THE DEVELOPMENT OF RECOMBINANT FUNGAL ENZYME COCKTAILS FOR THE HYDROLYSIS OF CELLULOSIC WASTE PRODUCTS

by

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Thesis presented in partial fulfilment
of the requirements for the Degree

of

MASTER OF SCIENCE
IN MICROBIOLOGY

in the Faculty of Science
at Stellenbosch University

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April 2014

Declaration

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Summary

Biofuels, such as bioethanol, provide an alternative, environmentally friendly transportation fuel. Renewable energy sources, such as lignocellulosic material, are therefore being explored for the production of biofuels, since they offer an attractive and sustainable source for bioconversion processes. However, the major obstacle in the use of lignocellulosic biomass is its recalcitrant nature, which decreases the enzyme accessibility to cellulose and thus affects the overall hydrolysis process. Current commercial enzyme cocktails are not yet sufficient to promote hydrolysis on an industrial scale, thus hampering biofuel production.

A number of cellulase enzymes are needed to act in synergy to obtain complete hydrolysis of lignocellulosic material. The enzymatic hydrolysis of cellulose requires the synergistic action of three cellulase enzymes namely endoglucanases, exoglucanases and β-glucosidases. However, cellulolytic organisms do not produce significant amounts of ethanol, whereas strong fermentative organisms don't produce enzymes for cellulose hydrolysis. A need has therefore arisen to develop recombinant technologies to obtain maximum production of cellulolytic enzymes that can be used (exogenously) in combination with a fermentative organism.

Paper sludge is a lignocellulosic waste material that is generated in large quantities by the pulp and paper industry. Non-hazardous paper sludge can be converted to fermentable sugars, which can then be fermented to bioethanol. Biological conversion of paper sludge requires no pre-treatment, making it an ideal substrate for industrial use. The development of enzyme cocktails for efficient hydrolysis of paper sludge is therefore important in the pursuit of second-generation bioethanol production.

A recombinant cellulase enzyme cocktail tailored for the degradation of paper sludge was developed using cellulases from recombinant *Aspergillus niger* and *Saccharomyces cerevisiae* strains. The recombinant strains were cultured and their supernatants used to develop an enzyme cocktail based on activity ratios. The core cellulases in the optimal cocktail included a cellobiohydrolase I, cellobiohydrolase II, endoglucanase and β-glucosidase. The enzyme cocktails were subsequently evaluated on triticale, Avicel and wheat bran.

The activities (in Filter Paper Units) for the final cocktails were 0.7 and 0.45 for the CbhI:CbhII:EgA:Bgl1 and CbhI:CbhII:EgA:Bgl2 cocktails, respectively. The optimum enzyme ratio (based on protein concentrations) for the CbhI:CbhII:EgA:Bgl1 cocktail was 7.4:6.6:1:208 and 7.4:6.6:1:41 for the CbhI:CbhII:EgA:Bgl2 cocktail. Overall, hydrolysis with the Bgl2 cocktail allowed for longer incubation times and an improved degree of saccharification when the enzyme concentration was doubled. Comparison of paper sludge hydrolysis results with those from Avicel hydrolysis highlight the need to tailor enzyme cocktails based on natural substrates.

Two industrial amylolytic *S. cerevisiae* yeast strains were compared in an SSF (10% wheat bran) process, using the Bgl2-cocktail. The maximum ethanol yield produced by *S. cerevisiae* S2[TLG, SFA] and *S. cerevisiae* MH1000[TLG, SFA], in the presence of the 1x enzyme cocktail, was 5.72 $g.l^{-1}$ and 5.45 $g.l^{-1}$, respectively. This study demonstrated that the addition of the recombinant cellulase cocktail improved the ethanol yields by 8.69% in the SSF process and that the *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA] strains efficiently converted starch to ethanol.

To our knowledge, this is the first report of the use of individual enzymes from recombinant strains, for the hydrolysis of paper sludge and wheat bran. This study has provided insight into the hydrolysis of cellulosic materials, using recombinant cellulase cocktails. The knowledge obtained could be applied in optimising lignocellulose hydrolysis, for efficient sugar release and ultimately improving ethanol production by recombinant yeast strains. This study also demonstrates the potential of using agricultural and industrial wastes as lignocellulosic feedstocks for biofuels production.

Opsomming

Biobrandstof, bv.bioetanol, bied 'n alternatief vir 'n omgewingsvriendelike vervoerbrandstof. Hernubare energiebronne, soos lignosellulose-ryke materiaal, word dus vir die produksie van biobrandstof ondersoek, aangesien hulle 'n aantreklike en volhoubare bron vir bio-omskakelingsprosesse bied. Die grootste struikelblok in die gebruik van lignosellulose-ryke biomassa is hul weerstandige natuur, wat die hidrolitiese proses beïnvloed. Huidige kommersiële ensiemmengels is onvoldoende vir substraathidrolise op 'n industriële skaal wat dus biobrandstofproduksie belemmer.

'n Aantal sellulase ensieme, in sinergistiese samewerking is nodig vir volledige hidrolise van lignosellulose-ryke materiaal. Die ensiematiese hidroliese van sellulose vereis die sinergistiese aksie van drie sellulase ensieme naamlik endoglukanases, eksoglukanases en β-glukosidases. Sellulolitiese organismes produseer egter nie beduidende hoeveelhede etanol nie, en fermenterende organismes produseer nie sellulolitiese ensieme nie. Hiervolgens het 'n behoefte ontstaan om rekombinante tegnologie te ontwikkel waardeur groot hoeveelhede ensieme geproduseer kan word, wat dan eksogenies in aanvulling tot 'n fermenterende organisme gebruik kan word.

Papierslyk is 'n lignosellulose-ryke afvalmateriaal wat in groot hoeveelhede deur die pulp-en-papierbedryf gegenereer word. Onskadelike papierslyk kan na fermenteerbare suikers omgeskakel word, wat dan na bioetanol gefermenteer kan word. Biologiese omskakeling van papierslyk vereis geen vooraf-behandeling nie en maak dit 'n ideale substraat vir industriële gebruik. Die ontwikkeling van 'n ensiemmengsel vir doeltreffende hidrolise van papierslyk is dus belanrik vir die nastrewing van tweede-generasie etanol produksie.

'n Rekombinante sellulase ensiemmengsel, aangepas vir die afbraak van papierslyk, is ontwikkel deur gebruik te maak van sellulases van rekombinante *Aspergillus niger* en *Saccharomyces cerevisiae* rasse. Die rekombinante stamme is gekweek en hul bostande gebruik om 'n ensiemmengsel, gebaseer op aktiwiteitsverhoudings, te ontwikkel. Die kern sellulases in die optimale mengsel sluit 'n sellobiohidrolase I, sellobiohidrolase II, endoglukanase en β-glukosidase in. Die ensienmengsels is geëvalueer op korog, Avicel en koringsemels.

Die aktiwiteite (in Filter Paper eenhede) vir die finale ensiemmengsels was 0,7 en 0,45 vir die CbhI:CbhII:EgA:Bgl1 en CbhI:CbhII:EgA:Bgl2 ensiemmengsel, onderskeidelik. Die optimum ensiem verhouding (gebaseer op proteïen konsentrasies) vir die CbhI:CbhII:EgA:Bgl1 ensiemmengsel was 7.4:6.6:1:208 en 7.4:6.6:1:41 vir die CbhI:CbhII:EgA:Bgl2 ensiemmengsel. Algehele, hidrolise met die Bgl2 ensiemmengsel het 'n beter graad van versuikering te weeggebring met “n toename in inkubasie tyd en ‘n verdubbelling in ensiem konsentrasiel. Vergelyking van papierslyk hidrolise resultate met dié van Avicel hidrolise beklemtoon die noodsaaklikheid daarvan om ensiemmengsels aan te pas gebaseer op natuurlike materiale.

Twee industriële amilolitiese*S. cerevisiae*-gisrasse is met mekaar vergelyking in 'n GVF (Gelyktydige Versuikering en Fermentasie) met 10 % koringsemels in die teenwooedigheid van die Bgl2-ensiemmengsel. Die maksimum etanolopbrengs deur *S. cerevisiae*S2 [TLG, SFA] en *S. cerevisiae* MH1000[TLG, SFA], in die teenwoordigheid van die sellulase ensiemmengsel, was 5,72 $g.l^{-1}$ en 5,45 $g.l^{-1}$, onderskeidelik. Hierdie studie het getoon dat die toevoeging van die rekombinante sellulase ensiemmengsel die etanol opbrengs verbeter met 8,69% in die GVF proses en dat die *S. cerevisiae* S2 [TLG, SFA] en MH1000 [TLG, SFA] rasse doeltreffend stysel na etanol omskakel.

Volgens ons kennis is dit die eerste verslag oor die gebruik van individuele ensieme vanaf rekombinante rasse vir die hidrolise van papierslyk en koringsemels. Hierdie studie lewer insig tot die hidroliese van sellulose-ryke materiaal deur rekombinante sellulase ensiemmengsels. Die kennis kan in die optimisering van lignosellulose hidrolise vir doeltreffende suikervrystelling en uiteindelik die verbetering van etanolproduksie deur rekombinante gisrasse toegepas word. Hierdie studie toon ook die potensiaal van landbou-en industriële afval as lignosellulose substrate vir biobrandstofproduksie.

Acknowledgements

I thank God for his countless blessings, protection and the abilities given to me to complete this study. My sincere thanks and appreciation is extended to the following people and institutions:

Prof. W.H. van Zyl, Department of Microbiology, Stellenbosch University, my supervisor for this study, for his encouragement and allowing me all the opportunities that I received during this study;

Dr S.H. Rose, Department of Microbiology, Stellenbosch University, my co-supervisor, for always believing in me, teaching me more than words can express and her continued support;

Dr L. Favaro, Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE), Padua University, who was my co-supervisor for part of this study, for his guidance and valuable knowledge in research methods;

My co-workers in the Bloom lab, for their support, scientific input and troubleshooting discussions;

The Staff of the Department of Microbiology, for all their assistance and administrative help;

Technical assistance from Mr Solomon, Department of Wood Science; Ms Rossouw, Process Engineering; Mrs Hugo, Microbiology Department, Stellenbosch University; Mr Fontana, Padua University;

The National Research Foundation (NRF) for financial assistance towards this research. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF;

Stellenbosch University for financial support;

My fiancé, Jamie Cripwell, for his love, continued support and encouragement;

My parents, Sue and Roy Dobson (to whom this thesis is dedicated), and my brothers Richard, Timothy and Arthur, for their love and faith in me to achieve my goals.

to my parents, Sue & Roy

Abbreviations

AFEX	Ammonia fibre expansion
ANOVA	Analysis of variance
BGL	β-glucosidase
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
CBM	Cellulose binding molecule
CBP	Consolidated bioprocessing
CbU	Cellobiose units
DNS	Dinitro salicylic acid
DS	Degree of saccharification
DW	Dry weight
EG	Endoglucanase
EC	Enzyme commission
FPU	Filter paper units
GENPLAT	Great lakes bioenergy research Centre enzyme platform
GH	Gycoside hydrolase
GRAS	Generally regarded as safe
HPLC	High performance liquid chromatography
HMF	Hydroxymethylfurfural
LAP	Laboratory analytical procedure
NERL	National energy research laboratory
*p*NP	*p*ara nitrophenyl
*p*NPC	*p*-nitrophenyl-*β*-D-cellobioside
*p*NPG	*p*-nitrophenyl-β-D-glucopyranoside
SDS-PAGE	Sodium dodecyl sulphate -polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
Xyn	Xylanase
2xMM	Double strength minimum media

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Chapter 1: General introduction and project aims

1. Introduction

In order to meet the world's energy demands, but knowing earth's limited resources, alternative fuels need to be generated as a supplement and replacement for fossil fuels. Bioethanol can be produced from a number of different raw materials, which can be grouped into three categories: simple sugars, starch and lignocellulose (Balat, 2011). Since the price and availability of raw materials (feedstock) are highly volatile, it affects the production costs of bioethanol and this in turn negatively effects the efforts to increase the production of alternative fuels. Renewable energy resources, such as lignocellulosic waste from agricultural and municipal sources, are economically viable feedstock options because of their abundance and low cost.

Although lignocellulose is an ideal carbon source, its recalcitrant nature presents a major obstacle for enzymatic hydrolysis (Saratale & Oh, 2012). The biological conversion of cellulose to glucose requires the synergistic action of three types of hydrolytic enzymes: (1) endoglucanases, which cleave internal β-1, 4-glucosidic bonds; (2) exoglucanases, for example cellobiohydrolases, that cleave disaccharide cellobiose from the end of the cellulose polymer chains; and (3) β-1, 4-glucosidases, which hydrolyse the cellobiose and other short cello-oligosaccharides to fermentable sugars. These enzymes need to be present in optimal ratios to prevent a bottleneck effect, which can lead to feedback inhibition of the enzymes. Optimising enzyme cocktails will therefore contribute towards our understanding of lignocellulose degradation and synergy between enzymes.

Existing commercial enzyme cocktails are limited in their specific activity and their role in cellulose degradation is poorly understood (Banerjee *et al*., 2010). Commercial enzyme preparations are complex and include many proteins that may be non-essential; this adds to the costs and presents a disadvantage to understanding lignocellulose hydrolysis. In addition, most commercial enzyme preparations have been optimised for acid pre-treated stover from corn and other grasses (Banerjee *et al*., 2010). Different pre-treatment methods, such as steam, hot water, ionic liquids, acids and alkaline peroxide, affect different feedstocks

dissimilarly. Therefore, the enzyme ratios in commercial enzyme cocktails are not optimal for all types of biomass.

Recently, Banerjee and co-workers (2010) studied different pre-treatment conditions for several common feedstocks to optimise the core set of enzymes needed for hydrolysis. Substrate composition is an important factor that affects bioconversion processes and it is therefore hypothesised that tailor-made enzyme cocktails are required for each individual substrate to maximise hydrolysis and improve ethanol yields during fermentation processes. Similar studies are required to enable a more economical and effective use of enzyme cocktails for bioethanol production from specific feedstocks.

Pulp and paper mills generate millions of tons of paper sludge annually, which is an environmental threat and its disposal represents an economical and environmental problem (Demarche *et al*., 2012). Current disposal options (landfills and incineration) are neither sustainable nor environmentally friendly. Alternative ways to utilise paper sludge therefore need to be explored, e.g. using it as a feedstock for bioethanol production. In comparison to other lignocellulosic materials, paper sludge has already had most of the lignin removed during the industrial pulping process. This is advantageous as the paper sludge can be used in bioconversion processes without a pre-treatment step.

Paper sludge typically contains 25% to 75% (dry weight) carbohydrates, with the remaining components being lignin, clays and fillers (Lynd *et al*., 2001). Paper sludge has a reported composition (on a dry weight basis) of 34.1% cellulose, 29.3% ash, 20.4% Klason lignin, 7.9% xylan, 4.8% protein and 3.5% fat (Marques *et al*., 2008). The cellulose content differs slightly depending on the pulping process and an analysis of 15 different batches of paper sludge (taken from numerous paper mills) had an average carbohydrate content of 42% (Lynd *et al*., 2001).

The first step in the conversion of paper sludge to ethanol is saccharification of paper sludge cellulose to reducing sugars by means of cellulases (Dwiarti *et al*., 2012). However, the high enzyme cost is currently preventing the commercial production of bioethanol from lignocellulosic feedstocks. One of the proposed methods for addressing the issue is to use simultaneous cellulose hydrolysis and yeast

fermentation of the paper sludge. This is estimated to decrease the paper sludge insoluble fraction by 60% (Demarche *et al*., 2012) and release fermentable sugars for ethanol production, without end-product inhibition of the cellulases. Marques *et al*. (2008) is one of several research groups that demonstrated that recycled paper sludge can be used as a substrate for yeast fermentations; in their study they used an initial paper sludge loading of 3% or 7.5% (w/v), expressed in terms of total carbohydrate mass. Furthermore, there are many other lignocellulosic materials, for example agricultural waste (such as triticale straw, wheat bran, corn stover and sugarcane bagasse) that are also receiving interest, with regards to bioethanol production (Chandel & Singh, 2011).

Wheat (*Triticum aestivum* L.) is an agricultural feedstock of interest for the conversion of wheat starch to ethanol (Favaro *et al*., 2012). However, this substrate also contains cellulose that can be hydrolysed for the release of additional fermentable sugars. Since wheat bran contains high levels of starch (10% to 20%) (Liu *et al*., 2010) and some cellulose (around 10%), the concept of simultaneously hydrolysing these components was evaluated using a recombinant cellulase enzyme cocktail, as well as amylolytic *Saccharomyces cerevisiae* yeast strains. The yeast *S. cerevisiae* can easily ferment hexose sugars, e.g. glucose to ethanol and has a robust nature, thus a recombinant strain expressing starch hydrolysing enzymes (amylases) would act as a saccharifying agent in the fermentation of starch and benefit the overall process.

An enzyme cocktail targeted towards paper sludge hydrolysis would offer an alternative to paper sludge disposal, offer a low cost feedstock option for the production of ethanol and pave the way for the production of other value-added products. In addition to optimising paper sludge hydrolysis, a cost effective process for wheat bran hydrolysis and fermentation to produce ethanol was investigated. The enzymatic saccharification of wheat bran, using cellulases and amylases was determined in a simultaneous saccharification and fermentation (SSF) process for evaluating the conversion of both the cellulose and starch components in the wheat bran to ethanol.

2. Aims of the study

The first aim of this study was to develop a recombinant cellulase enzyme cocktail for effective lignocellulose hydrolysis when using paper sludge as the raw material. Secondly, this study aims to evaluate this enzyme cocktail on other cellulosic materials, namely Avicel, pre-treated triticale straw and wheat bran.

To achieve these goals, the following objectives were followed:

(i) The cloning and over-expression of the *Aspergillus niger* β-glucosidase gene (*bgl1*) of in *A. niger*.
(ii) Development of a recombinant cellulase cocktail tailored for paper sludge hydrolysis.
(iii) Evaluation of recombinant enzyme cocktails on triticale straw, Avicel and wheat bran.
(iv) Comparison of ethanol production by two amylolytic *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG,SFA] strains on wheat bran in the presence of the CbhI:CbhII:EgA:Bgl2 recombinant enzyme cocktail.

The experimental design focused on optimising a recombinant cellulase cocktail in which the ratios of enzymes were optimised in terms of enzyme activity. The recombinant cellulase cocktail was evaluated on different substrates, as well as in an SSF study using wheat bran as the carbohydrate source (to demonstrate its potential as an industrial feedstock for bioethanol production). Amylolytic yeast strains were used in the SSF process for the fermentation of sugars, and to assist in the hydrolysis of the starch component of wheat bran.

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Chapter 2: Literature review

1. Lignocellulose for bioethanol

The use of lignocellulosic biomass for the production of alternative fuels has received much attention in the last two decades. This abundant cellulosic material is considered to be the largest renewable energy resource and can be classified into the following groups: forest residues, crop residues, waste paper and municipal solid waste (Balat, 2011). It has been estimated that plants produce 1.3 x 10^{10} metric tons of lignocellulose per annum; this energetically corresponds to about two-thirds of the world's energy requirement (Kim & Yun, 2006).

Agricultural waste materials are the preferred feedstock for biofuels, because their use as a feedstock does not complete with their use as a food source. Furthermore, they don't require additional land, as is the case for energy crops. Agricultural waste is inexpensive and no separation of waste is required (as is the case with municipal waste). The most abundant agricultural lignocellulosic residues are corncobs, corn stover, straw (wheat, rice and barley), sorghum stalks, coconut husks, sugarcane bagasse, switch grass, pineapple and banana leaves (Demain *et al*., 2005). Large amounts of lignocellulosic waste are also generated by the timber and the pulp and paper industries (Saratale & Oh, 2012).

Organisms that can utilise biomass as a carbon source are found amongst the archaea, bacteria, fungi, protists, plants and animals (including symbiotic gastrointestinal microbes). These microorganisms produce various lignocellulolytic enzymes that act on the (hemi-)cellulose backbone, hemicellulose substituents or cellulose-shielding lignin (Sweeney & Xu, 2012). The principle role of biomass-converting enzymes is to degrade polymeric cellulose or hemicellulose into simple sugars, which can then be metabolised by the microorganisms. The most economical way to produce cellulosic ethanol is by using a single organism or microbial consortium that is able to degrade the biomass and ferment the resulting sugars; this approach is called consolidated bioprocessing (CBP). CBP would signify a major development or low-cost biomass processing due to the economic benefits of process integration and the elimination of the high cost of enzyme additions (Den Haan *et al*., 2013a).

1.1. Structure and components of lignocellulose

The composition of lignocellulosic materials differs from one plant species to another. However, each type of lignocellulosic biomass can be divided into three main components: cellulose (30-50%), hemicellulose (15-35%) and lignin (10-20%) (Limayem & Ricke, 2012); pectin, proteins, ash, salt and minerals are also present (van Dyk & Pletschke, 2012). Covalent and hydrogen bonds firmly link the cellulose and hemicelluloses to the lignin component, resulting in a structure (Figure 1) that is highly robust and resistant to degradation treatments (Limayem & Ricke, 2012). Environmental and genetic influences affect the structural and chemical composition of lignocellulosic material (Balat, 2011), contributing to its highly variable nature.

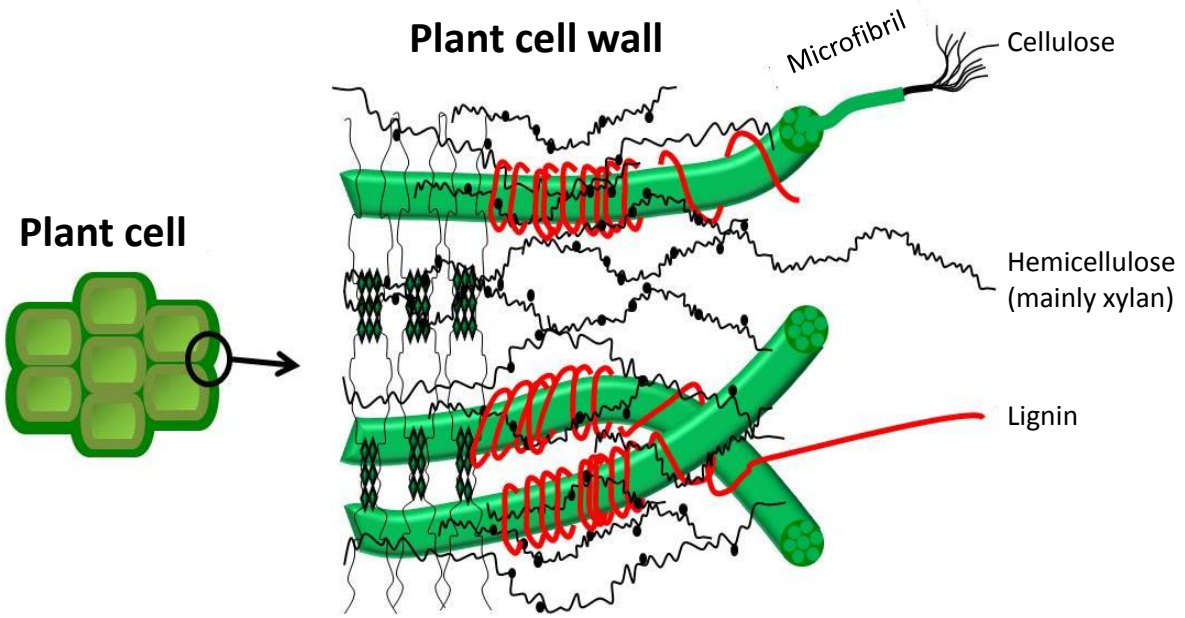

Figure 1: Structure of lignocellulosic plant biomass (Ratanakhanokchai *et al*., 2013).

Cellulose polymers are long chains that are packed together into microfibrils by hydrogen and Van der Waals bonds. Hemicellulose is a relatively amorphous branched polymer consisting of various sugars and along with lignin, encompasses these microfibils. Lignin is the most complex natural polymer (Verardi *et al*., 2012); it is composed of phenylpropane units that bind covalently (with cross-links) to hemicellulose. This results in cellulose being embedded tightly into the overall structure (Subhedar & Gogate, 2013). Since hemicellulose is more hydrophilic in comparison to cellulose, it can be hydrolysed more easily (Galbe & Zacchi, 2012).

The general structure of lignocellulosic material is the main factor that hinders its hydrolysis, because of physical and chemical barriers that are formed. As a result, the production of biofuel on an industrial scale cannot be achieved economically. The plant material requires an initial pre-treatment step to facilitate the bioconversion process. Pre-treatment is necessary for reducing the crystallinity of the cellulose, lowering the lignin and (hemi-)cellulose concentrations and increasing the exposed surface area for hydrolysis (Balat, 2011). This step is followed by enzymatic hydrolysis, during which the (hemi-)cellulose is converted into fermentable sugars (pentose and hexose).

1.2. Pre-treatment methods

In nature, there are several factors that assist in loosening up the structure of cellulose to make it more accessible to microbial cellulases (Seiboth *et al*., 1996). These include biotic and abiotic factors, the presence of other microorganisms and changes in ambient temperature and humidity. However, in biotechnological processes alternatives need to be found to replace these natural processes. Potential solutions would include optimising the type of biomass pre-treatment or improving the enzyme cocktail.

The three main types of pre-treatment methods, namely physical (mechanical, thermal), chemical or biological (enzymatic) can be employed to increase hydrolysis (Saratale & Oh, 2012). The type of pre-treatment affects the morphology and composition of the biomass, with the aim of removing lignin from the lignocellulosic material and making the substrate more susceptible to enzymatic hydrolysis (Figure 2). Even though there are high costs associated with the pre-treatment of biomass, the costs involved in the absence of pre-treatment are even greater (van Dyk & Pletschke, 2012).

The different types of chemical pre-treatment options include using alkali, acids, oxidising agents, solvents and gases. One of the most developed and commonly used types of chemical pre-treatments for lignocellulose is dilute acid hydrolysis (Balat, 2011). High reaction rates and improved cellulose hydrolysis can be achieved when using dilute sulphuric acid as a pre-treatment (Saratale & Oh, 2012). However, high costs are associated with this method, which are greater than the costs of some

physicochemical pre-treatment processes, such as steam explosion or ammonia fibre expansion (AFEX). Dilute alkali treatments are also advantageous, since they alter the lignin structure. Structural linkages are disrupted by swelling, which leads to an increased surface area, as well as a decrease in the degree of polymerisation and crystallisation of lignin (Saratale & Oh, 2012).

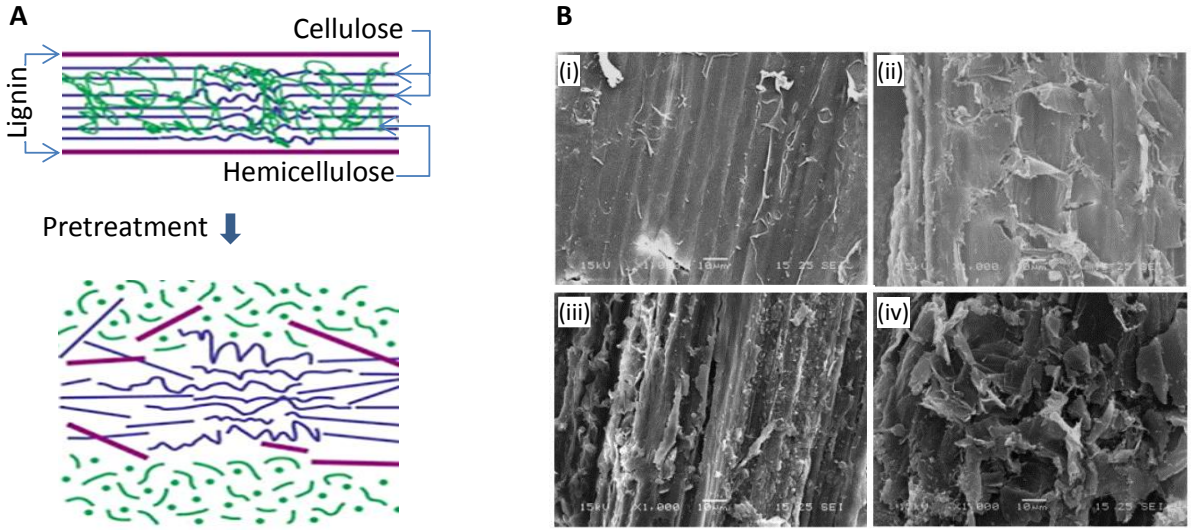

Figure 2: (**A**) Schematic representation of the structure of lignocellulose, before and after pre-treatment (Brodeur *et al*., 2011). (**B**) SEM of (i) untreated wheat straw (ii) untreated wheat straw after enzymatic hydrolysis at a cellulase loading of 25 FPU/g for 30 hours; (iii) wheat straw pre-treated at 121°C /15psi; (iv) wheat straw pre-treated at 121°C /15psi followed by enzymatic hydrolysis at a cellulase loading of 25 FPU/g for 30 hours (adapted from Han *et al*., 2012).

Biological pre-treatment is advantageous because of the low energy requirements, mild reaction conditions and environmental benefits. Biological pre-treatment is an alternative to chemical treatment and involves cellulolytic microorganisms, especially lignocellulolytic fungi. These microorganisms have a remarkable potential for uses in pre-treatment of cellulosic biomass, cellulase production and direct enzymatic hydrolysis (Fan *et al*., 2012).

White rot fungi are the preferred microorganisms for biomass pre-treatment because of their ability to degrade lignin (Fan *et al*., 2012). However, limitations to this process include a slow rate of hydrolysis and the use of the reducing sugars by the microorganism for growth, which results in the loss of carbohydrates needed for fermentations (Saratale & Oh 2012). Figure 3 shows a summary of the different pre-treatment technologies for lignocellulosic biomass and the methods that characterise them.

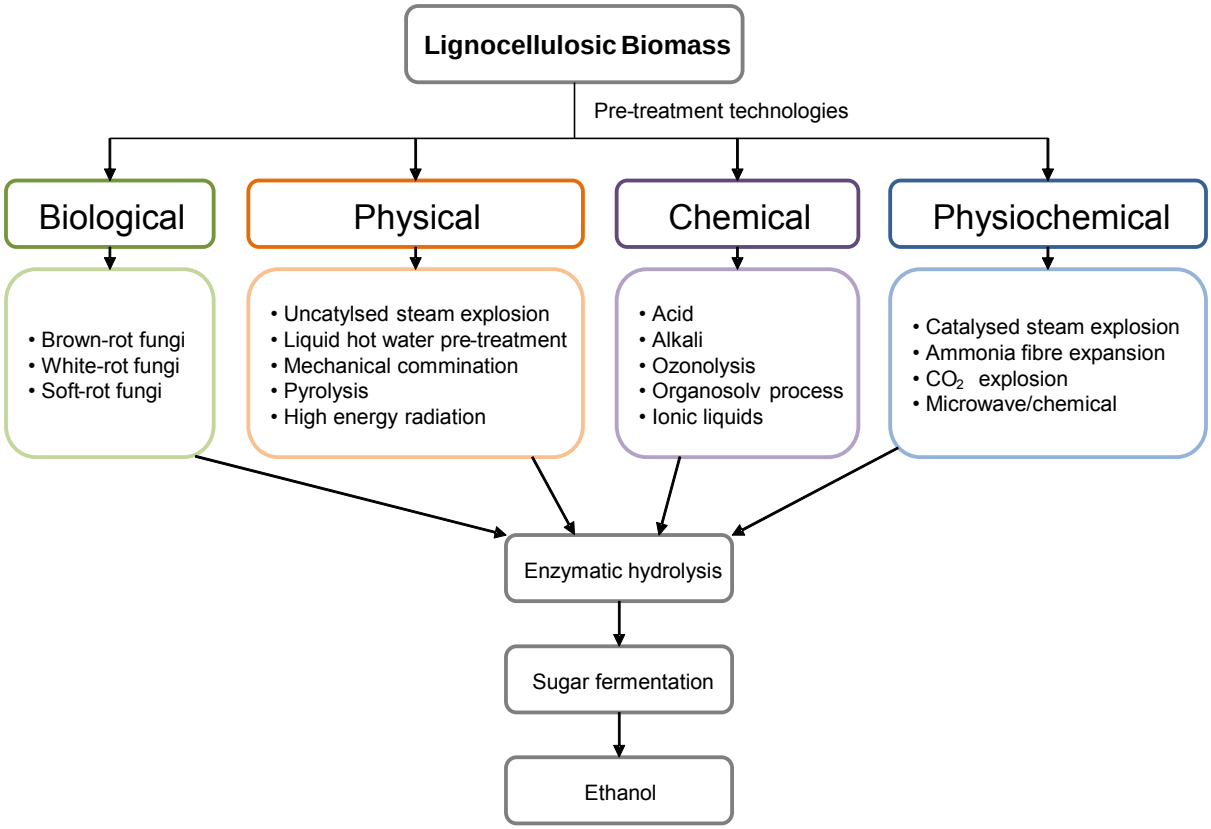

Figure 3: Pre-treatment technologies to produce bioethanol from lignocellulosic biomass (adapted from Subhedar & Gogate, 2013).

1.3. Microbial hydrolysis

Enzymatic lignocellulosic hydrolysis using microbial enzymes plays a fundamental role in carbon recycling and energy conversion. The use of microbial enzymes in the 1980s and 1990s caused the enzyme industry to prosper. Prior to this, most of the enzymes used had been derived from animal and plant sources; enzyme availability was low and prices were high (Demain & Vaishnav, 2009). Microbial enzymes provided economic advantages because cultivation of microbes was simpler and faster than that of plants and animals and their use allowed for the expansion of the enzyme industry. The initial drive behind the development of microbial enzyme technology was their use in biotechnological applications and the need for environmental sustainability (Demarche *et al*., 2012). Microbial enzymes have desirable properties that make them attractive biological agents for waste/pollutant treatment processes, as well as industrial applications.

Cellulolytic enzymes play a central role in the degradation of lignocellulosic biomass. The secretion of cellulases by fungi is superior to the amounts produced by bacteria, with *Trichoderma reesei* being one of the best studied cellulase secretors (Ahmed *et al*., 2009). Filamentous fungi secrete a variety of cellulolytic enzymes and can be isolated from the environment (through bioprospecting) where they inhabit ecological niches such as soil, living plants and lignocellulosic waste material. Their ability to secrete intra- and extracellular enzymes necessary for the degradation of various organic pollutants, helps these organisms to adapt their metabolism for different carbon and nitrogen sources (Saratale & Oh, 2012). These adaptations make fungi desirable for use in commercial applications. Furthermore, compared to plants and animals, microorganisms can be more easily manipulated through genetic engineering techniques to produce enzymes with improved properties and higher titres.

The majority of commercial cellulases are mesophilic enzymes produced by filamentous fungi such as *T. reesei* and *Aspergillus niger* (Jamil, 2009). Yet, thermostable enzymes (produced by thermophillic and extremophilic strains) are better suited to reactions that require high temperatures (Liu *et al*., 2012). Improvements in lignocellulosic processing can be achieved with continued research directed at enzymes that are able to tolerate acidic and high temperature conditions (Menon & Rao, 2012). This would allow for the incorporation of microbial hydrolysis under conditions that are typically associated with industrial applications, as well as the production of biofuels.

2. Cellulose

Cellulose is a biosynthetically produced linear polymer, consisting of D-anhydroglucopyranose molecules joined by β-1, 4-glycosidic bonds (Figure 4). It differs from starch, another type of polymer consisting of α-1, 4- linked glucose units, in that the anhydroglucose molecules are rotated 180° with respect to the adjacent molecules. This rotation causes a parallel orientation, which enables the chains to form a highly ordered crystalline structure (Zhang & Lynd, 2004).

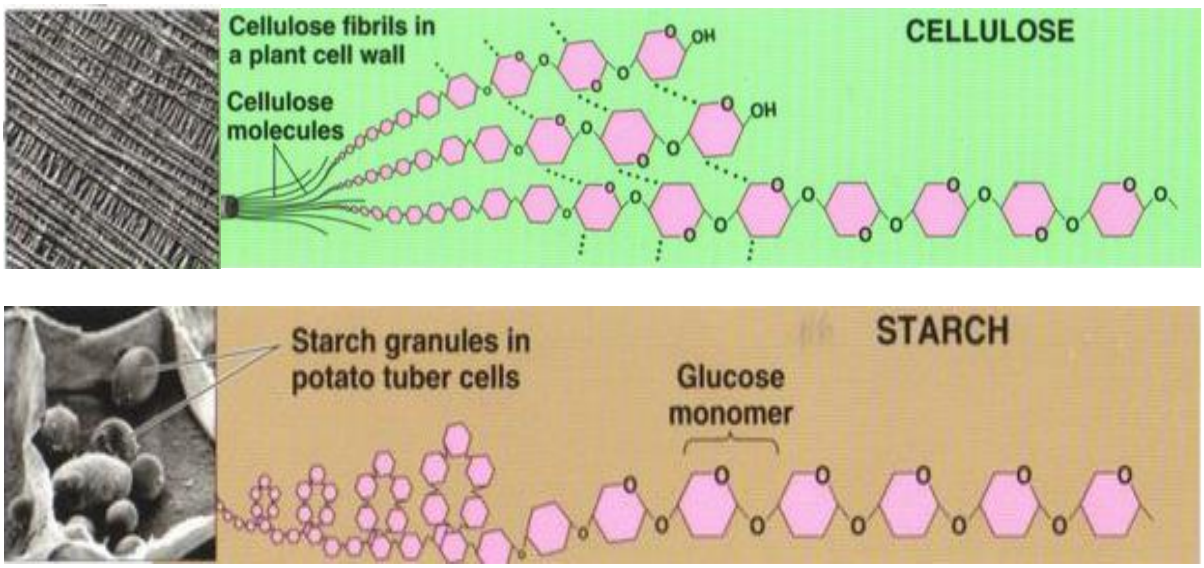

Figure 4: The structures of cellulose and starch. Cellulose and starch contain cellobiose and glucose, respectively, as the repeating unit (adapted from http://industrialsfloor.com/wp-content/uploads/2011/09/Cellulose-Molecular-Structure07.jpg).

Cellulose is the main component of plant biomass; it makes up 30-60% of feedstock dry matter and forms the framework of the cell (Balat, 2011). It is chemically homogeneous and available in highly pure forms. Natural cellulose molecules occur in elementary fibrils that are embedded in a matrix consisting of hemicellulose, pectin and lignin (Zhang & Lynd, 2004). Cellulose is insoluble in water and common solvents due to the strong intra- and interchain hydrogen bonding. It is also resistant to enzymatic hydrolysis (Galbe & Zacchi, 2012).

In cell walls of higher plants, the microfibrils form parallel lines, which characterise the crystalline regions, within which cellulose molecules are tightly packed. Cellulose also contains amorphous regions, in which the molecules are less compact and easier to degrade (van Dyk & Pletschke, 2012). The staggering of both the amorphous and crystalline regions gives strength to the cellulose structure. The global flow of carbon is greatly dependant on the cellulose production by photosynthetic higher plants and algae. However, cellulose production by non-photosynthetic organisms (certain bacteria, marine invertebrates, fungi, slime molds and amoebae) has also been reported in literature (Zhang & Lynd, 2004).

2.1. Cellulases

Cellulases are generally secreted as free molecules in filamentous fungi, actinomycetes and aerobic bacteria. They are important enzymes for the hydrolysis of cellulose and therefore play an essential role in a number of different industries, e.g. alternative fuels (Zhang & Lynd, 2004), textile, detergent, pulp and paper, as well as improving digestibility of animal feeds (Sukumaran *et al*., 2005). Due to their wide range of applications, cellulolytic enzyme systems have been studied extensively in various organisms.

The main hydrolytic enzymes (Figure5) for the hydrolysis of cellulose are endo-β-1,4-glucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BGL) (Mathew *et al*., 2008). The synergistic action of all three types of cellulases are employed by soft-rot and white-rot fungi to degrade the components of woody material (Wood and Garcia-campayo, 1990). On the other hand, the mechanisms of brown-rot fungi differ because many of these species do not produce and secrete cellobiohydrolase enzymes (Schilling *et al*., 2012). Brown-rot fungi mainly metabolise cellulose and hemicellulose and their cellulases are mostly limited to endoglucanases.

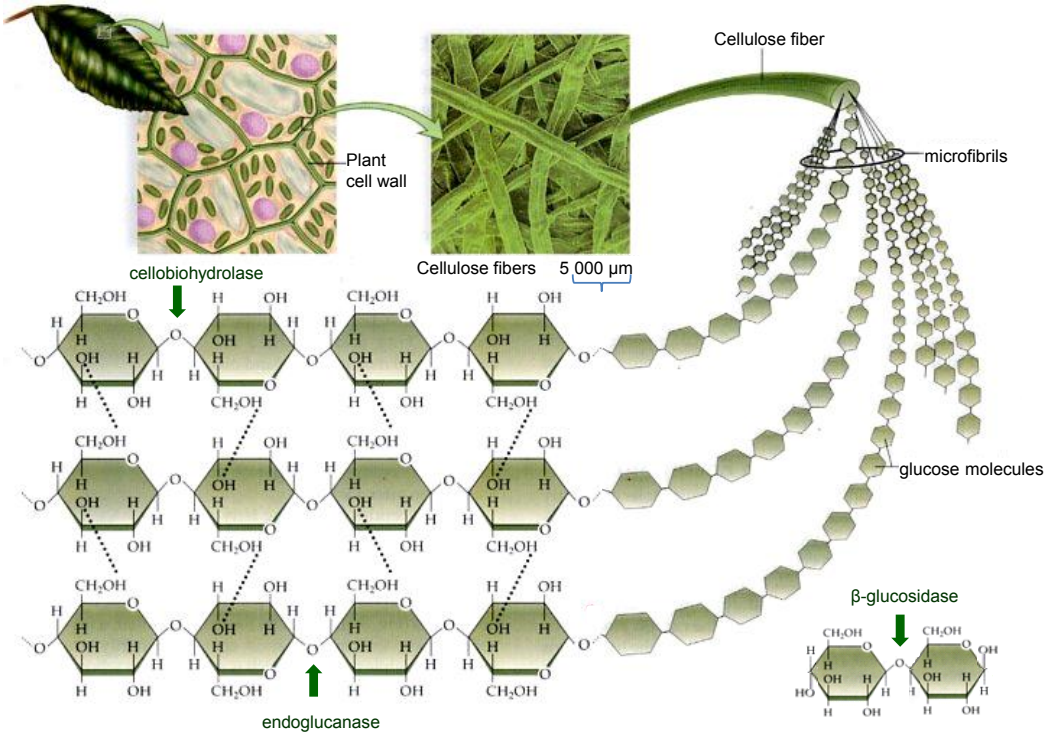

Figure 5: The structure of a cellulose molecule. The main hydrolytic enzymes involved in cellulose hydrolysis are included (by arrows) at their sites of action (adapted from http://industrialsfloor.com/doors-sliding-images/cellulose-molecular-structure-2.html).

Modularity is a key feature of lignocellulose-degrading enzymes, which allows them to be versatile in their actions. These enzymes have a catalytic core and many of them also have non-catalytic domains (Figure 6), which include carbohydrate-binding modules (CBMs), fibronectin 3-like modules, dockerins, immunoglobulin-like domains or functionally unknown "X" domains (Sweeney & Xu, 2012). CBMs direct the enzymes to the targeted carbohydrate substrate by promoting the association between enzyme and substrate (van den Brink & de Vries, 2011). These CBMs can also disrupt crystalline cellulose microfibrils to assist cellulase enzymes (Sweeney & Xu, 2012) and may also cause a disturbance in the substrate surface, which would allow the glucan chain to enter into the tunnel of the catalytic domain (Den Haan *et al*., 2013b).

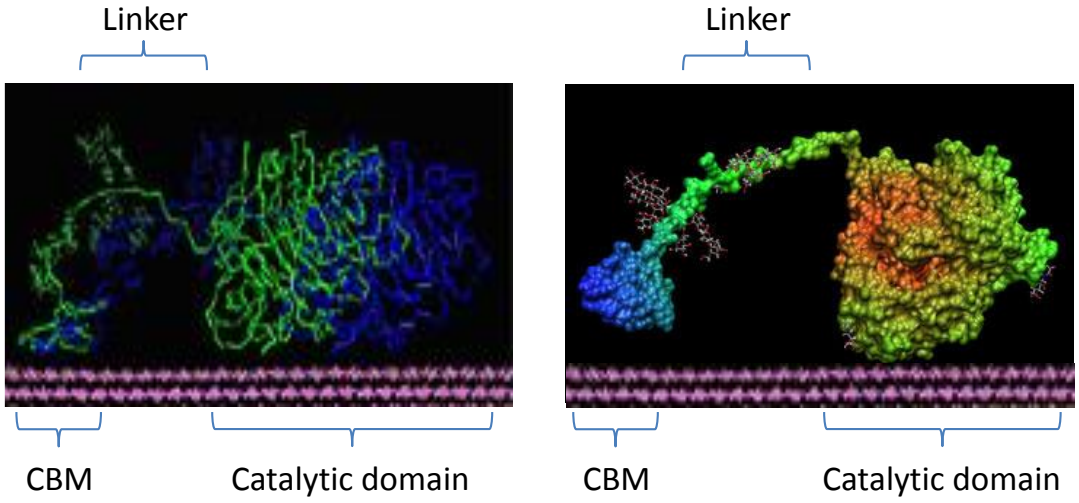

Figure 6: An artist's representation of the side view of a cellulase enzyme. Strands of cellulose are pulled up, ingested into the main "body" of the enzyme and digested into smaller pieces (http://www.nsf.gov/news/news_images.jsp?cntn_id=111097&org=NSB and http:wwwsdsc.edu/News%20Items/PR101305.htm).

Cellulases constitute a large percentage of the world's enzyme market, which is growing rapidly. Currently, researchers are investigating different aspects of cellulase enzyme technology, such as cellulase gene regulation and protein expression; development of recombinant strains expressing cellulases; physiological and biochemical studies; artificial cellulase complexes, as well the development of enzyme cocktails for efficient biomass hydrolysis (Mathew *et al*., 2008). Two of the main objectives for future cellulase production are to reduce the cost of cellulases, as well as to make these enzymes more effective for their role in hydrolysis (Sukumaran *et al*., 2005).

2.2. Classification

Public databases contain thousands of genes annotated as “cellulases” (Banerjee *et al*., 2010b) and more are added every year as genome sequencing improves with new technologies. Over the 12-year period from 1991 to 2003, the number of known glycosyl hydrolase gene sequences increased from 300 to more than10 000 (Zhang & Lynd, 2004). Cellulases form one of the largest groups of glycosyl hydrolases in terms of structural classification. This classification is based only on the variability of catalytic domains and does not consider variability in cellulose binding domains.

There are a number of other systems that have been used in the past to classify enzymes based on similarities in amino acid sequence (Seiboth *et al*., 1996) or catalytic activities. The International Union of Biochemistry and Molecular Biology (IUBMB) uses a numerical classification called the Enzyme Commission number (EC-numbers) system, which is based on the chemical reaction that the enzyme catalyses. The numbers correlate to enzyme activity and the system depends on biochemical characterisation of the proteins (e.g. hydrolases, lipases or esterses) to enable prediction and characterisation (Seiboth *et al*., 1996). For example, cellulases from *T. reesei* belong to the hydrolases and are grouped in EC 3, with cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21).

2.2.1. Cellobiohydrolase (CBH)

Degradation of crystalline cellulose is carried out primarily by CBHs (or exo-1, 4-beta-glucanases) (EC 3.2.1.91), making these enzymes indispensable to the industrial enzymatic hydrolysis of lignocellulose. Exemplary CBHs are found in Glycoside Hydrolase (GH) family 6, 7 and 48 (Sweeney & Xu, 2012) and are capable of degrading the crystalline parts of cellulose by cleaving off cellobiose molecules from the ends of the cellulose chains (Sørensen *et al*., 2011). There are two types of CBH enzymes that both produce cellobiose as main products: CBHI, which cleaves the cellulose chain at the reducing end, and CBHII, which cleaves at the non-reducing end (Boonvitthya *et al*., 2013). The “opposing” specificities of the two types of CBHs make them highly synergistic and cooperative in hydrolysing cellulosic substrates.

Amongst secreted proteins and enzymes produced by cellulolytic fungi, up to 70% (wt.) may be CBHs (Sweeney &Xu, 2012), which renders these organisms essentially CBH sources. CBHI and CBHII are the main components of the *T reesei* cellulase system, representing 60% and 20% of the total enzymes secreted on a mass basis (Lynd *et al*., 2002). It has also been shown that CBMs play an important role in ensuring the binding and processivity of these enzymes. However, both the CBHs are relatively slow at decreasing the degree of polymerisation of cellulose (Lynd *et al*., 2002).

2.2.2. Endo-β-1, 4-glucanase (EG)

Endoglucanases (EC 3.2.1.4) act on the solid cellulose substrate (Boonvitthya *et al*., 2013) and are thought to be the main enzymes responsible for decreasing the polymerisation of cellulose (Lynd *et al*., 2002). In contrast to CBH, EGs hydrolyse internal glycosidic bonds in the more amorphous regions of the cellulose in a random fashion. This results in a decreased degree of polymerisation, as well as the generation of new cellulose chain ends for CBH action (Sørensen *et al*., 2011). EG action prepares the substrate for cellobiohydrolases, making it easier for CBHI and CBHII to hydrolase the substrate.

There are two possible ways for EGs to prepare the substrate for CBHs and these lead to endo-exo synergism. Firstly, endo activity by EGs results in shortened cellulose chains being produced. Endoglucanase activity subsequently prevents CBHs getting trapped by cellulose chains that are physically blocked by cellulose microfibrils and lignin. Shorter cellulose chains increase the probability of CBHs hydrolysing a complete chain. Secondly, EGs also produce more “productive sites” for CBHs, thus the ratio between productive and non-productive bound CBH increases. These two mechanisms for endo-exo synergy can occur simultaneously (Karlsson *et al*., 1999).

Cellulolytic fungi can typically secrete EGs at around 20% (wt.) in their secretomes (Sweeney & Xu, 2012). Since there is a significant synergy between CBH and EG action, their co-presence and cooperation are important factors for highly effective industrial biomass-conversion involving enzymatic systems. In *T. reesei,* the expression levels of EGII (Cel5A) and CBHII (Cel6A) are particularly abundant and

these enzymes represent up to 10% and 20% (wt.), respectively, of the total secreted proteins. In addition, EGII has been shown to possess the highest catalytic efficiency amongst the *T. reesei* EGs (Boonvitthya *et al*., 2013).

2.2.3. β-glucosidase (BGL)

The β-glucosidases act in the liquid phase by hydrolysing mainly cellobiose to glucose, but they can also act on cellodextrins to a certain extent (Sørensen *et al*., 2011). BGL is considered the rate limiting enzyme in the cellulase complex, because it is inhibited by its own end product (glucose) and it drives hydrolysis to completion by eliminating cellobiose (which is a major inhibitor of CBH and EG enzymes). *T. reesei's* cellulase enzyme complex has been intensively investigated and although high levels of activity are displayed by some of the cellulases, these strains produce low quantities of BGL. Therefore, in order to achieve complete hydrolysis of cellulose, commercial preparations containing BGL are often used to supplement *T. reesei* cellulases, e.g. Novozyme 188 (which contains the BGL from *A. niger*) (Singhania *et al*., 2011).

The lack of sufficient BGL activity contributes to the bottleneck in the industrial conversion of lignocellulosic materials. The ideal BGL should be able to facilitate efficient hydrolysis at the appropriate operating conditions (Sørensen *et al*., 2013) and factors that need to be considered when evaluating BGLs include hydrolysis rate, inhibitors and stability. In order to have a profitable biomass conversion process, a high amount of glucose must be released. Therefore, BGL must not be inhibited by its end product, while at the same time maintaining high conversion rates in environments having high glucose concentrations.

2.2.4. Swollenin

Non-enzymatic proteins, such as swollenin (SWO1), also play a role in cellulose degradation. The swollenin protein (discovered in *T. reesei*) has sequence similarity to expansins (Chen *et al*., 2010), which are plant cell wall proteins that cause cell enlargement through loosening the structural components of the cell wall. Swollenin changes the structure of the cellulose by disrupting its rigid crystalline structures,

thus making it easier for enzymatic hydrolysis to occur (Saloheimo *et al*., 2002). An advantage of combining swollenin with classical cellulases is that it does not produce detectable amounts of reducing sugars and therefore will not contribute to feedback inhibition of the other enzymes.

3. Hemicellulose and hemicellulases

Hemicellulose is a group of complex polysaccharides consisting of glycol-units and glycosidic bonds. It has an amorphous structure made up of branches with short lateral chains, consisting of different sugars. In contrast to crystalline cellulose, which is strong and resistant to hydrolysis, hemicellulose can be more easily hydrolysed (Pérez *et al*., 2002). Common hemicelluloses include β-glucan, xylan, xyloglucan, arabinoxylan, mannan, galactomannan, arabinan, galactan, polygalacturonan. The enzymes that target these molecules include β-glucanase, xylanase, xyloglucanase, mannanase, arabinase, galactanase, polygalacturonase, glucuronidase, acetyl xylan esterase, as well as others. These enzyme are also produced by cellulolytic microbes for the effective degradation of lignocellulose (Sweeney & Xu, 2012).

Xylan is the main polysaccharide found in hemicellulose and a number of hydrolytic enzymes are required for complete hydrolysis of this molecule (Figure 7).The action of endo-1,4-β-xylanase results in oligosaccharides from the cleavage of xylan, whereas 1,4-β-xylosidase targets xylan oligosaccharides, producing xylose. Hydrolysis of the xylan backbone does not occur randomly, since the nature of the substrate (chain length and degree of branching) affects the accessibility of the bonds (Gírio *et al*., 2010). Xylanases play an important role in a number of different processes e.g. biopulping and bleaching; they are also attractive for waste/pollutant treatment processes (Demarche *et al*., 2012). These enzymes have been isolated from several ecological niches associated with plant material. A common xylanase producer is the white-rot fungus *Phanerochaete chrysosporium,* which has been shown to produce multiple endoxylanases (Pérez *et al*., 2002).

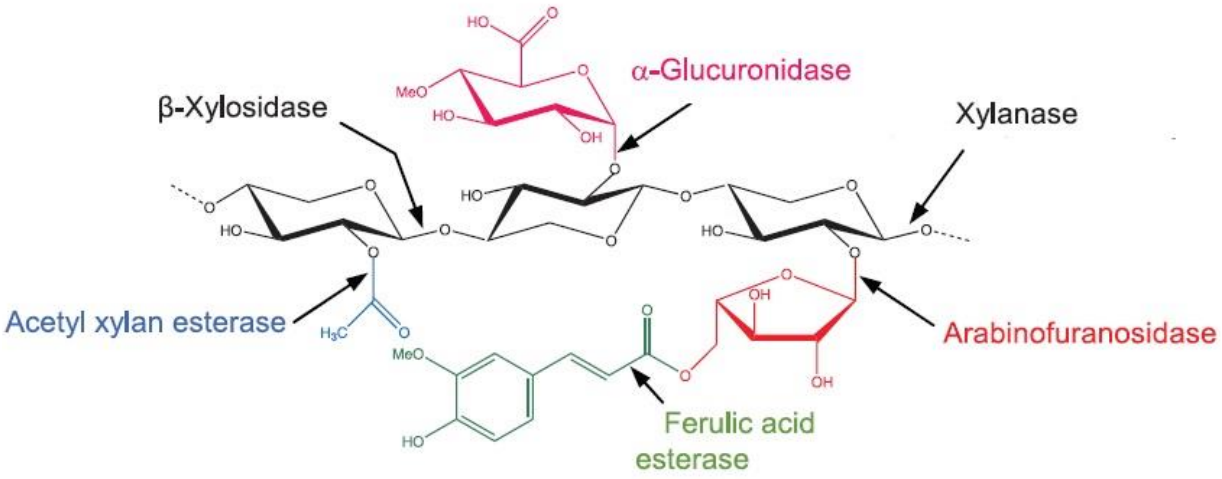

Figure 7: Schematic outline showing enzymes needed for hemicellulose hydrolysis (mainly xylan degradation) (DeBoy *et al*., 2008).

4. Enzyme production for cocktail development

Heterologous gene expression is used in many different industries for the production of a variety of important proteins. Some examples include pharmaceutical proteins of therapeutic interest e.g. interferon, interleukins; while others include commercial enzymes that are valued by several industries, e.g. food, textiles and laundry (Domínguez *et al*., 1998). Protein production by genetic recombination is the method of choice when it comes to obtaining pure proteins. Recombinant techniques, compared to methods of purifying proteins from natural sources, allow for more protein to be made with fewer contaminants (Ward & Swiatek, 2009).

Gene expression in heterologous systems, along with the subsequent advances in downstream processing technologies, allows for rapid and efficient protein/enzyme purification techniques. Initially, the commercial production of heterologous proteins was accomplished using *Escherichia coli* as host due to the vast understanding of its biochemical systems and the simplicity of genetic engineering in this host.

The advantages associated with the use of heterologous enzyme expression systems, compared to the cultivation of wild-type strains for enzyme production, include a shorter fermentation period, economical fermentations (inexpensive media) and the over-expression of proteins at high concentrations (Demarche *et al*., 2012). It is also beneficial to use a host that has “Generally Regarded as Safe” (GRAS) status, because products synthesised by these organisms are more easily accepted

by consumers, as opposed to products produced by other non-approved production hosts. Recombinant organisms are important hosts for producing proteins, several of which of have immense commercial value. Many studies are focusing on adapting expression systems to produce recombinant proteins efficiently and in a functional form (Macauley-Patrick *et al*., 2005).

4.1. Yeast expression systems

Microorganisms that are classified as 'yeast' provide an attractive option for the expression of recombinant proteins. Compared to *E. coli*, they are more advanced and are capable of secreting correctly folded and processed proteins (Verma *et al*., 1998). Some of the key advantages that yeasts have over other expression systems are that they are eukaryote microorganisms, they can grow on simple media and heterologous proteins can be secreted into the culture media

The common baker's yeast, *Saccharomyces cerevisiae,* has been used extensively for the expression of heterologous proteins and other valuable compounds (Ilmén *et al*., 2011). Engineered *S. cerevisiae* is the main host considered for bioethanol production from renewable resources due to its robust ethanol producing nature. *Kluyveromyces lactis* can use lactose and glucose as a carbon and energy source and is currently used for industrial scale production of valuable proteins (Colussi & Taron, 2005). It is an attractive host because of its ability to secrete high molecular weight proteins and its GRAS status (Domínguez *et al*., 1998). *Pichia pastoris* is a methylotrophic yeast that has also been developed into an efficient heterologous expression host (Cereghino & Cregg, 2000) and it has the ability to produce high titres of foreign proteins. Genetic manipulation techniques are similar to those for *S. cerevisiae* and protocols are already established for its genetic engineering.

It is important to choose the best suited experimental organisms for the type of heterologous expression required and to consider the advantages that the chosen expression system offers. When yeasts are considered, *S. cerevisiae* is the best known organism, but it does have a few drawbacks, including limited secretory capacities and hyperglycosylation. Non-conventional yeasts, on the other hand, offer alternative expression systems and may be chosen for better secretion efficiency.

4.2. *Aspergillus* as an expression host

The Aspergilli is an important group of filamentous ascomycetes fungi, first identified in 1729. The genus *Aspergillus* is found worldwide and consists of more than 180 officially recognised species (Ward *et al*., 2006). These species can be cultivated over a wide range of temperatures (10 to 50°C), pH (2 to 11), salinity (0 to 34%), water activity (0.6 to 1) and nutrient conditions (oligotrophic or nutrient-rich). In addition, these fungi have the ability to degrade a variety of different biopolymers, including starch, (hemi-)cellulose, pectin, xylan and proteins, which allows for cultivation on different plant materials, including renewable resources (Meyer *et al*., 2011) and industrial waste materials, such as bagasse (Rose & van Zyl, 2002). Advantages of using *Aspergillus* include the reduction in cultivating costs (compared to yeasts) and alternatives to using food resources as raw materials.

Aspergillus spp have been used as expression platforms for the production of several commodities, such as food ingredients, pharmaceuticals and enzymes (Ward *et al*., 2006). For more than a thousand years, Aspergilli have been used for food production and beverage processes (Flessner & Dersch, 2010). The use of *Aspergillus nidulans* as an expression host began in the early 1940s. Since then a number of other enzyme producing species have been for the focus of molecular studies, such as *A. niger*, *A. awamori* and *A. oryzae* (Ward *et al*., 2006).

Currently, several enzymes produced by *Aspergillus* spp are available commercially, including amylases, chymosin, glucose oxidases, catalases, cellulases, pectinases, lipases, proteases, phytases and xylanases (Flessner &Dersch, 2010). These enzymes benefit a number of different industries, which include the food, beverages, detergent, textile and the pulp and paper industry. Other advantages of *Aspergillus* as an expression host for biotechnological purposes, include existing industrial facilities, availability of easy biomass separation procedures, comprehensive knowledge about high-yield cultivations and efficient heterologous protein glycosylation (Fleissner &Dersch, 2010).

Compared to research conducted using *S. cerevisiae*, *Aspergillus* studies are less common. The discovery of native plasmids allowed for advances in the genetic manipulation of *S. cerevisiae* and *E. coli*. Unfortunately, the Aspergilli lack natural extra chromosomally replicating DNA elements (Lubertozzi & Keasling, 2009). The

lack of native plasmids and genetic tools has hampered the progress of using *Aspergillus* spp as an expression system. Yet, the remarkable secretion capacity of the Aspergilli outweighs that of other eukaryotic expression systems (such as yeast, algae or insect cells) and warrants more attention with regards to the heterologous expression of proteins. This has led to the recent increase in molecular knowledge of fungal genetics.

The advantages to using a fungal expression system (Su *et al*., 2012) include:

- produce large quantities of recombinant protein;
- secrete large proteins;
- produce extracellular proteins, which allows for simplified protein purification methods;
- contain glycosylation machinery;
- relatively inexpensive growth medium;
- approved by the FDA (Food and Drug Administration) United States as GRAS microorganisms;
- availability of transformation protocols and molecular biological tools for manipulating and engineering the chosen fungal host.

The main filamentous fungal hosts that are used for industrial production of biotechnology products and commercial enzymes are *Trichoderma* and *Aspergillus* spp. In addition, filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* are also suitable hosts for expressing heterologous genes (Su *et al*., 2012). The development of systems for heterologous expression in *Aspergillus* began soon after the first *Aspergillus* transformation (1983), for which vectors were constructed containing a selectable marker with fungal promoter and terminator sequences for gene expression (Lubertozzi &Keasling, 2009).

Although expression systems have been developed, controlled expression in *Aspergillus* carries more problems compared to expression using yeast and *E. coli*. The physiology of filamentous fungi is complex; a factor that has hindered the development and use of these microorganisms for the expression of recombinant proteins (Su *et al*., 2012). The thick cell wall that characterises filamentous fungi, as

well as their lack of ability to maintain (in a stable manner) a self-propagating plasmid, are obstacles in the development of effective transformation techniques, when compared to model organisms such as *E. coli* and *S. cerevisiae*. Episomal vectors are available, but they are seldom stable and tend to integrate after a number of generations. Therefore, *E. coli* systems are still extensively used in molecular biology.

Despite these drawbacks, *Aspergillus* species offer an important alternative host system for the expression of recombinant proteins. Aspergilli are robust fungi with industrial importance and can secrete large proteins in large quantities (Lubertozzi & Keasling, 2009). With these characteristics in mind, the objective of researchers is to eliminate the bottlenecks in heterologous protein expression in *Aspergillus*, as well as the optimisation of culture conditions, in order to optimise the levels of heterologous expression of proteins and metabolites.

5. Development of enzyme cocktails

The development of economically viable, alternative fuels currently requires the use of enzyme cocktails for the hydrolysis of lignocellulose into fermentable sugars. Enzymatic hydrolysis of lignocellulosic biomass is naturally a slow and complicated process, which involves many integrated events that facilitate the degradation of the heterogeneous substrate. Understanding the composition of lignocellulose (Figure 8) is required for cocktail development because several enzymes are required to work in synergy to degrade lignocellulosic substrates.

It is important to know and understand the advantages of different types of enzyme cocktails (Figure 9), such as customised cocktails of individual enzymes, commercial cocktails or a substrate-specific cocktail of enzymes (van Dyk & Pletschke, 2012). Enzyme cocktails need to be inexpensive and have a range of properties complementary to current cellulase systems (King *et al*., 2009). It is still debatable as to whether an enzyme cocktail should be adaptable to a wide range of cellulosic feedstocks (agricultural and forestry residues), or whether it should be tailor-made and optimised for specific feedstocks and or tailored for use in combination with a specific yeast strain.

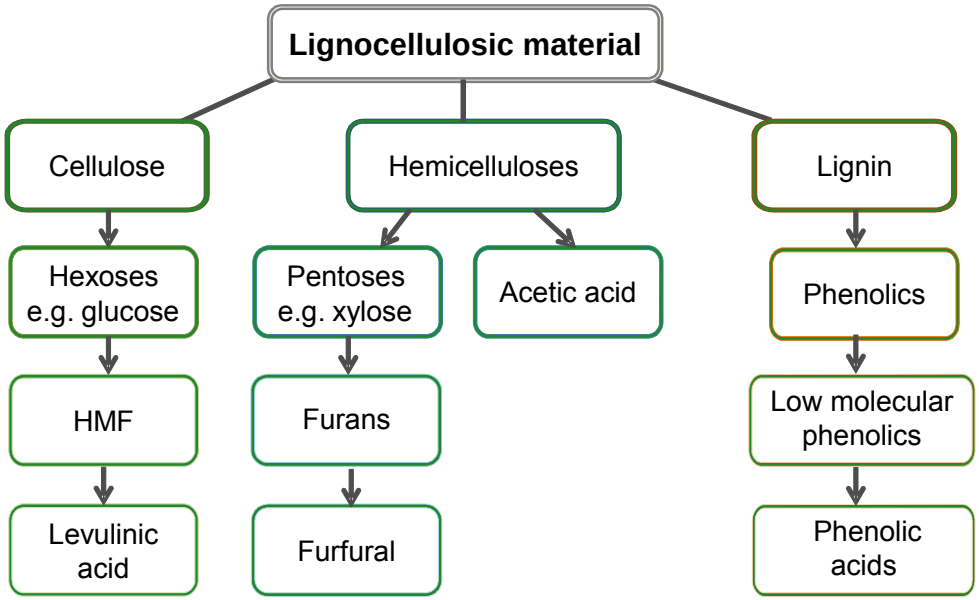

Figure 8: The main degradation products that occur after the hydrolysis of lignocellulosic biomass (adapted from Balat, 2011).

One of the main disadvantages to the use of individual enzymes is that pure enzymes are expensive as a result of the purification process. Subsequently, its commercial availability is also affected and these factors hinder studies examining the interactions between enzymes. On the other hand, the main shortcoming associated with the use of commercial enzyme cocktails is the lack of characterisation of the enzymes in the mixtures (van Dyk & Pletschke, 2012).

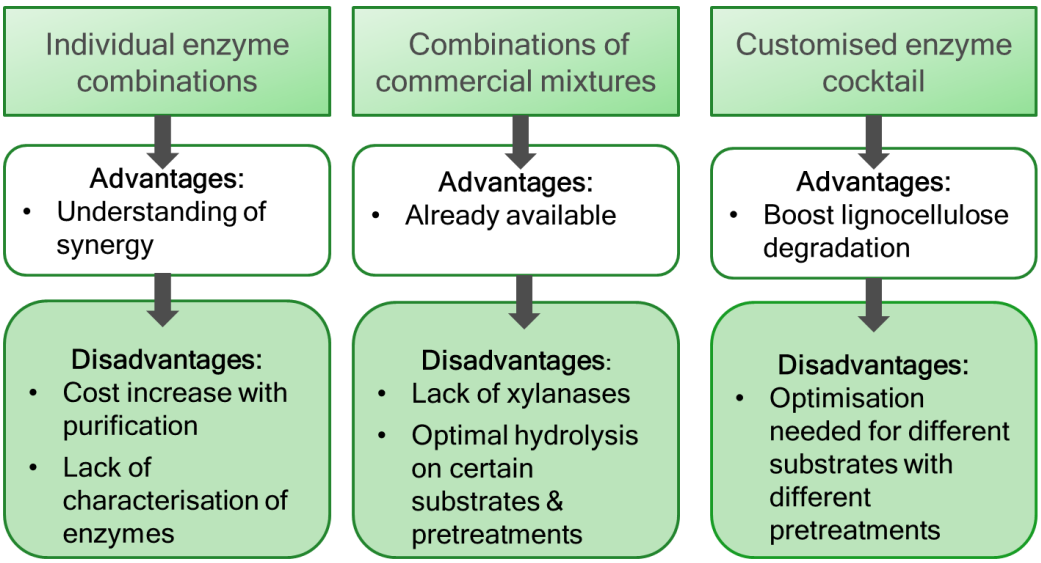

Figure 9: Bioconversion using enzyme synergy (adapted from van Dyk & Pletschke, 2012).

The biofuels industry (and other biotechnological applications) would benefit from more cost effective enzymes. Therefore, major enzyme producers (such as Genencor and Novozyme) aim to reduce the cost of enzymes by reducing production cost. A more effective enzyme cocktail would also generate a cost reduction, but requires an increased understanding of the interplay and synergistic interaction of different enzymes, as well as the precise role of the individual enzymes (Mathew *et al*., 2008).

The development of an ideal enzyme mixture (cocktail) should have the following desired outcomes (Banerjee *et al.,* 2010a):

- a large number of enzymes to achieve the release of major sugars e.g. glucose and xylose
- purified protein in large enough quantities, to allow comparative studies with the same batch of enzymes
- moderate to high throughput methods involving microtitre plates and liquid handling robots
- use of realistic lignocellulosic substrates during the enzyme optimisation stage

The use of customised enzyme cocktails containing individual enzymes, as well as crude commercial preparations, is well documented. Major enzyme companies, such as Novozyme and Genencor, have developed crude commercial enzyme cocktails with financial assistance from the US government (Banerjee *et al*., 2010b). However, a problem with these preparations is that the exact composition is unknown and as these cocktails may contain up to 80 proteins (e.g. Spezyme), they are less specific in their degradation abilities and are not optimised for specific biomass types.

Subsequently, Qing and Wyman (2011) reported the shortage of xylanase activity in commercial enzyme cocktails; this accessory enzyme may provide increased hydrolysis, depending on the specific substrate. Furthermore, it is difficult to achieve optimal enzyme ratios with commercial enzyme cocktails, since many non-essential enzymes are also present. Removing these could increase specific activity and lower the enzyme cost (van Dyk &Pletschke, 2012).

Several factors need to be considered when developing an optimal enzyme cocktail for a particular biomass. The pre-treatment method used is important as it affects the composition of the feedstock. Most enzyme cocktails have been optimised for acid pre-treated biomass, whereas there are fewer reports on cocktails tailored for alkaline pre-treated biomass (Gao *et al*., 2010). The next step in developing an enzyme cocktail is the optimisation of a "core" set of enzymes that can be used to find better replacement enzymes. This then forms the platform upon which accessory enzyme can be tested (Figure 10).

The core set is needed for significant release of free sugars from the lignocellulosic substrate. The core set developed by Banerjee *et al.* (2010a), for use on AFEX treated corn stover includes a cellobiohydrolase (CBH; GenBank CAA49596), an endoglucanase (EG; AAA34212), a β-glucosidase (BG; AAA18473), an endo-xylanase (EX; BAA89465) and a β-xylosidase (BX; CAA93248). Once the core set has been established, accessory enzymes can then be added individually in order to determine their contribution to the synergistic effects. Other strategies include multi-stage hydrolysis, new enzyme cocktails for biomass hydrolysis, as well as the addition of surfactants. These strategies have significantly enhanced the efficiency of enzyme hydrolysis of lignocellulosic materials (Maclellan, 2010).

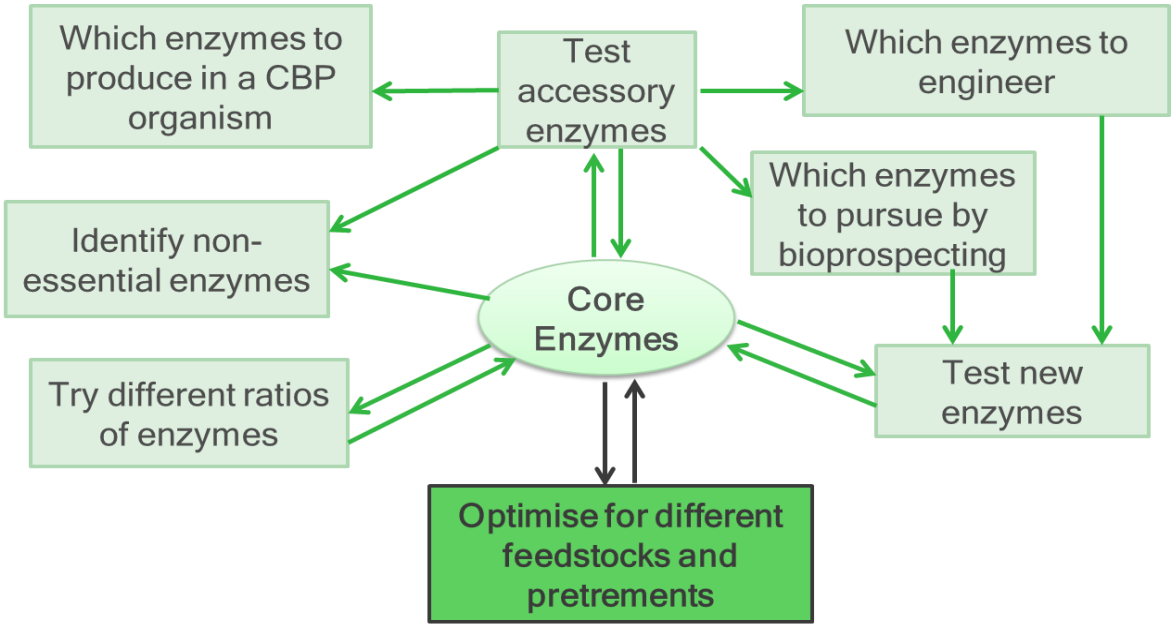

Figure10: Enzyme cocktail development: the importance of a core set of enzymes (adapted from Banerjee *et al*., 2010c).

BGL is commonly used to supplement enzyme cocktails for the efficient production of fermentable glucose (Chang *et al*., 2013). Many investigations have focused on the development of a highly efficient cellulase mixture for the effective hydrolysis of cellulosic materials. However, a recombinant fungal host that is able to secrete a cocktail of enzymes for the effective hydrolysis of cellulose has not yet been developed. In order to increase the level of bioethanol production for industrial use, the cost of BGL and other enzymes needs to decrease and their respective activities need to be improved.

5.1. Potential for engineering cellulosomes

Free cellulase systems usually contain individual enzymes that house a catalytic module, together with a cellulose-binding module (CBM); a cellulosomal system differs by having a scaffolding subunit containing a single CBM, together with numerous cohesin modules. The cohesion modules in turn binds strongly to a dockerin module borne by each cellulosomal enzyme (Bayer *et al*., 2007). Cellulosomes are associated with the cell surface and mediate cell attachment to the insoluble substrate, assisting in degradation upon which soluble products can then be absorbed. The presence of cellulosomes, however, does not mean that the organism will have cellulolytic activity. To date the majority of information shows that cellulosomes are mainly produced by anaerobic microbes (Fontes & Gilbert, 2010).

Bayer and colleagues (2007) highlight the potential for engineering cellulosomes as a means to increase enzyme synergy. These ‘designer cellulosomes’ have been proposed as a tool for understanding cellulosome action and recently substantial progress has been documented with regards to the production of “designer cellulosomes”. These are artificial enzymatic complexes that have been developed for the efficient degradation of crystalline cellulose. The aim of engineering artificial cellulosomes is to promote biomass waste solutions, as well as biofuel production (Vazana *et al*., 2012).

5.2. Great Lakes Bioenergy Research Centre Enzyme Platform

GENPLAT (Great Lakes Bioenergy Research Centre Enzyme Platform) is the latest technology that is being used for the development of enzyme cocktails, as well as the discovery and development of novel enzymes. This high-throughput analysis platform utilises individual purified enzymes, statistical experimental design, robotic pipetting of slurries and enzymes, as well as an automated colourimetric determination of the amount of sugars released (Banerjee *et al*., 2010a). The platform allows for the optimisation of cocktails containing pure enzymes for the release of glucose and xylose from different types of biomass.

Enzyme cocktails of high complexity are constructed using robotic pipetting into 96-well plates. This is followed by enzyme-linked colourimetric assays, which measure the released glucose and xylose. Since the design of the experiment can allow for many different enzyme combinations to be tested, it is easier to establish if an uncharacterised enzyme has an effect on hydrolysis. Banerjee and colleagues (2010a) have optimised an 11-component enzyme cocktail for corn stover pre-treated AFEX. This technique can be adapted to cocktails of 20 components, whereby cocktails can be optimised with fewer than 200 individual reactions.

In addition to core cellulases, lignocellulose-degrading fungi are also known to produce accessory enzymes that act on the linkages that are less abundant in plant material, e.g. arabinanases, galactanases, lyases, pectinases and several types of esterases. In the future, customised enzyme cocktails will probably contain different assessor enzymes, as well as non-enzymatic proteins (e.g. swollenin).

5.3. Enzyme ratios and synergy

The main focus in the development of synthetic enzyme cocktails is to learn which of the hundreds of known enzymes are important and in what ratios. The motivation behind evaluating specific ratios is to optimise substrate degradation and subsequently lower enzyme loading, resulting in a reduction in cost (Gottschalk *et al*., 2010). Enzyme ratios for the hydrolysis of different substrates are determined through synergy studies and the degree of synergy between different enzymes is defined as “the ratio between the activity of the mixture and the sum of

the individual activities on the same substrate" (Andersen *et al*., 2008). It can be reported as a percentage enhancement of activity (Gottschalk *et al*., 2010).

Synergy occurs when the observed action of two or more enzymes (acting together) is greater than the sum of their individual action (Wood & Garcia-Campayo, 1990). Two types of synergistic action are involved in cellulose hydrolysis: co-operation between endoglucanase and cellobiohydrolase (endo-exo synergism) and co-operation between two cellobiohydrolases (exo-exo synergism) (Wood & Garcia-Campayo, 1990). Several factors that affect the synergistic actions amongst cellulases include the properties of the substrate, the affinity of a particular component for that substrate and the concentration of the components in a cellulase mixture. Certain phenomena are not yet fully understood and further research is needed to understand the synergism in cellulase systems, for example the existence of stereospecific cellulases (Woodward, 1991).

Measuring the degree of synergy will assist in determining whether one enzyme is contributing to the ability of another enzyme to act on a specific substrate (van Dyk & Pletschke, 2012). Subsequently, the results can provide further information regarding the structure of a substrate, the mechanism of action of an enzyme, as well as information on the mechanisms of substrate hydrolysis. In theory, the optimal enzyme ratio needed to degrade the chosen substrate can be calculated. Advantages to knowing the synergy amongst enzymes, as well as the specific ratios of individual enzymes, will assist in the optimisation of enzyme cocktails. Only once an enzyme cocktail has been optimised, can the other factors (substrate loadings, enzyme loadings, inhibitors, adsorption and surfactants) be addressed.

5.4. Biocatalysts: strategies to improve enzymes

The world enzyme market is expanding at a rapid rate and is worth billions of dollars. The majority of industrial enzymes (75%) are hydrolases, followed by carbohydrolases (Mathew *et al*., 2008).The favourable properties of enzymes as biocatalysts make them desirable for many different industrial applications but the high prices associated with enzymes is a major limitation. Since enzymes are sold on a protein mass basis, strategies aim to increase the specific activity of the enzymes (Sørensen *et al*., 2013). This can be done by either manipulating different

host organisms or by improving enzyme characteristics. Recombinant techniques have been used for several decades for the production of enzymes and in 1993, recombinant processes contributed to more than 50% of the industrial enzyme market (Demain & Vaishnav, 2009).

Recent improvements in genetic engineering, recombinant DNA technologies and protein chemistry are providing researchers with powerful tools for understanding enzyme structure and function (Sanchez & Demain, 2011). In the future, enzymes will be designed to match specific requirements through the rational modification of enzymes that will introduce novel properties. Better suited enzyme will be developed through techniques such as protein engineering, gene shuffling and directed evolution (Sanchez & Demain, 2011). Ultimately, the production and commercialisation of newly synthesised biocatalysts will allow for a fundamental breakthrough and an expansion of the current enzyme market.

6. This study

The conversion of waste material (industrial and agricultural) to biofuels is a topic that has been widely investigated over the last few years. Cellulosic waste can be converted into sugars that can be fermented to ethanol using microorganisms (Sanchez & Demain, 2011). Currently, the majority of bioethanol is derived from sugar cane or starchy crops. However, the development of processes that utilise renewable lignocellulose materials (that do not compete with food sources) is required.

The most economical way to produce cellulosic ethanol is by using a single organism that is able to degrade the biomass, while fermenting the resulting sugars at the same time; this approach is called consolidated bioprocessing (CBP). The yeast *S. cerevisiae* is able to produce ethanol at high titres, but is unable to degrade lignocellulose. The construction of a strain engineered for lignocellulose hydrolysis would signify a major development for low-cost biomass processing due to the economic benefits of process integration and the elimination of the high cost of enzyme additions (Den Haan *et al*., 2013b). However, until the ideal strain is constructed, enzyme cocktails will need to be used for the hydrolysis of

lignocellulose. The optimisation of these cocktails for specific substrates or to supplement enzyme production by CBP yeast, will assist in reducing the overall bioconversion costs.

This study was motivated by the need to lower the cost of enzyme addition in order to economically use lignocellulosic biomass (in particular, paper sludge and wheat bran) as a feedstock for bioethanol production. Paper sludge contains high levels of cellulose and would require different cellulases for effective hydrolysis. Wheat bran on the other hand, contains some cellulose but a greater starch content and therefore both cellulases and amylases are required for hydrolysis.

The bioconversion of paper sludge will be advantageous in turning a waste disposal problem into an energy opportunity (Figure 11) and will not compete with food resources (Dwiarti *et al*., 2012). Around three million tons (dry weight) of paper sludge is produced per year (Fan & Lynd, 2007), therefore the development of technology for the enzymatic hydrolysis of paper sludge could become important in the pursuit of second-generation ethanol production in South Africa.

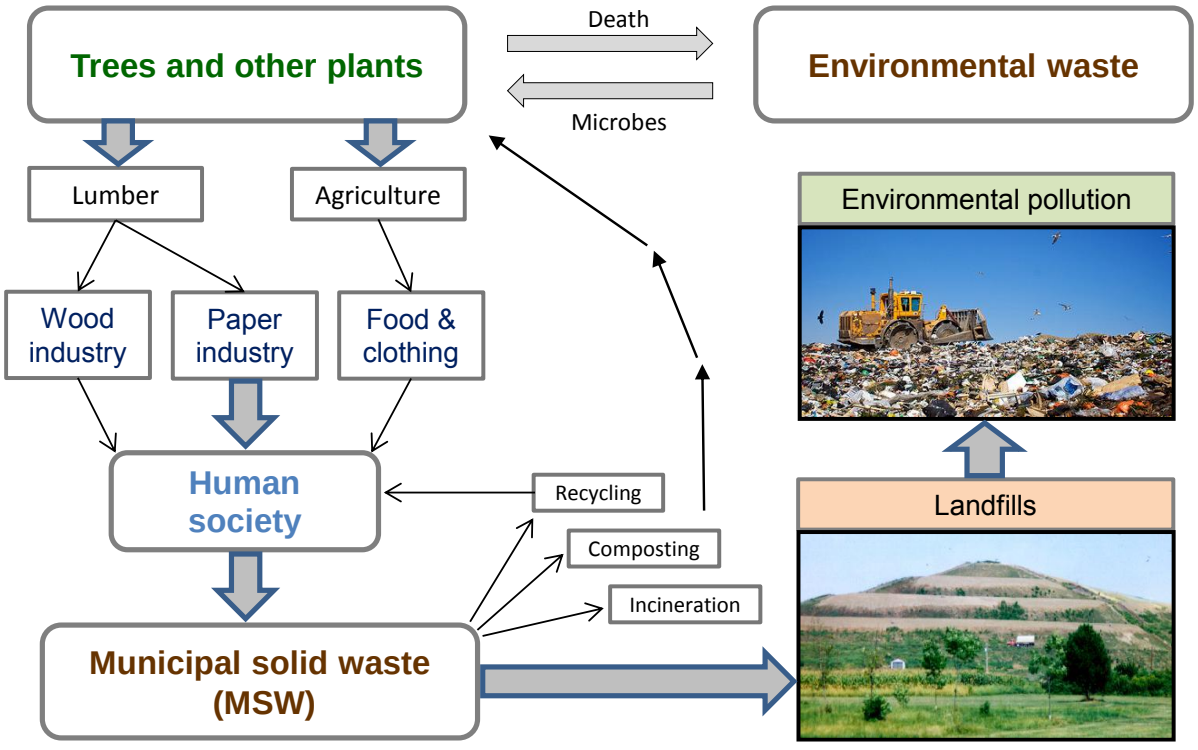

Figure 11: Fate of cellulose in the environment (adapted from Bayer *et al*., 2007).

Wheat bran represents an agricultural waste material that is produced by the milling of wheat. Wheat is a cereal that is used in many parts of the world and consists mainly of starch, arabinoxylans, cellulose, β-glucan, protein and lignin (Manikandan & Viruthagiri, 2009). Wheat bran has a lower cellulose content (around 10%) than paper sludge, but is also a promising feedstock for the production of second-generation biofuels due to its low cost and abundance. About 6.87 billion tons of wheat was produced worldwide in 2009 equating to about 1.7 billion tons of residual wheat bran that is potentially available for the production of ethanol (Favaro *et al*., 2013).

Enzyme-based technologies for biomass conversions are considered to be the most efficient way of processing biomass. However, current enzyme cocktails are not yet sufficient (in terms of specificity activities) to promote this process to an industrial scale for the economical production of biofuel; enzyme cost is also a major obstacle for lignocellulosic conversion. It has been predicted that with the increased use in lignocellulose (biomass conversion to ethanol), the demand for commercial cellulases will significantly increase, which will put pressure on the current cellulase-producing companies (Mathew *et al*., 2008). At the same time, developing efficient cellulase cocktails that can be used by local industries on specific feedstocks will curb possible monopolies by major enzyme companies, thus reducing the risk of unnecessary inflation of enzyme cost.

An increased understanding of microbial physiology and genetics of cellulase producers is also required to ensure that the demand does not exceed the ability to supply cellulases to industries. Furthermore, advances in producing hydrolytic enzymes on a large scale will contribute to reducing the cost of biofuels and will assist in promoting its acceptability. Since available feedstocks for use in biofuel production have diverse compositions, different pre-treatment methods are required and subsequently altered ratios of enzymes from a core set are needed. Once hydrolysis conditions have been optimised for a specific substrate, fermentation of the resulting sugars can be performed.

There is a lack of information on the hydrolysis of cellulosic substrates using recombinant enzymes. In this study, several recombinant enzymes were tested and used to develop a 4-enzyme cocktail tailored to paper sludge and evaluated on

different cellulosic substrates (Avicel, triticale straw and wheat bran). The Baker's yeast, *S. cerevisiae,* has the potential to be used in a CBP environment and it can be engineered to produce hydrolytic enzymes that can assist in hydrolysis of the feedstock; CBP also has the potential to provide the most cost effective method for cellulosic ethanol. In this study, two amylolytic *S. cerevisiae* strains were used in combination with a developed recombinant enzyme cocktail (containing Bgl2) to hydrolyse wheat bran for the production of ethanol.

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Chapter 3

The development of a recombinant fungal enzyme cocktail for the hydrolysis of paper sludge and evaluation on cellulosic substrates

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Manuscript in preparation for publication in *Journal of Industrial Microbiology and Biotechnology*

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Abstract

The pulp and paper industry generates significant quantities of non-hazardous paper sludge as a waste material. The low lignin content makes paper sludge an ideal feedstock for biological conversion to bioethanol. However, current enzyme cocktails have not been optimised to promote paper sludge conversion on an industrial scale. Customised cellulase enzyme cocktails (containing a cellobiohydrolase I, cellobiohydrolase II, endoglucanase I and a β-glucosidase) were developed for the hydrolysis of paper sludge and evaluated on Avicel and triticale straw. Individual enzymes were expressed by recombinant *Aspergillus niger* and *Saccharomyces cerevisiae* strains were used for hydrolysis trials with 2% substrate loading (w/v dry weight). Glucose concentrations were measured after 24 and 48 hours and the hydrolysis efficiency (degree of saccharification) calculated. The activities (in FPU) for the final cocktails were 0.7 and 0.45 for the CbhI:CbhII:EgA:Bgl1 and CbhI:CbhII:EgA:Bgl2 cocktails, respectively. The optimum enzyme ratio (based on protein concentrations) for the CbhI:CbhII:EgA:Bgl1 cocktail was 7.4:6.6:1:208 and 7.4:6.6:1:41 for the CbhI:CbhII:EgA:Bgl2 cocktail. Overall, hydrolysis with the Bgl2 cocktail allowed for longer incubation times and an improved degree of saccharification when the enzyme concentration was doubled. Comparison of paper sludge hydrolysis results with those from Avicel hydrolysis highlight the need to tailor enzyme cocktails based on natural substrates. To our knowledge, this is the first report on the use of individual enzymes from recombinant strains for the hydrolysis of paper sludge and triticale straw. Results show the importance of optimising enzyme cocktails and hydrolysis conditions, based on specific feedstocks.

Keywords: paper sludge • enzyme hydrolysis • enzyme cocktail • degree of saccharification• biofuels

1. Introduction

Biofuels offer many advantages in terms of economic, environmental and energy security compared to petroleum-based fuels (Balat, 2011). Agricultural lignocellulosic waste materials, e.g. corn stover, wheat straw, switch grass, rice straw and sugarcane bagasse can be used as feedstocks for biofuel production. Although progress has been made in optimising the hydrolysis of corn stover (Banerjee *et al.*, 2010b), rice straw (Singh & Bishnoi, 2012) and wheat straw (Banerjee *et al*., 2010c), enzyme cocktails for these substrates have not yet been completely optimised.

The pulp and paper industry generates millions of tons of paper sludge per year (Kang *et al*., 2010). This represents a large expense to the paper mills because it needs to be transported and disposed of, primarily in landfills or by incineration (Fan & Lynd, 2007). This is not only a financial burden to the industry, but also a source of various environmental problems. Although it is projected that a majority of ethanol is to be produced from natural lignocellulosic feedstock, paper sludge is a promising feedstock that needs to be considered for bioconversion processes (Zhu *et al.*, 2011). Furthermore, paper sludge can be used for bioconversion to ethanol without additional pre-treatment (Fan *et al*., 2003; Marques *et al*., 2008).

The cost of hydrolytic enzymes is a major challenge for the economically viable production of cellulosic ethanol (Banerjee *et al*., 2010b; Zheng *et al*., 2012). Although paper sludge can be converted to fermentable sugars (which can then be fermented to bioethanol), current enzyme cocktails are not efficient, hampering its industrial application. Current commercial cocktails are complex, only partially defined and not optimised for effective hydrolysis of a specific substrate (Banerjee *et al*., 2010b). Furthermore, some cocktails may include more than 80 proteins (Banerjee *et al*., 2010b), making it difficult to understand which enzymes (and in what ratios) are important for lignocellulosic hydrolysis. The substrate and pre-treatment conditions affect enzymatic hydrolysis, which necessitates the optimisation of enzyme cocktails for different pre-treatment/biomass combinations (Banerjee *et al*., 2010b).

Numerous organisms are capable of producing extracellular depolymerising enzymes, with fungi comparing better in terms of the variety of enzymes they secrete, as well as their enzyme yields (Bansal *et al*., 2011). The most commonly

used industrial cellulase producers are *Trichoderma* and *Aspergillus* species (Seidl & Seiboth, 2010; van Dyk & Pletschke, 2012). Fungi are generally not suitable for the fermentation industry because they have a low growth rate, require specific growth medium and do not produce ethanol (Chang *et al*., 2013). However, by engineering a yeast host to produce cellulases, the cellulolytic enzyme systems of fungi can be used for lignocellulosic hydrolysis. Yeasts are able to ferment various sugars for the production of ethanol and can subsequently be engineered as hosts for the cellulosic ethanol industry.

The hydrolysis of cellulose requires the synergistic action of exoglucanases (cellobiohydrolases), endoglucanases and β-glucosidases; these enzymes are involved in the release of glucose as the end product. The commercial enzyme preparations Novozyme 188 and Celluclast 1.5 are currently used in combination for the hydrolysis of cellulosic biomass on an industrial scale. Novozyme 188 (containing a β-glucosidase derived from *A. niger*) is widely used to supplement enzyme cocktails, such as Celluclast 1.5 (a commercial cellulase cocktail derived from *T. reesei*). Recently, a commercial enzyme preparation has been released that contains all three types of cellulases e.g., Cellic CTec (Sørensen *et al*., 2011).

The development of technology for the enzymatic hydrolysis of paper sludge is an important step in understanding the enzyme dynamics involved in hydrolysis. The aim of this study was to develop a cellulase enzyme cocktail, using enzymes produced by different recombinant fungal strains. The β-glucosidase (*bgl1*) of *Aspergillus niger* was over-expressed in *A. niger* D15 and the Bgl1 enzyme evaluated and included in one of the final cocktails. The additional cellulases were obtained from existing *A. niger* and *S. cerevisiae* strains that were previously constructed in this laboratory.

The first step in this developmental process was to define an optimal ratio of core enzymes. To do this, enzymes (cellobiohydrolases, endoglucanases and β-glucosidases) were added in a stepwise manner and the resulting hydrolysis efficiency (degree of saccharification) of paper sludge evaluated by measuring the release of glucose. The optimal enzyme ratio was then used to test different enzyme loadings, for the hydrolysis of different substrates, namely Avicel and triticale straw.

Avicel has traditionally been used as a model substrate for evaluating the performance of microbial cellulases systems because it is relatively well defined (Shao *et al*., 2011) and was therefore included in the study for comparative purposes. Avicel is free of inhibitors and less viscous than paper sludge (Zhang & Lynd, 2007). Triticale straw was also used for evaluating the enzyme cocktail because it represents an attractive agricultural lignocellulosic feedstock for bioethanol production. Triticale straw can be grown easily and the plant can tolerate dry and acidic soil conditions (Marković *et al*., 2011). Unlike Avicel, it is a natural substrate and was included in the evaluation of hydrolysis with regards to different types of cellulosic substrates.

The difference in the chemical composition and the percentage of crystalline cellulose in lignocellulosic materials can influence the susceptibility of the materials to hydrolysis. Therefore, the degree of saccharification was compared for the hydrolysis of the selected cellulosic materials; this was performed to indicate substrate specific factors among the recombinant enzymes. It also served to form a good platform for studies investigating enzyme synergy. Ultimately, the hydrolysis of paper sludge was the focus of enzymatic hydrolysis trials so that a cellulase cocktail could be developed and optimised for the release of fermentable sugars.

2. Materials and methods

2.1. Media and cultivation conditions

All plasmids were constructed and amplified in *Escherichia coli* DH5α (Takara Bio Inc.). The *E. coli* transformants were selected on Luria Bertani agar containing 100 $\mu g.ml^{-1}$ ampicillin and cultivated at 37°C in Terrific Broth (12 $g.l^{-1}$ tryptone, 24 $g.l^{-1}$ yeast extract, 4 $ml.l^{-1}$ glycerol, 0.1 M of phosphate buffer) containing 100 $\mu g.ml^{-1}$ ampicillin for selective pressure (Sambrook *et al*., 1989).

Minimal media containing 5 $g.l^{-1}$ yeast extract, 2 $g.l^{-1}$ casamino acids, 10 $g.l^{-1}$ glucose, 6 $g.l^{-1}$ $NaNO_3$, trace elements (Punt & van den Hondel 1992) and 0.01 M uridine (Sigma-Aldrich, Germany) was used to cultivate the *A. niger* D15 strain prior to transformation. All *A. niger* D15 transformants ($1x10^6$ $cells.ml^{-1}$) were cultivated at 30°C in 125 ml shake flasks (200 rpm) containing 25 ml double strength

minimal media (2xMM) and 100 $g.l^{-1}$ glucose to quantify enzyme activity (Rose & van Zyl, 2002, 2008).

The recombinant *S. cerevisiae* strains were aerobically cultivated on a rotary shaker (200 rpm) at 30°C in 125 ml Erlenmeyer flasks containing 25 ml synthetic complete (SC^{-URA}) medium (6.7 $g.l^{-1}$ yeast nitrogen base without amino acids (Difco laboratories), 20 $g.l^{-1}$ glucose and supplemented with appropriate amino acids). Yeast strains were maintained on agar plates with the same composition. Similarly, auto selective *S. cerevisiae* strains were cultured in YPD medium (10 $g.l^{-1}$ yeast extract, 20 $g.l^{-1}$peptone and 20 $g.l^{-1}$ glucose). Unless stated otherwise, all cultures were inoculated to a concentration of 1 x 10^6 $cells.ml^{-1}$.

2.2. Strains and plasmids

The genotypes of the bacterial and fungal strains, as well as the plasmids used in this study, are summarised in Table 1.

2.2.1. DNA manipulations

Standard protocols were followed for DNA manipulations and *E. coli* transformations (Sambrook *et al*., 1989). The enzymes used for restriction digests and ligations were sourced from Inqaba Biotec (South Africa) and used as recommended by the supplier. Digested DNA was eluted from 0.8% agarose gels using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA).

2.2.1. Plasmid construction

The pGT expression vector (Rose & van Zyl, 2002) was used for the over-expression of the *A. niger* β-glucosidase gene (*bgl1*) in *A. niger* D15. The genomic copy of the *bgl1* gene was obtained from *A. niger* ATCC 10664 and cloned into the *Not*I site of vector pGT. Sequence verification of the final vector construct was performed by the dideoxy chain termination method with an ABI PRISM™ 3100 Genetic Analyzer (CAF, Stellenbosch University).

Table 1: Strains and plasmids used in this study

Strains and plasmids	Genotype	Source for hydrolase	Reference
Strains			
A. niger	Wild type	N/A	ATCC10664
A. niger D15	*cspA1*, *pyrG1*, *prtT13*, *phmA*, a non-acidifying mutant of AB1.13	N/A	Wiebe *et al*., 2001
A. niger D15[*Xyn2*]pyrG	*gpd$_P$-xyn2-glaA$_T$; pyrG$_P$-pyrG-pyrG$_T$*	*T. reesei*	Rose & van Zyl, 2008
A. niger D15[EgI]	*gpd$_P$-eg1-glaA$_T$*	*T. reesei*	Rose & van Zy,l 2008
A. niger D15[EgA]	*gpd$_P$-eglA-glaA$_T$*	*A. niger*	Rose & van Zyl, 2008
A. niger D15[EgII]	*gpd$_P$-eg2-glaA$_T$*	*T. reesei*	Rose & van Zyl, 2008
A. niger[Bgl1]	*gpd$_P$-bgl1-glaA$_T$*	*A. niger*	This study
A. niger[GT]	*pyrG$_P$-pyrG-pyrG$_T$*	N/A	This study
S. cerevisiae Y294	*α leu2*-3,112 *ura3*-52 *his3 trp1-289*	N/A	ATCC 201160
S. cerevisiae Y294[CbhI]	*bla ura3/URA3 ENO1$_P$-cbhI-ENO1$_T$*	*T. emersonii*	Ilmén *et al*., 2011
S. cerevisiae Y294[CbhII]	*bla ura3/URA3 ENO1$_P$-cbhI-ENO1$_T$*	*C. lucknowense*	Ilmén *et al*., 2011
S. cerevisiae Y294[Bgl2]	*bla ura3/URA3 ENO1$_P$-bgl2-ENO1$_T$*	*P. chrysosporium*	Njokweni *et al*., 2012
S. cerevisiae Y294[Bgl3]	*bla ura3/URA3 ENO1$_P$-bgl3-ENO1$_T$*	*P. chrysosporium*	Njokweni *et al*., 2012
E. coli DH5α	*supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	N/A	Sambrook *et al*., 1989
Plasmids			
pUC19-PyrG	*blapyrG$_P$-pyrG-pyrG$_T$*	*A. nidulans*	This laboratory
pGT	*bla gpd$_P$-glaA$_T$*	N/A	Rose & van Zyl, 2008
pGT-Bgl1	*bla gdp$_P$-bgl1-glaA$_T$*	*A. niger*	This study

N/A: not applicable.

2.2.2. *A. niger* D15 transformations

The *A. niger* D15 strain was transformed by means of spheroplasts using lysing enzymes (Sigma-Aldrich, Germany) in accordance with Punt and van den Hondel (1992). The High Pure Plasmid Isolation Kit (Roche, Germany) was used to isolate plasmid DNA. Twenty microliters (2 µg) of pGT-Bgl1 vector DNA was co-transformed with10 µl (1 µg) of the pUC19-PyrG vector (containing the fungal marker) to obtain *A. niger* D15[Bgl1], whereas the reference strain *A. niger* D15[GT] was co-transformed with 20 µl (2 µg) of pGT vector DNA and 10 µl (1 µg) of pUC19-PyrG. Transformants were selected for growth in the absence of uridine (Sigma-Aldrich, Germany) and for the ability to produce dark haloes on SC plates containing 1 $g.l^{-1}$ esculin (Njokweni *et al*., 2012).

2.3. Liquid enzyme activity assays

The β-glucosidase activity (for selection and characterisation of Bgl1 transformants, as well as characterisation of Novozyme 188) was determined by the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) (Sigma-Aldrich, Germany), as described by Parry *et al*. (2001), with some modifications. A 50 µl sample of the appropriately diluted enzyme was incubated with 2 mM *p*NPG in 0.5 mM citrate phosphate buffer (pH 5.0) at 50°C for 5 min. The reaction was terminated by the addition of 100 µl of 1 M Na2CO3. The released *p*-nitrophenol (pNP) was measured colourimetrically at 400 nm (xMark™ Microplate Spectrophotometer, Bio-Rad, San Francisco, USA) and quantified with a *p*NP standard curve prepared under the same assay conditions. The CbhI activity was measured using the same pH and temperature, but with *p*-nitrophenyl-*β*-D-cellobioside (*p*NPC) as substrate, a 60 minute incubation and the addition of 1000 µl of 1 M Na_2CO_3. The *p*NP released was measured at 420 nm. Enzyme activity is defined as the amount of enzyme required to release 1 µmol of product per minute under the specified assay conditions.

The cellobiose-to-glucose conversion assay was used to standardise the activities of the different three β-glucosidases for the hydrolysis trials. The enzymes were incubated with 1% cellobiose (in 50 mM citrate phosphate buffer, pH 5) for 60 minutes at 50°C. The amount of glucose released was determined using the K-GLUC kit (Megazyme, Ireland), in accordance with the manufacturer's instructions.

Endoglucanase and endoxylanase activities were assessed colourimetrically by using the reducing sugar assay (Miller, 1959) with 1% carboxymethyl-cellulose (CMC) (Sigma-Aldrich, Germany) and 1% beechwood xylan (Roth, Germany), respectively, as substrates. The substrates were dissolved in 50 mM citrate phosphate buffer (pH 5) at 50°C. One unit of enzyme activity is defined as 1 µmol reducing sugar liberated per minute using glucose as the standard.

Total cellulase activity for the enzyme cocktails in the optimised ratios was determined using a method adapted from the filter paper assay (Ghose, 1987). Whatman No. 1 filter paper strips, 1.0 × 6.0 cm (approximately 50 mg) were incubated in 5 ml citrate phosphate buffer (pH 5) at 50°C for 60 minutes. The glucose released was determined using the K-GLUC kit (Megazyme, Ireland), in accordance to the manufacturer's instructions. One international filter paper unit (FPU) was defined as the amount of enzyme required to release 1 µmol of glucose per minute under the assay conditions. All assays were performed in triplicate using the final two cocktails as the samples.

2.4. β-glucosidase characterisation

The pH optimum of the Bgl1 transformant was determined at 50°C using 2 mM *p*NPG buffered at different pH values using 50 mM citrate phosphate buffer. The temperature optimum was determined by performing the assay with 2 mM *p*NPG (dissolved in 50 mM citrate phosphate buffer, pH5), at varying temperatures.

2.5. Protein analysis

Protein samples were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) to confirm the presence and size of the recombinant protein. Proteins were stained using the silver staining method (O'Connell and Stults 1997), using the broad-range Page Ruler Prestained SM0671 Protein Ladder (Fermentas, China) as molecular weight marker.

Protein concentrations were determined in duplicate with 20 µl solution A, 5 µl diluted enzyme sample and 200 µl Bio-Rad protein reagent solution B (Biorad, USA). Protein concentrations were determined colourimetrically at 750 nm (xMark^{TM} Microplate Spectrophotometer, Bio-Rad, San Francisco, USA), using bovine serum albumin (BSA) as standard. Specific activity was expressed as milligram of protein per ml.

2.6. Harvesting enzymes

The *A. niger* D15[EgI], D15[EgII], D15[EgA], and D15[Bgl1] strains from the laboratory database, were cultured in 25 ml 2xMM with 100 g.l^{-1} glucose (and 0.01 M uridine when required) for 3 days. The supernatants were collected by filtering the culture through Myracloth. The supernatant was lyophilised under vacuum using the Vertis bench top freeze dryer (SP Scientific, USA) and then stored at -20°C until required. The *S. cerevisiae* Y294[CbhI], Y294[CbhII], Y294[Bgl2] and Y294[Bgl3] strains from the laboratory database, were cultured in SC^{-URA}. The supernatants were harvested from the yeast cultures after four days of cultivation and lyophilised, as described above.

2.7. Analysis of substrates

Paper sludge was used without any pre-treatment. The same batch was used throughout the study and stored at -20°C in aliquots of 20 g. The percentage solids was calculated by drying the paper sludge at 50°C for 48 hours. The moisture content of the dried paper sludge was determined as per the National Renewable Energy Laboratory Analytical Procedure (NREL LAP) (Hammes *et al*., 2005). The paper sludge was dried to constant weight at 40°C, cooled (in a desiccator) and weighed. The moisture content was calculated as a percentage (%) based on dry biomass weight and ash content.

The acid insoluble lignin and ash content was determined by heating the dried residue from the quantitative hydrolysis in a muffle furnace at 575 ± 25°C for 4 hours. Ash content was calculated as a percentage of the initial oven dry weight. The acid soluble lignin content was determined colourimetrically using a UV-Visible

spectrophotometer at 240 nm with an absorptivity constant of 12. The acid-insoluble residue was considered as Klason lignin, after correction for the acid-insoluble ash.

Monomeric sugar composition of the acid hydrolysate was determined using the NREL LAP method recommended for determination of structural carbohydrates (cellulose and hemicellulose) and lignin in biomass (Sluiter *et. al*., 2006). The sugar content of the paper sludge was measured by high performance liquid chromatography (HPLC) separated on an Aminex HPX-87H column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). The column temperature was set to 65°C with5 mM sulphuric acid was used as mobile phase at a flow rate of 0.6 $ml.min^{-1}$. Sugars were detected with a reflective index (RI) detector (Shodex, RI-101) operating at 45°C. The pH of the samples was adjusted to between pH 2 and6 using 3 M KOH prior to filtering through a 0.22 µm pore size filter. The glucose, xylose, arabinose and cellobiose components, as percentages were calculated ($g.l^{-1}$) based on the dry weight.

For pre-treatment of triticale straw the biomass was impregnated with water and steam-exploded at 205 °C for 5 minutes and analysed using the same procedures as mentioned above. Avicel PH-101 (Sigma-Aldrich, Germany) was used for comparative purposes during the hydrolysis trials with the chemical composition supplied by the manufacturer.

2.8. Enzymatic hydrolysis of cellulosic materials

The enzymatic hydrolysis experiments were performed with different enzyme loadings in MC Cartney bottles containing 2% paper sludge; all substrate loadings were expressed as w/v dry weight. The lyophilised enzymes were dissolved in 0.05 M citrate phosphate buffer (10 $mg.ml^{-1}$) and the individual enzymes activities of the stock solutions determined. Different enzyme combinations were evaluated in terms of glucose release following hydrolysis of paper sludge. The 10 ml suspensions were incubated in a rotary incubator (8 rpm) at 30°C, 0.02% NaN_3 and 50 mM citrate phosphate buffer (pH 5). Where applicable, 5 µl of Novozyme 188 was added to convert the cellobiose to glucose (also to prevent feedback inhibition of the cellobiohydrolases). Supernatants were collected at 24 hour intervals (including time zero) and centrifuged at 13 000 rpm for 3 minutes.

The glucose content of the supernatant was determined using the K-GLUC kit (Megazyme, Ireland) according to the manufacturer's instructions. The hydrolysis efficiency for paper sludge conversion was determined by calculating the degree of saccharification (DS_{glucan}), which is based on the total sugar concentration in the final hydrolysate (values were normalised by deducting the initial glucose determined in the sample at time zero) relative to the initial cellulose content in the substrate.

2.9. Statistical analysis

Data from the hydrolysis trials was analysed by three ways factorial Analysis Of Variance (ANOVA) using Duncan test *post hoc* means differentiation.

3. Results

3.1. Recombinant strains

The *A. niger bg1* gene was cloned on to plasmid pGT (Figure 1) and transformed into *A. niger*. The *A. niger* D15 transformant containing the pGT vector alone (designated *A. niger* D15 [GT]) acted as negative control.

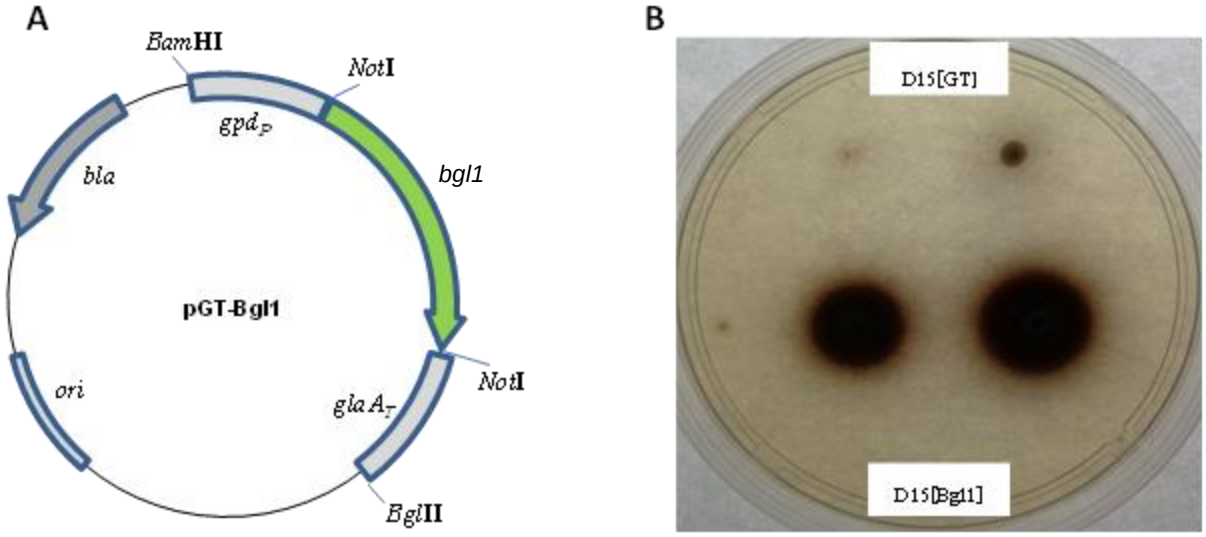


Figure 1: (**A**) Schematic representation of the pGT-Bgl1 vector used for over-expression of *bgl1* in *A. niger* D15. (**B**) Recombinant *A. niger* D15[Bgl1] displaying higher levels of extracellular β-glucosidase activity on SC agar plates containing 1 $g.l^{-1}$ esculin after 24 hours (left-hand side) and 48 hours (right-hand side) of incubation at 30°C.

Fifty *A. niger* D15 transformants were screened for extracellular β-glucosidase activity, using *p*NPG as substrate. The best β-glucosidase producing *A. niger* D15[Bgl1] strain was selected for further experiments. The remaining *A. niger* D15[Xyn2]pyrG, D15[EgI], D15[EgA], D15[EgII] strains and the *S. cerevisiae* Y294[CbhI], Y294[CbhII], Y294[Bgl2] and Y294[Bgl3] strains had previously been constructed (Table 1).

3.2. Characterisation of the *A. niger* D15[Bgl1]

The *A. niger* D15[Bgl1] strain was cultivated in 2xMM and the supernatant harvested after three days of cultivation. The supernatant was used to determine the optimum pH and temperature of Bgl1 (Figure 2). The best transformant was cultured (in triplicate) and the levels of extracellular β-glucosidase activity monitored over time (Figure 3A); at optimum conditions (pH 5 and 60°C) the *A. niger* D15[Bgl1] strain displayed a β-glucosidase activity of 5.81 nkats.ml^{-1} on day four, which is more than 5 times the level of activity displayed by the negative control strain, *A. niger* D15[GT].

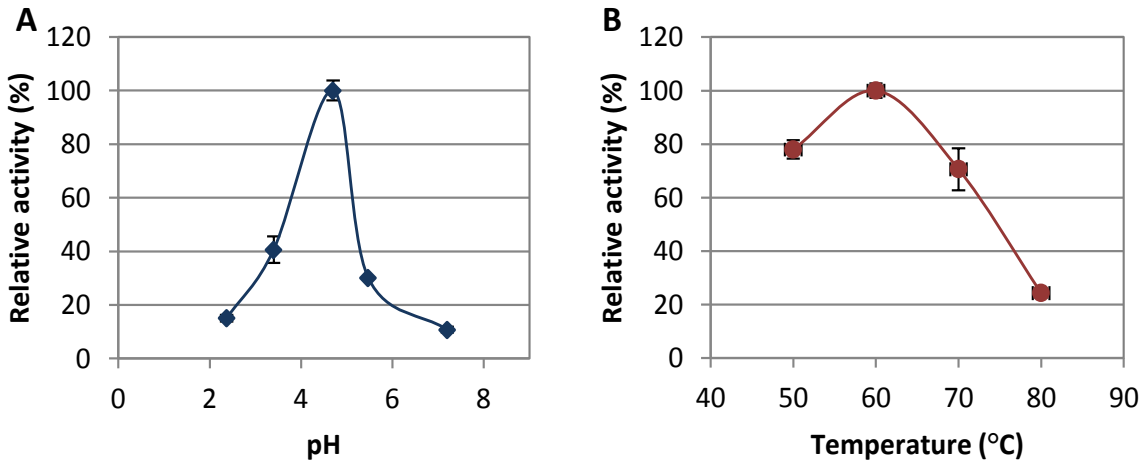


Figure 2: The effects of pH (**A**) and temperature (**B**) on the activity of the *A. niger* D15[Bgl1].

3.3. SDS-PAGE analysis

The supernatants of *A. niger* D15[GT], and D15[Bgl1) were collected after 72 hours of cultivation. Supernatants were lyophilised and 20 µg separated on a 10% SDS-polyacrylamide gel (Figure 3B). The recombinant Bgl1 exhibited a molecular mass of about 110 kDa.

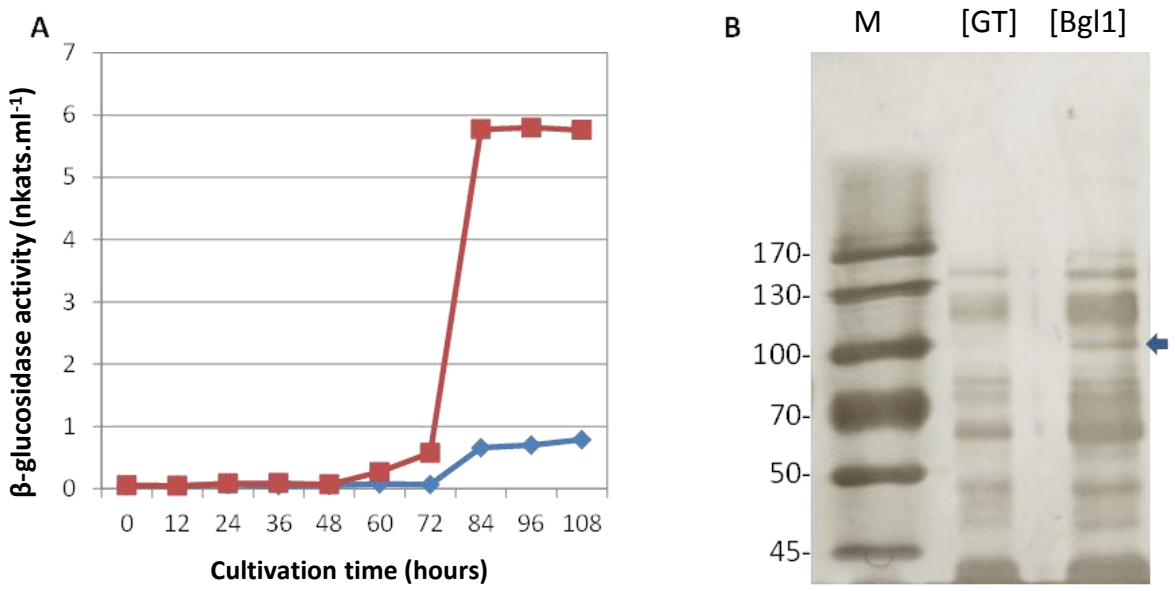


Figure 3: (**A**) Activity curve for the (■) *A. niger* D15[Bgl1] and (◆) *A. niger* D15[pGT] strains cultivated in 2xMM containing 10% glucose as carbon source. (**B**) The supernatants of the *A. niger* D15[Bgl1] and *A. niger* D15[GT] strains were separated by 10% SDS-PAGE and the protein species visualised by silver staining. Sizes of the molecular weight marker are depicted on the left hand side. M indicates the protein ladder and the arrow indicates the Bgl1 protein expressed by *A. niger* D15[Bgl1].

3.4. Composition of substrates

Paper sludge from a kraft process using softwood (*Pinus* sp), triticale straw (cultivar US2009) and Avicel PH-101were used as substrates for hydrolysis. Avicel was used without any treatments and contained 97% cellulose (based on dry weight). The main compositions were determined on a dry weight basis and are presented in Tables 2 and 3. The moisture percentage of paper sludge and triticale straw, based on a total weight basis, were approximately 79% and 68%, respectively. The equation below represents the degree of saccharification or hydrolysis efficiency, which is based on the release of glucan oligomers (Equation 1).

$$\textbf{Equation 1: } Ds_{glucan} = \frac{[glucose\ g.l^{-1}]\ x\ 0.9}{[cellulose\ g.l^{-1}]}\ x\ 100\%$$

A conversion factor of 0.9 (162/180) was applied due to the difference in the mass between the anhydroglucose ring and glucose, as a water molecule is added during the hydrolysis. The concentration of glucose used in Equation 1 represents soluble sugars after hydrolysis (soluble sugars determined at time zero are deducted).

Table 2: Chemical composition of paper sludge obtained from a kraft process

Component	Composition (% dry weight)	Component	Composition (% dry weight)
Ash	5.68 ± 0.12	Glucose	28.95 ± 0.27
Lignin (acid insoluble)	27.04 ± 0.93	Arabinose	0.57 ± 0.08
Lignin (acid soluble)	1.81 ± 0.02	Xylose	14.24 ± 1.85
Cellulose	34.06 ± 0.66	Cellobiose	8.20 ± 1.10

Table 3: The chemical composition of triticale straw before and after pre-treatment

Component	Composition (% dry weight of the raw triticale)	Composition (% dry weight of the pre-treated triticale)
Cellulose	36.68 ± 0.83	46.80 ± 1.09
Hemicellulose	24.96 ± 1.15	8.94 ± 1.24
Lignin	20.53 ± 0.67	28.12 ± 1.72
Ash	1.05 ± 0.10	3.11 ± 0.10
Extractives	12.07 ± 0.10	-

3.5. Enzymatic hydrolysis of paper sludge

Enzyme stocks were made by dissolving the freeze-dried enzyme in buffer. Table 4 shows the activity and protein concentrations of the different stocks used.

Table 4: Enzyme activity of enzymes stock solution used in the hydrolysis trials

Enzyme	Assay Substrate	Activity (nkats.ml^{-1})*	Protein concentration (mg.ml^{-1})**
EgA	CMC	198.66 ± 2.14	1.51 ± 0.14
EgI	CMC	176.60 ± 2.24	Not included
EgII	CMC	769.42 ± 6.54	Not included
Bgl1	*p*NPG	2.32 ± 0.01	24.16 ± 1.06
Bgl2	*p*NPG	11.90 ± 0.07	4.81 ± 0.16
Bgl3	*p*NPG	2.29 ± 0.49	Not included
Xyn2	Beechwood xylan	2188.67 ± 58.45	Not included
CbhI	*p*NPC	50.48 ± 2.20	8.60 ± 0.23
CbhII	-	Not determined	3.84 ± 0.29
Novozyme 188	*p*NPG	4115.46 ± 360	Not included

*Determined using a 10 mg.ml^{-1} stock of the freeze dried enzyme.
**Determined using the enzyme stock solution of the final cocktail.

Three endoglucanases were evaluated (10 mg.ml^{-1} stock diluted to 50 ± 5 nkats.ml^{-1}) when choosing the core enzymes for the enzyme cocktail. These endoglucanases were evaluated in different ratios, ranging from 1:2 to 2:1 based on enzyme activity (with 100 µl being the total volume of recombinant enzymes). The endoglucanases were combined with CbhI (50.48 ± 2.20 nkats.ml^{-1}) and 5 µl Novozyme 188 for hydrolysis of paper sludge. The control hydrolysis reaction contained only Novozyme 188. The CbhI:EgI in a 1:1 ratio and the CbhI:EgA in a 1:1 and 1:2 ratio yielded the best hydrolysis (Figure 4). The CbhI:EgI combination yielded more consistent results (smaller standard deviation) and was therefore selected for further study.

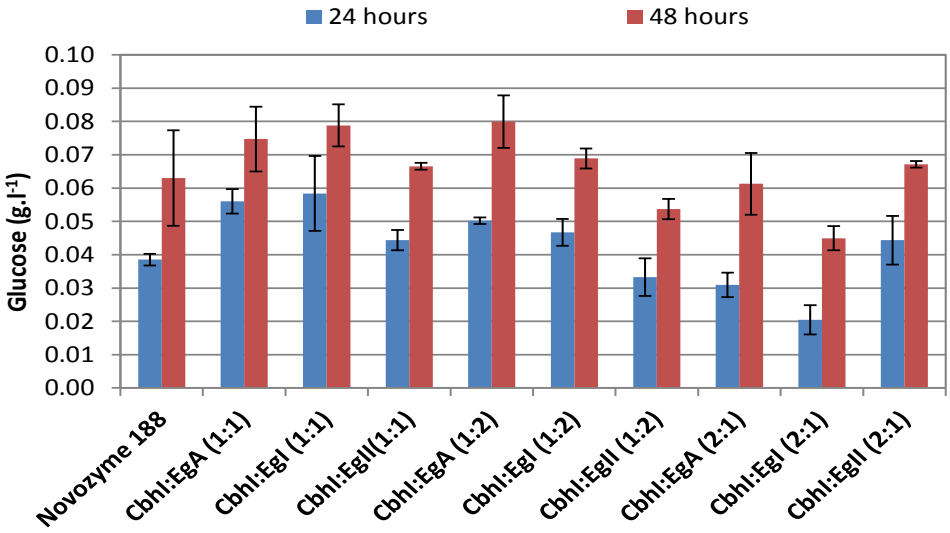
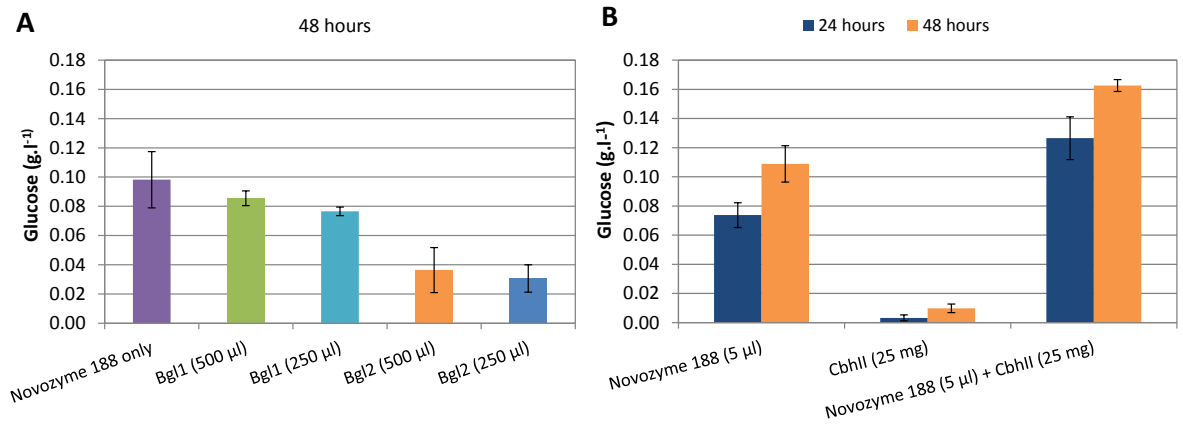


Figure 4: Glucose released by different CbhI:Eg ratios during the hydrolysis of 2% (w/v) paper sludge after 24 and 48 hours.

The activities of two different β-glucosidases were compared (standardised using the *p*NPG assay) and two different β-glucosidase loadings (500 µl and 250 µl of a 11.90 nkats/ml stock) were tested in a 3-enzyme cocktail; CbhI:EgI:β-glucosidase (Figure 5A). The ANOVA test confirmed a significant difference between the glucose yields from Bgl1 and Bgl2 when added with CbhI:EgI. Even at the highest enzyme loading, lower glucose yields were obtained with both β-glucosidases than with Novozyme 188 at 48 hours (Figure 5A). Novozyme 188 is a highly concentrated enzyme cocktail and was used as a benchmark for choosing a β-glucosidase enzyme loading.

The effect of CbhII addition (25 mg stock solution) supplemented with Novozyme 188 (5 µl) was determined on paper sludge at 50°C and compared to the controls, containing either Novozyme 188 or CbhII (Figure 5B). A 66.99% increase in the DS_{glucan} was obtained after 48 hours treatment with CbhII + Novozyme 188 (compared to the control with only Novozyme 188). The ANOVA test confirmed a significant increase in the glucose yield when CbhII was added to the hydrolysis reaction.



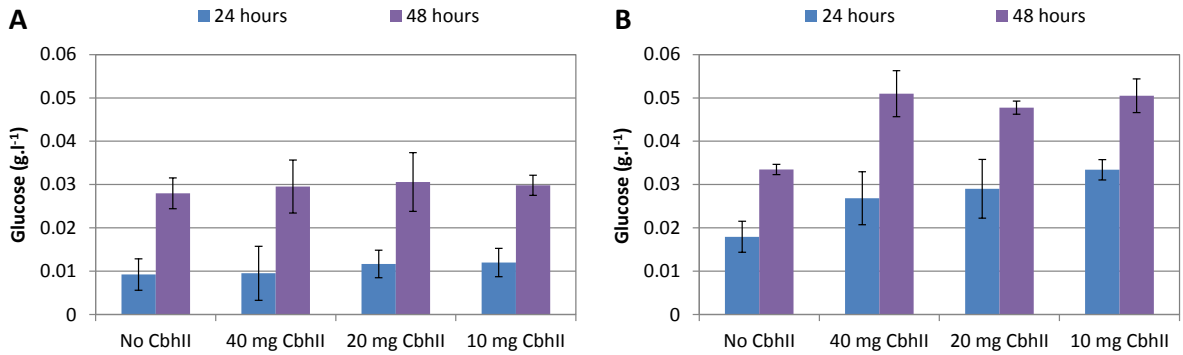
C

Parameter	F value	Probability of F value	Significance
β-glucosidase type (A)	74.75	<0.001	**
CbhII addition (B)	100.65	<0.001	**

Figure 5: (**A**) Paper sludge hydrolysis with two different β-glucosidases (Bgl1 and Bgl2), at two different enzyme loadings in the CbhI:EgI:β-glucosidase cocktail, relative to only Novozyme 188 (positive control). (**B**) The effect of CbhII in combination with Novozyme 188 on paper sludge hydrolysis. (**C**) Statistical evaluation by ANOVA of the effect of different β-glucosidase loading (only Bgl2) enzymes and CbhII addition (**p<0.01) after 48 hours.

The results for CbhII hydrolysis were used to establish a starting point for testing different CbhII loadings (Figure 6), i.e. using 10 mg, 20 mg and 40 mg of the freeze dried enzyme, as the concentrations for the CbhII stock solution. The highest previously tested β-glucosidase loading of Bgl2 (500 µl equivalent to 5 mg of Bgl enzyme), and a 10x increase in stock concentration (50 mg Bgl2) were used in combination with three different CbhII loadings. Results show that there was no significant difference in the glucose released from adding CbhII compared to the control, when the reactions only contained 5 mg Bgl2 (Figure 6A). However, when there was an increase in Bgl2 (50 mg of enzyme) a significantly higher glucose yield

was obtained from reactions that contained CbhII compared to the control (no CbhII) (Figure 6B). Furthermore, there was no significant difference between the glucose yields when the three different CbhII loadings (10 mg, 20 mg and 40 mg) were tested (Figure 6B), indicating that a loading of 10 mg of CbhII was sufficient (for the ratio being developed) and the use of more enzyme would be unnecessary and uneconomical.



C

Parameter	F value	Probability of F value	Significance
Enzyme combinations (A)	0.141	0.932	ns
Enzyme combinations (B)	17.470	<0.001	**
Different CbhII loadings (B)	0.601	0.578	ns

Figure 6: The effect of different CbhII loadings in the 4-enzyme cocktail containing (**A**) diluted Bgl2 (5 mg stock) and (**B**) undiluted Bgl2 (50 mg stock). (**C**) Statistical evaluation by ANOVA of the effect of different Bgl2loadings and CbhII addition after 48 hours (ns: not significant, $^{**}p<0.01$).

When the effect of the Xyn2 loading (using 0.05 mg, 0.10 mg, 0.15 mg) on paper sludge hydrolysis was tested (Figure 7A) in combination with the core enzymes (CbhI:CbhII:EgI:Bgl2), there were no significant differences between the different concentrations of Xyn2 addition, as determined by one-way ANOVA (*F* value = 0.335, probability of *F* value (*p)* = 0.801).

Previously, different endoglucanases were only evaluated in combination with CbhI (Figure 4). However, the synergy in the cocktail could be different due to the presence of Bgl2 and CbhII, therefore endoglucanase addition was re-evaluated. Another trial was thus conducted where Xyn2 was added to cocktail

CbhI:CbhII:endoglucanase:Bgl2 cocktail containing either EgI, EgA or EgII (Figure 7B). The CbhI:CbhII:EgA:Bgl2:Xyn2 cocktail yielded the best result, releasing 0.13 g.l^{-1} glucose from 2% (w/v) paper sludge after 48 hours (Figure 8).

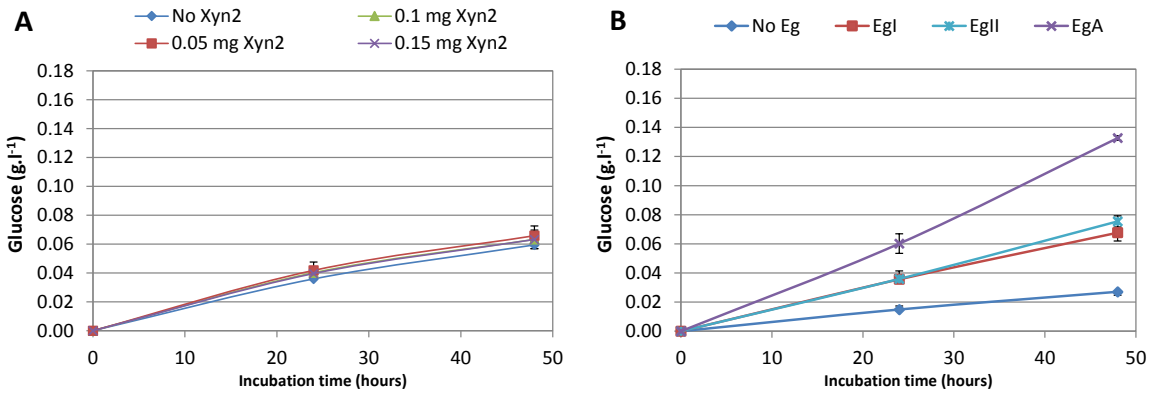


Figure 7: (**A**) The effect of different xylanase additions (Xyn2) to the (CbhI:CbhII:EgI:Bgl2:) cocktail on paper sludge hydrolysis. (**B**) The effect of the CbhI:CbhII:Bgl2:Xyn2 cocktail when added to different endoglucanases.

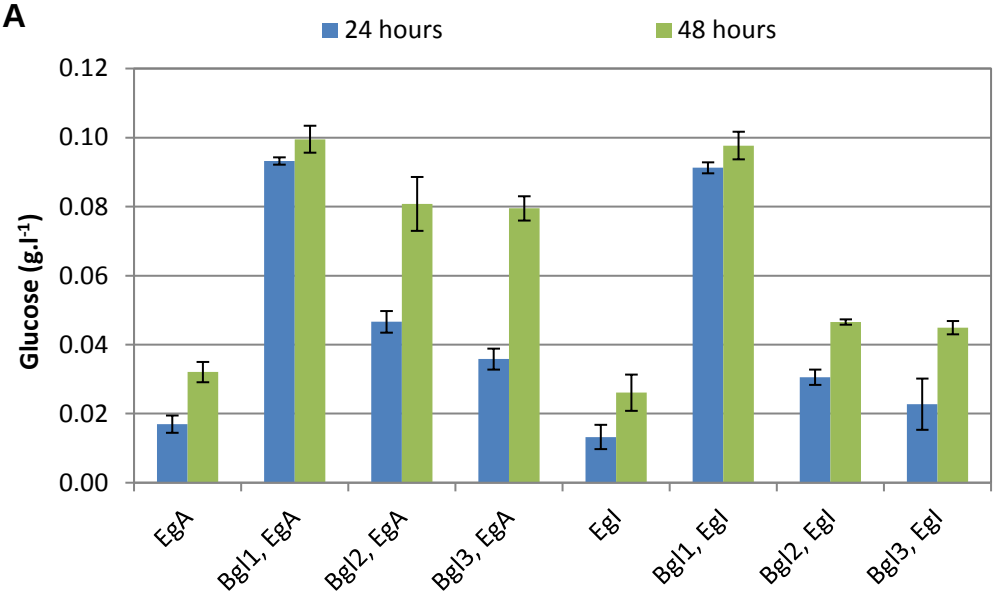
The synergy between different endoglucanases and different β-glucosidases was investigated; Bgl stocks were standardised to have the same activity (on pNPG) and endoglucanases stocks were standardised to 50 ± 5 nkats.ml^{-1} with a DNS assay (Figure 8). Bgl2 and Bgl3 have a higher affinity for *p*NPG, but Bgl1 has a preference for cellobiose (Table 5). Due to the differences in substrate affinity, the interactions and synergy between different β-glucosidase enzymes were examined in relation to the different endoglucanases. Bgl2 and Bgl3 indicated better synergy with EgA, compared to EgI. In addition, the combination of Bgl1 with EgI and EgA yielded similar results. This supported the inclusion of EgA as a core enzyme in the cocktail.

Table 5: Enzyme ratios when standardising β-glucosidase activity

Substrate	**β-glucosidase enzymes**		
	Bgl1[a]	**Bgl2[b]**	**Bgl3[c]**
*p*NPG	1.7	4.6	1
Cellobiose	2.2	2.5	1
*p*NPG: cellobiose ratio	1:1.2	1:0.54	1:1

[a] Bgl1: 300 mg.ml^{-1} stock; [b] Bgl2: 100 mg.ml^{-1} stock; [c] Bgl3: 300 mg.ml^{-1} stock

A



B

Parameter	F value	Probability of F value	Significance
β-glucosidase	290.28	<0.001	**
Endoglucanase	12.11	0.003	**
β-glucosidase x Endoglucanase	33.79	<0.001	**

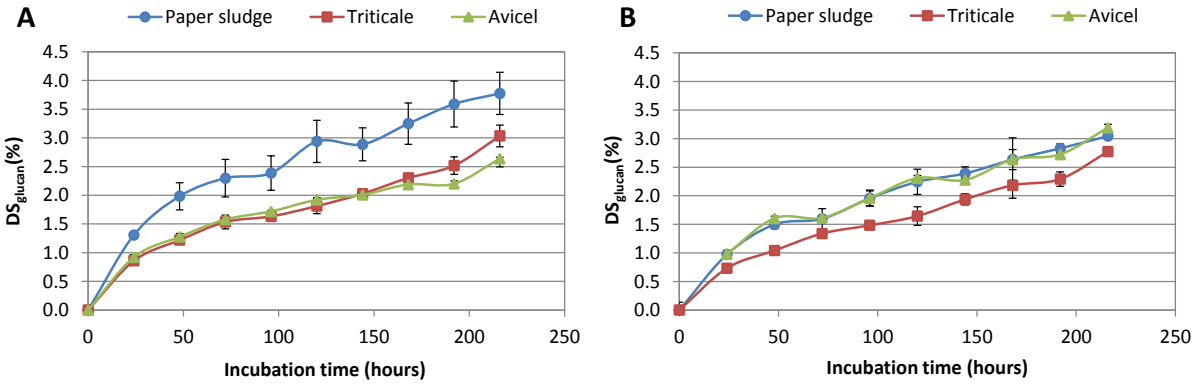
Figure 8: (**A**) Synergy between the different β-glucosidase and endoglucanase combinations. (**B**) Statistical evaluation by ANOVA testing the effect of different β-glucosidases, different endoglucanases and their interaction on paper sludge hydrolysis after 48 hours (**p<0.01).

3.6. Enzymatic hydrolysis of other substrates

Given that significant differences were found between the different β-glucosidases, endoglucanases, and their interaction on paper sludge (Figure 8), two cocktails were compiled for further testing, one containing CbhI:CbhII:**EgA**:Bgl1and the other containing CbhI:CbhII:**Egl**:Bgl1. These 4-enzyme cocktails were evaluated in hydrolysis trials containing paper sludge, pre-treated triticale straw and Avicel (2% substrate loading, based on dry weight).

The ANOVA test shows a highly significant relationship between substrate and endoglucanase interactions (Figure 9). A significant increase in the DS_{glucan} on paper sludge was observed for EgA (3.78%) relative to Egl (3.04%) when used in the 4-enzyme cocktail. There was no significant difference in the DS_{glucan} for triticale straw hydrolysis when using different endoglucanases. A significantly higher DS_{glucan}

of Avicel was observed with EgI relative to EgA; the DS_{glucan} was 3.19% and 2.64%, respectively, after 216 hours.



C

Parameter	F value	Probability of F value	Significance
Endoglucanase	2.88	0.116	ns
Substrate	14.87	<0.001	**
Endoglucanase x Substrate	18.72	<0.001	**
Paper sludge: EgA vs EgI	11.31	0.028	*
Triticale straw: EgA vs EgI	5.05	0.088	ns
Avicel: EgA vs EgI	45.38	0.003	**

Figure 9: The effect of a 4-enzyme cocktail CbhI:CbhII:Bgl1 with (**A**) EgA or (**B**) EgI on different substrates. (**C**) Statistical evaluation by ANOVA of the effect of different endoglucanases, different substrates and the combination of these parameters. (ns: not significant, *p<0.1,**p<0.01).

Once the ratio for the core enzymes had been established (referred to as the 1x enzyme cocktail), the effect of increasing enzyme loadings was tested on the hydrolysis of paper sludge (Figure 10). The enzyme concentration was increased, using the developed ratios for the CbhI:CbhII:EgA:**Bgl1** cocktail; paper sludge and triticale straw were used as substrates. Increasing the enzyme concentration of the CbhI:CbhII:EgA:**Bgl1** cocktail did not improve hydrolysis of 2% paper sludge and the 1x enzyme cocktail yielded the highest DS_{glucan} (Figure 10). However, when 2% triticale straw was used as substrate, the DS_{glucan} increased from 1.53% to 1.96% with the 2x enzyme cocktail (double strength), compared to the 1x enzyme cocktail. However, increasing the enzyme concentration 5-fold (5x enzyme cocktail) did not increase the DS_{glucan} of triticale straw.

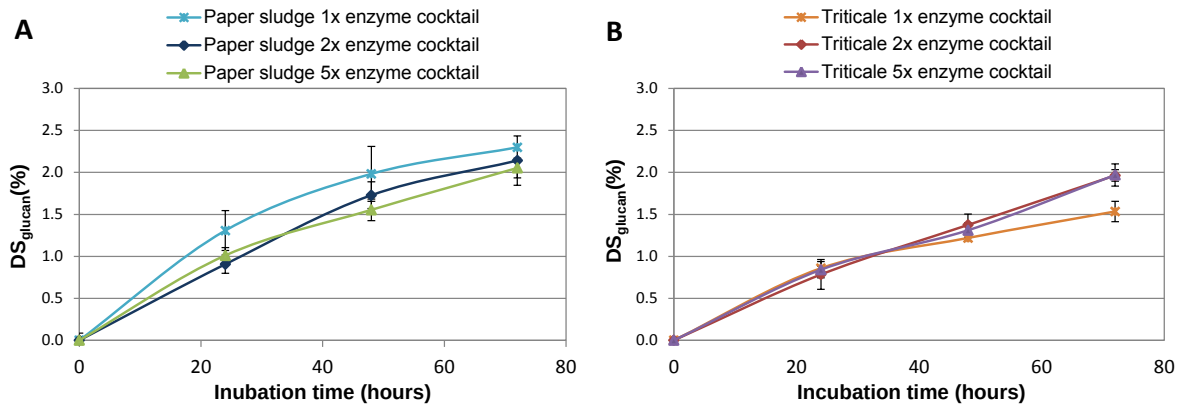


Figure 10: The effect of enzyme loading on the hydrolysis of (**A**) paper sludge and (**B**) triticale straw, using different concentrations of the 4-enzyme cocktail: CbhI:CbhII:EgA:Bgl1.

When Bgl2 was used in the 4-enzyme cocktail (CbhI:CbhII:EgA:**Bgl2**), a significant improvement in the DS_{glucan} on paper sludge was observed for the 2x enzyme cocktail (Figure 11). There was a significant difference between the glucose yields measured from the two cocktails at 264 hours (*F* value = 530.29, probability of *F* value (*p*) = <0.001). The final 1x enzyme cocktail (in a 10 ml working volume) contained 5 mg CbhI, 10 mg CbhII, 0.025 mg EgA and 50 mg β-glucosidase. Therefore, the final ratio for either cocktail containing CbhhI:CbhII:EgA:Bgl1/Bgl2 based on mg of freeze died enzyme is: 200:400:1:2000.

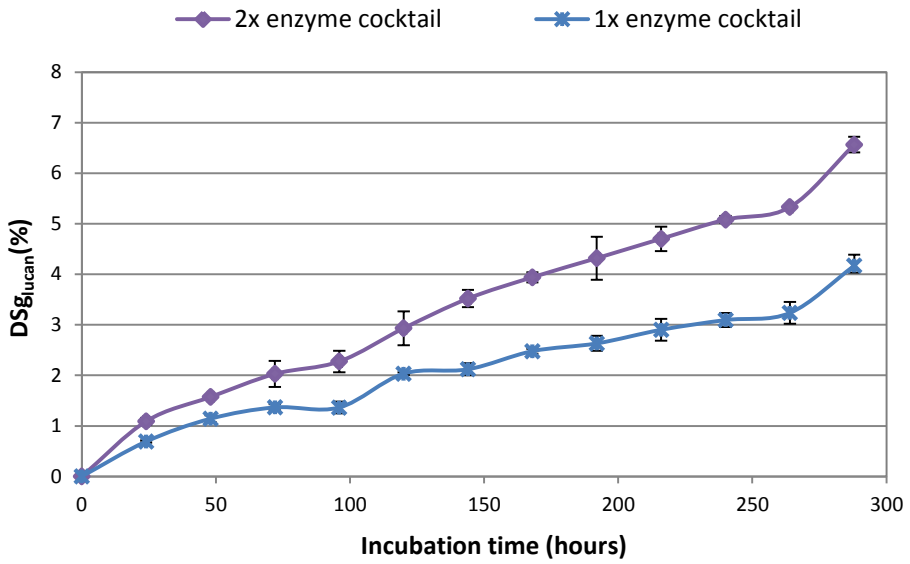


Figure 11: The effect of enzyme loading on paper sludge hydrolysis. The hydrolysis of 2% paper sludge with (∗) 1x enzyme cocktail or a (◆) 2x enzyme cocktail containing CbhI:CbhII:EgA:Bgl2 with a ratio of 7.4:6.6:1:41 (based on protein concentration).

The two final enzyme cocktails (containing either Bgl1 or Bgl2) were analysed in terms of activity and protein content (Table 6). Protein concentrations were determined in order to compare results with published data.

Table 6: Activity of the final CbhI:CbhII:EgA:β-glucosidase cocktails

Final cocktail*	Glucose ($mg.ml^{-1}$)**	Activity (FPU)***	Activity ($nkats.ml^{-1}$)	Protein ($mg.ml^{-1}$)
With Bgl1	5.60	0.70	11.67	13.00
With Bgl2	16.55	0.45	7.45	3.29

*CbhI:CbhII:EgA with the addition of a β-glucosidase
**Glucose in supernatant.
***FPU= µmol/(min.ml^{-1})/enzyme dilution.

The protein concentrations for the stock solutions of the different enzymes in the final 1x enzyme cocktails were used to calculate the protein ratio of the two different 4-enzyme cocktails (Table 7). The final protein ratio for the CbhI:CbhII:EgA:**Bgl1** is 7.4:6.6:1:208 and for CbhI:CbhII:EgA:**Bgl2** is 7.4:6.6:1:41. The large difference in the protein concentrations of the two different β-glucosidases (Bgl1 and Bgl2) is because the *A. niger* D15[Bgl1] strain was cultivated in a medium that contains yeast extract, which has a high protein content, whereas Bgl2 was isolated from *S. cerevisiae* and the growth medium was different.

Table 7: Determination of protein ratio in the final cocktail

Protein	CbhI[1]	CbhII[2]	EgA[3]	Bgl1[4]	Bgl2[5]
Stock solution ($mg.ml^{-1}$)	8.60	3.84	1.51	24.16	4.81
Cocktail*	0.430	0.380	0.076	12.100	2.400
Protein ratio	7.4	6.6	1	208	41

*protein (mg) in the final reaction volume of 10 ml.
[1] 50 µl in the final cocktail.
[2] 100 µl in the final cocktail.
[3] 50 µl in the final cocktail.
[4] 500 µl in the final cocktail.
[5] 500 µl in the final cocktail.

4. Discussion

Paper sludge is a waste material generated by the pulp and paper industry. It can be used as a low-cost feedstock for the production of second-generation biofuel (Gonzalez, 2012), with the process being feasible and profitable under the current market situation. As a result of the pulping process, the lignin content is low and the carbohydrate material has a high specific surface area (due to the fine fibres). This is advantageous because substrates with a high percentage of lignin require increased enzyme loadings due to the non-productive adsorption of enzymes to the lignin component. The high specific surface area benefits the enzyme accessibility, which means that high pre-treatment costs (which account for up to a third of total costs) can be avoided.

4.1. Developing recombinant enzyme cocktails

This study was designed to simulate an industrial process where different enzymes are added simultaneously to avoid additional steps that would result in additional costs.

Direct use: Paper sludge was used directly (as obtained from the supplier) for the hydrolysis reactions, without drying. Drying can result in a less efficient hydrolysis (Hendriks & Zeeman, 2009) because the pores collapse, which decreases the total surface area that is accessible to the enzymes (van Dyk & Pletschke, 2012). Furthermore, in an on-site biorefinery, the paper sludge would be used directly without any modification. Transport of dried material would be less expensive, but the drying process requires energy input, which would add to the cost of paper sludge as a feedstock for bioconversion processes.

Substrate loading: Paper sludge has a high percentage moisture and a 2% substrate loading thus requires 9.5 g of wet weight in a 100 ml working volume. The amount of paper sludge influences the viscosity of the hydrolysis/fermentation and subsequently effects mass transfer. This results in the released sugars being unable to disperse adequately though the medium (Prasetyo & Park, 2013). Since 2% substrate loading already yields a highly viscous solution, increased sludge loadings

were not evaluated; adequate mixing would not be possible and accurate sampling would be problematic.

Substrate compositions: Efficient saccharification of this biomass is the most crucial factor in ethanol production, because the fermentation process is dependent on the amount of sugar released and the rate of sugar production (Prasetyo & Park, 2013). The paper sludge and triticale straw compositions were therefore determined to obtain the cellulose content needed to calculate the hydrolysis efficiency (DS_{glucan}). The cellulose content of the paper sludge used in this study was 34.06 ± 0.66% (based on dry weight), which is similar to that obtained by Marques *et al*. (2008). The cellulose content of paper sludge batches can range between 20 to 70% (Fan & Lynd, 2007), but averages at 42% (dry weight basis) (Fan *et al*. 2003).

Recombinant enzymes: Large volumes of enzyme cocktails are required for the conversion of biomass to simple sugars, which can then be fermented to ethanol. The *A. niger* D15 strain is an ideal host for enzyme production due to its ability to secrete large amounts of enzyme (Rose & van Zyl, 2002, 2008). The heterologous enzymes are generally produced in a relatively pure form and can be used directly without the need for further purification. Similarly, the enzymes produced by recombinant *S. cerevisiae* Y294 strains are relatively free from other proteins, since *S. cerevisiae* has been reported to secrete low levels of native proteins when cultured in minimal media (Den Haan *et al*., 2013). In some hydrolysis studies where recombinant strains were used, the enzymes were concentrated and desalted with tangential flow filtration (Banerjee *et al*., 2010a). This step was omitted in this study, since it would not be practical on industrial scale and the additional steps would incur addition costs. Furthermore, if a CBP organism were to be used in an industrial process, the enzymes would be secreted into the fermenter as “crude enzyme” without purification or concentration. Therefore, no purification was undertaken and enzymes were used in their “crude” state.

Homologous expression of Bgl1: Cellulase preparations are typically supplemented with β-glucosidase (Novozyme 188) (Banerjee *et al*., 2010a, van Dyk & Pletschke, 2012) to maximise the sludge conversion and to prevent cellobiose accumulation, thus minimising product inhibition. Novozyme 188 is

obtained from *A. niger* and therefore the β-glucosidase gene (*bgl1* of *A. niger*) was selected for over-expression. The recombinant *A. niger* D15[Bgl1] strain was cultivated on 10% glucose, which resulted in catabolic repression of the native cellulases and hemicellulases and no endoglucanase and cellobiohydrolase activity could be detected. Novozyme 188, on the other hand, contained amylase (83 539 ± 2520 nkats.ml^{-1}) and xylanase activity (1 920 ± 110 nkats.ml^{-1}).

Enzyme stock solutions: Usually experiments of this nature are performed with commercial enzyme cocktails at fixed protein loadings, e.g. the hydrolysis of corn stover (Banerjee *et al*., 2010a). Protein concentrations can be deceptive (as was demonstrated in the protein ratio for the final cocktail), because different enzymes have different levels of specific activity (activity per gram of enzyme); not all of the enzymes contribute to the hydrolysis and commercial enzyme preparations can contain additional BSA as a stabiliser. Furthermore, media components, such as yeast extract, can affect protein concentration of unpurified enzymes, e.g. Bgl1 has a much higher protein concentration compared to Bgl2. Therefore, the enzyme activities (as opposed to protein concentrations) were taken into account when enzyme stock solutions were prepared. However, the protein ratios were determined to compare these results with previously published data.

4.2. Enzymatic hydrolysis

Effective enzymatic hydrolysis of paper sludge has previously been demonstrated using commercial enzymes (Marques *et al.*, 2008, Fan &Lynd, 2007, Kan *et al*., 2010). However, limited information is available on the use of customised enzyme cocktails containing recombinant enzymes. Developing an enzyme cocktail requires the testing of different enzymes in different ratios, until a core set is developed. Once the core set of enzymes is optimised for a specific feedstock, accessory enzymes can be tested in combination with the core set.

The aim of this study was to develop an enzyme cocktail customised for the hydrolysis of paper sludge. The initial emphasis was on optimising the ratio of the enzymes, rather than enabling maximum conversion, hence only a 48 hour hydrolysis was performed in preliminary trials. The initial cellobiohydrolase and endoglucanase combinations represented a starting point for combining the core

enzymes (in an appropriate ratio) (Figure 4). The CbhI:EgI (1:1), CbhI:EgA (1:2) and CbhI:EgA (1:1) yielded similar results (ratios are given in volumes and the activity was the same for a specific volume). However, after further hydrolysis experiments, it was established that EgA gave better results (1:1 ratio) compared to EgI with regards to paper sludge hydrolysis (Figure 7B).

Karlsson *et al*. (1999) studied the effect of different ratios of only CbhI and EgII from *T. reesei* for the hydrolysis of steam pre-treated willow. They concluded that the ratio between EgII and CbhI was not critical and a ratio of 40% EgII:60% CbhI showed similar conversion to 5% EgII:95% CbhI. Furthermore, hydrolysis using Avicel was included in their study to compare the synergistic effects on a different substrate; significant synergism between the enzymes was noted when using the willow substrate, but not on Avicel. This highlights the importance of “substrate” as a factor that affects enzyme synergy.

A more recent study by Várnai *et al*. (2010) used purified enzymes from *T. reesei* and commercial enzyme cocktails to develop different enzyme cocktails based on protein concentrations. Enzyme cocktails were then evaluated on a variety of lignocellulosic substrates. A Cbh:Eg:Xyn mixture was calculated to resemble the enzyme composition commonly present in *T. reesei* culture broths, containing 58.4% CbhI, 19.5% CbhII, 19.5% EgII and 2.7% XynII. Although this ratio shows a low EgII percentage in the mixture, it had a higher activity (2670 nkats.g^{-1} cellulose) compared to CbhI (384 nkats.g^{-1} cellulose); the CbhI:EgII activity ratio was 1:6. In the overall 1x enzyme cocktail from this study, the activity for CbhI and EgA were the same and the protein ratio for CbhI:EgA was 7.4:1. Therefore, although the amount of endoglucanase is often described as being relatively less than other enzymes, it is because protein concentrations and not enzyme activities are reported for enzyme loading.

The Bgl1 performed better than the Bgl2 and Bgl3 enzymes on paper sludge (Figure 5A and Figure 8). The effect of Bgl2 in combination with CbhII (Figure 6B) was noticeable. As expected, the Xyn2 did not contribute to the hydrolysis of paper sludge (Figure 7A); although an effect on hydrolysis was observed when different endoglucanases were also present in the reaction (Figure 8). This was attributed to the effect of the endoglucanase. Furthermore, combinations of endoglucanases and

β-glucosidases were evaluated in a 4-enzyme cocktail (Figure 8); Bgl2 and Bgl3 indicated good synergy with EgA, compared to EgI. However, when the glucose yields were compared for Bgl1 paired with EgA and Bgl1 paired with EgI, similar glucose yields were obtained (0.010 $g.l^{-1}$ and 0.098.$g.l^{-1}$, respectively).

Two almost identical core sets were therefore compiled with CbhI:CbhII:EgI/EgA:Bgl1 and evaluated on paper sludge, triticale straw and Avicel (Figure 9). The cocktail containing EgA released more glucose from paper sludge compared to the cocktail containing EgI. However, the EgI cocktail resulted in a slightly better hydrolysis of Avicel than the EgA cocktail. The different cocktails had a similar effect on pre-treated triticale straw. Therefore, the EgI cocktail is a consistent cocktail giving similar results for all three substrates.

Dissimilar trends were observed in the hydrolysis efficiency, depending on the type of β-glucosidase in the cocktail, as their binding affinities for *p*NPG and cellobiose are different. Both Bgl1 and Bgl2 were considered for final cocktails depending on the industrial/academic need. Although the incorporation of Bgl1 had an improved effect on the hydrolysis of paper sludge (Figure 8), increasing concentrations of a 4-enzyme cocktail containing Bgl1 did not increase the overall hydrolysis (Figure 10). Consequently, a 2 and 5-fold increase in enzyme loading resulted in a decrease in hydrolysis efficiency. However, when Bgl2 was included (Figure 11) a 2-fold increase in enzyme concentration (2x enzyme cocktail) increased hydrolysis by 67.34% and 63.56% after 72 and 288 hours, respectively. The enzyme dosage was not fully optimised for complete hydrolysis, since a further increase in CbhI, CbhII, Bgl2 and EgA concentrations might yield an even better result.

The addition of a xylanase as an accessory enzyme to the core set (EgI cocktail), did not increase hydrolysis (Figure 7A). For the xylanase to have an effect on the amount of glucose released, it should benefit the cellulases by removing the xylan that is blocking the sites of attack on the cellulose chain. The chemical procedures involved in the pulping process degrade and remove some of the xylan. The residual xylan is probably not attached to the cellulose and therefore did not interfere with the cellulases or the xylan was sufficiently hydrolysed by the EgI (Rose & van Zyl, 2002). The *T. reesei* EgI was included in this study because of its ability to hydrolyse a wide range of substrates, such as Avicel, carboxymethyl

cellulose (CMC), hydroxyethyl cellulose (HEC), barley β-glucan, acid-swollen amorphous cellulose, lichenan, xylan and even galactomannan (Rose & van Zyl, 2002). Additional xylanase activity might still have a beneficial effect when using another type of lignocellulose as the substrate. EgA and EgII do not degrade xylan and would therefore benefit from the addition of a xylanase to the enzyme cocktail.

Hydrolysis of paper sludge by the CbhI:CbhII:EgA:Bgl2 cocktail showed a continued increase in the degree of saccharification, over a long period of time (Figure 11). This means that the enzymes remain active for more than a week and should release a sufficient amount of glucose to enable fermentation by a *S. cerevisiae* strain in a separate hydrolysis and fermentation (SHF) process, as has been demonstrated by Zhu *et al*. (2011). It might also be beneficial to construct a recombinant *S. cerevisiae* strain that can continuously produce the core enzymes in the optimised ratio. The glucose can then be fermented upon release, minimising the loss of carbon due to contamination.

It would be a challenge to produce the enzymes in a specific ratio using only one host organism. The paper sludge hydrolysate can (in theory) also be used on-site to cultivate the strains used in this study to produce the individual enzymes (the strains listed in Table 1 do not require induction for enzyme expression). These enzymes can then be used to supplement the less-than-ideal cocktail produced by the single host scenario.

Paper sludge from the kraft process has features desirable for bioconversion processes. Several results from this study are seen as new developments, such as using recombinant enzymes in a hydrolysis process to develop a tailor-made enzyme cocktail for paper sludge hydrolysis. It can be concluded that different enzyme cocktails are required for different substrates, in order to accomplish production of cellulosic ethanol on a commercial scale.

5. Acknowledgements

The authors thank the National Research Foundation (NRF) for financial support, Mrs Lalie Kossatz (preparation and analysis of the pre-treated triticale straw), Mr Henry Solomon (assistance with paper sludge analysis), Ngodwana Mill Sappi Paper (paper sludge), Dr Maria Garcia, Mrs Manda Rossouw and Mrs Annatjie Hugo (technical assistance and HPLC).

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Chapter 4

The use of a defined cellulase cocktail for the hydrolysis and conversion of wheat bran to ethanol using amylolytic *Saccharomyces cerevisiae* strains

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Manuscript in preparation for publication in *Journal of Industrial Microbiology and Biotechnology*

The use of a defined cellulase cocktail for the hydrolysis and conversion of wheat bran to ethanol using amylolytic *Saccharomyces cerevisiae* strains

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Abstract

Wheat bran is generated from the milling of wheat and represents a promising feedstock for the production of bioethanol. Wheat bran consists of three main components: starch, hemicellulose and cellulose. The optimal conditions for wheat bran hydrolysis have been determined using a recombinant cellulase enzyme cocktail. Milling and sulphuric acid pre-treatment yielded similar results in terms of the hydrolysis efficiency. A substrate loading of 10% (w/v, dry weight) yielded the highest level of glucose, while a 2% substrate loading yielded the best hydrolysis efficiency (degree of saccharification), for unmilled wheat bran, i.e. no pre-treatment. Ethanol production by two industrial amylolytic *Saccharomyces cerevisiae* strains, S2[TLG, SFA] and MH1000[TLG, SFA], were compared in an Simultaneous Saccharification and Fermentation (SSF) using a 10% substrate loading. The two strains produced similar amounts of ethanol in the presence of the recombinant cellulase enzyme cocktail (5.72 ± 0.07 $g.l^{-1}$ and 5.45 ± 0.09 $g.l^{-1}$, respectively, after 72 hours). This study demonstrated that the addition of the recombinant cellulase cocktail improved the ethanol yields by 8.69% in the SSF process and that the *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA] strains efficiently converted starch to ethanol.

Keywords: wheat bran • cellulose •recombinant cellulase cocktail • amylolytic *S. cerevisiae* • starch • bioethanol

1. Introduction

The increase in oil and energy prices, as well as a depletion of fossil fuel reserves, has resulted in a global challenge to explore alternative energy sources. First-generation biofuels are largely produced from edible sugars and starches; second-generation biofuels are produced from non-edible plant materials; and third-generation biofuels are obtained from algae and other microbes (Sweeney & Xu, 2012; Limayem & Ricke, 2012). First-generation bioethanol has been used as a transportation fuel in Brazil since the 1970s (Chotěborská *et al*., 2004) and bioethanol programmes are currently being implemented in several other countries, e.g. the United States and China (Petrova & Ivanova, 2010). Since the existing supply of raw materials (sugar and grain) will not be sufficient over the long term due to the increasing demand for bioethanol (Hahn-Hägerdal*et al*., 2006), other biomass resources need to be explored and optimised.

Bioethanol, as a second-generation biofuel, can be obtained from cellulosic biomass, including agricultural and forestry residues, portions of municipal waste and herbaceous and woody crops (Wyman, 2007). The development of second-generation bioethanol from lignocellulosic biomass has many advantages, from both an environmental and energy perspective (Menon & Rao, 2012). Cellulosic ethanol offers greater environmental benefits and sustainability; however, the main concern is the economic viability of the bioconversion process (Menon & Rao, 2012). This process depends on innovative technologies that require low-cost enzymes (with high levels of activity), inexpensive feedstocks (that don't compete with food demands) and an efficient process design. Alternative feedstocks for bioconversion processes need to be exploited in a more efficient process that hydrolyses all the components, including lignocellulosic residues.

Bioethanol production is usually performed in three steps: 1) obtaining a solution of fermentable sugars; 2) fermentation of these sugars into ethanol; and 3) ethanol separation and purification, usually by distillation–rectification–dehydration (Mussatto *et al*., 2010). However, for lignocellulosic feedstocks, these steps are preceded by a pre-treatment step to improve substrate accessibility to the hydrolytic enzymes. Efficient and economical biofuel production can be carried out using the simultaneous saccharification and fermentation (SSF) process. In an SSF process,

glucose is used by the fermenting organism, thus removing the feedback inhibitor to cellulase activity (Philippidis *et al*., 1993); the result is an increased yield and rate of cellulose hydrolysis. This differs from separate hydrolysis and fermentation (SHF), in which product inhibition from glucose and cellobiose occurs, inhibiting the actions of the cellulolytic enzymes (Limayem & Ricke, 2012). The optimisation of cellulase cocktails and enzyme loading can improve the hydrolysis efficiency of lignocellulosic/starchy materials in an SSF process (Sun & Cheng, 2002).

Wheat bran (a by-product of the wheat milling industry) is the outer covering of the wheat grain and an attractive agricultural waste material for bioethanol production. It has a relatively diverse application in several industries (food, animal feed, medicine and fermentation industries) due to the high carbohydrate content (mostly fibres), protein and fats (Javed *et al*., 2012). Industrial wheat bran usually accounts for 14-19% of the grain and includes the outer coverings, the aleurone layer and what is left of the starchy endosperm (Palmarola-Adrados *et al*., 2005). The outermost covering, called the pericarp, contains insoluble dietary fibre (cellulose) and complex xylans (with a high arabinose to xylose ratio), lignin, ferulic acid, as well as other bioactive compounds (Javed *et al*., 2012).

The fibre fraction of bran is not utilised by many ethanol-producing plants, because current bioethanol strategies (using wheat bran as a feedstock) involve the conversion of only wheat starch into ethanol (Palmarola-Adrados *et al*., 2005). Utilization of both the starch and hemicellulose/cellulose part would increase the ethanol yield considerably, as well as improve the protein content of the resulting distiller's dry grain soluble (DDGS) (Amigun *et al*., 2011). The hemicellulose and cellulose components of wheat bran can thus be considered as a promising renewable substrate for biotechnological processes, especially bioethanol production (Chotěborská *et al*., 2004).

Yeast strain development is also an important factor to be considered in the development of industrial ethanol production (Mussatto *et al*., 2010). Yeasts, particularly *S. cerevisiae* strains, are commonly used because of their superior fermentative capacity, high tolerance to ethanol and other inhibitors (either formed during feedstock pre-treatment or produced during fermentation). Furthermore, they grow rapidly under anaerobic conditions (van Dijken *et al*., 1993), which are typical of

the environment in fermentation reactors. Since wheat bran contains a large percentage of starch (between 10-34%) (Favaro *et al*., 2012; Javed *et al*., 2012), it would be beneficial to include a starch-utilising yeast to also assist in the starch conversion process. The use of an amylolytic fermentative organism will not only produce ethanol, but its inclusion in an SSF process will decrease the reliance on commercial enzymes, specifically amylases.

The development of technologies for novel raw starch fermentation systems is important for reducing the production costs in a bioethanol plant. The aim of this study was to evaluate a recombinant cellulase cocktail (refer to chapter 3) on wheat bran. Two industrial amylolytic yeast strains, both secreting a glucoamylase and an α-amylase, were evaluated in an SSF process in the presence of a cellulase cocktail.

2. Materials and methods

2.1. Enzymes

Recombinant *Aspergillus niger* and *S. cerevisiae* strains were cultured, the supernatant lyophilised and used to develop an enzyme cocktail for cellulose hydrolysis (Chapter 3), hence forth referred to as a 1x enzyme cocktail (Table 1). In addition, a commercial β-glucosidase preparation, NS50010 (Novozyme, Denmark), containing 250 CbU.g^{-1} (Cellobiase Units.g^{-1} solution) was used as a supplementary enzyme (Table 2). The NS50010 preparation is hereafter referred to as Novozyme Bgl.

Table 1: Recombinant enzymes and protein concentrations in the 1x enzyme cocktail

Enzyme	Name	Source organism	Protein (mg.ml^{-1})
cellobiohydrolase I	CbhI	*Talaromyces emersonii*	43.74 ± 1.28
cellobiohydrolase II	CbhII	*Chrysosporium lucknowense*	3.84 ± 0.29
endoglucanase I	EgA*	*Aspergillus niger*	2.71 ± 0.04
β-glucosidase	Bgl2	*Phanerochaete chrysosporium*	4.81 ± 0.16

* EgA was expressed in *A. niger* D15, the other recombinant enzymes were expressed in *S. cerevisiae strains.*

Table 2: Characterisation of Novozyme Bgl in terms of activity and protein concentration

Novozyme Bgl	
β-glucosidase activity	7121 ± 195 nkats/ml
Amylase activity	92983 ± 2059 nkats/ml
Xylanase activity	1215 ± 6.56 nkats/ml
Protein concentration	192 ± 5.46 $mg.ml^{-1}$

2.1.1. Activity assays

For characterisation of Novozyme Bgl, the β-glucosidase activity was determined by the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) (Sigma-Aldrich, Germany), as described by Parry *et al*. (2001), with some modifications. A 50 µl sample of the appropriately diluted enzyme was incubated with 2 mM *p*NPG in 0.5 mM citrate phosphate buffer (pH 5.0). The reaction was carried out at 50°C for 5 minute and terminated by the addition of 100 µl of 1 M Na_2CO_3. The released *p*NP was measured colourimetrically at 400 nm (xMark™ Microtitreplate Spectrophotometer, Bio-Rad, San Francisco, USA) and quantified using a *p*NP standard curve (prepared under the same assay conditions.

Amylase and xylanase activities were assessed colourimetrically using the reducing sugar assay (Miller, 1959) and 0.2% soluble starch (Sigma-Aldrich, Germany) and 1% beechwood xylan (Roth, Germany), respectively, as substrates. The substrates were dissolved in 50 mM citrate phosphate buffer (pH 5) and hydrolysis reactions were carried out at 50°C for 5 minutes. All enzyme activities are expressed as nanokatals per millilitre ($nkat.ml^{-1}$), with one katal defined as the amount of enzyme required to release 1 mol of glucose or xylose per second under the specified assay conditions.

2.1.2. Protein concentration

The protein content of the enzyme preparations was determined with the Bio-Rad protein reagent (BioRad, USA), as directed by the manufacturer. Protein concentrations were determined colourimetrically at 750 nm using the xMark™ Microtitreplate Spectrophotometer (Bio-Rad, San Francisco, USA) and bovine serum albumin (BSA) as standard. Protein concentration was expressed as milligram of protein per ml (Table 1).

2.2. Amylolytic yeast strains

The recombinant *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA] strains (Stellenbosch University) were used in this study. Both strains contained the *TLG1*gene (glucoamylase from *Thermomyces lanuginoses*) expressed under the control of the *ENO1* promoter, and the *SFA1*gene (α-amylase from *Saccharomycopsis fibuligera*) expressed under the control of the *PGK1* promoter sequences. Both genes contained the coding region of the *XYN2* secretion signal (*T. reesei XYNSEC*) and were synthetically made and codon optimised (GeneArt) for expression in *S. cerevisiae*. The expression cassettes were integrated into the delta sequences on the genomes of the industrial *S. cerevisiae* S2 and MH1000 strains (Lee & Da Silva, 1997).

2.3. Feedstock

Wheat (*Triticum aestivum* L.) was cultivated in the area of Rovigo (Italy, 45°4'51''N, 11°47'38''E), harvested at 6 months, processed by Grandi Molini Italiani (Rovigo, Italy) and stored in plastic bags at 4°C. The geometric mean diameter of the wheat bran was 0.79 mm (Favaro *et al*., 2012).

2.4. Chemical analysis of wheat bran

The dry matter content of the wheat bran was obtained by drying triplicate samples for 48 hours in an oven at 100°C. Wheat bran was analysed in terms of ash, starch, hemicellulose, cellulose, lignin and protein content according to international standard methods (AOAC).

2.5. Pre-treatment

Raw wheat bran was grounded to a geometric mean diameter value of 0.45 mm, using a laboratory knife mill to obtain milled wheat bran. Unmilled and milled wheat bran were pre-treated with 1% sulphuric acid (w/w dry wheat bran) at 121°C. Dry matter concentration was adjusted to 51 $g.kg^{-1}$ with deionised water. Pre-treatment vessels were filled with 100 ml of the resulting slurry and autoclaved at 121°C for 30 minutes (Favaro *et al*., 2012).

2.6. Enzymatic hydrolysis

Initial enzymatic hydrolysis trials were performed on different wheat bran samples: unmilled wheat bran; unmilled, pre–treated wheat bran; milled wheat bran; and milled, pre-treated wheat bran. A 2% substrate loading was used with a 1x enzyme cocktail (enzyme loading adapted to a 5 ml working volume). Five microlitres of Novozyme Bgl was added after 65 hours of hydrolysis. Additional trials were then conducted to test different experimental parameters (Table 3). Hydrolysis trials were performed in a 5 ml working volume in McCartney bottles, with 0.05 M citric acid buffer (pH 5) and 0.02% NaN_3 (to prevent contamination). Reactions were incubated at 30°C in a laboratory rotary-shaker-incubator (10 rpm), with sampling (100 µl) at time zero and at regular intervals. All substrate loadings are expressed as w/v, based on dry weight.

Table 3: Summary of parameters for hydrolysis experiments examining the effects of substrate loadings and enzyme loadings on enzymatic hydrolysis of wheat bran

Experimental parameter	Substrate loading % (w/v)	Enzyme loading	Novozyme Bgl*
Substrate loading %	2, 5, 10	5, 10	10
Enzyme loading	1x enzyme cocktail	1x, 2x enzyme cocktail	1x, 2x enzyme cocktail
Novozyme Bgl*	5 µl at 72 hours	5 µl at 72 hours	5 µl at 0 hours

*Novozyme Bgl: 250 CbU per 100 g of wheat bran slurry (Favaro *et al*., 2013).

Supernatants were collected after centrifugation at 13000 rpm for 3 minutes. The glucose content of the samples was determined (in duplicate) using the Roche D-Glucose Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Absorbance readings were taken at 340 nm on a UV-Visible spectrophotometer (Boehringer Mannheim/R-Biopharm).

2.7. Fermentation studies on wheat bran

For both *S. cerevisiae* strains, inocula were prepared in 200 ml culture medium (6.7 $g.l^{-1}$yeast nitrogen base, 20 $g.l^{-1}$ peptone and 20 $g.l^{-1}$ glucose, 0.05 mM citric acid buffer, pH5) in 500 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (30°C) at 150 rpm for 60 hours. An SSF was performed using fermentation

medium (similar to the culture media, but containing only 0.5g.l^{-1} glucose), 10% unmilled wheat bran and an initial inoculum of 0.3 g DW.l^{-1} (corresponding to an absorbance of 1 at 600nm). Control fermentations were run in parallel to the SSF reactions using the fermentation medium, which was supplemented with 30 g.l^{-1} glucose. In addition, hydrolysis controls were run in parallel to the SSF reactions under the same conditions, without inoculum.

Different filter-sterilised enzyme combinations were compared using unmilled wheat bran: (1) no enzymes; (2) 1x enzyme cocktail; (3) 1x enzyme cocktail supplemented with Novozyme Bgl; and (4) Novozyme Bgl. Fermentations and hydrolysis reactions were conducted at a working volume of 50 ml (pH5) in a 55 ml serum bottle at 30°C. The fermentations were carried out for 10 days under oxygen-limited conditions and the bottles, equipped with a bubbling CO_2 outlet, were incubated at 30°C on a magnetic stirrer. Ampicillin (100 mg.l^{-1}) and streptomycin (75 mg.l^{-1}) were added at the start of the fermentation, as well as after 48 hours to prevent contamination.

Daily samples were taken during the course of fermentation and analysed for glucose, cellobiose and ethanol content, using ultra High Performance Liquid Chromatography (Nexera – Shimadzu Italia SRL, Milan, Italy) with a hydrogen column (Rezex R0A) at 60°C and5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml.min^{-1}. The compounds were detected with a refractive-index detector (RID 6A; Shimadzu, Kyoto, Japan). Experiments were performed in triplicate.

2.8. Data analysis

Data from the four hydrolysis trials was analysed by three ways factorial ANOVA (Analysis Of Variance) using Duncan test *post hoc* means differentiation.

3. Results

Wheat bran samples contained a dry matter (DM) content of 903.4 g.kg^{-1}and were analysed for cellulose and starch components (Table 4).

Table 4: Composition of wheat bran used in this study

Components (% of dry matter)	Substrate (wheat bran)			
	Unmilled	Unmilled pre-treated	Milled	Milled pre-treated
Cellulose	10.68	10.63	10.91	10.79
Hemicellulose	39.06	35.10	38.99	29.23
Ash	0.05	0.02	0.04	0.04
Lignin	4.98	4.70	5.08	4.63
Starch	11.01	11.01	11.01	11.01
Protein	17.94	17.86	17.88	17.75

3.1. Enzymatic hydrolysis

Hydrolysis trials were carried out to investigate the effect that different parameters (pre-treatment, substrate loading and enzyme loading) had on the enzymatic hydrolysis of wheat bran. Glucose concentrations were used to calculate the degree of saccharification, DS_{glucan} (Equation 1). DS_{glucan} represents the hydrolysis efficiency based on the release of glucan oligomers. A conversion factor of 0.9 (162/180) is applied due to the difference in the mass between the anhydroglucose ring and glucose, as a water molecule is added during the hydrolysis. The concentration of glucose used in Equation 1 represents soluble sugars after hydrolysis (soluble sugars determined at time zero are deducted). DS_{total} (Equation 2) was based on the total sugar concentration in the hydrolysate (corrected for glucose concentration measured at time zero) with respect to the initial cellulose and starch concentrations.

$$\textbf{Equation 1}: DS_{glucan} = \frac{[glucose\ g.l^{-1}]\ x\ 0.9}{[cellulose\ g.l^{-1}]}\ x\ 100\%$$

$$\textbf{Equation 2}: DS_{total} = \frac{[glucose\ g.l^{-1}]\ x\ 0.9}{[cellulose\ g.l^{-1} + starch\ g.l^{-1}]}\ x\ 100\%$$

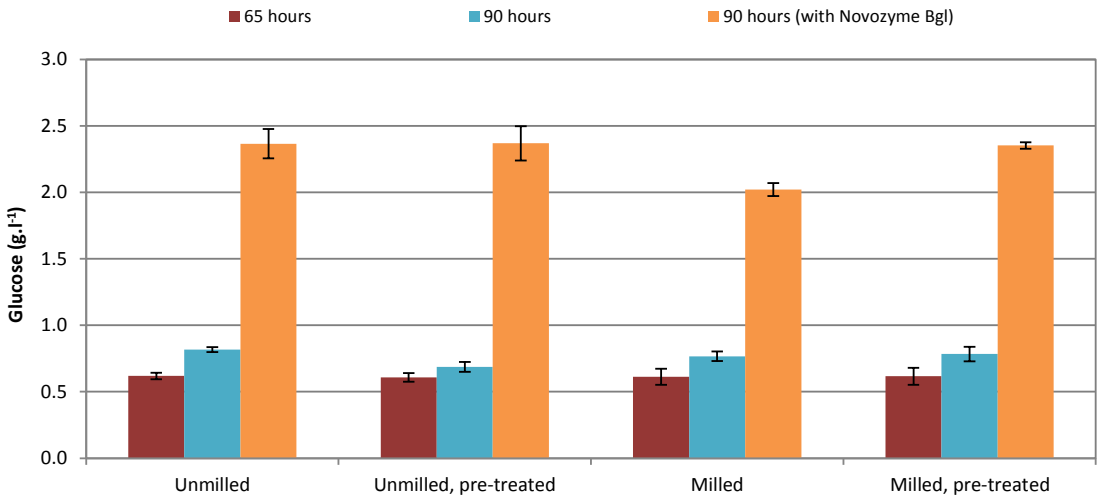
3.1.1. Effect of pre-treatment and Novozyme Bgl addition

The first phase of this study involved selecting the type of wheat bran substrate by comparing the effect of when different pre-treatment methods on hydrolysis. The ANOVA test did not reveal significant differences for wheat bran hydrolysis when the four types of wheat bran were compared (Figure 1). Furthermore, no significant

differences were detected with the interactions between factors, i.e. substrate and acid addition. Accordingly, at a substrate loading of 2%, milling and mild acid pre-treatment did not significantly influence the release of sugars from wheat bran.

To determine the residual glucose that could be released after 65 hours of incubation, one replicate from each hydrolysis reaction was treated with 5 µl of Novozyme Bgl (Figure 1) and incubated for an additional 25 hours. The addition of the commercial Novozyme Bgl to the unmilled wheat bran substrate resulted in a significantly higher glucose yield (2.37 $g.l^{-1}$) after 90 hours, compared to reactions that only contained the 1x enzyme cocktail (0.82 $g.l^{-1}$). It should be noted that Novozyme Bgl contained high amylase activity (Table 2) and that the starch content in wheat bran was about 11% (Table 3). Glucose released by the amylolytic activity thus masked the glucose released from cellobiose hydrolysis.

A



B

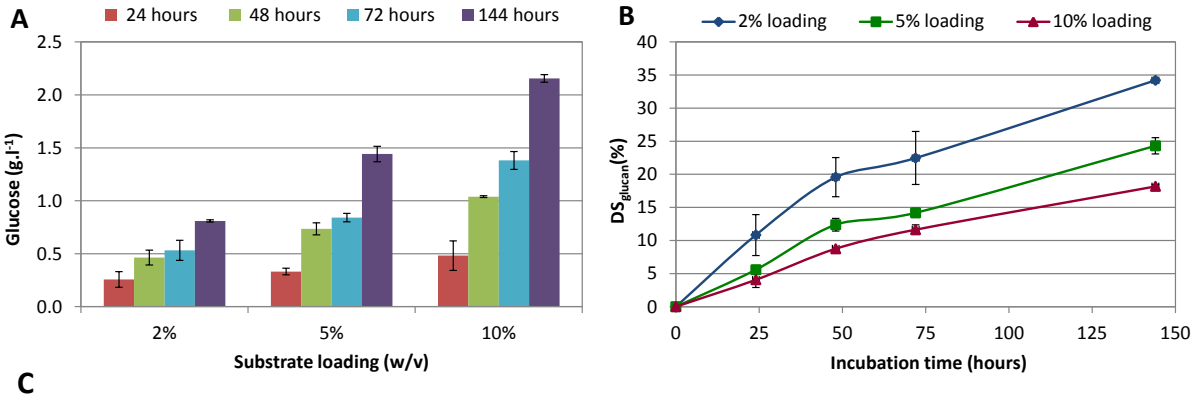
Parameter	F value	Probability of F value	Significance
Substrate	0.002	0.966	ns
Acid addition	0.054	0.819	ns
Substrate x Acid addition	0.177	0.679	ns

Figure 1: (**A**) Glucose yield ($g.l^{-1}$) determined after the hydrolysis of 2% (w/v) wheat bran with a 1x enzyme cocktail. Average values are from three replicates and standard deviations are reported. (**B**) Statistical evaluation by ANOVA of the effect of different substrates, acid addition and their interaction on hydrolysis after 65 hours (ns: not significant)

3.1.2. Effect of substrate loading

After establishing that pre-treatment had no significant effect on wheat bran hydrolysis (Figure 1), unmilled wheat bran (without pre-treatment) was used throughout for the rest of the study. Hydrolysis was subsequently performed with different substrate loadings and the glucose released, measured (Figure 2A). The degree of saccharification (DS_{glucan}) for wheat bran hydrolysis was determined for the hydrolysis of 2%, 5% and 10% substrate loadings (w/v), resulting in hydrolysis efficiencies of 34.20%, 24.30% and 18.16%, respectively, after 144 hours (Figure 2B). However, supplementation with Novozyme Bgl at 72 hours resulted in significantly higher glucose yields (Figure 3A).

The glucose yield at 144 hours (with Novozyme Bgl supplementation at 72 hours) (Figure 3A) was not comparable to the glucose released at 144 hours when Novozyme Bgl supplementation occurred at the beginning of the reaction (Figure 3B). The addition of the 1x enzyme cocktail (Figure 3B) resulted in an increase of 1.60 $g.l^{-1}$ at 144 hours, corresponding to a 10.40% increase in hydrolysis.



C

Parameter	*F* value	Probability of *F* value	Significance
Substrate loading	235.74	<0.001	**
Incubation time (h)	256.24	<0.001	**
Substrate loading x h	26.12	<0.001	**

Figure 2: Effect of three substrate loadings (2%, 5% and 10%) on the hydrolysis of wheat bran using a 1x enzyme cocktail. The glucose (**A**) from different substrate loadings and the degree of saccharification (DS_{glucan}) (**B**) was calculated for wheat bran hydrolysis at 2%, 5% and 10% substrate loadings. (**C**) Statistical evaluation by ANOVA of the effect of different substrate loadings, time (h) and their interaction on hydrolysis after 144 hours (**p<0.01).

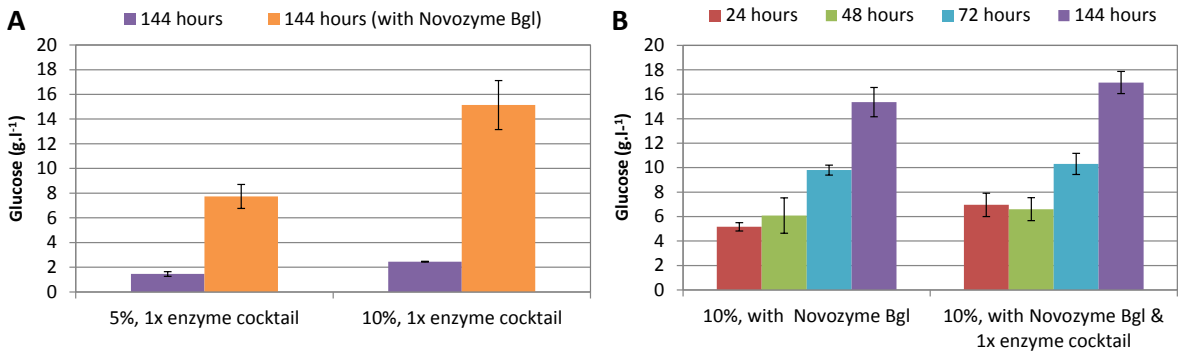


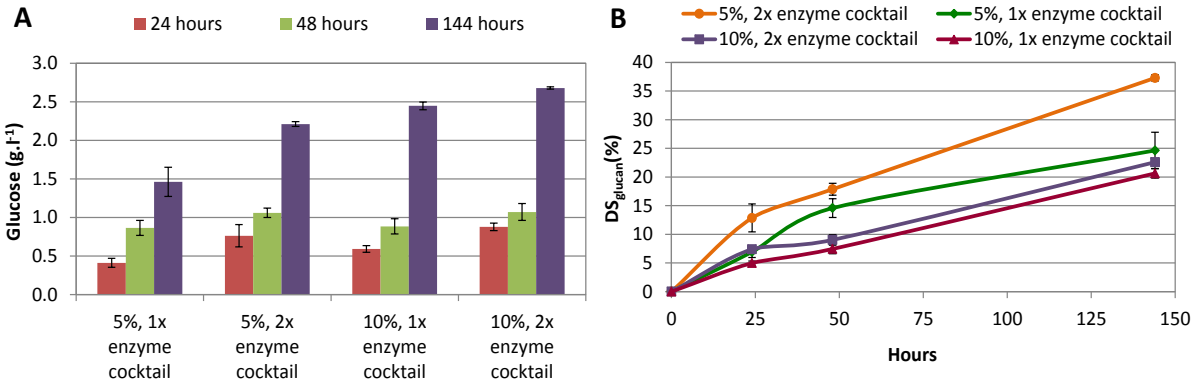
Figure 3: (**A**) Effect of Novozyme Bgl supplementation after 72 hours, on the hydrolysis of wheat bran. (**B**) The amount of glucose released over time (10% substrate loading) with Novozyme Bgl supplementation at the beginning of the reaction, as well as in combination with the 1x enzyme cocktail.

With regards to substrate loading, the best hydrolysis efficiency of 34.20% was obtained using 2% wheat bran (Figure 2A). However, the aim was to achieve the maximum release of glucose. Therefore, a 10% substrate loading would be better suited for a SSF process; even though the DS_{glucan} was low, the amount of glucose released would be high enough (more than 1 $g.l^{-1}$ after 48 hours) to support growth. Increasing the substrate loading above 10% was not possible, as the reaction mixture would became too viscous, compromising proper mixing.

3.1.3. Effect of enzyme loading (no Novozyme Bgl)

The effect of enzyme loading at two different substrate loadings was investigated (Figure 4A). When the enzyme loading was doubled (2x enzyme cocktail), the glucose yield after 24 hours increased by 85.72% and 48.48% for the 5% and 10% substrate loadings, respectively, relative to the 1x enzyme cocktail. At 144 hours, the increase was, 51.37% and 9.49%, respectively (Figure 4A).

When the efficiency of these reactions is considered (Figure 4B), it was observed that the highest degree of saccharification was achieved with a 2x enzyme cocktail and 5% substrate loading. Furthermore, the DS_{glucan} using a 10% substrate loading and 2x enzyme loading, was only 1.96% higher than for a 1x enzyme loading, whereas a 12.65% increase was noted when the enzyme concentration was doubled using a 5% substrate loading. The ANOVA test revealed a significant increase in the glucose yield when the substrate loading, the enzyme loading, or treatment time increased.



C

Parameter	*F* value	Probability of *F* value	Significance
Substrate loading	28.60	<0.001	**
Enzymatic loading	216.26	<0.001	**
Incubation time (h)	470.42	<0.001	**
Substrate loading x Enzymatic loading	<0.001	0.957	ns
Substrate loading x h	15.25	<0.001	**
Enzymatic loading x h	19.56	<0.001	**

Figure 4: Effect of substrate and enzyme loadings on enzymatic hydrolysis. (**A**) Experiments were carried out with 5% and 10% substrate loading (w/v) of unmilled wheat bran and two different enzyme loadings: 1x enzyme loading and a 2x enzyme loading. (**B**) The degree of saccharification (DS_{glucan}) of wheat bran at 5% and 10% substrate loadings with different enzyme loadings. (**C**) Statistical evaluation by ANOVA of the effect of substrate loading, enzymatic loading and incubation time (h), as well as their interactions on hydrolysis (ns: not significant; **p<0.01).

3.2. Fermentation studies on wheat bran

A substrate loading of 10% (w/v) was used for the SSF as it gave the best glucose yields in the hydrolysis trials (Figure 3). Ethanol production by the *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA]) strains was determined in fermentation media containing 30 $g.l^{-1}$glucose (Figure 5). Wheat bran from the Italian milling group, Grandi Molini Italiani typically contains 10% cellulose and 20% starch (equal to 30% carbohydrate on average).

The maximum ethanol concentrations were reached at 24 hours for both the *S. cerevisiae*S2 [TLG, SFA] and MH1000[TLG, SFA] strains, namely14.34 ± 0.15 $g.l^{-1}$ and 13.99 ± 0.16 $g.l^{-1}$, respectively. This represents 93.7% and 91.4%, respectively, of the theoretical maximum yield. All the glucose in the media was

metabolised within 18 hours of fermentation. The yeast strains did not display cellulase activity, since a constant cellobiose concentration of 0.06 g.l^{-1} was measured throughout the fermentation process (data not shown).

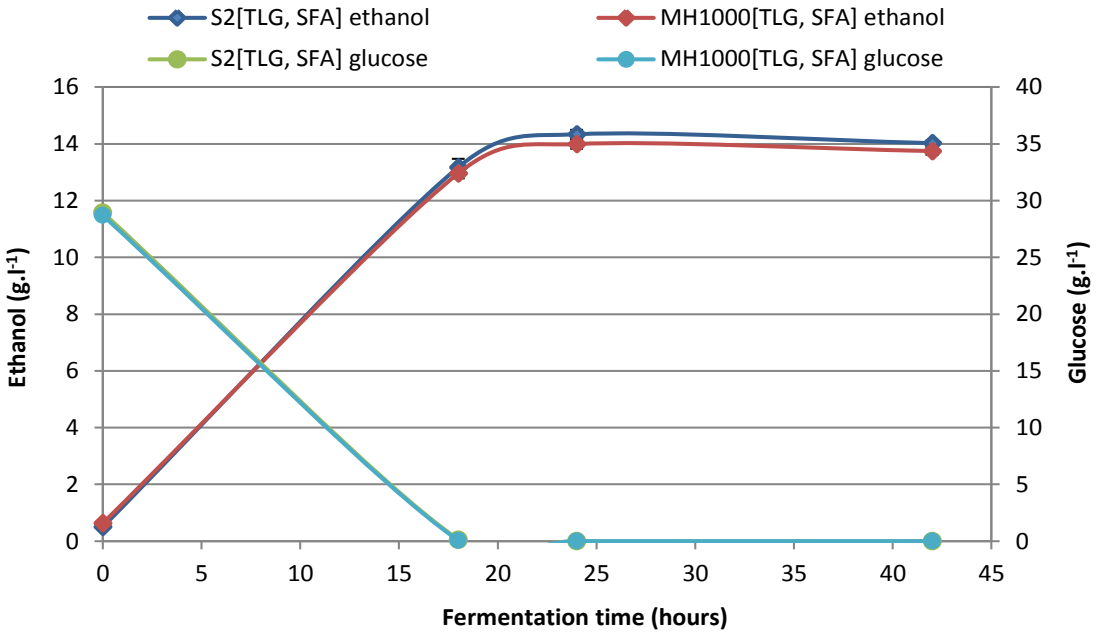


Figure 5: Ethanol production and glucose concentrations in the supernatant of *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA] strains, cultured anaerobically in the fermentation medium, which contained an initial 30 g.l^{-1} glucose.

The *S. cerevisiae* S2[TLG, SFA] strain in the presence of 1x enzyme cocktail supplemented with Novozyme Bgl yielded the highest ethanol concentrations in the SSF (7.21 ± 0.23 g.l^{-1}), after 72 hours (Figure 6B). The ethanol concentration was 0.33 g.l^{-1} higher than that obtained for the *S. cerevisiae* MH1000[TLG, SFA] strain under similar conditions (Figure 6B). The highest ethanol concentrations after 72 hours by the amylolytic strains in the presence of only the 1x enzyme cocktail, were 5.71 ± 0.07 g.l^{-1} and 5.45 ± 0.09 g.l^{-1} for *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA],respectively (Figure 6A). In contrast the ethanol concentrations when the only enzyme source was Novozyme Bgl, were 6.68 ± 0.15 g.l^{-1} and 6.52 ± 0.12 g.l^{-1} for *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA], respectively (Figure 6B).

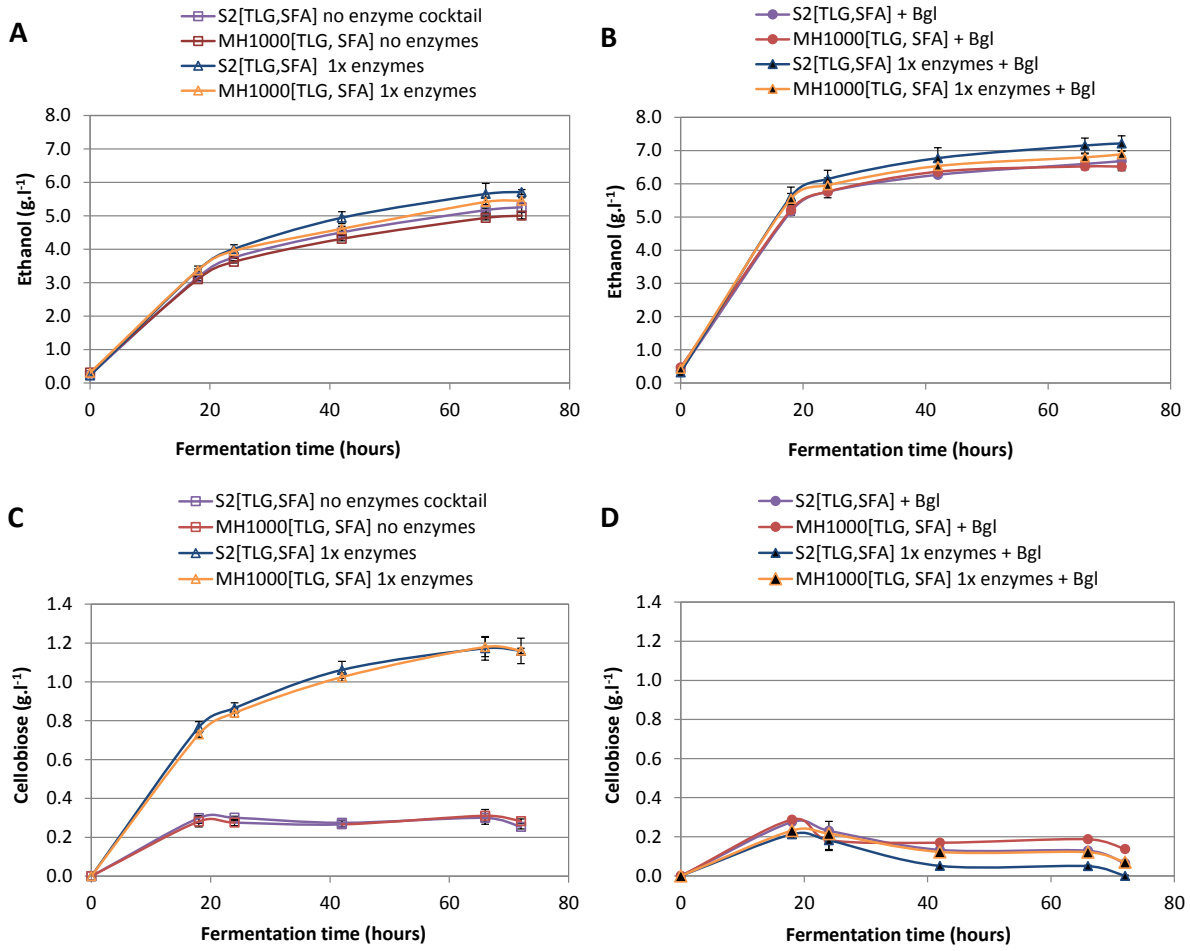


Figure 6: Fermentation products produced during the SSF process on unmilled 10% wheat bran, pH5 and 30°C by amylolytic *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA] strains. (**A**) Production of ethanol when 1x enzyme cocktail was added to reaction compared to control (no enzymes). (**B**) Ethanol produced when Novozyme Bgl was added to 1x enzyme cocktail compared to control (only Novozyme Bgl). (**C**) Cellobiose accumulation in reaction with 1x enzyme cocktail compared to control (no enzymes). (**D**) cellobiose accumulation when Novozyme Bgl was added to 1x enzyme cocktail compared to control (only Novozyme Bgl). Data shown is the mean values of three replicates and standard deviations are included.

An increase of 1.16 $g.l^{-1}$cellobiose can be noted when the 1x enzyme cocktail was added to the fermentation with both *S. cerevisiae* S2[TLG, SFA] and MH1000[TLG, SFA] (Figure 6C). Subsequently, the combination of Novozyme Bgl and the 1x enzyme cocktail allowed for complete hydrolysis of the cellobiose and thus prevented its accumulation in the SSF process (Figure 6D). Furthermore, cellobiose concentrations remained low (below $0.2g.l^{-1}$) for the reactions that only contained Novozyme Bgl as the enzyme source (Figure 6D).

In order to establish the cellobiose and glucose concentrations that resulted from the addition of the 1x enzyme cocktail and/or the Novozyme Bgl a hydrolysis reaction with the same conditions as the SSF (except no yeast strains) was run in parallel to the fermentation (Figure 7). High cellobiose concentrations were observed in hydrolysis reactions that contained the 1x enzyme cocktail (1.01 ± 0.02 $g.l^{-1}$), while all the cellobiose in the reaction was hydrolysed when the 1x enzyme cocktail was supplemented with Novozyme Bgl. Furthermore, the 1x enzyme cocktail supplemented with Novozyme Bgl yielded more glucose than the 1x enzyme cocktail by itself.

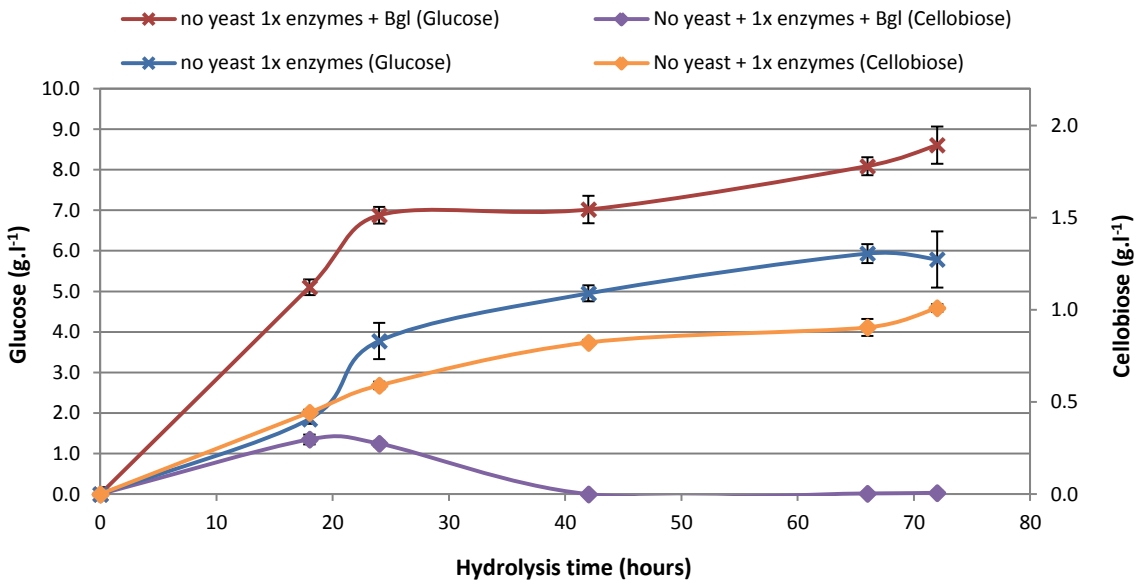


Figure 7: Glucose and cellobiose production from10% wheat bran loading, using a 1x enzyme cocktail and a 1x enzyme cocktail supplemented with Novozyme Bgl. Data shown is the mean values of three replicates and standard deviations are included.

4. Discussion

Wheat bran is a residual product from the flour industry and represents a promising feedstock for the production of bioethanol. It has a complex chemical composition, which includes carbohydrates, proteins, minerals and vitamins (Liu *et al*, 2010). The purpose of this study was to evaluate the possibility of converting wheat bran, a waste product with low cellulosic content, into bioethanol through SSF.

4.1. Recombinant enzyme cocktail and hydrolysis

This study was designed to evaluate wheat bran hydrolysis using a recombinant cellulase enzyme cocktail. The 1x enzyme cocktail contained CbhI:CbhII:EgA:Bgl2 with a protein ratio of 7.4:6.6:1:41 and a final protein concentration of 3.29 mg.ml^{-1}). The wheat bran was also evaluated as a substrate (where both the cellulose and the starch components were utilised) in an industrially simulated SSF process.

Substrate use: From an industrial point of view, wheat bran represents an inexpensive feedstock for bioconversion processes. The composition of wheat bran used in this study is similar to that used by Palmarola-Adrados *et al*. (2005). Inhibitors are released during sulphuric acid pre-treatment, which can have an effect on processes such as enzymatic hydrolysis and the fermentation of sugars by yeast strains (Liu *et al*., 2010). A study by Favaro *et al*. (2013) showed that mild pre-treatment produced only low concentrations of acetic acid and lactic acid, while other commonly known inhibitory by-products, such as furfural and 5-hydroxymethyl-2-furaldehyde (HMF), were not detected. Since mild pre-treatment improved enzymatic saccharification (95% of the theoretical yield) (Favaro *et al*., 2013), 1% sulphuric acid was used as pre-treatment option in this study.

Substrate hydrolysis: The ANOVA test did not reveal significant differences on wheat bran hydrolysis when the four types of wheat bran were compared (Figure 1). Therefore, unmilled wheat bran (preferred from an industrial perspective) was used for further hydrolysis experiments, as well as in the SSF study. Furthermore, pre-treatment processes add extra costs to the overall bioconversion process and if this step could be avoided, bioconversion costs can be reduced. A 2% substrate loading corresponds to 10.68% cellulose in the hydrolysis reaction. A low substrate loading was chosen to eliminate possible inhibition of the enzymes as a result of the accumulating glucose. The low cellulose content might explain why there was not a significant difference in glucose yields between the different pre-treatments (Figure 1); this might not be the case if a higher substrate loading was used to determine the effect of pre-treatment.

A 5% substrate loading resulted in a higher DS_{glucan}, compared to a 10% substrate loading (Figure 4).Enzymatic hydrolysis at high substrate loadings has been identified as one of the main bottlenecks that affects ethanol yield and titre (Zhu *et al*., 2011). An increase in substrate loading affects the slurry viscosity and therefore the degree of saccharification decreases. Due to stirring difficulties, reduction of the aqueous mobile phase and end product inhibition (Rodhe *et al*., 2011); defining the optimum loadings conditions are therefore crucial for maximising the hydrolysis of a substrate. When substrate loadings are increased, more glucose is released, which increases product inhibition (Zhu *et al*., 2011).

Although the recombinant cellulase enzyme cocktail contained a β-glucosidase, the activity of this enzyme was insufficient to prevent the accumulation of cellobiose, resulting in product inhibition of the endoglucanase and cellobiohydrolases (Figure 6C). Therefore, supplementation of the cocktail with Novozyme Bgl resulted in a decrease in cellobiose concentrations and an increase in glucose concentrations. The increase in glucose concentrations is attributed to the hydrolysis of cellobiose, as well as the hydrolysis of the starch in the wheat bran, due to the amylase activity present in Novozyme Bgl preparation (Table 2).

The β-glucosidase activity is a limiting factor during the hydrolysis of cellulose and this is evident in both the hydrolysis trials and the SSF study (Figure 3A and Figure 6A). The effect on cellulose and starch hydrolysis was investigated when Novozyme Bgl was added to different hydrolysis reactions (Table 2 and Figure 7). The maximum amount of glucose released from wheat bran is dependent on the time at which the β-glucosidase is administered (Figure 3). Therefore, in order to achieve optimal hydrolysis, supplementation should occur at the beginning of the hydrolysis experiment. Subsequently it was decided that Novozyme Bgl addition should be included in the SSF study at the onset of the experiment (Figure 6).

The main goal of the enzymatic hydrolysis trials was to investigate the effect of pre-treatment, substrate loading and enzyme loading on the hydrolysis of wheat bran for maximum glucose yield, regardless of the hydrolysis efficiency (DS_{glucan}). Therefore, even though the highest degree of saccharification was reached with 2% substrate loading (Figure 2), the glucose release from this loading would be

inadequate for an SSF process. Hence, a 10% substrate loading was used for the remainder of the study.

Degree of saccharification: The hydrolysis efficiency (DS_{glucan}) could not be calculated from reactions that contained Novozyme Bgl addition (Figure 2 and 3), because hydrolysis is attributed to both β-glucosidase and amylase activity (Table 2). Therefore, the DS_{total} (Equation 2) was considered to compare the hydrolysis efficiency when hydrolysis reactions contained Novozyme Bgl. At 72 hours during the hydrolysis trials (5 ml volume and 10% substrate loading), the DS_{glucan} for the efficiency of the1x enzyme cocktail was 11.63% (Figure 2B) and the DS_{total} for the efficiency of the 1x enzyme cocktail supplemented with Novozyme Bgl was 42.74% (Figure 3B).

Subsequently, the DS_{glucan} and DS_{total} were determined using the glucose concentrations obtained from the hydrolysis reactions that was run in parallel to the SSF process (50 ml volume) (Figure 7). The DS_{glucan} for the hydrolysis from the 1x enzyme cocktail was 10.74%, while the DS_{total} from the 1x enzyme cocktail supplemented with Novozyme Bgl was 35.70% (Figure 7);this is 7.60% and 16.47% lower, respectively, than for the preliminary hydrolysis trials (5 ml volume). This difference can be attributed to the differences in the experimental set up (different volumes and mixing conditions), as well as the method used to measure glucose yield (D-glucose kit versus HPLC).

4.2. Fermentation

The hydrolysis trials were conducted at 30°C, which is the optimal temperature for yeast cultivation. Subsequently, the conditions used for the SSF were the same as in the hydrolysis trails. The *S. cerevisiae* S2[TLG, SFA] and MH100[TLG, SFA] strains exhibit amylolytic activity, which provides promising fermentative abilities when using starchy feedstocks. To compare these two strains a fermentation was conducted using media that contained 30 $g.l^{-1}$ glucose and no wheat bran (Figure 5); both strains produced ethanol quickly and the maximum yield was measured at 24 hours, after which no increase in ethanol was observed.

The addition of the 1x enzyme cocktail to the fermentation resulted in an additional 0.4 $g.l^{-1}$ethanol being produced at 72 hours, compared to when no enzyme were added (Figure 6A). The addition of the 1x enzyme and Novozyme Bgl resulted in 35% increase in ethanol levels, with an average increase of 1.8 $g.l^{-1}$ ethanol (for the 2 strains) at 72 hours compared to when no enzymes were added to the fermentation reaction (Figure 6B). The addition of Novozyme Bgl did not significantly improve the ethanol yield when the 1x enzyme cocktail was used, despite the high levels of amylase activity. This indicates that the amylase activity produced by the yeast strains was sufficient for starch conversion. Traditionally, α-amylases and glucoamylases have been used in pre-treatment processes for starchy grains prior to fermentation. However, the use of amylolytic yeasts as the saccharifying agent could supplement or replace the use of commercial amylases, e.g. Stargen 001 (Genencor) for raw starch hydrolysis (Gibreel *et al*., 2009).

The SSF using wheat bran and the recombinant enzyme cocktail demonstrated that sufficient substrate hydrolysis could take place. However, the cocktail only contained four major activities, which are not sufficient for complete cellulosic hydrolysis. Additional accessory enzyme would be required for significant hydrolysis of lignocellulosics, such as wheat bran. The presence of the 1x enzyme cocktail facilitated an increase of 8.69% in ethanol yields; maximum ethanol yields were reached after 72 hours. Both the starch and cellulose components were hydrolysed during SSF, indicating that wheat bran is a promising feedstock for the production of bioethanol.

Not many studies have been conducted using wheat bran as substrate. It is a potential feedstock for ethanol production that does not require pre-treatment for hydrolysis (Favaro *et al*., 2013; Lui *et al*., 2010). It has been reported that wheat bran (pre-treated with diluted sulphuric acid) with the addition of *Clostridium beijerinckii* ATCC 55025, can result in an ethanol yield of 0.8 $g.l^{-1}$ within 72 hours (Lui *et al*., 2010). Therefore, it is worthwhile optimising current technologies, to improve ethanol production from wheat bran.

This study confirms that β-glucosidase activity is a limiting factor in the hydrolysis of wheat bran. Furthermore, statistical analysis shows that enzyme and substrate loading are two important parameters that need to be optimised for industrial applications.

5. Acknowledgments

The authors would like to thank the National Research Foundation (NRF) for financial support. This study was supported by the bilateral joint projects for the exchange of researchers within the "Executive Programme of Scientific and Technological Cooperation" between Italy and South Africa, at the Padua University and Stellenbosch University (Project Number ZA11MO2). We would also like to express our gratitude to Mr Federico Fontana for HPLC analysis, as well as Prof. Sergio Casella for the opportunity to work in his laboratory.

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Chapter 5: General discussion and conclusions

1. Discussion

Lignocellulose conversion technologies are currently uneconomical due to the high cost of hydrolytic enzymes. Commercial enzyme cocktails contain enzyme combinations that are not optimised for a specific substrate and may contain additional enzymes that are not required for hydrolysis of a particular type of feedstock. For example Novozyme 188 and Novozyme Bgl (NS50010 of Novozyme AS) contain significant amylase activity that is not required for the hydrolysis of lignocellulosic material (Shao *et al*., 2010). Similarly, these preparations contain β-glucosidase and xylanase activities that are not needed for starch hydrolysis (Pallapolu *et al*., 2011).

Defining the essential enzymes in the optimal ratio needed for the hydrolysis of a specific substrate will enable a more efficient and cost effective hydrolysis process. Defining the core set of enzymes requires the use of individual enzymes, which can be obtained through heterologous expression systems (Garvey *et al*., 2013). The use of tailor-made enzyme cocktails will provide insight into the hydrolysis of lignocellulose and the synergy between the individual enzymes. An understanding of the optimal enzyme combinations and ratios of these enzymes is essential to develop the ideal hydrolysis conditions for a specific substrate.

Lignocellulosic biomass that is obtained from industrial waste (e.g. paper sludge) or agriculture residues (e.g. wheat bran and triticale straw) represents an abundant, inexpensive and renewable feedstock. It is estimated that the pulp and paper industry in China produces around 4000 dry tons (dry weight) of paper sludge per day, while around three million tons of paper sludge is produced annually by the United States of America (Fan & Lynd, 2007). The approximately 500 kraft mills and thousands of other types of pulp and paper mills (Rashid *et al*., 2006) around the world can supply a sustainable feedstock for biofuel production. Paper sludge consists mainly of cellulose and does not require additional pre-treatment, making it an attractive feedstock for bioconversion to ethanol. Therefore, paper sludge was used as substrate for the development of an enzyme cocktail, which was subsequently evaluated on triticale straw, Avicel and wheat bran.

Factors such as enzyme ratios, enzyme and substrate loading, as well as pre-treatment options were investigated in this study. Pre-treatment methods generally increase the yield of inhibitors; however, most of the studies were conducted on substrates without any pre-treatment and the effect of inhibitors was thus omitted. One of the main goals of the study was to establish experimental conditions that yielded the highest amount of glucose as fermentable sugar for ethanol production during SSF.

2. Summary

- The β-glucosidase (*bgl1*) of *Aspergillus niger* was over-expressed in *A. niger* D15 and the Bgl1 enzyme evaluated and included in one of the final cocktails.
- During the development of the recombinant enzyme cocktail, it was noted that the different β-glucosidases displayed different affinities for cellulosic substrates. Bgl1 has a higher affinity for cellobiose, while Bgl2 has a preference for *p*NPG.
- The endoglucanases tested in the recombinant enzyme cocktail resulted in different DS_{glucan} values on the substrates evaluated (paper sludge, triticale straw and Avicel). The use of EgI yielded a more consistent DS_{glucan} for all the different substrates, compared to EgA.
- Increasing the concentration of the CbhI:CbhII:EgA:Bgl2 cocktail (2x enzyme cocktail)yielded significantly more glucose.
- Glucose yields of below 0.1 $g.l^{-1}$ obtained from paper sludge hydrolysis using a 4-enzyme cocktail (after 48 hours) were too low to support a SSF process.
- Higher glucose yields of greater than 1 $g.l^{-1}$ were obtained after 48 hours from wheat bran hydrolysis, using the 4-enzyme cocktail (CbhI:CbhII:EgA:Bgl2).
- The amylolytic *S. cerevisiae* S2[TLG, SFA] and MH1000 TLG, SFA] strains were compared in an SSF process and similar ethanol yields of 5.26 $g.l^{-1}$ and 5.72 $g.l^{-1}$ were obtained, respectively, indicating that the fermentative abilities of these strains are similar when combined with the 4-enzyme cocktail.

Objectives achieved during this study:

1. The *Aspergillus niger* β-glucosidase gene (*bgl1*) bgl1 was over-expressed in *A. niger*.
2. Recombinant enzyme cocktails were developed for the hydrolysis of paper sludge.
3. Evaluation of the cellulase cocktail on different substrates was performed and supports the need for tailored enzyme cocktails that can be used in combination with specific types of biomass for maximum release of sugars (without containing unnecessary enzymes). However, the enzyme activities were not high enough to release sufficient fermentable glucose. Therefore, the cocktails could not be tested in an SSF process using paper sludge as the feedstock
4. Comparison of the two amylolytic *S. cerevisiae* stains S2[TLG, SFA] and MH1000[TLG,SFA] in the SSF process showed that these strains facilitated the efficient release of sugars during the hydrolysis of starch; ethanol yields improved by around 10% when the 1x enzyme cocktail was added.

5. Current status and future research

Second-generation lignocellulosic biofuels are becoming a reality. The first commercial-scale cellulosic ethanol plant was opened in Italy at the end of 2012 (Gusakov, 2013), with the expectation that full-scale commercial biorefineries will soon be established worldwide. Currently, the enzymatic hydrolysis of cellulose is still considered to be the key step in the bioconversion of lignocellulosic biomass. At present, cellulases are only the third largest group of industrial enzymes produced worldwide. However, with the predicted expansion of the biofuels industry, cellulase is expected to become the largest group. The main producers of microbial cellulases have, in the past, been filamentous fungi, particular *T. reesei*. However, alternative eukaryote and prokaryote species need to be investigated for enzyme production (Mohanram *et al*., 2013). The characterisation of novel enzymes will also benefit the enzyme industry.

In order to be economically and practically effective, commercial cellulase cocktails need to be tailored for both biomass source and biomass pre-treatment. There are three main routes to obtaining novel and improved enzymes that can then be tested for cocktail development: bioprospecting, mining plant pathogens for hydrolytic enzymes and engineering enzymes through directed evolution, rational design and multifunctional chimeras (Mohanram *et al*., 2013). The latter strategies can result in improved enzymes and are recommended for enzyme cocktails tailored to lignocellulosic feedstocks.

This study was a starting point for the evaluation of paper sludge as a feedstock for enzymatic hydrolysis of cellulose to glucose. This efficiency of the enzyme cocktail was not evaluated in an SSF process, since glucose yields obtained from paper sludge were not high enough to sustain growth. There is therefore scope to improve the glucose yields in order to allow for ethanol to be produced during SSF. Washing the paper sludge prior to SSF could result in a lower ash content, which could improve glucose yields during the hydrolysis trials.

The low activity of the β-glucosidases (Bgl1 and Bgl2) in the recombinant enzyme cocktail resulted in a bottleneck in the hydrolysis of cellulose. The accumulation of cellobiose results in the feedback inhibition of endoglucanses and cellobiohydrolases. Therefore, improvements still need to be made to the enzyme cocktail, to increase the β-glucosidase activity. Alternatively, other β-glucosidases can be tested, or the specific activity/stability of the Bgl1 and Bgl2 can be improved through protein engineering.

The engineering of microbial systems to convert lignocellulose to bioethanol using the CBP concept will reduce conversion costs. A recombinant yeast strain that is able to produce a cellulase cocktail will be capable of utilising lignocellulose as the sole carbon source. Since this theoretical organism will produce its own cellulases, cellulose can be converted to fermentable sugars, which in turn will be utilised for ethanol production. Research focused on developing a CBP yeast host that expresses cellulases in an optimal ratio is still under construction. The relevance of this CBP yeast is that it will provide direct starch conversion to ethanol. Subsequently, overall costs can be reduced, which would result in a more

economical process and hopefully contribute significantly to advances in cellulosic ethanol production.

Fermentations using wheat bran were only done on a small scale (55 ml fermentation bottles) and future studies are needed to scale-up the SSF in a bioreactor. Although current methods for SSF are efficient, alternative strategies can be explored to improve ethanol production using this process. These need to be aimed at substrate pre-treatment and strain improvement. Experimental work needs to be undertaken to examine the relationship between feeding frequency and cellulase loading, as well as to characterise mixing energy requirements for wheat bran in relation to concentration and conversion. An economic impact study can also be conducted to establish the feasibility of integrating wheat bran into a biorefinery. This will allow for an economic model to be developed.

6. Conclusion

The pressing need for sustainable fuel production is the strong motivation for biomass derived fuels. Cellulosic ethanol is a type of biofuel that will partially assist in the solution to the energy crisis and its production is expected to have a significant impact on the global economy (in the next few years). This will directly impact many industries and promote the establishment of new biorefineries. Although cellulases have been extensively researched, crystalline cellulose hydrolysis is still not fully understood. It is critical therefore that technology for the conversion of biomass to fermentable sugars be optimised to clarify the hydrolysis process. An understanding of the hydrolysis process will lead to enzyme cocktails designed specifically for different biomass feedstocks. Tailored enzyme cocktails will require less enzymes, which will ultimately lead to a reduction in overall costs and improved hydrolysis results.

7. References

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