Evaluation of triticale straw as feedstock for the production of bioethanol in a SSF process

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Declaration

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Summary

The production of renewable liquid fuels such as bioethanol is currently at the forefront of scientific research, with a specific focus on production processes that are sustainable, inexpensive and environmentally friendly. Traditional biofuel feedstocks include maize, wheat, sugar and sugar beet, which can be easily converted to ethanol using hydrolytic enzymes and microorganisms. The focus has recently shifted to less expensive feedstocks, namely lignocellulosic biomass. Lignocellulose is found in all plants and byproducts or waste material from several industries can therefore be utilised for the production of lignocellulosic bioethanol, including paper sludge, wood chips, corn stover, sugarcane bagasse and straw.

One of the potential alternative feedstocks for bioethanol is triticale straw. Triticale (*Triticosecale rimpau*) is a robust and tolerant cereal crop that is cultivated worldwide and has desirable qualities such as disease and drought tolerance and the ability to grow on marginal land. It produces grain with high protein content suitable as food or animal feed, whereas the straw has little monetary value and is therefore an ideal feedstock for bioethanol production. The straw is more susceptible to enzymatic hydrolysis than other lignocellulosic sources such as wood, sugarcane bagasse and corncobs. However, little research has been done on the enzyme activities and dosages required to utilise triticale straw as bioethanol feedstock.

In this study, triticale straw was evaluated using steam-explosion pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) as processing pipeline. The conditions for steam-explosion were set at 203°C for 7 minutes, which improved the cellulose content of the straw by 6% and reduced the hemicellulose content by 17%. One kilogram of triticale straw produced 720 grams of water-insoluble solids (WIS), as well as a liquid fraction. Five commercial cellulase cocktails were evaluated for the hydrolysis of the WIS, with Spezyme® CP delivering the highest glucose yield (57%) at 15 FPU/g cellulose. Several strains of *Saccharomyces cerevisiae* were screened for their fermentative ability at 37°C and high glucose concentrations and Ethanol Red®, an industrial strain, and the wild-type strain L21 were selected for evaluation in an SSF setup. The combination of 15 FPU/g Spezyme® CP and Ethanol Red® yielded the best results on the triticale WIS, with 26.9 g/l ethanol produced after 144 hours, corresponding to 92% of the theoretical ethanol yield. The promising performance of triticale straw under laboratory conditions therefore supports further investigation on an industrial scale.

Opsomming

Die produksie van hernubare vloeibare brandstof, soos bio-etanol, is tans aan die voorpunt van wetenskaplike navorsing, met spesifieke fokus op produksieprosesse wat volhoubaar, goedkoop en omgewingsvriendelik is. Tradisionele bio-etanol voerstowwe sluit mielies, koring, suiker en suikerbeet in, wat maklik deur hidrolitiese ensieme en mikro-organismes na etanol omgeskakel kan word. Die fokus het onlangs na goedkoper voerstowwe, naamlik lignosellulosiese biomassa, verskuif. Lignosellulose word in alle plante gevind en neweprodukte of afvalmateriaal van verskeie nywerhede, insluitende papierslyk, hout splinters, mieliestronke, suikerrietbagasse en strooi kan dus vir die produksie van bio-etanol aangewend word.

Een van die potensiële alternatiewe voerstowwe vir bio-etanol is korogstrooi. Korog (*Triticosecale rimpau*) is 'n geharde en verdraagsame graansoort wat wêreldwyd verbou word. Dit het verskeie gewensde eienskappe, insluitende siekte- en droogtebestandheid en die vermoë om op marginale grond te groei. Dit produseer graan met 'n hoë proteïeninhoud wat as voedsel of veevoer geskik is, terwyl die strooi min geldwaarde het en dus die ideale voermateriaal vir bio-etanolproduksie is. Strooi is meer vatbaar vir ensiematiese hidrolise as ander lignosellulosiese bronne soos hout, suikerriet bagasse en mieliestronke. Nietemin is min navorsing op die ensiemaktiwiteite en dosering vir die benutting van korogstrooi as voerstof vir bio-etanol gedoen.

In hierdie studie word korogstrooi geëvalueer deur gebruik te maak van voorbehandeling met stoomontploffing, ensiematiese hidroliese en gelyktydige versuikering en fermentasie (GVF). Die toestande vir stoomontploffing was 203°C vir 7 minute, wat die sellulose-inhoud van die strooi met 6% verhoog en die hemisellulose-inhoud met 17% verminder het. Een kilogram korogstrooi het 720 gram totale onoplosbare vastestowwe (TOV) gelewer, asook ‘n vloeibare fraksie. Vyf kommersiële sellulasemengsels is vir die hidroliese van die TOV geëvalueer, waaronder Spezyme® CP die hoogste glukose-opbrengs (57%) met 15 FPU/g sellulose gelewer het. Verskeie stamme van *Saccharomyces cerevisiae* is vir hul fermentasievermoë by 37°C en hoë glukosekonsentrasies gesif en Ethanol Red®, ‘n industriële stam, sowel as die wilde-tipe L21 ras, is vir gebruik in ’n GVF-opstelling gekies. Die kombinasie van 15 FPU/g Spezyme® CP en Ethanol Red® het die beste opbrengs gelewer, met 26.9 g/l etanol wat na 144 ure geproduseer is, gelykstaande aan 92% van die teoretiese etanolopbrengs. Die belowende prestasie van korog strooi onder laboratoriumtoestande ondersteun verdere ondersoek op 'n industriële skaal.

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To my husband, **Eric**

Abbreviations

1G	First Generation
2G	Second Generation
3G	Third Generation
5-HMF	5-hydroxymethyl-2-furaldehyde
BGL	β-glucosidase
BSA	Bovine Serum Albumin
CBH	Cellobiohydrolases
CBP	Consolidated Bioprocessing
CMC	Carboxy-methyl cellulose
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic acid
EG	Endo-glucanases
FPU	Filter Paper Units
HPLC	High-Performance Liquid Chromatography
HMF	Hydroxy-methyl furfural
IU	International Unit
IUPAC	International Union of Pure and Applied Chemistry
LAP	Laboratory Analytical Procedure
NREL	National Renewable Energy Laboratory
*p*NPG	*p*-nitrophenyl-β-D-glucopyranoside
SC	Synthetic complete
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
YPD	Yeast Peptone Dextrose
WIS	Water-Insoluble Solids

Table of Contents

1. Introduction and aims

1.1. Introduction

There is an increasing demand for alternative fuels produced from sustainable sources to supplement and replace liquid fossil fuels. The proposed candidate is bioethanol, an alcoholic molecule that can be produced from the glucose found in sources such as sugarcane and sugarbeet, as well as starch feedstocks such as corn and wheat. The use of bioethanol would lead to job creation as it can be locally produced. It is also less expensive than fossil fuels and has lower carbon emissions (Sarkar *et al.,* 2012). However, the feedstocks generally used for first generation (1G) bioethanol production are expensive as they are also used for human consumption. Second generation (2G) bioethanol is produced from lignocellulosic biomass, which is an inexpensive, abundant and renewable (practically inexhaustible) source of carbon (Binod *et al*., 2011). Agricultural residues are of particular interest, since it is estimated that 491 billion litres of bioethanol can be produced annually from this resource (Sarkar *et al*., 2012). With the help of microbes and enzymes, agricultural residues can be fully utilised to produce enough bioethanol to provide liquid fuel to the world.

Lignocellulose is found in all plant types and consists of three main components, namely cellulose, hemicellulose and lignin. Cellulose is the key component (30-50%) and is formed by thousands of D-glucose molecules linked by β-1,4-glycosidic bonds to form long, linear polymer chains. Hemicellulose is formed primarily by 5-carbon sugars (xylan) linked in branched chains that envelop the cellulose fibres, whereas lignin is a polymer of phenolic molecules that are tightly linked to the cellulose and hemicellulose (Mosier *et al*., 2005). These components create a tight and rigid structure that strengthen plants and resist microbial invasion, but this recalcitrant nature also renders lignocellulosic bioethanol more difficult to produce compared to starch ethanol.

Lignocellulosic biomass has not yet been widely implemented as a bioethanol feedstock, mainly because of the high production cost (Sarkar *et al*., 2012; Gusakov, 2013). In the production of lignocellulosic ethanol, cellulose is the polymer of interest as it contains glucose monomers that can be fermented to ethanol by yeast. However, cellulose is interspersed by hemicellulose and lignin, making it inaccessible to enzymes. Pretreatment is therefore required to remove these obstacles and render the cellulose chains accessible for enzymatic hydrolysis. Despite the cost added by this process, it significantly improves

the hydrolysis of cellulose and is an essential step in the production of lignocellulosic ethanol (Alvira *et al*., 2010).

Another factor is the cost of cellulases used for enzymatic hydrolysis of lignocellulose (Gnansounou and Dauriat, 2010). Cellulases are enzymes that hydrolyse the β-1,4-glycosidic bonds between glucose molecules in the cellulose chain. The synergistic action of three types of cellulases is required to hydrolyse the cellulose polymers to glucose units, namely endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BGL). Endoglucanases nick the cellulose chains to create free ends and produce cello-oligosaccharides; cellobiohydrolases cleave cellobiose units from the free ends and β-glucosidases cleave the cellobiose units and other oligosaccharides into glucose molecules. Several microorganisms, including cellulolytic fungi and some bacterial species, produce these three enzymes (Lynd et al., 2002).

In large-scale cellulase production, filamentous fungi such as *Trichoderma reesei* and *Aspergillus niger* are routinely used to produce cellulolytic enzymes, but the production and purification of these enzymes are expensive (Mathew *et al*., 2008). Commercial cellulase cocktails have high enzyme activities and act on a wide variety of substrates, but it is unlikely that a commercial process that uses large amounts of expensive enzyme will be economically viable (Klein-Marcuschamer *et al*., 2012). This underlines the need to optimise the type and dosage of cellulases in the production of bioethanol from plant residues. Commercial cellulase cocktails have varying efficacies on different types of lignocellulosic substrates, as they contain enzymes with different substrate specificities (Lin *et al*., 2010). It is thus necessary to select commercial cocktails based on their specific enzyme activity and required dosage for a given substrate, to ensure the most effective use of this resource (Klein-Marcuschamer *et al*., 2012). Furthermore, additional specific enzyme activities required for optimal utilisation of the given substrate need to be identified and supplemented where possible.

Several yeast species have been used for the production of bioethanol, the most popular being *Saccharomyces cerevisiae*. Since there is great variation between strains of *S. cerevisiae*, some might be more effective at fermenting lignocellulosic hydrolysates, which typically include a mixture of several phenolic inhibitors and acids. In this study, several strains are screened and selected for desirable qualities such as ethanol and temperature tolerance, which will allow the fermentation process to be optimised.

Triticale (*Triticosecale rimpau*) is a hybrid cereal crop developed through genetic crossing of rye and wheat. It has several desirable qualities such as disease and drought resistance, low nutrient requirements and the ability to produce high grain yields even when cultivated

on marginal land (Mergoum *et al*., 2009). This crop has the potential for becoming a versatile source of grain for both human and animal consumption, as well as a bioethanol feedstock, since it can survive and perform well in the South African climate.

Straw from several crops have been extensively researched for their use in bioethanol production and in general, straws are considered some of the most important feedstocks of the future (Lal, 2008; Sarkar *et al*., 2012). The use of triticale straw as feedstock for bioethanol production could be a viable option in the South African context, since it is considered a waste product that will benefit from new market opportunities, and will not add cost to the production process. However, this will require a tailored and fine-tuned process to demonstrate its potential, with the identification of the relevant enzymes an important point of departure. The ideal scenario would be to have the necessary enzymes available in a commercial cocktail, with supplementation with those lacking as an alternative option to be considered. For example, a β-glucosidase is used in this study to optimise glucose release from the triticale straw substrate.

This study employs a simultaneous saccharification and fermentation (SSF) strategy to produce ethanol from a lignocellulosic substrate. This implies that enzymatic hydrolysis and fermentation take place simultaneously in the same reaction vessel. The substrate, cellulases and yeast are combined in a buffered medium, allowing the glucose that is released by the cellulases to be immediately consumed and fermented by the yeast. This strategy is effective because it removes the glucose from the environment and reduces its inhibition of the cellulases. This method is well established and commonly used for ethanol production from solid substrates.

1.2. Aims of the study

The aim of this study was to evaluate the use of triticale straw as a feedstock for bioethanol production, based on its enzymatic digestibility with commercial cellulase cocktails and its performance in an SSF model. The culmination of the investigation will be a single-step, optimised process for the production of bioethanol from triticale straw.

The following objectives were identified:

i. pretreatment and preparation of triticale straw for use in enzymatic hydrolysis and SSF;
ii. evaluation of commercial cellulase cocktails for hydrolytic activity on pretreated triticale straw;

iii. screening of wild-type and industrial *S. cerevisiae* strains for ethanol production at 37°C;

iv. SSF of triticale straw with a commercial cellulase cocktail combined with selected *S. cerevisiae* strains, and

v. impact of supplementing the commercial cellulase cocktail with a recombinant β-glucosidase expressed by *S. cerevisiae*.

2. Literature Review

2.1. Bioethanol

The continuing growth and development of the world and the expansion of technologies and industries have led to a point in global history where we are increasingly dependent on fossil fuels to feed energy supply. The world's oil consumption reached 93,25 million barrels per day in 2010, with South Africa's oil consumption at 553 000 barrels per day (www.cia.gov). However, this source of energy is finite – oil, coal and natural gas are becoming more coveted and thus more expensive. It is estimated that at the present economic growth rate, the world will have depleted all of the current crude oil sources within the next 35 years (www.iea.org). Despite this, the world's energy and oil demands continue to grow as technologies and industries advance. The consumption of fossil fuels also leads to the release of greenhouse gasses, which in turn contribute to an increase in global temperature. This increase in temperature is expected to dramatically change the global climate, with disastrous results, such as changes in weather patterns, devastating storms and a rise in the sea level (Sadorsky, 2009).

The need for alternative energy has been addressed by the production and usage of renewable energy sources in several countries (www.EthanolRFA.org). The integration of renewable energy into the current energy system will ensure a smooth transition to an energy system that is independent on fossil fuels such as coal, gas and oil. One of the world's biggest demands is petroleum – a liquid fuel produced from crude oil that powers almost all motorised vehicles and is the cornerstone of the transportation industry. Cost-effective petroleum sources are diminishing rapidly, generating a need for alternatives to feed the demand. Liquid biofuels obtained from sustainable local sources can supplement and ultimately replace petroleum as well as diesel, can reduce dependency on foreign oil, and reduce carbon emissions from internal combustion engines. One of these alternative liquid fuels is bioethanol. The global production of bioethanol tripled between 2000 and 2007, and 84,6 billion litres was produced in 2011, mostly from food crops. In the USA and Brazil, bioethanol is already being blended with petroleum for use in flexible-fuel vehicles. It is estimated that as long as oil prices remain high, there will be a substantial market for ethanol even if the production costs are not subsidised by government (Tyner, 2008).

The first stages of the energy revolution incited the production of liquid fuels from starch and sugar-rich sources such as corn, sugarcane and sugarbeets, generally referred to as

first generation (1G) biofuels (www.biofuelstp.eu). The process of producing ethanol from these sources is well-established and fairly straight-forward. For example, starch has a relatively simple structure and can be easily hydrolysed to fermentable sugars by starch-hydrolysing enzymes (amylases). However, the use of food crops for fuel could create a global food supply shortage if it became widely implemented (Tyner, 2008). Also, one of the biggest challenges to the widespread integration of bioethanol with petroleum fuels is the cost of production. Corn and wheat as feedstock, as well as the starch-hydrolysing enzymes, are very expensive. Therefore, the focus of liquid fuel production has shifted towards non-food feedstocks, specifically lignocellulosic biomass. These feedstocks are not in direct competition with food production and are cheap and abundantly available (Binod *et al*., 2011). In 2008, the annual production of lignocellulosic biomass worldwide was estimated at 1 x 10^{10} metric tonnes, in the form of wood, agricultural residues and bagasse (Sánchez and Cardona, 2008).

Fuels produced from sustainable feedstocks, such as lignocellulose, are referred to as second generation (2G) biofuels. The environmental impact of using 2G biofuels is significantly lower than 1G biofuels. Britain's National Non-food Crops Centre estimate the total net saving on greenhouse gas emissions at 25-87% for 1G biofuels when compared to fossil fuels, the exact number depending on the specific feedstock (www.nnfcc.co.uk). However, this number does not consider the significant amount of nitrous oxide that is released by nitrogen fertilisers during crop cultivation. 2G biofuels is estimated to save 90% on the total net greenhouse gas emissions when compared to fossil fuels (www.eea.europa.eu). It also produces no additional nitrous oxide since most lignocellulosic materials are waste products from other industries.

Lignocellulosic feedstocks for the production of 2G biofuels are significantly cheaper than those for 1G biofuels, and in some cases they have no monetary value. However, the process of producing ethanol from lignocellulosic materials is much more complex than that of 1G biofuels. Due to the physical and chemical characteristics of lignocellulose, several steps are to be completed before ethanol is obtained. These steps are illustrated in Figure 1.

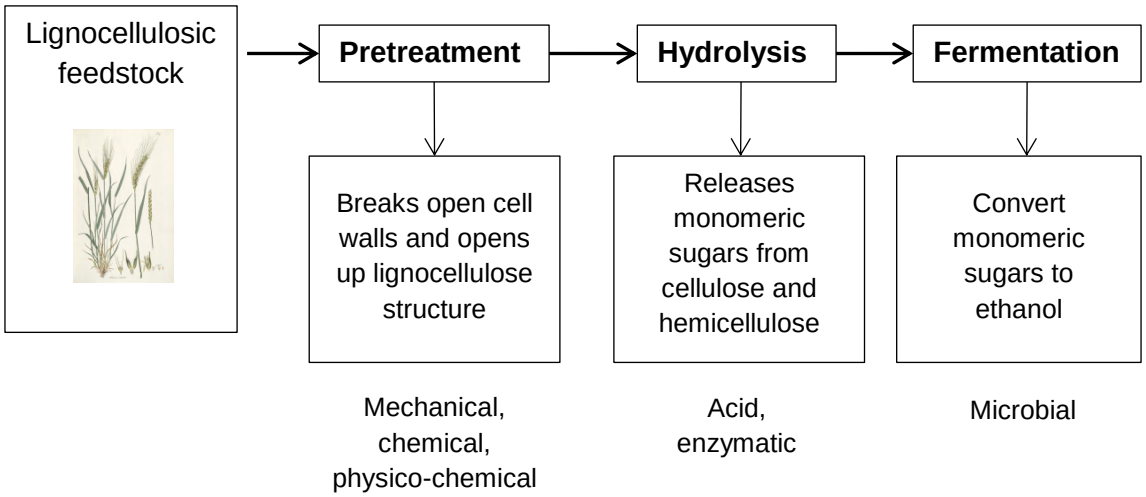


Figure 1: Processes involved in the complete conversion of lignocellulose to ethanol in the production of 2G biofuels.

2.2. Lignocellulose as 2G biofuel feedstock

Lignocellulosic biomass refers to plant material composed of cellulose, hemicellulose and lignin (Palmqvist and Hahn-Hägerdal, 2000). It is found in all plants as it forms the support structure of the plant cell wall. Sources of lignocellulose for biofuel production include agricultural and forestry residues (straw and bran, hardwoods and softwoods, bagasse) as well as wastes from industry such as paper sludge, as well as municipal solid waste. Lignocellulosic materials can be used to produce biofuels through enzymatic hydrolysis and fermentation of the resulting monomeric sugars to ethanol (Sarkar *et al*., 2012).

Cellulose is the main component of lignocellulose (30-50 % of dry weight) and consists of linear chains of hundreds to thousands of β-1,4-linked D-glucose monomers (Pérez and Mackie, 2001). The hydroxyl groups on the glucose molecules in one chain form hydrogen bonds with oxygen atoms on other chains, creating a tight, rigid structure. A bundle of cellulose chains form a microfibril, which combined provides strength to the structure of plant cell walls (Figure 2). In lignocellulose, hemicellulose is intertwined with these microfibrils. Hemicellulose (20-40% of lignocellulose dry weight) consists of branched chains of monomeric sugar units, including hexoses (glucose, mannose, galactose), pentoses (xylose and arabinose) and other acetylated sugars (Walker and Wilson, 1991). The hemicellulose chains are much shorter than those of cellulose and create a branched structure. These characteristics make it easier for hydrolysing enzymes to degrade hemicellulose compared to cellulose, since the branches in hemicellulose create more open ends for the attachment of hydrolysing enzymes.

Lignin is a highly complex, branched and cross-linked molecule composed of aromatic acids, which provides strength to the plant structure especially within the stems, branches, trunks and bark. Harder plant materials such as wood contain a larger proportion of lignin and have a different structure to those in materials such as straw or leaves (Han *et al*., 2010) (Table 1). Lignin is fully integrated into the lignocellulose structure together with cellulose and hemicellulose, as illustrated in Figure 2.

Table 1: Composition of some lignocellulosic materials (adapted from Sánchez, 2009).

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Sugarcane bagasse	32-44	27-32	19-24
Rice straw	32	24	18
Barley straw	31-34	24-29	14-15
Wheat straw	29-35	26-32	16-21
Rye straw	33-35	27-30	16-19

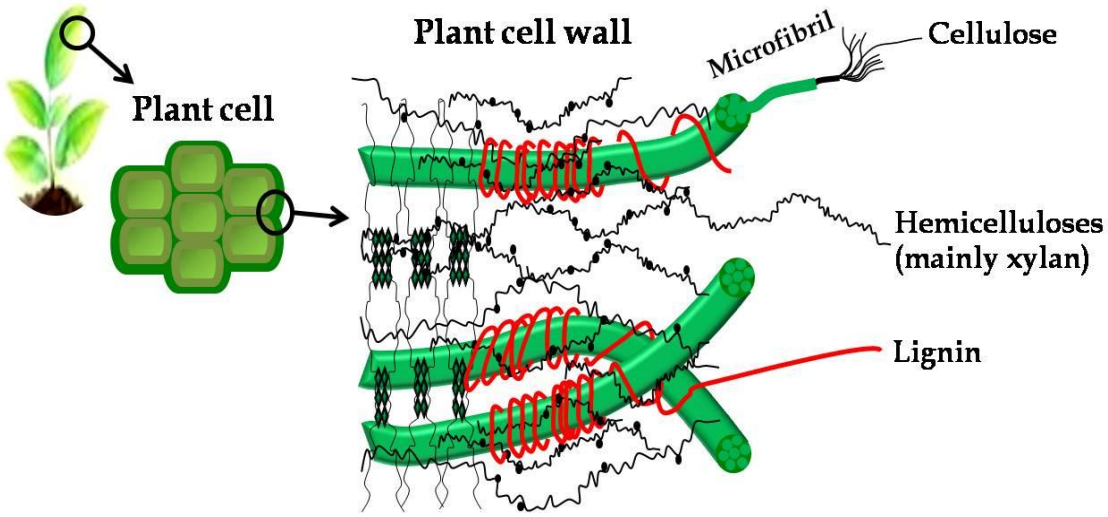


Figure 2: Illustration of the organisation of cellulose, hemicellulose and lignin within the plant cell wall (Ratanakhanokchai *et al*., 2013).

The abundance of lignin in wood poses a challenge when using these materials for bioethanol production, since one of the functions of lignin is to protect the plant from biological and enzymatic attack (Juhász *et al*., 2005). Despite the smaller amount of lignin in materials such as straw (Table 1), it still creates a significant barrier to such an extent that specialised strategies have to be employed to extract and utilise the cellulose.

Lignin hinders complete and efficient enzymatic hydrolysis of cellulose in several ways (Van Dyk and Pletschke, 2012), including:

- direct inhibition of cellulases;
- creating a physical barrier to enzymes;;
- non-specific adsorption of enzymes to lignin, which reduces the hydrolytic ability of the enzyme; and
- blocking the progression of an enzyme along the cellulose chain, effectively reversing the adsorption of the enzyme and stopping the hydrolysis.

Regardless of how lignin causes the reduction in bioconversion efficiency, the removal or disruption of lignin is an essential step to ensure maximum sugar yields from lignocellulose (Van Dyk and Pletschke, 2012). The exact composition and chemical structures vary between lignocellulosic materials, for instance softwood hemicellulose contains more glucose and mannose units compared to hardwoods, which in turn contain more xylose (Palmqvist and Hahn-Hägerdal, 2000). It is thus necessary to adapt the processing of each feedstock to suit specific requirements.

2.2.1. Agricultural residues

Agricultural residues are the remnants of crops after the edible portion of the plant has been harvested, or what is left after processing. These include the stalks, stems, leaves, husks, bagasse and seed pods of the plants (Lal, 2008). The residues are commonly used in soil amendment, as straw for thatching or as animal bedding. In Brazil, sugarcane bagasse is used in the sugar production plant for electricity and heat generation through burning (Sarkar *et al*., 2012).

Figure 3: Agricultural residues with potential as bioethanol feedstock (Anwar *et al.,* 2014)

Crops such as wheat, corn, rye and rice all leave significant amounts of straw behind after harvesting of the grains; 600-900 million tonnes of rice straw and more than 500 million tonnes of wheat straw is produced globally each year (Zhang *et al*., 2012; Sarkar *et al.,* 2012). Most of this straw is burned and incorporated into the soil to improve the mineral wealth (Zhang *et al.,* 2012). Over 90% of corn straw produced in the USA is left or burned in the field (Sarkar *et al*., 2012). It is debatable whether residues should be used to enrich soil after the harvest of crops (i.e. soil amendment) instead of fertilizers (Lal, 2008). The re-introduction of the residues into soil does not completely replace the nutrients, since it takes several years for the residues to break down in the soil and release its nutrient content. The traditional practice of burning the residues in the field also damages the microfauna and microflora in the soil, and causes air pollution (Curreli *et al*., 2002). This affects human and animal health, and is banned in many European countries (Sarkar *et al*., 2012). The practice of tillage, where the residues are reintroduced into the soil during ploughing, is also thought to be harmful to the soil ecosystem and quality. If these practises are discontinued, straw and other residues will maintain a low monetary value and be ideal for biofuel production.

Straw has significant potential as feedstock for biofuel production. It is abundantly available, inexpensive, generates low net greenhouse gas emissions and is not in direct competition with food production (Kumar *et al*., 2009; Lal, 2008). About 1 kg of straw is

produced for every 1,3 kg of wheat grain that is harvested (Zhang *et al.,* 2012). It is estimated that more than a billion tonnes of straw (combined rice, wheat and corn straw) is available worldwide for bioethanol production, which could potentially be converted to 150 billion litres of bioethanol (Sarkar *et al*., 2012). Since the cost of the feedstock is the main cost associated with lignocellulosic bioethanol production (representing around 38% of the production cost), using an inexpensive feedstock is essential to reducing the sale price of bioethanol (Gnansounou and Dauriat, 2010).

Using straw as feedstock eliminates the need for additional fertile land, water or energy to plant crops, which is the case with dedicated bioenergy crops such as switchgrass. The agricultural crops that produce straw also grow much faster than trees that could supply wood feedstocks. Straw is easier to hydrolyse than wood (due to its low lignin content) and will consequently require less severe pretreatment conditions, which in turn saves energy, time and chemical input (Curreli *et al*., 2002). All in all, a feedstock such as straw remains a viable, cheap, safe and economically friendly alternative to traditional feedstocks such as sugar, corn or grains (Lal, 2008).

2.3. Pretreatment

Lignocellulosic feedstocks require pretreatment since the enzymatic hydrolysis of cellulose is hampered by various physico-chemical, structural and compositional factors (Alvira *et al*., 2010). In the lignocellulose structure, the cellulose is intertwined with lignin and hemicellulose, making it inaccessible to hydrolysis. The highly crystalline structure of cellulose also hinders the hydrolysis process.

Pretreatment is any specialised treatment process through which the cellulose is released from the lignocellulosic structure to make it available to enzymatic hydrolysis. A good pretreatment process should achieve the following (Alvira *et al*., 2010; Kumar *et al*., 2009):

- disrupt the lignin and hemicellulose barrier to expose cellulose;
- decrease the crystallinity of the cellulose structure;
- increase the porosity and decrease particle size to create a larger accessible surface area;
- avoid the degradation or loss of sugars;
- limit the formation of inhibitory by-products that affect subsequent hydrolysis or fermentation; and
- be cost-effective.

Pretreatment changes the microscopic and macroscopic structure of the material and significantly increases the digestibility of the substrate. Figure 4 illustrates how pretreatment opens up the lignocellulose structure to expose cellulose and to reduce its crystallinity. Although it adds substantial cost to the production of ethanol from lignocellulose, it is an essential step to ensure the release of sugars during hydrolysis (Mosier *et al*., 2005).

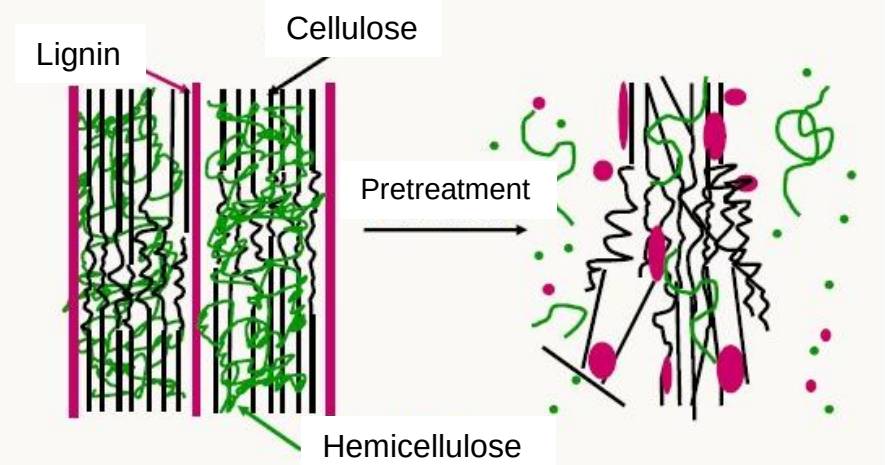


Figure 4: Schematic representation of the effect of pretreatment on the lignocellulose structure. The rigid structure of lignocellulose is disrupted, and the crystallinity of cellulose is reduced. Hemicellulose is partially degraded and lignin is removed or redistributed (adapted from Mosier *et al*., 2005).

There are different types of pretreatment techniques, including biological pretreatment, chemical treatment (concentrated acid, etc.), mechanical and steam explosion. The specific strengths and disadvantages of these techniques should be considered when choosing a pretreatment process. Each process is specifically suited to a substrate and the conditions for each technique needs to be fine-tuned to optimise the sugars released while minimising carbohydrate loss and inhibitor formation (Rivers and Emert, 1988; Ballesteros *et al*., 2006). Increasing the severity of the pretreatment process (eg. higher temperatures and higher pressures, addition of acids or other chemicals) may improve the digestibility of cellulose, but can also lead to sugar degradation or inhibitor formation. Decreasing the severity may not have these negative side-effects, but may also not produce high sugar yields if the lignocellulose structure is not sufficiently disrupted (Olofsson *et al*., 2008).

One of the most popular and effective pretreatment methods is steam explosion, which ensures high sugar recovery and good hydrolysis (Alvira *et al.,* 2010; Kumar *et al*., 2009). It

requires less hazardous chemicals, is not time-consuming and could be implemented on a commercial scale. Steam explosion requires 70% less energy than mechanical methods (e.g. milling or grinding) to achieve the same particle size reduction (Holtzapple *et al*., 1989). It is considered a physico-chemical pretreatment process, since it combines mechanical and chemical forces to effectively expose cellulose fibres. During this process, the material is treated and impregnated with highly pressurised, saturated steam (often with the addition of chemicals such as dilute acids or SO_2), after which the pressure is reduced abruptly. The process is initiated at temperatures of 160-260°C and pressures of 0.69 to 4.83 MPa for several seconds or minutes (depending on the material), before being exposed to atmospheric pressure.

The mechanical effect of steam explosion is that the material undergoes explosive decompression, which opens up the lignocellulose fibres and exposes amorphous cellulose. The chemical effect is called autohydrolysis, which is the process by which acetic acid is formed at high temperatures from acetyl groups present on the hemicellulose chains. This leads to the hydrolysis of hemicellulose and the release of various sugars in the hemicellulose, such as xylose, mannose and arabinose. Water also acts as an acid at high temperatures, assisting in this degradation process (Alvira *et al.,* 2010). Hemicellulose presents one of the main barriers to enzymatic hydrolysis by cellulases, therefore the removal of hemicellulose is crucial to obtaining the highest possible sugar yields (Yang *et al*., 2011). Lignin is also degraded, redistributed and removed from the substrate to some extent. However, the decrease in temperature after pretreatment leads to repolimerisation, causing some lignin to be reconstituted (Cantarella *et al*., 2004). Although lignin is not necessarily removed, it is displaced and its structural interference with enzyme activity is reduced (Kristensen *et al*., 2008). In terms of the carbohydrate composition of the substrate, steam explosion pretreatment generally leads to a 10 to 20% increase in cellulose, a 15 to 20% reduction of hemicellulose, but no drastic changes in the lignin content is observed (Kristensen *et al*., 2008).

2.3.1. Inhibitors

Although the degradation of some structural components during the pretreatment process is desired, the formation of several inhibitors is a drawback, since these products could inhibit both the enzymatic hydrolysis and subsequent fermentation (García-Aparicio *et al*., 2006; Palmqvist and Hahn-Hägerdal, 2000). The inhibition of these processes can lead to lower ethanol yields and lower productivity.

The degradation of hemicellulose through autohydrolysis produces acetic acid as well as xylose and several hexoses (glucose, mannose, galactose, etc.). The xylose is further converted to furfural (Figure 5), whereas the hexoses are converted to 5-hydroxymethyl furfural (HMF). Furfural and HMF can be degraded to formic acid (Palmqvist and Hahn-Hägerdal, 2000). The breakdown of lignin produces a wide variety of phenolic compounds that could also potentially affect downstream processes.

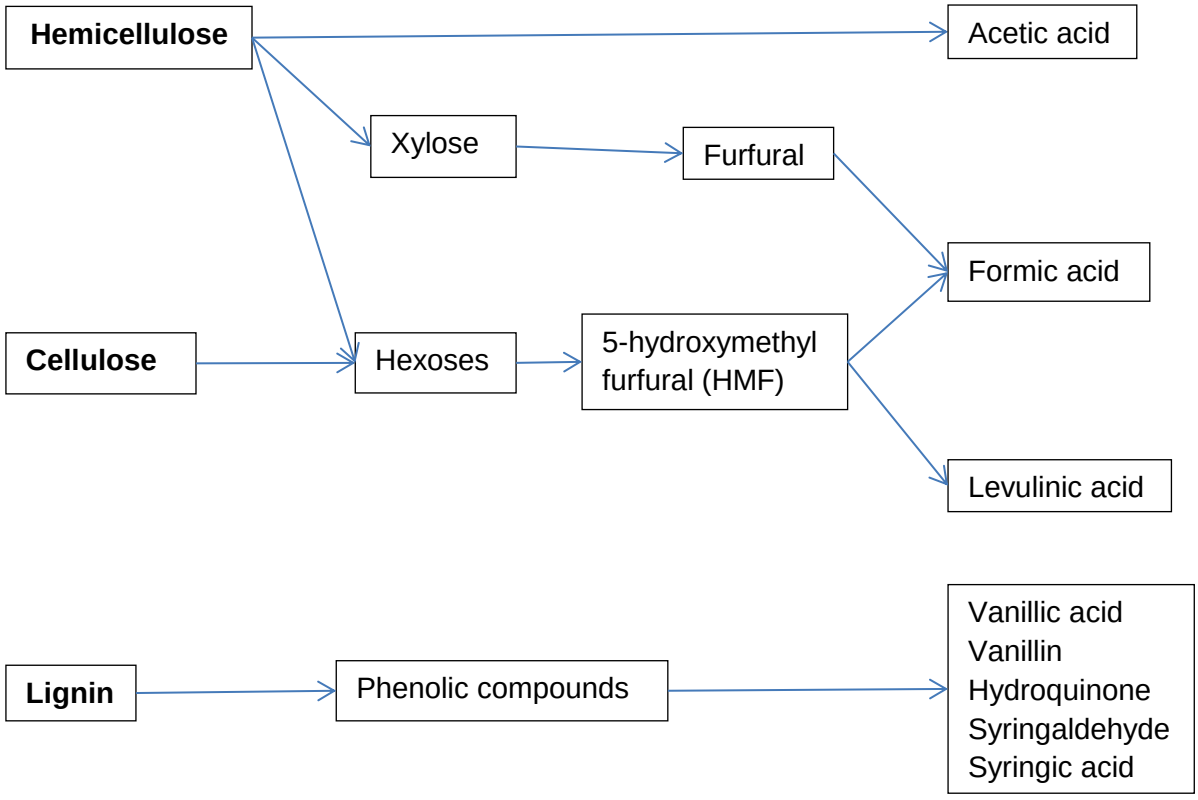


Figure 5: Compounds released during the degradation of lignocellulosic constituents during high temperature and pressure conditions, such as those required for steam explosion pretreatment (Adapted from Palmqvist and Hahn-Hägerdal, 2000).

Weak acids such as acetic, levulinic and formic acid have a significant effect on the micro-organisms that are used in the subsequent fermentation process, as it inhibits cell growth at high concentrations. It is therefore necessary to carefully consider how these byproducts will affect reactions that utilise living micro-organisms (Brown and Booth, 1991). Slight inhibition of yeast growth is desirable, since it will prevent the loss of sugars through

biomass formation by yeast. However, if ethanol production is negatively affected, the presence of inhibitory compounds must be avoided.

Low concentrations of weak acids have been found to have a stimulating effect on ethanol production by *Saccharomyces cerevisiae*, specifically acetic, formic and levulinic acid (Pampulha and Loureiro-Dias, 1989; Larsson *et al.,* 1998). Low acid concentrations (<100 mmol/l) increased the ethanol yield, but the yield decreased at higher concentrations. It was also reported that acetic, formic and levulinic acid had different toxicities at the same concentrations, indicating that they may have different membrane permeabilities (Larsson *et al*., 1998). Interestingly, low internal pH is reported to enhance the thermotolerance of *S. cerevisiae*, thought to be the effect of transcription activation of certain heat-shock genes (Coote *et al*., 1991).

Two theories have been proposed for the inhibitory mechanism of weak acids: the uncoupling effect and intracellular anion accumulation. The uncoupling effect is based on the theory that the cell uses an ATPase proton pump to remove protons from the cell when the intracellular pH decreases because of an influx of weak acids (Stouthamer, 1979; Verduyn *et al.*, 1992). This process depletes ATP; the growth of yeast in the presence of sorbic acid is reported to decrease the intracellular ATP levels 10-fold (Holoyaki *et al*., 1996). To maintain a neutral intracellular pH, the cell must generate additional ATP that under anaerobic conditions leads to the production of ethanol, at the expense of biomass production (Viegas and Sá-Correia, 1991). However, at high acid concentrations, the proton secreting capacity of the cell is overwhelmed, depleting the ATP and leading to the acidification of the cytoplasm. Unfortunately this theory is not supported by the fact that the anionic forms of acetic and formic acid are lipophobic, i.e. they would not cross the plasma membrane during growth on glucose (Casal *et al*., 1996). It is also unlikely that weak acids would deplete the proton motive force since each undissociated acid molecule leads to the import of only one proton (Russell, 1992).

The anion accumulation theory speculates that the anionic form of the acid is captured in the cell while undissociated acid diffuses into the cell until equilibrium is reached (Rottenberg, 1979). When the extracellular pH is low in *S. cerevisiae* cultures, anions continue to accumulate inside the cell to high levels as the yeast maintains a neutral intracellular pH (Russell, 1992). The activity of glycolytic enzymes seems to be affected by weak acids due to internal acidification and direct interference of the acid, leading to higher ethanol production rather than completion of the glycolytic pathway (Palmqvist and Hahn-Hägerdal, 2000).

Hydroxymethyl-furfural (HMF) and furfural are toxic compounds that lower saccharification yields and impact yeast growth (García-Aparicio *et al*., 2006; Panagiotou and Olsson, 2006). Sanchez and Bautista (1988) found that furfural at a concentration of 1.5 g/l caused inhibition of fermentation and biomass formation in *S. cerevisiae*. While HMF did not decrease the amount of ethanol produced, it did delay the start of ethanol production. Taherzadeh and colleagues (2000) observed the same inhibitory effect on fermentation at a concentration of 4 g/l furfural. However, furfural was rapidly converted by the yeast to furoic acid and furfuryl alcohol in both cases. Tomás-Pejó and colleagues (2008) noted that HMF and furfural could be used by xylose-fermenting yeast strains to overcome redox imbalances, which usually leads to the accumulation of xylitol. In the presence of HMF or furfural, the compounds act as electron acceptors, alleviating the redox imbalance and improving xylose fermentation.

The phenolic compounds that are released by the degradation of lignin also negatively affect enzyme activities. Ximenes and colleagues (2010) investigated the inhibitory activity of phenols released during liquid hot water treatment of distiller's grain. Vanillin, syringaldehyde, trans-cinnamic acid and 4-hydroxybenzoic acid all displayed significant inhibition of three different commercial cellulases. The removal of these compounds from the substrate is necessary to achieve the maximum cellulase activity and ethanol yields.

Despite all the research conducted on the effects of toxic compounds on enzymatic hydrolysis and fermentation, high levels of monomeric sugars and oligomers such as cellobiose have a much greater impact on enzymatic hydrolysis than any phenolic compound or acid (García-Aparicio *et al*., 2006). Glucose is known to inhibit the activity of β-glucosidases, while cellobiose displays strong inhibition of cellobiohydrolase enzymes (Xiao *et al.,* 2004). Since the hydrolytic activity of these types of enzymes is essential to achieving complete hydrolysis of cellulose, this inhibitory effect could significantly impact the efficiency of the entire process.

The material obtained from steam explosion pretreatment is often pressed and thoroughly washed to remove inhibitory compounds that will negatively affect cellulase activity (García-Aparicio *et al*., 2006). The liquid fraction (containing monomeric and short oligomeric sugars, as well as several by-products) and water-insoluble solids (WIS) (containing cellulose and hemicellulose chains) are separated to obtain the most digestable substrate. Unfortunately, this does not protect the process from the effects of product inhibition.

2.4. Lignocellulose hydrolysis

The structural characteristics that are proven to influence lignocellulose hydrolysis the most are the surface area and crystallinity of the substrate (Fan *et al*., 1980; Arantes and Saddler, 2010; Yang *et al*., 2011). Cellulose is a heterogeneous material with outer and inner surfaces, as well as amorphous and crystalline areas. The external surface area depends on the shape and size of the particles, which can be determined with a particle counter (Cowling and Brown, 1969; Lee and Fan, 1982). The internal surface area is determined by the porosity and capillary structure of the cellulose fibers. The internal surface area can be measured by analysing N_2 adhesion to a dry sample, or with solute exclusion on a wet sample (Fan *et al.,* 1980; Stone *et al.*, 1969).

Cellulose contains crystalline and non-crystalline (amorphous) regions with crystalline regions being more difficult to hydrolyse than the amorphous regions (Coughlan, 1985). The crystallinity of cellulose in a substrate is widely accepted as one of the most important factors influencing enzymatic hydrolysis. In crystalline cellulose microfibrils, the cellulose chains are tightly packed and cross-linked with hydrogen bonds, creating a rigid, inaccessible structure that cellulases are unable to penetrate. This means that the fraction of crystalline cellulose in a substrate is of particular interest. Cellulose crystallinity can be measured with X-rays and a crystallinity index (Crystalline cellulose/Total cellulose x 100) determined. Fortunately, most pretreatment methods that are used to overcome the recalcitrance of lignocellulose also disrupt crystalline cellulose and significantly reduce the crystallinity index of a substrate (Walker and Wilson, 1991).

2.4.1. Chemical hydrolysis

The release of monosaccharides from the lignocellulosic substrate (saccharification) can be achieved in several ways, including chemical hydrolysis, the most common of which is acid hydrolysis. During this process, the hydrogen bonds between cellulose chains are disrupted by concentrated acid (such as sulphuric acid), leaving the cellulose in a completely amorphous state, as it forms a homogenous gelatine with the acid. The acid is then diluted with water and the cellulose is rapidly hydrolysed to glucose at mild temperatures. Unfortunately, concentrated acid is toxic and corrosive, resulting in a high maintenance cost for the reactors. Although the treatment is rapid and easy to apply, it is plagued by non-selectivity (i.e. degradation of the released sugars) and the formation of inhibitory by-products such as caproic acid, caprylic acid, pelargonic acid and palmitic acid (Fan *et al.*, 1982; Palmqvist and Hahn-Hägerdal, 2000; Tran and Chambers, 1985). In

addition, the ethanol yields obtained with dilute-acid hydrolysis and subsequent fermentation are only 50-60% of the theoretical yield, since the acidic environment is unsuitable for the yeasts used to ferment the released sugars (Wyman, 1994). The hydrolysed materials must therefore be highly diluted or neutralised prior to fermentation, which further increases the processing costs.

2.4.2. Enzymatic hydrolysis

Enzymatic hydrolysis is an environmentally friendly process and does not cause the problems with corrosion linked to chemical hydrolysis, it also has lower energy costs and operate at milder conditions (Yang *et al*., 2011). Enzymatic hydrolysis yields higher monosaccharide sugars, since cellulase enzymes are highly substrate-specific and only catalyse the hydrolysis of cellulose, not the degradation of sugars, as is the case with acid hydrolysis (Parisi, 1989). Enzymes are also biodegradable, natural compounds and therefore offer environmentally friendly disposal options.

2.4.2.1. Cellulase production

Cellulases are used in several applications, for example, reducing the fibre content of animal feed and clarification of fruit juices. They are also used in the textile industry, the pulp and paper industry and in laundry detergents (Sukumaran *et al*., 2005). Most cellulases are produced by fungal species that have the natural ability to degrade and utilise cellulose (and hemicellulose) from plant material (Lynd *et al*., 2002). Enzyme addition for cellulose hydrolysis significantly contributes to the production costs of bioethanol on a commercial scale (Wyman, 2007). This is because the production of cellulases on an industrial scale is a highly specialised process. The expression of cellulase genes by the producing organism requires the genes to be induced, either by culturing on cellulose or by an inducer molecule such as lactose (Sukumaran *et al*., 2005). However, culturing on a solid substrate such as cellulose causes practical challenges and makes the process expensive. There has been much research into using lignocellulosic waste for culturing and production as cheaper substrates will help to reduce the cost of the production of cellulases (Juhász *et al*., 2005). It has also been found that the enzyme activity and characteristics are highly dependent on the nature of the carbon source on which it is produced. This means that using the same lignocellulosic feedstock for enzyme production and enzymatic hydrolysis could be beneficial (Sukumaran *et al*., 2005).

The most widely used cellulolytic fungus is *Trichoderma reesei*, which is used for the production of several commercial cellulase preparations (Yang *et al*., 2011; Peterson and Nevalainen, 2012). In an attempt to lower the costs of biofuel production, *T. reesei* and other organisms used for cellulase production have been extensively engineered and selected to maximise the amount and types of enzymes produced (Yang *et al*., 2011). Genetic modification of these organisms has helped to improve the quality of the cellulase cocktails that they produce. Desirable characteristics for cellulases include thermal stability, high specific activity and resistance to environmental inhibitors (Sukumaran *et al.,* 2005).

2.4.2.2. Classification of cellulases

Cellulases are enzymes that hydrolyse the β-1,4-D-glucan linkages in cellulose, and are classified as glycoside hydrolases (Mathew *et al*., 2008). The action of cellulases leads to the degradation of cellulose to glucose, cellobiose and cello-oligosaccharides. Most cellulases have a characteristic two-domain structure. These domains are the catalytic domain, which contains the catalytic site, and the carbohydrate-binding module, which facilitates the binding of the enzyme to the carbohydrate substrate. The two domains are connected through a peptide linker.

The complete and efficient hydrolysis of cellulose to glucose requires the synergistic action of three enzymes – endoglucanase, cellobiohydrolase (or exoglucanase) and β-glucosidase (Binod *et al*., 2011; Van Dyk and Pletschke, 2012; Lynd *et al*., 2002). Endoglucanase (EG) (endo-β-1,4-glucanase) targets cellulose chains within amorphous regions of the cellulose structure and randomly hydrolyses the internal β-1,4-glucosidic bonds. This exposes free ends that are accessible to cellobiohydrolase (CBH) (1,4-β-D-glucan cellobiohydrolase), which systematically cleaves off cellobiose units from the free ends of the cellulose chains. There are two kinds of CBH enzymes - CBHI cleaves cellobiose from the reducing ends, while CBHII cleaves cellobiose from the non-reducing ends. Beta-glucosidase (BGL) hydrolyses cellobiose into glucose and some are able to cleave individual glucose units from free chain ends of cellulose (Binod *et al*., 2011). With the combined activity of these three enzymes, the cellulose chains become progressively shorter until it is completely hydrolysed to glucose (Figure 6).

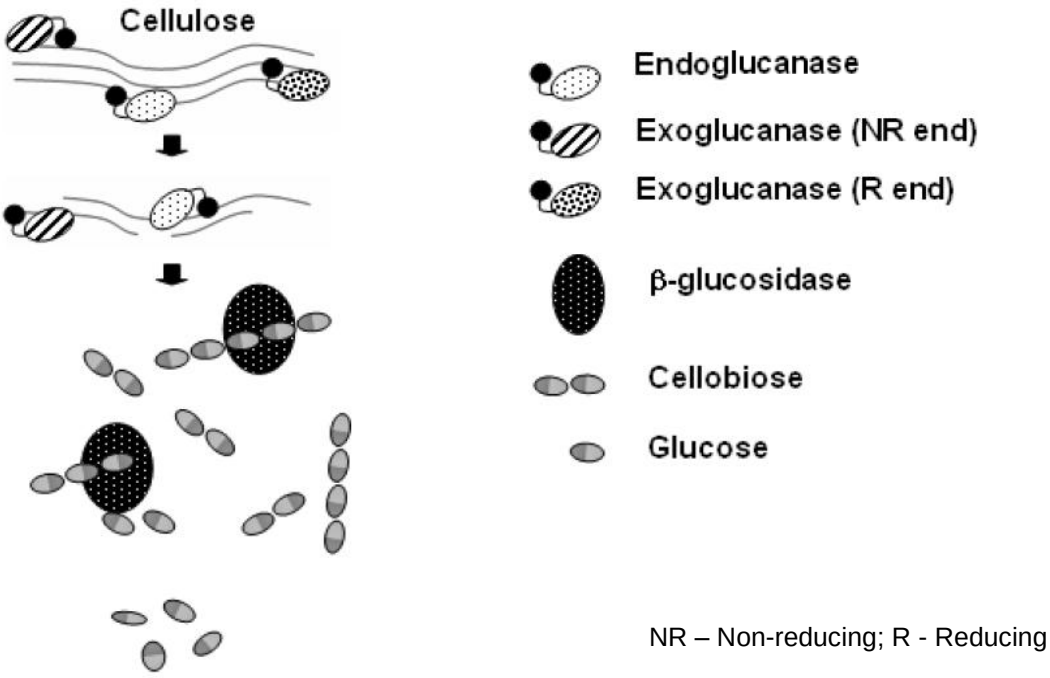


Figure 6: The role of cellulases during enzymatic hydrolysis of cellulose. Endoglucanase randomly hydrolyses the cellulose chains and creates free ends. Exoglucanases, or cellobiohydrolases, can cleave off cellobiose units from these free ends of the cellulose chains, which are then hydrolysed by β-glucosidases to deliver glucose molecules. The synergistic action of these enzymes reduces the degree of polymerisation until only glucose molecules are left (Mathew *et al*., 2008).

The CBHI-protein has a three-dimensional structure with four peptide loops that forms a tunnel-like structure on the surface (Lynd *et al*., 2002). CBHII only has two loops that form a shorter tunnel. These tunnel structures are essential in the hydrolysis of reducing and non-reducing ends of cellulose chains, as it allows the cellulose chain to move progressively through the tunnel structure as cellobiose molecules are removed from the end. EG-proteins have a similar three-dimensional structure, but contain shorter loops that form a cleft or groove-like structure (Figure 7). The open shape of the cleft allows the EG to bind in the middle of a cellulose chain, and is not limited to binding to free ends. This allows it to create “nicks” in the chain to which CBHI or II can bind.

The cellulase system of *T. reesei* contains at least two CHB enzymes - one each of CBHI and CBHII, which represents 60% and 20% respectively of the protein mass of the cellulase system (Lynd *et al*., 2002; Bezerra and Dias, 2005). It also contains five different EG species, but these represent less than 20% of the cellulase system. This means that a very small percentage of the cellulase enzymes produced by *T. reesei* are β-glucosidases.

It produces very low levels of BGL compared to other fungi such as *Aspergillus niger,* and they are more susceptible to product inhibition by glucose. This leads to *T. reesei* cellulase preparations supplemented with BGL from *A. niger* being most often used for industrial saccharification (Lynd *et al*., 2002; Sørensen *et al*., 2013).

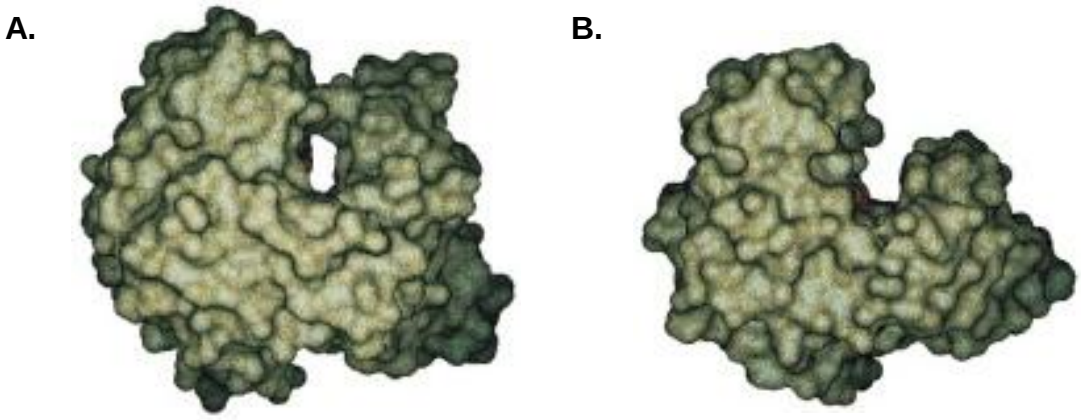


Figure 7: The three-dimensional structures of (A.) cellobiohydrolase, which forms a tunnel-like structure around the catalytic domain and (B.) endoglucanase, with a cleft-shaped structure (adapted from Varrot *et al*., 1999).

In addition to the three enzymes discussed above (EG, CBH and BGL), filamentous fungi also produce accessory enzymes and enzyme cofactors. Some commercial enzyme preparations contain up to 80 different proteins. These proteins may improve the action of the cellulase enzymes, since their presence significantly increase the release of monosaccharide sugars from lignocellulose (Banerjee *et al*., 2010; Gottschalk *et al.*, 2010).

2.4.2.3. Enzyme kinetics

The enzymatic hydrolysis of cellulose includes the following steps (Walker and Wilson, 1991):

1) transfer of the enzymes from the aqueous environment to the cellulose surface;
2) adsorption of enzymes to the cellulose chains, forming enzyme-substrate complexes;
3) hydrolysis of the cellulose;
4) transfer of the hydrolysis products to the aqueous environment; and
5) final hydrolysis to glucose.

These steps can all be influenced by the structural characteristics of the specific substrate, the nature of the specific enzymes and the effect of compounds such as phenols and ethanol.

The enzyme kinetics of cellulases indicate a dramatic decline in the rate of hydrolysis over time. This results in low sugar yields that cannot solely be attributed to product inhibition. Enzyme-related factors could include thermal instability, enzyme inactivation or inhibition, and loss of enzyme function (due to short enzyme half-lives), while substrate-related factors include the transformation of the cellulose into a less digestible form or the heterogeneous nature of the substrate (Yang *et al*., 2011). Other explanations for the drop in the hydrolysis rate include the most digestible parts of the substrate being hydrolysed preferentially by the enzymes, as well as "jamming" of the binding site of the enzyme (Desai and Converse, 1997; Yang *et al.,* 2006). However, as mentioned earlier, the strongest enzyme inhibition is the result of product inhibition from cellobiose and glucose.

2.4.2.4. Hemicellulose

In some cases, a large fraction of the lignocellulosic structure consists of hemicellulose, whose hydrolysis will add additional monosaccharides that could be fermented leading to a higher ethanol yield. Of particular interest is the polysaccharide xylan, which constitutes the largest fraction of hemicellulose and consists of D-xylose units linked by β-1,4-glycosidic bonds (Binod *et al*., 2011). Commercial xylanase preparations are available to increase the sugars yielded from enzymatic hydrolysis. Some fungi produce a wide array of xylanases including endoxylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, acetyl mannan esterase, ferulic acid esterase and acetyl-xylan esterase (Gottschalk *et al*., 2010). When these enzymes are present in the commercial cellulase enzyme preparations, the sugar yields can be significantly increased. Some pretreatment methods such as steam explosion remove nearly all hemicellulose from the substrate, negating the need to use hemicellulases in enzymatic hydrolysis.

2.5. Fermentation

Fermentation is the chemical reaction where sugars are converted to acids, gasses and/or alcohols. A reduced carbon source, such as glucose, is converted to acid or alcohol by the process of substrate-level phosphorylation in the absence of oxygen as an electron acceptor. The absence of oxidative phosphorylation in this process means that little energy

is obtained – only two ATP molecules are generated during fermentation of one glucose molecule, plus two ethanol and two CO_2 molecules (Figure 8).

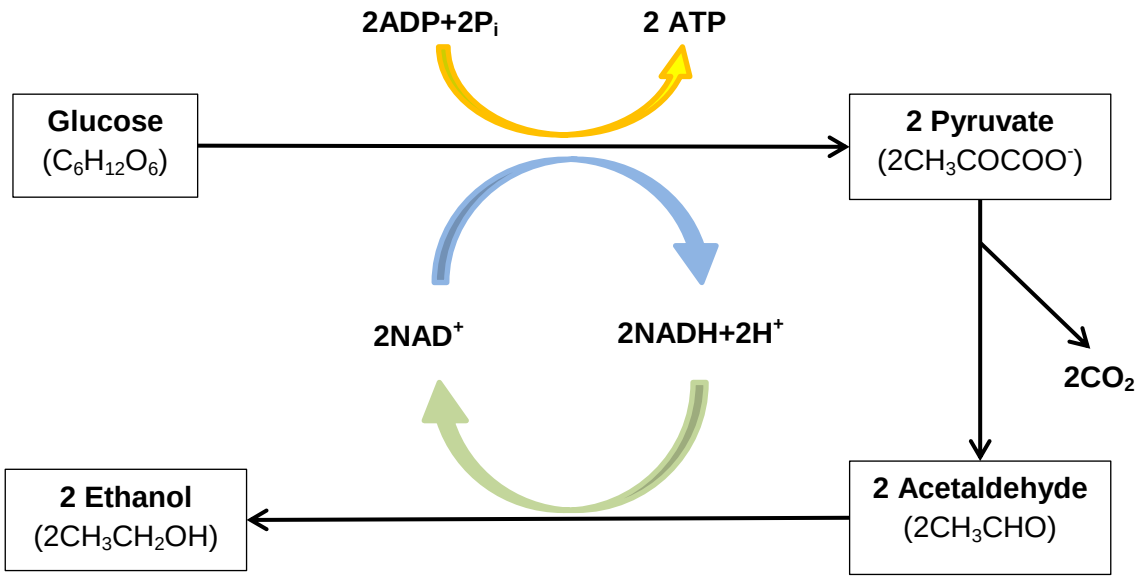


Figure 8: The chemical process of glucose fermentation where one glucose molecule is converted to two ethanol and two carbon dioxide molecules and generates two ATP molecules.

Microorganisms use the fermentation reaction to produce energy in oxygen-poor environments; therefore this process usually occurs under anaerobic conditions. However, some organisms use fermentation for ATP production despite the presence of oxygen. For example, the yeast *S. cerevisiae* will ferment glucose in aerobic conditions if the external glucose concentration is high. This phenomenon is called the Crabtree effect and is only observed in a few yeast and bacterial species. This characteristic of *S. cerevisiae* makes it ideal for the production of wine, beer, bread and bioethanol. It is commonly referred to as baker's yeast and is the most well-known and studied yeast, as well as the most popular microorganism for the production of ethanol. It displays high ethanol productivity, tolerance to high concentrations of ethanol and the inhibitory compounds that are released during pretreatment of lignocellulosic biomass (Olsson and Hahn-Hägerdal, 1993; Sanchez and Bautista, 1988). Several strains of *S. cerevisiae* have also been engineered to perform additional functions, such as the fermentation of pentoses, or adapted to further increase its tolerance to inhibitors and ethanol (Tomás-Pejó *et al*., 2008a). Since lignocellulose is a complex substrate that contains both cellulose and hemicellulose, enzymatic hydrolysis will

release several types of monomeric sugars. These include glucose, xylose, mannose, galactose and arabinose, of which glucose and xylose are the most prevalent. If the hemicellulose portion of the substrate is significant, the conversion of the saccharides to ethanol can increase the ethanol yield from the lignocellulosic substrate. This offers an opportunity to reduce the cost of cellulosic ethanol production by utilising all the available carbohydrates to produce ethanol (Sarkar *et al*., 2012).

The ethanol-producing yeast *S. cerevisiae* can efficiently ferment glucose, but wild type strains are unable to utilise or ferment xylose. Several other microorganisms have the ability to utilise xylose, and genes from these organisms can be introduced into *S. cerevisiae* (Tomás-Pejó *et al*., 2008a). This could provide *S. cerevisiae* strains with the ability to produce ethanol from xylose and therefor effectively increase the ethanol yield from biomass.

2.6. Bioethanol production from lignocellulose

2.6.1. Separate Hydrolysis and Fermentation

During Separate Hydrolysis and Fermentation (SHF), the enzymatic hydrolysis and yeast fermentation are performed individually. Each process has its own operating conditions, which can be optimised separately. Enzymatic hydrolysis with cellulases typically takes place at around 50°C and pH 5.0, while fermentation usually takes place at a maximum of 37°C and a pH of 6-7. The concept of separating these processes to perform at their individual optimal conditions is thus simple and this method is frequently used for ethanol production. However, a problem arises during the enzymatic hydrolysis phase when the production of glucose due to the hydrolysis of cellulose inhibits the cellulases, particularly β-glucosidases (Alfani *et al*., 2000; Tomás-Pejó *et al*., 2008a). Increased glucose levels result in feedback inhibition, leading to the build-up of cellobiose, which in turn strongly inhibits endoglucanases and cellobiohydrolases. This causes a decrease in the rate of hydrolysis, leading to longer reaction times and lower sugar yields, while also limiting the substrate loading of the reaction. The SHF process results in logistical problems, since this method takes longer and requires two bioreactors. In addition, the reaction mixture must be kept sterile during the hydrolysis step to prevent contamination by microorganisms that could affect the reaction or lead to a loss of carbon (Tomás-Pejó *et al*., 2008a).

2.6.2. Simultaneous Saccharification and Fermentation

The Simultaneous Saccharification and Fermentation (SSF) process involves combining enzymatic hydrolysis with fermentation, allowing both processes to occur concurrently (Alfani *et al*., 2000). This alleviates the problem with feedback inhibition experienced during SHF, since the fermentation reaction immediately removes the glucose released by the enzymatic hydrolysis. The process is faster and it requires only one reactor. However, combining the processes implies compromising on the reaction conditions, and as a result neither process performs optimally.

Several research groups have focussed on comparing SSF and SHF strategies, evaluating the ethanol yield and ethanol productivity of both. Alfani and colleagues (2000) found that in the two-step SHF process, the highest ethanol yield was close to 81% of the theoretical yield at hydrolysis and fermentation temperatures of 45°C and 37°C, respectively. The single-step SSF process at 37°C produced ethanol yields close to 68% of the theoretical yield. However, the SSF process required a much shorter process time of about 30 hours, as opposed to the SHF process of about 96 hours.

Tomás-Pejó and colleagues (2008) considered the effects of separating hydrolysis and fermentation when converting steam-exploded wheat straw to ethanol. Fermentations were performed using both the water-insoluble solids and whole slurry as substrate in SSF and SHF processes. The highest ethanol concentration (23.7 g/l) was obtained from whole slurry as substrate in the SSF process, using a recombinant *S. cerevisiae* strain with xylose-fermenting abilities. A yield of 22.6 g/l ethanol was produced in the SHF process, concluding that the SSF process was a more desirable option. It was also concluded that the use of the whole slurry was advantageous since it contained additional monosaccharides that could be fermented and thus contribute to the ethanol production.

2.6.3. Consolidated bioprocessing

One of the biggest obstacles in the large-scale production of bioethanol is the cost of producing the enzymes required for hydrolysis. A possible solution that has been investigated is engineering microorganisms such as *S. cerevisiae* that have high fermenting abilities, to also produce cellulases. This means that cellulase production, cellulose hydrolysis and fermentation can be combined in one single process, saving on space, time and cost. This concept is called consolidated bioprocessing (CBP) (Lynd *et al*., 2005). However, despite significant efforts to perfect this process, limited success has been

achieved, as yields obtained through CBP are not as high as the ethanol productivity of SSF processes. This is mainly a result of the metabolic burden on the organism when producing several different enzymes, as well as the difficulty of obtaining high levels of enzyme activity. The low recombinant protein levels and enzymes with reduced activity result in lower ethanol yields (Hasunuma and Kondo, 2012). Much attention is given to developing this technique and it is likely to become the dominant method for ethanol production with microorganisms in the future.

In some cases, lignocellulose hydrolysis can be improved by the addition of recombinant cellulolytic yeasts, while still relying on a fungal cellulase cocktail for the bulk of the hydrolysis activity (Shen *et al*., 2008). For instance, most crude cellulase enzyme cocktails have high endoglucanase and exoglucanase activity, but low β-glucosidase activity. This has a major impact on ethanol yields, since the reaction catalysed by the β-glucosidase is considered the most important as it mediates the product inhibition of exoglucanase and endoglucanase. The logical way to alleviate this problem is to supply β-glucosidase during hydrolysis, but this adds substantial cost to the production of ethanol. However, by using a recombinant yeast strain that expresses additional β-glucosidase, there is no additional cost to the process while the glucose and ethanol yields are increased.

Shen and colleagues (2008) used commercial cellulase preparations in conjunction with a recombinant *S. cerevisiae* strain during an SSF process with acid-pretreated corncobs as substrate. It produced 20g/l ethanol after 72 hours, which was close to the ethanol concentration when the parental strain was supplemented with 20 IU of β-glucosidase per gram substrate. However, the results differed when different commercial enzymes were used, highlighting the significance of selecting the right enzyme preparation for the specific substrate.

2.7. Triticale as 2G biofuel feedstock

Triticale (*Triticosecale rimpau*) is a cereal crop hybrid obtained from the crossing of wheat (*Triticum*) and rye (*Secale*) (Oelke *et al.*, 1989; Qualset and Guedes-Pinto, 1996). Its appearance and uses are similar to that of wheat (Figure 9). It originated in the late 19th century with breeding in Scotland and Sweden, and aimed to combine the large yields and high quality grain of wheat with the drought and disease tolerance of rye. Successful hybrids were also selected based on the high protein content of the grains (Oelke *et al*., 1989). It has only recently become a commercially viable crop and is considered to have significant potential as a starch-cellulose feedstock for bioethanol production (Mergoum *et*

al., 2009). Currently, triticale is cultivated in more than 30 countries on an area of over 3.7 million ha, and is especially popular in Europe and in parts of Canada and the USA (Figure 10) (Mergoum *et al*., 2009; Pejin *et al*., 2011). The leading producers of triticale include Germany, France, Poland, Australia and China. Its popularity is constantly growing, with a more than 50% increase in triticale grain production in the last 15 years.

Figure 9: Triticale crops (Source: www.agricool.net)

Figure 10: The distribution of triticale cultivation worldwide (Source: www2.mpiz-koeln.mpg.de)

The benefits of triticale include that it is easily grown on marginal land in nutrient-poor soil, it requires less water and fertilizer than wheat and is resistant to several crop diseases and environmental stresses. Furthermore, even when grown in harsh conditions, triticale still produces high grain yields and can be cultivated year-round (Schwarte *et al*., 2005; Tohver *et al.,* 2005). The grain is normally used as animal feed since it has a higher protein and calcium content than wheat, as well as a lower fibre content (Table 2), and is less expensive (Šramková *et al*., 2009). It can also be used in brewing, baking and cereals for human consumption (Oelke *et al*., 1989). Triticale produces more biomass overall than any other small-grain cereal, because of the high grain yields and taller plants that produce more straw per square kilometre (Mergoum *et al*., 2009).

Triticale is considered a valuable potential feedstock for bioethanol production from both starch and cellulose, where the cultivation of other common feedstocks such as wheat and barley is not feasible due to environmental conditions. Triticale outperforms even the best wheat cultivars on marginal land and in arid conditions (Tohver *et al.,* 2005). Even in areas where it is possible to cultivate other feedstocks, the yield advantage and crop stability of triticale under almost any conditions makes it the more desirable cereal crop (Mergoum *et al*., 2009).

Table 2: Composition of triticale and wheat grain (Oelke *et al.,* 1989; www.ars.usda.gov)

Component	% of dry matter	
	Triticale	**Wheat**
Protein	19.71	14.48
Fibre	3.10	14.02
Fat	1.61	1.77
Calcium	0.12	0.033
Phosphorus	0.44	0.331
Total sugars	5.74	0.47
Starch	67.78	68.26

In South Africa, the use of a suitable crop as feedstock is essential, as large areas of the country are subject to harsh climate conditions. For a feedstock to be economically viable, it would have to be produced locally on a large scale. This can contribute to South Africa's own bioethanol production industry, but also improve the lives of citizens that depend on farming for survival, since the grains can be used as food or animal feed, while the straw can be used or sold as feedstock. The widespread use of triticale in bioethanol production could create a market value that would encourage commercial and subsistence farmers to plant these crops for additional income.

The use of starch-rich **triticale grain** for bioethanol production has been investigated as an alternative to wheat grain. Tohver and colleagues (2005) found that using triticale grain as feedstock negated the use of commercial amylases to degrade starch and concluded that it is a more economical option than wheat and other grains. Vučurović and Pejin (2007) found that this characteristic of triticale grain is due to its high autoamylolytic activity, which means that the grain produces enough of its own amylases to degrade its starch. It was determined that the autoamylolytic quotient (a number representing the self-hydrolysing ability of grains) of triticale grain was ± 88%, depending on the specific variety. With a starch content of about 60%, this means that 30g ethanol per 100g of triticale grain could be obtained without the addition of commercial enzymes. Pejin and colleagues (2009) followed up on this research by comparing the grains of four wheat varieties with those of four triticale varieties. They determined that the autoamylolytic quotients of the wheat varieties ranged between 62.15 to 81.46%, compared to 94.24 to 99.55% in the triticale varieties. This unique characteristic of triticale grain gives it great value in the bioethanol context, since the cost of enzymes is one of the main constraints in the large-scale production of bioethanol from starch.

Triticale bran is another possible source of fermentable sugars in the form of starch, cellulose and hemicellulose. The bran is typically removed from the grains to reduce the fiber content and expand its use in animal feed (Figure 11). García-Aparicio and colleagues (2011) removed the starch from triticale bran and pretreated it with dilute acid before using a commercial cellulase cocktail (Spezyme® CP) and a β-glucosidase preparation (Novozym® 188) to release the monosaccharides. Despite the success of the experiments, the authors questioned whether the starch-removal step and the use of three different commercial enzymes in the process renders it too expensive to be commercially viable, especially considering that the bran represents only a small fraction of the whole crop.

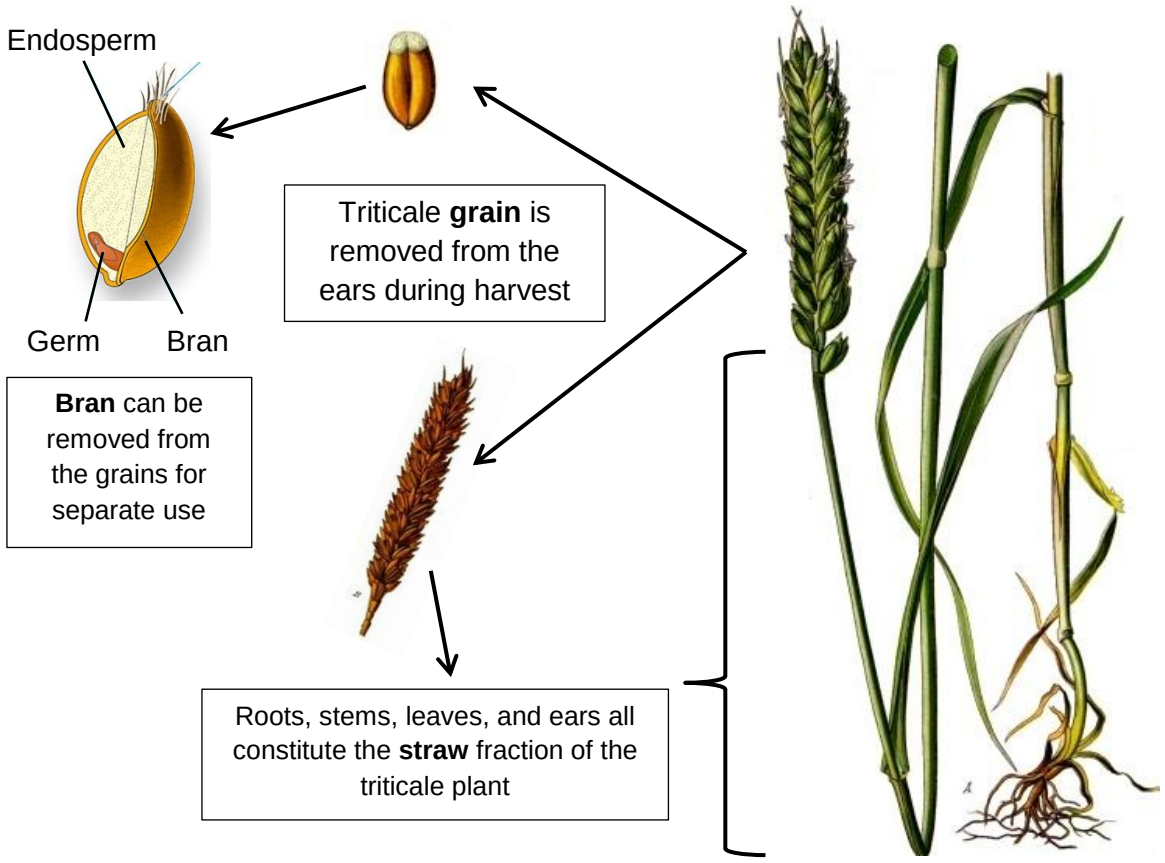


Figure 11: Parts of the triticale plant (adapted from www.oatbran.org)

The use of **triticale straw** in bioethanol applications is somewhat new and little research has been reported in this regard. There has been significant research on the use of other straw-producing crops such as wheat, rice and barley straw for the production of bioethanol, with promising results (Rivers and Emert, 1988; Alfani *et al*., 2000; Garćia-Aparicio *et al*., 2006; Tomás-Pejó *et al*., 2008a). These examples can be used to evaluate the relative potential of triticale straw, since some have similar physico-chemical characteristics to that of triticale (Table 3).

The composition of wheat straw and triticale straw is very similar (Table 3), and as such the data from research on wheat straw can be used as benchmark for triticale straw. As previously mentioned, Tomás-Pejó and colleagues (2008) used steam-exploded wheat straw and commercial cellulases in both an SSF and an SHF process. They reported up to 23.7 g/l ethanol in high-gravity SSF with a xylose-fermenting *S. cerevisiae* strain, which amounts to a 43% ethanol yield. Alfani and colleagues (2000) obtained 68% of the theoretical ethanol yield with SSF. Both used steam-exploded wheat straw, commercial cellulases and high-gravity (10% w/v solids loading) SSF with similar reaction conditions.

However, Alfani and colleagues used higher enzyme loadings and washed the material after pretreatment, removing many of the inhibitory compounds as mentioned in Section 2.3.1.

Table 3: Composition of various straw types including triticale (Adapted from Sánchez, 2009 and the results section of this study)

Straw type	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Rice straw	32	24	18
Barley straw	31-34	24-29	14-15
Wheat straw	29-35	26-32	16-21
Rye straw	33-35	27-30	16-19
Triticale straw	37	25	20

Ethanol production from other types of straw and using other pretreatment methods have also achieved high rates of enzymatic hydrolysis and high ethanol yields using different fermenting organisms (Wang *et al*, 1998; Curreli *et al*., 2002; Karimi *et al*., 2006; Zhu *et al*., 2006; Chen *et al*., 2008; Tomás-Pejó *et al.,* 2009). The research suggests that pretreated straw types are easily hydrolysed by cellulase enzymes and can produce high ethanol yields. However, it is essential to evaluate feedstocks individually and to adapt the specific processes to suit the feedstock's particular requirements. This includes the optimisation of pretreatment, selecting hydrolysing enzymes that have high activity on the substrate, and fine-tuning fermentation to obtain high ethanol yields.

2.8. Proposed study

Research on feedstocks such as rice and wheat straws have provided enough evidence to merit further investigation into other lignocellulosic crop residues. Triticale straw can be used for bioethanol production in the South African context, due to the hardy nature of this crop. However, the process of converting triticale straw to ethanol needs to be evaluated from field to fuel. This means applying standard industrial techniques and processes such

as steam-explosion pretreatment, enzymatic hydrolysis and fermentation to determine its potential as feedstock for the bioethanol industry.

Research has been conducted on the steam-explosion pretreatment and enzymatic hydrolysis of triticale straw at the Department of Process Engineering at Stellenbosch University (Pengilly, 2013). When compared to other lignocellulosic feedstocks, triticale straw is more readily hydrolysed by commercial cellulases and obtains higher glucose yields. The specific cell wall and cellulose structure, as well as the low lignin content, results in milder pretreatment requirements, which in turn leads to the production of fewer inhibitors. Pengilly (2013) found that the WIS fraction of pretreated triticale straw is the preferred substrate for enzymatic hydrolysis, rather than pressed or whole slurry. This substrate will therefore be prepared through pretreatment and washing to be used in SSF experiments according to industry standard methods.

Commercial cellulases from various sources (Optiflow™, Accellerase® 1500, Spezyme® CP, Celluclast® 1.5, Alternafuel® CMAX™) will be screened to achieve optimum glucose yields from the WIS substrate. Samples of these cellulase cocktails were obtained from the suppliers for research purposes by the Department of Process Engineering. All of the enzymes have been used in projects by several research groups and for several purposes and are commercially available (Table 4).

Table 4: Commercial cellulase cocktails for use in hydrolysis and SSF

Enzyme	Supplier	Organism	Reference
Celluclast® 1.5	Novozymes, Sigma Aldrich	*T. reesei*	García-Aparicio *et al*., 2006
Spezyme® CP	Genencor	*T. reesei*	Berlin *et al*., 2006
Optiflow™ RC 2.0	Genencor, DuPont	*T. reesei*	Da Cruz *et al*., 2012
Accellerase® 1500	Genencor	GM *T. reesei*	Lin *et al*., 2010
Alternafuel® CMAX™	Dyadic	*Myceliophthora thermophila*	Gusakov, 2013

The commercial cellulases selected for this project were produced and developed with different applications in mind. Spezyme® CP was developed for use in the starch fuel industry to remove non-starch carbohydrates from grains to improve starch hydrolysis. Celluclast® 1.5 was developed for use in the food industry, while Accellerase® 1500 and Alternafuel® CMAX™ was developed specifically for the cellulosic ethanol industry. Although the cellulases have different operational conditions and have been developed for different applications, the enzymes will all be evaluated under standardised conditions frequently used in SSF applications and as stipulated by the NREL protocol for SSF. As indicated in Table 5, the optimal hydrolysis conditions for most cellulase preparations used in research is 50°C and pH 5 (Sørensen *et al*., 2013).

Table 5: Characteristics of commercial cellulases

Enzyme	Reported activity	Optimum ranges	
		Temperature	pH
Celluclast® 1.5	Cellulase	NR	NR
Spezyme® CP	Cellulase, hemicellulose, β-glucanase	25-50°C	3.5-6.5
Optiflow™ RC 2.0	Cellulase	NR	NR
Accellerase® 1500	Endoglucanase, β-glucosidase, xylanase	50-65°C	4.0-5.0
Alternafuel® CMAX™	Cellulase, β-glucanase and other activities	35-80°C (optimum 55°C)	3.5-8.0 (optimum 6.0)

*NR – Not reported (information not included on product data sheet)

As shown in Table 4, all but one of the commercial cellulase cocktails used in this study are produced by *T. reesei*, whose cellulase preparations are known to lack sufficient β-glucosidase activity (refer to section 2.4.2.2). The lack of β-glucosidase activity in cellulase cocktails significantly hamper the ability to achieve high glucose and ethanol yields (Sørensen *et al*., 2013; Shen *et al*., 2008). The accumulation of cellobiose during hydrolysis, due to the lack of β-glucosidase activity, adversely affects the activity of endoglucanase and cellobiohydrolase enzymes. It should therefore benefit the hydrolysis of the triticale straw if additional β-glucosidase enzymes can be added to the enzymatic

hydrolysis process, which will reduce the inhibitory effects of cellobiose (Walker and Wilson, 1991; Mathew *et al*., 2008). However, adding commercial β-glucosidases will also add to the cost of the process, which should be avoided.

A fungal β-glucosidase enzyme, produced by an existing recombinant *S. cerevisiae* strain, will be used in hydrolysis and SSF processes. The gene encoding this enzyme (named *PcbglB*) was isolated from *Phanerochate chrysosporium*, a species of white-rot fungi that has cellulolytic abilities (Njokweni *et al*., 2012). The gene was obtained from a gene database and was selected because of high substrate specificity. The gene was codon-optimised for expression in *S. cerevisiae* and synthetically produced. The gene was cloned onto the yBBH1 vector plasmid with suitable marker genes, secretion signal, promoter and terminator. This plasmid was then used to transform the laboratory *S. cerevisiae* strain, Y294. This strain, named *S. cerevisiae* Y294[*Pcbgl1B*], was used for the production of the β-glucosidase enzyme in this study.

Several indigenous wild-type *S. cerevisiae* strains were isolated from sites in and around vineyards, as well as other areas in the Western Cape, as part of a wine research project in 1998 (Van der Westhuizen *et al*., 2000). These strains have since been evaluated and characterised in several ways, such as ethanol and inhibitor tolerance, temperature and pH tolerance, and fermentation capacity under various conditions. Of the 46 strains originally isolated, the 30 strains that performed best in previous ethanol tolerance and fermentation tests were screened in this study, as well as an industrial strain. Ethanol Red® is the industry grade *S. cerevisiae* strain, considered the number one yeast in North America for the production of fuel ethanol (www.lesaffre.com; www.fermentis.com). According to the production company, Ethanol Red® is a specially selected strain that was developed for the industrial ethanol industry. It reportedly has several desirable qualities, such as rapid fermentation, high ethanol tolerance, ethanol production at high temperatures (up to 40°C), and high cell viability especially in high gravity fermentations. This strain has been used for ten years in industrial ethanol production applications, and related research on bioethanol production from starch and lignocellulose (Devantier *et al*., 2005; Moreno *et al*., 2013).

The use of commercial cellulase cocktails, combined with selected wild-type and industrial yeast strains, as well as optimisation by addition of β-glucosidase, will allow us to evaluate the efficiency and viability of a single-step SSF process to convert triticale straw to ethanol. This research will confirm the potential use of triticale straw as a bioethanol feedstock. It will also combine the use of commercial enzymes with recombinant *in vivo*-produced enzymes to determine if the addition of specific enzyme activity can significantly improve hydrolysis, leading to increased ethanol yields.

3. Materials and Methods

The experimental design to mimic the typical processing of triticale straw can be divided into three separate stages: preparation of substrate, enzymatic hydrolysis and fermentation (Figure 12).

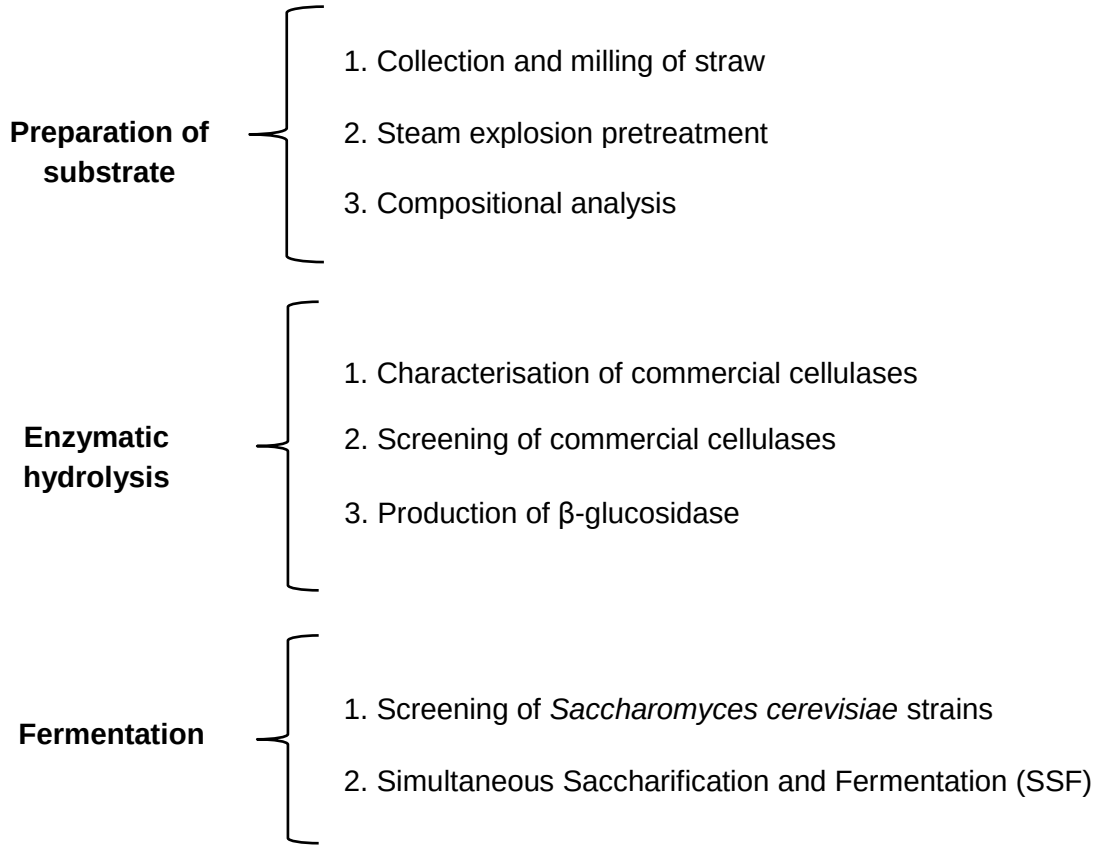


Figure 12: Experimental design for conversion of triticale straw to ethanol

All chemicals were of analytical grade and unless stated otherwise, sourced from Merck (Darmstadt, Germany). Values reported are averages of triplicate experiments (unless otherwise stated) with standard deviations included where relevant.

3.1. Substrate preparation

3.1.1. Collection and preparation of triticale straw

Triticale (*Triticosecale rimpau* cultivar US2009) was planted in November 2012 on the Welgevallen Experimental Farm (Stellenbosch University). The triticale grain was harvested on 24 April 2013 and the straw collected on 25 April 2013. The straw consisted mainly of the stems, leaves and “ears” of the triticale plant. The triticale straw was milled with a Condux-Werk type mill (Wolfgang bei Honou, Germany). The fractions with particle size between 0.38 and 10 mm was collected in 500 g batches in plastic bags and stored at room temperature until further use (Figure 13).

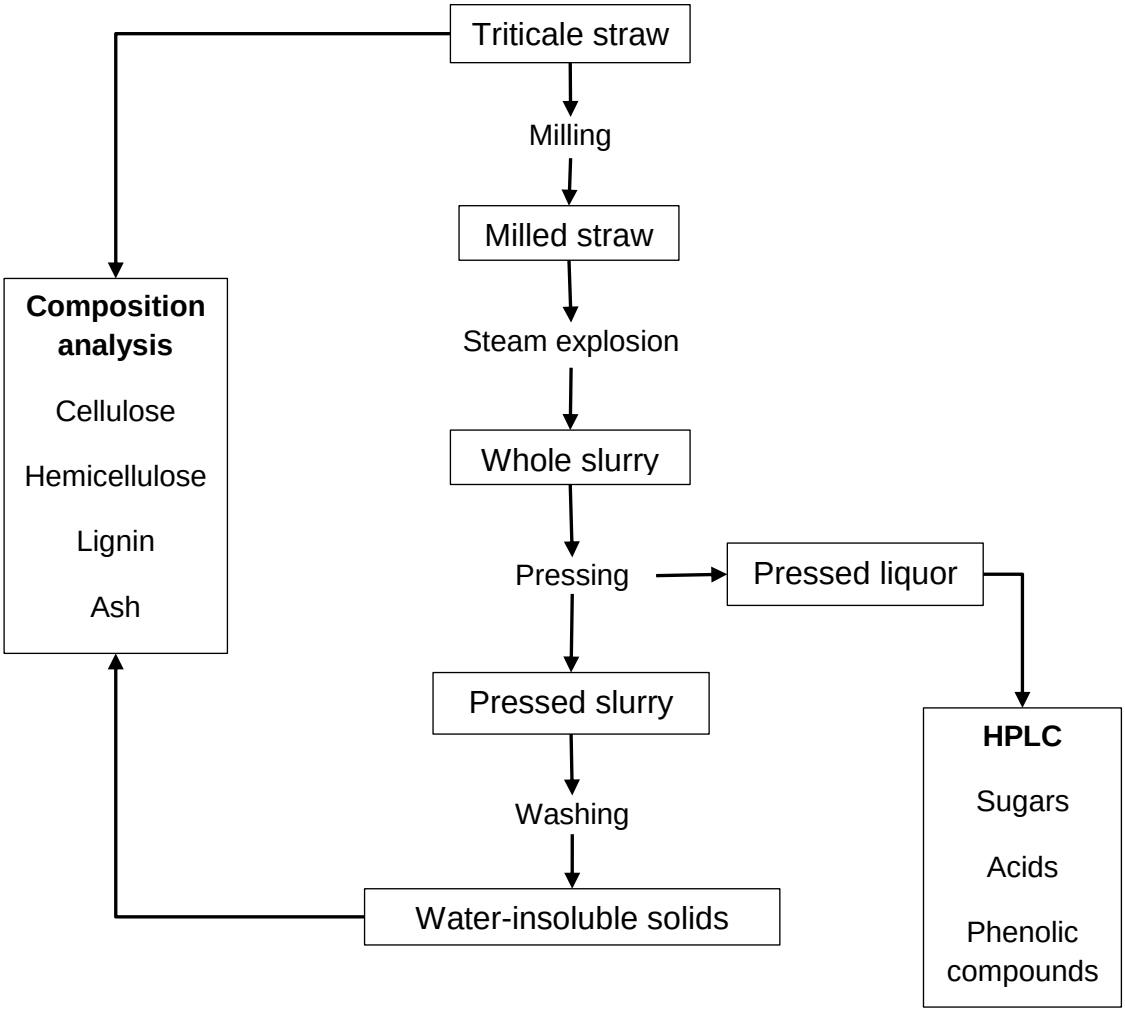


Figure 13: Experimental design for preparation of water-insoluble solids (WIS) from triticale straw.

3.1.2. Steam explosion

Following the experimental design (Figure 12) 500 g of milled straw was soaked overnight in 10 litres of water, after which the excess water is poured off to achieve straw moisture content of ± 45%. This water-impregnated straw is then subjected to steam explosion by means of a pilot-scale steamgun (Process Engineering at Stellenbosch University). The material was exposed to pressurised steam at the 203°C for 7 minutes, after which the pressure was released, causing explosive decompression of the material. These conditions were predetermined by optimisation studies at Stellenbosch University (Mr. RA Agudelo-Aguirre, personal communication). The pretreated material (slurry) was collected from the cyclone and allowed to cool.

The slurry was pressed with an industrial scale hydrolic press to a pressure of 2 US tonnes. The liquid (pressed liquor) obtained was collected. The solid pressed material was washed with 1 litre distilled water per 100 g of material and excess water removed to obtain the water-insoluble solids (WIS) with an approximate 70% moisture content.

3.1.3. Biochemical analyses

The structural carbohydrates and lignin content of the raw and pretreated materials were determined according to NREL's Laboratory Analytical Procedures (Sluiter *et al.*, 2008). Extractives were removed from raw material. The materials were subjected to an initial phase of acid hydrolysis with concentrated sulphuric acid (H_2SO_4) (72% w/w), followed by a second hydrolysis with diluted sulphuric acid (4% w/w), which resulted in the complete hydrolysis of all polymeric molecules, including cellulose and hemicellulose. The monomeric sugars in the cellulose and hemicellulose were released and the concentrations in the hydrolysate were quantified with HPLC analysis (Finnigan Surveyor RI Plus Detector, Thermo Scientific). These values were used to calculate the amount of cellulose and hemicellulose in the material. In calculating the amount of cellulose and hemicellulose in the original material, a conversion factor is applied to the values from the HPLC data. This accounts for the addition of water molecules to form monomeric sugars when complex carbohydrates are hydrolysed. The conversion factors are 1.11 for hexoses and 1.13 for pentoses.

The acid-soluble lignin content was determined by measuring the UV absorbance of the hydrolysate at 240 nm with a spectrophotometer (Pharmacia LKB Ultrospec III). The

insoluble lignin is represented by the solid residue remaining after total acid hydrolysis. The ash content was determined by calcination at 575°C for 4 hours.

The liquid fraction resulting from pressing of the whole slurry after pretreatment (pressed liquor) was analysed for sugars, acids and phenolic compounds with HPLC (Finnigan Surveyor RI Plus Detector, Thermo Scientific).

3.2. Enzymatic hydrolysis

3.2.1. Characterisation of commercial cellulase cocktails

Samples of selected commercial cellulase cocktails for experimentation were acquired from the department of Process Engineering at Stellenbosch University (Table 6). All are considered highly effective and some have been used extensively in bioethanol research.

Table 6: Commercial cellulase cocktails used in enzymatic hydrolysis trials and SSF

Enzyme	Supplier	Enzyme description	Reported activity
Celluclast® 1.5	Novozymes, Sigma Aldrich	Cellulase	700 Endoglucanase U/g
Spezyme® CP	Genencor	Cellulase, hemicellulose, β-glucanase	90 Genencor Cellulase Units /ml
Optiflow™ RC 2.0	Genencor	Cellulase	Not reported
Accellerase® 1500	Genencor	Endoglucanase, exoglucanase, β-glucosidase, hemicellulase	2200-2800 CMC U/g; 525-775 *p*NPG U/g
Alternafuel®CMAX™	Dyadic	Cellulase, β-glucanase and other activities	>15000 CMCase/g

3.2.1.1. Protein content determination

The protein content of all the commercial cellulase cocktails used in this study was determined using a standard Lowry Protein Concentration Determination Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as standard.

3.2.1.2. Cellulase activity on filter paper

Filter paper units (FPU) were determined according to the method described by Ghose (1987) and defined as the amount of enzyme that released 1 µmole (2 mg) of reducing sugars from 50 mg filter paper per ml per minute. The enzymes were diluted in 5 ml 0.05 M citrate buffer (pH 5.0) in test tubes, and placed in a 50°C waterbath for 2 minutes. Whatman no. 1 filter paper strips (± 50 mg each) were added to the tubes and incubated for 1 hour. The reaction was terminated with the addition of 5 ml dinitrosalicylic acid (DNS)-reagent and boiling for 5 minutes, which caused a colorimetric reaction. The absorbance was measured at room temperature with a microtitre plate reader (Biorad xMark™ Microplate spectrophotometer) at a wavelength of 540 nm. Standard curves were used to calculate the amount of enzyme that would release 2 mg of glucose-equivalents. The filter paper activity (FPA) was calculated as follows (Ghose, 1987; Adney and Baker, 1996):

$$Filter\ Paper\ Activity = \frac{0.37 *}{enzyme\ concentration\ to\ release\ 2.0\ mg\ glucose}\ units/ml$$

* The numerator (0.37) is derived from converting the 2.0 mg of monomeric sugars that are released to mmoles of glucose (2.0 ÷ 0.18016), the volume of the enzyme that is used in the assay (0.5 ml), and the incubation time (60 minutes) as follows:

$$\frac{(2.0\ mg\ glucose/\ 0.18016 mg\ glucose/\mu mol)}{(0.5\ m\ell\ enzyme\ dilution\ x\ 60\ minutes)} = 0.37\ \mu mol/minute/ml$$

3.2.2. Screening of commercial cellulase cocktails

3.2.2.1. Enzymatic hydrolysis setup

The WIS was used as substrate for enzymatic hydrolysis with selected commercial cellulase cocktails. This substrate contains mostly cellulose, lignin and some hemicellulose. No additional enzymes were added during the screening phase.

Enzyme and substrate (2% w/v solids loading) was combined in McCartney bottles in 20 ml 0.05 M citrate buffer (pH 5.0) with different enzyme dosages (5-30 mg protein/g dry weight substrate). The moisture content of the material was determined beforehand and was accounted for during weighing to obtain exact dry weights of substrate. Sodium azide was added at a concentration of 0.02% (w/v) to prevent microbial contamination. The bottles were placed inside hybridisation bottles, which were rotated at 7 rpm in a hybridisation oven (Amersham Life Science, Amersham, UK). The experiments were conducted at 50 and 37°C with 500 µl samples taken periodically during the 56 hour hydrolysis. Samples were immediately boiled (100°C for 5 minutes) to inactivate enzymes and stored at -20°C until further analysis.

3.2.2.2. Glucose yields

The glucose concentrations of the samples were determined using the K-GLUC GOPOD-format assay (Megazyme®, Ireland). Samples were centrifuged for 2 minutes at 100 000 rpm. The clear supernatant (15 µl of sample) and 250 µl of the GOPOD-reagent was combined in microtitre plate wells and incubated at 50°C for 15 minutes. The absorbance was measured at 510 nm with a xMark™ Microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) and the glucose concentration calculated from a standard curve. The amount (mg) of glucose released was used to calculate the glucose yields. Glucose yield (%) is thus defined as the portion of the total amount of glucose in the substrate that was released, and is calculated as follows:

$$Glucose\ yield\ \% = \frac{Reaction\ volume\ (ml)\ x\ Glucose\ concentration\ (mg/ml)}{Amount\ of\ glucose\ in\ the\ substrate\ (mg)}\ x\ 100$$

3.2.2.3. Xylose yields

Xylose concentrations in the hydrolysate were determined using the K-XYLOSE assay (Megazyme®, Ireland). The assay was conducted as specified by the manufacturer for microtitre plates. The glucose in the sample was removed by pre-incubation with hexokinase in the presence of excess ATP. The xylose mutarotase and xylose dehydrogenase were added which led to the release of one NADH from each xylose. The amount of NADH was measured at 340 nm and used to calculate the xylose concentration as follows:

$$Xylose\ concentration = \frac{\Delta\ A\ sample}{\Delta\ A\ standard}\ X\ [standard]\ g/l$$

Xylose yield (%) is defined as the portion of the total amount of xylose in the substrate that was released, calculated as follows:

$$Xylose\ yield\ \% = \frac{Reaction\ volume\ (ml)\ x\ Xylose\ concentration\ (mg/ml)}{Amount\ of\ xylose\ in\ the\ substrate\ (mg)}\ x\ 100$$

3.2.3. Production and evaluation of β-glucosidase

3.2.3.1. Production of PcbglB

The codon-optimised *PcbglB*-gene encoding a *Phanerochaete chrysosporium* β-glucosidase had previously been cloned into *S. cerevisiae* Y294 to yield the recombinant strain, *S. cerevisiae* Y294[Pcbgl1B] (Dobson *et al.,* 2014; Njokweni *et al*., 2012). The strain was maintained on SC^{-URA} agar plates (1.7 g/l yeast nitrogen base, 20 g/l glucose, 5 g/l ammonium sulphate and yeast synthetic drop-out medium supplements (Sigma-Aldrich, Germany)). It was cultured in 50 ml double strength SC^{-URA} medium (3.4 g/l yeast nitrogen base, 20 g/l glucose, 10 g/l ammonium sulphate, 3 g yeast synthetic drop-out medium supplements (Sigma Aldrich), 100 mg/l Ampicillin and 15 mg/l Streptomycin) in 125 ml flasks for 3 days on a rotary shaker at 30°C. After centrifugation the supernatant was lyophilised in a Virtis benchtop freeze dryer (SP Scientific, USA) and stored in an airtight container at -20°C.

3.2.3.2. Characterisation of PcbglB

A standard Lowry Protein Concentration Determination Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as standard was used to determine the protein content of a 20 mg/ml stock solution of the lyophilised enzyme. The β-glucosidase activity was determined with *p*NPG as substrate (Dobson *et al.,* 2014; Njokweni *et al*., 2012).

The optimum temperature for β-glucosidase activity was determined by measuring the amount of glucose released from cellobiose. The substrate (450 µl of a 1% cellobiose

solution) was combined with 50 µl of a stock solution of enzyme (20 mg/ml) dissolved in 0.05 M citrate buffer (pH 5). After 5 minutes at the relevant temperature, the reaction was terminated and the glucose concentration determined using the K-GLUC GOPOD-format assay (Megazyme®, Ireland). The optimum pH was determined similarly using 0.05 M citrate buffer with different pH values (pH 2, 3, 4, 5, 6 and 7) at 60°C.

3.2.3.3. Hydrolytic synergy of Spezyme® CP and PcbglB

Enzymatic hydrolysis experiments were conducted by combining Spezyme® CP at 15 FPU/g cellulose loading with different amounts of the recombinant PcbglB (500, 800 and 1000 IU/g cellulose). Hydrolysis of the WIS substrate was performed at 37°C in 20 ml volumes of 0.05 M citrate buffer (pH 5) containing 0.02% sodium azide and 2% solids (as described in section 3.2.2.1.). Glucose concentrations were determined with HPLC analysis (Finnigan Surveyor RI Plus Detector, Thermo Scientific).

3.3. Screening of *S. cerevisiae* strains

3.3.1. Screening of yeasts for ethanol production

Wild-type and industrial *S. cerevisiae* strains were maintained on YPD agar plates (10 g/l yeast extract powder, 20 g/l peptone and 20 g/l glucose). The first round of screening involved 30 different wild-type *S. cerevisiae* isolates that were evaluated for ethanol production. Fermentation reactions were conducted in 100 ml volumes of SC medium (1.7 g/l yeast nitrogen base, 5 g/l ammonium sulphate, 100 g/l glucose, 5% ethanol, 100 mg/l Ampicillin, 15 mg/l Streptomycin) in sealed fermentation bottles. The inoculum size was 1 x 10^5 cells/ml. The fermentations were carried out by incubation for 7 days on a rotary shaker at 30°C with regular sampling for HPLC analysis (Finnigan Surveyor RI Plus Detector, Thermo Scientific). The fermentations were repeated at 30°C and 37°C with the eight best fermenting strains from the first screening.

3.3.2. Comparison of wild-type and industrial strains

The three top fermenting wild-type strains from the second screening was selected for further study and compared with Ethanol Red®, an industrial *S. cerevisiae* fermenting strain. The fermentations were conducted in 100 ml volumes of SC media as specified in section 3.3.1, except the glucose concentration was increased to 200 g/l. Inoculum size was 1 x 10^5 cells/ml. The fermentations were continued for 13 days at 37°C during which samples were periodically taken and analysed with HPLC (Finnigan Surveyor RI Plus Detector, Thermo Scientific).

Since the results from the previous fermentations were not as desired, the fermentations were repeated with the same strains, with additional citrate buffer (pH 5; 0.05 M) and 10 g/l yeast extract powder added. These fermentations were only continued for 7 days, when there was no more reduction in glucose concentrations.

3.4. Simultaneous Saccharification and Fermentation (SSF)

The commercial cellulase cocktail (Spezyme® CP) and two yeast strains (L21 and Ethanol Red®), selected from the screenings were used for SSF in combination with the recombinant PcbglB, according to the NREL protocol for SSF with lignocellulosic substrates (Dowe and McMillan, 2001). The WIS-fraction of the steam-exploded triticale straw was used at a 10% solids loading, i.e. 4.3% cellulose. Spezyme® CP was added at a final dosage of 5 and 15 FPU/g cellulose and the PcbglB at a dosage of 35 IU/g cellulose. The experiments were conducted in 100 ml fermentation vessels in 50 ml of citrate buffer, containing 10 g/l yeast extract, 20 g/l peptone, 100 mg/l Ampicillin, and 15 mg/l Streptomycin. Cultures of the selected yeasts were grown in YPD (10 g/l yeast extract powder, 20 g/l peptone and 20 g/l glucose) at 37°C overnight and used to inoculate the SSF vessels at 10% (v/v) of the final volume. Magnetic stirrer bars were used to agitate the slurry at 400 rpm. The SSF vessels were capped and incubated at 37°C. Samples were periodically taken and analysed with HPLC (Finnigan Surveyor RI Plus Detector, Thermo Scientific).

Ethanol yields (%) were calculated as follows:

$$Ethanol\ yield\ (\%) = \frac{[Ethanol](g/l)\ x\ Reaction\ volume\ (l)}{Amount\ of\ cellulose\ in\ reaction\ (g) x\ 1.11\ x\ 0.51}\ x\ 100$$

* The value of 1.11 in the formula is the conversion factor of cellulose to glucose (1.11 g glucose released from 1 g cellulose) accounting for the addition of a water molecule to the glucose during hydrolysis of cellulose. The conversion of 1 g of glucose to ethanol results in 0.51 g ethanol, thus represented by a value of 0.51 in the formula above (Badger, 2002; Dowe and McMillan, 2001).

The amount of cellulose (in grams) in the reaction is calculated by multiplying the amount of substrate (dry weight) in the reaction in grams with the percentage of cellulose in the substrate (in this case, the WIS contains 42.75% cellulose), i.e.:

$$Amount\ of\ cellulose\ (g) = \frac{WIS\ (g\ dry\ weight)}{0.4275}$$

4. Results and discussion

4.1. Substrate preparation

4.1.1. Steam explosion pretreatment

The straw harvested from the Welgevallen Experimental Farm contained minimal amounts of contaminating weeds and grasses that generally grow in the field. However, these plants are not expected to negatively affect the experimental results as they also consist of lignocellulose and represent actual conditions encountered by farmers that cultivate triticale.

Steam explosion of 1 kg raw milled triticale straw produced ± 800 g (dry weight) whole slurry, which yielded ± 720 g (dry weight) of WIS after washing and pressing (Figure 14).

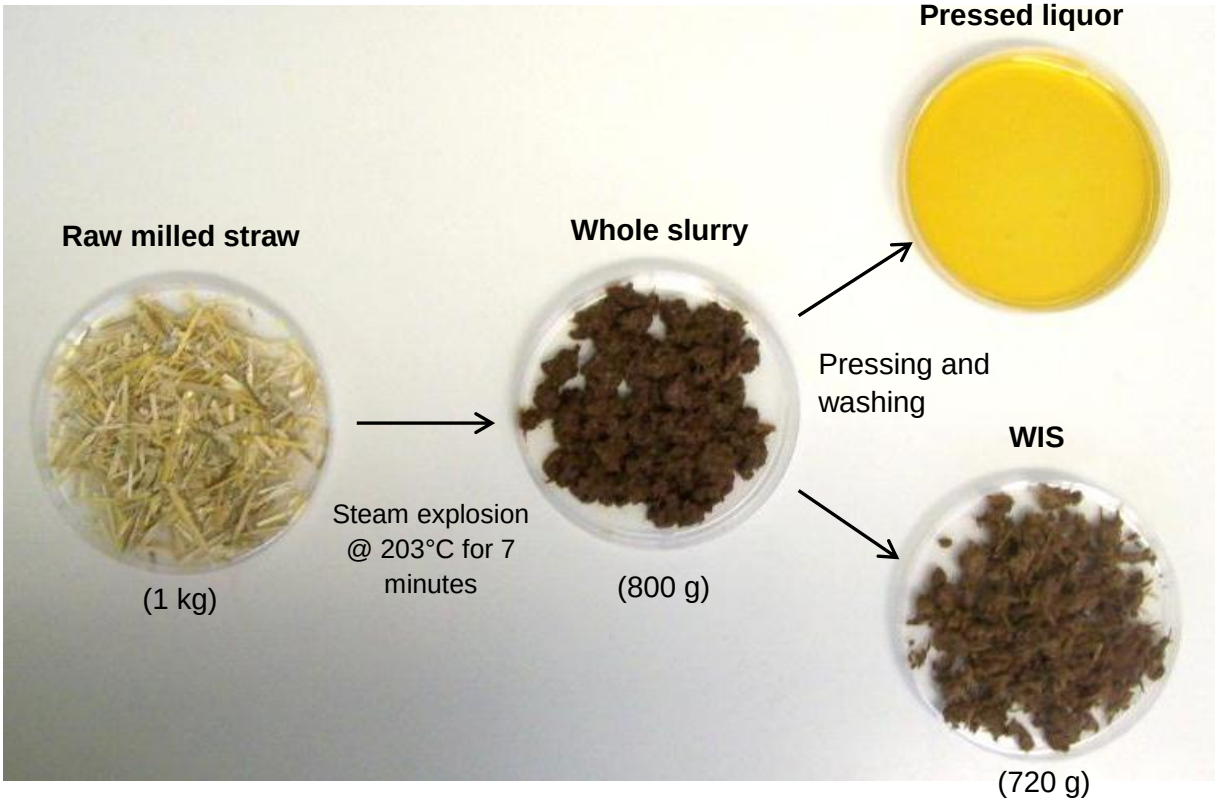


Figure 14: Images of raw and steam-exploded triticale straw. WIS = Water-Insoluble Solids

Steam explosion of the triticale straw increased the cellulose content of the substrate by more than 6%, while the hemicellulose content was reduced from 24.96% to 7.07% (Table 7). The reduced hemicellulose content will significantly influence the cellulases' access to the substrate and therefore enhance hydrolysis. The lignin content of the WIS was increased relative to the raw material, but had most likely been removed from its original position in the structure and this change could still improve the hydrolysing potential of the substrate (Cantarella *et al*., 2004; Kristensen *et al.,* 2008.).

Table 7: Composition of triticale straw before and after steam explosion at 203°C for 7 minutes

Component	% (g/100 g dry weight)	
	Raw material	**Pretreated material (WIS)**
Cellulose	36.68 ± 0.83	42.75 ± 2.44
Hemicellulose	24.96 ± 1.15	7.07 ± 0.87
Lignin	20.53 ± 0.67	30.84 ± 1.10
Ash	1.05 ± 0.10	3.28 ± 0.04
Extractives	12.07 ± 0.10	-

The ash content represents the proteins, fats, DNA, minerals and metals. This fraction is unlikely to be changed by pretreatment, because it is not affected in the same way as polymers such as cellulose. Although DNA and proteins will be denatured and destroyed during steam explosion, the remnants of these components will still be present in the ash fraction. Extractives consist of dust and dirt on the surface of the raw materials that accumulated on the plant during cultivation, or settled on the straw during milling. Removal of the extractives is essentially a "washing" procedure performed only on the raw material before chemical composition determination (the definition of WIS implies that it has already been washed).

The pressing of the whole pretreated slurry also produced a sugar and nutrient-rich liquid fraction that was frozen at -20°C for future evaluation. The sugars, acids and other compounds present in the pressed liquor were quantified to evaluate the effect of the steam explosion (Table 8). It contained minimal amounts of glucose, indicating that the cellulose fraction of the material is mostly intact. The liquor contained 4.41 g/l xylose and 1.12 g/l arabinose, confirming that hemicellulose was removed from the WIS. As expected,

acetic and formic acid were present in significant amounts as these compounds are formed from the degradation of hemicellulose (refer to section 2.3.1.). The phenolic compounds furfural and hydroxyl-methyl furfural were present in the liquor in significant amounts. The presence of acids and phenolic compounds was the main reason for washing the substrate, since these compounds can have an inhibitory effect on both cellulase activity and yeast growth (Taherzadeh *et al*., 2000; García-Aparicio *et al*., 2006). Glucose and cellobiose are also known to have a strong inhibitory effect on cellulases (Taherzadeh and Karimi, 2011). The WIS contained no detectable amounts of the compounds mentioned above, indicating that the washing of the pressed substrate was affective in removing potential inhibitors.

Table 8: Compounds in pressed liquor following steam explosion and pressing of whole slurry

Compound	Concentration (g/l)	Compound	Concentration (g/l)
Glucose	0.42 ± 0.050	Acetic acid	1.66 ± 0.030
Cellobiose	0.05 ± 0.001	Formic acid	0.31 ± 0.001
Xylose	4.41 ± 0.060	Furfural	0.49 ± 0.003
Arabinose	1.12 ± 0.050	Hydroxy-methyl furfural	0.52 ± 0.020

Pretreatment conditions should be optimised to suit the specific substrate. In this study, the change in the composition of the straw by steam explosion did not improve the cellulose content (g/dry weight) as much as expected. Other researchers have increased the cellulose content of wheat straw by up to 14.8% with steam explosion by extending the exposure time to 20 minutes while reducing the temperature to 180°C (Chen *et al*., 2011). Ballesteros and colleagues (2006) were able to improve the cellulose content of wheat straw from 30.2% to more than 63% with steam explosion at 180°C for 10 minutes with an acid catalyst. Tomás-Pejó and colleagues (2009) used pretreated wheat straw with a cellulose content of 71.2% to produce 36.2 g/l ethanol during SSF, highlighting the importance of having a good pretreatment method. Although the conditions used in this study falls within the typical range for straw pretreatment, there is a need to optimise the steam explosion of the triticale straw substrate to maximise the cellulose content, possibly with the addition of sulphuric acid as a catalyst. A well-optimised pretreatment method will lower the enzyme requirements of the substrate, which can significantly reduce the cost of

bioethanol production (Sørensen *et al*., 2013). Banerjee and colleagues (2010) found that the glucose released from a substrate is dependent on the pretreatment method, the enzyme cocktail used and the characteristics of the specific feedstock. Therefore, the efficiency of pretreatment will ultimately influence the ethanol yield obtained from the substrate (Ballesteros *et al*., 2006).

4.2. Enzymatic hydrolysis with commercial cellulases

4.2.1. Characterisation of enzyme cocktails

Filter paper activity was used as a standard for measuring cellulase activity as it is routinely used in most research protocols. The filter paper activity and protein content (Table 9) of the commercial cellulase cocktails used in this study were similar to results reported by other researchers (Tomás-Pejó *et al*., 2008a; Kovacs *et al*., 2009; Pengilly, 2013). However, the activities could not be compared to the information on the product information sheets for the enzymes, which report activity on different substrates (e.g. CMC, *p*NPG) and expressed in different units.

Table 9: Protein content and filter paper activity of commercial cellulase cocktails

Cellulase cocktail	Protein concentration (mg/ml)	Filter Paper Activity (FPU/ml)
Celluclast® 1.5	153.17 ± 12.75	51.93 ± 2.25
Spezyme® CP	120.14 ± 14.48	40.89 ± 2.25
Optiflow™ RC 2.0	182.24 ± 17.47	101.59 ± 9.95
Accellerase® 1500	91.18 ± 4.46	46.26 ± 0.58
Alternafuel® CMAX™ (Reconstituted powder; 1 g/10 ml)	82.48 ± 0.10	22.18 ± 1.45

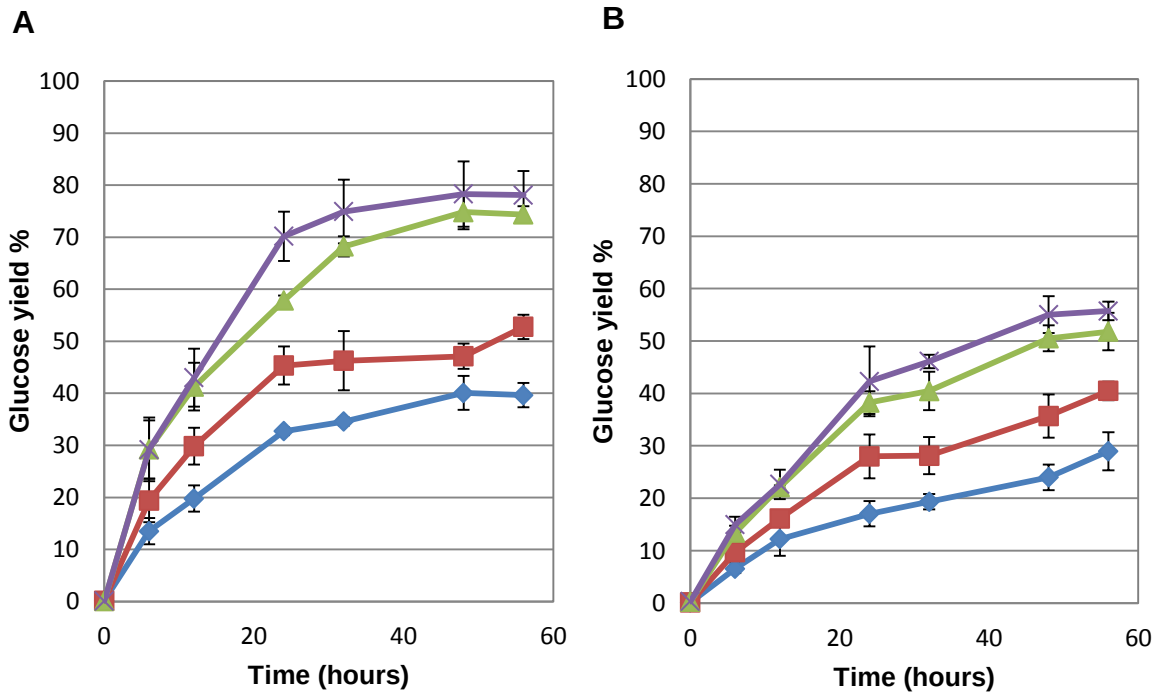
4.2.2. Small-scale hydrolysis trials

4.2.2.1. Glucose yields

a.) Celluclast® 1.5

Celluclast® 1.5 performed well at both temperatures, releasing 78% of the available glucose at 50°C and 56% at 37°C (Figures 15A and B). The curve shows rapid hydrolysis of the substrate up to 24 hours, where after the hydrolysis rate decreased, possibly due to inhibition of the enzymes because of glucose accumulation.

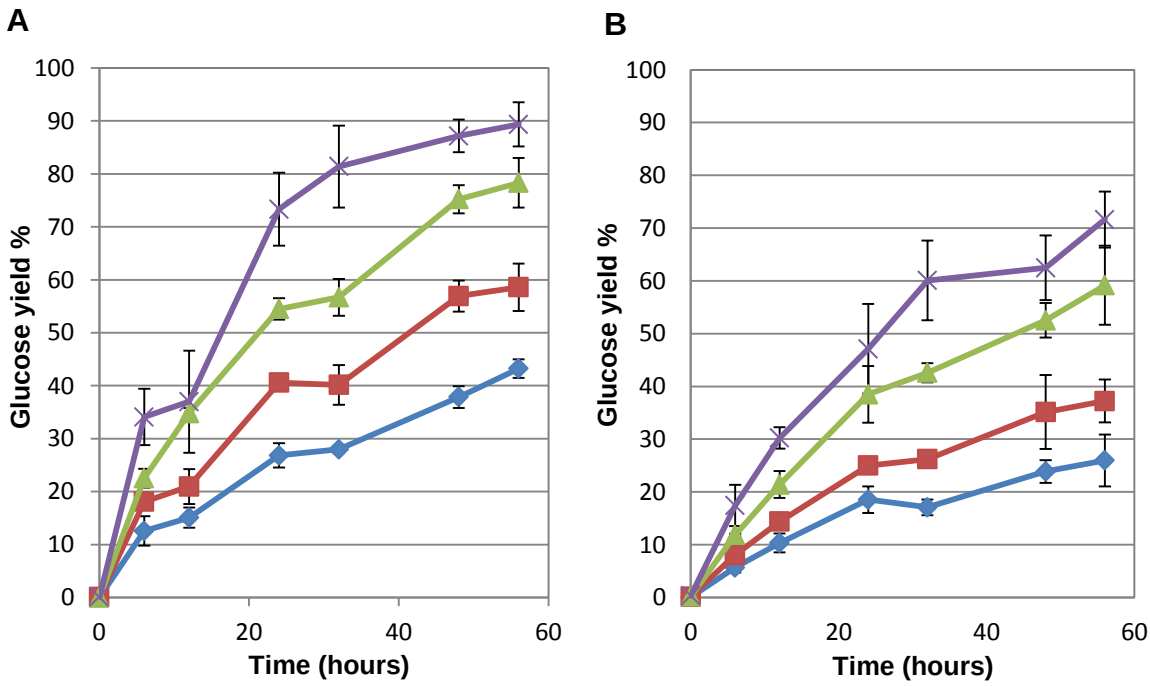
The glucose yields during hydrolysis of the triticale substrate was compared to the hydrolysis of other lignocellulosic substrates by Celluclast® 1.5, as previously reported for barley straw (94%) and *Brassica carinata* (99%) by other researchers (García-Aparicio *et al*., 2006; Ballesteros *et al.,* 2002). However, Celluclast® 1.5 was supplemented with Novozym® 188 (β-glucosidase activity) in these instances, resulting in much higher glucose yields.



Figures 15: Glucose yields during enzymatic hydrolysis of triticale WIS at (A) 50°C and (B) 37°C with Celluclast® 1.5 at enzyme dosages of (◆) 5 mg, (■) 10 mg, (▲) 20 mg and (×) 30 mg protein/g substrate. Error bars represent standard deviations of triplicate experiments.

b.) Spezyme® CP

Spezyme® CP released nearly 90% of the available glucose from the triticale WIS at 50°C after 56 hours (Figure 16A). After 32 hours, it surpassed the maximum yield obtained with Celluclast® 1.5 at the same temperature and enzyme dosage (Figures 15A and 16A). At an enzyme dosage of 30 mg protein/g substrate, Spezyme® CP yielded 81% hydrolysis at 50°C and 60% at 37°C after 32 hours (Figure 16B). Spezyme® CP is commonly used in research, usually paired with Novozym® 188 to supplement β-glucosidase activity (García-Aparicio *et al*., 2011; Yang *et al.,* 2011). However, it is mostly used to remove cellulose from starch substrates rather than for bioethanol production.

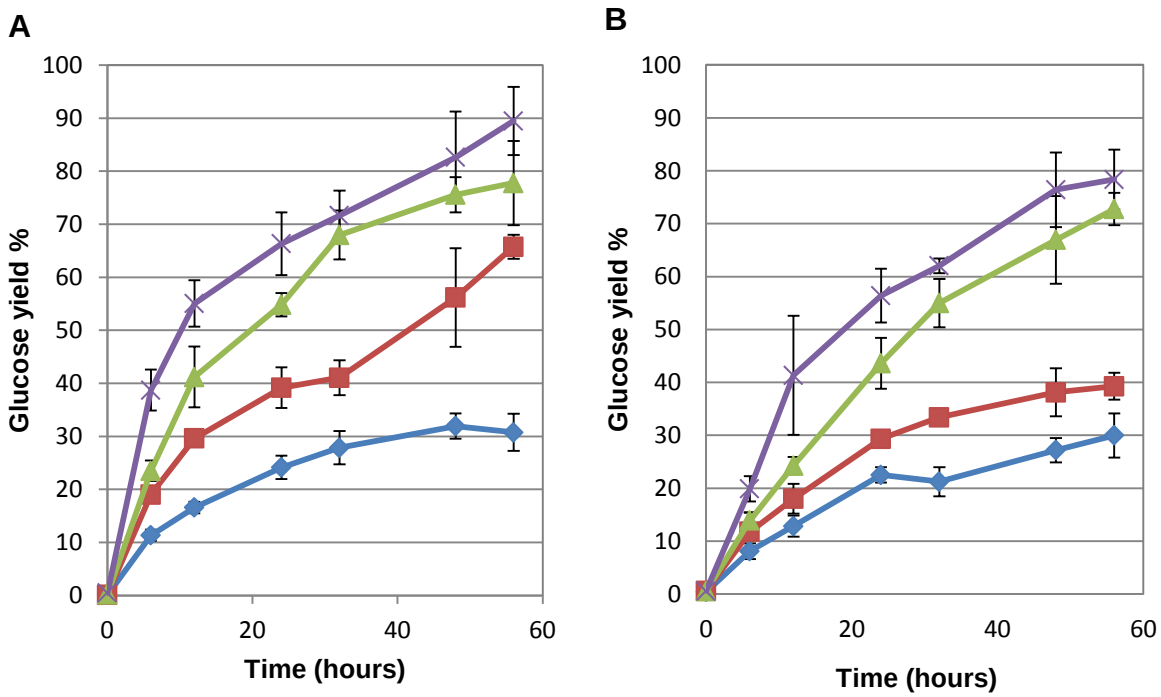


Figures 16: Glucose yields during enzymatic hydrolysis of triticale WIS at (A) 50°C and (B) 37°C with Spezyme® CP at enzyme dosages of (◆) 5 mg, (■) 10 mg, (▲) 20 mg and (×) 30 mg protein/g substrate. Error bars represent standard deviations of triplicate experiments.

c.) Optiflow™ RC 2.0

Optiflow™ performed well at 50°C, almost reaching a glucose yield of 90% with an enzyme dosage of 30 mg protein/g substrate (Figure 17A). In contrast to Celluclast® and Spezyme® CP, the yield reached at 37°C was only 11.12% lower than at 50°C, indicating that Optiflow™ might be suitable for SSF conditions where a lower temperature is used. Optiflow™ outperformed the other enzymes at 37°C using a high enzyme dosage, but the results were similar at the lower enzyme dosages (5 and 10 mg protein/g substrate).

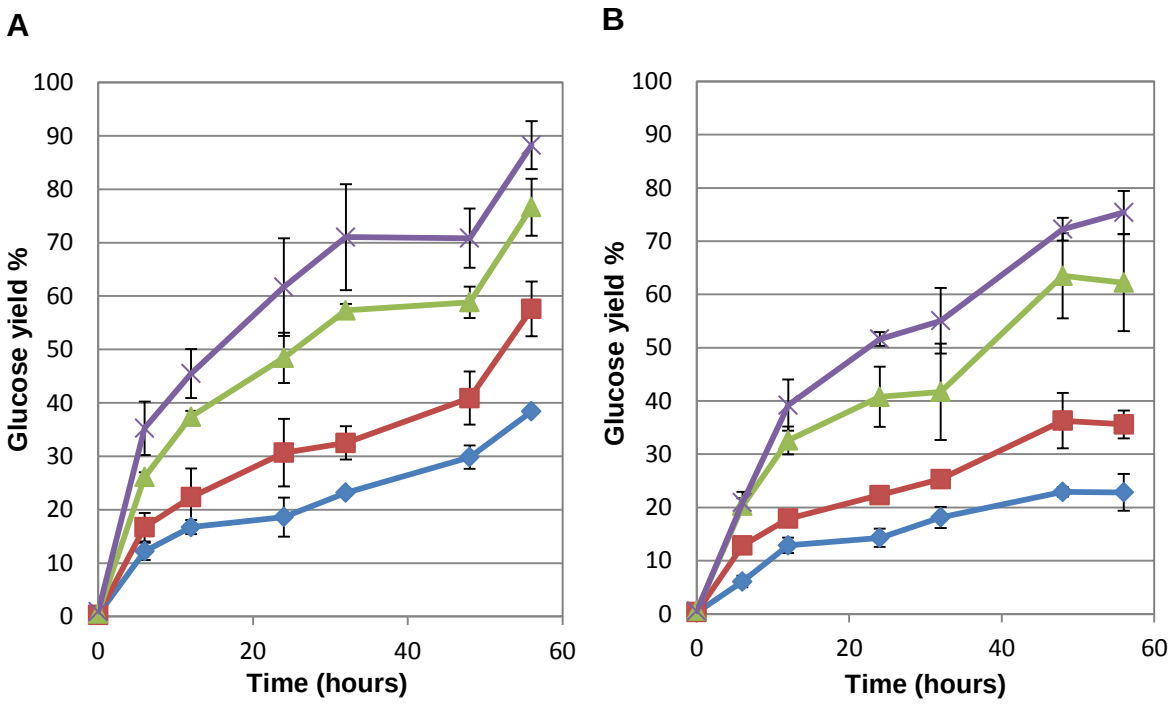
Other studies on lignocellulose hydrolysis employing Optiflow™ have shown good results, but the high glucose yields (>80%) were obtained with extremely high enzyme dosages (60 FPU/g cellulose) and Novozym® 188 supplementation (Da Cruz *et al.,* 2012) and can therefore not be directly compared to the results obtained in this study.



Figures 17: Glucose yields during enzymatic hydrolysis of triticale WIS at (A) 50°C and (B) 37°C with Optiflow™ at enzyme dosages of (◆) 5 mg, (■) 10 mg, (▲) 20 mg and (×) 30 mg protein/g substrate. Error bars represent standard deviations of triplicate experiments.

d.) Accellerase® 1500

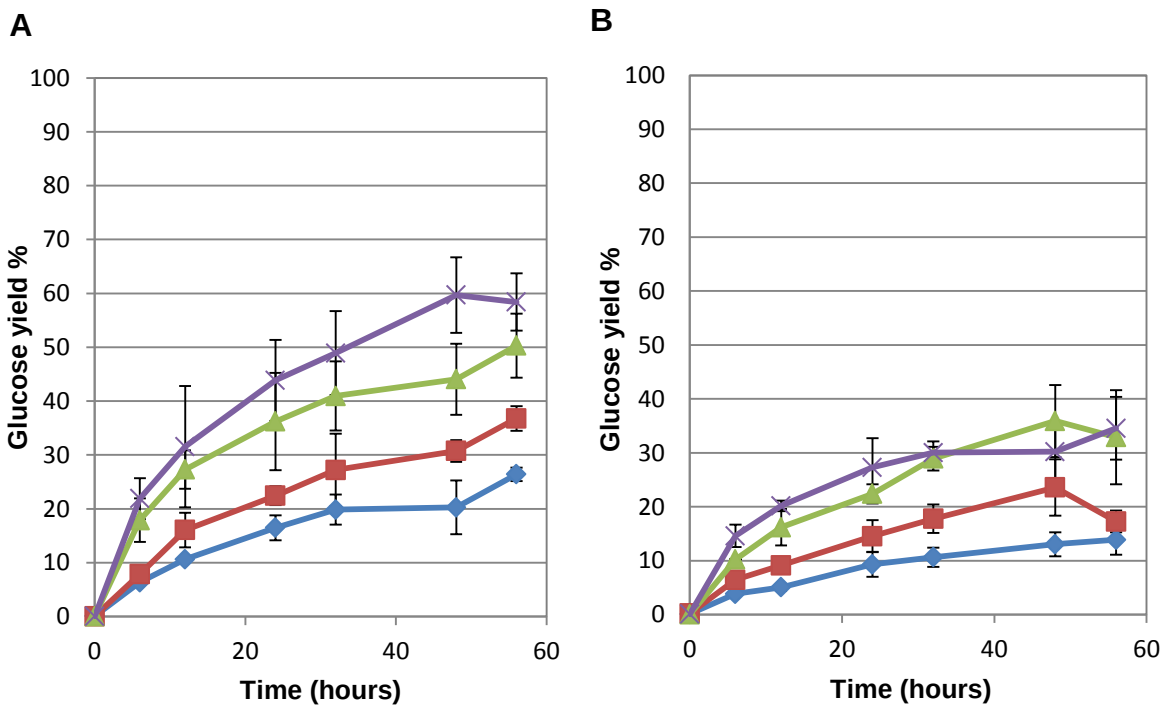
Accellerase® 1500 indicated trends similar to Optiflow™, releasing 88% of the available glucose at 50°C and 75% at 37°C after 56 hours (Figure 18). Other researchers have found that Accellerase® 1500 has higher a β-glucosidase activity than other commercial enzymes, and is also more resistant to inhibition by lignin derivatives and acids (Banerjee *et al.,* 2010; Lin *et al.,* 2010). Accellerase® 1500 is therefore expected to perform well on the whole slurry of pretreated materials that contain inhibitory compounds.



Figures 18: Glucose yields during enzymatic hydrolysis of triticale WIS at (A) 50°C and (B) 37°C with Accellerase® 1500 at enzyme dosages of (◆) 5 mg, (■) 10 mg, (▲) 20 mg and (×) 30 mg protein/g substrate. Error bars represent standard deviations of triplicate experiments.

e.) Alternafuel® CMAX™

Alternafuel® CMAX™ is part of a relatively new range of products from Dyadic and therefore no research results have yet been reported with this enzyme. Alternafuel® CMAX™ is supplied in a powder form and according to the product information sheet it should be able to effectively hydrolyse cellulosic substrates. However, it was outperformed by all the other cellulase cocktails at both temperatures (Figure 19) which might be ascribed to a different species of origin. Alternafuel® CMAX™ is produced by the thermophilic fungus *Myceliophthora thermophila*, while all the other enzymes are produced by *Trichoderma reesei.* It is possible that the specific enzymes in Alternafuel® CMAX™ is more susceptible to product inhibition by glucose than the enzymes produced by *T. reesei*. Since *M. thermophila* is thermophillic, the enzymes have a slightly higher optimum hydrolysis temperature compared to the other fungal cocktails, and therefore does not perform as well under the relatively low temperatures used for hydrolysis.

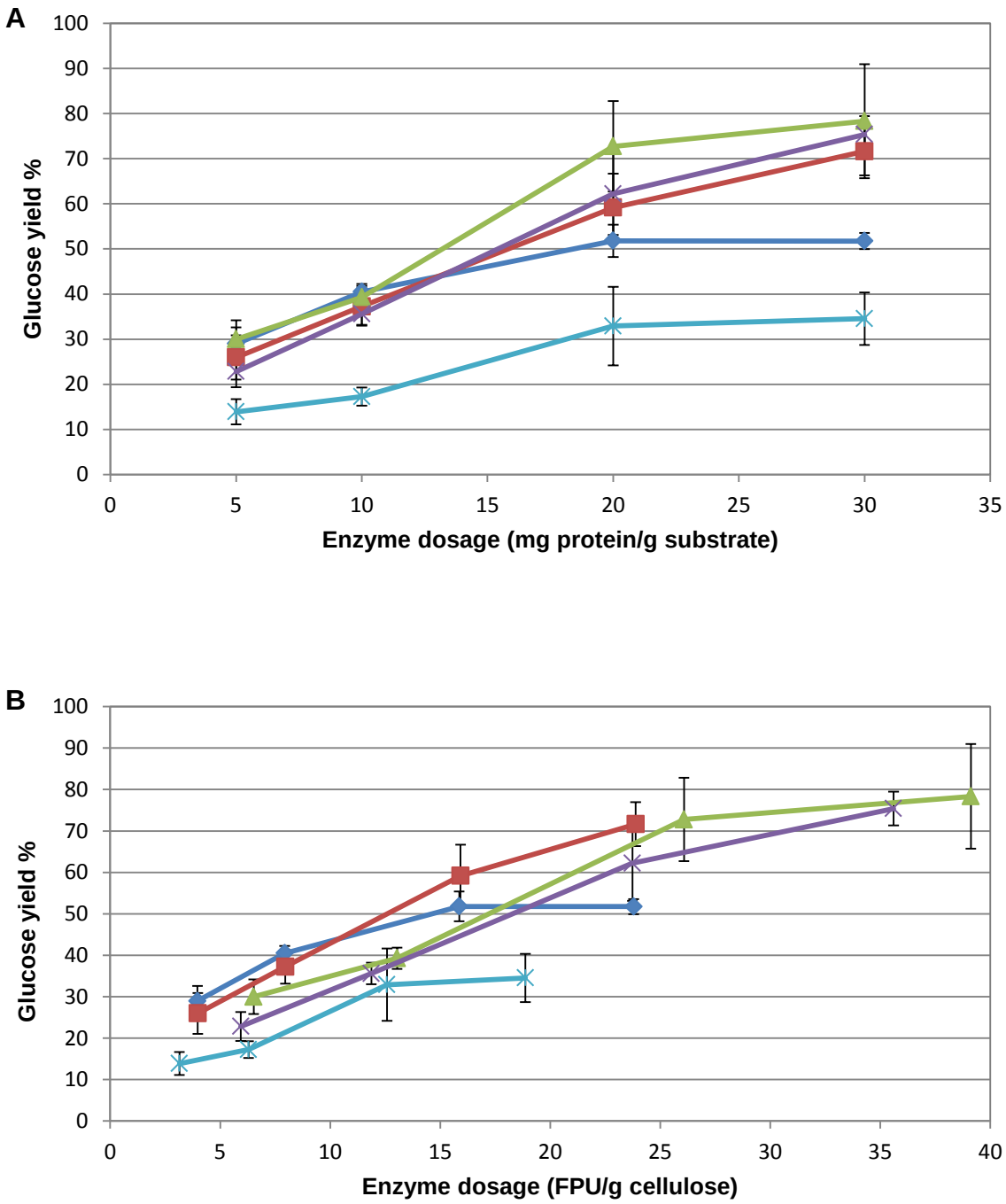


Figures 19: Glucose yields during enzymatic hydrolysis of triticale WIS at (A) 50°C and (B) 37°C with Alternafuel® CMAX™ at enzyme dosages of (◆) 5 mg, (■) 10 mg, (▲) 20 mg and (×) 30 mg protein/g substrate. Error bars represent standard deviations of triplicate experiments.

The enzymatic hydrolysis of steam-exploded triticale WIS was conducted at 50°C and 37°C; 50°C is the optimum temperature for enzyme activity of most commercial cellulases, while 37°C is the temperature proposed for SSF. Therefore, more consideration was given to results obtained at 37°C, although all the cellulase cocktails performed better at 50°C. The commercial cellulase cocktails were able to digest the pretreated triticale straw to varying degrees, depending on the temperature and enzyme dosages. The glucose concentration (a measurement of how well the cellulases were able to degrade the substrate) was determined and glucose yields (%) were expressed as a percentage of the maximum theoretical yield.

The results obtained from all the cellulase cocktails are summarised in Figures 20. Figure 20A shows the glucose yields in terms of enzyme dosage in mg protein per gram substrate, obtained from the hydrolysis trials at 37°C after 56 hours, whereas Figure 20B shows the same data for the enzyme dosage expressed as filter paper units per gram cellulose. This indicates the true activity of the enzymes on cellulose in the substrate, rather than relative to the amount of total proteins in the cocktail, which are not only cellulases.

At 37°C, Optiflow™ and Accellerase® obtained the highest yields at high enzyme dosages (> 25 FPU/g cellulose), with Optiflow™ reaching a maximum yield of 78% (Figure 20B). However, using an expensive cellulase cocktail at this high enzyme dosage will not be economically feasible in the industry, which typically apply enzyme dosages of 5 to 15 FPU/g cellulose, with 15 FPU/g cellulose often used as a standard dosage (Dowe and McMillan, 2001; Tomás-Pejó *et al*., 2008a). At an enzyme dosage of 15 FPU/g cellulose, Spezyme® CP performed the best of the five cellulase cocktails at 37°C, resulting in a 56% glucose yield (Figure 20B). Spezyme® CP was therefore selected for further evaluation in SSF.



Figures 20: Glucose yields (%) after enzymatic hydrolysis of triticale WIS at 37°C versus enzyme dosage expressed as (A) mg protein/g substrate or (B) FPU/g cellulose with: (◆) Celluclast® 1.5, (■) Spezyme® CP, (▲) Optiflow™, (×) Accellerase® 1500 or (✳) Alternafuel® CMAX™. Error bars represent standard deviations of triplicate experiments.

All enzymes showed a decrease in the rate of hydrolysis at some stage during the experiment, which is most likely the result of product inhibition by glucose. The hydrolysis experiments give an indication of not only the final amount of glucose released from the

substrate, but also how susceptible the enzymes in the cocktail might be to product inhibition.

Glucose yields during enzymatic hydrolysis did not show a direct correlation with the enzyme dosage (Figure 15-19). The same phenomenon was observed during SSF where a 3-fold increase in enzyme dosage only increased the ethanol yield by ± 20% (section 4.4). This has also been observed by other researchers and could be the result of spatial interference between enzymes competing for the same substrate (Karimi *et al*., 2006). With increased amounts of enzyme, the substrate becomes saturated and the addition of more enzymes will not increase the hydrolysis of the substrate. The saturation point of the substrate must therefore be determined to avoid using large amounts of expensive enzyme that will not significantly improve yields.

It is also worth mentioning that the large standard deviation for some of the data points in the enzymatic hydrolysis trials are the result of the heterogeneous nature of the substrate. Since the substrate is fibrous and contains almost 70% water, it is difficult to determine the actual amount of cellulose used in each experiment. The substrate can also not be homogenised by drying and milling, as this will change the digestibility of the material. This high level of variability is thus acceptable when working with complex lignocellulosic materials that have natural variation.

4.2.2.2. Xylose yields

The xylose yields obtained after 56 hours of hydrolysis is indicative of each cellulase cocktail's ability to degrade hemicellulose (Table 10), as xylose is a monosaccharide that forms part of hemicellulose. Xylanase activity is only specified for Spezyme® CP and Accellerase® 1500 in their product data sheets, however, some enzymes are said to have "other activities", which may include activity on hemicellulose. The results obtained indicate that all the cellulase cocktails contained some xylanase activity. The highest xylose yield obtained was 36%, which corresponded to a concentration of 0.58 g/l xylose (Spezyme® CP, 37°C).

Table 10: Xylose yields (%) after 56 hours of enzymatic hydrolysis with commercial cellulases at 30 mg protein/g substrate enzyme loading

Cellulase cocktail	Xylose yields (%)	
	50°C	37°C
Celluclast® 1.5	33.18 ± 9.55	27.26 ± 8.51
Spezyme® CP	25.78 ± 1.71	35.85 ± 3.39
Optiflow™ RC 2.0	34.06 ± 3.90	33.53 ± 8.70
Accellerase® 1500	24.73 ± 0.84	28.32 ± 7.38
Alternafuel® CMAX™	22.21 ± 1.06	25.20 ± 5.07

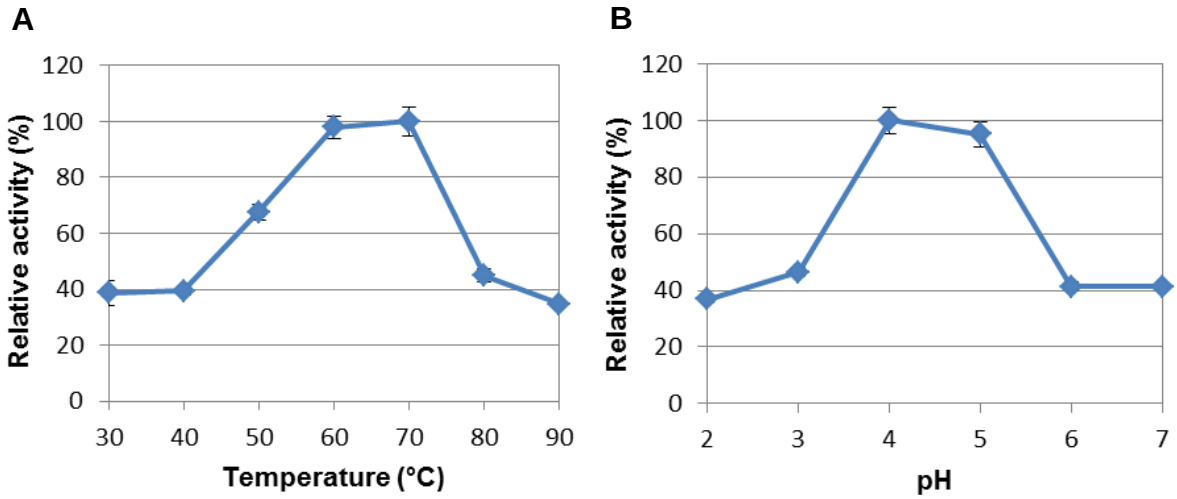
The xylanase activity of Spezyme® CP at 37°C could indirectly contribute to the high glucose yields obtained with this cocktail, since removal of xylan from the lignocellulosic structure could render cellulose more accessible to hydrolysis by cellulases. However, the low levels of xylose released during hydrolysis are not enough to make a significant difference in the ethanol that could be obtained from this substrate during fermentation. Even if an additional xylanase is added, the substrate does not contain enough xylan to raise the xylose concentration above 1.5 g/l. Also, *S. cerevisiae* is not naturally able to ferment xylose and must be genetically engineered to allow for pentose fermentation to ethanol. In an SSF experiment with a much higher solids loading (10%), the xylose concentration could increase to 7.4 g/l, in which case it may merit further investigation. In this study, however, the focus was on optimising glucose release and fermentation, not the fermentation of xylose.

4.3. Characterisation and evaluation of PcbglB

4.3.1. Characterisation of PcbglB

A 20 mg/ml stock solution of the freeze-dried PcbglB had a protein content of 1.05 mg/ml and β-glucosidase activity (on *p*NPG) of 1.932 IU/ml. This implies that 1 mg of freeze-dried enzyme contains 0.053 mg protein and represents 0.097 IU β-glucosidase. The optimum conditions for activity on cellobiose was 70°C and pH 4, with significant activity also

observed at 60°C and pH 5 (Figure 21 A and B). These results are comparable to those obtained by Dobson and colleagues (2014) who cloned and characterised the PcbglB enzyme in their research.



Figures 21: Relative β-glucosidase activity of PcbglB at different (A) temperatures and (B) pH values on cellobiose. Error bars represent standard deviations of triplicate experiments.

4.3.2. Hydrolytic synergy of Spezyme® CP and PcbglB

Most cellulase cocktails lack adequate β-glucosidase activity; therefore, it is common practice to supplement cocktails with additional β-glucosidases to reduce the accumulation of cellobiose in the slurry (Singhania *et al*., 2013; Sørensen *et al*., 2013). These cocktails, including Spezyme® CP, Optiflow™ and Celluclast® 1.5, are routinely combined with the commercial β-glucosidase, Novozym® 188, but this increases the cost of hydrolysis (García-Aparicio *et al.,* 2011; Berlin *et al*., 2006; Da Cruz *et al*., 2012). The combined activity of Spezyme® CP and the recombinant PcbglB was therefore evaluated to determine if the addition of β-glucosidase would increase glucose released from the substrate.

Spezyme® CP is produced by controlled fermentation of *T.reesei*. This fungi's cellulase secretome has been well characterised and it was shown that β-glucosidases constitute about 1% of the total cellulases produced by the fungus (Lynd *et al*., 2002). Pengilly (2013) also indicated that Spezyme® CP has lower β-glucosidase activity compared to other cellulase cocktails, which suggested that the addition of a β-glucosidase to Spezyme® CP would increase the amount of glucose released from the substrate. As shown in Figure 22, the addition of PcbglB did not have a significant impact on hydrolysis of the triticale WIS,

increasing it by a maximum of about 8%. This could indicate that Spezyme® CP has adequate β-glucosidase activity, despite previous reports that it contains 20-40 IU/ml, compared to 220 IU/ml in Optiflow™ (Golias *et al*., 2002; Pengilly, 2013). It is possible that the relatively low level of β-glucosidase activity is adequate, or that the inhibition by glucose nullifies the enzyme addition. Since the enzymes will not be subjected to inhibition by glucose in an SSF environment the combination of Spezyme® CP and PcbglB was still evaluated under these conditions (section 4.5).

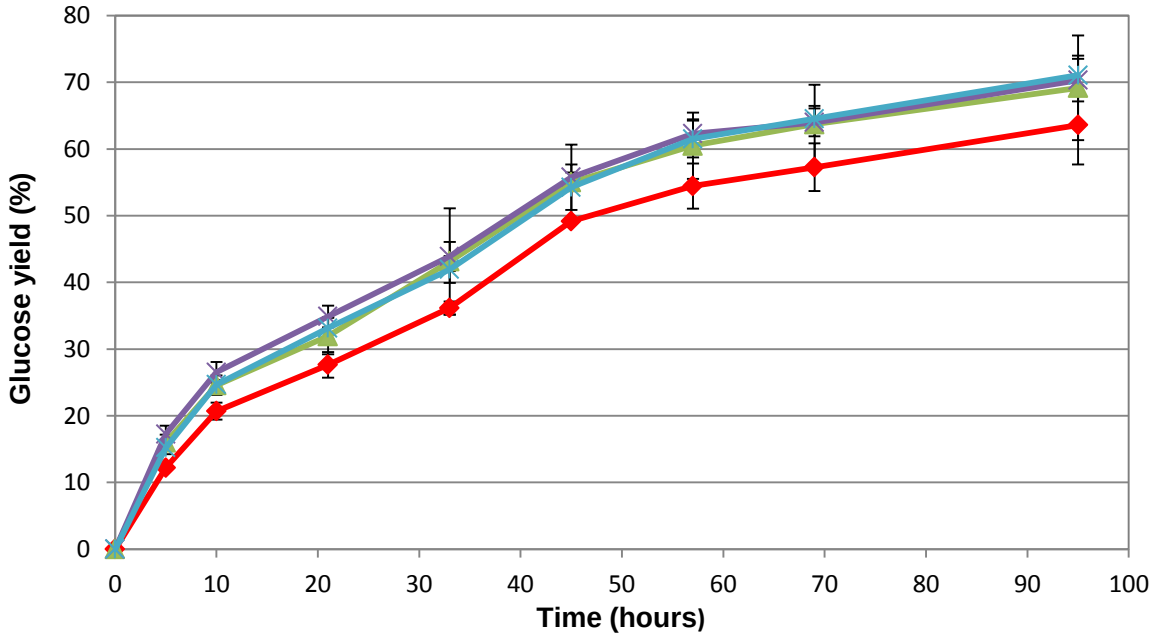


Figure 22: Glucose yield (%) after enzymatic hydrolysis of WIS with 15 FPU/g cellulose Spezyme® CP and a recombinant β-glucosidase enzyme, PcbglB: (◆) Spezyme® CP only; (✳) Spezyme® CP + 500 IU/g PcbglB; (✕) Spezyme® CP + 800 IU/g PcbglB; (▲) Spezyme® CP + 1000 IU/g PcbglB. Error bars represent standard deviations of triplicate experiments.

4.4. Screening of *S. cerevisiae* strains

Several indigenous wild-type *S. cerevisiae* strains were previously isolated from sites in and around vineyards, as well as other areas in the Western Cape (Van der Westhuizen *et al*., 2000). These strains have since been evaluated and characterised in terms of ethanol, inhibitor, temperature and pH tolerances, as well as fermentation vigour under various conditions. Based on those results (unpublished), 30 strains were selected for ethanol production at 30°C using 100 g/l glucose as carbon source and an initial ethanol

concentration of 50 g/l. The initial presence of ethanol will also provide an indication of the ethanol tolerance of the strains. The eight strains that produced the highest ethanol concentrations were *S. cerevisiae* L21, V3, VERG1, YI1, YI9, YI13, YI39 and YI61 (Figure 23).

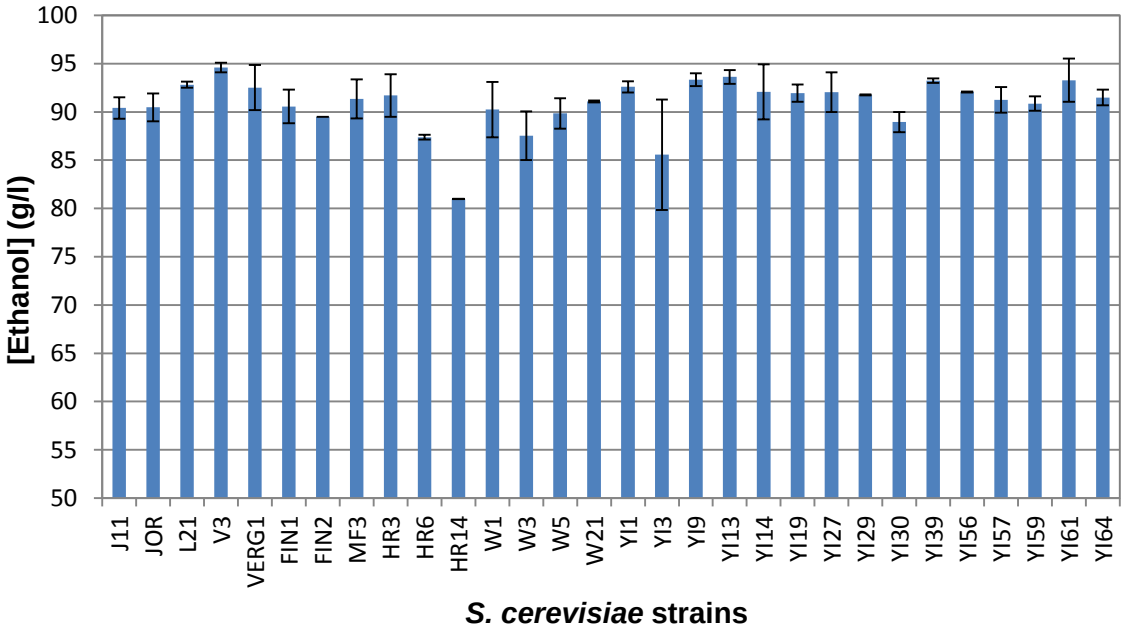


Figure 23: Ethanol concentrations after 7 days of fermentation with an initial 100 g/l glucose and 50 g/l ethanol by 30 wild-type *S. cerevisiae* strains on rotary shaker. Error bars represent standard deviations of duplicate experiments.

The fermentations were repeated at 30°C and 37°C on a multiple stirrer with the eight best performing strains mentioned above. Although most *S. cerevisiae* strains prefer fermentation at 30°C, the optimum conditions for SSF require a higher temperature (37°C) to also allow for cellulase activity. The *S. cerevisiae* L21, YI9 and YI13 strains performed best at 37°C, producing more than 55 g/l ethanol after 7 days of fermentation (Figure 24).

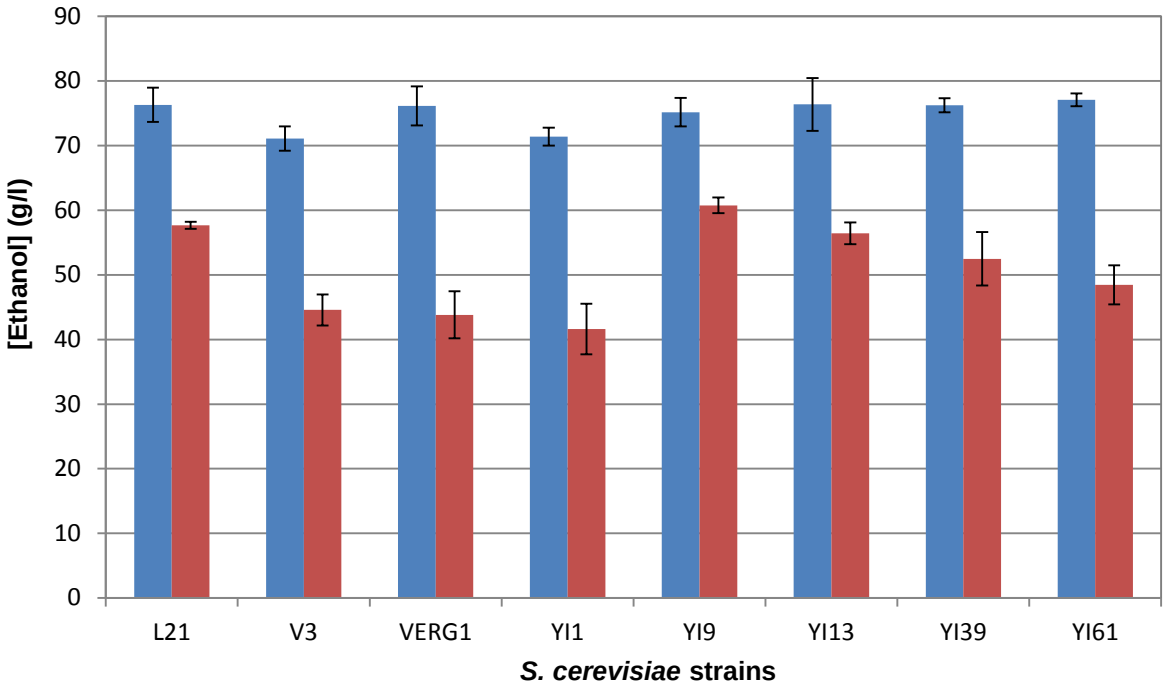
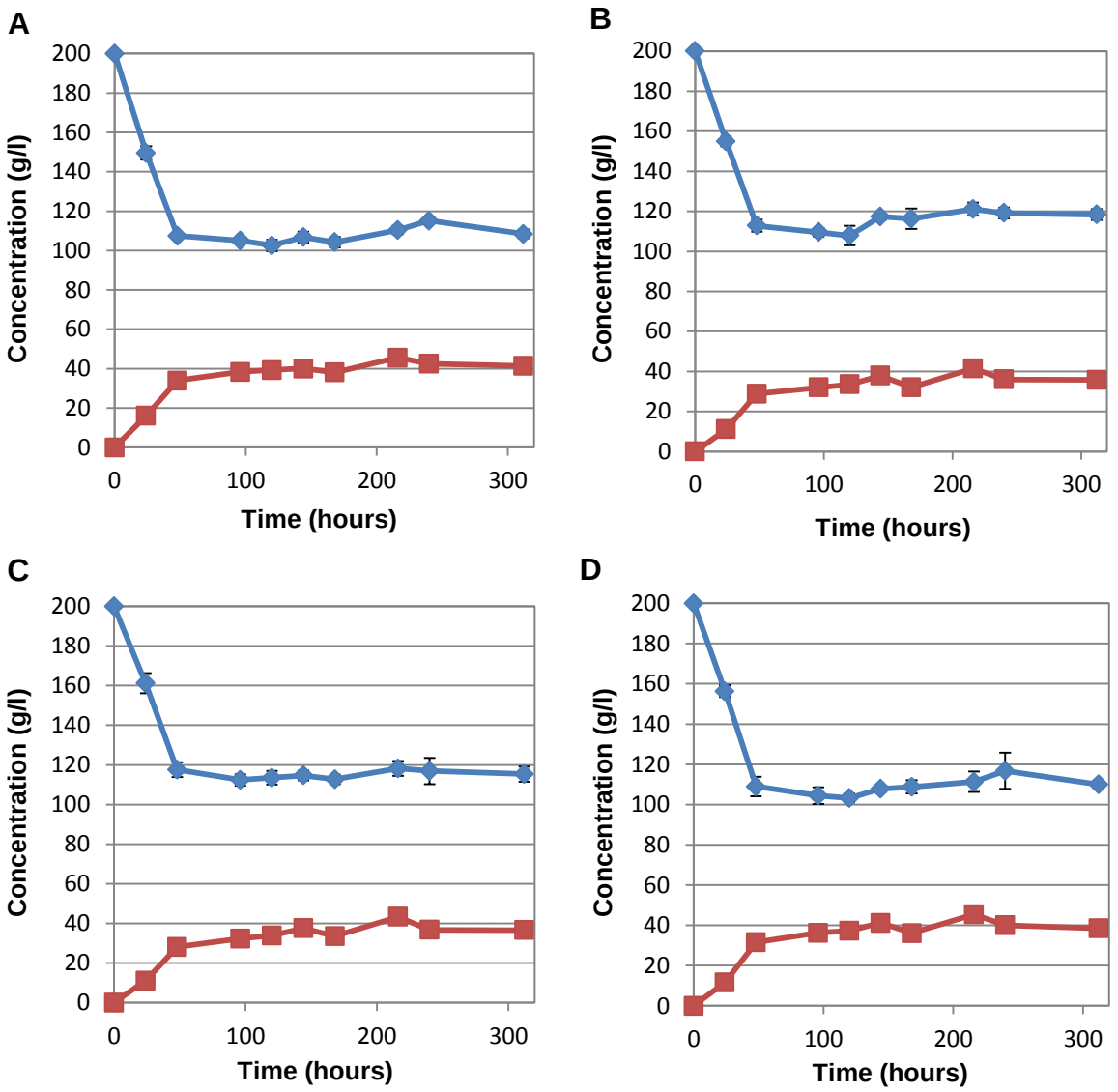


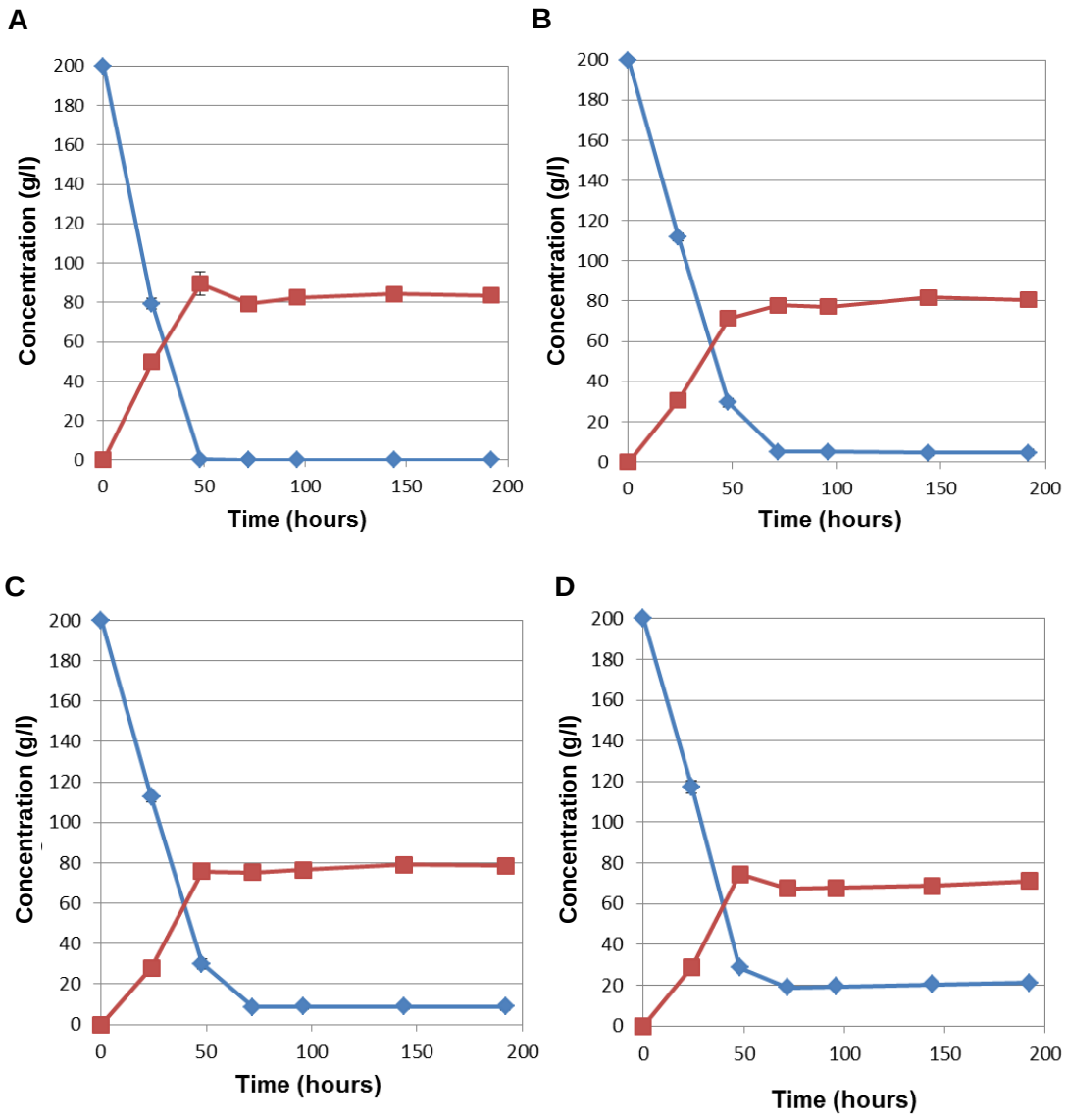
Figure 24: Ethanol production from 100 g/l glucose by selected *S. cerevisiae* strains after 7 days of fermentation at (■) 30°C and (■) 37°C with magnetic stirrers. Error bars represent standard deviations of triplicate experiments.

These three best-performing strains were compared to the industrial *S. cerevisiae* strain, Ethanol Red® (Fermentis®), at 37°C and with 200 g/l glucose as carbon source for 312 hours (13 days). None of the strains was able to ferment all of the glucose or reach high concentrations of ethanol (Figure 25). The residual glucose concentrations remained well above 100 g/l in all the strains, and ethanol concentrations did not surpass 50 g/l. This was ascribed to the SC-medium that does not supply the yeast cells with adequate sterols for growth under anaerobic conditions, as well as acidification, due to the production of H_2SO_4 from $(NH_4)_2SO_4$ in the medium (the pH dropped below 3). The acidic environment would also impact the growth and fermentation by the yeast.



Figures 25: Fermentation of (◆) glucose to (■) ethanol by *S. cerevisiae* strains (A) Ethanol Red®, (B) L21, (C) YI13 and (D) YI9. Error bars represent standard deviation of triplicate experiments.

The fermentations were repeated under the same conditions, but with additional yeast extract and 0.05 M citrate buffer (pH 5) to stabilise the pH and supply enough nutrients for yeast growth. This significantly improved the fermentation results obtained, with the Ethanol Red® strain consuming all the glucose, and producing 83.60 g/l ethanol after only 72 hours (Figure 26A). The wild-type strain L21 performed almost as well, consuming nearly all the glucose and producing 80.6 g/l ethanol after 72 hours (Figure 26B). Wild-type strains YI9 and YI13 failed to consume all the glucose, even after 192 hours. YI13 performed almost as well as L21 in terms of ethanol production, but consumed less glucose. Ethanol Red® and L21 were selected for the subsequent SSF experiments.



Figures 26: Fermentation of (◆) glucose to (■) ethanol by *S. cerevisiae* strains (A) Ethanol Red®, (B) L21, (C) YI13 and (D) YI9. Error bars represent standard deviation of triplicate experiments.

Glycerol and acetic acid are by-products often produced by yeasts during fermentation, especially in harsh or anaerobic conditions when the cells experience a redox imbalance (Tomás-Pejó *et al*., 2008a). However, this trait is undesirable as carbon is diverted away from ethanol production. Strains with lower glycerol and acetic acid production levels are preferred for bioethanol production. Three of the four strains mentioned above (YI9, L21 and Ethanol Red®) did not produce any detectable amounts of acetic acid, while strain YI13 produced minimal amounts (Table 11). All the strains produced glycerol, with strain L21 producing the least (5.91 g/l), adding to its preference for use in the SSF. Although

Ethanol Red® produced significant amounts of glycerol, it had also produced the most ethanol, which makes the possible loss of carbon irrelevant.

Table 11: Concentrations of by-products formed after 192 hours of fermentation by industrial and wild-type *S. cerevisiae* strains.

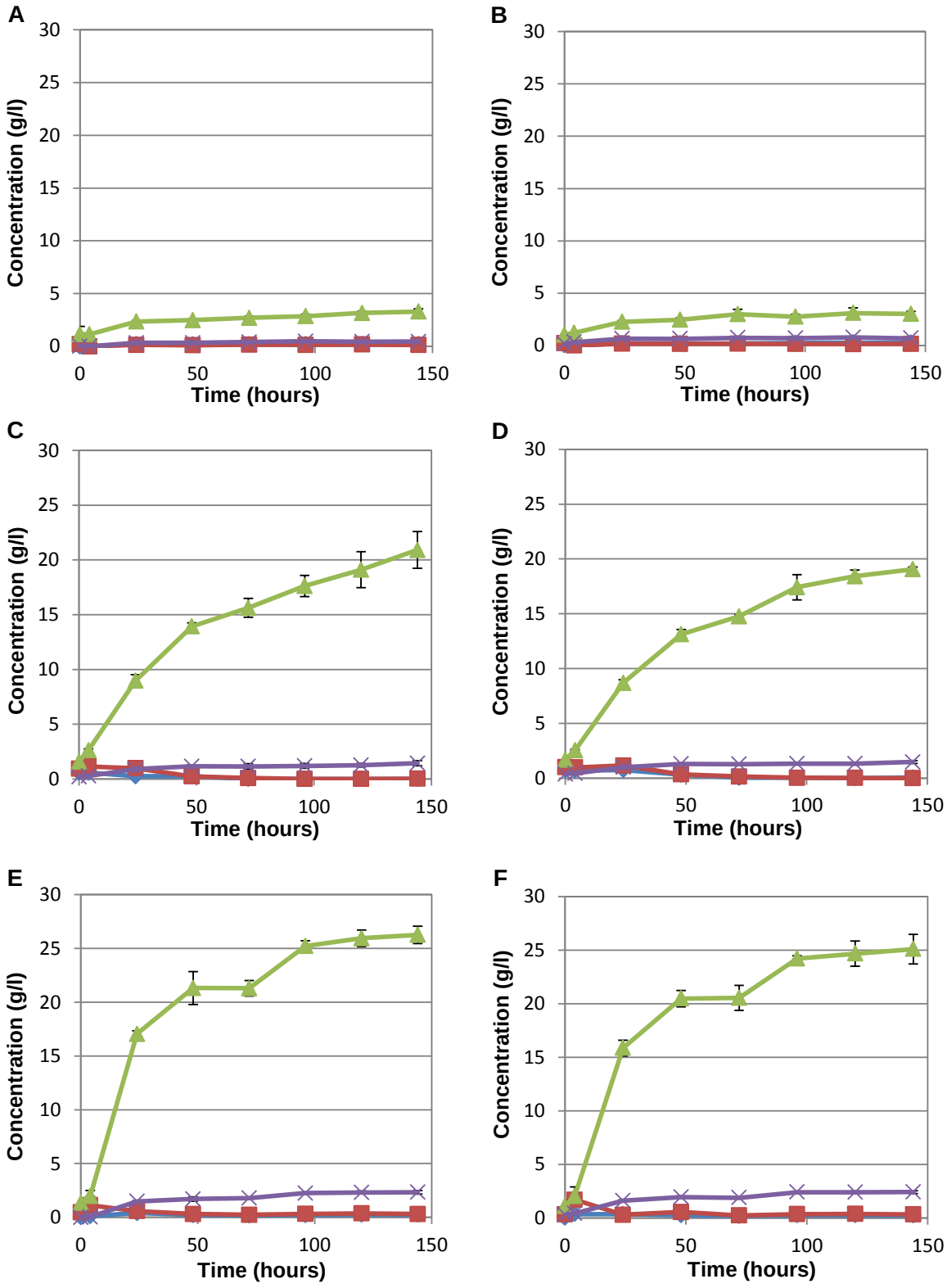
Strains	[Glycerol] (g/l)	[Acetic acid] (g/l)	[Ethanol] (g/l)
S. cerevisiae YI9	7.61 ± 0.29	ND	71.11 ± 1.50
S. cerevisiae YI13	6.79 ± 0.03	0.023 ± 0.02	78.59 ± 1.42
S. cerevisiae L21	5.91 ± 0.09	ND	80.61 ± 0.90
S. cerevisiae Ethanol Red®	7.11 ± 0.11	ND	83.60 ± 0.95

ND = not detected

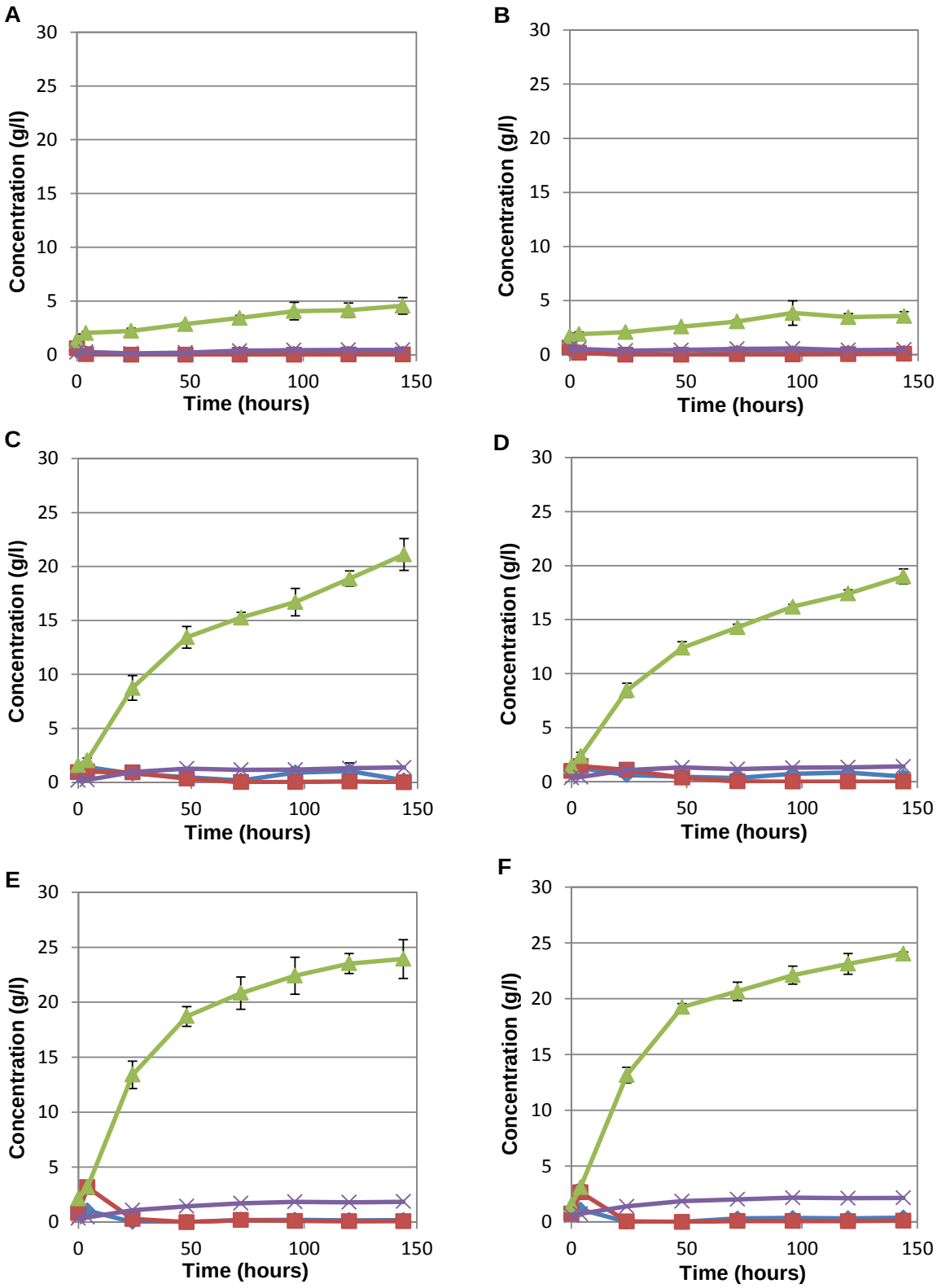
4.5. Simultaneous Saccharification and Fermentation (SSF)

The SSF resulted in efficient hydrolysis and production of ethanol from triticale straw WIS. The glucose and cellobiose concentrations remained low throughout the fermentation (< 1.5 g/l) due to the utilisation of glucose by the yeast (Figures 27 and 28). This created a favourable environment for maximum activity of the cellulases since they were not subject to product inhibition.

The highest ethanol concentration reached (26.9 g/l) was with the combination of the Ethanol Red® and 15 FPU/g cellulase of Spezyme® CP (Figure 27 E). Even with a lower cellulase dosage (5 FPU/g cellulose), Ethanol Red® produced ethanol of around 20 g/l (Figure 27C).



Figures 27: Concentrations of (◆) glucose, (■) cellobiose, (▲) ethanol and (×) glycerol in SSF of triticale WIS in the following combinations: (A) Ethanol Red® only; (B) Ethanol Red® + PcbglB; (C) Ethanol Red® + 5 FPU/g cellulose Spezyme® CP; (D) Ethanol Red® + 5 FPU/g cellulose Spezyme® CP + PcbglB; (E) Ethanol Red® + 15 FPU/g cellulose Spezyme® CP; (F) Ethanol Red® + 15 FPU/g cellulose Spezyme® CP + PcbglB. Error bars represent standard deviations of triplicate experiments.



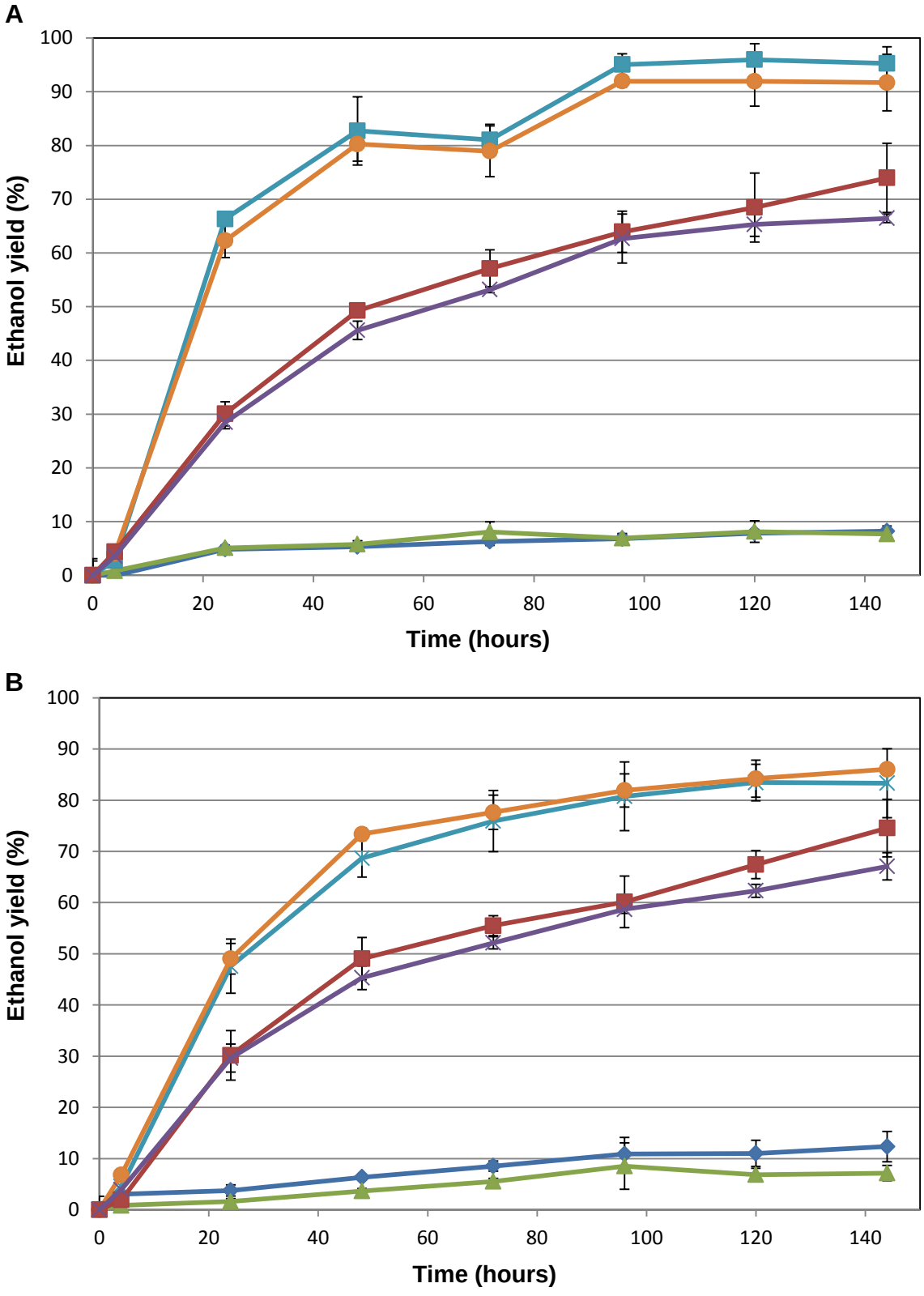
Figures 28: Concentrations of (◆) glucose, (■) cellobiose, (▲) ethanol and (×) glycerol in SSF of triticale WIS in the following combinations: (A) L21 only; (B) L21 + PcbglB; (C) L21 + 5 FPU/g cellulose Spezyme® CP; (D) L21, 5 FPU/g cellulose Spezyme® CP + PcbglB; (E) L21 + 15 FPU/g cellulose Spezyme® CP; (F) L21 + 15 FPU/g cellulose Spezyme® CP + PcbglB. Error bars represent standard deviations of triplicate experiments.

The wild-type strain L21 was also able to produce ethanol from the triticale WIS, although at slightly lower levels compared to Ethanol Red® (Figure 28). At high enzyme dosage (15 FPU/g cellulose), strain L21 produced 23.5 g/l ethanol compared to the 26.9 g/l produced by Ethanol Red® in the SSF experiments, but performed similarly with 5 FPU/cellulose enzyme. Ethanol Red® has been extensively engineered and selected for high ethanol and temperature tolerance, which is why it outperformed the wild-type strain. Nevertheless, there is potential for a wild-type strain such as L21 to also be engineered and utilised as an industrial strain.

The results from the SSF experiments indicate that the triticale substrate is easily digested and converted to ethanol, even at low cellulase dosages. An ethanol yield of 95.97% (% of maximum theoretical yield) was reached after 144 hours when Ethanol Red® was used in combination with 15 FPU/g cellulose Spezyme® CP (Figure 29A). At the lower dosage of 5 FPU/g cellulose, an ethanol yield of 73.96% was obtained.

The ethanol yield during SSF of triticale WIS with 15 FPU/g cellulose Spezyme® CP and Ethanol Red® reached a plateau after 96 hours, with a residual glucose concentration of 0.21 g/l. Most of the glucose had thus been fermented and no further ethanol was produced. This demonstrates rapid fermentation and hydrolysis when a high cellulase dosage and a yeast strain with good fermenting capabilities are employed. This quick reaction time would be a desirable trait in an industrial process. Even after 48 hours, the ethanol yield was substantial at nearly 83% (Figure 29A). The wild-type strain, L21, also produced high yields when combined with 15 or 5 FPU/g cellulose Spezyme® CP (86.04% and 74.57%, respectively) (Figure 29B).

Neither of the strains produced significant amounts of glycerol during the fermentation of triticale WIS (Figure 27 and 28). This is a desirable trait since glycerol production is a waste of carbon that could otherwise have been converted to ethanol. The ethanol yields obtained with both yeast strains (Figures 29) indicate that more than 85% of the available glucose was converted to ethanol, with only a minimal amount lost to cell growth and glycerol production.



Figures 29: SSF of triticale WIS with *S. cerevisiae* strains (A) Ethanol Red® and (B) L21, in the following combinations: (◆) yeast only; (▲) yeast + PcbglB; (■) yeast + 15 FPU/g cellulose Spezyme® CP; (●) yeast + 15 FPU/g cellulose Spezyme® CP + PcbglB; (■) yeast + 5 FPU/g cellulose Spezyme® CP; (×) yeast + 5 FPU/g cellulose Spezyme® CP + PcbglB. Error bars represent standard deviations of three separate experiments.

The combination of Ethanol Red® and Spezyme® CP (15 FPU/g cellulose) resulted in a 95.97% ethanol yield after 144 hours (6 days). Similar research on biofuel production from corn cobs reported higher ethanol yields (up to 98.8%), but much higher enzyme dosages (> 33 FPU/g cellulose) were employed (Shen *et al*., 2008) (Table 12). Research conducted by Tomás-Pejó and colleagues (2008a) on wheat straw with similar reaction conditions and enzyme dosages resulted in only a 75.5% ethanol yield, despite the higher cellulose content of their substrate and the addition of a commercial β-glucosidase. However, the distinguishing factor was the use of the whole slurry, which contains enzyme and fermentation inhibitors, resulting in a significantly lower ethanol yield. Their substrate could also be less digestible than our substrate. They also did not evaluate several enzyme cocktails to determine which would have the best activity on their specific substrate.

It should also be mentioned that results from an enzymatic hydrolysis trial alone is not a reliable estimation of the performance of an enzyme or substrate in SSF. In this study, the high ethanol yields obtained in SSF imply that the glucose yield would have to be close to 100%. However, the enzymatic hydrolysis predicted a maximum of only 59% hydrolysis, and the hydrolysis reached a plateau after 48 hours. The enzyme dosage optimisation should therefore not be trusted unless evaluated in combination with an SSF process. In the absence of product inhibition by glucose and cellobiose, the cellulases could have improved activity and be more enduring than is indicated by enzymatic hydrolysis trials. Isolated hydrolysis trials also do not give an indication of how acids, ethanol, lignin and phenolic compounds (released from lignin) will influence the specific cellulases in the SSF environment (Ximenes *et al*., 2010).

The addition of the PcbglB in the SSF experiments had no significant effect on the ethanol yields (Figures 29A and B), the rate of fermentation or the glucose and cellobiose concentrations (Figures 27 and 28). Even at a low Spezyme® CP dosage of 5 FPU/g cellulose the addition of PcbglB did not improve the ethanol yields. This was unexpected since β-glucosidases are routinely added to commercial processes to supplement cellulase cocktails. A possible explanation is that the amount of enzyme added was not enough to have a significant effect on the hydrolysis and that a much higher dosage should be used in future. However, the dosage used (35 IU/g cellulose) was already much higher than the 15 IU/g cellulose generally used in research (Tomás-Pejó *et al*., 2008a; García-Aparicio *et al*., 2011). It is also possible that the Spezyme® CP enzyme cocktail is sufficient for the complete hydrolysis of cellulose and cannot be improved upon by the addition of β-glucosidase activity. This might be especially true in the SSF experiments where there is a constant flow of chemical reactions that remove glucose and prevent product inhibition.

Although the lyophilised PcbglB retains high activity after storage at -20°C for several months, it is not known how stable the enzyme would be after several days of incubation at 37°C in a liquid medium. It might have been denatured after a few hours, rendering its addition obsolete. The PcbglB could also have been degraded by proteases produced by the yeast, as these strains were not protease deficient. However, this does not explain why the activity of Spezyme® CP was not affected. Also, PcbglB might not be suitable for the harsh conditions of the SSF setup, where it is subjected to inhibition and structural interference by phenolic compounds and ethanol. It is possible that the enzyme's activity at 37ºC is not enough to have a significant effect at the SSF temperature. Activity assays revealed that it only has around 40% activity between 30ºC and 40ºC (Figure 21A). An enzyme from a different origin might perform better under these conditions.

The results obtained in this study are similar to those obtained by other researchers who have produced bioethanol from various straw types with an SSF process. In a single-batch SSF setup with a pretreated straw substrate (and 10% solids loading), a final ethanol concentration of between 23 and 26 g/l, or ethanol yield of 70-85%, is the current benchmark (Tomás-Pejó *et al*., 2008a; Saha *et al*., 2011; López-Linares *et al*., 2014). However, in processes with higher substrate loadings, the final ethanol concentrations can be much higher although the yields might be similar (Chen *et al.,* 2008; Watanabe *et al.,* 2012). Table 12 summarises the results obtained by other researchers in similar studies with SSF reactions and lignocellulosic substrates.

The results of Shen and colleagues (2008) (98% ethanol yield) were obtained with a commercial cellulase supplemented with a recombinant β-glucosidase. However, the final ethanol concentration is much lower than is required for a commercial process. Some of the best results obtained by researchers have been with the use of higher solids loadings, high enzyme dosages (>30 FPU/g cellulose) and extreme pretreatment methods (Chen *et al.,* 2008) (Table 12). Although this approach can produce high ethanol yields and concentrations that will make distillation less expensive, the SSF process will be significantly less cost-effective. It is essential to find the balance between the efficiency and cost of the process to maintain a commercially viable production process.

Although the overall results from the SSF experiments are promising, the highest ethanol concentration reached was 26.9 g/l, which is well below the 40 g/l needed for cost-effective ethanol distillation (Zacchi and Axelsson, 1989; Park *et al*., 2013). The concentration of 26.9 g/l represents an ethanol yield of more than 90%, but even at 100% ethanol yield the concentration will not reach 40 g/l. The substrate does not contain enough cellulose that can be degraded and fermented to ethanol, therefore an ethanol concentration of 40 g/l cannot be attained. It is possible to use a higher substrate loading to increase the amount

of cellulose, however, this will increase the amount of toxic or inhibitory compounds in the slurry, which can have a negative impact on enzyme activity and yeast growth. Higher solids loading will also increase the viscosity and restrict agitation.

Table 12: Summary of results for bioethanol production with SSF processes previously reported

Substrate	Pretreatment	Solids loading	[Final ethanol]	Ethanol yield	Reference
Rice straw	Dilute acid	5%	12.4 g/l	73.6%	Karimi *et al.,* 2006
Corncobs	Acid	7%	20 g/l	98%	Shen *et al.,* 2008
Triticale straw	Steam explosion	10%	26.9 g/l	95.97%	This study
Rapeseed straw	Hydrothermal	10%	23 g/l	69%	López-Linares *et al*., 2014
Wheat straw	Steam explosion	10%	23.7 g/l	77.5%	Tomás-Pejó *et al*., 2008a
Wheat straw	Steam explosion	10%	25 g/l	68%	Alfani *et al*., 2000
Wheat straw	Steam explosion	10%	-	80%	Ballesteros *et al*., 2006
Wheat straw	Steam explosion and alkaline peroxide	16.7%	51.5 g/l	81%	Chen *et al.,* 2008
Rice straw	Alkali-treated	20%	38 g/l	84.7%	Watanabe *et al.,* 2012

As seen in Table 12, the highest ethanol concentrations are produced with high solids loading (Chen *et al.,* 2008; Watanabe *et al.,* 2012). Agitation of lignocellulosic substrates during SSF is essential because of its fibrous and viscous nature, especially in high gravity fermentations (Tomás-Pejó *et al*., 2008a). The reaction volume and reactor size will also

have a significant effect on the outcome of fermentation. The results from this study are reliable on laboratory scale while in the industry much larger volumes are required to make a process viable. In an ideal industrial setup, the SSF process would be conducted in a bioreactor with a large volume and good mixing and agitation capabilities, in which case the substrate loading can be increased and the increased viscosity can be managed (Jørgensen *et al.,* 2007). Alternatively, a fed-batch process could increase the final ethanol concentration without compromising the mixing of the slurry (Rudolf *et al*., 2005; Anwar *et al.,* 2014).

Other options to improve bioethanol production from triticale straw include increasing the cellulose content of the substrate (optimisation of steam-explosion) and the addition of laccases or phenoloxidases that degrade the lignin structure. As mentioned earlier, the use of a fed-batch process could be a means to obtain an ethanol concentration sufficient for distillation. Tomás-Pejó and colleagues (2009) were able to obtain an ethanol concentration of 36.2 g/l with a fed-batch process to produce ethanol from wheat straw, with a final substrate loading of 15% reached by addition of substrate every 12 hours during the SSF process.

5. General conclusions

5.1. Main findings of the study

Agricultural residues are considered some of the most interesting and promising feedstock options for bioethanol production (Sarkar *et al*., 2012; Anwar *et al*., 2014). Triticale in particular is a good alternative feedstock for bioethanol production in South Africa, since the crop is resistant to disease and can tolerate the harsh African climate. The grain produced from triticale is used as food or animal feed, while the straw is suitable for bioethanol production. The straw is a softer substrate than most woody lignocellulosic materials and does not require harsh pretreatment conditions associated with more woody feedstocks (Sun and Cheng, 2002; Tomás-Pejó *et al*., 2008a). This also implies that less inhibitors and acids are produced without compromising the digestibility of the cellulose. It has been suggested that genetic manipulation or selective breeding of this crop could change the lignin composition and further reduce its resistance to enzymatic hydrolysis (Fu *et al.,* 2011).

The results of this study confirmed that triticale straw is suitable for large-scale bioethanol production. This substrate was effectively processed in the steam-explosion pretreatment setup and the resulting WIS was hydrolysed to varying degrees by the commercial cellulase cocktails. However, the digestibility as well as the cellulose content of the WIS could be further improved by optimisation of the steam-explosion conditions. Ballesteros and colleagues (2006) demonstrated that a substrate that is easily digested and has high cellulose content, significantly improves its performance in an SSF process with higher ethanol yields that will ease distillation of the ethanol.

All five cellulase cocktails evaluated in this study were able to digest the triticale WIS to some degree, as measured by the release of glucose from the lignocellulosic substrate. Spezyme® CP displayed high cellulase activity and longevity even in the harsh SSF environment, and shows potential for use in an industrial setup. In the SSF experiments, different cellulase dosages were evaluated, although a lower dosage is usually preferred to reduce the processing cost (Viikari *et al*., 2012). The lower sugar yields from lower enzyme dosages could be negated by extending the reaction time to allow the substrate to be completely hydrolysed, but this is not always cost-effective. These challenges support the need for detailed process modelling specifically for triticale WIS to determine the optimal enzyme dosage and fermentation time required.

The choice of the fermenting organism is another important factor that will significantly affect bioethanol production from lignocellulose. Several of the wild-type *S. cerevisiae* strains that were evaluated showed good fermenting ability at 37°C, including YI9, YI13 and L21, whose ethanol production from glucose was close to that of Ethanol Red®. Mating and genetic manipulation of these strains could produce a strain on par with Ethanol Red®, with desirable traits such as ethanol, inhibitor and temperature tolerance that will perform well in an SSF environment. Other traits such as glycerol and acid production can also be reduced to improve ethanol yields. Some consideration has been given to the use of other yeast species for ethanol production, such as *Kluyveromyces marxianus*, and this could be a possible alternative to the traditional use of *S. cerevisiae*, in particular species that may have desirable qualities such as temperature tolerance (Tomás-Pejó *et al.,* 2009).

It can be concluded from the SSF results that the common practice of combining β-glucosidases with commercial cellulases is unnecessary when triticale WIS is used as feedstock under the conditions described in this study. The common belief that more β-glucosidase activity is required is likely based on data from isolated hydrolysis trials, where high glucose yields are difficult to achieve due to product inhibition. This study showed that cellulase cocktails should be evaluated in the absence of product inhibition, such as those associated with SSF.

The SSF experiments resulted in a 95.97% ethanol yield, or 26.9 g/l ethanol, which is similar to the results reported by other researchers (Ballesteros *et al.,* 2006; Tomás-Pejó *et al.,* 2008a). This study provides proof of concept that triticale straw is a viable substrate for bioethanol production. Some improvements can still be made to the overall process design to adapt it for an industrial application, including steam-explosion optimisation, increased solids loading, enzyme dosage optimisation and the implementation of a fed-batch process.

The following conclusions can be drawn from this research:

- triticale straw has significant potential as feedstock for bioethanol production;
- all five commercial cellulase cocktails evaluated displayed hydrolytic activity on triticale straw WIS, while some performed better than others;
- Spezyme® CP displayed high activity on the triticale WIS and performed well in the SSF setup;
- *S. cerevisiae* strain L21 performed similarly to Ethanol Red®, a robust fermenting *S. cerevisiae* strain developed for ethanol production in an SSF process utilising a lignocellulosic substrate;

- enzyme dosage, substrate loading and pretreatment optimisation are required to fine-tune the SSF conditions and obtain maximum ethanol concentrations with minimal inputs of cellulases, substrate and energy.

5.2. Future research

The sustainable future of energy supply relies on the ability to find alternative ways to produce renewable liquid fuels to provide energy for everyday industrial and domestic use. It is essential to develop solutions that can be implicated on a large scale and that will not have unforeseen consequences, such as the food crisis predicted as a result of ethanol production from corn (Demirbas, 2011). Lignocellulosic biomass is the bioethanol feedstock of the future and the use of lignocellulose in liquid fuel production will solve the food versus fuel debate; it is also a renewable and sustainable feedstock (Goldemberg, 2007; Tomás-Pejó *et al.,* 2008b). Since the cost of feedstock represents the largest portion of the cost of bioethanol, an inexpensive source is essential. As a bioethanol feedstock, triticale has obvious advantages because of its robust growth capacity, grain yields and straw production. The low monetary value of the straw makes it an ideal lignocellulosic feedstock.

While the ethanol concentrations obtained through SSF can be increased through optimisation of the fermentation process, it is the ethanol yield from lignocellulose will ever reach 100% as the fermenting yeast also uses the glucose for biomass production, respiration and glycerol production. However, attention should be given to upscaling the SSF process for industrial evaluation to ascertain the techno-economic efficiency in terms of enzyme dosages and ethanol yields. Optimised pretreatment and improved agitation during SSFcould decrease the required enzyme dosage, which will decrease the cost of commercial ethanol production.

The main concern regarding the use of cellulases for lignocellulosic ethanol is the cost of their production and purification. The expression of cellulases by recombinant yeasts (such as in consolidated bioprocessing) is the most elegant solution to this dilemma, as the same organism will produce the enzymes and ferment the glucose to ethanol (Den Haan *et al*., 2007). The research in this area has focussed on yeast expression and secretion systems, but has not yet achieved the same success as traditional SSF processes. However, substantial progress has been made in recent years and it is likely that the first phases of commercial lignocellulosic bioethanol production will be based on traditional SSF processes (Olson *et al*., 2012). The consolidated bioprocessing strategy could then be

adopted at a later stage when it has been optimised to achieve industrial standards. In the meantime, the cost of cellulase production can be reduced by using less expensive production techniques (recombinant strains, alternative substrates, less expensive purification techniques, etc.) and the use of cellulases can be minimised by dosage optimisation. It is also essential to match specific enzyme cocktails to a given substrate, taking the substrate specificity of the cellulases and substrate composition into consideration. Discovering and identifying novel cellulases can contribute to improved hydrolysis of various substrates, in addition to enzyme modification through directed evolution or protein engineering.

The most significant obstacle to the global introduction of bioethanol to replace fossil fuels is that its production cost is still too high. Major advances in terms of cultivating, collecting and converting lignocellulosic biomass are required to reduce these costs. Developing a biorefinery industry around lignocellulosic biomass could support current strategies to make cellulosic ethanol a reality (Demirbas, 2011). New possibilities are discovered nearly every day that could be vital to the development of a cost-effective and viable process for bioethanol production from lignocellulose. Although the foundation has been laid for bioethanol production, its global application on commercial scale will rely on fine-tuning and optimising the existing technologies.

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