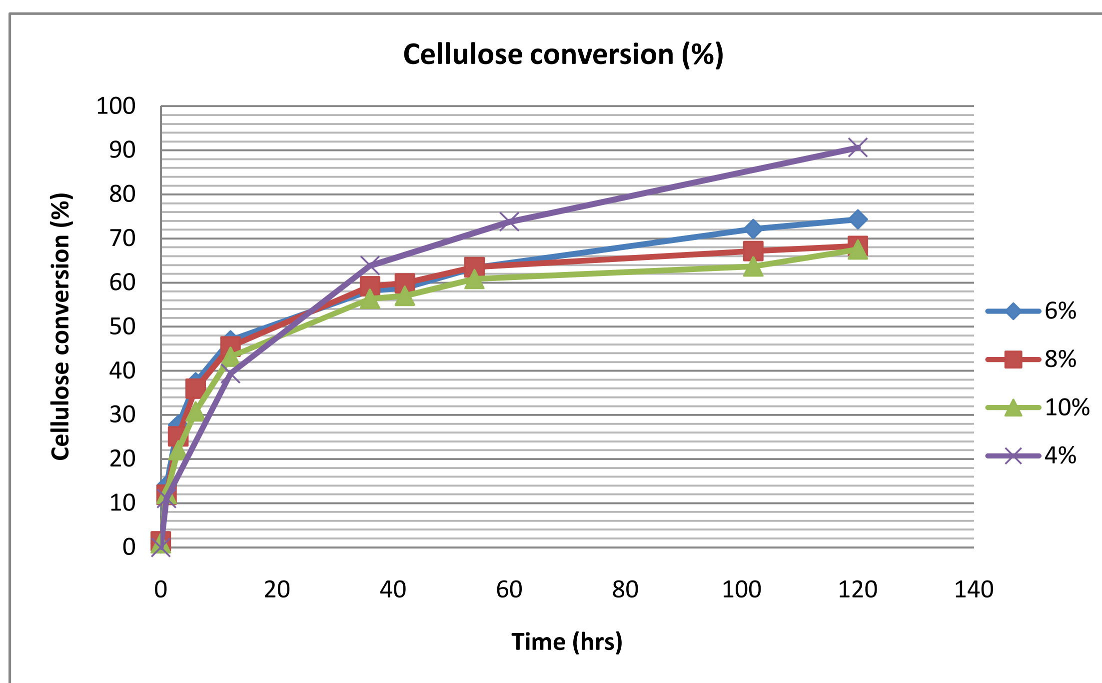


Figure 11: Cellulose conversion through enzymatic saccharification of 4%, 6%, 8% and 10% (w/v) initial WIS with the optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS).

The inverse relationship between cellulose conversion and solid loading has been attributed to end product inhibition (Holtzapple *et al*., 1990; Philippidis *et al*., 1993; Tolan and Foody, 1999; Xiao *et al*., 2004) and/or inhibition by lignocellulosic inhibitory compounds such as furfural and HMF (García-Aparicio *et al*., 2006). Other researchers attributed this phenomenon to increased mass transfer resistance and/or other effects related to the increased content of insoluble solids, such as non-productive adsorption of enzymes (Rosgaard *et al*., 2007). However, the specific mechanism(s) responsible for the decreasing hydrolytic efficiency have not been fully elucidated (Kristensen *et al*., 2009). Despite the decrease in convertibility, it is critical to use high solid loadings for economical bioethanol production as increasing solid loading results in higher sugar concentrations and concomitant high ethanol concentrations, which are necessary for reduced processing costs (Zacchi and Axelsson, 1989).

4.5. Conclusions

From the results obtained in this work, it can be concluded that steam explosion pretreatment is a suitable option for the conversion of sugarcane bagasse

into ethanol as it significantly hydrolysed hemicellulose and increased the enzymatic digestibility of the cellulose remaining in the biomass. Furthermore, synergism between saccharolytic enzymes was exhibited and it was confirmed that the optimized saccharolytic enzyme mixture is an available and efficient paradigm for hydrolysis of lignocellulosic substrates. It is, however, critical to use the right proportions of the supplementary activities while avoiding a strategy involving addition of the supplementary enzymes in excess, even if their production cost is to be ignored. The highest conversion of cellulose to glucose of 93% was achieved at a solid loading of 2% (w/v) WIS whilst the lowest of 67.5% was achieved at 10% (w/v) WIS, depicting an inverse relationship between cellulose conversion and solid loading. Although cellulose conversion was observed to decrease with increasing solid loading, the economic advantages of high solid loadings outweigh this partial offset.

4.6. Acknowledgements

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CHAPTER 5

EVALUATION OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) AND CONSOLIDATED BIOPROCESSING (CBP) OF STEAM EXPLODED SUGARCANE BAGASSE BY DIFFERENT CELLULOLYTIC, HEMICELLULOLYTIC AND XYLOSE FERMENTING RECOMBINANT *S. CEREVISIAE* STRAINS

Evaluation of Simultaneous Saccharification and Fermentation (SSF) and Consolidated Bioprocessing (CBP) of steam exploded sugarcane bagasse by different cellulolytic, hemicellulolytic and xylose fermenting recombinant *S. cerevisiae* strains

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Keywords: sugarcane bagasse, Simultaneous Saccharification and Fermentation (SSF); Consolidated Bioprocessing (CBP); *Saccharomyces cerevisiae*; bioethanol

5.1. Abstract

In this study, the optimised saccharolytic enzyme mixture determined in Chapter 4 was used to supplement enzyme activities of recombinant yeast strains co-expressing several cellulases and xylanases in Simultaneous Saccharification and Fermentations (SSFs). The ability of a cellulolytic, a xylan-degrading and a xylose-utilizing recombinant *S. cerevisiae* strain to separately and synergistically hydrolyse and ferment bagasse to ethanol in a one-step "consolidated bioprocessing" (CBP) where hydrolysis and fermentation of polysaccharides would be mediated by a single microorganism or microbial consortium without added saccharolytic enzymes, was also evaluated. Although the recombinant yeast strains were able to hydrolyse and ferment the substrate, it was at lower rates than in the SSFs. Thus, the concept of CBP of steam exploded bagasse was proven feasible.

5.2. Introduction

The need to mitigate the twin crises of Peak Oil and climate change has necessitated the development of alternative energy sources that should preferably be renewable and carbon-free or of low-carbon (Tsoskounoglou *et al*., 2008). This convergence of market pressure (supply instability and high oil prices) and concern for the environment (the greenhouse effect) has driven a headlong rush to biofuels. Lignocellulosic biomass, such as agricultural byproducts, presents an inexpensive, abundant and diverse source of sugar for fermentation into the sustainable transportation fuel ethanol. Sugarcane bagasse is the fibrous residue obtained after extracting the juice from sugarcane (*Saccharum officinarum*) in the sugar production process. The South African sugar industry generates approximately 6 million tonnes of sugar cane bagasse annually from crushing about 21 million tonnes of sugarcane (Mashoko *et al*., 2008). Most of the bagasse produced in the sugar industry is used as a fuel for generating the energy required by the sugar mills. However, with improvements in the thermal efficiency of combustion units, the energetic demands of sugar factories could be satisfied with reduced amounts of bagasse. Therefore, a surplus of bagasse would become available for alternative uses, including ethanol production.

There are three major steps in the process of converting lignocellulosic materials into ethanol: (i) thermochemical pretreatment - a preprocessing step that improves enzyme access to the cellulose; (ii) enzymatic saccharification - use of cellulases and on some occasions hemicellulases; and (iii) fermentation of the released sugars by specialized organisms (Gray *et al*, 2006). Steam explosion (STEX) is the most commonly used method for the pretreatment of lignocellulosic materials. This pretreatment technology has been extensively investigated and found to be effective on sugarcane bagasse and several other lignocellulosics (Kaar *et al*., 1998; Martin *et al*., 2002a, 2006).

The main obstacle preventing a large-scale utilization of lignocellulose for liquid fuel production is the requirement of uneconomically high enzyme loadings to achieve high saccharification yields, along with long process times due to a rapid decrease of the hydrolysis rate. This has been attributed to non-productive binding of cellulases and hemicellulases with lignin and other portions of the lignocellulose as well as inhibition by carbohydrate oligomers, the released sugars and their

degradation products (Boussaid and Saddler 1999; Palonen *et al*., 2004; Xiao *et al*., 2004; García-Aparicio *et al*., 2006; Kumar and Wyman, 2008). It has, however, been shown that optimizing the composition of the saccharifying enzyme mixture, by supplementation with accessory enzymes such as xylanases, ferulic acid esterases and laccases, can reduce the concentration of enzyme needed, thus reducing the cost and rendering enzymatic saccharification economically feasible (Berlin *et al*., 2007; Kim *et al*., 2008; Zhou *et al*., 2009). Although the composition of enzymes influences hydrolysis, it is apparent that the efficacy of enzymatic complexes is inextricably linked to the structural characteristics of the substrate, e.g. cellulose crystallinity, degree of cellulose polymerization, surface area and content of lignin, and/or the modifications that occur as saccharification proceeds. Thus it becomes paramount to optimize saccharolytic enzyme complexes for each lignocellulosic substrate and pretreatment technology.

The final stage of lignocellulose bioconversion to ethanol is the fermentation of sugars released during saccharification. In the Simultaneous Saccharification and Fermentation (SSF) configuration, the glucose produced during saccharification is fermented simultaneously by microorganisms present in the media. Thus SSF combines enzymatic hydrolysis of lignocellulosic material and fermentation in one bioreactor. The principal advantage of SSF compared to Separate Hydrolysis and Fermentation (SHF) is that the inhibitory effect of glucose and cellobiose on cellulases is minimized by keeping a low concentration of these sugars in the media, thereby increasing the rate of hydrolysis.

For lignocellulosic ethanol to be economically viable, microorganisms that can ferment both hexose (glucose, mannose and galactose) and pentose (xylose and arabinose) sugars will be essential. Currently, there are no naturally occurring microorganisms that can ferment all these sugars, but this hurdle could be solved by appropriate metabolic engineering. The ultimate process would be a one-step "consolidated bioprocessing" (CBP) of lignocellulose to ethanol, where the four biologically mediated transformations, i.e. production of saccharolytic enzymes (cellulases and hemicellulases); hydrolysis of carbohydrate components in biomass to sugars; fermentation of hexose sugars; and fermentation of pentose sugars (Lynd *et al*., 2005), would be mediated by a single microorganism or microbial consortium without added saccharolytic enzymes (van Zyl *et al*., 2007).

Microorganisms with the properties required for CBP are not currently available, but efforts for their development are underway. One strategy being applied involves engineering non-saccharolytic organisms that exhibit high product yields and titers to express a heterologous saccharolytic system enabling lignocellulose utilization. The yeast *Saccharomyces cerevisiae* is an attractive host organism for this strategy given that it is a proven ethanol-producer, exhibits tolerance to inhibitors commonly found in hydrolyzates resulting from biomass pretreatment, enjoys GRAS (Generally Regarded as Safe) status, and has well-established tools for genetic manipulation (van Zyl *et al.*, 2007).

In the present study, the use of an optimized enzyme cocktail in a SSF using various yeast strains was evaluated. Furthermore, the effect of substrate concentration on the SSF process was investigated. Finally, an attempt at CBP of steam exploded sugarcane bagasse by a consortium of recombinant *S. cerevisiae* strains was made.

5.3. Materials and methods

5.3.1. Substrate and chemicals

Raw bagasse was supplied by TSB Sugar, Mpumalanga, South Africa. All chemicals, media components and supplements used are of analytical grade and were obtained from BDH Chemicals Ltd. (Poole, England), Merck (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, U.S.A).

5.3.2. Pretreatment of sugarcane bagasse

Steam Explosion (STEX) pretreatment was generously performed at CIEMAT Biomass Unit (CIEMAT- Renewable Energies Department, Av. Complutense, 22, 28040 Madrid, Spain) as described in chapter 3 (section 3.3.2.). The solid fraction (Water Insoluble Solids (WIS)) was water-washed and used in the fermentation studies.

5.3.3. Compositional analysis of untreated and STEX pretreated bagasse

The carbohydrate, lignin, ash and extractives composition was determined as previously described (Chapter 3, 3.3.6.).

5.3.4. Enzymes

Celluclast® 1.5L (74 FPU/ml) and Novozyme 188 (740 IU/ml) were acquired from Novozymes A/S (Bagsvaerd, Denmark). Filter paper (FPU) and β-glucosidase activities were determined by the method of Ghose (1987). A previously optimized enzyme mixture (20 FPU Celluclast + 30 IU β-glucosidase) was used for the SSFs. Protein concentrations were determined using the Bio-Rad Bradford protein assay (Bio-Rad laboratories, München, Germany) as described in chapter 4 (section 4.3.5.).

5.3.5. Organisms and culture conditions

The *S. cerevisiae* strains that were screened for their ability to hydrolyse and grow on sugarcane bagasse are listed in Table 1. All strains were cultivated in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and strains expressing the *Saccharomycopsis fibuligera* BGL1 were at times cultured on YPC medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L cellobiose). Strain Y118 co-expressing the *S. fibuligera* BGL1 and the *Trichoderma reesei* EGI was cultured on YP-PASC medium (10 g/L yeast extract, 20 g/L peptone, 10 g/L PASC). Yeast strains were routinely cultured in 250 mL Erlenmeyer flasks containing 100 mL medium on a rotary shaker at 150 rpm and 30 °C.

Table 1: Yeast strains to be used in hydrolysis and fermentation

Strain	Heterologous enzyme	Designation	Reference
S. cerevisiae D_5A+Xi+Xk	Co-producing of xylose isomerase and xylulokinase	[1]D_5A+Xi+Xk	Dept. Microbiology, University of Stellenbosch
S. cerevisiae Y294 (*fur1*::*LEU2* pDLG56)	Co-producing of both XYN2 and XLN D	[2]Y294+pDLG56	La Grange *et al*. (2001)
S. cerevisiae Y118	Co-producing β-glucosidase, endoglucanase, CBH1 and CBH2	[3]Y118	Dept. Microbiology, University of Stellenbosch
S. cerevisiae Y294	None	Y294	ATCC 201160
S. cerevisiae D_5A	None	D_5A	National Renewable Energy Laboratory (NREL)

[1]Strain D5A +Xi+Xk is the *S. cerevisiae* D_5A strain co-expressing the *Bacteroides thetaiotaomicron* xylose isomerase (*Xi*) and an overexpressed xylulokinase (*XKS1*) gene.
[2]Strain Y294 +pDLG56 is the *S. cerevisiae* Y294 co-expressing the *Aspergillus niger* β-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes.
[3]Strain Y118 is the *S. cerevisiae* Y294 strain co-expressing the *Saccharomycopsis fibuligera* β-glucosidase *(bgl1)*, a synthetic version of the *T. reesei* endoglucanase (*eg1*), the *T. reesei* cellobiohydrolase *(cbh2)* and the *Humicola grisea* cellobiohydrolase *(cbh1)* genes.

5.3.5.1. Activity measurements for recombinant cellulases and xylanases

The recombinant yeast strain Y118 containing the *T. reesei* EGI was screened for carboxymethyl-cellulose (CMC) degrading ability by patching on SC^- medium containing 0.1% (w/v) CMC (Sigma) with 20 g/L glucose as carbon source. After 48 h of growth, colonies were washed off the plate and the remaining CMC was stained with 0.1% Congo red and de-stained with 1% (w/v) NaCl. Extracellular endoglucanase activity was indicated by clearing zone formation. Endoglucanase activity was quantified as described by Bailey *et al*. (1992) in citrate buffer (0.05 M, pH 5, 50˚C) with 1% CMC as substrate.

β-Glucosidase activity was measured by incubating appropriately diluted cells or supernatant with 5 mM of p-nitrophenyl-β-D-glucopyranoside (pNPG) in citrate buffer (0.05 M, pH 5.4, 55˚C) for 2 min (van Rooyen *et al*., 2005). The p-nitrophenol released from pNPG was detected at 405 nm after adding 1 ml of 1 M Na_2CO_3 to raise the pH and stop the reaction. All enzymatic assays were done in triplicate and expressed in units per mg dry cell weight (DCW) (Meinander *et al*., 1996) where one unit was defined as the amount of enzyme required to produce 1 mmol of p-nitrophenol or reducing sugar per minute under the assay conditions. Total cellulase activity was determined as filter paper (FPU) activity by the method of Ghose (1987).

Cellobiohydrolase activity was determined by the high throughput Avicel conversion assay procedure. Appropriately diluted cells or supernatant were incubated with Avicel (2%) in NaAc buffer (3 M, pH 5.0) supplemented with 0.02% NaN_3 and β-glucosidase in a 96 deep-well microtiter plate at 35˚C.

Yeast strains were screened for xylan degrading ability after being plated on SC^{-Ura} medium or SC medium containing 0.2% of 4-*O*-methyl-D-glucurono-D xylan–remazol brilliant blue R (RBB)–xylan (Sigma) as the carbon source. β-Xylanase cleaves RBB-xylan into a colourless product producing a clearing zone around a colony. Endo-β-1,4-xylanase activity was assayed by the method described by Bailey *et al*. (1992) with 1% birchwood glucuronoxylan (Sigma) as the substrate at 50˚C. Appropriate dilutions of the cell-free culture solution in 50 mM sodium citrate buffer (pH 5.3) were used as the enzyme source.

The strain D_5A+Xi+Xk was tested for its xylose fermenting ability by observing growth in shake flasks containing YPX medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose).

5.3.6. Protein assays

Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, München, Germany) as directed by the manufacturer for the microplate assay. A standard curve was made with 0.2 to 1.5 mg/ml BSA.

5.3.7. Fermentations

5.3.7.1. Simultaneous Saccharification and Fermentation (SSF)

The SSF reaction mixture consisted of 4% w/v WIS, 1% w/v yeast extract, 2% w/v peptone, 0.05 M citrate buffer (pH 4.8), 20 FPU/g cellulase (Celluclast® 1.5L), 30 IU/g β-glucosidase (Novozymes 188) and a 10% strain D5A inoculum (starting A_{600} of 0.5). Aerobic fermentations were performed in 125 mL conical flasks with a 50 mL working volume at 30°C and a mixing rate of 150 rpm. Samples for HPLC analysis (2 mL) were withdrawn directly from the fermentation broth using a pipette. Anaerobic fermentations were carried out in 100ml Schott Duran bottles fitted with screw caps with a rubber septum embedded in the middle. The Schott Duran bottles were incubated at 30°C on a magnetic stirrer with mixing at 150 rpm. Sampling was done through an epidural needle and an additional hypodermic needle was used for carbon dioxide venting. At time of transfer, the culture was diluted to ensure the SSF flasks were inoculated with a starting A_{600} = 0.5 cells.

5.3.7.2. Consolidated Bioprocessing (CBP)

The CBP reaction mixture consisted of the same constituents as those of the anaerobic SSF excluding the enzymes and the yeast strains Y118, D_5A+Xi+Xk, and/or Y294+pDLG56 as inoculums. Samples (2 mL) were withdrawn directly from the fermentation broth through the epidural needle and analysed for glucose, xylose, arabinose, cellobiose, acetic acid, glycerol and ethanol by HPLC.

5.3.8. Analytical methods

High performance liquid chromatography was conducted on a Surveyor Plus liquid chromatograph from Thermo scientific consisting of a LC pump, autosampler and RI detector. The Rezex RHM polymer-based column (300 x 7.8 mm) was used to separate glucose, xylose, arabinose, cellobiose, acetic acid, glycerol and ethanol concentrations in 0.22 μm filtered samples. The column temperature was maintained

at 40°C using a Gecko 2000 column heater. The mobile phase was HPLC grade water at a flow rate of 0.6 ml/min.

5.3.9. Calculations

The % theoretical ethanol yield or % cellulose conversion was calculated using the following formula:

$$\textit{Cellulose conversion (\%)} = \frac{[EtOH]_f - [EtOH]o}{0.51\ (f\ [Biomass]\ 1.111)} \times 100\% \qquad (1)$$

Where:

$[EtOH]_f$	Ethanol concentration at the end of the fermentation (g/L) minus any ethanol produced from the enzyme and medium
$[EtOH]_o$	Ethanol concentration at the beginning of the fermentation (g/L) which should be zero
[Biomass]	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose fraction of dry biomass (g/g)
0.51	Conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast
1.111	Converts cellulose to equivalent glucose

5.4. Results and discussion

5.4.1. Chemical composition of substrates

The chemical composition of untreated and uncatalysed STEX pretreated bagasse (WIS) are discussed in chapter 3 (section 3.3.6.).

5.4.2. Shake flask fermentations

Figure 1 shows the comparative fermentation of WIS by the yeast strain D_5A and related recombinant strains in a SSF configuration. The highest ethanol yield of 7.3 g/L was achieved with the recombinant strain D_5A+Xi+Xk after 60 hours. *S. cerevisiae* is naturally unable to utilise the 5-carbon sugar D-xylose present in the WIS, and must thus be engineered to ferment it. The strain D_5A+Xi+Xk contains the xylose isomerase (XI) pathway in which D-xylose is directly isomerised to D-xylulose by xylose isomerase (XI; EC 5.3.1.5). The D-xylulose is then phosphorylated to D-xylulose-5-phosphate by xylulokinase (XK; EC 2.7.1.17) before being assimilated via the non-oxidative pentose phosphate pathway (PPP) by strain D5A +Xi+Xk. Thus,

the higher ethanol yield could have been due to the ability of this strain to ferment some of the xylose in the WIS hydrolysate.

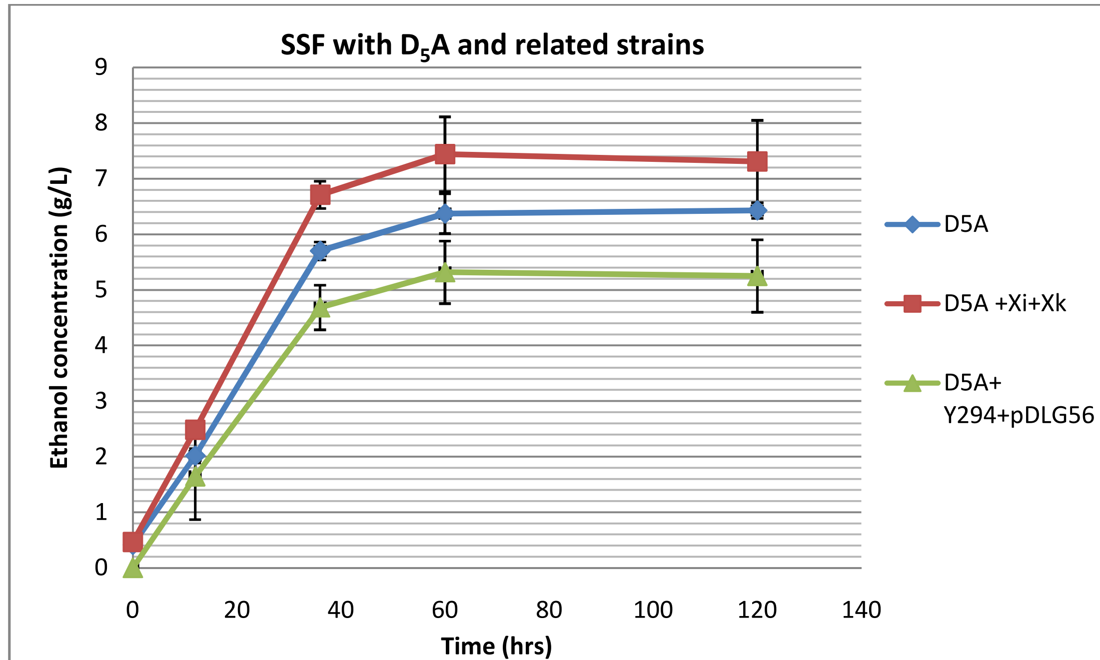

Figure 1: Ethanol production in SSFs of 4% (w/v) WIS with the yeast strain D_5A and related recombinant strains under aerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

SSF with strain D_5A and a mixed culture (50:50) of strain D_5A and strain Y294 +pDLG56 produced a maximum of 6.4 g/L and 6.1 g/L ethanol respectively. The strain Y294+pDLG56 co-expresses both the *A. niger* β-xylosidase (*xlnD*) and the *T. reesei* xylanase II (*xyn2*) genes and is thus capable of degrading xylan to D-xylose since xylan is hydrolyzed to xylo-oligosaccharides by endo-β-xylanase (EC 3.2.1.8), following which β-D-xylosidase (EC 3.2.1.37) hydrolyzes xylo-oligosaccharides to D-xylose. However, neither of the strains in the mixed culture could utilize xylose and coupled to strain Y294 +pDLG56 being a less robust fermentor that probably led to the lower ethanol yield observed for these strains.

Figure 2 shows the comparative fermentation of WIS by the yeast strain Y294 and related recombinant strains. Strains Y118 and Y294+pDLG56 produced a maximum ethanol concentration of 3.4 g/L, whilst strain Y294 only produced (2.7 g/L).

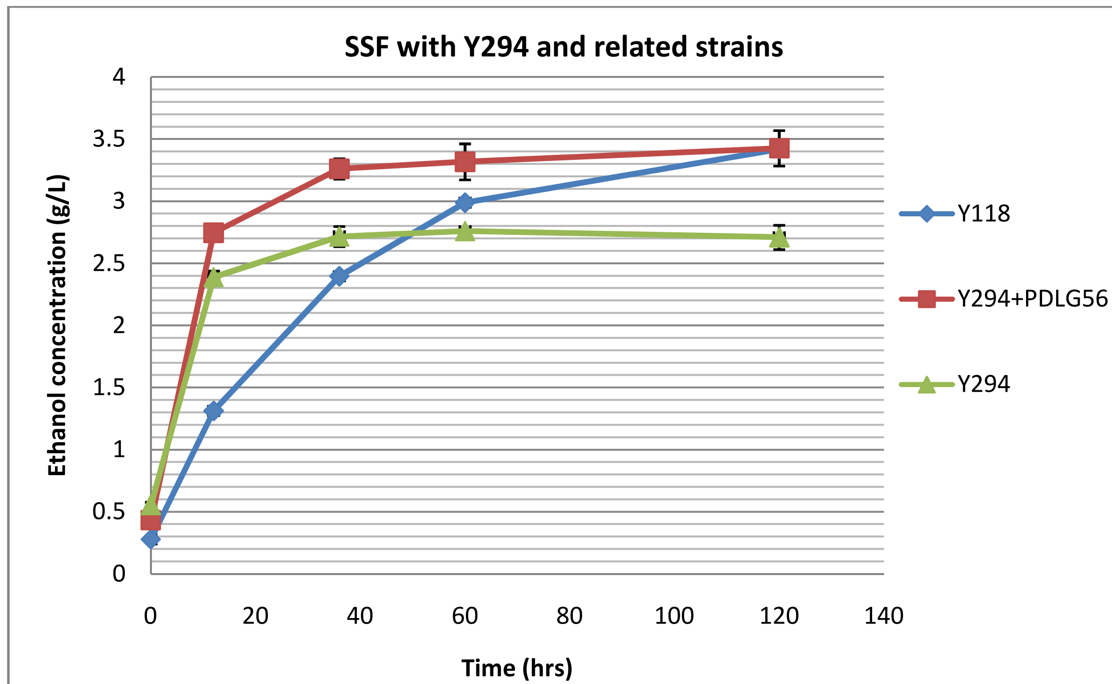

Figure 2: Ethanol production over time in SSFs of 4% (w/v) WIS with the yeast strain Y294 and related recombinant strains under aerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

Since strain Y118 expresses a reconstructed cellulolytic system, it supplemented the activity of the saccharolytic enzyme mixture thus enhancing cellulose hydrolysis and increasing the ethanol yield compared to the parent strain Y294. As described above, the strain Y294+pDLG56 expresses a xylanolytic system that could act synergistically with the saccharifying enzyme mixture resulting in better enzymatic saccharification and a concomitant higher ethanol yield compared to the parent strain Y294.

From Figures 1 and 2 it is clear that SSFs with strain D_5A and its recombinant strains yielded much higher ethanol yields compared to SSFs with strain Y294 and its recombinants. Strain D5A is an industrial strain which is more robust and exhibits better tolerance to high sugar concentrations, ethanol and inhibitory compounds commonly found in hydrolysates resulting from biomass pretreatment. Strain Y294 is a laboratory strain that is more susceptible to fermentation stresses hence the lower ethanol yields.

Fermentation kinetics of the SSF with the best ethanol producer (strain D_5A +Xi+Xk) are represented in Figure 3. A phenomenon that was observed with all the fermentations is that, although it was expected that after an initial adaptation phase, the glucose concentration would be reduced to zero, it remained high (5–6 g/L) increasing continuously. Similar observations have been reported previously (Bollok *et al*., 2000; Sharma *et al*., 2007). It can be noted that WIS was hydrolyzed quickly (but continuously) to glucose at higher rates than yeast utilization resulting in higher glucose accumulation during SSF. The maintenance of a high glucose concentration during the SSFs indicated continued cellulolytic activity, but unsatisfactory sugar utilisation by the yeast, which resulted in a rather poor ethanol yield. The cessation of ethanol production indicated the cessation of yeast fermentability, which is suggestive of inhibition of the yeast by inhibitory compounds in the hydrolysate.

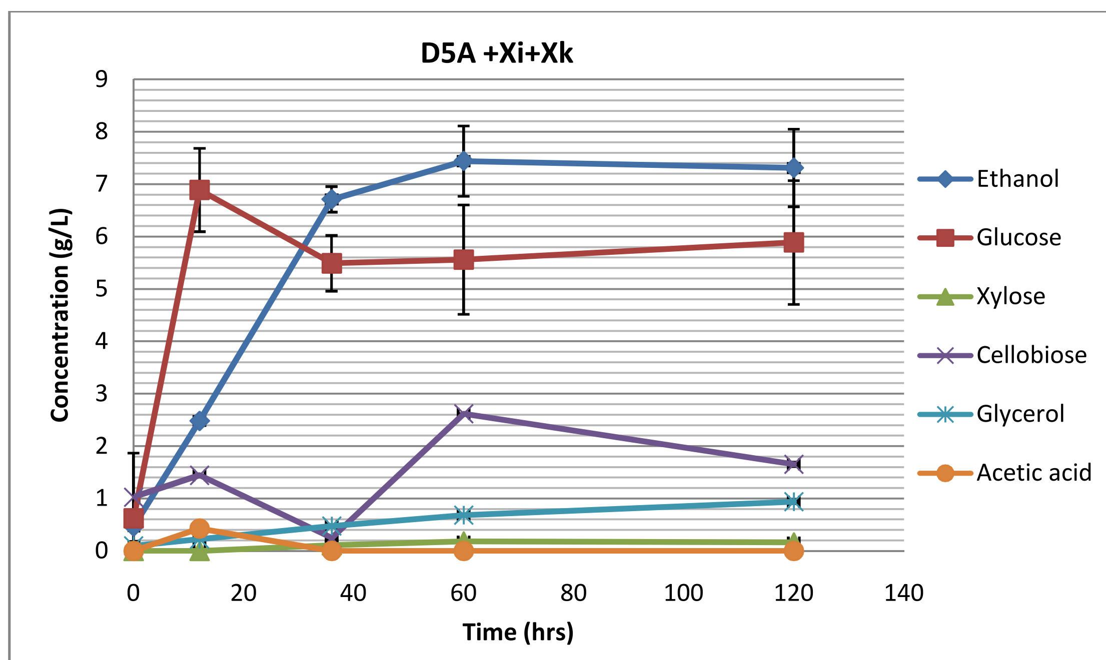

Figure 3: SSF kinetics for 4% (w/v) WIS with the yeast strain D_5A+Xi+Xk under aerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

Since yeasts are susceptible to inhibitors in enzymatic hydrolysates (Klinke *et al*, 2004), the adaptation of strains to the hydrolysate and the use of efficient detoxification methods (Martin *et al*., 2002b; Mussato and Roberto, 2004; Chandel *et*

al., 2007) should be investigated in future. The potential of using adapted strains or strains that have been genetically engineered to withstand high inhibitor concentrations as a tool for improving the tolerance of yeast strains to inhibitors in lignocellulose hydrolysates has been reported (Larsson *et al*., 2001a,b; Martin *et al*., 2007) and shown to decrease the need for more extensive measures such as detoxification. The cellobiose concentration fluctuated during the SSF reaching a maximum of 2.6 g/L (after 60 hours) and a minimum of 0.2 g/L indicating that the β-glucosidase was active throughout the SSF. Glycerol and acetate remained at concentrations below 1 g/L, indicating that the efficiency of ethanol conversion was quite satisfactory. The xylose concentration was negligible, which was expected as there was no xylanase activity added to the SSF.

Figure 4 shows ethanol production from WIS as the sole carbon source using the CBP configuration. The highest ethanol yield (considering the initial ethanol concentration) was achieved with a mixed culture fermentation with strains Y118 and Y294+pDLG56. As has already been discussed strain Y118 is a cellulolytic yeast whilst strain Y294 +pDLG56 is a hemicellulolytic strain. Synergistic interactions between the cellulases and hemicellulases led to better cellulose hydrolysis compared to strain Y118 alone. However, the maximum ethanol concentration after 7 days, was very low 0.412 g/L, but this is not insignificant considering that *S. cerevisiae* cannot naturally grow on a lignocellulosic substrate as complex as sugarcane bagasse, let alone produce ethanol from it. Better yields could be attained if the cellulolytic and hemicellulolytic systems are reconstructed in a more robust industrial yeast strain rather than the laboratory strain Y294.

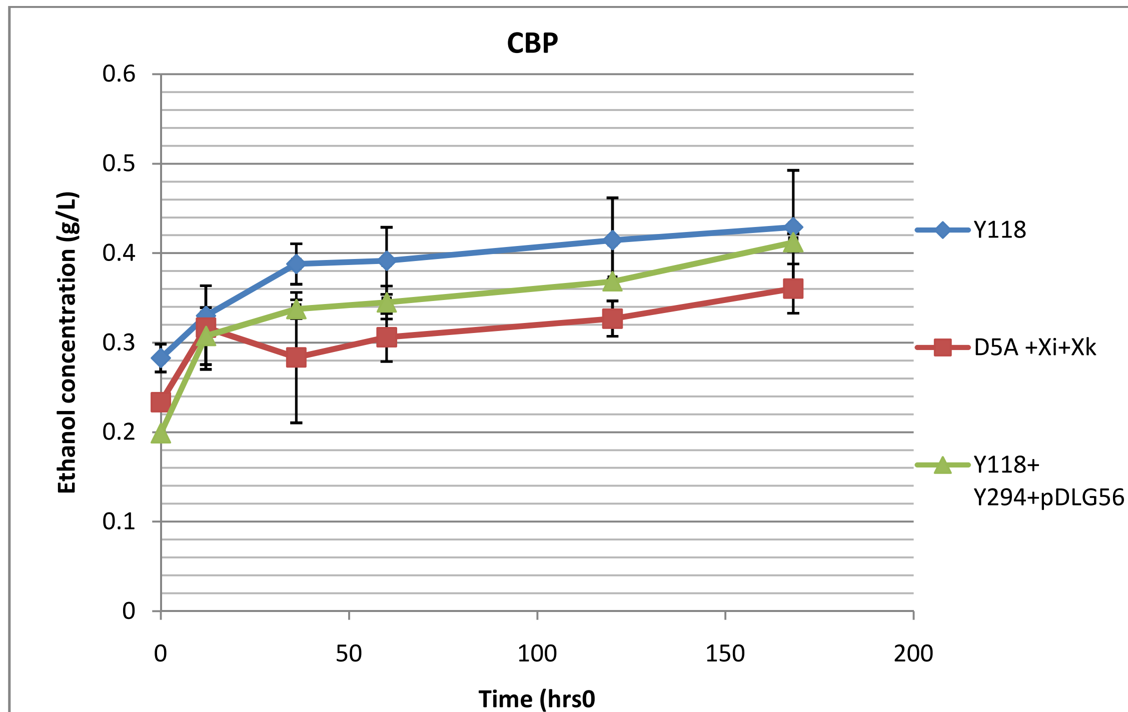

Figure 4: Ethanol production over time in CBP of (4%) w/v WIS with different recombinant yeast strains.

Fermentation kinetics of the CBP of WIS with strain Y118 and the mixed culture of strains Y118 and Y294+pDLG56 are represented in Figures 5 and 6. In both fermentations, it can be noted that there was an overall cellobiose accumulation. Cellobiose reached a maximum concentration of 2 g/L. Since no saccharolytic enzymes were added the cellobiose was certainly released by the heterologous cellulases (cellobiohydrolase 1 and 2, and endoglucanase 1) expressed by strain Y118. However, the yeast expresses only very low β–glucosidase levels such that the cellobiose was not efficiently converted to glucose and glucose concentration remained below 0.1 g/L. The accumulated cellobiose could have also resulted in significant cellulase inhibition (Philippidis *et al.*, 1993). Ethanol concentration remained low as little glucose was released and *S. cerevisiae* cannot utilise the cellobiose that accumulated. Hence, a strategy to improve β–glucosidase expression by strain Y118 could increase the ethanol yield.

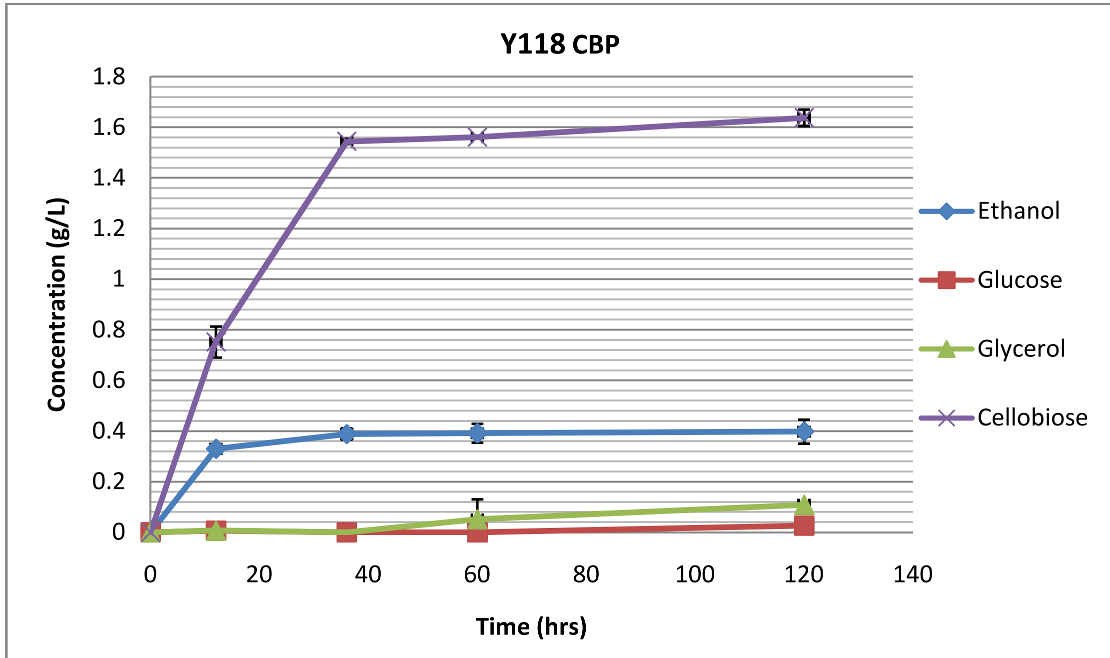

Figure 5: CBP kinetics for 4% w/v) WIS with strain Y118. No extracellular enzymes were added.

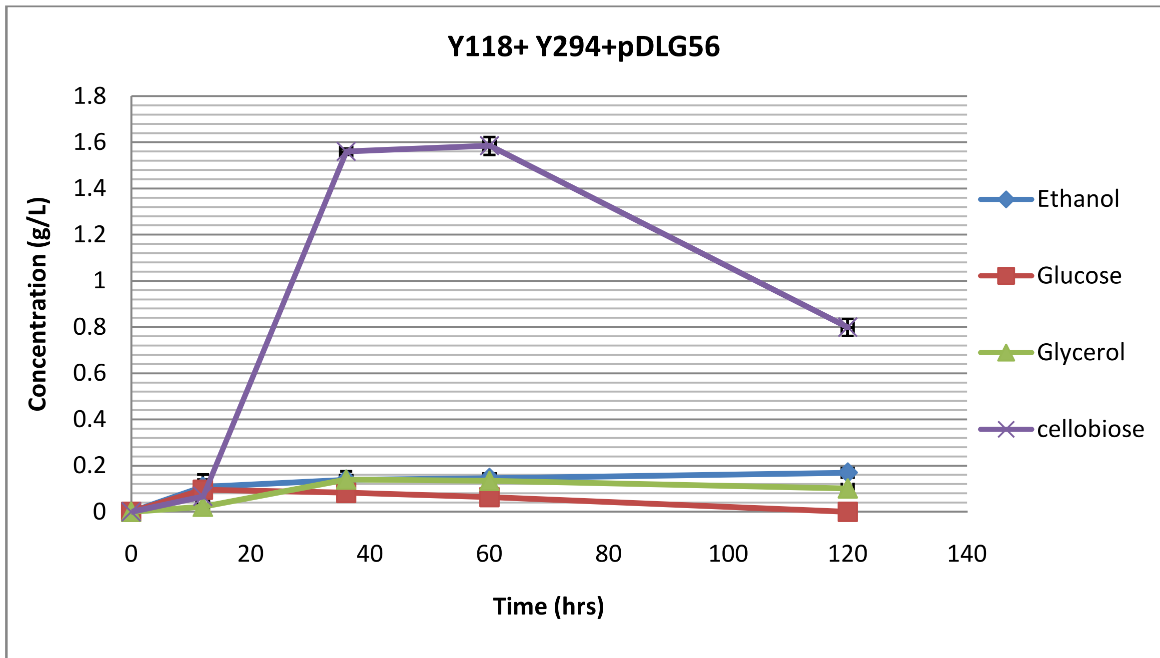

Figure 6: CBP kinetics for 4% (w/v) WIS with strains Y118 and Y294+pDLG56. No extracellular enzymes were added.

The effect of using the cellulolytic yeast strains Y118 and Y294 in SSFs at increasing cellulase loadings was investigated to explore the potential of using a

reduced cellulase loading in SSF with the recombinant strain and ascertain the ability of strain Y118 to heterologously express functional cellulases capable of hydrolyzing biomass. The results for the comparative SSFs are shown in Figure 7.

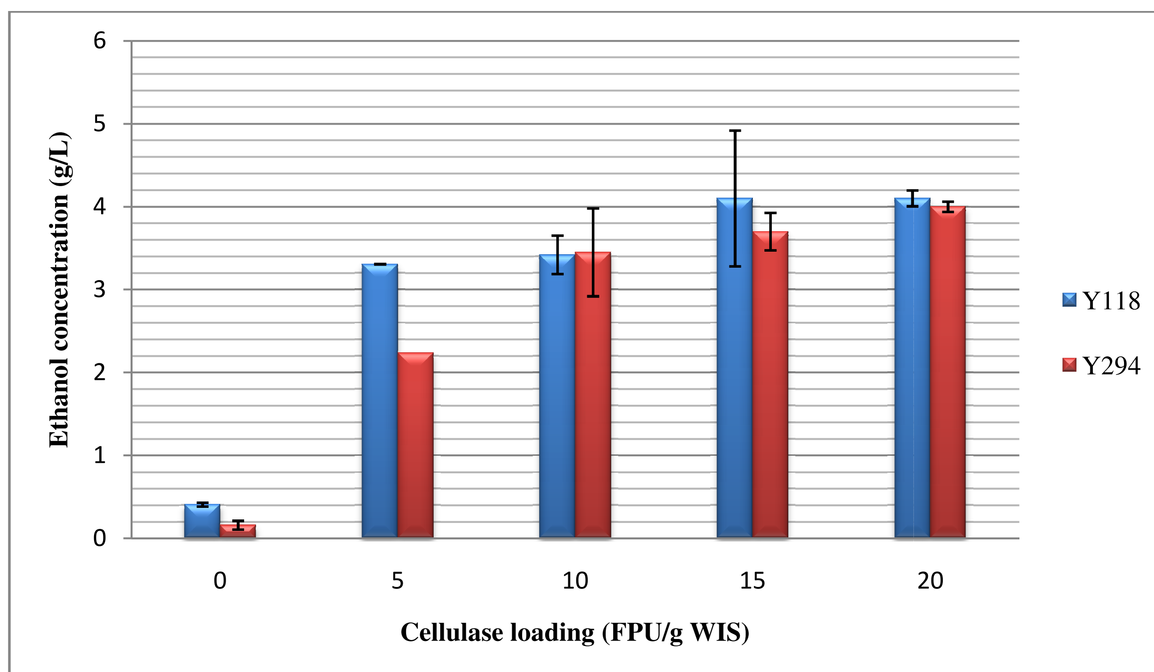

Figure 7: SSF of 4% (w/v) WIS with strains Y118 and Y294 at different cellulase loadings under aerobic conditions for 72 hours. Results represent the mean values from two separate experiments; error bars represent the standard deviation.

Figure 7 clearly shows that at low cellulase loading, the recombinant yeast produced more ethanol from WIS than the parent strain Y294. This is due to the fact that strain Y118 heterologously produced endoglucanase, β–glucosidase and cellobiohydrolases which supplemented celluclast cellulase activity, thereby leading to higher glucose productivity and concomitant higher ethanol yields. This differentiation decreased with increasing cellulase concentration, with a maximum of about 4 g/L ethanol being produced by both strains Y118 and Y294 at 20 FPU/g WIS cellulase loading. At higher cellulase loading the saccharification step seizes to be the limiting factor as the cellulase becomes saturated thus the insignificance of the yeast strain of choice.

5.4.3. Anaerobic fermentations

Anaerobic fermentations of WIS resulted in a much higher ethanol yield compared to the shake flask SSFs (Figures 8 and 9). SSFs with strain D5A and MH1000 at 5%

(w/v) resulted in similar ethanol yields after 72 hours, ~ 14 g/L (Figures 8 and 9), and thus the yeast strain D_5A was chosen for further investigations on the effect of substrate concentration on SSF under anaerobic conditions.

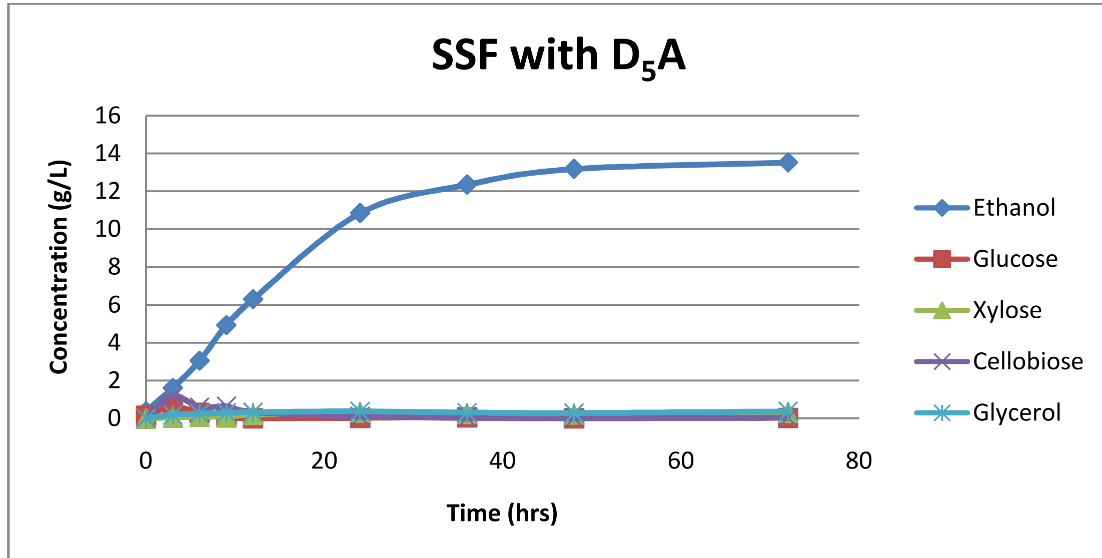

Figure 8: SSF of 5% (w/v) WIS with the yeast strain D_5A under anaerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

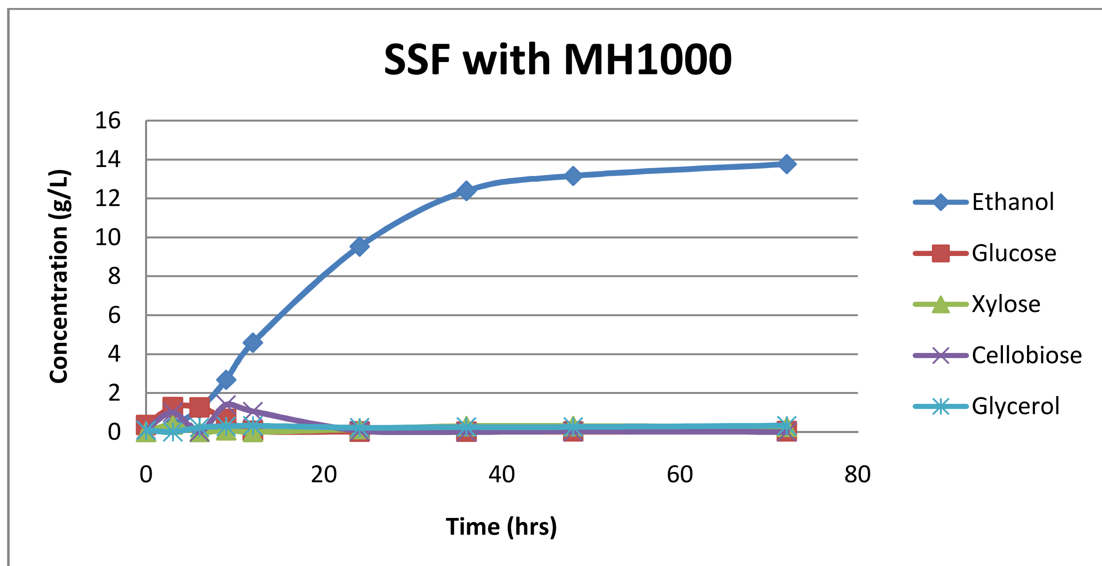

Figure 9: SSF of 5% (w/v) WIS with the yeast strain MH1000 under anaerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

The fermentation kinetics show that glucose, glycerol and acetate remained at concentrations below 1 g/L, indicating that the efficiency of ethanol conversion was quite satisfactory. The cellobiose concentration also remained low, indicating that the β–glucosidase remained active throughout the SSF. The xylose concentration was negligible, which was expected as there was no xylanase activity added to the SSFs. Similar results were obtained with 8% and 10% (w/v) WIS (figures 10 and 11).

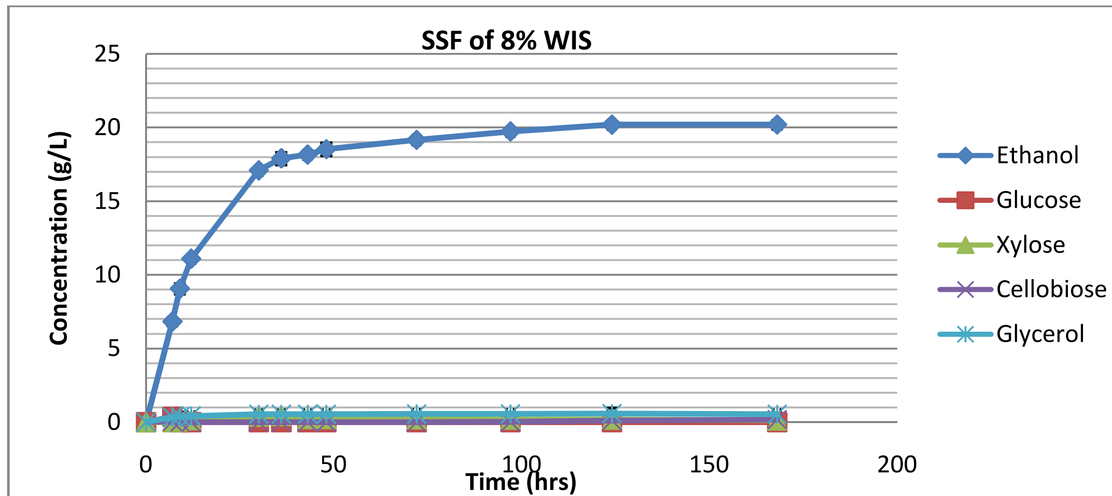

Figure 10: SSF of 8% (w/v) WIS with the yeast strain D_5A under anaerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

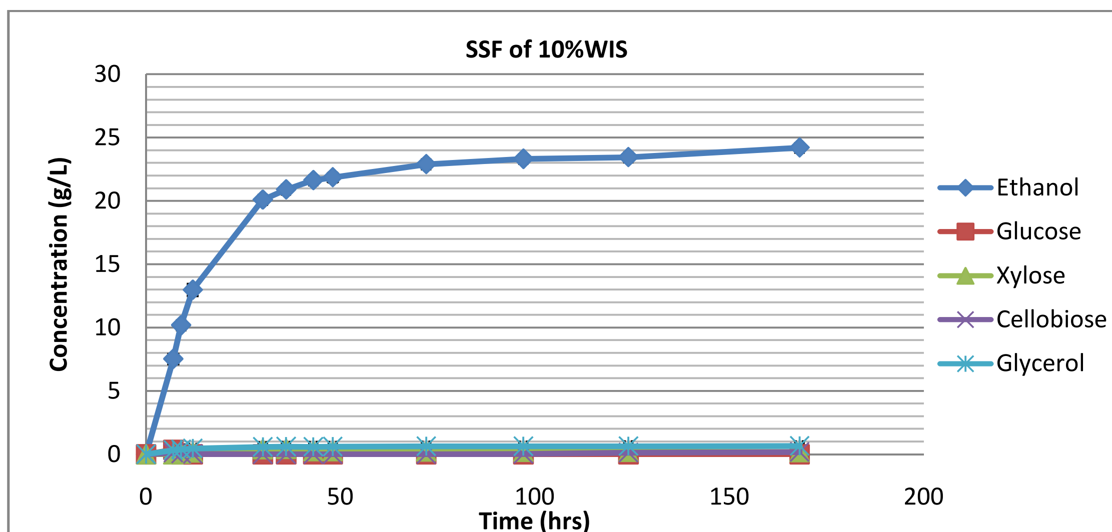

Figure 11: SSF of 10% w/v WIS with the yeast strain D_5A under anaerobic conditions. A previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS) was used in the SSF.

A comparison of SSF at increasing substrate concentration clearly shows a decrease in cellulose conversion (expressed as percentage of ethanol released in relation to potential cellulose in the WIS, Equation 2) (Figure 12). The ethanol yield after 72 hours of fermentation at WIS concentrations of 5%, 8% and 10% was 82%, 73% and 70%, respectively. The decrease in ethanol yield at high WIS concentrations has been shown to be a combined effect of increased mass transfer resistance and increased inhibitor concentration in the fermentation broth (Hoyer *et al*., 2008).

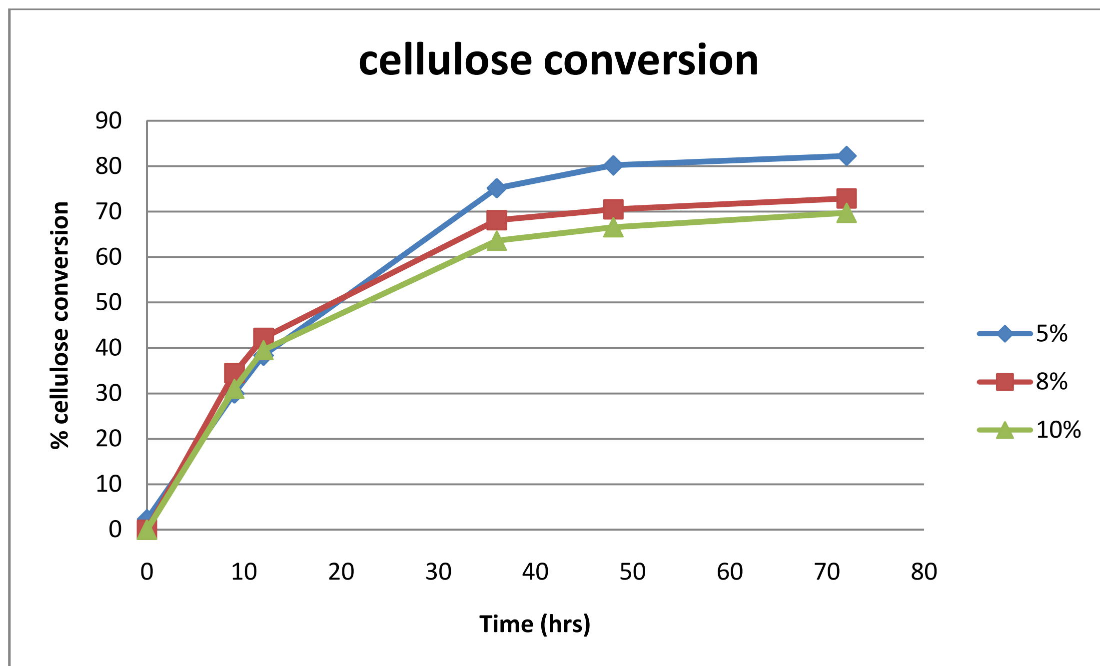

Figure 12: Influence of different WIS concentration on SSF with strain D5A. The SSFs were carried out with the addition of the previously optimized enzyme mixture (20 FPU Celluclast® 1.5L + 30 IU β-glucosidase /g WIS).

Although ethanol productivity was inversely proportional to the substrate concentration, it is important to consider that maintaining a high ethanol concentration is imperative for energy balance and economic viability of bioethanol production. Techno-economic evaluations have mentioned that a minimal final ethanol concentration of 4% (v/v) is required in the final fermentation slurry, before distillation, for economical bioethanol production (Zacchi and Axelsson, 1989). A higher ethanol concentration results in a reduction in the energy demand in the final distillation of the fermentation broth.

5.5. Conclusions

From the results obtained in this work, it can be concluded that steam explosion pretreatment followed by SSF of the WIS with *S. cerevisiae* and an optimised enzyme mixture is a suitable process for the conversion of sugarcane bagasse into ethanol. Microbial growth on sugarcane bagasse and utilization of the reducing sugars from enzymatic hydrolysis during SSF were observed to a significant degree. High ethanol yields reaching a maximum cellulose conversion of 82% were achieved under anaerobic conditions. Although ethanol productivity was observed to decrease with increasing substrate concentration, the economic advantages of high solid loadings outweigh this partial offset. Consolidated bioprocessing of STEX bagasse was also demonstrated. The cellulolytic yeast strains were able to autonomously multiply on sugarcane bagasse and concomitantly produce ethanol, though at very low yields (0.4 g/L). CBP could be improved if the cellulolytic and hemicellulolytic systems are reconstructed in a more robust industrial yeast strain rather than the laboratory strain Y294 used in this study. Overall, tremendous efforts are required to vastly improve the fermentation ability of the yeast strains so as to achieve a high ethanol yield from the utilization of bagasse. In the future, the adaptation of strains to the hydrolysate and the use of more efficient detoxification methods should be investigated.

5.6. Acknowledgements

TSB Sugar is gratefully acknowledged for supplying the sugarcane bagasse. The Department of Renewable Energy, CIEMAT (Madrid, Spain), assisted with the Steam Explosion (STEX) pretreatment of bagasse. The authors also thank Genencor and Novozymes for supplying the enzyme preparations.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

General discussion and conclusions

6.1. General Discussion

The adverse effects of the greenhouse gas emissions coupled with the dwindling global oil reserves has compelled society to contemplate on the development of renewable alternative energy sources that are carbon-free or of low carbon. The conversion of biomass sugars to transportation biofuels seems to be taking precedence as an attractive substitute for petroleum-based liquid fuels. However the practical impediments brought about by the recalcitrance of lignocellulosic material to enzymatic hydrolysis have impeded the commercial production of lignocellulosic ethanol. Thus, there currently is widespread research on optimization of the biomass-to-ethanol process. Fittingly, this research was aimed at process optimizing the bioconversion of sugarcane bagasse to bioethanol from pretreatment to ethanol fermentation.

6.1.1. Steam Explosion Pretreatment

This pretreatment technology has been extensively investigated and found to be effective on several lignocellulosics (Kaar *et al*., 1998; Martin *et al*., 2002, 2006). Two steam explosion pretreatment schemes, at different conditions, were investigated in this study as pretreatment conditions can modify the substrate composition resulting in a great effect in the subsequent enzymatic saccharification and fermentation steps. The first scheme involved uncatalysed steam explosion (STEX) of sugarcane bagasse at a temperature of 210°C for a residence time of 5 minutes (severity factor = 3.49) whilst in the second scheme STEX was catalysed by SO_2 at 188°C for 10 minutes (severity factor = 3.59).

Overall, STEX by both schemes was shown to be an effective technology for the pretreatment of sugarcane bagasse as it resulted in a high extraction of the hemicellulose fraction yielding a highly hydrolysable water insoluble solids (WIS) and generally low inhibitor concentrations in the prehydrolysate, with the exception of the 5 g/L acetic acid that, depending on the yeast strain, can lower specific growth rate and result in a lower ethanol production rate (Phowchinda *et al*., 1995). Uncatalysed

steam explosion, however, resulted in higher solid (64%) and glucose (98%) recoveries in the WIS fraction whilst SO_2 catalysed STEX yielded a higher xylose recovery (75.1%) in the prehydrolysate. Similar overal sugar yields (determined taking into account the sugar solubilized during pretreatment and the sugar released during the enzymatic hydrolysis of the WIS) were achieved with uncatalysed (39.7g / 100 g bagasse (dw)) and SO_2 catalysed [42.2 g/ 100 g bagasse (dw)] pretreatment. However, since the uncatalysed STEX explosion resulted in a higher glucose recovery in the WIS, the substrate from this pretreatment was used for the optimisation of both enzymatic saccharification and fermentation. Degradation products inhibit both enzymatic hydrolysis and growth of fermentative microorganism (Laser *et al*. 2002; Martin *et al*. 2002a), thus limiting their formation resulted in a highly fermentable substrate.

6.1.2. Enzymatic hydrolysis

Although the combination of enzymes influences hydrolysis, it is apparent that the efficacy of enzymatic complexes is inextricably linked to the structural characteristics of the substrate, e.g. cellulose crystallinity, degree of cellulose polymerization, surface area, lignin content and/or the modifications that occur as saccharification proceeds (Mansfield *et al*., 1999). It was therefore vital to design an optimized saccharolytic enzyme cocktail for the hydrolysis of the steam exploded bagasse. The statistical significance of the effect of cellulases, β-glucosidase and xylanase dosages on cellulose digestibility on enzymatic hydrolysis yield was determined by ANOVA, which revealed that the regression was statistically significant at a 95% confidence level in the case of cellulase activity (FPU). A cellulose conversion of close to a 100% was achieved after 120 hours at a critical enzyme ratio of 20 FPU cellulase, Celluclast® 1.5L, and 30 IU β-glucosidase, Novozyme 188 (Novozymes A/S, Bagsvaerd, Denmark) at 2% (w/v) WIS. At this critical enzyme combination an overall sugar yield of 53 g/100 g raw material was attained at 5% (w/v) WIS. This optimal enzyme mix was used to investigate the effect of increasing substrate concentration on enzymatic hydrolysis of steam exploded bagasse. An inverse relationship was observed between cellulose conversion (%) and substrate loading with the highest conversion of cellulose to glucose of 93% being achieved within 24 hours at a solid loading of 2% (w/v) WIS and the lowest of 67.5%

being achieved in 120 hours at 10% (w/v) WIS. This phenomenon appears to be a generic or intrinsic feature of lignocellulose conversion (Kristensen *et al*., 2009).

6.1.3. Fermentability

Fermentation of the reducing sugars from enzymatic hydrolysis during anaerobic SSF was observed to a significant degree. Throughout all anaerobic fermentations with the yeast strain D_5A, the glucose and cellobiose levels remained insignificant (less than 1 g/L) and a maximum ethanol yield of 82% was achieved at 5% (w/v) substrate concentration. Consolidated bio-processing of STEX bagasse was also demonstrated. The cellulolytic yeast strains were able to consolidatively bio-process steam exploded sugarcane bagasse, though at very low titres (0.4 g/L). For CBP to be economical there still is need for tremendous efforts in developing a yeast strain that can effectively degrade xylan and concomitantly utilize the released xylose. Ethanol yield was found to be inversely proportional to substrate concentration with the lowest ethanol yield of 70% being achieved in the SSF at a WIS concentration of 10% (w/v). This phenomenon, also observed with enzymatic hydrolysis, has been attributed to the conjugated effect of increased mass transfer resistance and increased inhibitor concentration at higher substrate loadings (Hoyer *et al*., 2002). To mitigate the effect of increased mass transfer resistance reactors with better mixing should be developed whilst inhibitor tolerance could be enhanced through the use of several strategies including hydrolysate detoxification (Martin *et al*., 2002b; Mussato and Roberto, 2004; Chandel *et al*., 2007; Cheng *et al*., 2008), improvement of *S. cerevisiae* strain tolerance via directed evolution or adaptative strategies (Martin *et al*., 2007), use of a careful fermentation control (process design strategy), and targeted metabolic engineering for improved yeast tolerance towards specific inhibitors (Almeida *et al*., 2008a; Almeida *et al*., 2008b; Alriksson *et al*., 2010; Guadalupe Medina *et al*., 2010).

6.2. Conclusions

The work presented in this thesis presents sufficient evidence to indicate that sugarcane bagasse is a good substrate for bioethanol production by using steam explosion pretreatment followed by SSF using the optimized enzyme mixture (20

FPU Celluclast® 1.5L: 30 IU β-glucosidase /g WIS). Sugarcane bagasse is cheap, abundant and rich in carbohydrates. Moreover, logistic problems are minimal, as it is available in high amounts at sugar mill sites and its utilisation helps to solve the disposal problem for sugar mills. Synergism within the cellulase systems was demonstrated as higher collective activity than the sum of the activities of individual enzymes was exhibited. This confirmed that the optimized cellulase mixture is an available and efficient paradigm for hydrolysis of steam exploded sugarcane bagasse. However it was also shown that it is critical to use the right proportions (20 FPU Celluclast® 1.5L: 30 IU β-glucosidase /g WIS) of the supplementary activities while avoiding a strategy involving addition of the supplementary enzymes in excess, even if their production cost is to be ignored.

Microbial growth on sugarcane bagasse and utilization of the reducing sugars from enzymatic hydrolysis during SSF was observed to a significant degree with a maximum ethanol yield of 82% achieved at 5% WIS (w/v). Moreover, consolidated bio-processing (CBP) of STEX bagasse was demonstrated. The cellulolytic yeast strain was able to autonomously multiply on sugarcane bagasse and concomitantly produce ethanol, though at very low titres (0.4 g /L). CBP could be improved if the cellulolytic and hemicellulolytic systems are reconstructed in a more robust industrial yeast strain rather than the laboratory strain Y294 investigated in this study. Overall, tremendous efforts are required to vastly improve the fermentation ability of the yeast strains, with particular emphasis on xylose utilization, so as to achieve a high ethanol yield from the utilization of bagasse. Reactors with better mixing should be utilized to mitigate the negative effect of increased mass transfer resistance observed in enzymatic hydrolysis and SSF at high solid concentrations.

6.3. Recommendations for future work

Further improvements of the process configuration are necessary to reduce the production cost of bioethanol. This can be achieved by further research on the following topics:

- Evaluation of nutrient components of STEX bagasse to identify excess and limiting nutrients so as to be able to reduce additional nutrient requirements in fermentation media.

- Reconstruction of cellulolytic pathway in more robust *S. cerevisiae* strain or adaptation of current strains to lignocellulose hydrolysates.

- Detoxification of prehydrolysate, adaptation of yeast to hydrolysate or metabolic engineering for inhibitor tolerance.

- Development of a more effective stirring scheme so as to achieve adequate mixing and reduce mass transfer resistance at high substrate concentrations which are necessary for economical feasibility.

- Complete substrate utilization is a prerequisite for economical bioethanol production. Thus there's still much work needed to be done to construct a highly efficient pentose fermenting *S. cerevisiae* strain.

- Investigate effect of surface active additives (surfactants) on CBP by cellulolytic strain. Kumar and Wyman (2009) demonstrated a 64% increase in glucose release from enzymatic hydrolysis of avicel when low cellulase loadings were used. Borjesson *et al*. (2007) further demonstrated extensive adsorption of cellobiohydrolase 1 and endoglucanase with only 10% and 13% of enzyme left unadsorbed respectively. Since the recombinant yeast strain Y118 expresses low titers of these enzymes supplementation with additives could result in significant improvement in enzymatic hydrolysis and concomitant higher ethanol productivity.

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