CONFERRING LIGNOCELLULOLYTIC CAPACITY TO A SUPERIOR INDUSTRIAL YEAST STRAIN FOR SECOND-GENERATION BIOETHANOL PRODUCTION

SIMULTANEOUS SECRETION OF SEVEN LIGNOCELLULOLYTIC ENZYMES BY AN INDUSTRIAL YEAST STRAIN ENABLES EFFICIENT ETHANOL PRODUCTION FROM MULTIPLE POLYMERIC SUBSTRATES

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Summary

Awareness of climate change encourages evolution of products towards more sustainable alternatives. One major driver for global warming is the extensive and increasing use of fossil fuels. To exonerate this issue, biofuels are investigated as potential fuel alternatives, of which bioethanol is one of the most promising biofuels. Second-generation bioethanol is produced from lignocellulosic biomass that originates from plant material. A major hurdle in the production of bioethanol with second-generation feedstocks is the high cost of the enzymes for saccharification of the lignocellulosic biomass into fermentable sugars. Simultaneous saccharification and fermentation with *Saccharomyces cerevisiae* yeast that secretes a range of lignocellulolytic enzymes might address this problem, ideally leading to consolidated bioprocessing. However, it has been unclear how many enzymes can be secreted simultaneously and what the consequences would be on the C6 and C5 sugar fermentation performance and robustness of the second-generation yeast strain. In this work, we have successfully expressed seven secreted lignocellulolytic enzymes, namely endoglucanase, β-glucosidase, cellobiohydrolase I and II, xylanase, β-xylosidase and acetylxylan esterase, in a single second-generation industrial *S. cerevisiae* strain. This AC14 strain was constructed by consecutive genomic integrations of the seven heterologous genes encoding these lignocellulolytic enzymes with CRISPR/Cas9. This method enabled efficient genome engineering in a polyploid, industrial *S. cerevisiae* strain. The resulting strain exhibited a high secreted activity on multiple polymeric substrates, reaching 94.5 FPU/g CDW *in vitro* and enabling direct conversion of lignocellulosic substrates into ethanol *in vivo* without preceding enzyme treatment. Neither glucose nor the engineered xylose fermentation were significantly affected by the enzyme secretion. This AC14 strain can therefore serve as a promising industrial platform strain for development of yeast cell factories for production of other bio-based chemicals which can significantly reduce the enzyme cost for saccharification of lignocellulosic feedstocks.

Samenvatting

Bewustzijn van klimaatverandering stimuleert een evolutie van producten richting meer duurzame alternatieven. Een belangrijke drijfveer voor het broeikaseffect is het uitgebreide en toenemende gebruik van fossiele brandstoffen. Om dit probleem te omzeilen worden biobrandstoffen onderzocht als potentiële vervangers, waarvan bioethanol één van de meest belovende biobrandstoffen is. Tweede-generatie bioethanol wordt geproduceerd van lignocellulose dat oorpronkelijk afkomstig is van plantmateriaal. Een belangrijke hindernis in de productie van bioethanol met tweede-generatie grondstoffen is de hoge kost van de enzymen voor de saccharificatie van de lignocellulose biomassa tot fermenteerbare suikers. Gelijktijdige saccharificatie en fermentatie met *Saccharomyces cerevisiae* gist die een assortiment aan lignocellulolytische enzymen secreteert, zou dit probleem kunnen oplossen, waarbij dit idealiter leidt tot geconsolideerde bioprocessing. Het is echter onduidelijk hoeveel enzymen gelijktijdig gesecreteerd kunnen worden en wat de gevolgen zouden zijn voor de fermentatieprestatie met C6 en C5 suikers en voor de robuustheid van de tweede-generatie giststam. In dit werk hebben we zeven gesecreteerde lignocellulolytische enzymen, namelijk endoglucanase, β-glucosidase, cellobiohydrolase I en II, xylanase, β-xylosidase en acetylxylaan esterase, succesvol tot expressie gebracht in één enkele tweede-generatie industriële *S. cerevisiae* stam. Deze AC14 stam werd geconstrueerd door middel van opeenvolgende genomische integraties van de zeven heterologe genen die coderen voor deze lignocellulolytische enzymen met behulp van CRISPR/Cas9. Met deze methode werd efficiënte genomische modificatie in een polyploïde, industriële *S. cerevisiae* stam mogelijk gemaakt. De resulterende stam vertoonde een hoge gesecreteerde activiteit op meerdere polymerische substraten, bereikte 94.5 FPU/g CDW *in vitro* en maakte onmiddellijke conversie van lignocellulose substraten in ethanol mogelijk *in vivo* zonder voorafgaande enzymebehandeling. Noch glucose, noch de ingebouwde xylose fermentatie werden significant aangetast door de secretie van de enzymen. Deze AC14 stam kan dus dienen als veelbelovende industriële platform stam voor de ontwikkeling van gistcelfabrieken voor de productie van bio-gebaseerde chemicaliën die de enzymekost voor saccharificatie van lignocellulose grondstoffen significant kan reduceren.

Abbreviations

1G	First-generation
2G	Second-generation
3G	Third-generation
ALP	Alkaline phosphate
AXE	Acetylxylan esterase
AXEXYL	Bifunctional acetylxylan esterase / xylanase
BGL	ß-glucosidase
BXL	ß-xylosidase
Cas	CRISPR-associated proteins
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CBP	Consolidated bioprocessing
CellG5	4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-cellopentaoside
Chr.	Chromosome
CMC	Carboxymethyl cellulose
CO	Carbon monoxide
CO_2	Carbon dioxide
CPY	Carboxypeptidase Y
CRISPR	Clustered regularly interspaced palindromic repeats
CTI	ß-cellotetraitol
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DSB	Double strand break
EC	Enzyme Commission
EG	Endoglucanase
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ESCRT	Endosomal sorting complexes required for transport
EU	European Union
FPU	Filter paper units
g CDW	Gram cell dry weight
GHG	Greenhouse gas
GMO	Genetically modified organism
GRAS	Generally regarded as safe
gRNA	Guide RNA
gTME	Global transcription machinery engineering
gTOW	Genetic tug-of-war
HDR	Homology directed repair
HMF	5-hydroxymethyl-2-furaldehyde
HPLC	High-performance liquid chromatography
IS	Integration site
kb	Kilobases
kDa	Kilodalton
LB	Luria-Bertani
LPMO	Lytic polysaccharide monooxygenase
MOPS	3-(N-morpholino)propanesulfonic acid
MU	4-methylumbelliferone
MULac	4-methylumbelliferyl-beta-D-lactopyranoside

$NADH/NAD^+$	Nicotinamide adenine dinucleotide
$NADPH/NADP^+$	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-homologous end joining
OD	Optical density
OST	Oligosaccharyl transferase
PAM	Protospacer adjacent motif
PASC	Phosphoric acid swollen cellulose
PCR	Polymerase chain reaction
pHBAH	p-hydroxybenzoic acid hydrazide
PMT	Protein *O*-mannosyltransferases
pNPA	p-nitrophenyl acetate
pNPC	4-nitrophenyl β-D-cellobioside
pNPG	4-nitrophenyl ß-D-glucopyranoside
pNPX	4-nitrophenyl β-D-xylopyranoside
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SHF	Separate hydrolysis and fermentation
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment receptor
SNP	Single nucleotide polymorphism
SPC	Signal peptidase complex
SRP	Signal recognition particle
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
U	Units
UPR	Unfolded protein response
XYL	Xylanase
XylX6	4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-4^5-glucosyl-xylopentaoside
YNB	Yeast nitrogen base
YP	Yeast extract – Bacto-peptone

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General aim and structure of this work

Plant-based chemicals are extensively explored as alternatives for petrol-based products. For the highly polluting petroleum-based transport fuels, research is focusing on development of second-generation biofuels, such as bioethanol. Second-generation bioethanol is produced from highly abundant and cheap lignocellulosic biomass using baker's yeast *Saccharomyces cerevisiae* and its most favorable trait, namely fermentation. This organism provides a good foundation for development of an agent for consolidated bioprocessing (CBP). This strategy infers that the lignocellulosic material is degraded to fermentable sugars by lignocellulolytic enzymes that are produced by the same host that also facilitates the fermentation process of the fermentable sugars to ethanol. This strategy is industrially appealing because of the current high enzyme cost required for saccharification of lignocellulosic biomass. However, *S. cerevisiae* originally lacks some characteristics to act as a CBP agent and to efficiently convert the second-generation feedstocks into ethanol. *S. cerevisiae* lacks the production and secretion of lignocellulolytic enzymes that degrade the biomass to fermentable sugars. Lignocellulose consists of cellulose, hemicellulose and lignin. These fractions consist of C6 sugars, such as glucose, and C5 sugars, such as xylose. *S. cerevisiae* excellently ferments C6 sugars, but is unable to ferment C5 sugars, which can account for up to 40% of the total sugar concentration. Furthermore, to ensure efficient fermentation of these sugars, a robust strain that withstands high concentrations of inhibitory compounds and other stress factors is required. To date, *S. cerevisiae* has been successfully engineered for efficient xylose fermentation as well as inhibitor and stress tolerance. On the other hand, to date, no yeast strain has been developed with the capacity to produce all essential lignocellulolytic enzymes for the breakdown of cellulose and hemicellulose.

This work aims to express and secrete four essential cellulases, namely endoglucanase, cellobiohydrolase I, cellobiohydrolase II and ß-glucosidase, and three hemicellulases, namely xylanase, ß-xylosidase and acetylxylan esterase, in an industrial xylose-fermenting, inhibitor-tolerant *S. cerevisiae* strain. To this purpose, genes originating from different species encoding (hemi-)cellulases are expressed in yeast and evaluated for specific enzymatic activity. This comparison enables the selection of the genes encoding for the most active proteins at industrially relevant conditions. Multiple copies of the selected genes are consecutively integrated in the genome of the industrial yeast strain using CRISPR/Cas9 methodology. With this methodology we are able to specifically target integration sites in the genome of the host organism to minimize side effects and optimize heterologous gene expression. The developed yeast strains are investigated for specific traits with regards to fermentation performance and heterologous (hemi-)cellulase activity. The final enzyme-secreting yeast strain would be able to degrade the lignocellulosic biomass into fermentable sugars by secretion of seven lignocellulolytic enzymes. Successful development of this strain brings us one step closer to an efficient CBP agent for the production of bioethanol and it can serve as a good platform strain for production of other bio-based chemicals.

The structure of this work is described in different chapters. The first chapter is devoted to sketching the bigger picture, introducing the work supported by a literature overview and emphasizing the importance and industrial relevance of this study. The following chapters are focusing on the consecutive genomic integration of several heterologous lignocellulolytic enzymes and the evaluation of the developed strains. Chapter two deals with the utilization of cellobiose to increase ethanol production using industrial *S. cerevisiae* strains that express and secrete a heterologous ß-glucosidase, even in real-life cellulosic hydrolysate. Chapter three discusses how consecutive expression of heterologous xylanolytic enzymes, namely ß-xylosidase and xylanase, enabled the

degradation of multiple xylan substrates to increase ethanol production during fermentation. The fourth chapter deals with the simultaneous secretion of three cellulases, namely endoglucanase, cellobiohydrolase I and cellobiohydrolase II. The fifth chapter discusses how xylan degradation could be improved by expression of an auxiliary hemicellulase, namely acetylxylan esterase, and evaluates the developed AC14 strain for its lignocellulolytic capacity towards multiple polymeric substrates. The sixth and final chapter gives the conclusions and some remarks on this work and its results. Furthermore, some future perspectives are proposed related to this study.

Chapter 1 - Literature overview

1. Green alternatives for fossil fuels

Sustainability of consumer products is the key driver for innovative development. This innovation is also observed in production of chemicals, as for example the shift from petro-based plastics to more sustainable and biodegradable compounds (Álvarez-Chávez et al., 2012). A text book example of chemical unsustainability is the use of fossil fuels. These fuels are subject to major instabilities such as price fluctuations, oil monopolies and the environmental impact (Caspeta et al., 2013). Greenhouse gas (GHG) emissions originating from fossil fuels are directly linked to many climate related issues (Change, 2014; Houghton et al., 1991). The transportation sector accounts for 60% of the world's consumption of fossil fuels and contributes 19% to the total energy-related carbon dioxide (CO_2) emissions and more than 70% of carbon monoxide (CO) (Aditiya et al., 2016; Balat and Balat, 2009). This sparks the interest for green and renewable alternatives for transport fuels, which in the case of fossil fuels can be biofuels, such as biodiesel, biogas and bioethanol (Gupta and Verma, 2015). Another green alternative on the rise is use of electricity to power vehicles. However, a major hurdle remains the size and capacity of the batteries, especially for heavy-duty road transport, marine vessels and airplanes. Also, supply of lithium is highly dependent on the import from countries where it is mined like Bolivia. This implies an unknown price variability, which is passed on the consumer's wallet. Moreover, it takes several hours to charge an electric vehicle while filling up a vehicle with biofuel takes five minutes. Nonetheless, combination of electricity and biofuel can solve a lot of the mutual disadvantages. These arguments may be a reason to look into hybrid vehicles that use electricity where possible and depend on biofuel to take over the more energy consuming processes.

1.1. Biofuels and bioethanol

Biofuels are defined as liquid or gaseous fuel derived from biomass (Demirbas, 2008). The European Union (EU) aims to have 10% of transport fuels of every EU country from renewable sources by 2020 (EUParliament, 2009). Currently, a Belgian Royal decree states that petrol fuels should contain minimally 4% from renewable source, whereas for diesel fuels this is 6%. In 2018, the Renewable Energy Directive II was endorsed by the European Parliament which targets the use of at least 14% of energy provided by renewable sources by 2030. However, the share of first-generation biofuels should not exceed 7%. These legislations and the fact that biofuels have a similar energy content as fossil fuels and are considered carbon-neutral, makes them a good alternative for petroleum-based fuels (Lee et al., 2008). Bioethanol can be applied as a gasoline additive or substitute (Balat and Balat, 2009; Demirbas, 2009) and was described as one of the most promising alternatives to conventional fossil fuels (Hasunuma et al., 2013) and is the focus of this work.

1.1.1. First-generation biofuels

The main producers of bioethanol are Brazil and the United States of America (USA), that are responsible for more than 90% of the total bioethanol production worldwide (Sims et al., 2010; Zabed et al., 2017). Both countries established efficient bioethanol production processes for first-generation (1G) feedstocks. 1G bioethanol is mainly produced from edible biomass, such as sugarcane, corn and sugar beets (Lee and Lavoie, 2013). Brazilian bioethanol is mainly produced from sugarcane juice and molasses, whereas in the USA bioethanol is produced mainly from corn starch feedstocks (Limayem and Ricke, 2012). Use of 1G feedstocks

commenced the ‘food versus fuel’ debate, since these biomasses can serve as food or animal feed (Cassman and Liska, 2007; Tenenbaum, 2008; Zhang et al., 2010b). However, use of food crops for fuel production did not affect the price of the corresponding food produced (Ajanovic, 2011; Zilberman et al., 2012). 1G bioethanol production is well-developed in several countries, but the edible feedstock dependence led to the development of second-generation (2G) bioethanol.

1.1.2. Second-generation biofuels

Where 1G biofuels provoke the debate of using food crops as fuel, 2G biofuels invalidate this matter since these are produced from lignocellulosic biomass, *e.g.* wood chips, corn stover and agricultural or municipal solid waste (Graham-Rowe, 2011; Maly and Degen, 2001), resulting in more sustainable and cheaper production (Caspeta et al., 2013; Taha et al., 2016; Tilman et al., 2009). Lignocellulose is an inexpensive carbon-rich biomass originating from plants that is widely available (Naik et al., 2010; Robak and Balcerek, 2018). For example, use of rice straw for ethanol production is estimated to yield around 205 billion liters of ethanol on a yearly basis (Sarkar et al., 2012). This agricultural waste is currently burnt on the fields without any added value (Binod et al., 2010).
However, large-scale production of 2G bioethanol is not yet economically viable due to multiple technological and cost barriers (Taha et al., 2016). Significant improvements need to be made in different areas to obtain competitive production processes and reduce the overall costs (Lynd et al., 2017). An improved understanding of the available feedstocks can enable an enhanced yield, optimized process parameters and eventually reduced production costs (Sims et al., 2010). In addition, pretreatment methods of lignocellulosic biomass can be optimized to achieve these purposes (Alvira et al., 2010; Jiang et al., 2019). Development of new and/or improved enzymes and enzyme cocktails is essential for an efficient hydrolysis (Binod et al., 2019). Recycling these enzymes can also help to reduce the production costs (Jørgensen and Pinelo, 2017; Yuan et al., 2018). Furthermore, fermentation organisms can be improved for specific traits to obtain efficient bioethanol production of lignocellulosic biomass. It can be greatly enhanced by conferring xylose fermentation capacity to the organism, as well as tolerance to fermentation inhibitors (Sims et al., 2010). It is clear that many factors can improve the production process and eventually lower the cost of bioethanol. There is a scientific consensus that taking these factors into account would make bioethanol competitive and industrially excelling (Aditiya et al., 2016; Lynd et al., 2017; Mohr and Raman, 2013; Taha et al., 2016; Zabed et al., 2017). This research potential was the motive to initiate this project and to reveal why we were devoted to enhance the production of 2G bioethanol.

1.1.3. Third-generation biofuels

Next to 1G and 2G biofuels, a third-generation (3G) biofuel production has been established. 3G bioethanol is produced from algal biomass, mostly relying on the high lipid content (Liang et al., 2009). The benefit of algae as substrate is found in the growth yield that is distinctively faster than that of lignocellulosic biomass (Chen et al., 2011). However, growing algae requires a large volume of water, which raises geographical and processing issues (Lee and Lavoie, 2013).

2. Lignocellulosic biomass as substrate for 2G bioethanol

Lignocellulose is a cheap, abundant and renewable resource of plant origin. It is a complex carbohydrate structure composed of different sugar polymers, namely cellulose and hemicellulose, and aromatic polymers, namely lignin (Fig. 1.1). In the plant cell wall, hydrogen and Van der Waals bonds link 20 to 300 cellulose chains together, thereby creating microfibrils. These are subsequently packed together by the same chemical bonds to form cellulose fibers, which are covered by hemicellulose and lignin (Meng and Ragauskas, 2014; Taha et al., 2016). These three components are present in different amounts depending on the plant species and its cell wall maturity (Table 1.1).

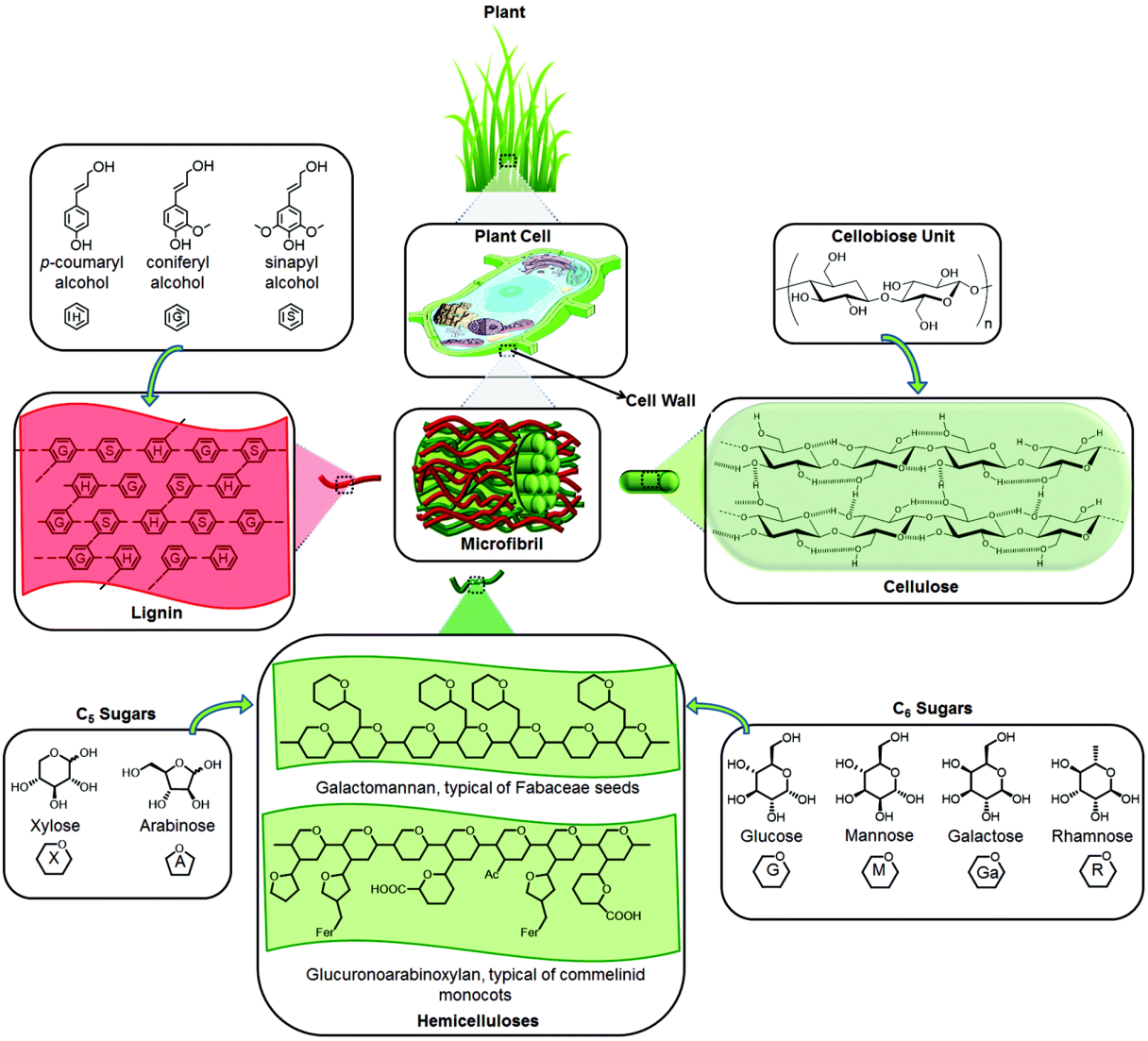


Figure 1.1. Schematic overview of the structural composition of lignocellulose. Plant cell walls are composed of microfibrils that consist of cellulose, hemicellulose and lignin. (Isikgor and Becer, 2015)

2.1.1. Cellulose

Cellulose is the most abundant component of lignocellulose, which also makes it the most plentiful carbon source on Earth (Behera et al., 2017). Cellulose is a linear polysaccharide that consists of D-glucose subunits linked by ß-1,4-glycosidic bonds and extensive hydrogen bonds (Fig. 1.1). The repeating unit of cellulose is cellobiose, which consists of two glucose molecules linked with a ß-1,4-glycosidic bond (Haghighi Mood et al., 2013; Isikgor

and Becer, 2015). Hydrogen bonds enhance the interaction between different cellulose chains, resulting in crystalline fibrils. While the majority of cellulose is present in the crystalline form, the amorphous form is more susceptible to enzymatic degradation (Aditiya et al., 2016; Kumar et al., 2008). Cellulose is responsible for 40% of the dry mass of the secondary cell wall and 15-30% of the dry mass of the primary cell wall in plants and contributes to the structural support and rigidity of plants (Zhao et al., 2012). Not only the total amount of cellulose differs between plant species, but also the degree of polymerization (DP) varies between plant types. This typically ranges between 10.000 and 15.000 glucose units (Bajpai, 2016; Ratanakhanokchai et al., 2013).

Table 1.1. Chemical composition of different lignocellulosic biomasses in terms of cellulose, hemicellulose and lignin. (Adapted from Isikgor and Becer (2015))

Lignocellulosic biomass		Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	Popular	50.8-53.3	26.2-28.7	15.5-16.3
	Oak	40.4	35.9	24.1
	Eucalyptus	54.1	18.4	21.5
Softwood	Pine	42.0-50.0	24.0-27.0	20.0
	Douglas fir	44.0	11.0	27.0
	Spruce	45.5	22.9	27.9
Agricultural waste	Wheat straw	35.0-39.0	23.0-30.0	12.0-16.0
	Barley hull	34.0	36.0	13.8-19.0
	Barley straw	36.0-43.0	24.0-33.0	6.3-9.8
	Rice straw	29.2-34.7	23.0-25.9	17.0-19.0
	Rice husks	28.7-35.6	12.0-29.3	15.4-20.0
	Oat straw	31.0-35.0	20.0-26.0	10.0-15.0
	Ray straw	36.2-47.0	19.0-24.5	9.9-24.0
	Corn cobs	33.7-41.2	31.9-36.0	6.1-15.9
	Corn stalks	35.0-39.6	16.8-35.0	7.0-18.4
	Sugarcane bagasse	25.0-45.0	28.0-32.0	15.0-25.0
	Sorghum straw	25.0-40.0	24.0-27.0	15.0-21.0
Grasses	Grasses	25.0-40.0	25.0-50.0	10.0-30.0
	Switchgrass	35.0-40.0	25.0-30.0	15.0-20.0

2.1.2. Hemicellulose

Hemicelluloses are the second most abundant polysaccharides next to cellulose occurring in plant cell walls, but unlike cellulose these polymers are not chemically homogenous. This heterogenous constituent of lignocellulose consists of a branched homo- or heteropolymeric backbone substituted with various side groups and mainly provides structural strength by linking cellulose fibers into microfibrils and cross-linking with lignin (Fig. 1.1) (Isikgor and Becer, 2015). The composition of the hemicellulosic fraction is highly variable and dependent on the plant species and on the type of plant (Table 1.1). The backbone is most commonly composed of D-xylose, D-mannose and D-glucose linked with ß-1,4-glycosidic bonds and is called xylan, mannan or glucan, respectively. The heteropolymeric nature of this component is caused by the high variability in side chains on the xylan, mannan or glucan backbone. The most frequent substitutions are D-galactose, L-arabinose,

(methylated) D-glucuronic acid and D-galacturonic acid (Carvalheiro et al., 2008; Timell, 1967). Also, commonly found side groups are acids, like acetic acid, *p*-coumaric acid or ferulic acid (Binod et al., 2019).
Xylan is the most frequent hemicellulose component in plant materials (Puls, 1997). Hardwood hemicellulose mainly consists of glucuronoxylan. This xylan is substituted with glucuronic acid, which is often methylated. Another fraction of hardwood hemicellulose is xyloglucan, glucan substituted with D-xylose. Glucuronoxylan also occurs in herbaceous hemicellulose, in which it is often substituted with L-arabinose. Softwoods, on the other hand, mainly contain galactoglucomannan, a mannose and glucose main chain with D-galactose substitutions, and glucuronoarabinoxylan (Puls, 1997; Scheller and Ulvskov, 2010; Timell, 1967).

2.1.3. Lignin

Lignin is an aromatic heteropolymer that forms a complex matrix shielding cellulose and hemicellulose, contributing to the biomass recalcitrance of lignocellulose and occurring mainly in secondary thickened cell walls (Vanholme et al., 2008). It offers several protective functions, *e.g.* rigidity, impermeability and it enhances the resistance against microbial attack and oxidative stress (Sanchez, 2009). It has the ability to form large structures by cross-linking polymers of phenolic monomers. Phenyl alcohols that are used as monomers for lignin production are coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol (Fig. 1.1) (Boerjan et al., 2003; Isikgor and Becer, 2015). Due to the close association of lignin with cellulose fibers and because of its ability to form covalent bonds with some hemicelluloses, it protects (hemi-)cellulose against microbial and chemical degradation (Martinez et al., 2005). This protective role of lignin also decreases the rate of the enzymatic hydrolysis of lignocellulose, which is detrimental to the bioethanol production process (Meng and Ragauskas, 2014). Lignin impairs enzymatic hydrolysis by irreversible adsorption of enzymes and by formation of inhibitory undesirable by-products in bioethanol production, such as phenolic alcohols. These degradation products originating from the pretreatment process of lignin, *e.g.* phenolic compounds, have inhibitory effects on the lignocellulolytic enzymes and on the metabolism of the fermentation organism (Ask et al., 2012). However, lignin can potentially serve as a self-sustaining energy source for biorefineries and production of various specialty chemicals (Ragauskas et al., 2014).

3. Production process of 2G bioethanol

The production process of second-generation bioethanol majorly affects the quality of the product and cost efficiency of the production (Aditiya et al., 2016). The general process always consists of pretreatment, hydrolysis, fermentation and distillation steps. The specific setup chosen for each step alters the production cost and the product quality. Additionally, since 2G bioethanol can be produced from various lignocellulosic biomasses, the different sugar contents also alter the final bioethanol yield (Demirbaş, 2005).

3.1. Pretreatment

The first step in bioethanol production is the pretreatment of the lignocellulosic substrate. The objective of this step is to physically reduce the size of the biomass, thereby creating more accessible, less crystalline substrate

prior to hydrolysis, resulting in more susceptible material for enzymatic decomposition (Fig. 1.2) (Mosier et al., 2005; Patel et al., 2007; Sanchez and Cardona, 2008).

An efficient pretreatment aims to result in immediate availability of fermentable sugars, to avoid loss or degradation of the sugars formed, to limit the formation of inhibitors, to reduce energy demands and to lower the overall processing cost (Aditiya et al., 2016; Gupta and Verma, 2015). Many different pretreatment methods have been developed to obtain the most accessible and enzyme-susceptible biomass and avoid negative side effects, such as formation of inhibitory compounds (Palmqvist and Hahn-Hägerdal, 2000a; Palmqvist and Hahn-Hägerdal, 2000b). Pretreatment of hemicellulose is known to easily form inhibitors such as 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde. These compounds hinder fermentation performance later in the process (Agbor et al., 2011). Lignin provides a protective role for (hemi-)cellulose by impairing enzymatic degradation. This makes optimal delignification without formation of inhibitory compounds a major target for efficient pretreatment methods as this makes the substrate more susceptible for hydrolysis using lignocellulolytic enzymes (Bajpai, 2016). There are three types of pretreatment processes, namely the physical, chemical and biological pretreatment.

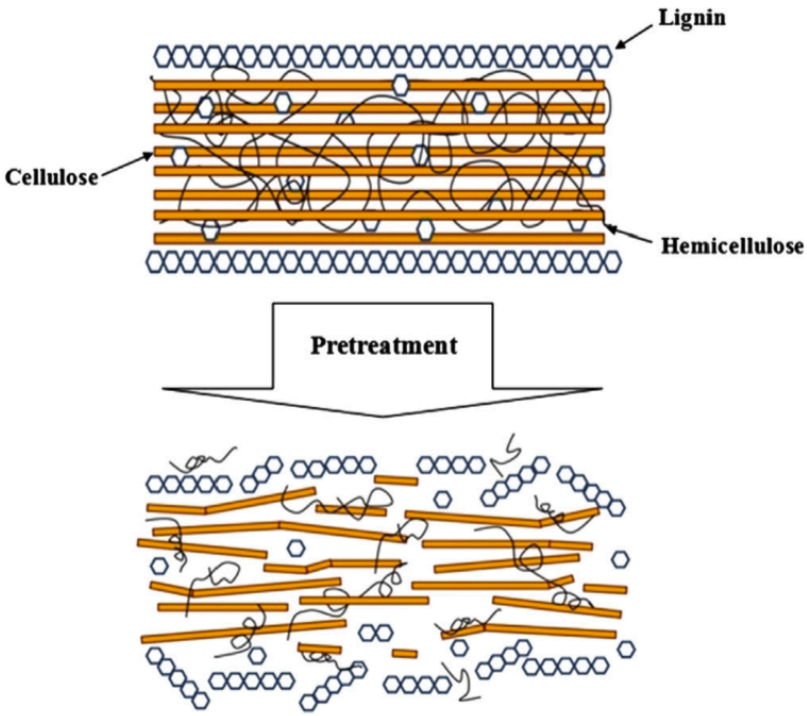


Figure 1.2. Schematic representation of pretreatment of lignocellulosic material, resulting in more susceptible material for enzymatic degradation. (Haghighi Mood et al., 2013)

Firstly, physical or mechanical pretreatment reduces the substrate size through material breaking methods like cutting, chipping or milling. This results in reduced cellulose crystallinity and improved downstream processing (Sun and Cheng, 2002). However, this pretreatment is energy consuming and can account for one third of the total energy consumption of the ethanol production process (Aden et al., 2002), making it unlikely to be used intensively at full-scale production (Gupta and Verma, 2015).

Secondly, various chemical pretreatment methods have been developed to treat lignocellulosic biomass (Sun and Cheng, 2002; Zheng et al., 2009). Dilute acid pretreatment was deemed to be most suitable for commercial scale

application (Sims et al., 2010). Although this method generates degradation products and a low final concentration of fermentable sugars, it also generates few inhibitors and provides less corrosion problems than concentrated acid (Aditiya et al., 2016). Dilute acid pretreatment has been identified as optimal for rye straw (Sun and Cheng, 2005), silvergrass (Guo et al., 2008), rice straw (Aditiya et al., 2015) and durian seed (Ghazali et al., 2016). A comparison of pretreatment methods with acid or other chemicals was made by Silverstein and colleagues (2007). This study indicated the different advantages and disadvantages of diverse methods.

A combination of physical and chemical pretreatment, called physicochemical pretreatment, is used for instance in steam explosion. This method exposes the biomass at high temperature and pressure to generate steam that will serially explode the fibers when the biomass is depressurized (Brownell et al., 1986; Grous et al., 1986; Kaar et al., 1998). Steam explosion has been assessed at pilot scale using sugarcane bagasse, indicating good reproducibility (Rocha et al., 2012). Ammonia fiber explosion relies on the same principle as steam explosion but uses ammonia as explosion reagent as investigated for switchgrass (Alizadeh et al., 2005), corn stover (Teymouri et al., 2004) and municipal solid waste (Holtzapple et al., 1992). Comparing ammonia fiber explosion and dilute acid pretreatment indicated a superior effect with ammonia fiber explosion over dilute acid (Mathew et al., 2016). However, this method was inefficient for biomass with high lignin content and costly due to the large amount of ammonia needed (Aditiya et al., 2016).

The final pretreatment method is the biological process, in which microorganisms are employed to degrade lignocellulosic feedstock to the amorphous form (Sarkar et al., 2012; Sun and Cheng, 2002). Typical microorganisms used for this biological pretreatment are brown rot, soft rot and white rot fungi (Lee et al., 2007; Sindhu et al., 2016; Singh et al., 2008). These organisms display strong hydrolytic and ligninolytic function (Sanchez, 2009; Saritha et al., 2012). This method is attractive because of the sustainability, safety and low energy requirement (Sindhu et al., 2016). However, pretreatment of lignocellulosic biomass with biological agents results in a much slower production rate than other pretreatments, what makes this approach unfavorable for large-scale processes (Saritha et al., 2012; Sharma et al., 2019). On the other hand, combining biological with mild physical or chemical pretreatment was beneficial for various lignocellulosic feedstocks (Yu et al., 2009).

3.2. Hydrolysis

One requirement for the pretreatment of lignocellulosic biomass is to increase the susceptibility of the polysaccharides to enzymatic degradation during the hydrolysis step. Breakdown of the polymeric substrates into monomeric, fermentable sugars requires various lignocellulolytic enzymes.

Hydrolysis of the cellulosic fraction of lignocellulose involves three classes of glycosyl hydrolase enzymes: endoglucanases (endo-1,4-ß-glucanase, EC 3.2.1.4, EGs), exoglucanases or cellobiohydrolases (exo-1,4-ß-glucanase, EC 3.2.1.91, CBHs) and ß-glucosidases (EC 3.2.1.21, BGLs) (Fig. 1.3). These three classes work sequentially and synergistically by hydrolysis of ß-1,4-glycosidic bonds to completely degrade cellulose. Endoglucanases cleave the cellulose chains at random internal positions with low crystallinity (amorphous regions), thereby creating more free-chain ends. These free ends are attacked by exoglucanases, specifically cellobiohydrolases that show activity towards reducing (CBHI) or non-reducing (CBHII) chain ends. This hydrolytic reaction results in release of cellobiose, which in turn is hydrolyzed into two glucose units by ß-

glucosidase. Commercial cellulosic substrates include Avicel, a crystalline and densely packed cellulose, and phosphoric acid swollen cellulose (PASC), the amorphous version that is created by swelling crystalline cellulose using phosphoric acid. The latter is abundantly used because of its higher degradability with cellulases.

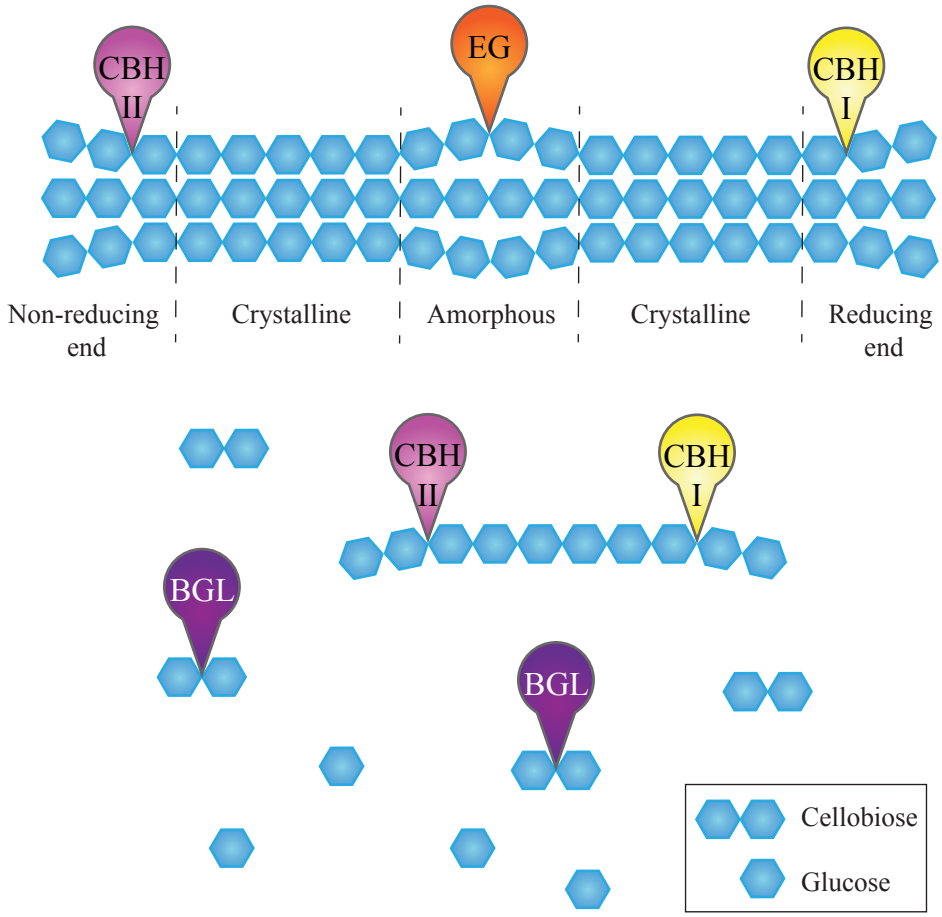


Figure 1.3. Schematic representation of the structure of cellulose and the cellulolytic enzymes required for its complete degradation. EG: endoglucanase, CBHI: cellobiohydrolase I, CBHII: cellobiohydrolase II, BGL: ß-glucosidase.

Hemicellulose degradation requires multiple enzymes depending on the plant source. When the hemicellulosic fraction consists mainly of xylan, as is the case for hardwood and herbaceous biomass, two major enzymes are required for breakdown of the backbone of xylose units: xylanases (endo-1,4-ß-xylanase, EC 3.2.1.8, XYLs) and ß-xylosidases (xylan-1,4-ß-xylosidase, EC 3.2.1.37, BXLs) (Fig. 1.4). Xylanases degrade the xylan backbone by hydrolyzing ß-1,4-glycosidic bonds between random xylose units, creating xylooligosaccharides of various shorter chain lengths. Sequentially, ß-xylosidase is employed to degrade xylobiose and short xylooligosaccharides into monomeric xylose units. When the hemicellulosic fraction mainly consists of mannan, as observed in softwoods, three enzymes are essential for its breakdown: mannanase (1,4-β-D-mannan mannohydrolase, EC 3.2.178), β-mannosidase (1,4-β-D-mannopyranoside hydrolase, EC 3.2.1.25), and β-glucosidase (EC 3.2.1.21) (Moreira and Filho, 2008). Mannanases catalyze the random cleavage of ß-1,4-linked mannose units, creating new chain ends. The released mannosides are attacked at the non-reducing end by ß-mannosidases, resulting in monomeric mannose. As the mannan backbone also consists of a small portion of glucose units, ß-glucosidase is used to attack the glycosidic bond between a glucose unit and a mannose unit (Malgas et al., 2015; van Zyl et al., 2010).

Despite the sugar composition of the backbone, all hemicelluloses are heavily substituted. These side chains impair the backbone hydrolysis by the major xylanolytic or mannanolytic enzymes. Various auxiliary enzymes have been identified for complete hydrolysis of hemicellulose, including α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), feruloyl esterase (EC 3.1.1.73), and acetylxylan esterase (EC 3.1.1.72) (Arnling Baath et al., 2018; Malgas et al., 2015; Saha, 2003). These accessory enzymes mainly act on the side chains, such as acetylxylan esterase that attacks the acetyl groups of acetylated xylan (Fig. 1.4), thereby debranching and increasing the susceptibility of the sugar backbone and commencing complete hemicellulosic hydrolysis (Laca et al., 2019; Wyman et al., 2005a). Although more enzymes are essential for a complete hydrolysis of hemicelluloses, it is typically more easily degraded than cellulose due to its more amorphous characteristics (Aditiya et al., 2016).

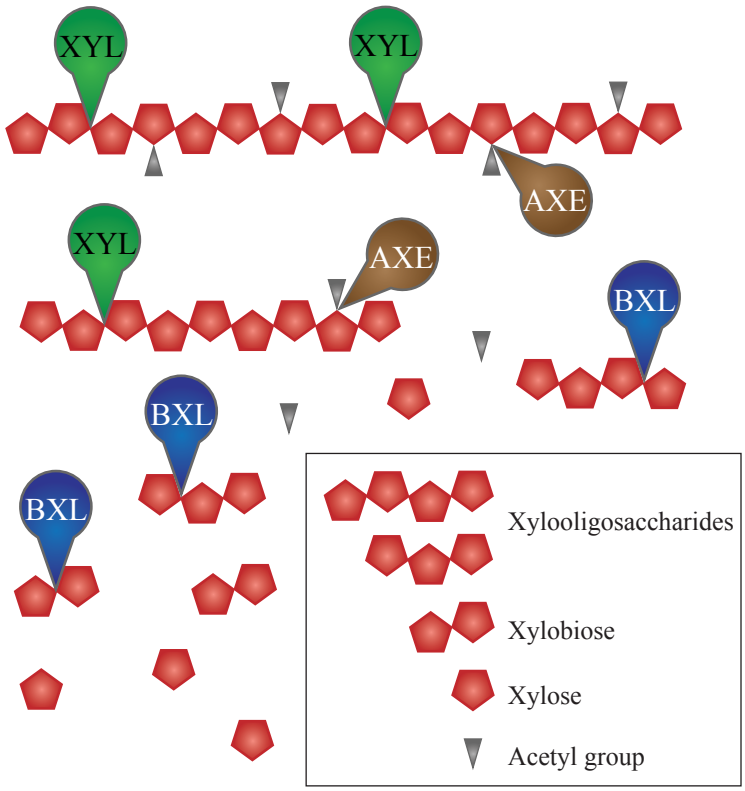


Figure 1.4. Schematic representation of the structure of acetylated xylan and the hemicellulolytic enzymes required for its complete degradation. XYL: xylanase, BXL: ß-xylosidase, AXE: acetylxylan esterase.

Lignin enhances bonds between (hemi-)cellulosic fibers, thereby hindering the approachability and binding of glycosyl hydrolase enzymes (Jeoh et al., 2007; Wyman et al., 2005b). In aqueous solution, lignin adsorbs, binds and precipitates proteins, impairing their enzymatic activity and affecting the overall hydrolysis (Kawamoto et al., 1992; Lu et al., 2002; Pareek et al., 2013; Yang and Wyman, 2006). The presence of lignin during hydrolysis is thus highly unfavorable. Delignification is prioritized during pretreatment, resulting in an enhanced hydrolysis of the feedstock (Yuldashev et al., 1993). However, enzymatic treatment with laccase (oxidoreductase, EC 1.10.3.2) removes lignin from various lignocellulosic biomasses (Chen et al., 2012a; Gutierrez et al., 2012; Rico et al., 2014). Unlike the classical pretreatment methods, enzymatic delignification selectively targets and cleaves the specific phenolic moieties of lignin, avoiding formation of inhibitory compounds and forming natural

mediators that take part in the oxidation of non-phenolic moieties of lignin molecules (Astolfi et al., 2005; Chandra Rajak and Banerjee, 2015; Eggert et al., 1996; Johannes and Majcherczyk, 2000).

3.2.1. Synergistic activity of enzymes

All these hydrolytic and ligninolytic enzymes are strongly influenced by each other as they depend on their products, resulting in a high enzyme synergy. Synergism is defined as "the ratio of the rate or yield of product released by enzymes when used together to the sum of the rate or yield of these products when the enzymes are used separately in the same amounts as they were employed in the mixture" (Kumar and Wyman, 2009). Synergy of enzymes is highly dependent on the ratio of the enzymes involved (Nidetzky et al., 1994) and on the enzyme specific characteristics and substrate properties (Wang et al., 2015). It was suggested that the standard activity assays for enzymes using synthetic substrates do not correlate with the hydrolytic capacity of these enzymes for different lignocellulosic substrates (Chundawat et al., 2008). Synergy studies are usually performed with pure enzymes on defined substrates (Van Dyk and Pletschke, 2012). However, optimization of hydrolysis of complex, lignocellulosic substrates for crude or commercial enzyme mixtures has been attempted (Banerjee et al., 2010a; Banerjee et al., 2010b; Selig et al., 2009).

Many lignocellulolytic enzymes have been studied for their synergistic action with other hydrolytic enzymes and have been extensively reviewed by Van Dyk and Pletschke (2012). Synergy between cellulolytic enzymes has been observed between different cellobiohydrolases attacking reducing and non-reducing ends (Boisset et al., 2000), between endoglucanases (Zhou and Ingram, 2000), between endoglucanases and cellobiohydrolases (Boisset et al., 2001; Zhang et al., 2010a) and between cellobiohydrolases, endoglucanases and ß-glucosidases (Qi et al., 2007; Zhang and Lynd, 2004). With respect to hemicellulases, different types of synergy have been identified, namely homeosynergy, heterosynergy and antisynergy (Bhat and Hazlewood, 2001; Coughlan and Hazlewood, 1993). Homeosynergy occurs between enzymes cleaving the hemicellulose main chain, whereas heterosynergy occurs between the enzymes attacking the backbone and auxiliary enzymes hydrolyzing the side groups. Antisynergy is when the activity of an enzyme lowers the action of another hemicellulase, *e.g.* removal of arabinose side chain of arabinoxylan by α-arabinofuranosidase inhibits the action of arabinoxylanase, which only exhibits activity towards a main chain with an arabinose substituent (Brigham et al., 2018).

The complexity of hemicelluloses and the spectrum of enzymes required for their complete hydrolysis complicate the synergy studies regarding hemicellulases. The backbone cleaving enzyme is commonly heterosynergistically linked to debranching enzymes as it requires removal of abundant side chains to hydrolyze the main chain (Vardakou et al., 2004). Many more studies have been performed to elucidate the synergistic action of different hemicellulases (Van Dyk and Pletschke, 2012).

Lignocellulose degradation requires a combination of cellulases and hemicellulases, suggesting that these different enzyme classes work in synergy for complete hydrolysis of lignocellulose. It has been established that, regardless of the xylan concentration in lignocellulosic material, addition of xylanase enhanced hydrolysis by cellulases (Bura et al., 2009; García-Aparicio et al., 2007). Other studies indicated strong synergies between cellulases and hemicellulases on complex lignocellulosic substrates (Hu et al., 2013; Selig et al., 2008a; Van Dyk and Pletschke, 2012).

3.2.2. End-product inhibition

Although lignocellulolytic enzymes show strong synergy and dependence on each other, they are also known to be easily inhibited by high concentrations of hydrolysis end-products (Mandels and Reese, 1965). It was suggested that inhibition of sugars has a greater impact on hydrolysis than the inhibitors formed during pretreatment (Garcia-Aparicio et al., 2006).

Cellulases specifically are strongly inhibited by degradation products of cellulose, namely cellobiose and glucose (Andric et al., 2010; Teugjas and Väljamäe, 2013). The inhibitory effect is highly dependent on the substrate (Gusakov and Sinitsyn, 1992) and the specific enzyme (Murphy et al., 2013). Since product inhibition often only occurs when a high concentration of the end products is generated (Hsieh et al., 2014), an excess of ß-glucosidase can be added to relieve feedback inhibition by cellobiose (Van Dyk and Pletschke, 2012). Products of hemicellulose hydrolysis, including xylose, xylooligosaccharides, galactose and mannose, also act as strong inhibitors of cellulases (Qing et al., 2010; Xiao et al., 2004; Ximenes et al., 2010). Combination of hemicellulases and cellulases might thus be unfavorable for cellulose hydrolysis and require optimization to minimize the formation of inhibitory products (Aditiya et al., 2016; Kont et al., 2013).

Although hemicellulase inhibition has been investigated to a lesser extent, similar trends have been observed. Various ß-xylosidases experienced end-product inhibition in the presence of xylose (Banka et al., 2014; Chen et al., 2012b; Yan et al., 2008) and xylanase was subject to product inhibition caused by xylobiose and xylooligomers (Ball and McCarthy, 1989).

3.2.3. Enzyme cost

To date, enzymatic hydrolysis of lignocellulosic biomass is accomplished by addition of commercially produced enzyme cocktails. Although limited information on the cost of lignocellulolytic enzymes is publicly available (Gusakov, 2011; Klein-Marcuschamer et al., 2010; Lynd et al., 2008), there is no consensus on the contribution of enzyme costs to the economics of 2G bioethanol production. Some argue that it remains a major hurdle for production of biofuels (Brijwani et al., 2010; Cherry and Fidantsef, 2003; Fang et al., 2009), while others estimate the cost to be relatively low or claim that due to technological innovations this cost will decrease (Aden and Foust, 2009; Schubert, 2006). Nonetheless, Klein-Marcuschamer and colleagues (2012) concluded that the vast majority of the literature to date significantly underestimated the contribution of enzyme costs to biofuel production. The cost of enzymes was estimated to be highly variable depending on the enzyme production process. Off-site enzyme production accounts for 28% of the total cost. This decreases to 22% by manufacturing the enzymes on-site and to 10% by using an integrated approach, where the enzymes are produced on-site using the same feedstock for enzyme and ethanol production (Johnson, 2016). Different companies are manufacturing commercially available enzyme cocktails to facilitate hydrolysis at large scale. Most cocktails contain enzymes produced by fungi, such as *Trichoderma reesei* and *Aspergillus niger* (Saini et al., 2015). The producing companies therefore focus on improvement of enzyme activity. However, these advances only affect biorefinery capital costs by reducing the saccharification time and they do not address the enzyme production costs, reflected

as operating costs (Klein-Marcuschamer et al., 2012). Recycling of the hydrolytic enzymes can lower the production cost of bioethanol (Sims et al., 2010), but evolution towards an approach called consolidated bioprocessing entails no operating costs or capital investment for the production and purchase of enzymes (Hamelinck et al., 2005; Taherzadeh and Karimi, 2007).

3.3. Fermentation

During the fermentation step, the sugars released during the pretreatment and enzymatic hydrolysis are converted to the product of interest by a fermentation agent. For the production of ethanol, specifically, sugars from the hydrolysate are converted to ethanol and CO_2. Theoretically, one gram of glucose or xylose is fermented to 0.51 g of ethanol and 0.49 g of CO_2 (Lynd, 1996). Various factors, such as temperature, pH and initial solid and microbial load, significantly affect the fermentation performance of different agents (Zabed et al., 2017). A commonly used bacterial agent for industrial fermentation is *Zymomonas mobilis* (Mohagheghi et al., 2002; Robak and Balcerek, 2018). This bacterium efficiently converts glucose, fructose and sucrose to ethanol. The most commonly used yeast species, *S. cerevisiae*, also has the natural ability to ferment hexose sugars to ethanol. However, both species are incapable of fermenting pentose sugars. Alternative organisms that possess the ability to utilize pentoses, like *Candida shehatae* and *Pichia stipitis*, are vulnerable to fermentation inhibitors, acidic environment and high ethanol concentrations, resulting in inefficient ethanol production (Hahn-Hagerdal et al., 2007). Nevertheless, many studies have been focusing on engineering and optimizing the fermentation efficiency of different microbial species (Aditiya et al., 2016).

3.4. Distillation

Distillation enables recovery of ethanol and other dilute volatile products by classical countercurrent vapor/liquid mass transfer (Robak and Balcerek, 2018). Ethanol obtained by fermentation of biomass-based streams is contaminated with residual solids, like unconverted polysaccharides, ash and breakdown products of lignin (Madson and Lococo, 2000). During distillation, ethanol is separated, concentrated and purified. However, high purity ethanol can only be obtained by drying and dehydration (Robak and Balcerek, 2018). To reduce distillation costs, a sufficiently high concentration of ethanol (4.5% (w/v)) has to be reached (Lennartsson et al., 2014).

4. Production process configurations

The bioethanol production process can be conducted in different configurations (Taherzadeh and Karimi, 2007). The general process still consists of pretreatment, hydrolysis, fermentation and distillation. The configurations, that are discussed here, are connected to improved hydrolysis and fermentation processes (Fig. 1.5) (Saini et al., 2015). Every configuration requires more abilities of the fermentation organism and advanced setups reduce the process cost and simplify the production system (Parisutham et al., 2014).

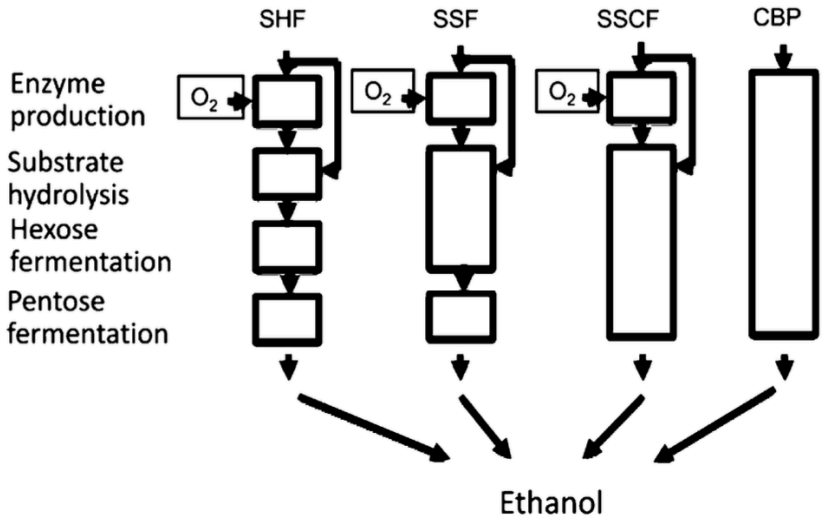


Figure 1.5. Different hydrolysis and fermentation configurations for production of bioethanol. The different biologically-mediated events can be performed separately or simultaneously. Each box represents one bioreactor in the processing configuration. SHF: separate hydrolysis and fermentation, SSF: simultaneous saccharification and fermentation, SSCF: simultaneous saccharification and co-fermentation, CBP: consolidated bioprocessing. (Adapted from Saini et al. (2015))

4.1. Separate hydrolysis and fermentation (SHF)

Separate hydrolysis and fermentation (SHF) indicates that hydrolysis and fermentation steps are performed separately (Fig. 1.5). This permits to take full advantage of the optimal conditions for every step. Hydrolysis is usually performed at 45-50°C depending on the origin of the manufactured enzymes (Olsson et al., 2006; Saha et al., 2005). Fermentation, on the other hand, is conducted at a lower temperature, since the optimal fermentation temperature for most ethanol-producing microorganisms is between 30-37°C (Kim et al., 2008; Lin et al., 2012). However, SHF requires different reactors to perform the hydrolysis, glucose fermentation and pentose fermentation of the biomass (Taherzadeh and Karimi, 2007). An additional disadvantage of this configuration is the lower final sugar release during hydrolysis as the liberated sugars induce product inhibition of the hydrolytic enzymes (Philippidis and Smith, 1995). Finally, the risk of contamination in SHF is relatively high, since the hydrolysis takes a long time and the hydrolysate becomes the perfect environment for microbial contamination (Saini et al., 2015; Sarkar et al., 2012). Despite these drawbacks, this setup has been established for conversion of amongst others paper sludge (Peng and Chen, 2011), rice straw (Abedinifar et al., 2009) and mesquite (Gupta et al., 2009).

4.2. Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF), on the other hand, implies that the hydrolysis and hexose fermentation are performed simultaneously in the same vessel (Fig. 1.5), thereby reducing the capital costs (Taherzadeh and Karimi, 2007). This also minimizes the product inhibition by depolymerized sugars on the lignocellulolytic enzymes, since they are immediately fermented without additional steps and low concentrations of these sugars can be maintained (Balat et al., 2008; Sun and Cheng, 2002). SSF lowers the risk of microbial contamination because of simultaneous sugar consumption and ethanol formation (Neves et al., 2007). The benefits of SSF are improved ethanol production and requirement of less enzymes (Eklund and Zacchi, 1995;

Wingren et al., 2003). However, with this configuration the different optimal conditions for hydrolysis and fermentation cannot be maintained. The optimal temperature for an SSF process using an enzyme mixture from *T. reesei* and *S. cerevisiae* as fermentation organism was suggested to be around 38°C, a compromise between optimal conditions for SHF (Tengborg, 2000). Hydrolysis is often the rate-limiting step in SSF (Philippidis and Smith, 1995). To optimize the hydrolysis, studies have investigated the use of thermotolerant microorganisms in order to be able to raise the temperature closer to the hydrolysis optimum (Ballesteros et al., 2004; Hari Krishna et al., 2001; Hong et al., 2007). An additional issue during SSF is ethanol inhibition of lignocellulolytic enzymes (Viikari et al., 2012). It was suggested that the enzymatic activity on cellulose decreases linearly with increased ethanol concentrations (Wu and Lee, 1997). However, ethanol inhibition was observed to be reversible (Chen and Jin, 2006) and less potent than inhibition by sugar products (Bezerra and Dias, 2005).

4.3. Simultaneous saccharification and co-fermentation (SSCF)

SSF can be expanded to simultaneous saccharification and co-fermentation (SSCF). This strategy includes the fermentation of glucose and pentose sugars such as xylose in one reactor (Fig. 1.5), avoiding the separation of sugars (Morales-Rodriguez et al., 2011; Teixeira et al., 2000). For this purpose, different species can be co-cultured, of which one ferments the glucose, predominantly *S. cerevisiae*, and one ferments the xylose fraction, such as *Candida tropicalis* or *Scheffersomyces stipitis* (Liu et al., 2019; Suriyachai et al., 2013). A monoculture of one microorganism capable of utilizing xylose is also an option, *e.g. Z. mobilis* (McMillan et al., 1999). Furthermore, ethanol-producing microorganisms have also been engineered for utilization of xylose, implying that one single microorganism can be used for the co-fermentation of hexose and pentose sugars. Recombinant *E. coli* was used in SSCF to produce ethanol from corn stover (Kim et al., 2006). Ethanol production with engineered xylose-utilizing *S. cerevisiae* strains for SSCF has been demonstrated with various lignocellulosic substrates, such as paper sludge (Zhang and Lynd, 2010) and corn stover (Jin et al., 2012; Liu and Chen, 2016; Öhgren et al., 2006; Zhu et al., 2014a).

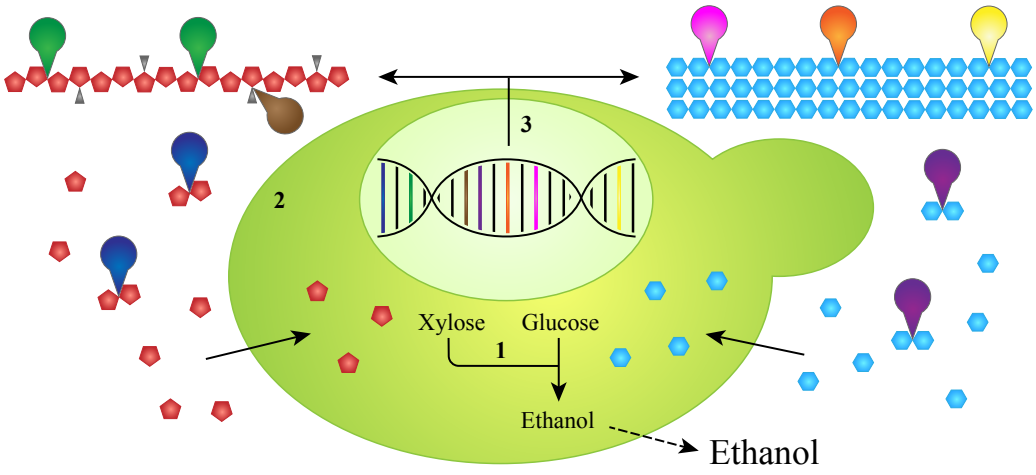
4.4. Consolidated bioprocessing (CBP)

All configurations to date require a separate enzyme manufacturing unit, either off-site or on-site. The final process configuration is consolidated bioprocessing (CBP), which is the holy grail for development of microbial cell factories. CBP implies that the production of lignocellulolytic enzymes, hydrolysis of the pretreated biomass and fermentation of both hexose and pentose sugars are performed in the same reactor by one single organism or a co-culture of different species (Fig. 1.5) (Balat, 2011). This has a lower production cost due to less process steps and vessels required, but also because of the absence of external enzyme cost (Lynd et al., 2005; Olson et al., 2012; Taherzadeh and Karimi, 2007). Brethauer and Studer (2014) combined the enzyme-producing *T. reesei* with a glucose-consuming *S. cerevisiae* strain and a xylose-utilizing *S. stipitis* strain in a co-culture to achieve ethanol production with a yield of 67% per gram of sugar from pretreated wheat straw. However, one single microorganism that is compatible for CBP does not yet exist and current research focuses on two different paths to obtain a CBP microorganism (Lynd et al., 2005). One approach starts with a native enzyme producer and modifies this organism to become an efficient ethanol producer. Bacterial species, such as *Clostridium*

thermocellum, and filamentous fungi, such as *Neurospora crassa*, have been investigated for this purpose (Hasunuma et al., 2013; Lee, 1997). However, these enzyme producers exhibit a slow fermentation rate and low ethanol yield (Szczodrak and Fiedurek, 1996). To achieve an efficient ethanol production process, another approach complements an excellent ethanologenic organism, such as *S. cerevisiae*, with xylose-utilization and lignocellulolytic capacity (van Zyl et al., 2007).

5. *Saccharomyces cerevisiae* as cell factory for CBP

S. cerevisiae is used as a cell factory for production of bioethanol and other bio-based chemicals. This native ethanologenic organism exhibits several other traits that are essential for the development of a CBP microorganism (den Haan et al., 2015). For bioethanol production specifically, it displays high ethanol yield and productivity, high ethanol and inhibitor tolerance and a general robustness for industrial processes. These traits are readily occurring in industrial *S. cerevisiae* strains (van Zyl et al., 2007). However, some traits need to be engineered into this organism to obtain an efficient CBP host (Fig. 1.6).



CBP requirements for *S. cerevisiae* cell factories

1. Utilization of pentose sugars, in particular xylose
2. Overall robustness to tolerate high ethanol and inhibitor concentrations
3. High level expression and production of lignocellulolytic enzymes

Figure 1.6. Schematic overview of a potential CBP *S. cerevisiae* cell factory. This requires a strain that (1) is able to ferment xylose, (2) exhibits an overall robustness towards ethanol and inhibitory compounds and (3) expresses and produces lignocellulolytic enzymes to a high extent. Different colored symbols indicate different enzymes.

Despite its native, excellent ability to ferment hexose sugars, *S. cerevisiae* is unable to utilize pentose sugars (Olson et al., 2012). Concurrent fermentation of sugars would also enhance ethanol production rate. Additionally, *S. cerevisiae* does not produce enzymes with lignocellulolytic properties and is inferior for high level expression and secretion of heterologous genes and their encoding proteins (Xu et al., 2009). Next to these essential traits, there are some desirable traits that encourage the use of *S. cerevisiae* for development of a CBP organism. It is generally regarded as safe (GRAS), recyclable, requires minimal nutrient supplementation and is very amenable

to DNA manipulation (van Zyl et al., 2007). The fermentation efficiency and industrial robustness of this organism provide a preferred baseline for industrial scale 2G ethanol production, which can be expanded to a CBP organism provided that certain essential adjustments are accomplished.

6. Pentose utilization

One of the most important prerequisites for development of a CBP organism is the utilization of pentose sugars, such as xylose and arabinose (Kwak and Jin, 2017). These sugars, derived from the hemicellulosic fraction of lignocellulose, can count up to 25% of sugarcane bagasse and other raw materials (Lee, 1997). Whereas in most biomass materials arabinose is found in low concentrations of 1-5%, xylose comprises up to 20% and is the most copious sugar in the hemicellulosic fraction of hardwoods and herbaceous biomass (Aristidou and Penttilä, 2000). The significance of this pentose fraction prioritized the development of efficiently xylose-utilizing organisms for bioethanol production.

Initial research focused on improving a natively pentose-fermenting organism, such as *Pachysolen tannophilus*, *Pichia stipitis* or *Candida shehatae*, for industrial traits required for 2G bioethanol production (Avanthi et al., 2016). However, these organisms exhibit low ethanol- and inhibitor tolerance, poor sugar consumption rate and formation of xylitol instead of ethanol. These drawbacks and the steadily improving technologies for genetic engineering of *S. cerevisiae* stimulated the development of xylose-utilizing yeast strains. *S. cerevisiae* possesses the capacity of excellent hexose fermentation, but lacks the ability to ferment pentoses. There are two possible ways to confer the capacity to ferment xylose to *S. cerevisiae* (Fig. 1.7).

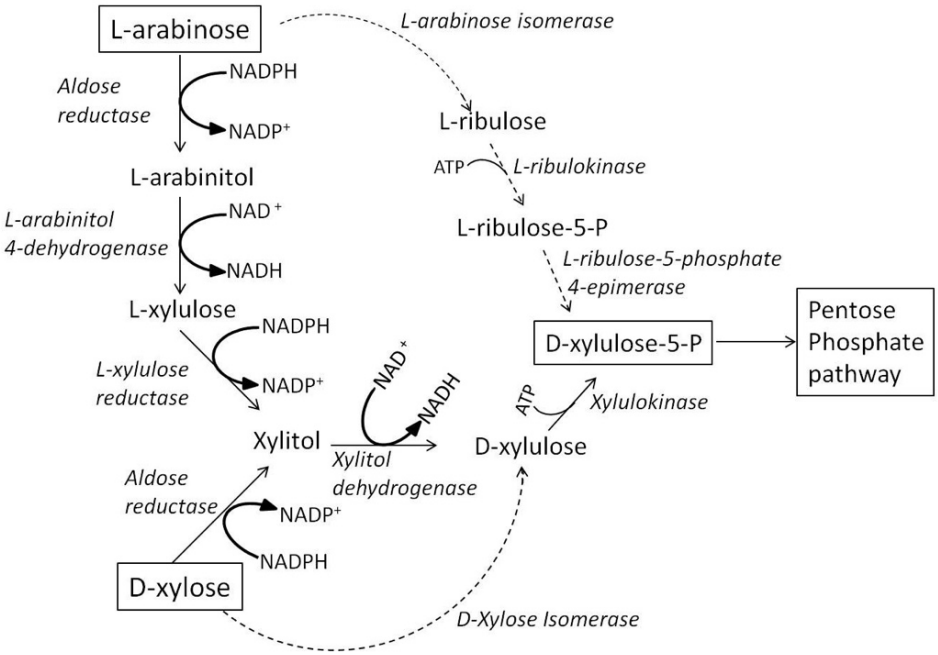


Figure 1.7. Engineering pentose utilization in yeast. The oxidoreductase pathways are indicated with solid arrows while isomerization pathways are shown in broken line arrows. Xylose isomerase bypasses the oxidation/reduction steps of xylose utilization, resulting in a single step process. Xylulose-5-phosphate enters into the pentose phosphate pathway (Demeke, 2013).

The first requires the expression of two heterologous key enzymes in the fungal xylose oxidoreductase pathway, namely xylose reductase and xylitol dehydrogenase (Cai et al., 2012; Kötter and Ciriacy, 1993). Co-utilization of xylose and arabinose has been engineered into laboratory and industrial *S. cerevisiae* strains by expression of the xylose oxidoreductase pathway genes and the bacterial arabinose pathway consisting of the genes for L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase (Bettiga et al., 2009; Garcia Sanchez et al., 2010; Karhumaa et al., 2006). However, the oxidoreductase approach to achieve xylose utilization induces a redox imbalance, potentially leading to xylitol accumulation and inefficient xylose fermentation (Cunha et al., 2019).

This redox imbalance can be circumvented by implementing a bacterial one-step xylose isomerase pathway (Moyses et al., 2016). This approach has been established frequently by successful expression of bacterial xylose isomerases in 2G industrial yeast strains that have been further improved for lignocellulosic inhibitor tolerance (Brat et al., 2009; Demeke et al., 2013a; Demeke et al., 2013b; Diao et al., 2013; dos Santos et al., 2016; Kuyper et al., 2005a; Moyses et al., 2016; Smith et al., 2014; Vilela et al., 2015; Zhou et al., 2012).

7. Industrial robustness for stress factors (adapted from Deparis*, Claes* et al., (2017))

Overcoming the major disability of pentose utilization in *S. cerevisiae*, revealed other weaknesses that could potentially be improved. Industrial production processes put a multitude of stresses on yeast cell factories. Physical and chemical extracellular stresses include temperature, osmotic pressure and the presence of lignocellulosic biomass derived inhibitors. Furthermore, producing recombinant proteins in yeast cell factories can create a protein burden on the cell and engineering novel metabolic pathways can cause redox imbalances.

7.1. Environmental stress factors

Alcoholic fermentation is a well-established industrial process where several factors can affect yeast performance and most of them are also relevant in other bio-based fermentations.

7.1.1. High ethanol concentration

In order to make the distillation of ethanol economically viable, industry usually requires a minimal concentration of 4-5% (v/v) ethanol. Lower ethanol concentrations dramatically increase the energy demand for distillation (Stampe et al., 1983; Zacchi and Axelsson, 1989). In the United States, corn ethanol production often reaches a final ethanol concentration of 16-18%, while Brazilian sugar cane ethanol falls in the range of 8-11% (Basso et al., 2008; Shapouri and Gallagher, 2005). The production processes are becoming more advanced resulting in higher ethanol yields and titers and the industry is thus in need of yeast strains with higher maximal ethanol accumulation capacity (Alexandre et al., 2001; Dinh et al., 2009; Hirasawa et al., 2007; Lewis et al., 2010; Watanabe et al., 2007a).

7.1.2. High osmotic pressure and ion toxicity

Osmotic stress in bioethanol production is caused by high sugar concentrations and/or high salt concentrations. In 1G bioethanol production, osmotic pressure is mostly caused by the concentrated streams of about 35% sugar that are used and are required to reach ethanol titers of 16-18% (Bertrand et al., 2016). In 2G bioethanol production, it is difficult to reach high concentrations of fermentable sugars. They are usually limited to about 12% sugar so that an ethanol titer of only 5-7% is reached. Hence, osmotic pressure caused by high sugar concentration is usually not a problem in 2G bioethanol production. On the other hand, only part of the solids can be converted into fermentable sugars. Increased solids loading requires additional pumping power and proper mixing is impaired when the viscosity in the reactor is too high (Fan et al., 2003). At high solids loading, the cellulolytic enzymes also start to become inhibited (Kristensen et al., 2009). Solids mainly affect the enzymatic hydrolysis because of enzyme adsorption to their surface while they have less effect on the yeast fermentation.
High sodium salt concentrations present in the 2G bioethanol production process, due to the pretreatment method or to procedures used to detoxify the hydrolysate, could also compromise the fermentation rate and yield (Casey et al., 2013). Removing the salt is not a good option because of the costs involved (Sanchez and Cardona, 2008). Furthermore, wastewater treatment and water recycling practices can also result in accumulation of (sodium) salt in pipelines and fermentors. The different salts do not only cause osmotic stress to the cell, but can also cause ion toxicity (Hohmann, 2002; Hohmann et al., 2007; Millati et al., 2002; Ranatunga et al., 2000; Wei et al., 2013). Some of the feedstocks contain, besides potassium and sodium salts, also (heavy) metal salts (Kaushal et al., 1981; Wythes et al., 1978).

7.1.3. High temperature

Thermotolerance is another highly desirable trait in the yeast fermentation industry. Appropriate cooling of the fermentors is required because of the exergonic fermentation process (Wheals et al., 1999). Higher tolerance to temperature overshoots and a higher mean fermentation temperature would provide more consistent fermentation performance, reduced contamination risks, lower ethanol distillation costs, a higher fermentation rate and thus reduced fermentor sizes and lower capital costs, and reduced cooling/heating expenses (Hamelinck et al., 2005; Stephen et al., 2012). High thermotolerance is even more important in advanced processes of 2G bioethanol production. In SSF, the optimum fermentation temperature of most *S. cerevisiae* strains is 30-35°C while the optimum temperature of the lignocellulolytic enzymes is 45-55°C (Choudhary et al., 2016). Hence, higher thermotolerance of the yeast allowing fermentation temperatures of 40°C or higher is highly beneficial for an SSF or CBP process. As a result, the amount of the enzymes needed for hydrolysis of the sugar polymers can be reduced significantly (Kawaguchi et al., 2016).

7.1.4. Weak acids

The most abundant and inhibitory weak acid present in lignocellulosic biomass is acetic acid. Its level varies considerably with the composition of the biomass. 2G feedstocks for bioethanol production have a variable composition in terms of cellulose, hemicellulose and lignin content. Also, the type of pretreatment process has a considerable effect on the level of acetic acid (Du et al., 2010a; Kristensen et al., 2009; Zha et al., 2012). Many

pretreatment methods release acetyl groups from the hemicellulosic fraction. The resulting acetic acid in unbuffered hydrolysates causes sluggish fermentations or may inhibit fermentation completely (Bellissimi et al., 2009; Graves et al., 2006; Narendranath et al., 2001; Palmqvist et al., 1999; Palmqvist and Hahn-Hägerdal, 2000b). Similar toxicity problems are caused by other organic acids, such as levulinic acid and formic acid, which are conversion products of hexose and pentose sugars, respectively (Horvat et al., 1985; Rasmussen et al., 2014). Genome-wide screens have revealed many genetic elements important for high acetic acid tolerance (Mira et al., 2010b; Mira et al., 2010c). A major role in this respect is played by the Haa1 transcription factor that mediates expression of multiple genes required for high acetic acid tolerance (Fernandes et al., 2005; Mira et al., 2010a). In previous studies, the transcription factor Haa1 was already linked to weak acid tolerance (Fernandes et al., 2005; Mira et al., 2010a), as was *VMA7* (Kawahata et al., 2006). Meijnen and colleagues (2016) identified a unique point mutation in the *HAA1* gene product (G1517A) that is sufficient by itself to increase acetic acid tolerance in the xylose fermenting industrial *S. cerevisiae* strain GSE16-T18. The point mutation considerably reduced the lag phase of fermentation with GSE16-T18 HAA1* in the presence of acetic acid.

7.1.5. Other chemical inhibitors

A first class of chemical inhibitors present in lignocellulose hydrolysates are the furaldehydes, such as furfural and 5-hydroxymethyl-2-furfural (HMF), which are conversion products of hexoses and pentoses, respectively. This conversion is mainly due to pretreatment methods applying high heat and high pressure. HMF is less inhibitory compared to furfural (Taherzadeh et al., 2000).

A second class of chemical inhibitors are the phenolic compounds derived from the lignin in lignocellulosic biomass. Lignin is a complex organic polymer of phenolic compounds, including acids, aldehydes and alcohols (Campbell and Sederoff, 1996). Coniferyl aldehyde has been identified as the most toxic phenolic compound in the lignin fraction and there seems to be a correlation between specific chemical classes and toxicity (Adeboye et al., 2015; Adeboye et al., 2014). Lignin is very hydrophobic and binds to cellulose, preventing cellulase access for its hydrolysis. In addition, lignin also interacts with the cellulose-binding module of cellulases further reducing hydrolytic efficiency (Eriksson et al., 2002; Kumar et al., 2012; Vermaas et al., 2015). Moreover, genes that are involved in aromatic acid conversion, such as *PAD1* and *FDC1,* have been shown to increase tolerance to furaldehydes and to enhance final ethanol yield (Larsson et al., 2001; Mukai et al., 2010). The validation of this approach in an industrial context, however, has not been reported.

7.2. Metabolic stress

Using *S. cerevisiae* as a cell factory for the production of 2G bioethanol and bio-based chemicals requires a microorganism that can cope with a high level of metabolic stress. Bio-based chemicals are generally not natively produced in the high amounts required for an industrial production process. Engineering of metabolic pathways and overexpression of recombinant enzymes can lead to diverse stress factors, especially protein burden, possible redox imbalances and product inhibition.

7.2.1. Protein burden

Strong overexpression of recombinant proteins in order to create a superior yeast cell factory can cause a metabolic burden or protein burden on the cell. This can be caused by the high demand for energy, reducing power and amino acid building blocks required for the production of the high levels of recombinant proteins, and/or competition between expressed proteins at the level of the protein biosynthetic machinery, and/or the secretion pathway. Another problem can be protein crowding in a confined cellular space (Gopal et al., 1989; Janes et al., 1990; Nevoigt, 2008; Sardonini and DiBiasio, 1987; Srienc et al., 1986). As a result, the overexpression of proteins can cause an impaired specific growth rate, delayed fermentation start-up, reduced fermentation rate and/or yield, decreased respiration capacity or reduced biomass yield. The cost of protein production in bacteria is commonly attributed to protein translation and ribosomal activity is considered to be a major limiting factor for the growth rate of the cells (Emilsson and Kurland, 1990; Kurland, 1992; Scott et al., 2010; Scott and Hwa, 2011; Scott et al., 2014; Vind et al., 1993).

Only a limited amount of insight in the molecular mechanisms of protein burden in eukaryotic cells is available (Hauf et al., 2000; Lang et al., 2009). Some mechanistic insights were suggested and published, but due to the complexity of heterologous gene expression and protein production, these mechanisms cannot be unified for different proteins (Bolognesi and Lehner, 2018; Eguchi et al., 2018). The concept of metabolic burden in *S. cerevisiae* was investigated and elaborated by Görgens and colleagues (2001). They compared *S. cerevisiae* strains expressing a heterologous xylanase (*XYN2*) from *Trichoderma reesei* and quantified the metabolic effects of expression of plasmid-based constructs behind a glycolytic promoter (*ADH2* or *PGK1*). Their research showed no significant burden on the cells when the heterologous gene is expressed from a multicopy plasmid. However, when the gene was expressed behind a glycolytic promoter, a reduction in maximum specific growth rate, biomass yield and specific glucose consumption was observed. They describe the metabolic effect of foreign gene expression as ‘disproportionally large with respect to the amount of heterologous protein produced’. The most obvious reasons for their findings are the increased energy demand and/or the competition for limiting amounts of transcription or translation factors, precursors and energy (Görgens et al., 2001). However, alternative explanations such as negative interference of the foreign, heterologous protein or the artificial recombinant protein with cellular factors important for growth and/or fermentation cannot be ruled out. The effect of recombinant gene expression on transcription and protein translation in *S. cerevisiae* was studied by Kafri and colleagues (2016). They showed that the processes that limit protein production depend on the growth conditions, that ribosomal activity is not universally limiting in rapidly growing cells and that cells can adapt the abundance of endogenous proteins to meet the metabolic burden at least to some extent.

In *Schizosaccharomyces pombe*, recombinant protein production can be limited by lipid biosynthesis, TCA cycle activity and the supply of NADPH and ATP. Another important factor is the nutrient composition of the medium that can strongly influence protein secretion (Klein et al., 2014). A similar effect was detected in *S. cerevisiae*, in which supplementation of the growth medium with amino acids improves growth and protein production (van Rensburg et al., 2012). Furthermore, high gene copy numbers may strongly affect the metabolic activity of the cell (Klein et al., 2014; Lin et al., 2013). To determine the copy number limit for overexpression of a target gene Makanae and colleagues developed a method named ‘genetic tug-of-war’ (gTOW). Using this genetic method,

they concluded that the yeast cell is robust to a copy number increase by up to 100 in more than 80% of its protein-coding genes. Also, they identified 115 dosage-sensitive genes of which a significant number are genes involved in cytoskeletal organization and intracellular transport. This dosage sensitivity was suggested to be caused by protein burden and stoichiometric imbalances (Makanae et al., 2013).

7.2.2. Redox factor imbalance

Another frequently encountered stress factor in metabolically engineered cells is a disturbance in the balance of redox factors. The preference of enzymes for specific cofactors leads to a depletion and/or accumulation of for instance NAD(P)H. Hence, metabolic engineering of yeast requires appropriate modelling of redox homeostasis with correct prediction of cofactor usage (Nevoigt, 2008).

The first efforts to engineer xylose fermentation into *S. cerevisiae* involved expression of xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) of *Pichia stipitis*. This created a slow-growing strain with a low fermentation rate on xylose (Jeffries and Jin, 2004). Since Xyl1 prefers NADPH over NADH and Xyl2 only uses NAD^+ (Rizzi et al., 1989; Verduyn et al., 1985), it led to accumulation of NADH (Jeffries, 2006).

The modification of cofactor specificity is often a target in metabolic engineering, as was the case in pentose utilization with the identification of mutant xylose reductase enzymes that have a higher affinity for NADH than for NADPH (Jeppsson et al., 2006; Watanabe et al., 2007b). Another approach was the engineering of xylitol dehydrogenase for $NADP^+$ cofactor specificity (Watanabe et al., 2007c).

Recent research on 2,3-butanediol production was also confronted with the issue of redox imbalance. It was caused by deletion of the *PDC* genes, with the aim of abolishing ethanol production for enhancing the flux into the 2,3-butanediol production pathway (Kim et al., 2013). In this case, the cofactor imbalance led to production of glycerol instead of 2,3-butanediol, because the disequilibrium was balanced by conversion of NADH to NAD^+ (Kim et al., 2016; Remize et al., 1999). However, it could be corrected by expressing NADH oxidase from *Lactococcus lactis* (*noxE*), obtaining a maximal 2,3-butanediol yield (Kim et al., 2016; Kim et al., 2015; Kim and Hahn, 2015).

Another approach is the expression of a transhydrogenase, that is natively absent in yeast (Bruinenberg et al., 1985), catalyzing the following reaction: $NADH + NADP^+ \rightarrow NAD^+ + NADPH$ (Sauer et al., 2004). Implementation of transhydrogenase activity can balance the disturbed redox state in metabolically engineered yeast as shown by Suga and colleagues (Suga et al., 2013). They overexpressed three enzymes, *i.e.* Mae1, Mdh2 and Pyc2, that have a transhydrogenase-like activity in a xylose-fermenting *S. cerevisiae* strain. This allowed proper control of the redox state. However, expression of a bacterial transhydrogenase in yeast resulted in a significant decrease in maximal specific growth rate, biomass and ethanol yield (Nissen et al., 2001).

7.2.3. Toxic metabolites

Industrial-scale production of bulk and fine chemicals requires high titers of the end product (Stephanopoulos, 2007). High concentrations of these chemicals are often toxic to the producing host cell (Keasling, 2010). Imbalances introduced by suboptimal metabolic engineering often lead to accumulation of metabolic intermediates that may also be toxic to the cells. An example is the frequent accumulation of the toxic

intermediate acetaldehyde in engineered yeast cell factories (Bae et al., 2016; Ng et al., 2012). This cytotoxic compound, that is also produced in low quantities during alcoholic fermentation, diffuses poorly across the plasma membrane, which leads to intracellular accumulation and inhibition of sensitive reactions and enzymes (Aranda and del Olmo, 2004).

One way to address this type of metabolic stress is through upregulation of efflux pumps (Kell et al., 2015; Sheng and Feng, 2015). Overexpression of membrane exporters can enhance the secretion of cytotoxic products. For instance, production of short branched-chain fatty acids is enhanced by overexpression of the native Pdr12 efflux pump (Yu et al., 2016). Overexpression of the *S. cerevisiae* Snq2, Pdr5 and Pdr15 transporters was shown to increase secretion of and tolerance to alkanes (Ling et al., 2013). Similarly, expressing the Abc2 and Abc3 membrane transporters of *Yarrowia lipolytica* in *S. cerevisiae* also improved its tolerance to alkanes (Chen et al., 2013).

Recent studies have explored another potential solution to address toxic effects of intermediary metabolites using the concept of 'metabolons'. This strategy has actually been adapted from nature, where biosynthetic enzymes are sometimes physically associated in large complexes (Jorgensen et al., 2005; Singleton et al., 2014; Trantas et al., 2009; Zhang et al., 2006). This spatial localization does not only facilitate the transfer of intermediates between the consecutive enzymes of a pathway due to channeling of the metabolites, but it also prevents the diffusion of the intermediates away from the enzyme complex keeping their overall level below a concentration that otherwise could become toxic for the cells (Siddiqui et al., 2012). Minimizing the diffusion of toxic intermediates while stimulating their rapid conversion to less toxic constituents through sequestration within an enzyme complex might thus improve cell growth and especially the final yield of the product of interest (Chen and Silver, 2012; Jorgensen et al., 2005; Roze et al., 2011; Srere, 1987).

8. Production of lignocellulolytic enzymes

Next to xylose fermentation and overall industrial robustness, a CBP organism requires the capability to express heterologous genes and produce the encoded enzymes to a high extent. This is essential firstly for production of bio-based chemicals and thus metabolic engineering of the strain to produce these compounds and secondly, for the production of enzymes with lignocellulolytic properties to achieve hydrolytic degradation of the 2G feedstocks.

S. cerevisiae is, as described earlier, an ethanologenic organism possessing an excellent native fermentation performance with hexoses and after recent genetic modifications, also with xylose. However, it lacks the genes that encode enzymes with lignocellulolytic properties, which is essential to develop a CBP organism that is able to perform biomass hydrolysis and fermentation in one step. For this purpose, *S. cerevisiae* has been genetically engineered for lignocellulolytic capacity. Three strategies have been explored to construct lignocellulolytic yeasts (Fig. 1.8).

First, enzymes can be secreted in the growth medium, which allows unlimited secretion of free enzymes (den Haan et al., 2015). Moreover, the free enzymes are able to penetrate the lignocellulosic biomass, increasing the accessibility of the substrate, which is a critical factor in enzymatic hydrolysis (Arantes and Saddler, 2010; Resch et al., 2013). This strategy requires high level secretion of heterologous enzymes, implying that the signal peptide

of the heterologous gene is recognized and cleaved for secretion (Xu et al., 2017). Many studies have identified potential genes to enhance the poor secretion capacity of *S. cerevisiae* (Kroukamp et al., 2013; van Zyl et al., 2014). However, this strategy results in diffusion of the enzymes in the medium, causing no direct benefit to the producing cells and hindering enzyme recycling (Liu et al., 2018a). Nonetheless, this strategy has been applied widely to obtain *S. cerevisiae* strains that secrete lignocellulolytic enzymes (den Haan et al., 2007; Ilmén et al., 2011; Lee et al., 2017; Penttilä et al., 1988; Takada et al., 1998; Tang et al., 2013; van Rooyen et al., 2005; Wong et al., 1988).

With the second strategy the enzymes are immobilized on the yeast cell wall. This keeps the enzymes and their catalytic products in close proximity to the organism. This results in improved synergistic action, lowers the risk of microbial contamination and enables enzyme recycling (Matano et al., 2013; Yamada et al., 2013a). However, the amount of enzyme displayed on the cell wall is strictly correlated to the cell surface area. Additionally, tethered enzymes may exhibit inefficient activity and reduced conversion compared to secreted systems (Liu et al., 2015). Attachment of the enzymes to the cell wall requires a fusion of the target enzyme to an anchoring protein, such as Aga2 (Fujita et al., 2002; Matano et al., 2012) or Sed1 (Inokuma et al., 2014). Many studies investigated this strategy to obtain efficient lignocellulose degradation with an *S. cerevisiae* strain with cell wall displayed enzymes (Bae et al., 2015; Fujita et al., 2004; Ishii et al., 2016; Matano et al., 2012; Murai et al., 1997; Nakatani et al., 2013; Sakamoto et al., 2012; Yeasmin et al., 2011).

The final strategy tethers the enzymes on the cell wall assembled in the nanostructure of a mini(hemi-)cellulosome. In these multi-enzymatic complexes, different types of lignocellulolytic enzymes are assembled on structural scaffoldin subunits through non-covalent protein-protein interactions between the docking modules (dockerins) and the complementary modules (cohesins) (Hasunuma et al., 2013). The recombinant complexes expressed in *S. cerevisiae* are often based on the natural complexes occurring in cellulolytic bacterial species such as *Clostridium* sp. or *Ruminococcus* sp. (Doi and Tamaru, 2001; Goyal et al., 2011; Wen et al., 2010). The scaffoldin also contains a carbohydrate-binding module to attach the complex directly to its substrate. This enables efficient synergistic degradation of biomass due to a combination of directing the enzymatic complex to its substrate and the spatial proximity of the enzymes to their substrates and/or products (den Haan et al., 2013a; Fierobe et al., 2002). This strategy is mainly applied because of the belief that the efficiency of lignocellulose degradation is defined not by the activity of the individual enzymes, but by their synergistic action to overcome its recalcitrant nature (Lilly et al., 2009). The engineering of *S. cerevisiae* with both cellulosomes and xylanosomes has been well-studied (Fan et al., 2012; Hyeon et al., 2010; Liang et al., 2014; McClendon et al., 2012; Srikrishnan et al., 2013; Sun et al., 2012; Tsai et al., 2009).

To date, none of the developed lignocellulolytic *S. cerevisiae* strains hydrolyzed the majority of the cellulosic fraction of the feedstock without addition of exogenous enzymes (den Haan et al., 2015). Furthermore, high activity of heterologous enzymes induced substantial burden on the secretion pathway (Ilmén et al., 2011). Development of a lignocellulolytic *S. cerevisiae* strain should be attempted without negatively affecting ethanol yield and productivity, but also without impacting the native and previously engineered traits, such as hexose and pentose fermentation, growth rate and overall robustness (van Zyl et al., 2007). In this work, we attempt to create a yeast that secretes the recombinant lignocellulolytic enzymes as described in the first strategy (Fig. 1.8A).

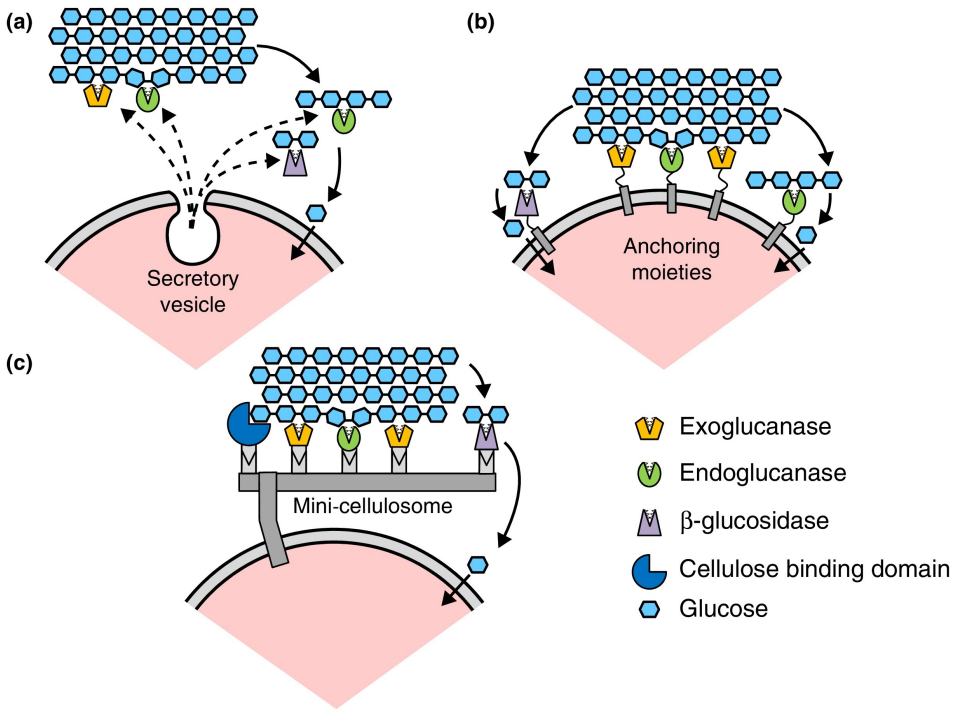


Figure 1.8. Schematic representation of the different recombinant cellulase engineering strategies to degrade cellulose. (a) Production of secreted enzymes that allow random free access to insoluble cellulosic materials. (b) Anchoring of cellulases to the cell wall. (c) Tethering of cellulases to the cell wall in minicellulosomes. Tethering strategies result in restricted access to insoluble cellulosic materials but allow improved synergism and recycling of tethered enzymes with cells. (den Haan et al., 2015)

9. Protein secretion by *S. cerevisiae*

S. cerevisiae gained a role as an upcoming expression host for production of recombinant proteins for biopharmaceutical, agricultural and enzymatic industries (Demain and Vaishnav, 2009). To produce these mature proteins, the gene product has to go through various intracellular compartments before definite release from the cell. This makes the secretory pathway of yeast an interesting field of study to potentially enhance the protein secretion. Secretory expression of heterologous genes is often subject to several bottlenecks that limit secreted yield. To understand the possible improvements, the protein secretion pathway of yeast is introduced in this section. A schematic overview of the pathway is depicted in Fig. 1.9.

When a gene is transcribed translated and if it is destined for secretion, the soluble peptide is translocated to the endoplasmic reticulum (ER). This is facilitated by a cleavable amino acid sequence of around 7-12 hydrophobic residues, called the secretion signal or signal peptide (Blobel and Sabatini, 1971). This signal peptide is recognized and bound by the signal recognition particle (SRP). A complex of a ribosome with the SRP delivers the nascent protein into the ER through the Sec61 protein-conducting channel (Hann and Walter, 1991; Pool, 2005), resulting in co-translational translocation.

In the ER, several processes occur to initiate quality control and folding of the soluble peptide. Immediately when entering the ER, the signal peptide can be cleaved by the signal peptidase complex (SPC) (Meyer and Hartmann, 1997; YaDeau et al., 1991). However, secretion of recombinant proteins with a signal peptide mostly relies on Kex2 protease activity (Hou et al., 2012). This protease acts later in the Golgi and recognizes the (K/R)-R motif

to cleave the signal peptide (Rockwell et al., 2002; Wilcox and Fuller, 1991). Next to cleavage of the signal peptide, post-translational modifications occur and folding starts. The nascent cleaved proteins undergo an initial glycosylation process in the ER. Glycosylation is the biosynthesis of glycans, linked to proteins, and unlike DNA and protein synthesis, this extremely complex process is not template-driven and may be branched (Varki et al., 2009). In eukaryotes, these protein modifications have many important functions, such as in cell physiology, protein targeting, protein recognition, protein folding, protein solubility, protein stability and protection against proteases (Roth et al., 2010; Varki, 1993). The most prominent types of protein glycosylation in yeast are *N*- and *O*-glycosylation (Spiro, 2002). *N*-linked glycosylation is accomplished by linking a sugar glycan tree to an *N*-acetylglucosamine that is anchored to an asparagine residue in a motif of N-X-S/T, where X can be any amino acid except proline (Bause, 1983). This type of glycosylation is conducted by an ER-resident oligosaccharyl transferase (OST) (Burda and Aebi, 1998). *O*-linked glycosylation is catalyzed by protein *O*-mannosyltransferases (PMT) and occurs at hydroxyl groups of serine and threonine residues (Strahl-Bolsinger et al., 1999). PMTs transfer one mannose to the respective residue in the ER, but additional mannoses can be added later in the Golgi (Loibl and Strahl, 2013). Furthermore, the nascent protein starts forming disulfide bonds and initiates folding in the ER. Folded proteins exit the ER in two different ways based on the correctness of the folding. Firstly, the proteins can be guided into the ER-associated degradation pathway (ERAD). Misfolded proteins are detected and targeted by lectins and ubiquitin ligases for transfer to the cytosol and proteasomal degradation (Hou et al., 2012; Jakob et al., 1998; Xu et al., 2004). When unfolded and/or misfolded proteins accumulate in the lumen of the ER, the unfolded protein response (UPR) is activated (Shamu et al., 1994). This aims to normalize cellular functions by halting protein translation, degrading misfolded proteins and activation of signaling pathways resulting in increased production of chaperones involved in protein folding. Cells deal with this stress response by the splicing of *HAC1* mRNA. The translated Hac1 transcription factor translocates to the nucleus and activates target gene transcription by binding a UPR-specific upstream activating sequence, such as the ER-resident chaperones *KAR2*, *PDI1* and *FKB2* (Okamura et al., 2000; Patil and Walter, 2001). Overexpression of the active, spliced *HAC1* gene increased overall protein production in different cases and is thus an interesting target for yeast engineering for protein production (Guerfal et al., 2010; Payne et al., 2008; Valkonen et al., 2003). Secondly, the correctly folded proteins exit the ER towards the Golgi. Therefore, the proteins need to be recognized by receptors for export in COPII vesicles. Via diffusion, these vesicles traverse from the ER to the Golgi (Preuss et al., 1992), where soluble N-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs) facilitate the fusion of transport vesicles with the organelles (Van Zyl et al., 2016). Once the peptides reach the Golgi apparatus, the proteins undergo a maturation process with the change from the *cis* cisternae to the *trans* cisternae. Glycosylated proteins are mannosylated on *N*- and *O*- linked glycans, which signals for trafficking to the secretion pathway (Ecker et al., 2003). As described earlier, in the Golgi peptides are also cleaved with three different proteases (Kex1, Kex2 and Ste13), depending on the recognition sites (Cooper and Bussey, 1989; Julius et al., 1983; Rockwell et al., 2002). This enables further maturation of proteins, activation of catalytic activity and conformational changes (Hou et al., 2012). The modifications made in the Golgi strongly affect the post-Golgi trafficking.

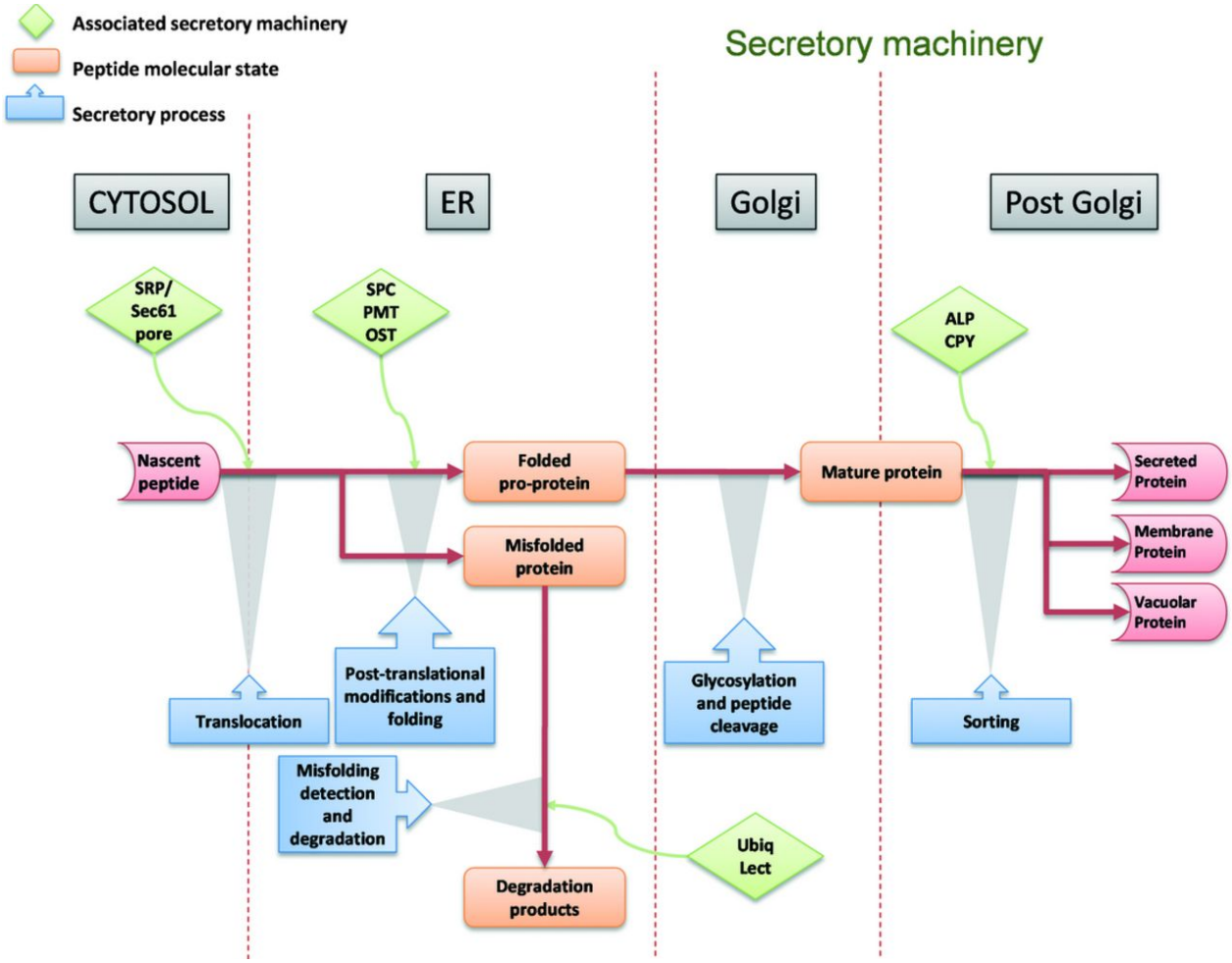


Figure 1.9. Overview of the secretory pathways and machinery of yeast. The nascent peptide is folded and modified through different cellular organelles and mechanisms. SRP, signal recognition particle; SPC, signal peptidase complex; PMT, protein O-mannosyltransferase; OST, oligosaccharyl transferase; Ubiq, ubiquitin; Lect, Lectin; ALP, alkaline phosphate pathway; CPY, carboxypeptidase Y pathway. (Hou et al., 2012)

Proteins exiting the Golgi are sorted depending on the final destination of the protein. This can be retrograde transport from the *cis*-Golgi to ER via COPI vesicles (Bednarek et al., 1995) or transport to the endosome or vacuole. The latter can be conducted via the carboxypeptidase Y (CPY) pathway (Jung et al., 1999), the alkaline phosphate (ALP) pathway (Piper et al., 1997) or via endosomal sorting complexes required for transport (ESCRT) (Henne et al., 2011). Recombinant proteins that lack a sorting signal are automatically sorted via the CPY pathway (Cowles et al., 1997). Alternatively, proteins can follow the exocytotic pathway from the *trans*-Golgi. Two types of vesicles have been distinguished based on their density (Harsay and Bretscher, 1995). Light density secretory vesicles are known to carry cell membrane proteins directly to the cell membrane. High density secretory vesicles, on the other hand, transport soluble proteins. These vesicles move from the *trans*-Golgi to the endosome and then to the cell membrane for secretion (Gurunathan et al., 2002). Secretion of the protein cargo is also facilitated by SNAREs that enable fusion of the vesicles with the plasma membrane (van Zyl et al., 2014). Research is focusing on engineering different targets in the secretion pathway to obtain optimal production processes and maximal yields using yeast as a host. This has been extensively reviewed by Hou et al. (2012) as well as Idiris et al. (2010). Davison and colleagues (2020) recently reviewed rational engineering of yeast to enhance recombinant cellulase secretion specifically.

10. Strain engineering for development of CBP organisms (adapted from Deparis*, Claes* et al., (2017))

S. cerevisiae provides excellent platform strains for conversion into cell factories. The species already possesses several traits that are required for cell factories, such as high fermentation rate, high propagation rate, high general robustness, high tolerance to alcohols and ease of genetic modification. These traits are even more pronounced in the many industrial strains that have been developed over the years, including those for production of bioethanol. *S. cerevisiae* can be improved for increased tolerance to certain stress factors, for xylose utilization and for enhanced heterologous gene expression and protein production through random genetic modification followed by selection or through targeted genetic modification based on previous knowledge of genetic determinants underlying possible enhancements.

10.1. Random genome engineering

Random genetic modification can be achieved by random mutagenesis, genome shuffling, whole-genome hybridization or evolutionary adaptation, each time followed by selection of superior strains under a specific condition (David and Siewers, 2015; Jullesson et al., 2015; Steensels et al., 2014). A disadvantage of all these approaches is that they can only be used for selective traits for which selection is possible based on growth rate or maintenance of viability. However, they generally lead to non-GMO strains that can be brought rapidly into industrial application.

10.1.1. Evolutionary engineering

Evolutionary engineering is a non-directed methodology to improve yeast strains for a specific trait by subjecting the cells to a continuous selective pressure. In repetitive batch cultivations or chemostat cultivation under selective pressure, genetic diversity is generated through spontaneous mutagenesis and the fitter cells are continuously selected because of faster multiplication or better survival. The most adapted lineage(s) will finally dominate the entire culture after prolonged cultivation (Dykhuizen and Hartl, 1983; Sauer, 2001).

Evolutionary engineering has been applied to improve several industrially important traits in *S. cerevisiae*, such as substrate utilization, product formation and stress tolerance. Ethanol fermentation with different carbon sources was enhanced successfully. Rapid xylose utilization is one of the main requirements for yeast strains used in second-generation bioethanol production. This challenge has been addressed extensively using evolutionary engineering (Demeke et al., 2013a; Kuyper et al., 2005b; Liu and Hu, 2010; Sonderegger and Sauer, 2003; van Maris et al., 2007; Zhou et al., 2012). Other carbon sources for which utilization has been improved by evolutionary engineering include arabinose (Garcia Sanchez et al., 2010; Wisselink et al., 2007) and lactose (Guimaraes et al., 2008). Furthermore, improvement of several stress tolerance characteristics has been achieved successfully by evolutionary engineering, such as freeze tolerance (Teunissen et al., 2002), ethanol tolerance (Stanley et al., 2010) and even multistress resistance (Cakar et al., 2005).

It is highly relevant for industrial application to improve multiple traits at the same time by applying different selective pressures simultaneously in order to achieve the best possible combination of the individual adaptations.

Wright and colleagues (2011) performed evolutionary engineering in medium with acetic acid and xylose to obtain a superior xylose-fermenting yeast strain under industrial conditions. Performing repetitive batch cultivations directly in lignocellulosic hydrolysates selects evolved strains that show increased tolerance to all inhibitors present in the hydrolysates (Koppram et al., 2012; Tomas-Pejo et al., 2010).

10.1.2. Genome shuffling

Genome shuffling is extensive inbreeding of two or more yeast strains after which the strain(s) with the desirable trait is obtained by selection for faster growth or maintenance of higher viability under a specific condition of interest. Recombination of desirable mutant genes into a single strain can generate strongly improved phenotypes (Nicolaou et al., 2010). In *S. cerevisiae*, this technique has been applied successfully to enhance tolerance to specific stress factors. Shi and colleagues (2009) enhanced thermotolerance, ethanol productivity and ethanol tolerance using genome shuffling.

Eukaryotic hybrids are unstable due to diversion of the genetic background of the parents (Giudici et al., 2005). To address this issue, researchers have attempted to make genome shuffling more efficient in several ways. For instance, the number of potentially useful mutations was increased by ethyl methane sulfonate mutagenesis followed by sexual and asexual reproduction instead of protoplast fusion (Hou, 2009). This methodology was applied successfully to improve ethanol productivity (Hou, 2010) as well as stress tolerance (Pinel et al., 2011) and fermentation performance (Zheng et al., 2011a). In other research, acetic acid tolerance in *S. cerevisiae* was enhanced using drug resistance marker-aided genome shuffling, which was proven to be advantageous for highly efficient selection of the genome shuffled mutants (Zheng et al., 2011b). Demeke and colleagues (2013a) developed an industrial D-xylose fermenting strain through genome shuffling by sporulation and mass mating of an ethyl methane sulfonate mutagenized segregant and its parent, followed by evolutionary adaptation.

Genomic DNA extraction from one parent followed by its transfer into the other parent also allowed recombination of the two genomes. To allow a second round of shuffling, the strain obtained can be transformed again with the whole genome of the first parent. Such a modified interspecies genome shuffling methodology was applied to construct a xylose-fermenting *S. cerevisiae* strain through transformation with the whole genome of *P. stipitis* (Zhang and Geng, 2012).

10.1.3. Global transcription machinery engineering (gTME)

Another technology to phenotypically improve cell factories is global transcription machinery engineering (gTME). This non-directed approach is based on mutagenesis of transcription factors and/or cofactors (Nevoigt, 2008). Altering key regulatory proteins generates a new transcription profile by reprogramming gene transcription. This was first accomplished by Alper and colleagues (Alper et al., 2006; Ding et al., 2009), who created a strain with increased ethanol tolerance caused by an altered TATA-binding protein Spt15. Three mutations in *SPT15* (F177S, Y195H and K218R) improved the performance compared to the control in the presence of high ethanol concentrations. These findings were challenged by Baerends and colleagues (2009) who could not observe the same effect in leucine prototrophic strains and ascribed the original effects on ethanol tolerance to alleviation of leucine auxotrophy. On the other hand, Yang and co-workers (2011) used the same

approach with the Spt15 transcription factor and also successfully demonstrated improved ethanol tolerance in rich medium with specific mutant alleles. However, they also used only leucine auxotrophic strains. Besides enhancement of ethanol tolerance, gTME has also been applied successfully in *S. cerevisiae* to improve xylose utilization (Liu et al., 2010), fermentation in corncob hydrolysates (Liu et al., 2011) and to enhance co-fermentation of xylose and glucose (Liu et al., 2008). This implies that gTME may be a promising technique for strain improvement but that more positive results especially with industrial strains and industrially-relevant conditions are required.

10.2. Targeted genome engineering

Since the discovery of recombination in yeast (Orr-Weaver et al., 1981; Szostak et al., 1983), many molecular technologies have been developed to introduce recombinant DNA in order to alter specific *S. cerevisiae* traits (Fraczek et al., 2018). Targeted genetic modification tools enable specific and efficient engineering of yeast. Basic yeast research has produced a wealth of information on genetic determinants of tolerance to specific stress factors, improved xylose utilization and enhanced heterologous gene expression and protein production. Most of this research has been performed with laboratory yeast strains which are generally less robust than industrial yeast strains.

Targeted genetic engineering requires specific tools to precisely target a locus or loci in the host's genome without affecting other genes and/or traits. Some of these specific tools are well-studied in yeast, such as Cre-*loxP*-recombinase (Delneri et al., 2000; Sauer, 1987), phiC31 integrase (Thomason et al., 2001), transcription activator-like effector nucleases (TALENs) (Beurdeley et al., 2013; Gan et al., 2018; Jakociunas et al., 2015b) and, more recently, CRISPR/Cas9 (Giersch and Finnigan, 2017; Stovicek et al., 2017). As opposed to the random approaches, targeted genetic modifications generally lead to GMO strains, either cisgenic (containing extra or modified species-own DNA only) or transgenic (containing heterologous DNA) organisms. In this study, phiC31 integrase recombination and CRISPR/Cas9 were explored for integration of heterologous DNA in the genome of *S. cerevisiae*. Therefore, these targeted genome engineering tools are described more in detail.

10.2.1. PhiC31 integrase

PhiC31 integrase is a DNA recombinase that is derived from *Streptomyces* phage φC31. This targeted recombination is a wide-spread mechanism used by temperate bacteriophages and other autonomous genetic elements to integrate in the bacterial genome (Rausch and Lehmann, 1991). The phiC31 integrase enzyme mediates recombination between two attachment sequences, namely *attB* (occurring in bacteria) and *attP* (occurring in phages). When both sites are present, the DNA recombinase will recombine *attB* and *attP* to hybrid attachment sites, *attL* and *attR*. These newly created sites are no longer recognized by phiC31 integrase, making this an irreversible modification mechanism (Chompoosri et al., 2009). However, phiC31 integrase does not require perfect *attP* site conservation, disabling the use of this method for specific genome targeting (Sclimenti et al., 2001), which was addressed by using a negative selection strategy to exclude random integration (Yu et al., 2014). This methodology has found applications in genome editing of microorganisms, insect cells and mammalian cells (Chompoosri et al., 2009; Katz et al., 1991; Michele, 2006).

10.2.2. CRISPR/Cas9

Genome editing is highly dependent on the efficacy of the engineering methodology used. In recent years, a very precise genetic targeting and modification technology, that is based on clustered regularly interspaced palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), has gained wide-spread application in many species because of its high efficacy and flexibility (Doudna and Charpentier, 2014).

CRISPR/Cas9 is based on a specific genomic sequence that is targeted by a guide RNA (gRNA). This gRNA guides the endonuclease Cas9, derived from *Streptococcus pyogenes*, towards this target site with a protospacer adjacent motif (PAM), where the Cas9 cleaves the host DNA by a double strand break (DSB) (Fig. 1.9) (Cui et al., 2018). This is lethal to the cells unless this DSB is repaired. Yeast cells can repair genomic discrepancies in two different ways. The DSB can be fixed by non-homologous end joining (NHEJ), possibly resulting in gene knock-out caused by an indel mutation at these specific sites, potentially leading to frameshift or non-synonymous mutations at the target site (Islam, 2019). The second way to repair a DSB is by homology directed repair (HDR). This implies the use of a template donor DNA that is homologous or highly similar to the targeted region of the DSB. This strategy is often used to replace genomic alleles or integrate heterologous genes in that specific target site (Musunuru, 2017).

Genome engineering in *S. cerevisiae* reached a new level of efficiency with CRISPR/Cas9, as described by DiCarlo and co-workers (2013), and also strongly facilitated targeted genome engineering in industrial yeast strains (Stovicek et al., 2015). This technology can be applied for introducing both single and multiple marker-free gene knock-outs (Bao et al., 2015; Mans et al., 2015), as was the case for establishing high mevalonate production in yeast (Jakociunas et al., 2015a). Besides gene knock-outs, successful CRISPR/Cas9 mediated gene integration can be achieved, which is often required in development of yeast cell factories for bio-based chemical production (Jakociunas et al., 2016). Marker-free integration of single or multiple genes, or even entire metabolic pathways, has become possible in a single step with the application of CRISPR/Cas9 combined with homologous recombination. Evaluation of a gene library by direct genomic insertion in a diploid strain in order to identify the most active variants of a cellodextrin transporter for increased cellobiose utilization was achieved in a single step (Ryan et al., 2014).

Adaptation of the original CRISPR/Cas9 method by combination with *in vivo* assembly using homologous recombination (CasEMBLR) makes it more versatile (Jakociunas et al., 2015b). The applicability of this approach was validated by introduction of the carotenoid pathway through insertion of 15 DNA fragments in multiple loci and through creating a tyrosine producing strain by simultaneously knocking out two genes and integrating ten DNA fragments (Jakociunas et al., 2015b). This methodology of combining CRISPR/Cas9 with *in vivo* assembly has been used for introduction of several traits in yeast. Horwitz and co-workers (2015) chromosomally integrated an entire 11-gene pathway for muconic acid production and Tsai and colleagues (2015) constructed a xylose-fermenting strain in this way.

Another recent study designed specific guide RNA sequences to target multiple ∂-sites (Di-CRISPR) (Shi et al., 2016). This allows simultaneous integration of multiple copies in different positions in the yeast genome. The authors successfully integrated 18 copies of a 24 kb combined xylose utilization and (R,R)-2,3-butanediol

production pathway with high efficiency in a single marker-free step in the ∂-elements, thus creating a yeast strain that produced (R,R)-2,3-butanediol directly from xylose (Shi et al., 2016). This approach is thus of great interest for integrating multiple copies of a gene or a pathway in a single step into the genome.

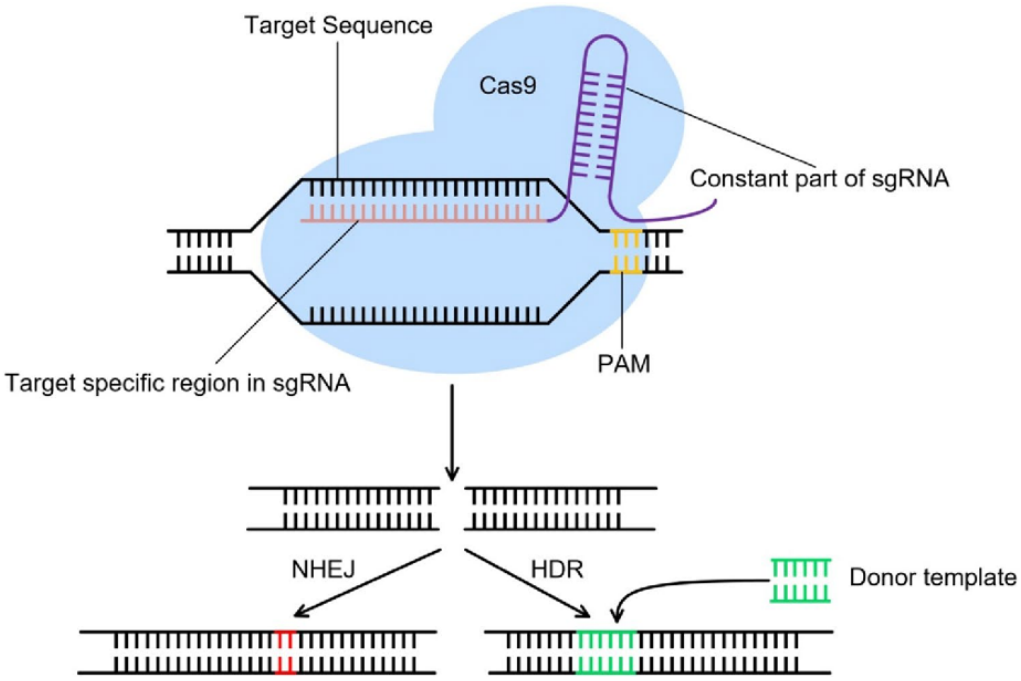


Figure 1.10. Schematic overview of the CRISPR/Cas9 genome editing system. A genomic sequence with a protospacer adjacent motif (PAM) is targeted by the endonuclease Cas9, which is guided by a guide RNA (gRNA) and creates a double strand break (DSB). This DSB can be repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR) by the use of a donor template. (Cui et al., 2018)

Overview - Development of the industrial cellulolytic and xylanolytic *Saccharomyces cerevisiae* strain AC14 that simultaneously secretes seven lignocellulolytic enzymes

In this work, a cascade of *S. cerevisiae* strains was created using CRISPR/Cas9-mediated genetic engineering. Seven strains were developed originating from MD4, an industrial xylose-fermenting, inhibitor-tolerant strain for second-generation (2G) bioethanol production. Consecutive genomic integrations of heterologous genes encoding cellulases and hemicellulases, discussed in Chapters 2-5, finally resulted in the AC14 strain. The series of consecutive integrations is summarized in Fig. O.1.

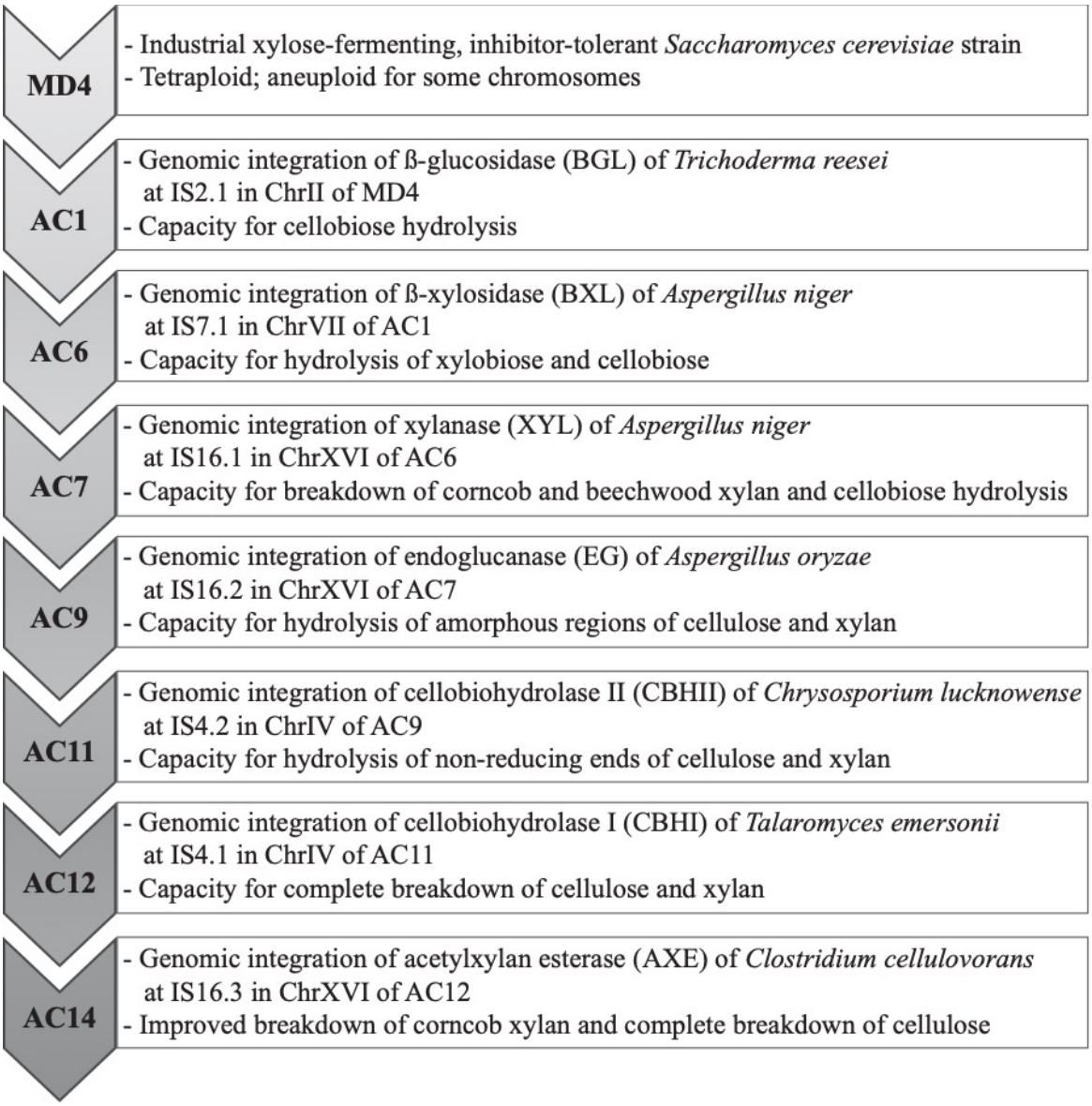


Figure O.1. Summary of consecutive genomic integrations of seven heterologous genes encoding cellulases and hemicellulases, resulting in the AC14 strain.

The developed xylanolytic and cellulolytic AC14 strain and all its ancestors were evaluated for enzyme activities with specific substrates as well as combined cellulase and hemicellulase activity with polymeric substrates. Also, the fermentation performance of this strain was assessed in medium containing various polymeric substrates. Furthermore, DNA from AC14 was used for whole genome sequence analysis to determine the copy number of all heterologous genes and to estimate the ploidy of the strain.

Chapter 2 - Consumption of cellobiose in cellulosic hydrolysate increases ethanol production of industrial yeast strains expressing a heterologous ß-glucosidase

1. Introduction

Breakdown of cellulose requires four different enzymes, *i.e.* endoglucanase, cellobiohydrolase I, cellobiohydrolase II and ß-glucosidase. The focus of this chapter is on ß-glucosidase and how this enzyme enables cellobiose utilization of industrial yeast strains.

ß-glucosidase (ß-D-glucoside glucohydrolase, EC 3.2.1.21, BGL) is an enzyme that catalyzes the hydrolysis of terminal, non-reducing ß-D-glucosyl residues thereby releasing ß-D-glucose. This implies that BGLs target alkyl and aryl ß-D glucosides, such as methyl ß-D-glucoside and p-nitrophenyl ß-D-glucoside, but also glycosides consisting of only carbohydrate residues, such as cellobiose (Machida et al., 1988).

BGL is a very widespread enzyme amongst all branches of the tree of life (Woodward and Wiseman, 1982). These enzymes are well-described in bacteria and fungi, where their main purpose is to provide the host with consumable sugars. Plants have this enzyme for ß-glucan synthesis, pigment metabolism and fruit ripening (Singhania et al., 2013) and make use of BGL to protect themselves against pathogens. For example, the 'fire blight' disease of apple and pear trees is caused by *Erwinia amylovora*. This bacterium produces BGL to breakdown the plant cell wall. However, these plants produce a ß-D-glucoside that is hydrolyzed by the pathogen's BGL to a toxic aglycone which can prevent fire blight (Kerppola et al., 1987). In mammalian cells and humans, BGL is essential for hydrolysis of glucosyl ceramides. Deficient BGL leads to a non-neuropathic lysosomal storage disorder, known as Gaucher's disease (Lieberman et al., 2007).

Production of second-generation bioethanol is highly dependent on the hydrolysis of the feedstock and the saccharification efficiency. To obtain monomeric sugars from these feedstocks an extensive treatment is necessary to breakdown the long polymeric chains of cellulose and hemicellulose. After a chemical and/or mechanical pretreatment, enzymatic hydrolysis is essential to convert the accessible carbohydrate polymers to fermentable sugars. In commercial enzyme cocktails required for hydrolysis of lignocellulosic biomass, there is usually a lower amount of ß-glucosidase present (Del Pozo et al., 2012), which makes it the rate limiting cocktail enzyme in the saccharification process (Rani et al., 2015). If BGL becomes limited during hydrolysis of lignocellulosic biomass, cellobiose will accumulate and inhibit the activity of the other cellulases, thereby decreasing the saccharification efficiency (Tang et al., 2013). Cellobiose was found to mainly affect the activity of exoglucanases, especially cellobiohydrolase I (Du et al., 2010b). Addition of extra exogenous BGL eliminates this unwanted inhibition (Ma et al., 2011; Sukumaran et al., 2009; Xiao et al., 2004). However, to alleviate the product inhibition of cellobiose without raising the enzyme cost and thus also production cost, the fermentation organism can be engineered to express and secrete BGL. This increases cellobiose hydrolysis rate and overall saccharification efficiency. Some organisms have an endogenous BGL-producing ability, such as *Clavispora sp.* (Liu et al., 2012b). However, these organisms are often less ethanol tolerant and accumulate lower ethanol levels what makes the production process economically inviable. Therefore, we opted to confer cellobiose hydrolysis capacity to an ethanologenic *S. cerevisiae* strain. Several approaches for expression of heterologous BGL-encoding genes in *S. cerevisiae* have been explored, but commonly observed problems were that the enzyme was stuck intracellularly (Adam et al., 1995; Ferreira et al., 2010; Raynal and Guerineau, 1984) or that insufficient BGL was secreted and quantified to obtain a proper cellobiose hydrolysis rate (Kohchi and Toh-e, 1986; Rajoka et al., 1998).

One possibility to deal with these issues is the "intracellular BGL-cellodextrin transporter" strategy, where genes encoding a cellodextrin transporter and an intracellular ß-glucosidase are expressed in a single yeast cell. This results in import of cellobiose by the transporter followed by its hydrolysis in the cell. Expression of an intracellular BGL and cellodextrin transporter from *Neurospora crassa* in *S. cerevisiae* resulted in efficient growth and fermentation on cellodextrins (Galazka et al., 2010). The use of these heterologous genes has been studied extensively and showed promising results for expression in *S. cerevisiae* (Bae et al., 2014; Kim et al., 2014; Lee et al., 2013). It was observed that recombinant strains created with this strategy were able to simultaneously utilize xylose and cellobiose for ethanol production (Ha et al., 2011; Li et al., 2010). In addition, the lactose permease from *Kluyveromyces lactis* was investigated for its relevance in cellodextrin transport and intracellular utilization of cellobiose (Sadie et al., 2011). However, use of this transport facilitator was dependent on oxygen levels and needs to be optimized for use in fermentation (Njokweni et al., 2012). Combining the "intracellular BGL-cellodextrin transporter" strategy with the expression of other cellulases, *i.e.* endoglucanase and cellobiohydrolase, displayed on the cell surface resulted in an improved fermentation of phosphoric acid swollen cellulose (PASC) (Yamada et al., 2013b).

Furthermore, BGL-encoding genes have been expressed in *S. cerevisiae* to obtain secreted BGL. This "secretion" strategy would allow the enzymes to come in contact with the substrate and thus would guarantee a higher conversion rate than using the "intracellular BGL-cellodextrin transporter" strategy. Many BGL-encoding genes from different origins have been expressed and secreted in *S. cerevisiae*. Cummings and Fowler (1996) were able to express *bgl1* from *Trichoderma reesei* and obtained a recombinant *S. cerevisiae* strain that actively secreted BGL. A similar result was obtained when *bglB* from *Candida wickerhamii* was expressed (Skory et al., 1996). Machida and colleagues (1988) obtained an *S. cerevisiae* strain that was able to hydrolyze cellobiose when Bgl1 from *Saccharomycopsis fibuligera* was secreted. This result was further supported with secretion of *Sf*Bgl1 during fermentation on corn cob hydrolysate with a laboratory strain (Tang et al., 2013) and with the industrial NAN-27 strain (Shen et al., 2008). Expression of the promising *bgl1* from *Saccharomycopsis fibuligera* was also compared with BGLs from other origins and in different strategies. It was reported that *Sf*Bgl1 was trapped in the periplasm and that fusing its sequence to a cell wall anchoring protein increased the cellobiose utilization of the cell (van Rooyen et al., 2005).

Recombinant *S. cerevisiae* strains were able to grow on PASC upon co-expression of a BGL-encoding gene from *S. fibuligera* and an EG-encoding gene from *T. reesei* (den Haan et al., 2007). Jeon and colleagues (2009b) were able to create a strain that fermented ß-glucan, PASC and carboxymethyl cellulose (CMC) to ethanol upon co-expression of *bgl1* from *S. fibuligera* and the endoglucanase E from *Clostridium thermocellum*. Combination of expression of *cbh1* and *bgl1* from *Aspergillus aculeatus* in *S. cerevisiae*, however, did not result in specific activity towards cellulosic substrates and required an EG to be able to hydrolyze Avicel (Takada et al., 1998).

In this chapter, we aim to obtain an industrial xylose-fermenting inhibitor-tolerant *S. cerevisiae* strain that secretes ß-glucosidase and has the capacity to hydrolyze cellobiose and ferment the released glucose immediately to ethanol. For that purpose, a heterologous gene encoding a ß-glucosidase is genomically integrated using CRISPR/Cas9. This genomic integration is conducted in two different yeast strains, namely GSE16-T18 HAA1* and MD4. GSE16-T18 HAA1* is an industrial strain that was optimized for xylose utilization and inhibitor

tolerance, specifically acetic acid tolerance (Demeke, 2013; Demeke et al., 2013a; Demeke et al., 2013b; Meijnen et al., 2016). The MD4 strain was derived from a cross between the diploid GSE16-T18 HAA1* and diploid Ethanol Red. This resulted in a tetraploid, xylose-utilizing and inhibitor-tolerant yeast strain. The developed recombinant strains are characterized for the secreted specific BGL activity and evaluated by fermentation performance.

2. Results

2.1. Evaluation of *Sf*Bgl1 and *Tr*Bgl1 in GSE16-T18 HAA1*

Initially, two different BGL-encoding genes were expressed and evaluated in an industrial second-generation bioethanol producing strain. The *bgl1* gene from *Trichoderma reesei* (*Trbgl1*) and the *bgl1* gene from *Saccharomycopsis fibuligera* (*Sfbgl1*) were investigated previously for their characteristics (Chauve et al., 2010; Gundllapalli et al., 2007) and their ability to confer cellobiose hydrolysis capacity to *S. cerevisiae* strains (van Rooyen et al., 2005). The enzymes and their characteristics are listed in Table 2.1.

Table 2.1. Evaluated heterologous ß-glucosidases and their properties

Gene	Organism	Enzyme	Uniprot	pH and temperature optimum	Size (kDa)	References
bgl1	*Trichoderma reesei*	*Tr*Bgl1	Q12715	4.6; 65°C	78.4	(Barnett et al., 1991; Chen et al., 1992; Mach et al., 1995)
bgl1	*Saccharomycopsis fibuligera*	*Sf*Bgl1	P22506	5.0; 50°C	220	(Machida et al., 1988)

The nucleotide sequence of these genes was codon optimized (Appendix Table A2.1) and cloned on a multicopy plasmid flanked by the strong *TEF1* promoter and *CYC1* terminator. Plasmid-based expression of *Trbgl1* and *Sfbgl1* in GSE16-T18 HAA1* showed that 1% (w/v) cellobiose is consumed in 24 h and 10 h, respectively (Fig. 2.1A). However, when the specific secreted activity of the enzymes was measured with 4-nitrophenyl ß-D-glucopyranoside (pNPG) as substrate, it was observed that *Sf*Bgl1 is not secreted but stuck in the cells since the activity is higher in the cell extract than in the cell-free medium (Fig. 2.1B). This indicates that although *Sf*Bgl1 showed a faster cellobiose utilization rate, the secreted specific activity was very low. Therefore, the project was continued with *Tr*Bgl1 to obtain high secreted specific BGL activity.

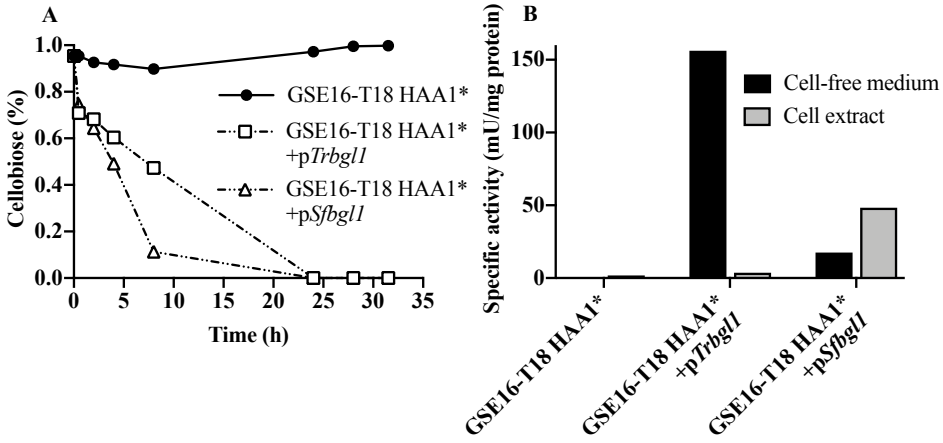


Figure 2.1. Cellobiose hydrolysis and specific BGL activity upon plasmid-based expression of *Trbgl1* and *Sfbgl1* in GSE16-T18 HAA1*. (A) Cellobiose hydrolysis during growth of GSE16-T18 HAA1* and GSE16-T18 HAA1* containing a plasmid with *Trbgl1* and *Sfbgl1* in SC with 1% (w/v) cellobiose (35°C, 50mL, 1g CDW/L). (B) Specific BGL activity measured with pNPG as substrate in the cell-free medium and in the cell extract (35°C, pH 5.5, pNPG).

2.2. pH and temperature optimum of *Tr*Bgl1

The pH and temperature optimum of *Tr*Bgl1 were determined upon expression in GSE16-T18 HAA1* to ensure BGL activity at the industrially relevant conditions. The pH optimum of *Tr*Bgl1 was pH 5.0 (Fig. 2.2A) and the temperature optimum was determined to be around 70°C (Fig. 2.2B). These optimal conditions were identified in cell-free medium containing the secreted BGL as well as native secreted proteins. However, since the used substrate pNPG is highly specific for cellobiose activity and this activity is not detected for the parent strain MD4, this method enabled the specific identification of optimal *Tr*Bgl1 conditions in this mixture.

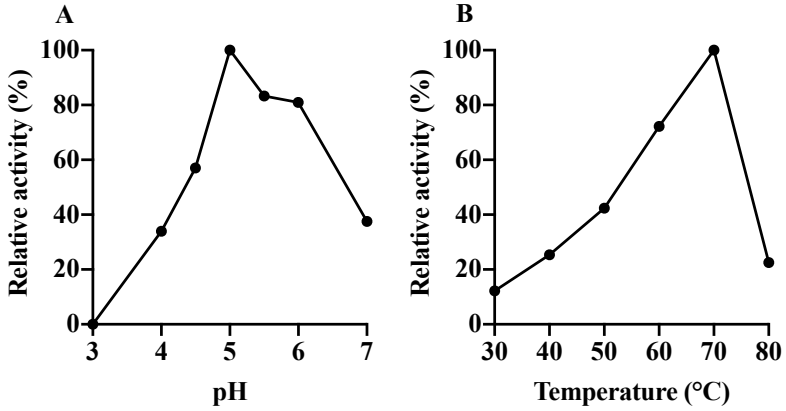


Figure 2.2. Relative activity of *Tr*Bgl1 in cell-free medium with pNPG as substrate to determine optimal conditions. (A) pH dependence measured at 35°C. (B) Temperature dependence measured at pH 5.5.

2.3. Genomic integration of *Trbgl1* in GSE16-T18 HAA1* and evaluation of enzyme activity, secretion and fermentation performance of the resulting strains

To obtain stable expression and secretion of *Tr*Bgl1 in GSE16-T18 HAA1*, the gene was genomically integrated. Firstly, two copies of *Trbgl1* were integrated using φC31 integrase in neutral site IS4.1 at chromosome IV (Table M2 in Materials and Methods). Additionally, four extra copies were genomically integrated using CRISPR/Cas9. For that purpose, an integrative cassette containing *Trbgl1* flanked by the strong constitutive *TEF1* promoter and *CYC1* terminator in tandem was created to target IS2.1 at chromosome II (Fig. 2.3A and Table M2 in Materials and Methods), which was integrated using the standard CRISPR/Cas9 protocol. The strains were checked for genomic integration in all alleles using PCR with two primers flanking the integration cassette, C-2827 and C-2828 for IS4.1 and C-431 and C-432 for IS2.1 (Table M3 in Materials and Methods). Successful integration was identified when a band of 8 kb was amplified as observed for lane 2 and 3 of Fig. 2.3B and lane 3 of Fig. 2.3C. No integration should be detected by amplifying a band of 1.5-2 kb as observed for lane 1 in Fig. 2.3B and for lane 1 and 2 in Fig. 2.3C.

After genomic integration of two copies using φC31 integrase and 4 additional copies using CRISPR/Cas9, the recombinant strains were evaluated for their cellobiose hydrolysis capacity. First, the secreted specific BGL activity of these strains with pNPG as substrate was determined. BGL activity was absent for GSE16-T18 HAA1*. Two copies of *Trbgl1* induced a specific BGL activity of 63 mU/mg secreted protein. This was further increased with the addition of four extra copies, where the activity reached 229 mU/mg secreted protein (Fig. 2.4).

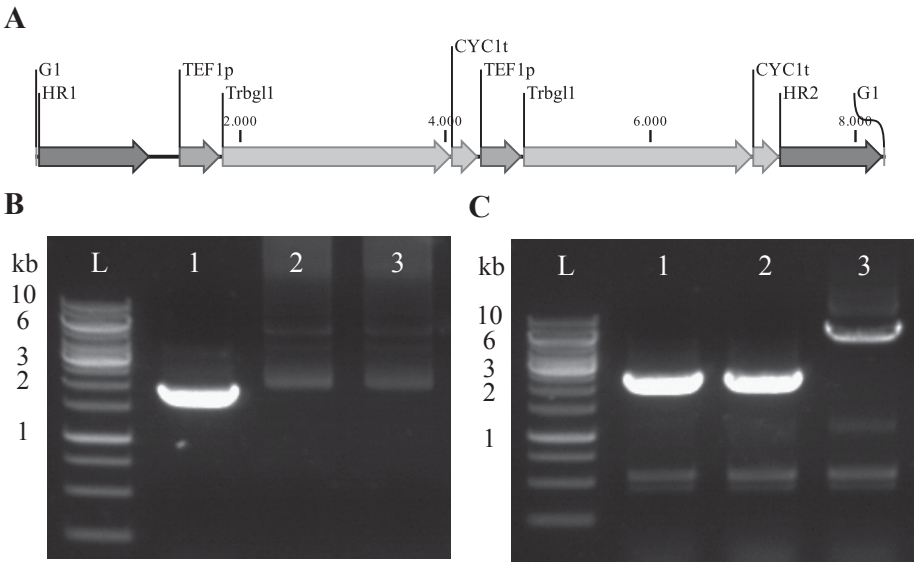


Figure 2.3. *Trbgl1* tandem cassette and its genomic integration in GSE16-T18 HAA1*. (A) Schematic overview of the tandem cassette of *Trbgl1* used for genomic integration using CRISPR/Cas9. (B) Gel electrophoresis of PCR product using two primers flanking the integration cassette to check genomic integration of *Trbgl1* at IS4.1 after using the φC31 integrase technique with GSE16-T18 HAA1* (lane 1), GSE16-T18 HAA1* containing two copies of *Trbgl1* (lane 2) and GSE16-T18 HAA1* containing six copies of *Trbgl1* (lane 3). Lane L is GeneRuler 1 kb DNA Ladder. (C) Gel electrophoresis of PCR product using two outside primers to check genomic integration of *Trbgl1* at IS2.1 after using the CRISPR/Cas9 technique for the same strains.

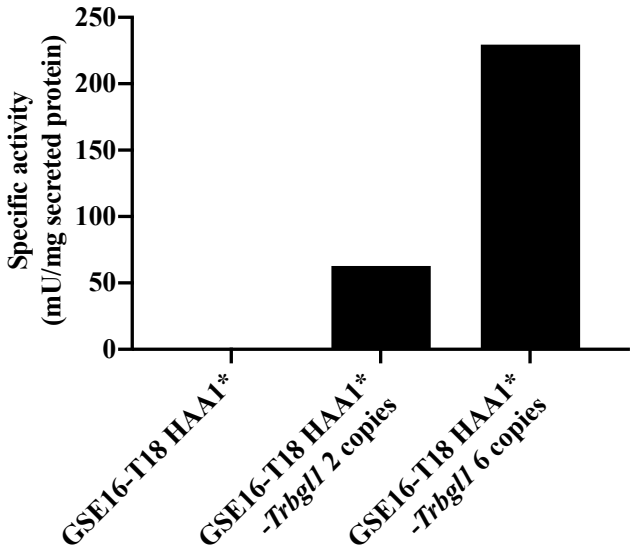


Figure 2.4. Specific secreted activity of *Tr*Bgl1 upon genomic integration of different *Trbgl1* copy numbers in GSE16-T18 HAA1*. Measured with pNPG as substrate (35°C; pH 5.5, pNPG). Average of three technical repeats is shown.

The secreted proteins of these strains were separated with SDS-PAGE. Coloring using silver staining indicated that a band of 78 kDa appeared in the strains harboring *Trbgl1* (Fig. 2.5). More copies of the gene also induced a stronger band and thus more secreted *Tr*Bgl1 enzyme.

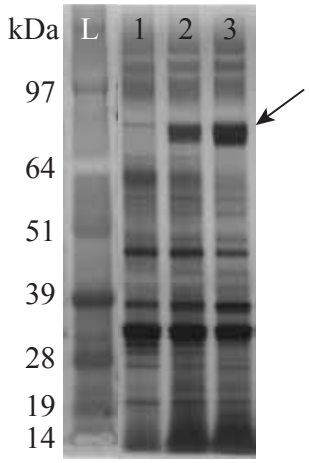


Figure 2.5. Silver staining of SDS-PAGE of cell-free medium of constructed strains. GSE16-T18 HAA1* (lane 1), GSE16-T18 HAA1* containing two copies of *Trbgl1* (lane 2) and GSE16-T18 HAA1* containing six copies of *Trbgl1* (lane 3). Arrow indicates *Tr*Bgl1. Lane L is SeeBlue Plus2 Pre-stained Protein Standard.

These results were validated during fermentation of YP with 6% (w/v) glucose, 4% (w/v) xylose and 1% (w/v) cellobiose, allowing a maximal weight loss of 5.5%. Glucose and xylose were added to eliminate the possibility of a negative effect due to integration of the heterologous gene. Cellobiose is a specific substrate for BGL and can thus be hydrolyzed and converted to ethanol when BGL activity is detected. The weight loss indicated that the parent strain GSE16-T18 HAA1* only consumed glucose and xylose. Furthermore, the weight loss increased with 0.5% for the two transformants containing *Trbgl1*, indicating that 1% (w/v) cellobiose has been converted to ethanol. The strain containing two copies consumed all cellobiose in 125 h, while the strain containing six copies of *Trbgl1* induced the same result but in 50 h (Fig. 2.6).

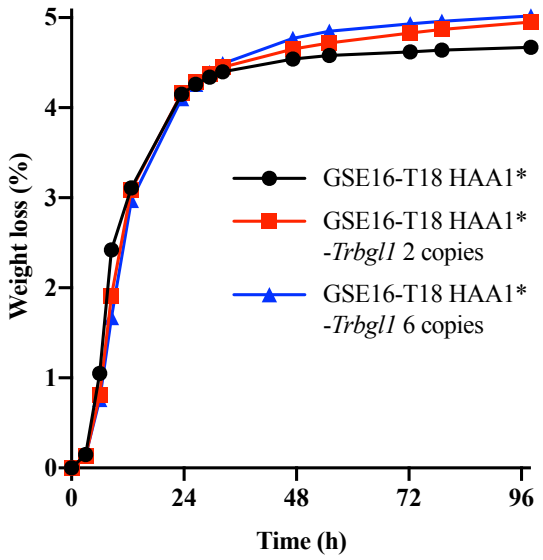


Figure 2.6. Fermentation performance measured by weight loss due to CO_2 release of constructed strains. GSE16-T18 HAA1*, GSE16-T18 HAA1* containing two copies of *Trbgl1* and GSE16-T18 HAA1* containing six copies of *Trbgl1* in YP with 6% (w/v) glucose, 4% (w/v) xylose and 1% (w/v) cellobiose (35°C, 50mL, 1g CDW/L).

2.4. Genomic integration of *Trbgl1* in MD4

After these results, we also tested the gene in another industrial xylose-fermenting inhibitor-tolerant yeast strain, MD4, that is more consistent in terms of fermentation performance, less flocculent and easier to work with. For that reason, all further strain developments were done in this strain background. Since this strain was developed from a cross between two diploid strains, MD4 is overall a tetraploid strain, but has some aneuploid chromosomes (Table 2.3).

To obtain an MD4 strain expressing *Trbgl1*, the tandem cassette of *Trbgl1* (Fig. 2.3A) was integrated at IS2.1 using the same plasmids and CRISPR/Cas9 approach as for integration in GSE16-T18 HAA1*. Genomic integration in all alleles was confirmed using PCR (Fig. 2.7) with primers C-431 and C-432 (Table M3 in Materials and Methods). No integration of *Trbgl1* resulted in a band of around 2 kb, as observed with DNA from MD4. If the tandem cassette of *Trbgl1* was integrated, this showed a band around 8 kb, as observed for the developed strain that was named AC1.

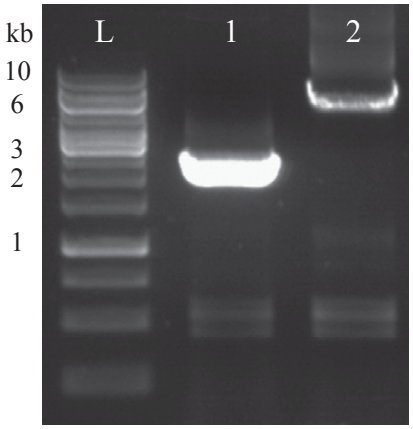


Figure 2.7. Gel electrophoresis of PCR product to check genomic integration of *Trbgl1* at IS2.1 using two primers flanking the integration cassette. MD4 (lane 1) and AC1 (lane 2). Lane L is GeneRuler 1 kb DNA Ladder.

2.5. Whole genome sequence analysis of MD4 and AC1

Since MD4 was pentaploid for chromosome II where IS2.1 is located, we hypothesized that the resulting strain, AC1, harbored 10 copies of *Trbgl1*. To investigate this, AC1 was sent for Illumina whole genome sequencing at BGI (Hong Kong). The data were analyzed to obtain the exact ploidy of chromosome II and thus the exact copy number of *Trbgl1* in AC1 using in-house scripts, as described in Materials and Methods.

First, the SNP frequency for each chromosomal position of chromosome II was compared between MD4 and AC1 (Fig. 2.8A, B). Every dot on these figures represents a SNP compared to the S288c reference mapping. For MD4, the distribution of SNPs was observed around every 20 % (Fig. 2.8A). This means that the data points are distributed over the red lines with 0.2-0.4-0.6-0.8 indications. This distribution can only be obtained if the occurring SNPs can be present in 1, 2, 3, 4 or 5 alleles, indicating that MD4 is pentaploid for chromosome II. For AC1, on the other hand, the distribution of SNPs was observed around every 25 % (Fig. 2.8B). This can only be obtained if the SNPs are present in up to 4 alleles. Therefore, AC1 was considered to be tetraploid for chromosome II and thus lost one allele due to the genomic integration of *Trbgl1*.

Second, the count of reads that cover entire chromosome II was compared with the coverage of the reads that sequenced the tandem cassette with *Trbgl1* in AC1 (Fig. 2.8C). The average coverage of both was fairly similar, which taught us that the tandem cassette appeared once per allele. Combining these results, the whole genome sequence analysis showed that AC1 carried four tandem cassettes and thus eight copies of *Trbgl1* at IS2.1. An overview of the genomic properties determined by whole genome sequencing of MD4 and AC1 is listed in Table 2.2.

Table 2.2. Overview of genomic properties as determined by whole genome sequence analysis of MD4 and AC1. Ploidy of all chromosomes and coverage ratio of the integrated heterologous gene *Trbgl1* compared to the coverage of its host chromosome, determining its approximate copy number.

	MD4			AC1		
Chr.	Ploidy	Ratio coverage tandem *Trbgl1*/ chromosome	Copy number	Ploidy	Ratio coverage tandem *Trbgl1*/ chromosome	Copy number
I	5n	-	-	5n	-	-
II	5n	-	-	4n	~1.0x	8 BGL
III	4n	-	-	4n	-	-
IV	5n	-	-	5n	-	-
V	4n	-	-	4n	-	-
VI	4n	-	-	4n	-	-
VII	5n	-	-	5n	-	-
VIII	4n	-	-	4n	-	-
IX	5n	-	-	5n	-	-
X	3n	-	-	4n	-	-
XI	4n	-	-	4n	-	-
XII	5n	-	-	4n	-	-
XIII	4n	-	-	4n	-	-
XIV	4n	-	-	4n	-	-
XV	4n	-	-	4n	-	-
XVI	5n	-	-	5n	-	-

Chr.: chromosome, -: not applicable

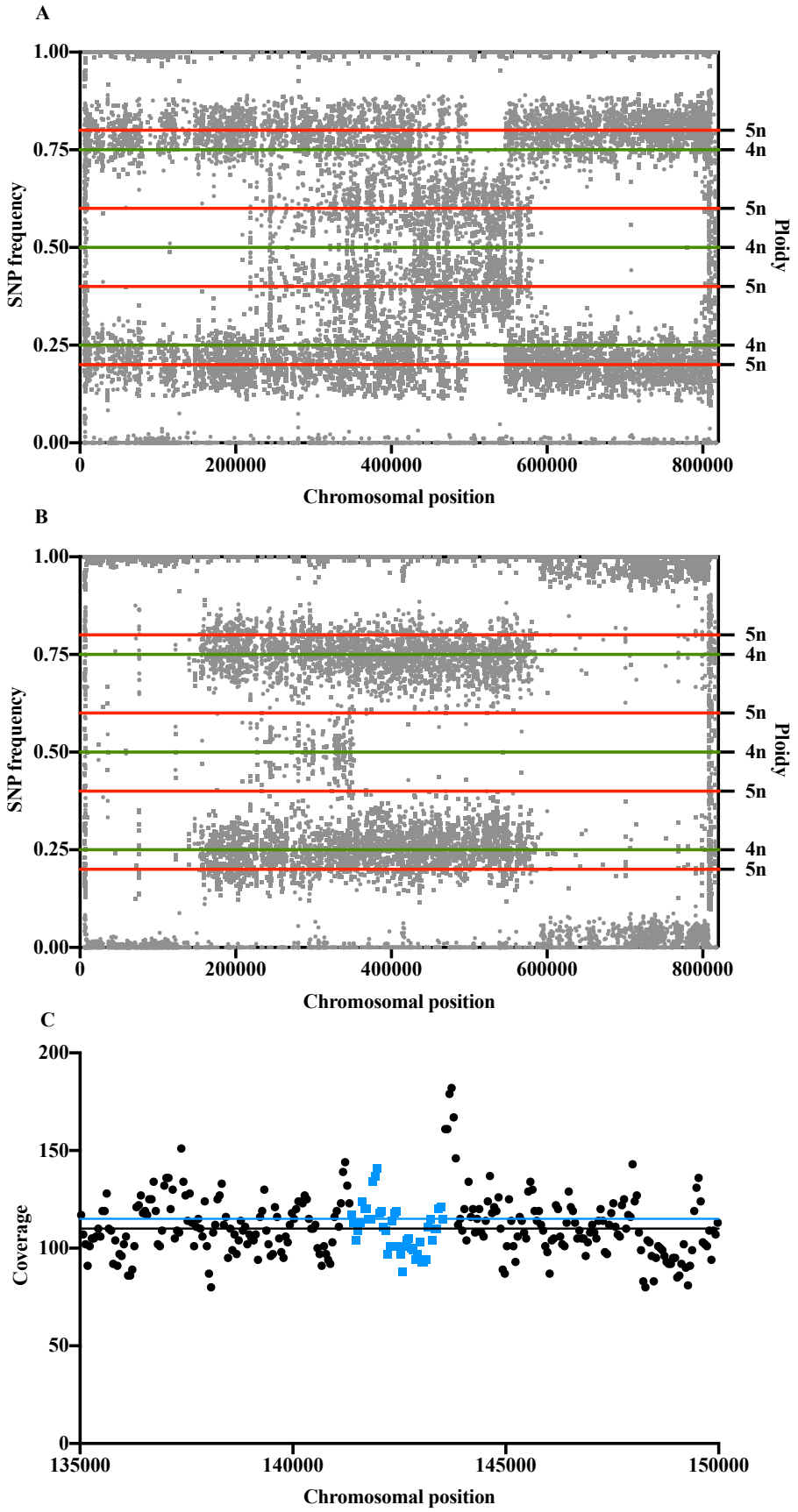


Figure 2.8. SNP frequency and read coverage as determined by whole genome sequence analysis of MD4 and AC1. (A) SNP frequency of chromosome II of MD4 plotted against chromosomal position and the resulting ploidy (4n, green; 5n, red). (B) SNP frequency of chromosome II of AC1 plotted against chromosomal position and the resulting ploidy (4n, green; 5n, red). (C) Coverage of the reads in *Trbgl1* (blue squares) and the surrounding genes (black circles) in AC1.

2.6. Evaluation of enzyme activity, secretion and fermentation performance of AC1

The BGL-secreting AC1 strain was subjected to a series of characterization experiments. The specific activity of secreted BGL was assessed in cell-free medium with pNPG as substrate. No activity was observed with MD4, whereas AC1 displayed a specific activity with pNPG of 19.1 U/g CDW or 523 mU/mg secreted protein (Fig. 2.9A and Appendix Fig. A2.1). Secreted BGL was visualized using SDS-PAGE and silver staining. This showed a band appearing with AC1 at 78 kDa, which is the size of *Tr*Bgl1 (Fig. 2.9B).

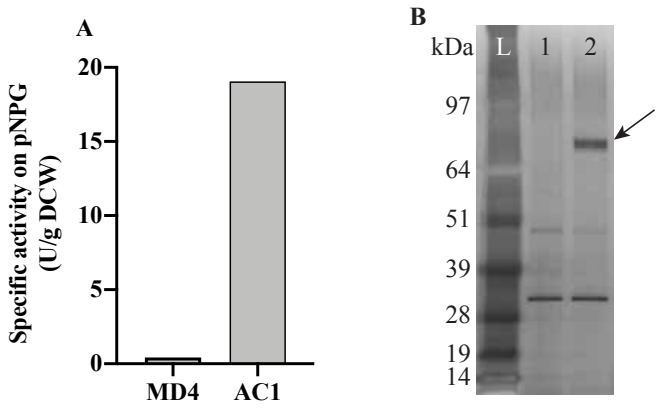


Figure 2.9. Evaluation of secreted *Tr*Bgl1 in MD4 and AC1. (A) Specific secreted BGL activity in cell-free medium on pNPG substrate (35°C, pH 5.5, pNPG). Average of three technical repeats is shown. (B) Silver staining of SDS-PAGE with cell-free medium of MD4 (lane 1) and AC1 (lane 2). Arrow indicates *Tr*Bgl1. Lane L is SeeBlue Plus2 Pre-stained Protein Standard.

Secreted BGL activity was also assessed during fermentation with cellobiose. With YP containing 2% (w/v) cellobiose, no weight loss was detected for MD4 as opposed to AC1 (Fig. 2.10). On the other hand, AC1 was able to hydrolyze 2% (w/v) cellobiose to glucose and convert the glucose to ethanol in about 100 h, reaching a final weight loss of 1.1%. Note that higher initial sugar levels result in increased weight loss and more significant differences.

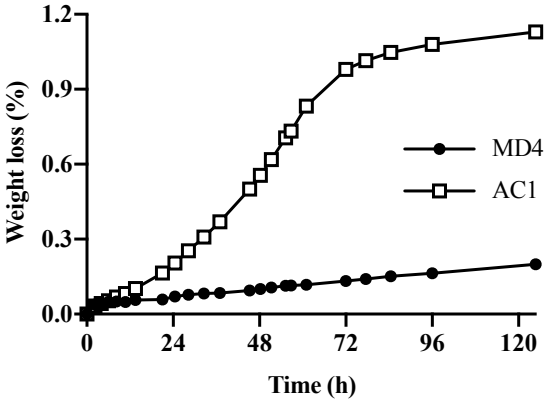


Figure 2.10. Fermentation performance measured by weight loss due to CO_2 release of MD4 and AC1. Fermentation with MD4 and AC1 containing eight copies of *Tr*bgl1 in YP with 2% (w/v) cellobiose (35°C, 50mL, 1g CDW/L).

There was no difference between MD4 and AC1 for the utilization of glucose or xylose in a YP medium containing 5% (w/v) glucose, 4% (w/v) xylose and 1% (w/v) cellobiose. On the other hand, cellobiose was only

fermented by AC1, causing a higher final weight loss than with MD4 and ethanol production of 6.1% (v/v) (Fig. 2.11A, B). Maximal weight loss when all sugars would be consumed is 5%.

When the concentration of cellobiose in this rich medium was increased, it was completely utilized and fermented to ethanol. Fermentation of YP with 4% (w/v) glucose, 2% (w/v) xylose and 2% (w/v) cellobiose with MD4 and AC1 showed a more distinct difference between the performance of both strains. In this case, 2% (w/v) cellobiose was converted to ethanol in 100 h (Fig. 2.12), resulting in the calculated maximal weight loss of 3.9%.

Finally, AC1 was also evaluated on more industrially relevant substrate, namely paper pulp hydrolysate. This substrate was provided by the Bio Base Europe Pilot Plant (BBEPP) and consisted of pretreated paper and cardboard waste streams. After pretreatment and hydrolysis performed at BBEPP, this hydrolysate contained around 8% (w/v) glucose, 2% (w/v) xylose and 0.8% (w/v) cellobiose. To prevent contamination and further activity of the commercial enzyme cocktail during the fermentation, the material was pasteurized for 1 h at 90°C. Fermentation of this material with MD4 and AC1 indicated that AC1 resulted in an additional ethanol production of 0.3% (v/v) which was a result of cellobiose utilization, albeit partial (Fig. 2.13). This hydrolysate is an example of industrially used media, which often contain cellobiose and thus using a cellobiose-utilizing strain increases the ethanol production.

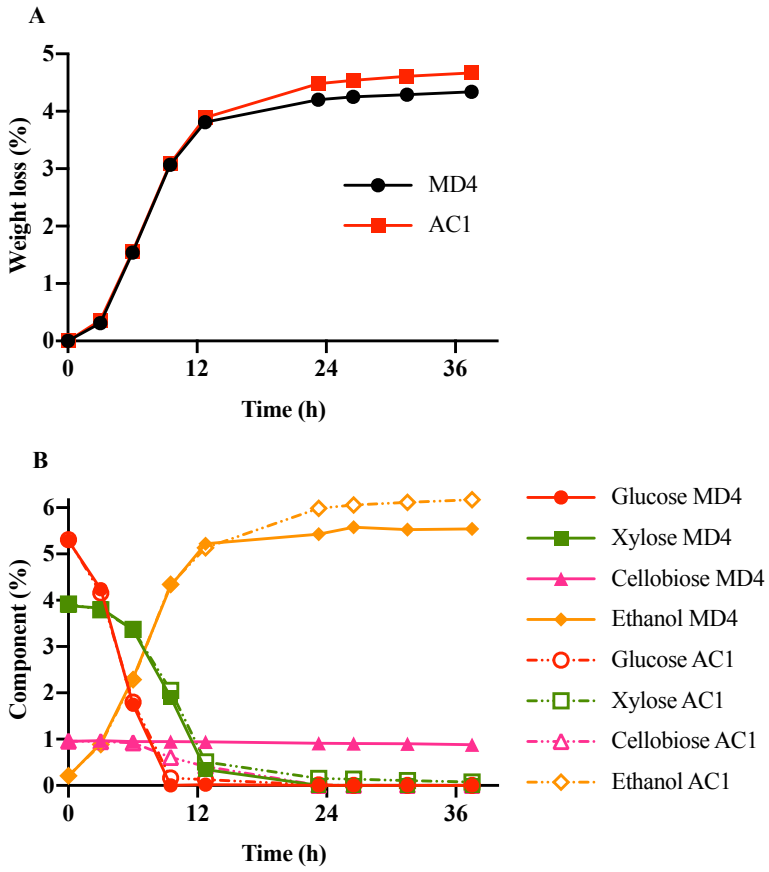


Figure 2.11. Fermentation performance of strains MD4 and AC1 in YP containing 5% (w/v) glucose, 4% (w/v) xylose and 1% (w/v) cellobiose (35°C, 60mL, 1g CDW/L). (A) Weight loss during fermentation with MD4 and AC1. (B) Sugar consumption (w/v) (glucose, red; xylose, green; cellobiose, magenta) and ethanol production (v/v) (orange) during fermentation with MD4 (full lines and symbols) and AC1 (dotted lines and open symbols).

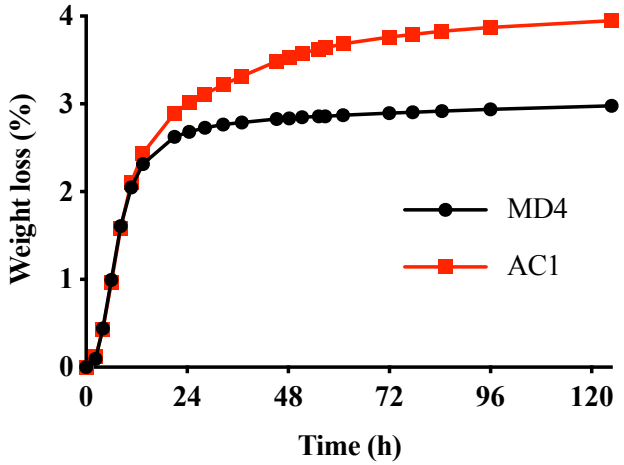


Figure 2.12. Fermentation performance measured by weight loss due to CO_2 release of AC1 compared to MD4. Fermentation with MD4 and AC1 in YP containing 4% (w/v) glucose, 2% (w/v) xylose and 2% (w/v) cellobiose (35°C, 50mL, 1g CDW/L).

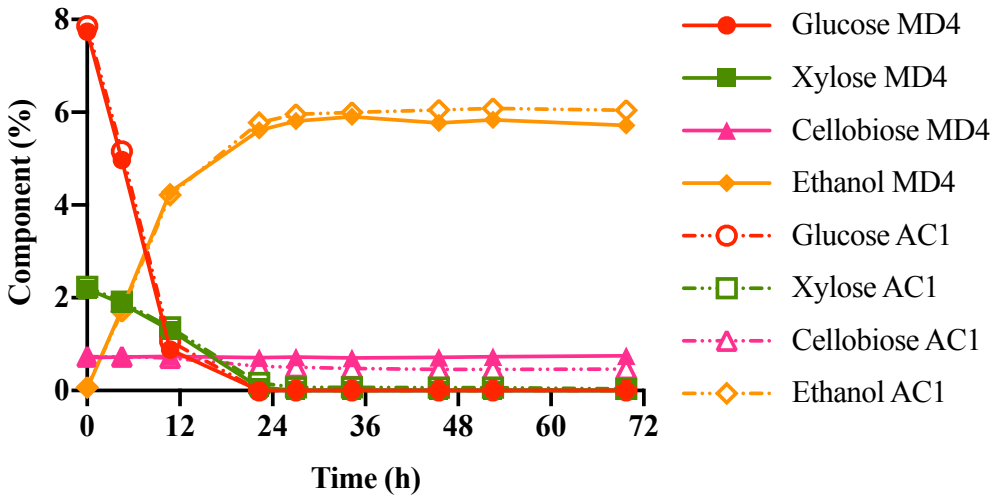


Figure 2.13. Sugar consumption and ethanol production of MD4 and AC1 during fermentation of paper pulp hydrolysate (35°C, 60mL, 1g CDW/L). Sugar consumption (glucose, red circles; xylose, green squares; cellobiose, magenta triangles) and ethanol production (orange diamonds) during fermentation with MD4 (full lines and symbols) and AC1 (dotted lines and open symbols).

3. Discussion

ß-glucosidase is the final enzyme in the cascade of cellulose hydrolysis. The conversion of cellobiose to glucose is an essential step that can only be performed by BGLs, which makes them indispensable for use of lignocellulosic biomass as feedstock for second-generation bioethanol production. Additionally, due to low amounts of BGL present in commercial enzyme cocktails (Del Pozo et al., 2012), exogenous secretion of BGL by *S. cerevisiae* was investigated to prevent cellobiose accumulation and thus cellulase inhibition (Tang et al., 2013).

Initially, two heterologous BGL-encoding genes from different origins were evaluated for expression and secretion in *S. cerevisiae*. In the first experiments, Bgl1 from *S. fibuligera* seemed to exhibit a faster cellobiose hydrolysis capacity compared to Bgl1 from *T. reesei*. However, the use of *Sf*Bgl1 was dubious due to possible IP protection (US20110129888A1; US20110177542A1), which has to be avoided in commercial industrial strains. Also, after evaluation of the specific BGL activity, it was observed that *Sf*Bgl1 was not secreted as low activity was detected in the cell-free medium. The cell extract, on the other hand, displayed higher specific BGL activity, which indicates that the enzyme is trapped in the cell. This can be accredited to the big size of *Sf*Bgl1 (220 kDa) and poor enzyme secretion. Furthermore, it is known that cellobiose cannot be transported to the cytosol by non-recombinant *S. cerevisiae* (Galazka et al., 2010; Sadie et al., 2011). Therefore, the hypothesis was that *Sf*Bgl1 is stuck in the periplasm, where diffusion of cellobiose can reach the trapped enzymes, as suggested by van Rooyen and colleagues (2005). Hydrolysis of cellobiose in the periplasm has a benefit of fast and efficient glucose uptake as the glucose is released in the near proximity of the cell, similar as displaying minicellulosomes on the cell wall (Fan et al., 2012; Tsai et al., 2009). However, not being able to secrete BGL means that the cellobiose hydrolysis rate is highly dependent on the viscosity of the medium and stirring in large-scale reactors. The bigger the fermentor, the more the cells will be gradually distributed and if the cells do not reach the cellobiose, no additional glucose will be released. For that reason, it is essential to mimic industrial conditions as closely as possible (Takors, 2012).

The optimal pH and temperature for *Tr*Bgl1 were determined to assess the relevance in industrial conditions. The pH optimum of the enzyme at 35°C was pH 5.0. This is very close to the pH value that can be found in industrial hydrolysates, which usually vary between pH 4.7 and pH 5.5. The optimal temperature of *Tr*Bgl1, on the other hand, was determined to be 70°C at pH 5.5. At the industrially relevant conditions, which would be around 32°C-35°C, the relative activity of *Tr*Bgl1 is lowered to 30%. Studying literature taught us that there are only few BGLs known that display a high activity at low temperatures. These enzymes are commonly originating from the digestive tract of animals (Li et al., 2014; Zhang et al., 2017). Enzyme activity at low temperatures can also be enhanced by random mutagenesis and selection of the concerning genes and their relevant phenotype (Lebbink et al., 2000). However, the *Tr*Bgl1 enzyme still displayed a considerable activity on pNPG and cellobiose at lower temperature. To optimize the cellobiose hydrolysis capacity of AC1, it would be interesting to perform an extensive screening of recombinant BGL enzymes. Most likely, this would result in a high variability in secreted specific BGL activity. This difference might be related to secretion efficiency, glycosylation or incorrect folding of the heterologous enzymes. Furthermore, *N*- and *O*-linked hyperglycosylation tends to affect the secretion and activity of heterologous enzymes expressed in *S. cerevisiae* (Hernandez et al., 2017; Tang et al., 2016). Also, the

heterologous gene was codon optimized for expression in *S. cerevisiae*, but the codon usage is known to affect translation elongation, efficiency and protein folding (Brule and Grayhack, 2017; Hanson and Coller, 2018). Screening and comparison of genes originating from different species or different codon optimized variations may result in improvement of specific secreted BGL activity and cellobiose hydrolysis.

This research initially focused on an industrial *S. cerevisiae* strain called GSE16-T18 HAA1*. First, genomic integration using φC31 integrase resulted in a strain harboring two copies of *Trbgl1* at IS4.1. This strain was then used for integration of additional copies of *Trbgl1* at IS2.1 using CRISPR/Cas9. This gradual increase in copy number provided insight in its influence on the enzyme activity. Specific secreted activity of BGL with pNPG as substrate increased from 63 mU/mg secreted protein to 229 mU/mg secreted protein when four additional copies of *Trbgl1* were expressed. This enhanced activity was also observed during fermentation where cellobiose was consumed significantly faster with GSE16-T18 HAA1* containing six copies of *Trbgl1* than with its ancestor containing two copies. This can be attributed to an increased amount of *Tr*Bgl1 in the medium, which was observed using SDS-PAGE and silver staining.

After these results, we have decided to switch to another industrial yeast strain called MD4. This parental strain was less flocculent and more consistent in terms of fermentation performance as determined in previous research. An additional benefit of using MD4 is the ploidy of this strain. Being mainly tetraploid with some aneuploid chromosomes, this allows for direct genomic integration of high copy numbers. Chromosome II is pentaploid in MD4, therefore this was one of the prioritized chromosomes for integration of heterologous genes using CRISPR/Cas9 to obtain high copy numbers. However, after genomic integration of the *Trbgl1* tandem cassette flanked by the *TEF1* promoter and *CYC1* terminator, whole genome sequence analysis of AC1 indicated that one allele of chromosome II was lost. This loss of an aneuploid chromosome can be related to strain stability. Since the coverage of the integrated cassette was similar to the coverage of the genes surrounding IS2.1, we concluded that AC1 harbored eight copies of *Trbgl1*. Expression of eight copies in AC1 resulted in a specific secreted activity of 19.1 U/g CDW, or 523 mU/mg secreted protein. Comparing this to the GSE16-T18 HAA1* containing six copies of *Trbgl1*, an increase of around 300 mU/mg secreted protein is observed which can be attributed to the extra copies or to the strain background. It was already shown in the same background that additional copies increase the enzyme secretion and thus specific activity. Increased heterologous enzyme expression and secretion efficiency can also be attributed to the strain background. Whereas GSE16-T18 HAA1* is a diploid, very flocculent strain, MD4 is a tetraploid strain that is easier to work with.

The activity of secreted *Tr*Bgl1 with AC1 was detected in both *in vitro* and *in vivo* assays. The specific BGL activity and fermentation performance in YP medium indicated the high cellobiose hydrolysis capacity of AC1. This was also evaluated in industrial relevant hydrolysate. The fraction of cellobiose present in this paper pulp hydrolysate was low around 0.8% (w/v). However, in this hydrolysate, only 0.4% (w/v) of this cellobiose was utilized and converted to ethanol in 70 h. On the other hand, since all cellobiose was consistently used in all previous experiments with AC1, it is assumed that the cellobiose present in the paper pulp hydrolysate is consumed by AC1 and that the leftover residue that is still detected using HPLC is another compound that cannot be consumed and has the same retention time as cellobiose.

In this chapter, we were able to create an industrial xylose-fermenting inhibitor-tolerant *S. cerevisiae* strain, AC1, that has the capacity to hydrolyze cellobiose and convert the released glucose to ethanol. This strain was created by genomic integration of *bgl1* from *Trichoderma reesei* using CRISPR/Cas9. AC1 was capable of utilizing cellobiose because of the high specific BGL activity. *Tr*Bgl1 was shown to be secreted and visually detected using SDS-PAGE. This enzyme not only displayed a high activity in *in vitro* assays, but also during fermentation of multiple synthetic media and paper pulp hydrolysate.

Chapter 3 - Conferring xylanolytic capacity to an industrial xylose- and cellobiose-utilizing *S. cerevisiae* strain by expression of heterologous genes encoding a ß-xylosidase and multiple xylanases

1. Introduction

Lignocellulose consists of lignin, cellulose and hemicellulose. The latter is fractionated in xylan, mannan, galactan and arabinan, depending on the amount of D-xylose, D-mannose, D-galactose and L-arabinose present (Beg et al., 2001; Girio et al., 2010). The major portion of hemicellulose present in plant material is xylan, which is a heteropolymer consisting principally of xylose and arabinose (Bastawde, 1992). The xylan backbone is composed of xylose residues linked by ß-1,4-glycosidic bonds. This backbone is known to be highly substituted with different side groups. The percentage of xylan in plant cell walls is dependent on the source. Hardwood and grasses contain up to 30 % xylan, while softwoods are usually limited to 10 % (Kulkarni et al., 1999; Motta et al., 2013).

To obtain fermentable sugars from this heteropolymeric substrate, multiple enzymes are essential for hydrolysis. Breakdown of the xylan backbone requires xylanase (endo-1,4-ß-xylanase, EC 3.2.1.8, XYL) and ß-xylosidase (xylan-1,4-ß-xylosidase, EC 3.2.1.37, BXL). Xylanases randomly catalyze the hydrolysis of the ß-1,4-glycosidic links in xylan, resulting in xylooligosaccharides of various chain lengths. ß-xylosidase acts on xylobiose and higher xylooligosaccharides with decreasing specific affinity, thereby liberating fermentable D-xylose molecules (Bastawde, 1992; Collins et al., 2005).

Xylanolytic enzymes are widely distributed and have been reported in prokaryotes, such as rumen bacteria, and eukaryotes, such as insects, snails and even germinating plant seeds (Dekker and Richards, 1976; Taiz and Honigman, 1976). These enzymes, in particular the microbial xylanases, have been studied extensively because of their widespread potential industrial use. In the paper and pulp industry, microbial enzymes are used for bio-bleaching of pulp (Perez et al., 2002). In the food industry, the released xylose can be converted into xylitol that can be used as a valuable sweetener (Parajó et al., 1998). Furthermore, xylooligosaccharides were shown to have prebiotic effect. Since they cannot be hydrolyzed or absorbed in the beginning of the human gastrointestinal tract, they can affect the growth and activity of several bacteria in the colon and thus improve the host's health (Roberfroid, 1997; Vázquez et al., 2000).

The industrial application that is of interest for this work is the hydrolysis of xylan from lignocellulosic biomass into xylose molecules for the production of bio-based chemicals. This is essential, not only to add carbon value to the production chain, but also to increase the exposure of cellulose to cellulases (den Haan et al., 2013a). Clearance of xylooligosaccharides is also proven to be important to relieve the inhibition of these molecules on xylanases and cellulases (Qing et al., 2010; Zhang et al., 2012).

Our aim is to use an *S. cerevisiae* strain that expresses and secretes xylanolytic enzymes to hydrolyze xylan and ferment the carbon to ethanol. However, this organism naturally lacks the ability to consume and ferment pentose sugars as xylose, which is essential for effectivity of xylanolytic enzymes. Therefore, the initial MD4 strain that was used for this project, was constructed by engineering xylose-utilization capacity. Expression of a heterologous xylose isomerase from *Clostridium phytofermentans* conferred the ability to ferment xylose to this industrial strain (Demeke et al., 2013a). This enables the use of strain MD4 that was developed from previously reported strains (Demeke et al., 2013a; Meijnen et al., 2016) for expression and evaluation of xylanolytic enzyme-encoding genes.

Expression of many different microbial xylan-degrading enzyme genes for different industrial applications has been extensively studied in several host organisms, such as *E. coli*, *P. pastoris* and *Aspergillus sp.* (Ahmed et al., 2009). In *S. cerevisiae* specifically, most research was performed with regard to production of bio-based chemicals from second-generation feedstocks. Cell-surface display of xylanolytic enzymes was explored to obtain xylan-degrading *S. cerevisiae* strains. Expression of *xylA* from *A. oryzae* and *xyn2* from *T. reesei* resulted in cell-surface co-display of BXL and XYL, respectively. This recombinant strain was able to directly produce ethanol from birchwood xylan (Katahira et al., 2004). Sakamoto and colleagues (2012) constructed an *S. cerevisiae* strain that co-displayed a BXL encoded by *xylA* from *A. oryzae*, a XYL encoded by *xyn2* from *T. reesei* and a BGL encoded by *bgl1* from *A. aculeatus* on the cell surface. This recombinant organism was able to produce ethanol from rice straw hydrolysate directly. The cell-surface approach has also been extended by creating a hemicellulosome or xylanosome, where the hydrolytic enzymes are docked on a scaffoldin. This would promote the synergy between the enzymes because of their spatial proximity and enzyme-substrate targeting (Bule et al., 2018; Fontes and Gilbert, 2010). Cells displaying a hemicellulosomes with a BXL from *A. niger* (XlnD), a XYL from *T. reesei* (Xyn2) and an α-L-arabinofuranosidase from *A. niger* (AbfB) targeted arabinoxylan in particular. This resulted in direct hydrolysis and fermentation of birchwood xylan to ethanol (Sun et al., 2012). Similar synergistic saccharification of birchwood xylan was observed when the xylanosome consisted of a BXL (XlnDt) from *A. niger*, a XYL (XynAc) from *Thermomyces lanuginosus* and an acetylxylan esterase (AwAXEf) from *A. awamori* (Srikrishnan et al., 2013). However, limited data are available on the secretion of xylanolytic enzymes by *S. cerevisiae*. Expression of a BXL-encoding gene *xlnD* from *Aspergillus niger* and a XYL-encoding gene *xyn2* from *T. reesei* resulted in a recombinant strain able to secrete the enzymes necessary for degradation of birchwood xylan to xylose (La Grange et al., 2001; La Grange et al., 1996).

In this chapter, we aim to construct an industrial xylose-fermenting *S. cerevisiae* strain with the capacity to hydrolyze the main constituent of hemicellulose, namely xylan. For that purpose, heterologous genes encoding a ß-xylosidase and multiple xylanases have been evaluated and integrated in the genome of the AC1 strain, which was engineered for cellobiose hydrolysis capacity in the previous chapter. The constructed strains have been evaluated for secreted enzyme activity and *in vivo* performance during fermentation of xylan from different sources.

2. Results

2.1. Characteristics of selected xylanolytic enzymes *An*XlnD and *Tr*Xyn2

Promising results obtained in previous research with the ß-xylosidase XlnD from *Aspergillus niger* and the xylanase Xyn2 from *Trichoderma reesei* identified these enzymes as potential candidates for construction of a xylanolytic strain on the way to a CBP organism (La Grange et al., 2001). A summary of the most important characteristics of these enzymes is shown in Table 3.1. The genes encoding these enzymes were codon-optimized and expressed in a multicopy plasmid flanked by the strong *TEF1* promoter and *CYC1* terminator for evaluation in the industrial MD4 strain. The codon-optimized nucleotide sequence of the genes is provided in Appendix Table A3.1.

Table 3.1. Overview of the main characteristics of the selected BXL *An*XlnD and XYL *Tr*Xyn2.

Gene	Organism	Enzyme	Uniprot	pH and temperature optimum	Size (kDa)	References
xlnD	*Aspergillus niger*	*An*XlnD	O00089	3.2; 60°C	87.2	(La Grange et al., 2001; Pérez-González et al., 1998; Selig et al., 2008b; van Peij et al., 1997)
xyn2	*Trichoderma reesei*	*Tr*Xyn2	P36217	4.5-5.5; 60°C	24.0	(La Grange et al., 1996; Tenkanen et al., 1992; Torronen et al., 1992)

The pH and temperature optimum of the chosen heterologous BXL and XYL was determined to ensure relevance for industrial conditions during a CBP process. For this purpose, the specific activity of *An*XlnD and *Tr*Xyn2 was determined with 4-nitrophenyl-β-D-xylopyranoside (pNPX) and 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-4^5-glucosyl-xylopentaoside (XylX6) as substrate, respectively. This was performed with MD4 strains harboring the genes encoding these enzymes on a multicopy plasmid.

For *An*XlnD, a pH optimum of around 3.5 at 35°C and temperature optimum of around 70°C at pH 5.5 were observed (Fig. 3.1A, B). For *Tr*Xyn2, on the other hand, a pH optimum of around 4.5 at 35°C and temperature optimum of around 50°C at pH 5.5 were measured (Fig. 3.1C, D).

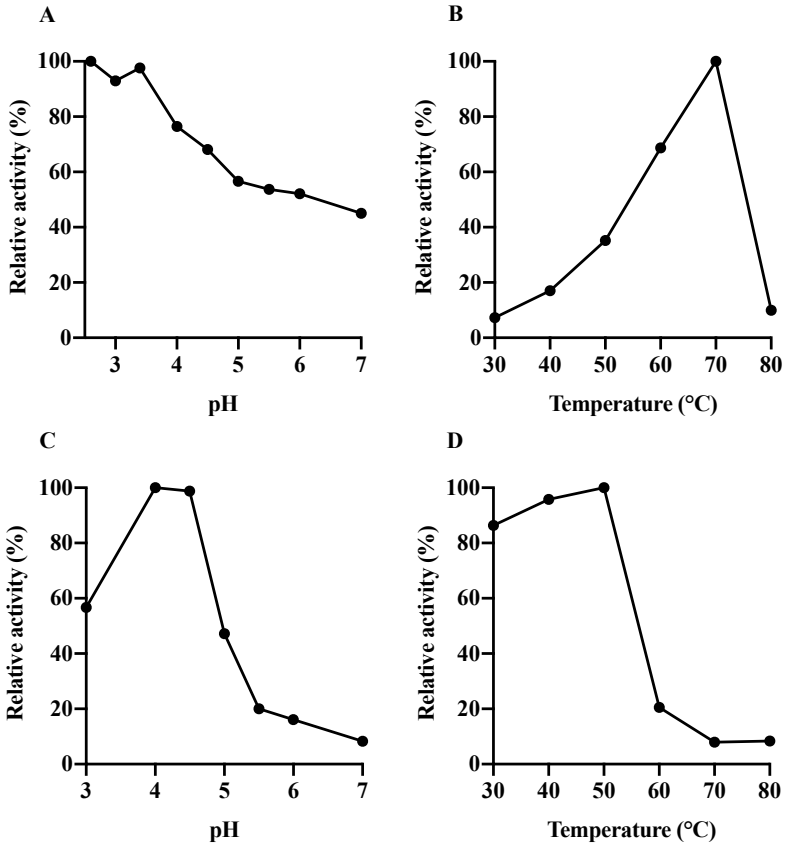


Figure 3.1. Determination of optimal conditions for activity of *An*XlnD and *Tr*Xyn2. (A) Relative activity to determine pH optimum of *An*XlnD at 35°C. (B) Relative activity to determine temperature optimum of *An*XlnD at pH 5.5. (C) Relative activity to determine pH optimum of *Tr*Xyn2 at 35°C. (D) Relative activity to determine temperature optimum of *Tr*Xyn2 at pH 5.5.

2.2. Genomic integration of BXL- and XYL-encoding genes *AnxlnD* and *Trxyn2* in AC1

Upon plasmid-based expression of the heterologous genes, the encoded enzymes exhibited a significant activity on specific synthetic substrate. Therefore, these genes were integrated in the genome of AC1, the xylose- and cellobiose-utilizing strain created in Chapter 2, to obtain stable expression. For this purpose, a cassette of the genes, both flanked by the strong constitutive *TEF1* promoter and *CYC1* terminator, was cloned on a multicopy plasmid that served as donor DNA for repairing the double-strand break caused by CRISPR/Cas9 (Fig. 3.2A). Using this methodology, integration site IS7.1 at chromosome VII (Table M2 in Materials and Methods) was targeted for integration of multiple copies of the *AnxlnD-Trxyn2* cassette. Genomic integration of the cassette in all alleles was checked with PCR using two primers flanking the integration cassette, C-4330 and C-4331 (Table M3 in Materials and Methods). Successful integration in all alleles was identified when a band of approximately 6 kb was observed, as for lane 2 (Fig. 3.2B). The resulting strain, harboring BGL-encoding *Trbgl1*, BXL-encoding *AnxlnD* and XYL-encoding *Trxyn2*, was named AC2.

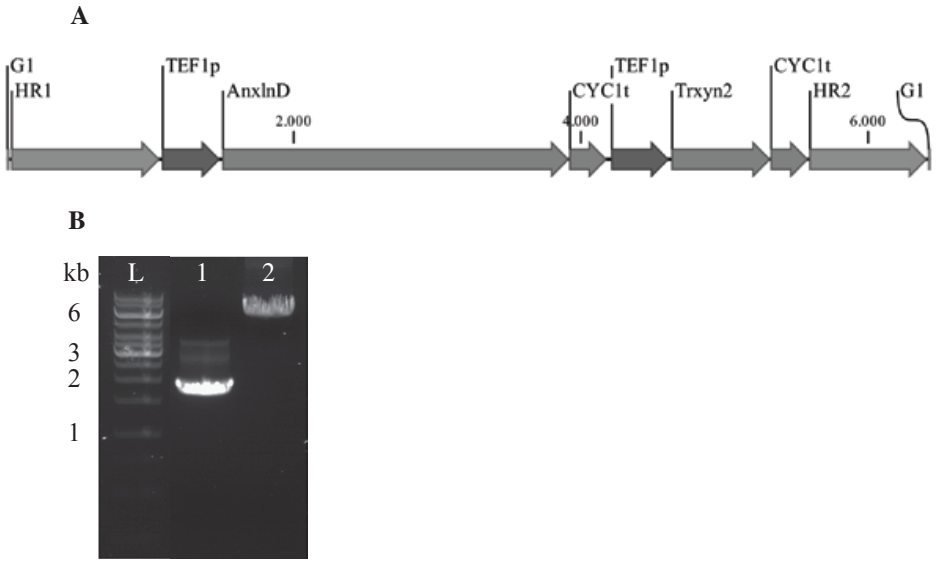


Figure 3.2. Genomic integration of *AnxlnD-Trxyn2* cassette in AC1. (A) Schematic overview of *AnxlnD-Trxyn2* integration cassette. (B) Gel electrophoresis result after PCR using two outside primers on DNA from AC1 (lane 1) and AC2 (lane 2) to check integration of cassette in all alleles. Lane L is GeneRuler 1 kb DNA Ladder.

2.3. Determination of copy number and ploidy of AC2 as a result of whole genome sequence analysis

The created AC2 strain was sent for Illumina whole genome sequencing to determine the exact copy number of the integrated cassette. For this purpose, the ploidy of the concerning chromosome, in this case chromosome VII, was determined using in-house scripts, as described in Materials and Methods. Therefore, the SNP frequency for each chromosomal position of chromosome VII was compared between AC1 and AC2 (Fig. 3.3A, B). Every dot on these figures represents a SNP compared to the S288c reference mapping. For AC1, the distribution of SNPs was observed around every 20 % (Fig. 3.3A). This means that the data points are distributed over the red lines with 0.2-0.4-0.6-0.8 indications. This distribution can only be obtained if the occurring SNPs can be present in 1, 2, 3, 4 or 5 alleles, indicating that AC1 is pentaploid for chromosome VII. For AC2, on the other hand, the distribution of SNPs was observed around every 25 % (Fig. 3.3B). This can only be obtained if the SNPs are present in up to 4 alleles. Therefore, AC2 was considered to be tetraploid for chromosome VII and thus lost one allele during the genomic integration of the *AnxlnD-Trxyn2* cassette.

Furthermore, the count of reads that cover chromosome VII was compared with the coverage of the sequence reads of the integration cassette in AC2 (Fig. 3.3C). The average coverage of both was fairly similar, which taught us that the *AnxlnD-Trxyn2* cassette appeared once per allele. Combining these results of whole genome sequence analysis showed that AC2 has four *AnxlnD-Trxyn2* cassettes integrated at IS7.1. An overview of the genomic properties determined by whole genome sequencing of AC1 and AC2 is listed in Table 3.2.

The *Trbgl1* tandem cassette, that was initially integrated in AC1, was also evaluated in AC2, but no difference in chromosomal ploidy or copy number of the cassette was observed, indicating that AC2 retained the eight copies of *Trbgl1*.

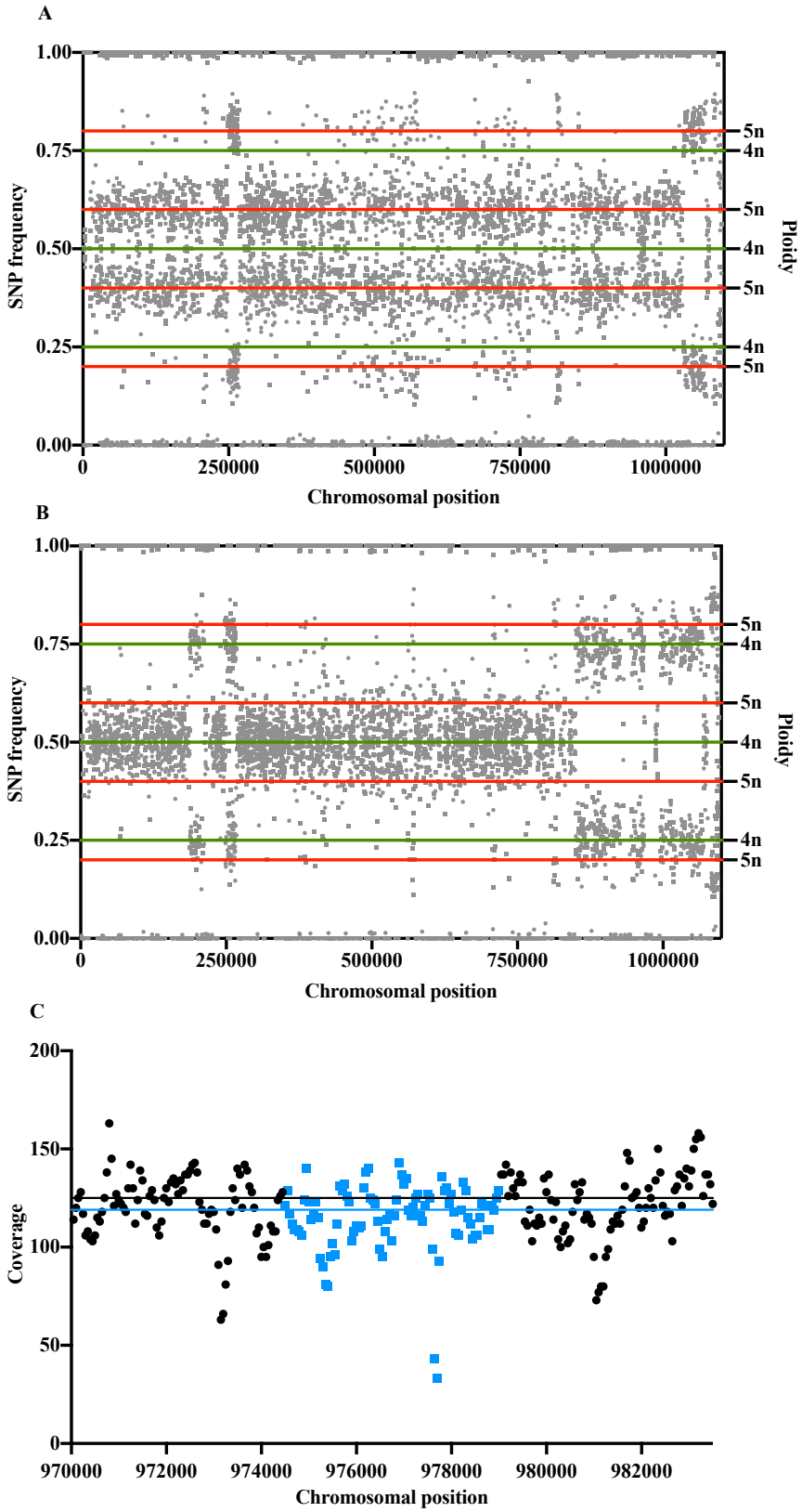


Figure 3.3. Determination of copy number of *AnxlnD-Trxyn2* cassette upon integration in AC2. (A) SNP frequency of chromosome VII of AC1 plotted against chromosomal position and the resulting ploidy (4n, green; 5n, red). (B) SNP frequency of chromosome VII of AC2 plotted against chromosomal position and the resulting ploidy (4n, green; 5n, red). (C) Coverage of the reads in *AnxlnD-Trxyn2* cassette (blue squares) and the surrounding genes (black circles) of chromosome VII in AC2.

Table 3.2. Overview of genomic properties as determined by whole genome sequence analysis of AC1 and AC2. Ploidy of all chromosomes and coverage ratio of the integrated heterologous gene compared to the coverage of its host chromosome, determining its approximate copy number.

	AC1			AC2		
Chr.	Ploidy	Ratio coverage gene/chromosome	Copy number	Ploidy	Ratio coverage gene/chromosome	Copy number
I	5n	-	-	5n	-	-
II	4n	~2.0x	8 BGL	4n	~2.0x BGL	8 BGL
III	4n	-	-	4n	-	-
IV	5n	-	-	5n	-	-
V	4n	-	-	4n	-	-
VI	4n	-	-	4n	-	-
VII	5n	-	-	4n	~1.0x BXL ~1.0x XYL	4 BXL 4 XYL
VIII	4n	-	-	4n	-	-
IX	5n	-	-	5n	-	-
X	4n	-	-	4n	-	-
XI	4n	-	-	4n	-	-
XII	4n	-	-	4n	-	-
XIII	4n	-	-	4n	-	-
XIV	4n	-	-	5n	-	-
XV	4n	-	-	4n	-	-
XVI	5n	-	-	5n	-	-

Chr.: chromosome, -: not applicable

2.4. Evaluation of enzyme activity and fermentation performance of AC2

The AC2 strain was evaluated for several characteristics to ensure that the final strain would not be tampered in any essential phenotypic features. The activity of the BGL that was expressed in AC1 was assessed to guaranty that the expression, secretion and/or activity were not negatively affected by consecutive integration of other heterologous genes with CRISPR/Cas9. The secreted BGL activity on pNPG remained constant after integration of the BXL-XYL cassette in AC2 (Fig. 3.4A). Secondly, the expressed BXL and XYL enzymes were assessed for high specific secreted activity. *An*XlnD activity was measured with pNPX as substrate and indicated a secreted activity of 88 mU/mg secreted protein (Fig. 3.4B). Activity of *Tr*Xyn2 was determined with XylX6 as substrate and reached around 6.5 mU/mg secreted protein (Fig. 3.4C). However, this activity was very low and highly variable, making the result dubious.

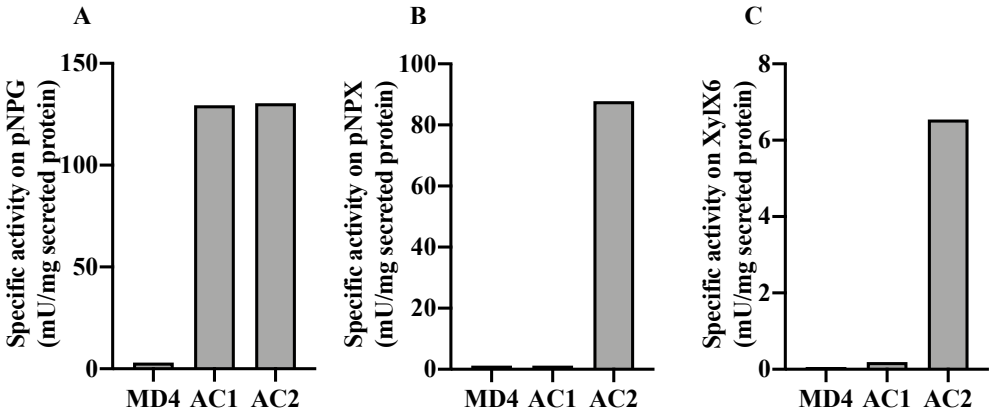


Figure 3.4. Secreted specific activity of all enzymes expressed by MD4, AC1 and AC2 (35°C, pH 5.5). (A) BGL activity determined with pNPG as substrate. Average of three technical repeats is shown. (B) BXL activity determined with pNPX as substrate. Average of three technical repeats is shown. (C) XYL activity determined with XylX6 as substrate. Average of three technical repeats is shown.

The fermentation performance of AC2 was assessed in different synthetic media. When MD4, AC1 and AC2 were used for fermentation of YP with 4% (w/v) corncob xylan to evaluate combined XYL and BXL activity, no weight loss was detected for the parent strains MD4 and AC1. AC2, on the other hand, fermented corncob xylan to ethanol, albeit partially, resulting in 1.5% weight loss after 100 h (Fig. 3.5). Maximally 2% weight loss could be obtained after consumption of all corncob xylan, indicating some residual corncob xylan after fermentation.

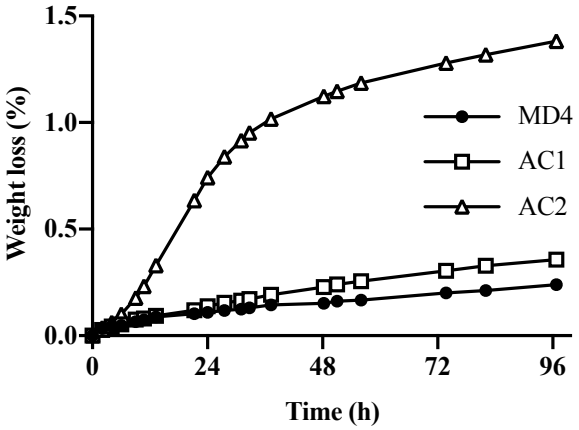


Figure 3.5. Fermentation performance evaluated by weight loss due to CO_2 release. Fermentation with MD4, AC1 and AC2 in YP with 4% corncob xylan (35°C, 50 mL, 1 g CDW/L).

Fermentation performance of MD4, AC1 and AC2 was also compared in YP medium complemented with 4% (w/v) glucose, 2% (w/v) xylose, 2% (w/v) cellobiose and 2% (w/v) corncob xylan, allowing a maximal weight loss of 5%. Glucose, xylose and cellobiose were added to eliminate the possibility of a negative effect due to integration of the new heterologous genes. MD4 was able to ferment the free sugar fraction of glucose and xylose, reaching the expected weight loss of around 3% (Fig. 3.6A, B). AC1 additionally fermented the cellobiose, reaching a final weight loss of 4% (Fig. 3.6A, C). The corncob xylan could only be fermented with AC2. However, it was not completely utilized with 0.5% (w/v) residual corncob xylan left in the fermentation broth.

No difference is observed between MD4 and AC2 with regard to the fermentation rate of glucose, xylose and cellobiose (Fig. 3.6A, D).

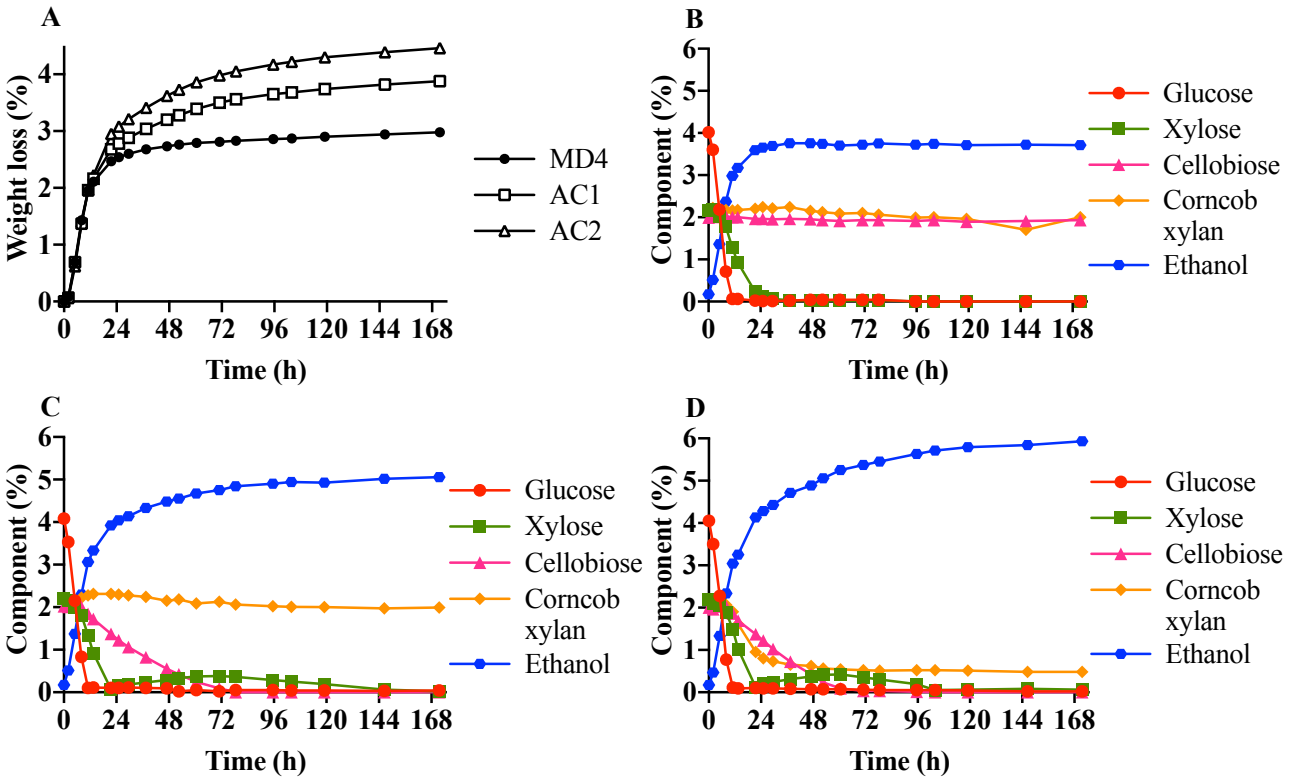


Figure 3.6. Fermentation performance of constructed strains. Fermentation in YP with 4% (w/v) glucose, 2% (w/v) xylose, 2% (w/v) cellobiose and 2% (w/v) corncob xylan (35°C, 70 mL, 1 g CDW/L). (A) Weight loss during fermentation due to CO_2 release with MD4, AC1 and AC2. (B) Ethanol production and sugar consumption during fermentation with MD4, (C) AC1 and (D) AC2.

2.5. Evaluation of three different heterologous xylanases

The low secreted specific activity of *Tr*Xyn2 and the residual corncob xylan after fermentation provoked the question whether this may be improved by adding extra copies of *Trxyn2* or use of another xylanase. To investigate this hypothesis, two extra genes encoding XYL were evaluated for their secreted activity upon plasmid-based expression in MD4. The evaluated genes, their encoded enzymes and their origins are listed in Table 3.3 and the codon-optimized nucleotide sequence of the genes is shown in Appendix Table A3.2. Determination of secreted activity of the three XYL-encoding genes, indicated a minimal XYL activity for the previously used *Tr*Xyn2 compared to XlnB from *A. niger*, which showed a significantly higher activity with 165 mU/mg secreted protein (Fig. 3.7).

Table 3.3. Three heterologous XYLs evaluated upon expression in MD4

Abbreviation	Gene	Species	Uniprot
XYL1	*xyn2*	*Trichoderma reesei*	P36217
XYL2	*xlnB*	*Aspergillus niger*	P55330

XYL3	*xynD*	*Talaromyces funiculosus*	Q5ZNB1

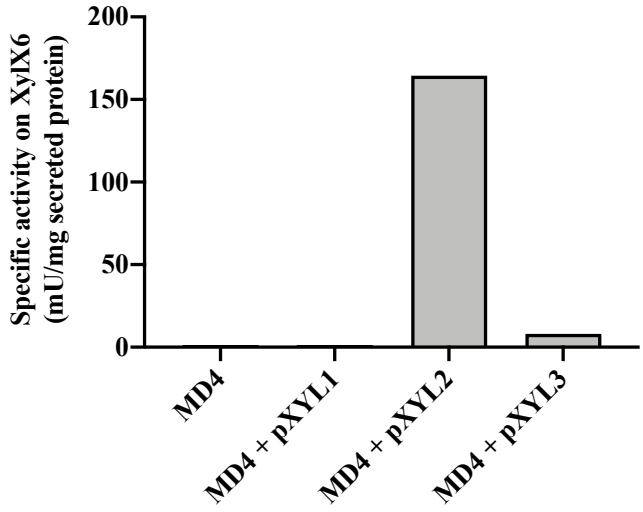


Figure 3.7. Secreted specific XYL activity on XylX6 substrate measured with MD4 and MD4 containing three different XYLs on a multicopy plasmid (35°C, pH 5.5, XylX6). Average of three technical repeats is shown.

2.6. Construction of AC6 containing *AnxlnD* and AC7 containing *AnxlnD* and *AnxlnB*

Considering these results, new strains were created from AC1 with different BXL and XYL levels. AC6 was constructed by genomic integration of *xlnD* from *A. niger* encoding BXL flanked by the *TEF1* promoter and *CYC1* terminator at IS7.1. This AC6 strain was used to create AC7, a strain harboring the promising *xlnB* xylanase from *A. niger* flanked by the strong constitutive *TEF1* promoter and *CYC1* terminator at IS16.1 (Table M2 in Materials and Methods). The main characteristics of *An*XlnB are listed in Table 3.4. Genomic integration of the heterologous genes in all alleles was checked with PCR using two primers flanking the integration cassette, *i.e.* C-4330 and C-4331 for checking at integration site IS7.1 and C-8549 and C-8550 for IS16.1 (Table M3 in Materials and Methods). Successful integration in all alleles was identified when a band of approximately 6 kb was observed for integration of *AnxlnD* (Fig. 3.8A) and 3 kb for integration of *AnxlnB* (Fig. 3.8B). The integration check of *AnxlnD* showed successful integration in AC6 and AC7. Since this concerns consecutive integrations, a positive result for integration of *AnxlnB* is only obtained for AC7. To investigate the hydrolysis action of both XYLs, *Tr*Xyn2 and *An*XlnB, together, strain AC2-XYL2 was created from AC2, harboring additional *AnxlnB* at IS16.1.

Table 3.4. Overview of the main characteristics of xylanase XlnB from *Aspergillus niger*

Gene	Organism	Enzyme	Uniprot	pH and temperature optimum	Size (kDa)	References
xlnB	*Aspergillus niger*	*An*XlnB	P55330	5.0; 50°C	24.1	(Deng et al., 2006; Korona et al., 2006; Levasseur et al., 2005)

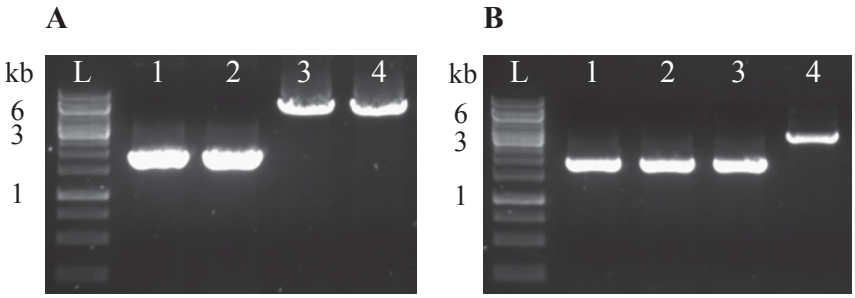


Figure 3.8. Genomic integration of *AnxlnD* and *AnxlnB*. Gel electrophoresis result of PCR on DNA from MD4 (lane 1), AC1 (lane 2), AC6 (lane 3) and AC7 (lane 4) using two outside primers to check genomic integration of (A) BXL-encoding *AnxlnD* and (B) XYL-encoding *AnxlnB*.

2.7. Evaluation of fermentation performance of all constructed xylanolytic strains

All the constructed strains were evaluated during fermentation in YP with 2% (w/v) glucose, 2% (w/v) xylose, 2% (w/v) cellobiose and 2% (w/v) corncob xylan, allowing a maximal weight loss of 4%. This indicated a similar fermentation performance for MD4, AC1 and AC2 as previously observed (Fig. 3.6A). However, the AC6 strain, that does not express any XYL and only harbors a BXL encoding gene, fermented the same level of corncob xylan as AC2 expressing *Trxyn2*, AC7 expressing *AnxlnB* and AC2-XYL2 expressing both (Fig. 3.9). This indicated that the corncob xylan that was used during this fermentation consists mainly of xylooligosaccharides that can be hydrolyzed by the expressed BXL, *An*XlnD, without requirement of xylanase activity. Expression of both XYLs in AC2-XYL2 seemed to slightly delay the rate of xylose fermentation and did not result in more xylan breakdown and was therefore not used in further experiments.

Furthermore, the strains were evaluated for degradation of beechwood xylan during fermentation. Xylan extracted from this source has a higher degree of polymerization than corncob xylan and its hydrolysis thus requires high xylanase activity. Fermentation performance was measured by weight loss in YP with 2% (w/v) glucose and 2% (w/v) beechwood xylan with the constructed strains. Maximal weight loss of 2% could be obtained if all sugars were consumed. The expected weight loss of 1% was detected due to glucose consumption for MD4 and AC1, which do not have any xylanolytic capacity. AC6, with only a BXL, was able to ferment a very low amount of beechwood xylan, indicating a low concentration of short chain xylooligosaccharides present. AC2, with a BXL and the *Tr*Xyn2 as XYL, was able to consume more beechwood xylan in 175 h, reaching a final weight loss of 1.2%. However, the better performing AC7, with a BXL and the *An*XlnB XYL, utilized beechwood xylan much faster than AC2 (Fig. 3.10).

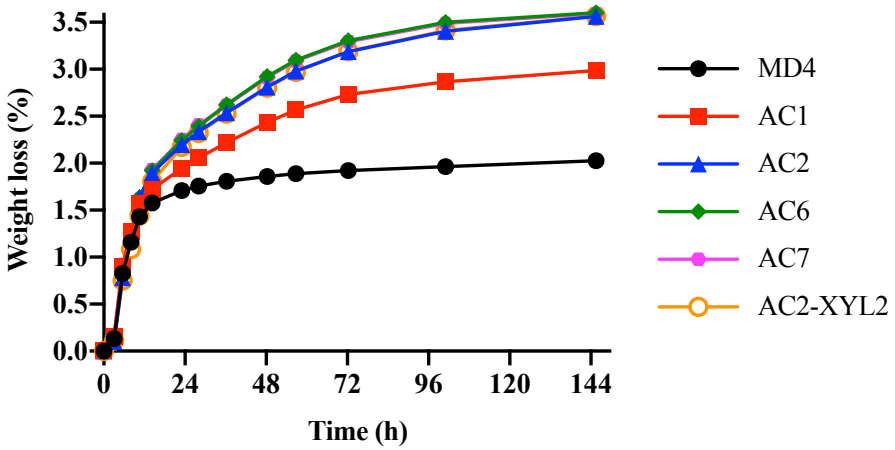


Figure 3.9. Fermentation performance evaluated by weight loss due to CO_2 release. Fermentation with strains MD4, AC1, AC2, AC6, AC7 and AC2-XYL2 in YP with 2% (w/v) glucose, 2% (w/v) xylose, 2% (w/v) cellobiose and 2% (w/v) corncob xylan (35°C, 60 mL, 1 g CDW/L).

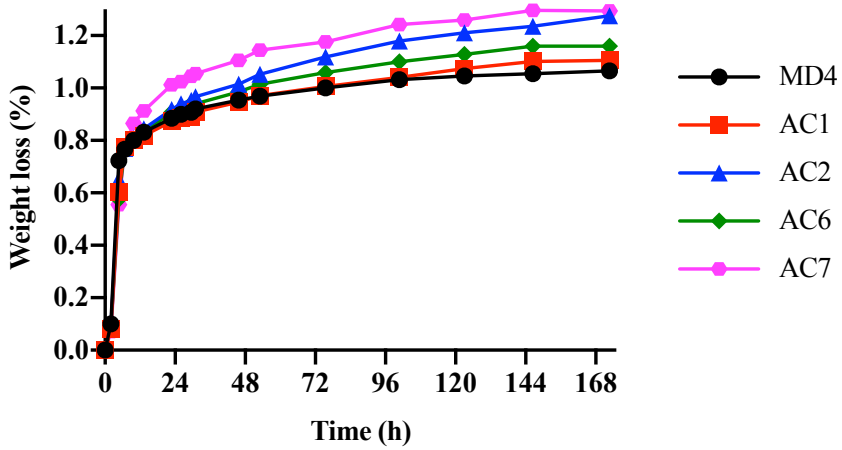


Figure 3.10. Fermentation performance evaluated by weight loss due to CO_2 release. Fermentation with strains MD4, AC1, AC2, AC6 and AC7 in YP with 2% (w/v) glucose and 2% (w/v) beechwood xylan (35°C, 10 mL, 1 g CDW/L).

2.8. Evaluation of specific activity of *An*XlnD and *An*XlnB produced by AC7

The faster fermentation of beechwood xylan with AC7 demonstrated a higher specific activity of *An*XlnB than of *Tr*Xyn2. To specifically assess the secreted activity of BXL and XYL produced by AC6 and AC7, the cell-free medium of the constructed strains was added to pNPX and XylX6 substrates, respectively. MD4 and AC1 do not exhibit activity on any of the substrates, while AC6 gained BXL specific activity of 12.8 U/g CDW or 315 mU/mg secreted proteins on pNPX. AC7 retained this BXL activity and additionally gained XYL specific activity of 30.2 U/g CDW or 930 mU/mg secreted proteins on XylX6 (Fig. 3.11B and Appendix Fig. A3.1). The secreted BGL activity that was conferred to AC1 by genomic integration of eight copies of *Trbgl1* was retained by AC6. However, after genomic integration of the XYL-encoding *AnxlnB* in AC7, the BGL activity dropped to 65% of that of AC1 and AC6 (Fig. 3.11A). Because of the higher observed specific activity and fermentation performance on beechwood xylan of *An*XlnB, the experiments were continued with AC7 with *An*XlnD instead of AC2 with *Tr*Xyn2.

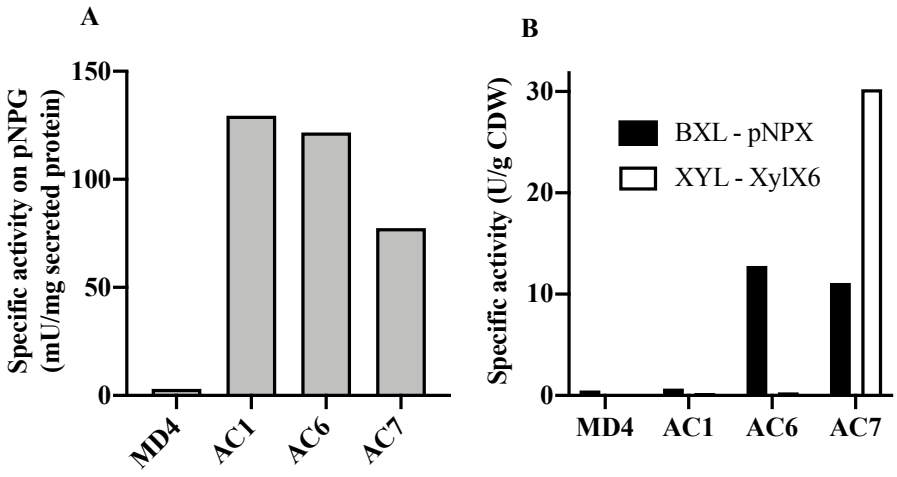


Figure 3.11. Specific activity of BGL, BXL and XYL with the constructed strains MD4 till AC7 after genomic integration of *AnxlnD* and *AnxlnB* (35°C, pH 5.5). (A) Secreted BGL activity with pNPG as substrate. Average of three technical repeats is shown. (B) Secreted BXL activity was measured with pNPX as substrate. Secreted XYL activity was measured with XylX6 as substrate. Average of three technical repeats is shown.

2.9. Evaluation of fermentation performance of AC7 compared to MD4, AC1 and AC6

The AC6 and AC7 strains were also evaluated for fermentation performance in more complex YP medium with 2% (w/v) glucose, 2% (w/v) xylose, 1% (w/v) cellobiose and 2% (w/v) beechwood xylan, allowing a maximal weight loss of 3.5%. Weight loss and ethanol production showed that AC1 and AC6 both utilize more carbon than MD4 and that AC7 consumes even more sugars than AC1 and AC6 (Fig. 3.12A, B). Sugar consumption analysis indicated that there is no difference in glucose or xylose fermentation from MD4 till AC7 (Fig. 3.12C, D). Cellobiose consumption is observed from AC1 till AC7 and does not seem to be affected from AC1 to AC6, but there appears to be a small effect in AC7 (Fig. 3.12E). Beechwood xylan consumption was not observed in MD4 and AC1. AC6 appeared to have a very low xylanolytic activity on beechwood xylan. AC7, on the other hand, indicated also in more complex medium a significant hydrolysis of beechwood xylan, albeit partial (Fig. 3.12F). The hydrolyzed beechwood xylan was fermented to ethanol, increasing the ethanol production of AC7 with 0.8% (v/v) compared to MD4 and by 0.3% (v/v) compared to AC1 and AC6 (Fig. 3.12A).

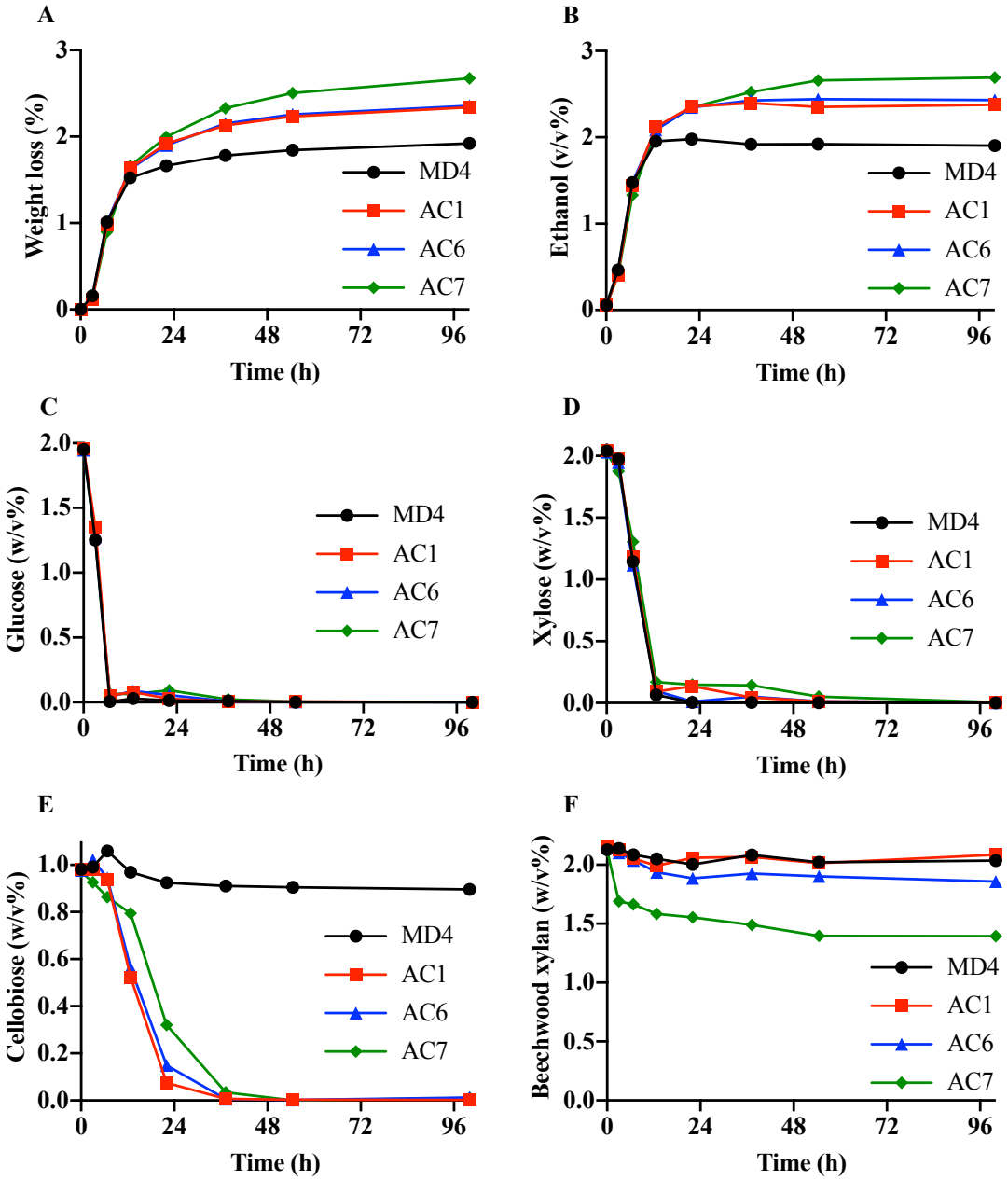


Figure 3.12. Fermentation performance of AC7 compared to MD4, AC1 and AC6. Fermentation with strains AC6 and AC7 compared to MD4 and AC1 in YP with 2% (w/v) glucose, 2% (w/v) xylose, 1% (w/v) cellobiose and 2% (w/v) beechwood xylan (35°C, 60 mL, 1 g CDW/L). (A) Weight loss due to CO_2 release. (B) Ethanol production. (C) Glucose consumption. (D) Xylose consumption. (E) Cellobiose consumption. (F) Beechwood xylan consumption.

3. Discussion

In this chapter, we engineered a range of xylose- and cellobiose-fermenting *S. cerevisiae* strains with the capacity to hydrolyze xylooligosaccharides and natural xylan, coming from both corncob and beechwood. The constructed strains were evaluated for secreted enzyme activity, specifically for BGL, BXL and XYL, and for fermentation performance in synthetic media containing xylan from different sources.

The first constructed strain, AC2, harbored the ß-xylosidase *xlnD* from *Aspergillus niger* and the xylanase *xyn2* from *Trichoderma reesei*. To create a strain that harbors as many copies as possible using one step CRISPR/Cas9 engineering, one of the pentaploid chromosomes of AC1, namely chromosome VII, was targeted for integration of the *AnxlnD-Trxyn2* cassette. The exact copy number of the heterologous genes after genomic integration was determined by whole genome sequence analysis. The ploidy of all chromosomes of AC2 was calculated and the same conclusion was made as in Chapter 2. Integration of foreign DNA in one of the pentaploid chromosomes of this mainly tetraploid, industrial strain, resulted in loss of one allele, probably improving the strain stability. Since the ploidy of chromosome VII changed to tetraploid and the coverage of the *AnxlnD-Trxyn2* cassette was similar to the average coverage of the neighboring genes, AC2 was determined to harbor four copies of *AnxlnD* and four copies of *Trxyn2*.

These enzymes were previously reported to have a high activity and good secretion in *S. cerevisiae* (Katahira et al., 2004; La Grange et al., 2001; La Grange et al., 1996; Sakamoto et al., 2012; Srikrishnan et al., 2013; Sun et al., 2012). This explains our interest for using these heterologous genes to build towards a CBP organism. Expression of the genes encoding BXL and XYL induced specific activity of the enzymes secreted in the medium. These activity measurements were used to determine the optimal pH and temperature. *An*XlnD showed an optimal pH at 3.5, but the enzyme activity appears to be relatively stable over a broad pH range. This enzyme, however, showed a high temperature dependence. *An*XlnD showed an optimal temperature of 70°C and its relative activity decreased to 15% at 35°C. For most lignocellulolytic enzymes the optimal temperature to obtain maximal activity is higher than the temperature that can be used during production processes of CBP. This is due to the lower optimal growth and fermentation temperature of *S. cerevisiae*. Currently, research is being performed to increase the thermotolerance and the optimal fermentation temperature of this organism which would benefit amongst others from the relative activity of all heterologous enzymes with lignocellulolytic properties produced by the CBP organism (Hasunuma and Kondo, 2012).

For *Tr*Xyn2 activity, on the other hand, a pH optimum of 4.5 and temperature optimum of 50°C were determined, which is closer to the optimal conditions for current CBP processes. However, the detected XYL activity with *Tr*Xyn2 was very low and highly variable. This raised some doubt on the intrinsic activity and/or secretion of this enzyme. Literature implied that the secretion signal sequence of 33 amino acids was recognized and cleaved at the Kex2-like Lys-Arg residues, resulting in efficient secretion in *S. cerevisiae* (La Grange et al., 1996). Additionally, the *Tr*Xyn2 activity was evaluated previously in the medium of xylanase-producing cells. At 50°C, they observed a maximal activity of 1,577 nkat/mL, which is approximately 95 U/mL. The activity that we measured during this study was around 6.5 mU/mg secreted protein or 0.002 U/mL cell-free medium. This low activity can be explained by a difference in codon usage, which can result in misfolding of the mature protein (Brule and Grayhack, 2017; Hanson and Coller, 2018) or by a strain background dependent effect. An alternative

explanation for this difference can be related to experiment conditions, especially incubation temperature. Where La Grange and colleagues (1996) used the optimal enzyme temperature of 60°C, we performed the activity assay at a much lower and more industrially relevant temperature of 35°C. Additionally, a copy number effect cannot be excluded since they expressed *Trxyn2* on a multicopy plasmid and we integrated four copies in the genome. The relatively low activity was firstly overlooked, since an apparent efficient breakdown of corncob xylan was observed. However, when the AC6 strain, that only expressed the *An*XlnD BXL, fermented this substrate at a same rate and to the same extent, we hypothesized that the used corncob xylan consists mainly of xylooligosaccharides and that an efficient *An*XlnD was able to consume this substrate without assistance from *Tr*Xyn2. This hypothesis was confirmed by chromatograms generated by HPLC, which showed multiple peaks with the retention time of xylooligosaccharides with a various degree of polymerization. 33% of the total corncob xylan concentration was even determined to be xylobiose. This indicates that this substrate is excellent for evaluation of BXL and that our *An*XlnD enzyme has significant activity on xylooligosaccharides with different degree of polymerization. However, to tackle the issue of low xylanase activity on corncob xylan, xylan from another source, namely beechwood, was used for evaluation of all the constructed strains expressing genes encoding xylanolytic enzymes. The HPLC chromatograms of beechwood xylan showed one peak indicating a high degree of polymerization of this substrate. During fermentation of beechwood xylan, no or little hydrolysis was detected with AC6, indicating that this substrate exists of long chains of xylan since *An*XlnD has no xylanase activity. Subjecting this substrate to fermentation with AC7 containing *An*XlnB XYL, however, appeared to liberate smaller xylooligosaccharides and xylobiose that were converted to xylose by *An*XlnD and further to ethanol by the yeast strain. Also, AC2 harboring *Trxyn2* seemed to have this effect on beechwood xylan, but this enzyme activity had a delay of 70 h compared to *An*XlnB. The significantly higher secreted activity of the *An*XlnB xylanase made this the evident enzyme to build a CBP organism with AC7 as a logical platform strain compared to AC2.

The specific BGL activity of AC6 did not differ from that of AC1. However, the consecutive genomic integration of *AnxlnB* in chromosome XVI of AC7 resulted in a 35% decrease in BGL activity and slight delay in cellobiose hydrolysis during fermentation. Multiple explanations can be the reason for this decreased activity. Expression of this specific xylanase from *A. niger* can have a negative effect on the BGL from *T. reesei*, thereby lowering its activity or specificity. However, a more logical explanation would be that the application of CRISPR/Cas9 induced some chromosomal rearrangements and/or loss of copies of *Trbgl1*. We observed before that CRISPR/Cas9 can cause genomic changes. In Chapter 2, we established in the GSE16-T18 HAA1* background that the copy number of *Trbgl1* plays an important role in its secreted activity. A lower copy number would explain the sudden decrease in BGL activity *in vitro* and during fermentation. This hypothesis was supported by the whole-genome sequence analysis of the final strain (Chapter 5), where three of the eight copies of *Trbgl1* were lost. A plausible explanation for the loss of these copies is the instability of the integrated *Trbgl1* tandem construct. Since *S. cerevisiae* is known for its good homologous recombination, use of an identical sequence of 4 kb is doomed to be unstable and loop out when the strain is further engineered.

The developed AC7 strain displayed a high specific BXL and XYL activity upon genomic integration of *AnxlnD* and *AnxlnB* with measurements of 11.1 U/g CDW and 30.2 U/g CDW, respectively. The BXL activity gained by

AC6 remained constant after integration of the xylanase in AC7. A high specific BXL and BGL activity with AC7 indicated a strong expression, proper folding and good secretion of the enzymes, making them promising targets for conferring and improving xylanolytic capacity.

Despite the high specific activity of the integrated xylanolytic enzymes, there is a residual fraction of xylan left after fermentation. 25% of corncob xylan and 75% of beechwood xylan appears to be unhydrolyzed and unused. This may be due to the composition of the concerning xylan, since it is known that the xylan backbone is highly substituted with different side groups, such as arabinose and acetyl groups (Rennie and Scheller, 2014). Side groups like this make the backbone inaccessible to xylanase and lower the enzyme efficiency (Puls et al., 1991). To improve the xylan degradation, accessory xylanolytic enzymes can be expressed to liberate the side groups from the xylan backbone, making it more accessible for hydrolysis. Expression of these auxiliary enzymes is highly dependent on the target group and complete breakdown of xylan including all its side groups takes up to seven accessory enzymes (Poutanen et al., 1991). Additionally, *S. cerevisiae* lacks the natural ability to consume arabinose and thus engineering it for arabinose fermentation may increase the xylan utilization, especially arabinoxylan and arabinan (Becker and Boles, 2003; Richard et al., 2003; Wisselink et al., 2007). Also, expression of additional copies of *AnxlnD* and/or *AnxlnB* might affect the xylan breakdown and hydrolysis. However, this is believed to affect the rate of hydrolysis but not the final consumed concentration.

In this chapter, we constructed several *S. cerevisiae* strains, that were previously engineered for xylose- and cellobiose-utilization, with xylanolytic capacity. Genomic integration of four copies of the ß-xylosidase XlnD from *Aspergillus niger* and four copies of the xylanase Xyn2 from *Trichoderma reesei* led to the AC2 strain that was able hydrolyze corncob xylan, that mainly consists out of xylooligosaccharides. Genomic integration of the ß-xylosidase XlnD from *Aspergillus niger* and the xylanase XlnB from *Aspergillus niger* resulted in the AC7 strain that was capable of breaking down beechwood xylan that is composed of longer chain xylan, albeit partial. The hydrolysis capacity during fermentation and specific activity of all heterologous enzymes produced by AC7 (BGL, BXL and XYL) indicates that this is a good platform strain for further expression of cellulase enzymes for complete breakdown of cellulose and eventually for production of bio-based chemicals using lignocellulosic biomass in CBP.

Chapter 4 - Conferring complete cellulolytic capacity to an industrial xylanolytic cellobiose-utilizing *S. cerevisiae* strain by simultaneous secretion of a heterologous endoglucanase, cellobiohydrolase I and cellobiohydrolase II

1. Introduction

Cellulose often comprises that largest fraction of lignocellulosic biomass. Depending on the source, cellulose reaches up to 35-50% of plant dry weight. This makes it the most abundant component of plant biomass on Earth (Behera et al., 2017). The abundancy and low cost of this biological resource make it an interesting component for production of bio-based chemicals (Lynd et al., 2008; Zhang, 2008). However, use of this substrate for production of bio-based compounds requires enzymatic hydrolysis to completely break down cellulose.

Cellulases are enzymes that catalyze the hydrolysis of ß-1,4 linkage in cellulose chains and can be categorized in three main types: endoglucanases (endo-1,4-ß-glucanase, EC 3.2.1.4, EGs), cellobiohydrolases (exo-1,4-ß-glucanase, EC 3.2.1.91, CBHs) and ß-glucosidases (EC 3.2.1.21, BGLs). All three types of glycosyl hydrolases are essential for the conversion of cellulose chains into glucose. EGs randomly act on the amorphous regions of cellulose, thereby creating more reducing and non-reducing ends. CBHs cleave off cellobiose from the crystalline ends of cellulose chains. Reducing ends are attacked by CBHI, while CBHII acts on the non-reducing ends. The released cellobiose is in its turn hydrolyzed to glucose by BGL (Obeng et al., 2017; Zhang and Zhang, 2013).

Applications of cellulases are widespread with uses in paper and pulp industry, textile industry and laundry detergents (Jayasekara and Ratnayake, 2019). In food processing industry, the enzymes are applied to drying coffee beans to hydrolyze the cellulose to increase the quality of the coffee beans (Uzuner and Cekmecelioglu, 2019). Moreover, addition of cellulases during the extraction stage of olive oil would increase the phenolic content and its antioxidant activity, thereby enhancing the extraction and quality of olive oil (Sharma et al., 2015).

Next to these industrial applications, cellulases also reveal medical relevance. These enzymes are used to treat phytobezoars. A phytobezoar is a mass of indigestible material from seeds and fruit and vegetable fibers that is found in the gastrointestinal tract of patients with impaired gastric motility or digestion (Jayasekara and Ratnayake, 2019; Kramer and Pochapin, 2012). To prevent surgical treatment, cellulases are injected to hydrolyze the indigestible mass and provide comfort to the patients (Gold et al., 1976; Kramer and Pochapin, 2012).

The industrial application we are targeting with this work is to improve the use of lignocellulosic biomass for production of bio-based chemicals, in particular ethanol. We aim to create an *S. cerevisiae* strain that produces and secretes cellulases to enhance cellulose hydrolysis and increase the fermentable carbon in the hydrolysate, and to decrease the use of costly commercial enzyme cocktails for this purpose (Magrey et al., 2018). Capacity to hydrolyze cellulosic material has been engineered in this organism previously (Liu et al., 2018a).

Co-display of cellulolytic enzymes on the cell surface of *S. cerevisiae* has been investigated to avoid diffusion of secreted enzymes in the fermentation broth (Liu et al., 2018a). A recombinant strain displaying the Eg2 from *Trichoderma reesei* and Bgl1 from *Aspergillus aculeatus* was able to grow on ß-glucan as sole carbon source (Fujita et al., 2002). Moreover, additional display of Cbh2 from *T. reesei* on the cell surface allowed direct ethanol production from amorphous phosphoric acid swollen cellulose (PASC) (Fujita et al., 2004; Yanase et al., 2010) and high-solids lignocellulosic materials (Matano et al., 2012). An *S. cerevisiae* strain co-displaying four enzymes, namely *T. reesei* Eg2, *Talaromyces emersonii* Cbh1, *Chrysosporium lucknowense* Cbh2 and *Saccharomycopsis fibuligera* Bgl1, exhibited high cell-to-cellulose adhesion ability resulting in high hydrolysis efficiency. This cellulase-displaying strain was capable of direct fermentation of rice straw to ethanol (Liu et al., 2016).

Combined display of cellulolytic enzymes on a miniscaffoldin on the cell surface would benefit the conversion rate due to spatial proximity of the enzyme cascade (Tsai et al., 2009). Assembly of this minicellulosome on the cell surface of *S. cerevisiae* was obtained by use of dockerin-tagged cellulases (EG, CBH and BGL) produced by a consortium of *Escherichia coli* (Tsai et al., 2009) and also of four different engineered yeasts (Kim et al., 2014; Tsai et al., 2010). The consortium including the recombinant strains carrying minicellulosomes directly produced ethanol from PASC (Kim et al., 2014; Tsai et al., 2010). Comparison of uni-, bi- and trifunctional minicellulosomes on the surface of *S. cerevisiae* cells indicated that trifunctional complexes enhanced enzyme-enzyme synergy and enzyme proximity synergy. Moreover, trifunctional minicellulosomes gave these strains the ability to ferment PASC to ethanol (Wen et al., 2010). Construction of minicellulosomes consisting out of two individual miniscaffoldins increased the display level of cellulolytic enzymes and showed high hydrolytic activity against microcrystalline cellulose (Fan et al., 2012).

Secretion of cellulases by *S. cerevisiae* has been established in single, dual or triple strategies. Single enzyme secretion is mainly focused on BGL, which can hydrolyze the cellobiose fraction thereby lower the use of costly enzyme cocktails. Also, additional BGL activity reduces the product inhibition on other cellulases (see Chapter 2). Dual enzyme secretion was obtained by production of BGL and EG. Den Haan and colleagues (2007) created an *S. cerevisiae* strain that was able to grow on amorphous PASC by expression of an endoglucanase (*eg1*) from *T. reesei* and a ß-glucosidase (*bgl1*) from *S. fibuligera*. Similarly, a recombinant yeast overexpressing an EG (*engD*) from *Clostridium cellulovorans* and a BGL (*bgl1*) from *S. fibuligera* produced ethanol from ß-glucan as sole carbon source (Jeon et al., 2009a). Replacing EngD with endoglucanase E from *C. thermocellum* resulted in a recombinant yeast strain that was able to ferment ß-glucan, carboxymethyl cellulose (CMC) and PASC (Jeon et al., 2009b). Furthermore, more stable expression through genomic integration of two cellulase genes *eg3* and *bgl1* from *T. viride* encoding an EG and a BGL, respectively, enabled direct fermentation of amorphous CMC by *S. cerevisiae* (Gong et al., 2014). Triple cellulase expression was reported by Hong and colleagues (2014). They developed a yeast strain with enhanced CBH activity and the capacity to grow on crystalline cellulose and ferment pretreated corncob hydrolysate to ethanol. The recombinant strain was created by ∂-sequence-mediated integration of an EG (*eg2*) from *T. reesei*, a BGL (*bgl1*) from *A. aculeatus* and three CBH-encoding genes, namely *cbh1* and *cbh2* from *T. reesei* and additional *cbh1* from *A. aculeatus* (Hong et al., 2014). Khramtsov and colleagues (2011) engineered an industrial wine strain by genomic integration of genes encoding an EG (*eg2*) and a CBHII (*cbh2*) from *T. reesei* and a BGL (*bgl1*) from *A. aculeatus*. The industrial recombinant strain efficiently fermented pretreated corn stover to ethanol (Khramtsov et al., 2011). An *S. cerevisiae* strain overexpressing a multifunctional single-enzyme-system-three-cellulase gene (*sestc*), isolated from stomach tissue of *Ampullaria gigas*, was able to produce ethanol with wheat bran as sole carbon source (Yang et al., 2016). Similarly, Liu and colleagues (2018c) compressed all three essential cellulases into one synthetic trifunctional cellulase gene. Synthetic linking of the three catalytic domains of an EG, a CBH and a BGL and expression of this construct in *S. cerevisiae* resulted in increased activity of the trifunctional cellulase compared to the enzymes separately (Liu et al., 2018c). Finally, co-fermentation with strains secreting different cellulases was investigated for reducing the commercial enzyme load. A mixed culture of four strains, of which one secreting Eg2 from *T. reesei*, one secreting Cbh1 from *Chaetomium thermofilum*, one secreting Cbh2 from *C. lucknowense* and one

secreting Bgl1 from *S. fibuligera*, was used for co-fermentation of Avicel and ß-glucan resulting in increased ethanol production compared to the wild type strains (Lee et al., 2017).

In this chapter, we aim to confer full cellulolytic capacity to an industrial, xylanolytic and cellobiose-utilizing *S. cerevisiae* strain. For this purpose, heterologous genes encoding endoglucanase, cellobiohydrolase I and cellobiohydrolase II have been expressed in the target strain. The resulting strains have been evaluated for activity of secreted EG, CBHI and CBHII with specific substrates and for the combined activity with complex polymeric substrates. Furthermore, fermentation performance of the strains has been evaluated in synthetic medium containing polymeric substrates.

2. Results

2.1. Selection and genomic integration of a heterologous gene encoding EG

Development of a strain fully capable of hydrolyzing cellulose, requires production and secretion of endoglucanase, exoglucanase and ß-glucosidase. For this purpose, multiple heterologous genes encoding EG and CBHs were evaluated for expression, secretion and enzyme activity upon plasmid-based expression in MD4. BGL activity was already engineered by expression of *Trbgl1* in AC1, which was the basis to develop AC7 with BXL and XYL.

First, the nucleotide sequence of six heterologous endoglucanases (Table 4.1) was codon-optimized for expression in *S. cerevisiae* (Appendix Table A4.1) and flanked by the strong constitutive *PGK1* promoter and *PRM9* terminator, followed by evaluation for secreted EG activity upon plasmid-based expression in MD4 (Fig. 4.1). Note that we opted to change from the *TEF1* promoter to the *PGK1* promoter and from the *CYC1* terminator to the *PRM9* terminator. Both options are strongly expressed constitutive and should result in high expression levels in the beginning of the fermentation with high sugar and low ethanol levels. The reason for this change was to avoid exhaustion of the *TEF1* promoter as this may result in lower protein production. The highest specific activity with synthetic substrate 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-cellopentaoside (CellG5) was observed for *celB*-encoded EG from *Aspergillus oryzae* (337.6 mU/mg secreted protein).

Table 4.1. Overview of evaluated heterologous genes encoding endoglucanases

Abbreviation	Gene	Species	Uniprot
EG1	*celA*	*Paenibacillus barcinonensis*	O08342
EG2	*end1*	*Butyrivibrio fibrisolvens*	P20847
EG3	*egl1*	*Trichoderma reesei*	P07981
EG4	*egl2*	*Trichoderma reesei*	P07982
EG5	*eg5A*	*Phanerochaete chrysosporium*	Q66NB7
EG6	*celB*	*Aspergillus oryzae*	Q2TX26

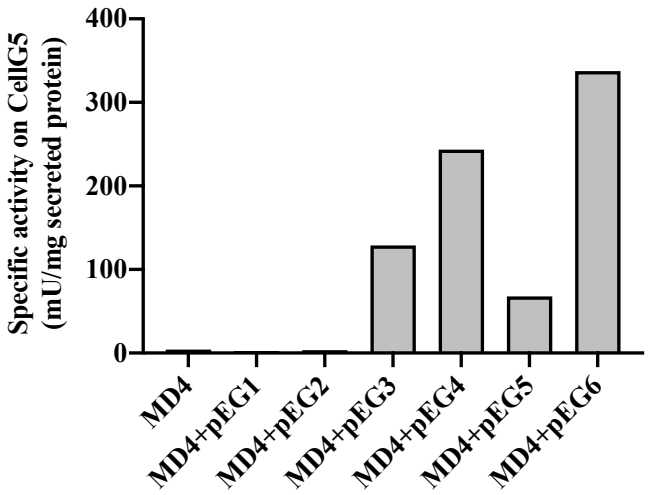


Figure 4.1. Screening of six heterologous EG-encoding genes for secreted specific activity (35°C, pH5.5, CellG5). Secreted specific activity determined with CellG5 as substrate upon plasmid-based expression in MD4. Average of three technical repeats is shown.

EG encoded by *celB* from *Aspergillus oryzae* (*Ao*CelB) was selected for integration in the xylanolytic, cellobiose-utilizing AC7 strain, obtained in Chapter 3. The main characteristics of endoglucanase *Ao*CelB are listed in Table 4.2. Genomic integration of this EG in all alleles of IS16.2 at chromosome XVI (Table M2 of Materials and Methods) of AC7 was checked by PCR using two primers flanking the integration cassette, D-431 and D-432 (Table M3 of Materials and Methods). Successful integration of *AocelB* was identified with a band of 5.5 kb as observed for the newly created strain AC9, in lane 5 (Fig. 4.2). The resulting AC9 strain gained *Ao*CelB activity with a specific activity of 18.1 U/g CDW or 581 mU/mg secreted proteins with the synthetic substrate CellG5 as measured with the cell-free culture medium (Fig. 4.3 and Appendix Fig. A4.1).

Table 4.2. Overview of main characteristics of endoglucanase CelB from *Aspergillus oryzae*.

Gene	Organism	Enzyme	Uniprot	pH and temperature optimum	Size (kDa)	References
celB	*Aspergillus oryzae*	*Ao*CelB	Q2TX26	4.0; 45°C	44.4	(Kitamoto et al., 1996; Kotaka et al., 2008; Yamane et al., 2002)

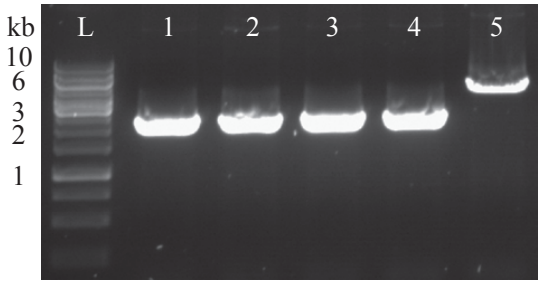


Figure 4.2. Electrophoresis gel of PCR product to check genomic integration of *AocelB* at IS16.2. PCR was performed with two primers flanking the integration cassette to obtain a negative band of 2 kb, as for MD4, AC1, AC6 and AC7 (lanes 1-4) or a positive band of 5.5 kb, as for AC9 (lane 5). Lane L is GeneRuler 1 kb DNA Ladder.

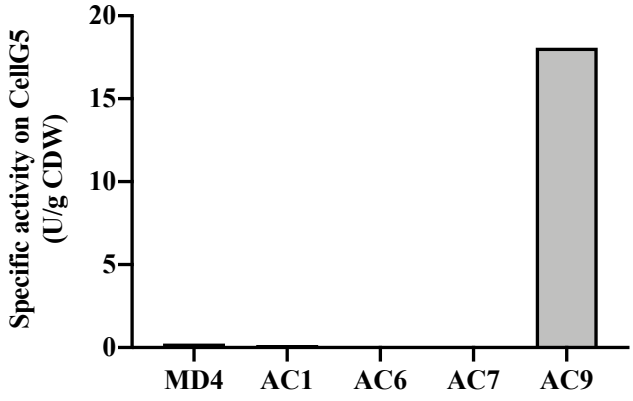


Figure 4.3. Evaluation of secreted EG activity upon genomic integration of *AocelB* (35°C, pH5.5, CellG5). Specific activity of secreted EG in strain AC9 with CellG5 as substrate after genomic integration of *AocelB* compared to strains MD4, AC1, AC6 and AC7, which lack EG activity. Average of three technical repeats is shown.

2.2. Selection and genomic integration of a heterologous gene encoding CBHII to develop AC11

Next, six heterologous genes encoding a cellobiohydrolase II (Table 4.3) were codon-optimized (Appendix Table A4.2), flanked by the strong *PGK1* promoter and *PRM9* terminator and evaluated for secreted activity upon plasmid-based expression in MD4 (Fig. 4.4). Due to a lack of specific CBHII activity measurements, PASC is used as substrate to estimate the CBHII activity. CBHII will hydrolyze the PASC, liberating cellobiose and thus create more reducing ends, which are quantified using the pHBAH reducing sugar assay with cellobiose as standard curve. The highest secreted CBHII activity with PASC was observed for CBHII2, the *cbh2b*-encoded CBHII from *Chrysosporium lucknowense* (30.3 mU/mg secreted protein) (Fig. 4.4).

Table 4.3. Overview of evaluated heterologous genes encoding cellobiohydrolase II.

Abbreviation	Gene	Species	Uniprot
CBHII1	*cbh2*	*Trichoderma reesei*	P07987
CBHII2	*cbh2b*	*Chrysosporium lucknowense*	EMBL bank ENA: HH793136.1
CBHII3	*cel7*	*Cochliobolus heterostrophus*	Q8J215
CBHII4	*cbh2*	*Chaetomium thermophilum*	G0SD43
CBHII5	*cel3*	*Agaricus bisporus*	P49075
CBHII6	*cel6A*	*Magnaporthe oryzae*	G4MM92

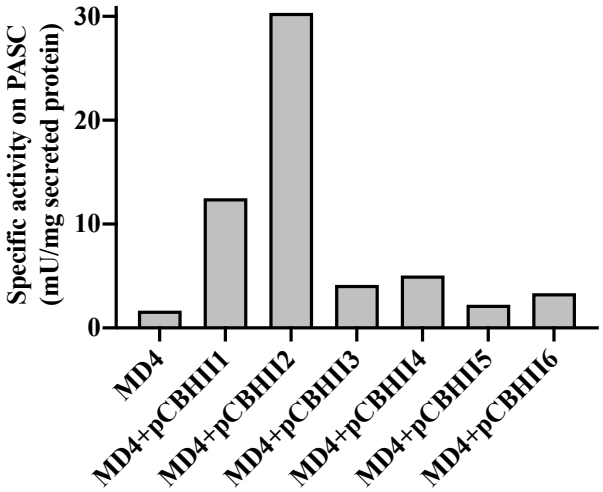


Figure 4.4. Screening of CBHII-encoding genes (35°C, pH5.5, PASC). Evaluation of six heterologous CBHII-encoding genes for highest secreted specific activity with PASC as substrate upon plasmid-based expression in MD4. Average of three technical repeats is shown.

CBHII encoded by *cbh2b* from *Chrysosporium lucknowense* (*Cl*Cbh2b) was selected for integration in the xylanolytic, cellobiose-utilizing AC9 strain that shows high EG activity, obtained in section 4.2.1. The main characteristics of cellobiohydrolase II *Cl*Cbh2b are listed in Table 4.4. Genomic integration of this CBHII in all alleles of IS4.2 at chromosome IV (Table M2 of Materials and Methods) of AC9 was proven by PCR using two primers flanking the integration cassette, C-2829 and C-2900 (Table M3 of Materials and Methods). Successful integration of *Clcbh2b* was identified with a band of 3.5 kb as observed for the newly created strain AC11 in lane

6 (Fig. 4.5). The resulting strain could not be tested for specific CBHII activity due to background activity of earlier integrated BGL and EG enzymes and will be evaluated after integration of CBHI.

Table 4.4. Overview of main characteristics of cellobiohydrolase II Cbh2b from *Chrysosporium lucknowense*.

Gene	Organism	Enzyme	EMBL bank ENA	pH and temperature optimum	Size (kDa)	References
cbh2b	*Chrysosporium lucknowense*	*Cl*Cbh2b	HH793136.1	NR; NR	51.1	(den Haan et al., 2013b; Ilmén et al., 2011; Lee et al., 2017; Liu et al., 2017)

NR: not reported

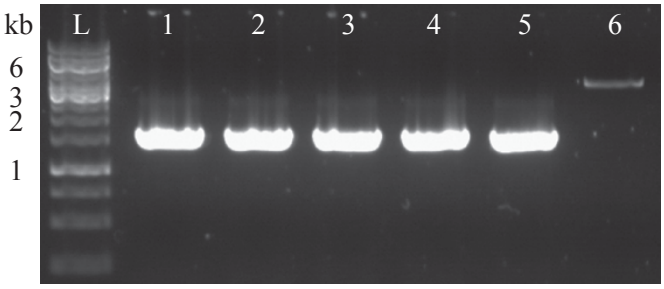


Figure 4.5. Electrophoresis gel of PCR product to check genomic integration of *Clcbh2b* at IS4.2. PCR was performed with two primers flanking the integration cassette to obtain a negative band of 1.5 kb, as for MD4, AC1, AC6, AC7 and AC9 (lanes 1-5) or a positive band of 3.5 kb, as for AC11 (lane 6). Lane L is GeneRuler 1 kb DNA Ladder.

2.3. Selection and genomic integration of a heterologous gene encoding CBHI to develop AC12

Finally, six heterologous genes encoding a cellobiohydrolase I (Table 4.5) were codon-optimized for expression in *S. cerevisiae* (Appendix Table A4.3), flanked by the strong *PGK1* promoter and *PRM9* terminator and evaluated for secreted activity upon plasmid-based expression in MD4 (Fig. 4.6). Secreted CBHI activity was measured with PASC as substrate to determine the degradation to cellobiose and thus more reducing ends. The highest secreted activity with PASC was observed for CBHI3, the *cbh1*-encoded CBHI from *Talaromyces emersonii* (46.3 mU/mg secreted protein) (Fig. 4.6A). Additionally, specific CBHI activity can be measured using 4-methylumbelliferyl-β-D-lactoside (MULac) as substrate. This method also indicated *Te*Cbh1 as the enzyme with highest activity on MULac with 20.5 µM 4-methylumbelliferone (MU) released per min (Fig. 4.6B).

Table 4.5. Overview of evaluated heterologous genes encoding cellobiohydrolase I.

Abbreviation	Gene	Species	Uniprot
CBHI1	*cbh1*	*Aspergillus aculeatus*	O59843
CBHI2	*cbh1*	*Trichoderma reesei*	P62694
CBHI3	*cbh1*	*Talaromyces emersonii*	Q8TFL9
CBHI4	*cbh1*	*Humicola grisea*	P15828
CBHI5	*cbhB*	*Aspergillus niger*	Q9UVS8
CBHI6	*xynA*	*Penicillium funiculosum*	Q8WZJ4

CBHI encoded by *cbh1* from *Talaromyces emersonii* (*Te*Cbh1) was selected for integration in the xylanolytic, cellobiose-utilizing AC11 strain that shows high EG activity and secretes CBHII, obtained in section 4.2.2. The main characteristics of cellobiohydrolase I *Te*Cbh1 are listed in Table 4.6. Genomic integration of this CBHI in all alleles of IS4.1 at chromosome IV (Table M2 of Materials and Methods) of AC11 was checked by PCR using two primers flanking the integration cassette, C-2827 and C-2828 (Table M3 of Materials and Methods). Successful integration of *Tecbh1* was identified with a band of 3.5 kb as observed for the newly created strain AC12 in lane 7 (Fig. 4.7).

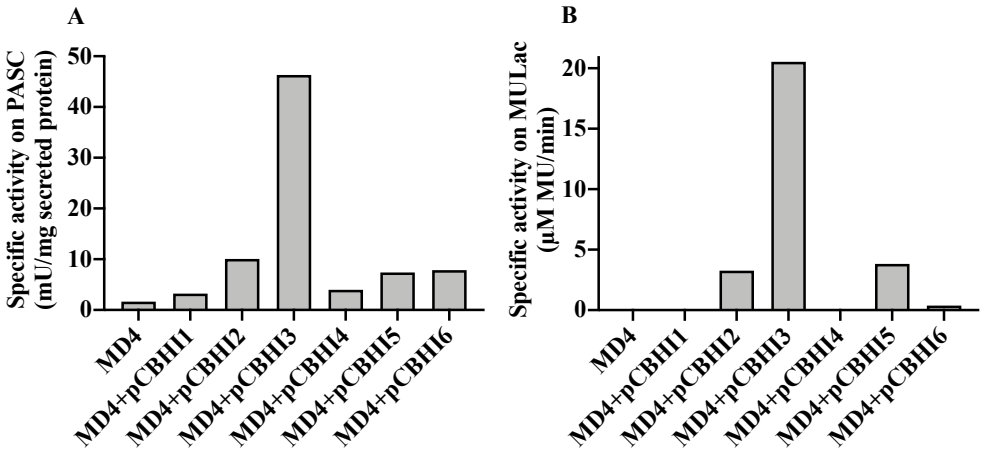


Figure 4.6. Screening of six heterologous CBHI-encoding genes for high secreted CBHI activity upon plasmid-based expression in MD4 using different substrates (35°C, pH5.5). (A) PASC. Average of three technical repeats is shown. (B) MULac.

Table 4.6. Overview of main characteristics of cellobiohydrolase I Cbh1 from *Talaromyces emersonii*.

Gene	Organism	Enzyme	Uniprot	pH and temperature optimum	Size (kDa)	References
cbh1	*Talaromyces emersonii*	*Te*Cbh1	Q8TFL9	5.0; 55°C	48.7	(den Haan et al., 2013b; Grassick et al., 2004; Ilmén et al., 2011; Komor et al., 2012; Liu et al., 2015; Liu et al., 2017)

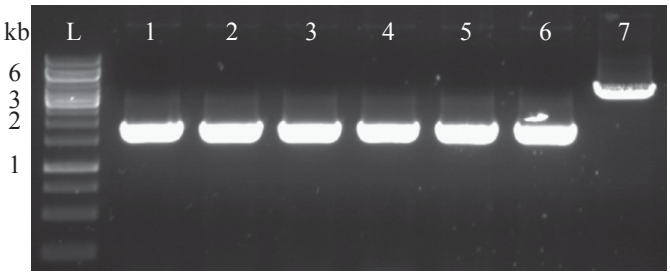


Figure 4.7. Electrophoresis gel of PCR product to check genomic integration of *Tecbh1* at IS4.1. PCR was performed with two primers flanking the integration cassette to obtain a negative band of 1.5 kb, as for MD4, AC1, AC6, AC7, AC9 and AC11 (lanes 1-6) or a positive band of 3.5 kb, as for AC12 (lane 7). Lane L is GeneRuler 1 kb DNA Ladder.

2.4. Evaluation of secreted enzyme activity and fermentation performance of developed cellulase-secreting strains

After performing successive genomic integration of EG *AocelB*, CBHII *Clcbh2b* and CBHI *Tecbh1* in strain AC7, resulting in strains AC9, AC11 and AC12, respectively, the performance of the developed strains was evaluated in terms of secreted enzyme activity and fermentation capacity.

The enzyme specific activity with AC12 was determined for BGL, BXL, XYL and EG with pNPG, pNPX, XylX6 and CellG5 as substrates, respectively. AC12 showed to have retained all enzyme specific activities previously engineered in this study (Fig. 4.8A). Due to high background activity of previously integrated enzymes in AC12 on substrates to determine CBH activity, this specific activity could not be tested. However, the combined cellulase activity was estimated by performing hydrolysis of CMC with cell-free medium of AC12 and determining the released glucose concentration using the pHBAH reducing sugar assay (Fig. 4.8B). Expression of only BGL in AC1 conferred very low activity on CMC. Additional expression of other cellulases increased the hydrolysis of CMC. Addition of EG in AC9 induced proper hydrolysis of CMC, which could be increased by expression of a CBHII in AC11. The optimum was reached with strain AC12 expressing all four essential cellulases for breakdown of cellulose with a combined activity of 139.8 mU/mg secreted protein (Fig. 4.8B).

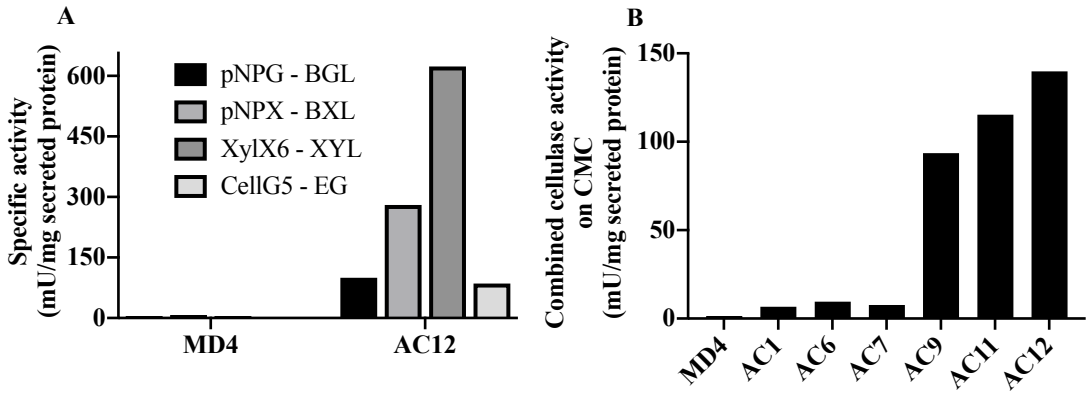


Figure 4.8. Determination of recombinant enzyme activity secreted by AC12 (35°C, pH5.5). (A) Specific activity of BGL, BXL, XYL and EG with pNPG, pNPX, XylX6 and CellG5 as substrates, respectively, with MD4 and AC12. Average of three technical repeats is shown. (B) Combined cellulase activity with CMC as substrate with a range of strains from MD4 till AC12. Average of three technical repeats is shown.

The fermentation performance of strain AC12 was then compared with that of MD4 in YP medium with 2% (w/v) glucose, 2% (w/v) xylose, 2% (w/v) cellobiose, 2% (w/v) corncob xylan and 2% (w/v) CMC. Glucose, xylose, cellobiose and corncob xylan were added to eliminate the possibility of a negative effect due to integration of the new heterologous genes. CMC is a polymeric compound that requires EG, CBHI, CBHII and BGL to be degraded. Consumption of all sugars would result in a maximal weight loss of 5%. Strain MD4 showed a weight loss of only 1.9%, whereas strain AC12 reached a weight loss of 3.5% (Fig. 4.9A). Strain MD4 only consumed glucose and xylose and produced 1.98% (v/v) ethanol (Fig. 4.9B). Strain AC12, on the other hand, consumed glucose, xylose and cellobiose completely. It also partially consumed the corncob xylan with 0.4% (w/v) apparently remaining. CMC, on the other hand, was degraded only very slowly with 1.7% (w/v) remaining in the

fermentation broth after 100 h. Although this result appears to be relatively small, the trend was consistent over several independent experiments. A representative of three fermentations showing consistent results is displayed. The more extensive consumption of the sugars by AC12 resulted in a much higher ethanol production of 3.8% (v/v) after 100 h (Fig. 4.9C).

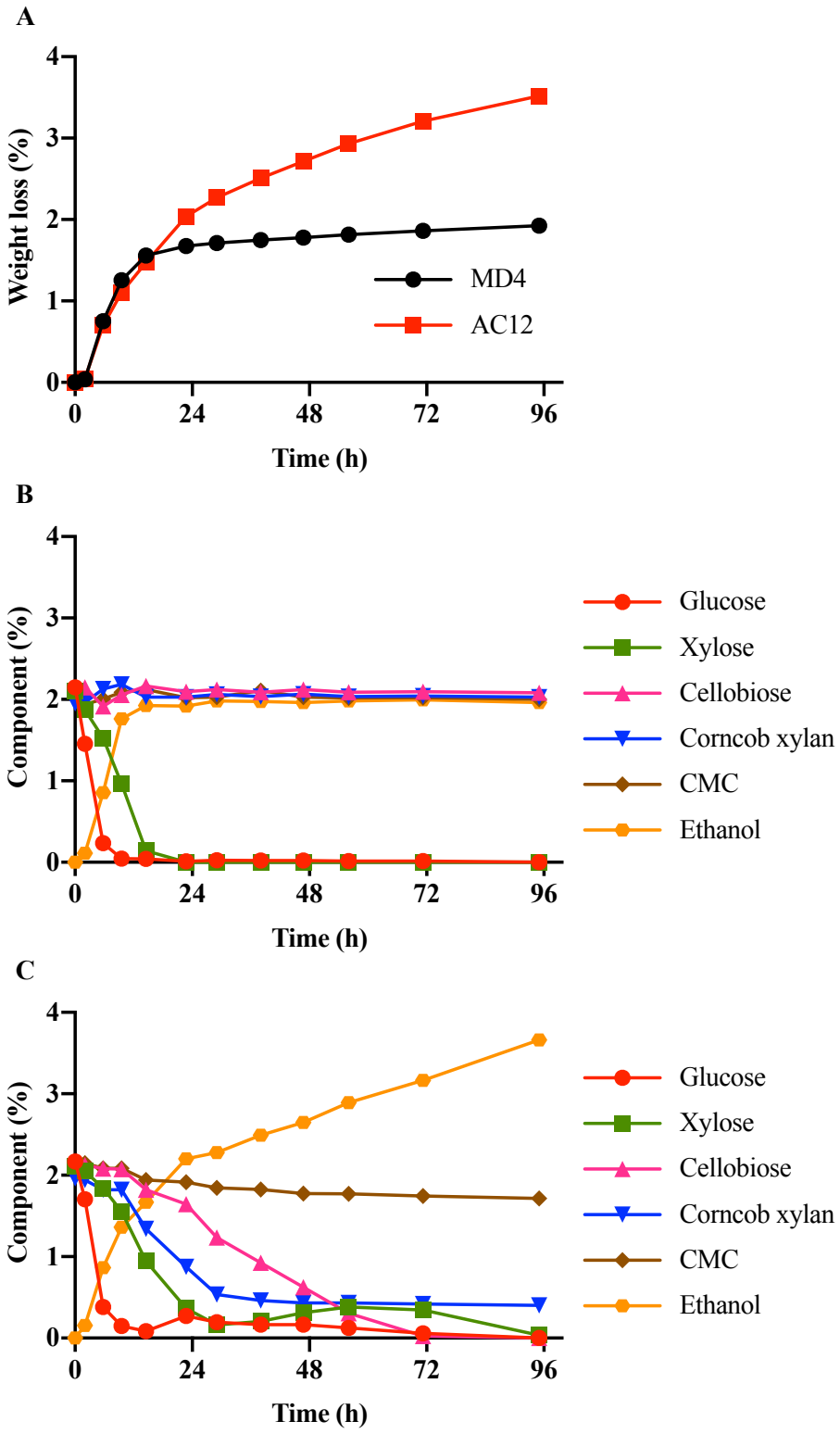


Figure 4.9. Fermentation performance of strains MD4 and AC12 in YP medium with 2% (w/v) glucose, 2% (w/v) xylose, 2% (w/v) cellobiose, 2% (w/v) corncob xylan and 2% (w/v) CMC (35°C, 70 mL, 1 g CDW/L). (A) Weight loss during fermentation with MD4 and AC12. (B) Detailed analysis of fermentation with MD4 and (C) AC12.

3. Discussion

Genomic integration of heterologous genes encoding an EG CelB from *Aspergillus oryzae*, a CBHII Cbh2b from *Chrysosporium lucknowense* and a CBHI Cbh1 from *Talaromyces emersonii* in the strain AC7, which was previously engineered for expression and secretion of a BGL, BXL and XYL, led to the development of AC12. This strain is an industrial *S. cerevisiae* strain that successfully expresses the six essential enzymes for the breakdown of the cellulosic and hemicellulosic fraction of second-generation feedstock, also referred to as the "six-pack" strain.

After screening of six heterologous genes encoding EG, six heterologous genes encoding CBHI and six heterologous genes encoding CBHII, the enzymes with the highest secreted activity were consecutively integrated in the genome of AC7. The developed strains were evaluated for *in vitro* enzyme specific activity. AC9 gained the capacity to break down CellG5 substrate with high efficiency. This property, as well as the previously engineered BGL, BXL and XYL activity, was retained by the "six-pack" strain AC12. Furthermore, the combined cellulase activity was determined with CMC as substrate. This indicated absence of any breakdown of CMC by the MD4 strain, while secretion of BGL by AC1 resulted in a minor hydrolysis of CMC. Consecutive integration of an EG in AC9, a CBHII in AC11 and a CBHI in AC12 showed a gradual increase in hydrolysis and thus enhanced breakdown of CMC, indicating that all genes synergistically contribute to reach the maximal activity of AC12.

The "six-pack" strain AC12 was also evaluated *in vivo* for fermentation performance in synthetic medium containing multiple polymeric substrates. Comparison of sugar consumption and ethanol production of AC12 and MD4 revealed no significant difference in glucose and xylose utilization. AC12 additionally consumed cellobiose and a part of the corncob xylan, confirming the intrinsic retained BGL, BXL and XYL activity. MD4, that does not express any cellulases, showed no consumption of CMC during fermentation. AC12, on the other hand, revealed a slow breakdown of CMC during fermentation. However, consumption of 0.3% (w/v) CMC in 100 h exposed a weakness of the "six-pack" strain. This observed hydrolysis rate is too slow for industrial relevance and the cellulase activity needs to be improved to obtain a potential CBP organism.

The three heterologous genes encoding EG, CBHI and CBHII that were selected for genomic integration in AC7, were the result of a confined screening. Six genes encoding each of the cellulases were selected based on literature and on their enzymatic properties. However, more elaborate screening and comparison of genes originating from different species or different codon optimized variations may result in further improvement of secreted enzyme activity and thus of the breakdown efficiency of CMC or another lignocellulosic feedstock.

Models of secreted cellulases show a strong synergism between endo- and exoglucanases for cellulose hydrolysis. Hu and colleagues (2015) identified CBHs as the major contributors to the breakdown process of cellulose. Also, Liu and colleagues (2017) investigated the transcription levels of heterologous cellulases in an optimized cellulolytic *S. cerevisiae* strain and observed the highest transcription levels for CBHI and EG. Furthermore, CBHI was shown to be more active and effective in degrading crystalline cellulose (Igarashi et al., 2011) and Avicel (Liu et al., 2017) than CBHII. These findings are consistent with the observations made in nature, as the endo-exocellulase system of *T. reesei* consists of around 60% CBHI, 20% CBHII and 12% EG (Zhang and Lynd,

2004). This indicates that the primary target for improving the cellulolytic capacity of AC12 should be enhancing the expression, secretion and for activity of CBHI and EG.

Since CBHI activity forms a bottleneck for synergistic hydrolysis of cellulose by cellulases, this requires a high specific enzyme activity to obtain a potential CBP organism. In this study, the CBHI specific activity measured using MULac was determined for six heterologous CBHI-encoding genes upon plasmid-based expression in MD4. This indicated a release of 20.5 µM 4-methylumbelliferone (MU) per min using cell-free medium of the MD4 strain containing a multicopy plasmid harboring *Tecbh1* (Fig. 4.6B). Ilmén and colleagues (2011) performed similar assays with the same *Tecbh1*-encoded CBHI upon expression in a multicopy plasmid. A screening of 14 different CBHI-encoding genes also indicated that *Te*Cbh1 exhibited the highest activity upon expression in *S. cerevisiae*. Overexpression of *Te*Cbh1 in *S. cerevisiae* Y294 resulted in an activity around 100 µM MU per min (Ilmén et al., 2011). However, due to different growth conditions and gene copy numbers, it is difficult to compare their acquired data to our results. Enzyme activity is highly dependent on several factors, such as the codon optimization and the copy number of the heterologous gene which is highly variable with overexpression using a multicopy plasmid. In the work of Ilmén and colleagues (2011), the *S. cerevisiae* Y294 strain they used was a laboratory strain, compared to the industrial MD4 strain that we used. Different strain backgrounds are known to exhibit differences in protein secretion and/or glycosylation (Liu et al., 2012a; Vieira Gomes et al., 2018).

Improvement of *Te*Cbh1 activity was attempted by fusing the protein to a carbohydrate binding module (CBM) from various fungal sources, since the natural *Te*Cbh1 enzyme lacks this module (Ilmén et al., 2011). This CBM promotes the association of the enzyme to the substrate and enhances hydrolysis of crystalline cellulose (Boraston et al., 2004; Zhang and Lynd, 2006). The fusion protein was observed to have increased CBHI activity with crystalline cellulose. However, addition of the CBM to *Te*Cbh1 reduces the protein production level, suggesting that the CBM affected the CBH1 production, secretion, folding and/or glycosylation (Ilmén et al., 2011; van Zyl et al., 2015).

Measuring and quantifying the enzyme specific activity of CBHI and CBHII are current inconveniences in cellulase research. When these enzymes are produced by a strain that expresses no other cellulase genes, the activity can be determined using polymeric substrates such as PASC, Avicel or CMC, followed by estimation of the released reducing sugar content. Specific CBHI activity can also be determined using the fluorescent substrate MULac. However, upon expression of multiple cellulase genes in one strain, the background activity caused by the BGL and EG enzymes often interferes with the measurements using specific substrates such as MULac, ß-cellotetraitol and pNPC. The most correct way to determine the combined activity of cellulases is the estimation of sugar release from filter paper. The established filter paper units (FPU) are universal and easily comparable. This assay and the results of AC12 will be discussed in Chapter 5.

In this chapter, we created a range of strains leading up to the first reported industrial “six-pack” strain AC12, an *S. cerevisiae* strain that successfully expresses the six essential enzymes for the breakdown of the cellulosic and hemicellulosic fraction of second-generation feedstock. This strain gained the capacity to hydrolyze CMC in *in vitro* tests. However, in *in vivo* fermentations the activity of the expressed cellulases was deemed to be insufficient for proper CMC degradation and conversion to ethanol.

Chapter 5 - Improved xylan degradation by expression of an auxiliary acetylxylan esterase in the industrial xylanolytic and cellulolytic “six-pack” strain AC12 and evaluation of the resulting AC14 strain

1. Introduction

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. Hemicellulose is the second major cell wall polysaccharide and contributes to strengthening the cell wall by interacting with cellulose and lignin (Scheller and Ulvskov, 2010). It comprises up to 20-40% of lignocellulose depending on the plant source, making it the second most abundant polysaccharide in nature (Saha, 2003). Hemicellulose is a heterogenous polysaccharide containing pentose and hexose sugars, mainly D-xylose, L-arabinose, D-mannose and D-galactose. The major component of hemicellulose in softwood is mannose, while in herbaceous and hardwood mainly xylan and thus xylose (de Vries and Visser, 2001). This work focuses on xylan from the hemicellulosic fraction of lignocellulose.

The heterogenous nature of hemicellulose requires a spectrum of hemicellulase enzymes for complete depolymerization to monomer sugars. As discussed in Chapter 3, the two main enzymes for hydrolysis of the xylan backbone are xylanase (endo-1,4-ß-xylanase, EC 3.2.1.8, XYL) and ß-xylosidase (xylan-1,4-ß-xylosidase, EC 3.2.1.37, BXL). However, to obtain complete depolymerization, a synergistic action of a spectrum of hemicellulases is required, *i.e.* α-glucuronidase (α-glucosiduronase, EC 3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55), arabinase (endo-α-L-arabinase, EC 3.2.1.99), α-D-galactosidase (EC 3.2.1.22), feruloylxylan esterase (EC 3.1.1.73) and acetylxylan esterase (EC 3.1.1.72, AXE) (Dodd and Cann, 2009; Juturu and Wu, 2013). These auxiliary enzymes are attacking different substituents on the xylan backbone, increasing the accessibility of other enzymes to the substrate, revealing the strong synergism between hemicellulases (Bhattacharya et al., 2015; Van Dyk and Pletschke, 2012). In this chapter, we focus on acetylxylan esterases (AXEs) to improve the breakdown of acetylated xylan.

Xylan chains are often substituted with acetyl groups as acetylated xylan forms a dense and rigid layer on cellulose, whereas deacetylated xylan forms a swollen, hydrated structure (Jaafar et al., 2019). Presence of acetyl groups is also responsible for the solubility of xylan in water (Sunna and Antranikian, 1997). Removal of acetyl groups increases the accessibility of xylan to xylanase and thus improved xylan hydrolysis (Zhang et al., 2011). In hardwood xylan, up to 60-70% of the xylose residues are acetylated (Saha, 2003; Timell, 1967), while softwoods are not acetylated (Sunna and Antranikian, 1997). Acetylxylan esterases are enzymes that catalyze the deacetylation of *O*-acetylated xylan at the C-2 and C-3 positions of xylose residues. Hydrolysis of the acetyl ester bond by AXE results in acetic acid and deacetylated xylan (Biely et al., 1985; Pouvreau et al., 2011; Tenkanen, 1998).

AXEs work in high synergistic interaction with other hemicellulases, such as XYL, BXL and α-arabinofuranosidase (Bachmann and McCarthy, 1991). XYLs and BXLs are unable to hydrolyze glycosidic bonds between substituted xylose residues and cleaving off the side chains improve the rate and extent of depolymerization (Biely et al., 1986; Poutanen and Puls, 1989; Poutanen et al., 1991). Two main sources for the production of AXEs are *Aspergillus* sp. and *Trichoderma* sp. (Panda and Gowrishankar, 2005; Poutanen et al., 1990). It was observed that the degree of acetylation of oat spelt xylan dramatically affects the enzymatic digestibility of this substrate by a XYL from *T. reesei* (Mitchell et al., 1990). Kormelink and colleagues (1993) reported that steamed birchwood xylan was not degraded with BXL and XYL enzymes from *A. niger*. In combination with an acetylxylan esterase, however, xylan was depolymerized, but these oligomers were only

released after most of the acetyl groups were removed from the steamed birchwood xylan by AXE (Kormelink et al., 1993). There has been establishment of a strong link between the activity of xylanase and acetylxylan esterase. These enzymes were shown to work in a very synergistic way (Cybinski et al., 1999; Puls et al., 1991). However, AXE is not always reliant on XYL and is able to deacetylate xylan independently, but the XYL activity was enhanced by AXE activity (Kam et al., 2005).

Expression of heterologous AXE-encoding genes in *S. cerevisiae* has not been reported yet. However, expression of heterologous genes encoding an AXE has been explored in bacterial and other fungal species (Juturu and Wu, 2013). In yeast specifically, expression of *axe* from *A. ficuum* in *Pichia pastoris* enabled deacetylation of hardwood xylan and induced a specific activity that was 100-fold greater than the native production organism (Chung et al., 2002). *P. pastoris* was also engineered with a recombinant *axe* from *A. oryzae*, resulting in release of acetic acid from birchwood xylan (Koseki et al., 2006). Furthermore, expression and site-directed mutagenesis of *axeA* from *A. awamori* in *P. pastoris* provided insight in essential residues for the deacetylation capacity of AxeA (Koseki et al., 2005). Finally, expression of *axe1* from *A. niger* in the plant *Arabidopsis thaliana* resulted in an elevated acetyl esterase activity and reduced xylan acetylation. This did not alter the cell wall composition of the plant significantly, but the xylan was more easily degradable by xylanases (Pawar et al., 2016).

Multiple investigations on xylanolytic enzymes uncovered bifunctional activity as acetylxylan esterase and xylanase. *Clostridium cellulovorans* is an organism that produces a large extracellular polysaccharolytic complex, called a cellulosome, that displays all cellulases bound on a scaffoldin protein (Doi and Tamaru, 2001). One of the cellulosomal enzymes of *C. cellulovorans* is XynA. This protein consists of a catalytic domain with high xylanase activity (Kosugi et al., 2001), a dockerin domain and an acetylxylan esterase domain. This bifunctional enzyme exhibited a broad substrate specificity and released acetic acid from acetylated xylan, but also other acetylated substrates (Kosugi et al., 2002). Pai and colleagues (2010) identified XynS20E from the rumen fungus *Neocallimastix patriciarum* as a bifunctional acetylxylan esterase/xylanase with high specific XYL and AXE activities on birchwood xylan. Similarly, the bifunctional acetylxylan esterase/xylanase enzymes Xyn11A and XynD were isolated and characterized from the rumen bacterium *Pseudobutyvibrio xylanivorans* (Cepeljnik et al., 2006) and from *Cellulomonas fimi* (Millward-Sadler et al., 1994), respectively.

In this chapter, we aim to develop an improved xylan-degrading strain originating from the xylanolytic and cellulolytic “six-pack” strain AC12, that successfully expresses and secretes a heterologous acetylxylan esterase, albeit with bifunctional activity as xylanase. For this purpose, a screening has been performed to evaluate seven heterologous genes encoding AXEs. The best performing AXE-encoding gene has been integrated in the genome of AC12 and the resulting strain has been evaluated for xylanolytic activity and fermentation performance.

2. Results

2.1. Evaluation of seven heterologous genes encoding uni- and bifunctional acetylxylan esterases for fermentation performance and enzymatic activity

To obtain an improved xylan-degrading strain by means of conferring acetylxylan esterase activity to the "six-pack" strain AC12, seven heterologous genes encoding an acetylxylan esterase (AXE) or a bifunctional acetylxylan esterase/xylanase (AXEXYL) were selected for evaluation of activity (Table 5.1) and the nucleotide sequence was codon-optimized for expression in *S. cerevisiae* (Appendix Table A5.1).

Table 5.1. Overview of evaluated heterologous genes encoding unifunctional acetylxylan esterases (AXEs) and bifunctional acetylxylan esterase/xylanases (AXEXYLs).

Enzyme	Abbreviation	Gene	Species	Uniprot
Acetylxylan esterase	AXE1	*axeA*	*Talaromyces purpureogenus*	Q8NJP6
Acetylxylan esterase	AXE2	*axeA1*	*Prevotella ruminicola*	D5EV35
Acetylxylan esterase	AXE3	*axe1*	*Trichoderma reesei*	Q99034
Bifunctional acetylxylan esterase/xylanase	AXEXYL1	*xynS20E*	*Neocallimastix patriciarum*	B8YG19
Bifunctional acetylxylan esterase/xylanase	AXEXYL2	*xynA*	*Clostridium cellulovorans*	Q8GH59
Bifunctional acetylxylan esterase/xylanase	AXEXYL3	*xyn11A*	*Pseudobutyrivibrio xylanivorans*	P83513
Bifunctional acetylxylan esterase/xylanase	AXEXYL4	*xynD*	*Cellulomonas fimi*	P54865

The genes were cloned on a multicopy plasmid flanked by the strong *TEF1* promoter and *CYC1* terminator and transformed in AC2, the strain expressing BGL *Trbgl1*, BXL *AnxlnD* and XYL *Trxyn2*, created and discussed in Chapter 3. This strain exhibited high BXL activity and low XYL activity, which makes it optimal for evaluating the synergistic AXE activity. The strains were evaluated for fermentation performance in YP with 4% (w/v) corncob xylan upon plasmid-based expression in AC2. Maximal weight loss during fermentation of 4% (w/v) corncob xylan would be 2%. The AC2 strain carrying an empty vector fermented the corncob xylan partially because of the high concentration of xylooligosaccharides, as discussed in Chapter 3. Overexpression of the unifunctional AXEs in AC2 improved the breakdown of corncob xylan to a minimal extent. AXE3, encoded by the gene *axe1* from *T. reesei*, exhibited the highest activity. Comparison of the bifunctional acetylxylan esterase/xylanase enzymes showed the highest activity for AXEXYL2, the *xynA* from *C. cellulovorans*, and thus also the best fermentation performance (Fig. 5.1). Although this result appears to be relatively small, the trend was consistent over several independent experiments. A representative of two fermentations showing consistent results is shown.

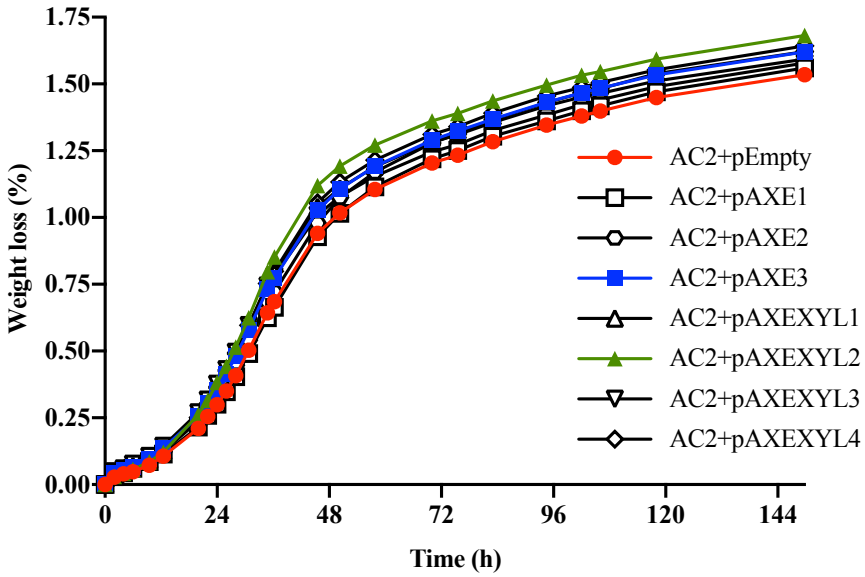


Figure 5.1. Fermentation performance of AC2 strains carrying multicopy plasmids encoding the three AXEs and four AXEXYLs in YP with 4% (w/v) corncob xylan (35°C, 50 mL, 1 g CDW/L). AC2 strain containing the empty plasmid (red circles) indicated the ancestral fermentation performance. AXE3 (blue squares) and AXEXYL2 (green triangles) showed the best improved performance of the uni- and bifunctional enzymes, respectively. All other genes are shown in black.

Additionally, MD4 strains with the plasmid-expressed enzymes were evaluated for enzymatic activity. The specific AXE activity was attempted to be measured using p-nitrophenyl acetate (pNPA), however, due to lack of specific substrates, no AXE specific assay could be established. The activity of the bifunctional genes, on the other hand, was estimated by measuring the XYL specific activity which should correlate with the AXE activity. XYL activity was determined in cell-free medium for secreted enzymes and in cell extract for intracellular enzymes from MD4 strains carrying the multicopy plasmids with the four heterologous genes encoding bifunctional enzymes. The AXEXYL2 enzyme, encoded by *xynA* from *C. cellulovorans*, displayed the highest specific xylanase activity with the synthetic 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-4^5-glucosyl-xylopentaoside (XylX6) substrate, both for the secreted enzyme measured in cell-free medium (14.0 mU/mg secreted protein) and for the intracellular enzyme measured in cell extract (110.7 mU/mg crude protein) (Fig. 5.2).

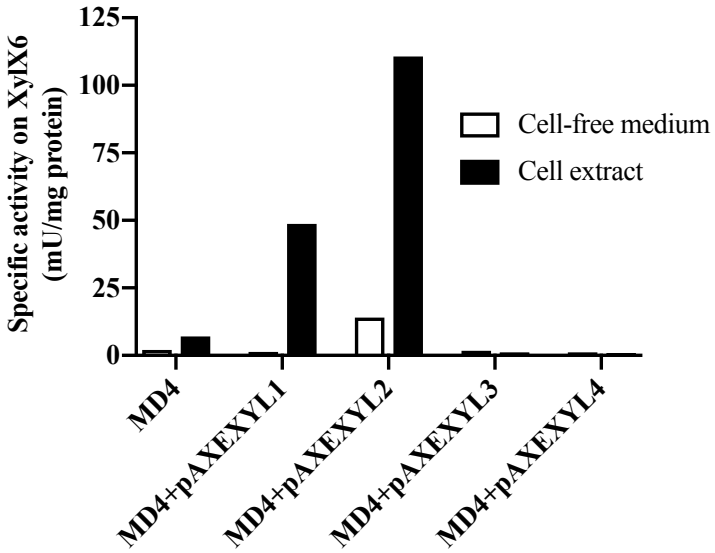


Figure 5.2. Screening of bifunctional AXEXYL-encoding genes for xylanase specific activity (35°C, pH 5.5, XylX6). Evaluation of four heterologous genes encoding AXEXYLs for highest specific activity in cell-free medium and cell extract with XylX6 as substrate upon plasmid-based expression in MD4. Average of three technical repeats is shown.

2.2. Selection and genomic integration of *CcxynA* in AC12

Evaluation of the three unifunctional AXEs and four bifunctional AXEXYLs for fermentation performance and for intrinsic enzymatic activity indicated AXEXYL2, the enzyme encoded by *xynA* from *C. cellulovorans*, as the most promising for improvement of xylan breakdown in "six-pack" strain AC12. An overview of the main characteristics of this *Cc*XynA enzyme is listed in Table 5.2.

Table 5.2. Overview of main characteristics of the bifunctional acetylxylan esterase/xylanase XynA from *Clostridium cellulovorans*.

Gene	Organism	Enzyme	Uniprot	pH and temperature optimum	Size (kDa)	References
xynA	*Clostridium cellulovorans*	*Cc*XynA	Q8GH59	5.0; 60°C	57.0	(Kosugi et al., 2001; Kosugi et al., 2002)

This gene was selected for integration in the genome of AC12 at IS16.3 (Table M2 in Materials and Methods) in chromosome XVI using CRISPR/Cas9. Integration of *CcxynA* flanked by the *TEF1* promoter and *CYC1* terminator in all alleles was proven by PCR with two primers flanking the integration cassette, C-9404 and C-9405 (Table M3 in Materials and Methods), followed by gel electrophoresis. A band of 5 kb identified successful integration of *CcxynA* in all alleles as observed for the newly created strain AC14 in lane 8 (Fig. 5.3). All other strains (lanes 1-7) showed no genomic discrepancy at the integration site (Fig. 5.3).

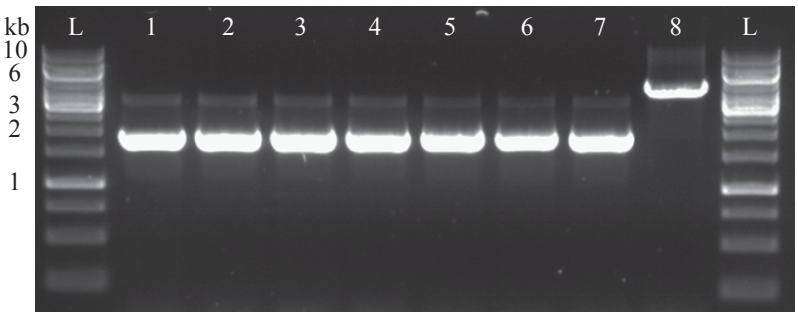


Figure 5.3. Electrophoresis gel of PCR product to check genomic integration of *CcxynA* at IS16.3. PCR was performed with two primers flanking the integration cassette to obtain a negative band of 1.7 kb, as for MD4, AC1, AC6, AC7, AC9, AC11 and AC12 (lanes 1-7) or a positive band of 5 kb, as for AC14 (lane 8). Lanes L are GeneRuler 1 kb DNA Ladder.

2.3. Fermentation performance of AC14 expressing *CcxynA* in corncob xylan

Since the resulting AC14 strain already possessed xylanase activity, no specific activity of *Cc*XynA could be determined. However, the strain was evaluated for fermentation performance in YP with 2% (w/v) glucose and 2% (w/v) corncob xylan. The acetic acid concentration was measured using HPLC and we observed that expression of *CcxynA* in AC14 resulted in an increase in the level of acetic acid in the beginning of the fermentation compared to the fermentation with AC12 (Fig. 5.4A). The breakdown of corncob xylan was also enhanced from 1.55% (w/v) with AC12 to 1.7% (w/v) with AC14 after 50 h (Fig. 5.4B). Although these results appear to be relatively small, the trend was consistent over several independent experiments. A representative of three fermentations showing consistent results is shown.

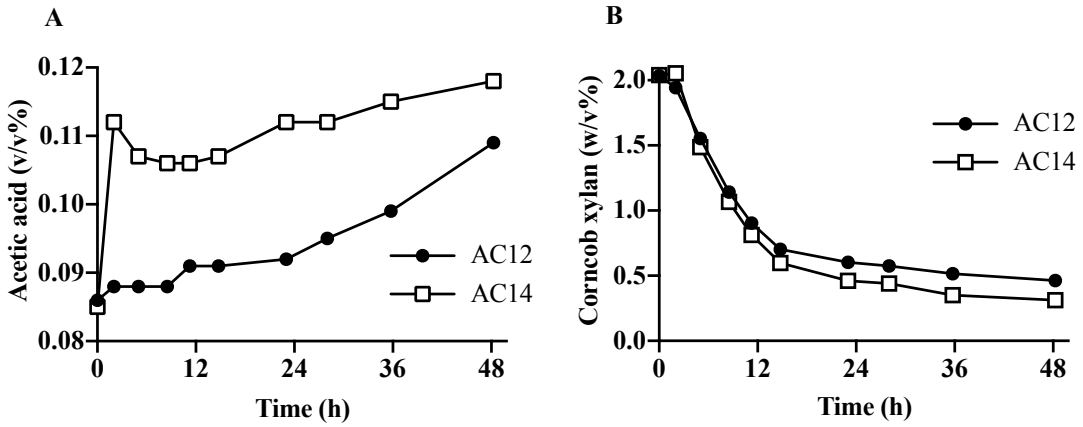


Figure 5.4. Fermentation performance of AC14 expressing *CcxynA* compared to AC12 in YP with 2% (w/v) glucose and 2% (w/v) corncob xylan (35°C, 60 mL, 1 g CDW/L). (A) Acetic acid level during the fermentation of corncob xylan with AC14 and AC12. (B) Consumption of corncob xylan with AC14 and AC12.

2.4. Evaluation of specific activity of all secreted lignocellulolytic enzymes in all consecutively created AC strains

To evaluate all genomically integrated heterologous enzymes secreted by the consecutive strains, the specific activity of BGL, BXL, XYL and EG was assessed with 4-nitrophenyl-β-D-glucopyranoside (pNPG), 4-nitrophenyl-β-D-xylopyranoside (pNPX), 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-4^5-glucosyl-xylopentaoside (XylX6) and 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-D-cellopentaoside (CellG5) as

substrates, respectively (Fig. 5.5). Specific BGL activity of *Tr*Bgl1 dropped from 19.1 U/g CDW in AC1 to 12.4 U/g CDW in AC14. Specific BXL activity of *An*XlnD was 12.8 U/g CDW in AC6 and remained relatively constant in the subsequently constructed strains. The same trend was observed for XYL activity of *An*XlnB, which was 30.2 U/g CDW in AC7 and for EG activity of *Ao*CelB which was 18.1 U/g CDW in AC9. The corresponding specific activity values in mU/mg secreted protein for all substrates are provided in Appendix Fig. A5.1.

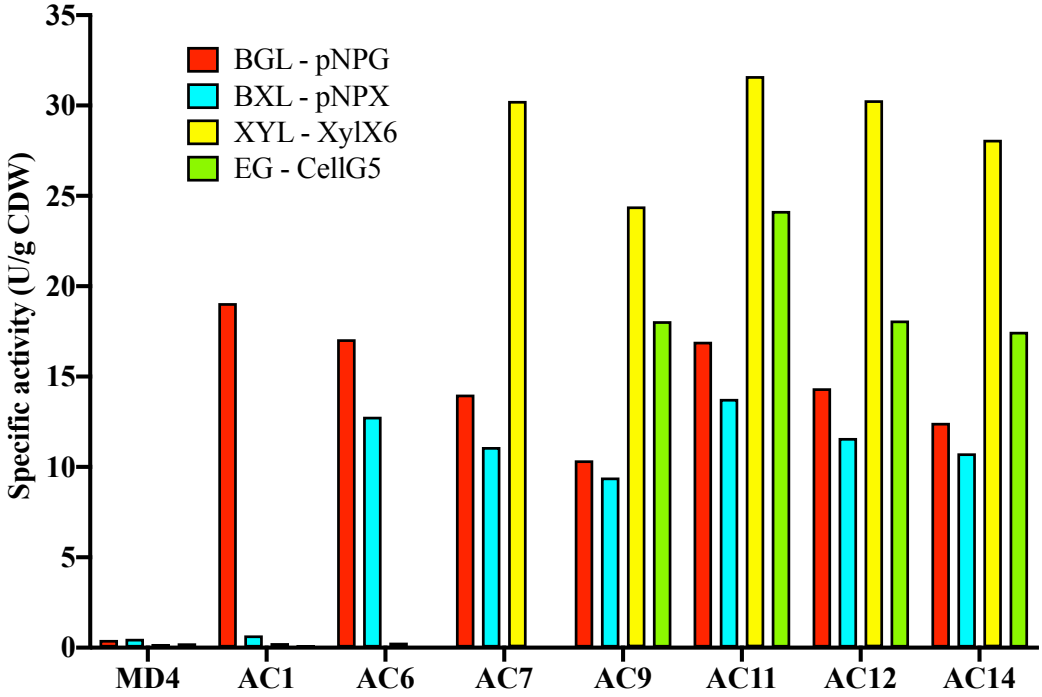


Figure 5.5. Overview of the specific activity of secreted lignocellulolytic enzymes in the different strains constructed from MD4 till AC14 (35°C, pH 5.5). BGL, BXL, XYL and EG activity with pNPG, pNPX, XylX6 and CellG5 as substrates, respectively. Average of three technical repeats is shown.

Since specific determination of *Cl*Cbh2b, *Te*Cbh1 and *Cc*XynA activity with 4-methylumbelliferyl-beta-D-lactopyranoside (MULac), 4-nitrophenyl β-D-cellobioside (pNPC), ß-cellotetraitol or p-nitrophenyl acetate (pNPA) as substrates was not possible due to background activity by the previously expressed heterologous enzymes (data not shown), the combined activity was measured with more complex (hemi-)cellulosic substrates, including carboxymethyl cellulose (CMC) (Fig. 5.6A), phosphoric acid swollen cellulose (PASC) (Fig. 5.6B), Avicel (Fig. 5.6C) and beechwood xylan (Fig. 5.6D). Activity with CMC and PASC as substrates was observed from AC9 on and remained constant at about 0.055 U/g CDW and 0.025 U/g CDW, respectively (Fig. 5.6A, B). Activity with Avicel as substrate increased with every integrated cellulolytic enzyme reaching its maximum in the final AC14 strain with a combined activity of 0.0061 U/g CDW (Fig. 5.6C). When beechwood xylan was used as substrate, combined activity was measured from AC7 on and remained relatively constant at about 1.3 U/g CDW in the subsequent strains (Fig. 5.6D). To assess the combined activity with pure cellulosic substrate, a filter paper assay was performed. The results are shown in Figure 5.6E and indicate that filter paper unit activity increased after integration of cellulase enzymes BGL, EG and CBHs. After genomic integration of seven (hemi-)cellulolytic enzymes in AC14, a combined activity of 94.5 FPU was obtained. The specific activity values in

mU/mg secreted protein for all substrates are provided in Appendix Fig. A5.2 and the conversion rates of cell-free medium from all strains compared for all substrates are listed in Appendix Table A5.2.

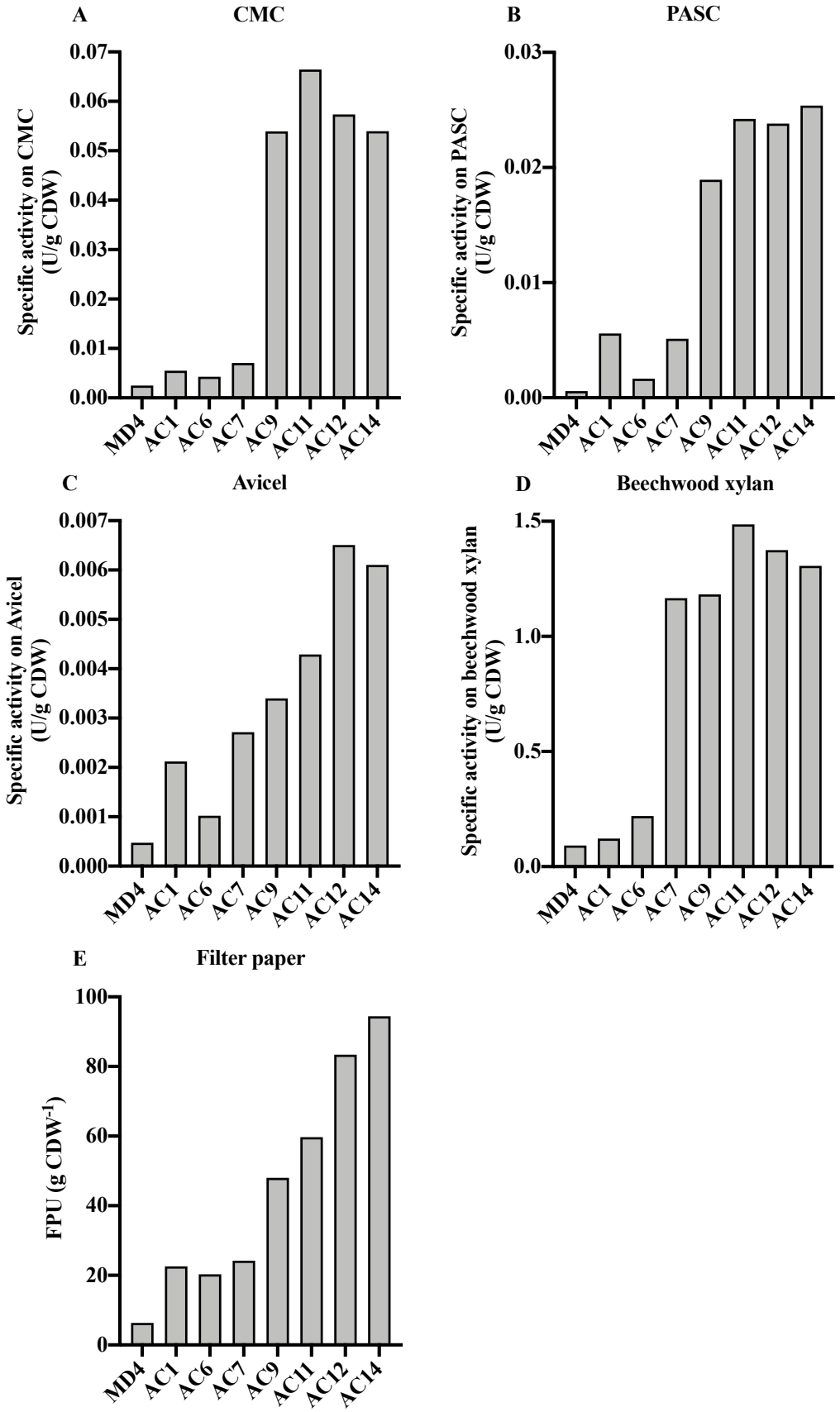


Figure 5.6. Combined hydrolytic activity measured with multiple polymeric substrates for all constructed strains from MD4 till AC14 (A-D: 35°C, E: 50°C, A-E: pH 5.5). (A) CMC. (B) PASC. (C) Avicel. (D) Beechwood xylan. (E) Filter paper units. Average of three technical repeats is shown.

2.5. Fermentation performance of AC14 compared to ancestor MD4 with various polymeric substrates

The fermentation performance of AC14 was evaluated in comparison with the initial MD4 strain. The strains were tested in synthetic YP medium with various polymeric substrates.

During fermentation of YP containing 2% (w/v) glucose, 2% (w/v) xylose, 1% (w/v) cellobiose and 2% (w/v) beechwood xylan, MD4 was able to ferment all glucose and xylose, resulting in an expected final weight loss due to CO_2 production of 1.9% and an ethanol production of 1.9% (w/v) (Fig. 5.7A, B). Fermentation of this medium with AC14, on the other hand, indicated complete consumption of glucose, xylose and cellobiose. Additionally, beechwood xylan was hydrolyzed partially, resulting in a final weight loss of 2.65% and an ethanol production of 2.7% (w/v) (Fig. 5.7A, C). However, a slight decrease in xylose fermentation rate was observed for AC14 compared to MD4. The maximal weight loss during this fermentation would be around 3.5% if all sugars were consumed, indicating that improved xylan breakdown can increase the ethanol production.

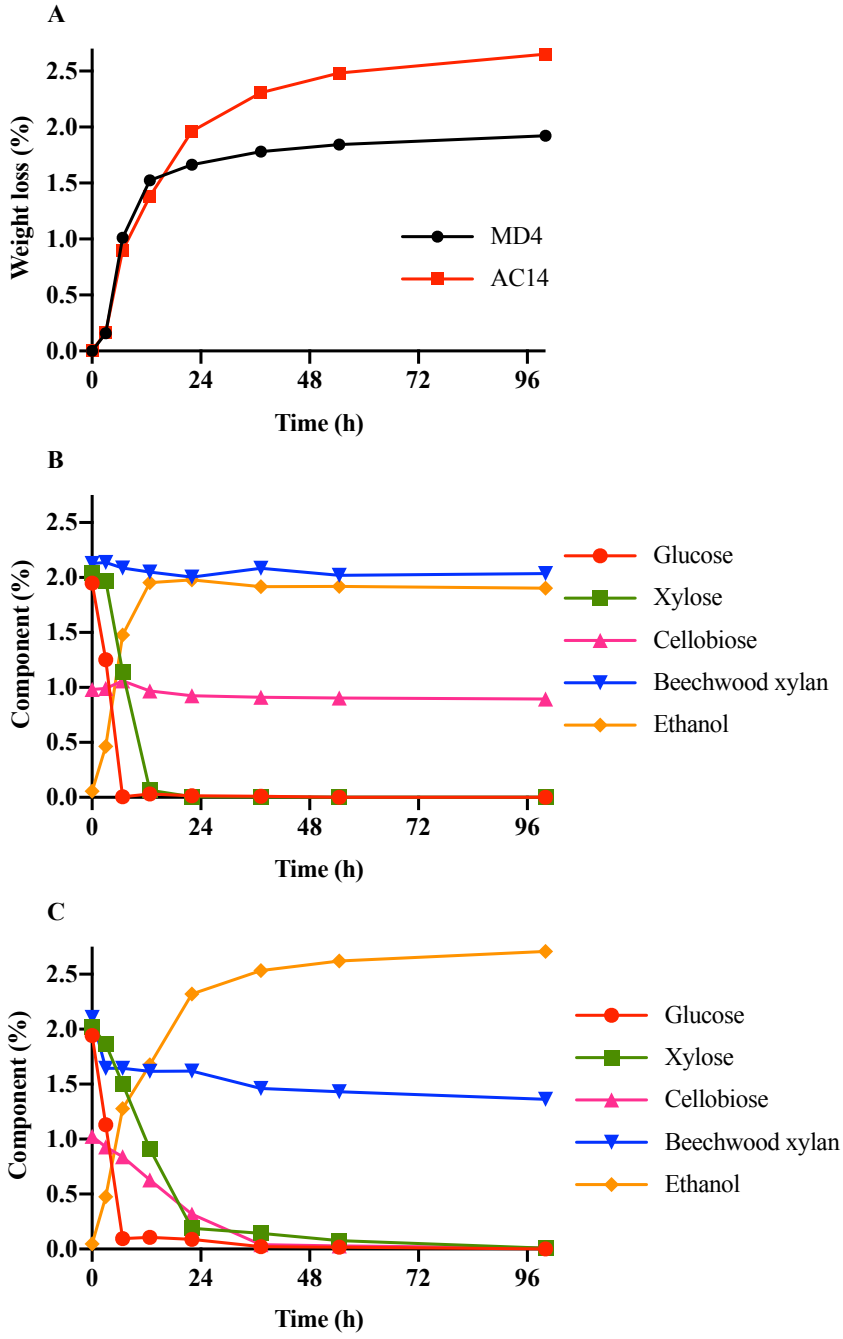


Figure 5.7. Fermentation performance of strains MD4 and AC14 in YP medium with 2% (w/v) glucose, 2% (w/v) xylose, 1% (w/v) cellobiose and 2% (w/v) beechwood xylan (35°C, 70mL, 1g CDW/L). (A) Weight loss during fermentation with MD4 and AC14. (B) Sugar consumption and ethanol production during fermentation with MD4 and (C) Sugar consumption and ethanol production during fermentation with AC14.

Additionally, these strains were evaluated in medium containing cellulosic polymers. During fermentation of YP with 2% (w/v) glucose and 2% (w/v) CMC, MD4 fermented the glucose fraction, resulting in a weight loss of 0.9%, as expected. AC14 additionally fermented a small portion of the CMC, resulting in a weight loss of 1.02% (Fig. 5.8). This indicated that 0.25% (w/v) CMC was depolymerized and fermented to ethanol with AC14. There is room for improvement in CMC hydrolysis, since the maximal weight loss during this fermentation would be around 2% if all glucose and CMC were consumed.

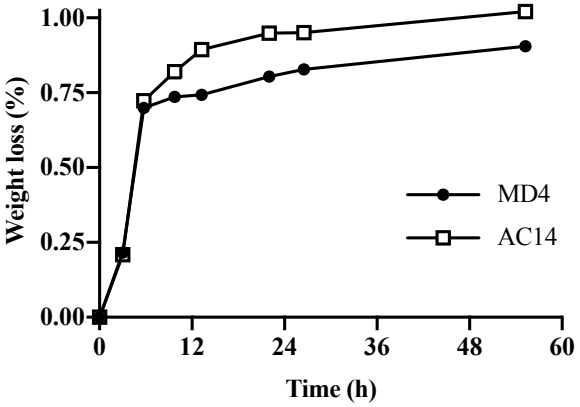


Figure 5.8. Fermentation performance of AC14 compared to MD4 measured by weight loss due to CO_2 production during fermentation of YP with 2% (w/v) glucose and 2% (w/v) CMC (35°C, 10mL, 1g CDW/L).

2.6. Whole genome sequence analysis for copy number determination of the heterologous genes inserted in AC14

Comparative analysis of chromosomal coverage and SNP frequency using in-house scripts, as described in Materials and Methods, confirmed proper integration of the seven (hemi-)cellulolytic genes in AC14 and revealed that MD4 was aneuploid, with an average ploidy of 4n (Table 5.3). Several pentaploid chromosomes were present (I, II, IV, VII, IX, XII and XVI). They were prioritized for integration of the lignocellulolytic genes to obtain a higher copy number. On the other hand, we observed frequent loss of one copy of a chromosome when genes were integrated using the CRISPR/Cas9 methodology. This was for instance the case after integration of BGL in chromosome II, with one allele of chromosome II being lost in AC1 (Chapter 2). Similarly, integration of XYL, EG and AXE in chromosome XVI reduced its chromosomal copy number resulting in four copies for each of the genes in the genome of AC14. Integration of CBHI, CBHII and BXL did not affect chromosomal copy number and their genes are thus present in five copies. A reduction in the number of BGL copies was observed after consecutive integration of the other genes. Whereas AC1 contained eight copies (Chapter 2), AC14 only retained five. This is in accordance with the results of the activity measurements and was verified using PCR amplification (data not shown). Using primers in opposite directions inside the *Trbgl1* tandem cassette, the amplified band indicating that at least in one allele the tandem cassette is still present. However, using two primers flanking the *Trbgl1* cassette, the amplified band indicated the size of one copy of *Trbgl1*, indicating that the tandem cassette was no longer present in at least one allele. Finally, for several chromosomes an additional spontaneous loss or gain of copy number was observed in the AC14 strain after all genes had been integrated, probably resulting in a genomic more stable strain (*e.g.* chromosome X and XII).

Table 5.3. Overview of genomic properties as determined by whole genome sequence analysis of MD4 and AC14. Ploidy of all chromosomes and coverage ratio of the integrated heterologous gene compared to the coverage of its host chromosome, determining its approximate copy number.

	MD4			AC14		
Chr.	Ploidy	Ratio coverage gene/ chromosome	Copy number	Ploidy	Ratio coverage gene/ chromosome	Copy number
I	5n	-	-	5n	-	-
II	5n	-	-	4n	~1.2x BGL	5 BGL
III	4n	-	-	5n	-	-
IV	5n	-	-	5n	~1.0x CBHI ~1.0x CBHII	5 CBHI 5 CBHII
V	4n	-	-	4n	-	-
VI	4n	-	-	4n	-	-
VII	5n	-	-	5n	~1.0x BXL	5 BXL
VIII	4n	-	-	4n	-	-
IX	5n	-	-	5n	-	-
X	3n	-	-	4n	-	-
XI	4n	-	-	4n	-	-
XII	5n	-	-	4n	-	-
XIII	4n	-	-	4n	-	-
XIV	4n	-	-	4n	-	-
XV	4n	-	-	4n	-	-
XVI	5n	-	-	4n	~1.0x XYL ~1.0x EG ~1.0x AXE	4 XYL 4 EG 4 AXE

Chr.: chromosome, -: not applicable

3. Discussion

Since both corncob and beechwood xylan were only hydrolyzed partially during fermentation with the cascade of recombinant xylanolytic AC strains (Chapter 3), improvement of the xylan breakdown was a target for development of a superior xylanolytic strain. To obtain an improved depolymerization of xylan, expression of genes encoding enzymes displaying acetylxylan esterase function was explored. After screening of seven heterologous enzymes, the bifunctional acetylxylan esterase/xylanase *xynA* from *Clostridium cellulovorans*, exhibiting the best performance during fermentation and in activity assays, was selected for genomic integration in the "six-pack" strain AC12. The resulting AC14 strain exhibited acetylxylan esterase activity towards corncob xylan, causing increased hydrolysis of corncob xylan. The rapid increase in the acetic acid level indicated a high acetylxylan esterase activity of *Cc*XynA on corncob xylan at the beginning of the fermentation.

However, fermentation of beechwood xylan with AC14 did not show an increased breakdown (data not shown). It is known that different xylan sources have a different composition, substitution pattern and acetylation (Deutschmann and Dekker, 2012; Ebringerová, 2005; Saha, 2003; Scheller and Ulvskov, 2010). In hardwood xylan, *O*-acetyl-(4-*O*-methylglucurono)xylan was found to be the most abundant constituent (Timell, 1967). In beechwood xylan specifically, out of 100 xylose residues 35 were *O*-acetylated with 1/3 on the C-2 position and 2/3 at the C-3 position (Teleman et al., 2002). With a degree of polymerization (DP) around 60, beechwood xylan was found to have long xylan chains (Teleman et al., 2002). In agricultural biomass, specifically corncob xylan, the DP was observed to be around 8, which is much lower (Kabel et al., 2002) and per 100 xylose residues 49 were substituted with an acetyl group (Van Dongen et al., 2011). These studies indicate that corncob xylan has a lower DP and higher acetylation level than beechwood xylan. There is high variability in substrate specificity for AXEs. Some AXEs prefer substrates with high molecular weight and thus high DP, while others only show activity towards acetyl xylobiose (DP 2) (Biely et al., 1986; Poutanen et al., 1990). Bifunctional acetylxylan esterase/xylanase XynA from *C. cellulovorans*, specifically, shows a 50% lower activity on hardwood xylan, *e.g.* birchwood xylan, compared to acetylated xylan (Kosugi et al., 2002). These arguments can explain why *Cc*XynA has a higher specificity for xylan with low DP and high acetylation and therefore no activity was detected on beechwood xylan. To confirm this hypothesis, xylans obtained from different sources should be compared for their characteristics and for breakdown using a strain producing *Cc*XynA.

It remains a challenge to determine acetylxylan esterase activity in the engineered strains. We attempted to measure the specific activity with p-nitrophenyl acetate (pNPA) as substrate. However, due to high native esterase secretion by *S. cerevisiae*, purification of the desired enzyme is required. Therefore, alternative activity measurement assays have been developed in which highly acetylated xylan is created from commercial xylans (Johnson et al., 1988; Khan et al., 1990). This acetylated xylan should be specific for acetylxylan esterases, which enables specific AXE activity measurement using bromothymol blue (Martinez-Martinez et al., 2007). This assay would allow for more elaborate screening of AXE-encoding genes for high esterase activity upon expression in *S. cerevisiae*.

In this study, we selected four enzymes that were described to have bifunctional activity as acetylxylan esterase and xylanase. Since AXE activity could not be measured specifically, the XYL activity was measured and correlated to the presumable AXE activity. This indicated that the *Cc*XynA enzyme is mainly present in the

intracellular environment and less in the cell-free medium. Therefore, one way to further improve the AXE activity and thus xylan hydrolysis using this enzyme, is by enhancing the secretion of *Cc*XynA. Secretion of recombinant proteins has been improved in many ways in *S. cerevisiae* (Hou et al., 2012; Idiris et al., 2010). Secretion of one specific enzyme can be enhanced by addition of a secretion signal that is known to be cleaved and processed for secretion by the host. *Cc*XynA and all other genes expressed in the AC14 strain were integrated with their native secretion signal. In *S. cerevisiae*, multiple signal peptides were explored to enhance secretion of heterologous proteins. Fusing the signal peptide from *AGA2*, *CRH1*, *PLB1* and *MFα1* to *LacA* was observed to improve the secretion of LacA in *S. cerevisiae* compared to the wild type sequence of *LacA* (Mori et al., 2015). Heterologous protein secretion was even further improved by engineering cellulases with the signal peptide of *OST1* (Besada-Lombana and Da Silva, 2019) or *SED1* (Inokuma et al., 2016). Other ways to improve enzyme secretion are tuning the promoter strength (Alper et al., 2005), optimization of the copy number of the heterologous gene (Kroukamp et al., 2017) and engineering the heterologous protein, taking codon bias into account (Brule and Grayhack, 2017; Hanson and Coller, 2018; Huang and Shusta, 2005).

The resulting AC14 strain retained the enzyme specific activity and fermentation capacity over the consecutive genomic enhancements. In fermentation medium containing 2% (w/v) glucose, 2% (w/v) xylose, 1% (w/v) cellobiose and 2% (w/v) beechwood xylan, the degradation of beechwood xylan by AC7, observed in Chapter 3, indicated that 25% of the initial concentration (*i.e.* 0.5% of 2%, w/v) was hydrolyzed in around 50 h. Also, the breakdown of CMC with AC12, observed in Chapter 4, indicated that 12.5% of the initial CMC concentration (*i.e.* 0.25% of 2%, w/v)) was hydrolyzed in around 60 h. Repetition of this fermentation with strain AC14 showed an equal rate of beechwood xylan and CMC hydrolysis.

In conclusion, a strain simultaneously expressing, producing and secreting seven lignocellulolytic enzymes has been developed. This AC14 strain inherited the capacity to secrete the six essential enzymes for breakdown of cellulose and hemicellulose from the “six-pack” strain AC12 and additionally gained acetylxylan esterase activity by expression of the heterologous *xynA* from *Clostridium cellulovorans*. This enzyme released acetic acid from the xylan backbone of corncob xylan, thereby increasing the extent to which this substrate was hydrolyzed during fermentation.

Chapter 6 - Concluding remarks and future perspectives

One of the most important obstacles hindering wide-spread establishment of industrial production of biofuels and bio-based chemicals with lignocellulosic biomass as feedstock is the huge cost of the commercial enzyme cocktails required for saccharification of the lignocellulose, representing about 20 - 30% of the ethanol cost (Johnson, 2016; Klein-Marcuschamer et al., 2012). One way to lower the enzyme requirement is to have at least part of the enzymes produced by the yeast used for the fermentation. The main challenge here is to have all the required enzymes expressed at adequate levels by the yeast and to maintain all other traits essential for efficient fermentation of lignocellulose hydrolysates, in particular the property of xylose fermentation that is also dependent on high expression of a heterologous enzyme, bacterial xylose isomerase.

In this study, we have successfully integrated seven heterologous genes encoding lignocellulolytic enzymes using the CRISPR/Cas9 technology in an industrial xylose-fermenting and inhibitor-tolerant *S. cerevisiae* strain. The final strain, AC14, is to our knowledge the first industrial 2G yeast strain that successfully expresses and secretes seven enzymes with lignocellulolytic capacity. Our work shows that at least seven genes encoding heterologous lignocellulolytic enzymes can be integrated in multiple copies in the *S. cerevisiae* genome without negatively affecting the fermentation capacity of the resulting strain, including the heterologous xylose fermentation capacity. A total of 32 copies of heterologous genes, of which 18 copies flanked by the *TEF1* promoter and *CYC1* terminator and 14 copies flanked by the *PGK1* promoter and *PRM9* terminator, have been introduced in the same genome. Although copies of aneuploid chromosomes got lost during the strain construction, the final strain, which was largely tetraploid with some pentaploid chromosomes, appeared to be stable at least during the time course of our studies. The developed strains were subjected to stability studies by evaluating the specific activity of the recombinant proteins after repeatedly transferring the strains from -80°C to fresh plates and vice versa. The specific activity was not affected by these serial transfers, indicating that the created strains are stable for the expression of the heterologous genes and the production of the recombinant proteins. Polyploid and aneuploid genomes are generally less stable than diploids and haploids, which makes them susceptible to rapid adaptation (Hickman et al., 2015; Selmecki et al., 2015). Aneuploidies are usually highly unstable if not maintained by strong selective forces (Kumaran et al., 2013; Zhu et al., 2012; Zhu et al., 2016; Zhu et al., 2014b). The loss of excess aneuploid chromosomes, resulting in a mainly tetraploid AC14 strain, may thus ensure higher genomic stability of the strain. Growth and propagation were also not affected significantly by the high-level secretion of heterologous enzymes.

The consecutive integration of new enzymes generally did also not compromise the expression, secretion or activity of previously integrated enzymes, except for BGL. This indicates that the upper limit of copy number and expression level of heterologous secreted enzymes in at least the specific industrial *S. cerevisiae* strain that we used in our work has not been reached yet. Probably, the copy number and expression level of the current enzymes, as well as of additional enzymes, can be further increased. Previously, it has been suggested that high levels of engineered secreted enzymes would result in metabolic stress and protein burden, compromising the fermentation capacity of the host yeast strain (Görgens et al., 2001; Kafri et al., 2016; van Rensburg et al., 2012). Our work shows that robust 2G industrial yeast strains might tolerate much higher levels and numbers of secreted enzymes compared to laboratory or 1G industrial yeast strains than previously anticipated.

There is one exception where further integration of lignocellulolytic enzyme-encoding genes affected the copy number of a previously integrated gene, namely *bgl1* from *Trichoderma reesei* (*Trbgl1*). Whole genome sequence analysis of AC1 revealed the presence of eight copies of *Trbgl1* at position IS2.1. After consecutive integration of additional genes using CRISPR/Cas9 to create AC14, whole genome sequence analysis revealed a loss of 3 copies of *Trbgl1*. This was also observed in the specific BGL activity *in vitro*, where 35% of the activity was lost from AC1 to AC14. This may be due to instability of the insertion construct as the cassette integrated in AC1 contained a tandem construct of *Trbgl1* flanked by the *TEF1* promoter and *CYC1* terminator. As these tandem sequences are 100% identical, they may have looped out copies using homologous recombination, possibly resulting in a strain with higher genomic stability.

The AC14 strain showed high secreted activity with enzyme-specific substrates and with some more complex substrates, especially corncob and beechwood xylan. This resulted in rapid utilization of these substrates in a mixed-substrate fermentation. On the other hand, the utilization of the other complex substrates, CMC, PASC, Avicel and filter paper, was much slower pointing to insufficient activity of at least one of the rate-limiting enzymes. The most likely candidates are CBHI and CBHII, which displayed the lowest activity *in vitro*. Hence, the secreted activity of these enzymes has to be increased further to support a significant reduction in the requirement of commercial enzyme cocktail and to finally arrive at a CBP organism. On the other hand, the implementation of an enzyme-secreting yeast strain allows for application of SSF at least in the second part of the biomass saccharification and fermentation process, *i.e.* a first part with a pure enzymatic saccharification process at higher temperature and a second part at lower temperature in which the saccharification is continued together with the fermentation by the yeast. SSF reduces feedback inhibition of monomeric sugars on the hydrolytic enzymes (Xiao et al., 2004), causes maintenance of a very low free sugar level, strongly reducing the risk of bacterial contamination and associated losses in yield, titer and productivity.

The AC14 strain produced about 94.5 FPU/g cell dry weight during propagation of the yeast in 50 ml YNB medium with 2% glucose, reaching a final OD_{600} of 15 or about 3 g CDW/L. At this moment, it is unclear whether utilization of this yeast in fermentations using an initial yeast pitching rate of 1 g CDW/L, free glucose and xylose level of 10-20% and fermentation time of about 48 h will result in the same secreted enzyme production. Depending on the feedstock used, the requirement for commercial enzyme cocktail varies greatly, with simple feedstocks like paper pulp requiring much less enzyme cocktail compared to more complex and more recalcitrant feedstocks, like bagasse, wheat straw and wood (Modenbach and Nokes, 2013). Hence, the application of enzyme-secreting yeast may be in first instance most promising for reducing the commercial enzyme load in fermentation processes using simple feedstocks like paper pulp.

In spite of this, further improvement of the total secreted enzyme activity is highly desirable. It is known that *S. cerevisiae* is a poor protein secretor compared to other microbial cell factories, such as *Pichia pastoris* (Ahmad et al., 2014) and *Trichoderma reesei* (Nevalainen et al., 2005; Penttilä, 1998). However, protein secretion in *S. cerevisiae* can be enhanced significantly in different ways: increasing copy number of the genes, increasing protein secretion efficiency, for example by adjusting specific exocytotic SNARE proteins that play an essential role in protein trafficking (van Zyl et al., 2014) and/or other components of the protein secretion machinery (Tang et al., 2017), by random mutagenesis and selection of better secretors (Eriksen et al., 2013) or by evolutionary

adaptation based on faster proliferation of better secretors (Lee et al., 2016). Optimization of glycosylation is also a possible target, since *N*- and *O*-linked hyperglycosylation tend to affect the secretion and activity of heterologous enzymes expressed in *S. cerevisiae* (Hernandez et al., 2017; Tang et al., 2016). The heterologous genes that we have used were codon optimized for expression in *S. cerevisiae*, since codon usage is known to affect translation elongation, translation efficiency and protein folding (Brule and Grayhack, 2017; Hanson and Coller, 2018). However, codon optimization does not always guarantee optimal expression and/or translation of heterologous genes (Cripwell et al., 2019; Lanza et al., 2014). Therefore, screening and comparison of genes originating from different species or different codon optimized variations may result in further improvement of secreted enzyme activity and thus of the breakdown efficiency of the lignocellulosic biomass. Especially, screening and/or discovery of lignocellulolytic enzymes with a lower temperature optimum can enhance the intrinsic enzyme activity under industrially relevant conditions (Kasana and Gulati, 2011).

Saccharification efficiency can also be improved by optimizing the balance between the different lignocellulolytic enzyme activities. Models for secreted cellulase activity indicate strong synergism between endo- and exoglucanases for efficient cellulose hydrolysis (Jalak et al., 2012; Kostylev and Wilson, 2014). Higher endoglucanase activity creates more substrates for the exoglucanases. Hu and colleagues (Hu et al., 2015) identified the CBHs as major contributors in the breakdown process of cellulose. Also, Liu and colleagues (Liu et al., 2017) investigated the transcription levels of heterologous cellulases in an optimized cellulolytic *S. cerevisiae* strain and observed the highest transcription levels for CBHI and EG. CBHI was shown to be more active and effective in degrading crystalline cellulose (Igarashi et al., 2011) and Avicel (Liu et al., 2017) compared to CBHII. This is consistent with observations on natural enzyme secretors, as the endo-exocellulase system of *T. reesei* consists out of around 60% CBHI, 20% CBHII and 12% EG (Zhang and Lynd, 2004). Hence, the primary target for further improvement of the cellulolytic capacity of AC14 should be enhancement of the expression, secretion and activity of both CBHI and EG.

Furthermore, the lignocellulolytic capacity of AC14 can be further improved by expression of additional genes encoding additional auxiliary hemicellulases, mannanases and lytic polysaccharide monooxygenases (LPMOs). As discussed in Chapter 5, hardwood xylan consists of *O*-acetyl-(4-*O*-methylglucurono)xylan, which is a highly substituted xylose backbone. To obtain complete hydrolysis of this substrate a spectrum of auxiliary enzymes is required to cleave off these side chains and increase the accessibility of other xylanolytic enzymes to xylan (Bhattacharya et al., 2015; Van Dyk and Pletschke, 2012). These auxiliary enzymes, *i.e.* α-glucuronidase (α-glucosiduronase, EC 3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55), arabinase (endo-α-L-arabinase, EC 3.2.1.99), α-D-galactosidase (EC 3.2.1.22) and feruloylxylan esterase (EC 3.1.1.73), can be a target to improve the partial breakdown of corncob and beechwood xylan by AC14 (Gao et al., 2011; van Zyl et al., 2007). Mannan is, next to xylan, the second most abundant hemicellulosic fraction constituting the majority in softwood (Chauhan et al., 2012). To hydrolyze this substrate, the strain can be engineered for mannanase activity by expression of an endomannanase (endo-ß-1,4-mannanase, EC 3.2.1.78) and ß-mannosidase (EC 3.2.1.25) (Ishii et al., 2016; Liu et al., 2018b; Prima et al., 2017; von Freiesleben et al., 2018). Combination of xylanase and mannanase even resulted in improved, synergistic hydrolysis of softwood xylan (Varnai et al., 2011). Other accessory enzymes that can improve the lignocellulolytic capacity of AC14 are lytic polysaccharide

monooxygenases (LPMOs). These enzymes deconstruct a range of different substrates, such as cellulose, hemicellulose and starch, through oxidative action (Hemsworth et al., 2015; Isaksen et al., 2014; Johansen, 2016). Oxidation of glycosidic bonds in these substrates cleaves the chains and boosts the activity of conventional glycoside hydrolases (Dimarogona et al., 2013; Horn et al., 2012). LPMOs have been identified as copper-dependent enzymes (Quinlan et al., 2011). A single copper ion binds and activates oxygen for the oxidative reaction (Frandsen et al., 2016; Hemsworth et al., 2015; Johansen, 2016). However, LPMOs require molecular oxygen for activity, which may be affected depending on the substrate composition and concentration of dissolved oxygen in the hydrolysate (Johansen, 2016). LPMO activity was shown to improve the breakdown of lignocellulosic substrates, such as delignified softwood from spruce (Koskela et al., 2019), Avicel (Arfi et al., 2014) and PASC (Liang et al., 2014; Villares et al., 2017). Therefore, LPMOs may be an interesting target with broad substrate specificity for enhanced lignocellulolytic activity of AC14.

In my opinion, the created AC14 strain can currently be able to act as a CBP host for bioethanol production from simple polymeric substrates, such as paper pulp, provided that the cellobiohydrolase activity can be improved. Screening for more active heterologous genes encoding for CBHI and CBHII can significantly improve the specific CBH activity and thus also the synergistic combined cellulase activity. This screening for high activity can be focused on different codon optimized genes encoding the same enzyme or on evaluation of the specific activity of different enzymes at low temperature. In my point of view, this should be a priority for future research based on the AC14 strain. To develop a strain fully capable of CBP of more complex polymeric substrates, such as bagasse, that can lower the use of expensive commercial enzyme cocktails, the overall secretion profile of the strain needs to be optimized for production of recombinant proteins. Overexpression of *HAC1* can reportedly lead to an increased recombinant protein production in yeast. This has been investigated extensively and might improve the overall secretion of the (hemi-)cellulases produced by AC14. Therefore, prioritizing this approach in future research might result in an easy way to enhance the CBP properties of the strain. Secretion of recombinant proteins can be also be enhanced easily by adding a secretion signal that is recognized for cleavage by the host. Also, engineering protein secretion machinery by overexpression of the SNARE genes *SED5* and *SS01/2* affecting the vesicles from ER to Golgi and from Golgi to plasma membrane, respectively, is an easy and well-established way to improve protein secretion in yeast. Additionally, hyperglycosylation can be avoided by eliminating glycosyltransferases, avoiding lipid-linked oligosaccharide assembly and/or overexpressing endo-N-acetylglucosaminidases. These modifications can all individually or together enhance recombinant protein secretion and thus activity of the recombinant (hemi-)cellulases. However, engineering the overall secretion profile of the strain will be more laborious and most likely needs to be optimized for different strains and heterologous genes. Finally, expression of the integrated genes can be optimized for their optimal ratio. On the other hand, this approach is highly dependent on the chosen enzymes and the strain background. Therefore, this appears to be one of the final modifications that I would execute to enhance and optimize the lignocellulolytic activity of the strain.

In our work, we have developed an enzyme-secreting yeast strain for production of ethanol from lignocellulosic biomass. However, the high cost of the enzyme cocktails required for saccharification also applies to the use of yeast for the production of bio-based chemicals with lignocellulosic biomass. Yeast strains producing a large

variety of such chemicals are currently being developed (Chen and Dou, 2016; Isikgor and Becer, 2015; Kawaguchi et al., 2016). Hence, highly efficient enzyme-secreting industrial 2G yeast strains will also constitute promising platform strains for the development of efficient cell factories for production of bio-based chemicals with higher added value using lignocellulosic biomass.

In conclusion, we have been able to develop a 2G industrial yeast strain, AC14, with efficient secretion of seven lignocellulolytic enzymes, reaching 94.5 FPU of secreted enzyme activity/g CDW of yeast. This enabled the yeast to use both cellulosic and hemicellulosic substrates for fermentation. The fermentation activity of the yeast, including the artificially engineered heterologous xylose fermentation capacity, was not significantly compromised by the combined enzyme secretion. The AC14 strain will serve as a promising platform strain for further improvement of secreted enzyme activity. This should allow to reduce the load and high cost of the commercial enzymes required for biomass saccharification significantly and may lead to development of efficient strains for production of bioethanol and bio-based chemicals with CBP using second-generation feedstocks.

Publications

Quinten Deparis*, Arne Claes*, María R. Foulquié-Moreno, Johan M. Thevelein, Engineering tolerance to industrially relevant stress factors in yeast cell factories, *FEMS Yeast Research*, Volume 17, Issue 4, June 2017, fox036, https://doi.org/10.1093/femsyr/fox036

Arne Claes, Quinten Deparis, María R. Foulquié-Moreno, Johan M. Thevelein, Simultaneous secretion of seven lignocellulolytic enzymes by an industrial second-generation yeast strain enables efficient ethanol production from multiple polymeric substrates, *Metabolic Engineering*, Volume 59, May 2020, Pages 131-141, ISSN 1096-7176, https://doi.org/10.1016/j.ymben.2020.02.004.

Marija Stojiljkovic, Arne Claes, Quinten Deparis, Mekonnen Demeke, María R. Foulquié-Moreno, Johan M. Thevelein. Whole-genome transformation of yeast causes rare mutations with a single causative SNP enhancing acetic acid tolerance. Submitted

* = equal contribution

Materials and methods

Strains and plasmids

Strains and plasmids used in this study are listed in Table M1.

Table M1. Yeast strains and plasmids used

	Description	Source
Yeast strains	**Genotype**	
Saccharomyces cerevisiae GSE16-T18 HAA1*	Industrial 2G xylose-fermenting and inhibitor-tolerant strain. GSE16-T18 with c.1517 G > A mutation in both copies of *HAA1*	(Meijnen et al., 2016)
GSE16-T18 HAA1*-*Trbgl1* 2 copies	GSE16-T18 HAA1* IS4.1::*Trbgl1* Industrial xylose-fermenting inhibitor-tolerant strain GSE16-T18 HAA1* that expresses 2 copies of ß-glucosidase	This study
GSE16-T18 HAA1*-*Trbgl1* 6 copies	GSE16-T18 HAA1* IS4.1::*Trbgl1* IS2.1::*Trbgl1-Trbgl1* Industrial xylose-fermenting inhibitor-tolerant strain GSE16-T18 HAA1* that expresses 6 copies of ß-glucosidase	This study
Saccharomyces cerevisiae MD4	Industrial 2G xylose-fermenting and inhibitor-tolerant strain. Derivative of a cross between GSE16-T18 and Ethanol Red (4n-5n), further improved by evolutionary adaptation for efficient xylose fermentation and high inhibitor tolerance.	Derived from (Demeke et al., 2013a)
AC1	MD4 IS2.1::*Trbgl1* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase	This study
AC2	AC1 IS7.1::*AnxlnD-Trxyn2* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase and xylanase	This study
AC2-XYL2	AC2 IS16.1::*AnxlnB* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase and xylanase *Tr*Xyn2 and *An*XlnB	This study
AC6	AC1 IS7.1::*AnxlnD* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase and ß-xylosidase	This study
AC7	AC6 IS16.1::*AnxlnB* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase and xylanase	This study
AC9	AC7 IS16.2::*AocelB* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase, xylanase and endoglucanase	This study
AC11	AC9 IS4.2::*Clcbh2b* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase, xylanase, endoglucanase and cellobiohydrolase II	This study
AC12	AC11 IS4.1::*Tecbh1* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase, xylanase, endoglucanase, cellobiohydrolase II and cellobiohydrolase I	This study
AC14	AC12 IS16.3::*CcxynA* Industrial xylose-fermenting inhibitor-tolerant strain MD4 that expresses ß-glucosidase, ß-xylosidase, xylanase, endoglucanase, cellobiohydrolase II, cellobiohydrolase I and acetylxylan esterase	This study
Plasmids		
pBevy-nat-phiC31i	Multicopy plasmid with NatMX and phiC31 integrase	(Wallace-Salinas et al., 2014)

pTEF-Cas9-KanMX	Single copy, vector backbone p414-TEF1p-Cas9-CYC1t with auxotrophic marker replaced by KanMX	(DiCarlo et al., 2013; Holt et al., 2017)
pgRNA-uni-NAT (p59)	Multicopy, vector backbone p426-SNR52p-gRNA.CAN1.Y-SUP4t with auxotrophic marker replaced by NatMX, used for cloning of gRNA sequences to target specific integration sites	This study
p59 G1-mk20	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and IS2.1 (ATCAACCACAGTGAACGCCG)	This study
p59 G1-AD7	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and IS7.1 (TAGCATCGTGCATGGGATAG)	This study
p59 G1-IS16.1	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and IS16.1 (GTTAACTCTGAATGTATGCG)	This study
p59 G1-IS16.2	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and IS16.2 (GTAGAATAAGTGTTTCGGAT)	This study
p59 G1-mk119.1	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and three alleles of IS4.2 in MD4 (AGACCTTCAGTTCTGACGCA)	This study
p59 G1-mk119.2	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and one allele of IS4.2 in MD4 (GACCTTCAGTTCTTGACGCA)	This study
p59 G1-mk114	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and IS4.1 (CAAAAGCGACACGTCGTCTG)	This study
p59 G1-IS16.3	pgRNA-uni-NAT backbone with NatMX and two gRNA sequences targeting G1 (GGCTGATTTTCGCAGTTCGG) and IS16.3 (CCTGCAGAGTTTGCATCTAG)	This study
p426hph	Backbone of donor plasmid containing HPH marker and different marker-site dependent homologous regions for integration by homologous recombination and flanking a gene encoding one of the secreted lignocellulolytic enzymes, expressed either from the TEF1 promoter and CYC1 terminator (BGL, BXL, XYL and AXE), or from the PGK1 promoter and PRM9 terminator (EG, CBHI and CBHII).	This study
p426hph-mk20-TrBGL1tandem	p426hph backbone with HPH marker and a tandem repeat of *Trbgl1* flanked with *TEF1* promoter and *CYC1* terminator. The cassette is flanked with homologous regions of IS2.1	This study
p426hph-AD7-AnXlnD	p426hph backbone with HPH marker and *AnxlnD* flanked with *TEF1* promoter and *CYC1* terminator. The cassette is flanked with homologous regions of IS7.1	This study
p426hph-AD7-AnXlnD-TrXyn2	p426hph backbone with HPH marker, *AnxlnD* flanked with *TEF1* promoter and *CYC1* terminator and *Trxyn2* flanked with *TEF1* promoter and *CYC1* terminator. The cassette is flanked with homologous regions of IS7.1	This study
p426hph-IS16.1-TrXyn2	p426hph backbone with HPH marker and *Trxyn2* flanked with *TEF1* promoter and *CYC1* terminator. The cassette is flanked with homologous regions of IS16.1	This study
p426hph-IS16.1-XYL2	p426hph backbone with HPH marker and *AnxlnB* flanked with *TEF1* promoter and *CYC1* terminator. The cassette is flanked with homologous regions of IS16.1	This study
p426hph-IS16.2-EG6	p426hph backbone with HPH marker and *AocelB* flanked with *PGK1* promoter and *PRM9* terminator. The cassette is flanked with homologous regions of IS16.2	This study
p426hph-IS4.2-CBHII2	p426hph backbone with HPH marker and *Clcbh2b* flanked with *PGK1* promoter and *PRM9* terminator. The cassette is flanked with homologous regions of IS4.2	This study
p426hph-IS4.1-CBHI3	p426hph backbone with HPH marker and *Tecbh1* flanked with *PGK1* promoter and *PRM9* terminator. The cassette is flanked with homologous regions of IS4.1	This study
p426hph-IS16.3-AXEXYL2	p426hph backbone with HPH marker and *CcxynA* flanked with *TEF1* promoter and *CYC1* terminator. The cassette is flanked with homologous regions of IS16.3	This study

Media and culture conditions

Yeast was grown at 30°C in YPD medium (1% yeast extract granulated (Merck), 2% bacteriological peptone (Oxoid), 2% glucose). When preparing agar plates, 1.5% agar granulated (Difco) was added to the solution. This solution was sterilized by autoclaving. The standard medium used in the experiments was YPD, unless stated otherwise. If necessary, YPD growth medium was supplemented with 100 µg/mL of nourseothricin, 200 µg/mL of geneticin, and/or 300 µg/mL of hygromycin B. YPD agar solution was poured in sterile petri dishes and solidified by drying at room temperature.

Yeast was grown at 30°C in minimal YNB medium (0.17% YNB (ThermoFisher) and 0.5% ammonium sulphate (Sigma)) for activity measurements. The pH was adjusted to 5.5 and the medium was autoclaved before addition of 2% glucose and 8.6 mM citric acid- 22.8 mM sodium phosphate buffer.

Escherichia coli TOP10F' cells were grown at 37°C in LB medium (2% LB broth (Sigma)). When preparing agar plates, 1.5% agar granulated (Difco) was added to the solution. Before usage, the solution was autoclaved. If necessary, LB growth medium was supplemented with 100 µg/mL ampicillin.

Molecular biology methods

DNA of yeast was purified by resuspending cells in TE buffer (pH 8) containing glass beads (0.75 mm). After addition of an equal amount of PCI (phenol-chloroform-isoamyl alcohol 25:24:1), the cells were lysed using a FastPrep®-24 (MP Biomedicals) for 20 s at 6 m/s (Hoffman and Winston, 1987). The obtained DNA was further purified using ethanol precipitation if required.

Polymerase chain reaction (PCR) was performed with Q5 (New England BioLabs) or ExTaq (TaKaRa) polymerases for cloning or sequencing. Standard Taq (New England BioLabs) and Taq E were used for checking and colony PCR.

Sanger sequencing was performed externally by Genetic Sequencing Facility of VIB (Antwerp, Belgium) or by Eurofins Genomics (Ebersberg, Germany). Sequences were analyzed using CLC Main Workbench software (QIAGEN Bioinformatics).

Plasmid digestion was performed as suggested by the supplier of the restriction enzymes (New England BioLabs). Dephosphorylation was done with FastAP thermosensitive alkaline phosphatase (Thermo Scientific).

Amplified DNA or digested plasmids were purified using the Wizard® SV Gel and PCR Clean-Up kit (Promega). Plasmid isolation from *E. coli* was done with the NucleoSpin® Plasmid Easy Pure kit (Macherey-Nagel).

DNA concentrations were measured using the NanoDrop® ND-1000 spectrophotometer (Isogen).

E. coli TOP10F' cells were made competent using the $CaCl_2$ method followed by transformation with heat shock (Sambrook et al., 1989).

Transformation of yeast was performed according to the LiAc/SS carrier DNA/PEG protocol established by Gietz and Schiestl (Gietz and Schiestl, 2007) including a heat shock at 42°C for 30 min.

Plasmid construction using Gibson assembly

Plasmid construction was performed according to the NEBuilder® HiFi DNA Assembly protocol (Gibson et al., 2009). 0.06 pmol of amplified and purified insert was combined with 0.02 pmol of purified digested plasmid and

an equal volume of NEBuilder® HiFi DNA Assembly Master Mix. After 1 h at 50°C, 10 µL of the assembly reaction was transformed in *E. coli*.

Evaluation and selection of heterologous genes encoding secreted lignocellulolytic enzymes

Previous reports on cellulases and hemicellulases successfully expressed and secreted by *S. cerevisiae* were used for initial selection of the heterologous genes to be expressed in the 2G industrial yeast strain MD4. Optimum temperature and pH were used as additional selection characteristics because in CBP hydrolysis and fermentation are performed at a temperature of 32-35°C and a pH range of 4.5-5.5. Three xylanases, four bifunctional acetylxylan esterases/xylanases, six endoglucanases, six CBHIs and six CBHIIs were evaluated for highest secreted specific activity in the MD4 strain. The heterologous genes were codon optimized for expression in *S. cerevisiae* using an online tool (https://eu.idtdna.com/CodonOpt). All nucleotide sequences of the evaluated and selected genes are provided in Appendix.

CRISPR-Cas9 protocol

The general CRISPR-Cas9 protocol was used to integrate the heterologous genes in the genome (Jakociunas et al., 2016; Ran et al., 2013). The genomic integration sites and their gRNA sequences are listed in Table M2. The respective p426hph donor plasmid and the Cas9 plasmid (pTEF-Cas9-KanMX) were transformed simultaneously in the target strain using the heat shock protocol (Gietz and Schiestl, 2007). After selection in the presence of both hygromycin B and geneticin, one of the transformant colonies was used for subsequent transformation with the corresponding gRNA plasmid (pgRNA-uni-NAT) and new transformants selected based on nourseothricin resistance. Proper genomic integration of the gene construct in all (4 or 5) alleles was confirmed using colony PCR. The primers used to verify integration are listed in Table M3. The transformants were cured of all plasmids and single colonies were picked using micromanipulation.

Table M2. The seven chromosomal insertion sites in strain MD4 used for integration of the genes encoding the secreted lignocellulolytic enzymes

Name	Chr.	gRNA sequence 5'->3'	Cutting site in genome 'yeast 2015'	Locus
IS2.1	II	ATCAACCACAGTGAACGCCG	140631^140632	Between *FUI1* and *PRE1*
IS4.1	IV	CAAAAGCGACACGTCGTCTG	867906^867907	Between *UME6* and *MSS4*
IS4.2	IV	AGACCTTCAGTTCTGACGCA GACCTTCAGTTCTTGACGCA	980437^980438	Between *EXG2* and YDR261W
IS7.1	VII	TAGCATCGTGCATGGGATAG	974537^974538	Between *PFK1* and YGR240C
IS16.1	XVI	GTTAACTCTGAATGTATGCG	525273^525274	Between *SWI1* and *HST2*
IS16.2	XVI	GTAGAATAAGTGTTTCGGAT	621736^621737	Between YPR027C and *YOP1*
IS16.3	XVI	CCTGCAGAGTTTGCATCTAG	882440^882441	Between *JIP1* and YPR170C

Chr. – Chromosome; IS – Integration site

Table M3. Primers used for verification of genomic integration of heterologous genes in all alleles at the specific integration site of the target strain

Lab number	Sequence	Name	Purpose
C-431	GAGTGATGGCGGATTTGAGTGC	mk20-Ch-Fw	Check integration at IS2.1
C-432	CTTCTTCCACGGACAAGTATTTC	mk20-Ch-Rv	Check integration at IS2.1
C-2827	CTCGTTGGTTGCAGTATACT	mk114-Ch-Fw	Check integration at IS4.1
C-2828	CGTGGAAGTTGCTGTTACTG	mk114-Ch-Rv	Check integration at IS4.1
C-4330	GGAGCAGACATCACTAAACG	AD7-Ch-Fw	Check integration at IS7.1
C-4331	GCCACAACCAAGTGAGATAC	AD7-Ch-Rv	Check integration at IS7.1
C-8549	CAGTAACAACAACCACAAGG	IS16-Ch-Fw	Check integration at IS16.1
C-8550	CGAGGAAATTCCTCGAAAGG	IS16-Ch-Rv	Check integration at IS16.1
D-431	GTCGAGAGGTTCAAATGTTG	YPR028W check_Fw	Check integration at IS16.2
D-432	CCAGCAGCCATATAACCTAC	YPR028W check_Rv	Check integration at IS16.2
C-2829	CTGACCTTGTGATGTTTCAA	mk119-Check-Fw	Check integration at IS4.2
C-2900	GTGTGTCATAATACTGCATC	mk119-Chnew-Rv	Check integration at IS4.2
C-9404	AAACAAGTACAGTGCTGACG	IntCheck_QDIS16_L_fw	Check integration at IS16.3
C-9405	TTTGTCCTTTATCGTAGAGG	IntCheck_QDIS16_R_rv	Check integration at IS16.3

Measurement of secreted enzyme activity

Cells were inoculated at an OD_{600} of 1.0 and grown for 24 h in YNB with 2% glucose supplemented with 8.6 mM citric acid- 22.8 mM sodium phosphate buffer to maintain the pH at about 5.5. The cell culture was centrifuged and the cell-free supernatant collected and used for enzyme activity measurements to determine secreted activity. Furthermore, in some cases enzyme activity was determined in cell extracts, which were made by first resuspending the cells after collection by centrifugation in citric acid-sodium phosphate buffer pH 5.5. Then, glass beads (0.75 mm) were added and the cells lysed using a FastPrep-24 for 20 s at a speed of 6 m/s. Cell debris were pelleted and the cell extract collected and used for enzyme activity measurements to determine total intracellular activity. Protein concentrations, both secreted and intracellular, were determined using the Lowry method with a BSA standard.

Enzyme activity measurements were performed using specific substrates. The prospective industrial conditions at the beginning of a CBP fermentation were mimicked to assess enzyme activity (35°C and pH 5.5). Specific activity of BGL and BXL was measured using 4-nitrophenyl-ß-D-glucopyranoside (pNPG, Sigma) and 4-nitrophenyl-ß-D-xylopyranoside (pNPX, Megazyme), respectively. 25 µL of 20 mM substrate solution and 50 µL 43.1 mM citric acid- 113.8 mM sodium phosphate buffer pH 5.5 were combined each time in a 96-well plate. 25 µL of enzyme sample was added and the reaction performed at 35°C for 15 min in a Grant-Bio microplate shaker. After addition of 100 µL of Na_2CO_3 (0.2 M), the absorbance was measured at 400 nm using a spectrophotometer. Attempts were made to measure secreted acetylxylan esterase activity and CBHII activity

with a similar protocol, but different substrates using p-nitrophenyl acetate (pNPA, Sigma) and 4-nitrophenyl β-D-cellobioside (pNPC, Sigma), respectively.

Specific activity of XYL and EG was detected with the XylX6 kit and CellG5 kit (Megazyme), respectively. The protocol was adapted for small-scale measurements in 96-well plates by adding 10 µL of substrate solution to 40 µL of 43.1 mM citric acid- 113.8 mM sodium phosphate buffer pH 5.5. After addition of 20 µL enzyme sample, the plate was put in a Grant-Bio microplate shaker at 35°C for 10 min. 100 µL of Na_2CO_3 (0.2 M) was added and the absorbance measured at 400 nm using a spectrophotometer.

Secreted activities for the strains producing CBHI enzymes were determined using soluble 4-methylumbelliferyl-β-D-lactoside (MULac, Sigma). For the assay 50 µl dialyzed culture supernatant was added to 50 µL sodium acetate buffer (50 mM, pH 5.5) containing 2.0 mM MULac and incubated at 35°C and 800rpm in a microplate incubator. Reactions were stopped after 10 min of incubation by adding 100 µl 0.2M Na_2CO_3. Liberation of 4-methylumbelliferone was detected by fluorescence measurement (excitation wavelength = 365 nm, emission wavelength = 445 nm) with a Synergy spectrofluorometer in a black, flat bottom microtiterplate (Nunclon). This reading was performed at 6.75mm from the bottom. Methylumbelliferone (MU, Sigma) dissolved in 0.2 M Na_2CO_3 was used to set up a standard curve up to 0.5 µM. Culture supernatants were diluted as required to keep emission values within this linear range.

To determine secreted activity of CBHII produced by the strains, the substrate ß-cellotetraitol (CTI, Megazyme) was explored. 25 µL of 6 mM CTI solution and 50 µL 43.1 mM citric acid- 113.8 mM sodium phosphate buffer pH 5.5 were combined each time in a 96-well plate. 25 µL of enzyme sample was added and the reaction performed at 35°C for 15 min in a Grant-Bio microplate shaker. After addition of 100 µL of Na_2CO_3 (0.2 M), the reducing sugar content was determined using 4-hydroxybenzoic acid hydrazide (pHBAH, Sigma).

The combined secreted activity of all enzymes was measured using 150 µL of 1% (w/v) substrate solution, either phosphoric acid swollen cellulose (PASC), Avicel (Sigma), carboxymethyl cellulose (CMC, Sigma), corncob xylan (Carl Roth) or beechwood xylan (Carl Roth). PASC was prepared as described by Kim and colleagues (2012). All substrates were resuspended in 43.1 mM citric acid- 113.8 mM sodium phosphate buffer pH 5.5. 100 µL of cell-free supernatant with the secreted enzymes was added and the reaction performed at 35°C for 24 h in the case of PASC, Avicel and CMC. In the case of corncob and beechwood xylan it was performed for 5 h.

The sugars released were measured with the reducing sugar assay using 4-hydroxybenzoic acid hydrazide (pHBAH, Sigma) (Mellitzer et al., 2012). For that purpose, a stock solution was prepared of 5% (w/v) pHBAH in 0.5% (v/v) HCl, which was filtered over a 0.2 µm filter to remove insoluble material. Glucose, cellobiose and xylose were used as standards. To determine the reducing sugar content, the 96-well plate used for the activity measurement was centrifuged at 4000 rpm for 5 min and 50 µL of the supernatant was transferred to a PCR tube containing 150 µL pHBAH solution diluted 1:4 (v/v) with 0.5 M NaOH. This solution was heated at 95°C for 5 min in a PCR apparatus to develop the color after which 150 µL was transferred to a fresh 96-well plate for readout at 410 nm using a spectrophotometer.

Filter paper units (FPU) were determined by adding a piece of filter paper (Whatman nr. 1, 1 x 0.6 cm) and 100 µL of 43.1 mM citric acid- 113.8 mM sodium phosphate buffer pH 5.5 in a 1.5 mL microcentrifuge tube. After addition of 100 µL cell-free supernatant with the secreted enzymes, the tubes were placed in a heat block at 50°C

for 4 h. The glucose released was measured with the pHBAH reducing sugar assay (Mellitzer et al., 2012) and the FPU estimated as described previously (Adney and Baker, 2008).

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining

Cells were grown for 24 h in YNB with 2% glucose supplemented with 8.6 mM citric acid- 22.8 mM sodium phosphate buffer to maintain the pH at about 5.5. The start OD_{600} was 1.0. After 24 h, the cell culture was centrifuged and the cell-free supernatant collected. This cell-free supernatant was used as such for protein visualization using SDS-PAGE. First, the supernatant was added in equal volume to sampling buffer (250 mM Tris pH8, 50 mM ß-mercaptoethanol, 10% SDS, 0,5% bromophenol blue, 50% glycerol) and boiled for 5 min at 90°C. These samples were loaded on a NuPAGE™ 4-12% Bis-Tris gel and placed in a Bio-Rad XCell SureLock™ machine. SeeBlue Plus2 Pre-Stain standard (Thermo Fisher Scientific) was used as a reference ladder. The gel was run for 60 min at 180 V in 1X NuPAGE™ 3-(N-morpholino)propanesulfonic acid (MOPS) SDS Running buffer (Thermo Fisher Scientific) and washed three times with MilliQ water for 5 min. The gel was stained with the SilverXpress™ Silver Staining kit (Thermo Fisher Scientific) to visualize protein bands with a concentration higher than 0.5 ng. This staining was performed as suggested by the supplier.

Evaluation of fermentation performance

Yeast was grown for propagation in YP with 2% glucose for 48 h, OD_{600} measured, and the cells were pelleted by centrifugation. Fermentations were performed with an initial pitching rate of 1 g CDW/L (OD_{600} of 5), based on the OD_{600} measured at the end of propagation. The composition and volume of the fermentation medium varied with the purpose of the fermentation and are specified in the figure legends. Fermentations were performed at 35°C with continuous stirring at 120 rpm. Weight loss was measured and samples of fermentation medium were taken using a needle and syringe through an impermeable membrane.

High-performance liquid chromatography (HPLC) analysis

HPLC was used to determine concentrations of glucose, xylose, cellobiose, beechwood xylan, corncob xylan, CMC, ethanol, acetic acid and glycerol during fermentation experiments. HPLC analysis was performed using a Shimadzu Nexera2 HPLC combined with an Agilent MetaCarb H87 column (300 x 7.8 mm) run at 70°C. 10 µL of cell-free fermentation medium was run with 5 mM H_2SO_4 as eluent at a flow rate of 0.7 mL/min. Compounds were measured with refractive index detection and analyzed with LabSolutions software (version 5.86, Shimadzu Corporation).

Whole genome sequence analysis

Genomic DNA of yeast strains was extracted using MasterPure Yeast DNA purification kit (Epicentre, Lucigen) and sent to Eurofins Genomics (Germany) for next-generation sequencing with Illumina (HiSeq2500) platform. The library consisted of 500 bp inserts and sequencing was done with pair-end reads of 125 bp. The reads were mapped against the S288c reference genome (R64-2-1; SGD) and coverage of AC1, AC2 and AC14 was

determined using samtools and compared with MD4 using NGSEP (Duitama et al., 2014). Variants were called using NGSEP and SNP frequency was determined using in-house scripts.

Appendix

1. Figures

Appendix Chapter 2

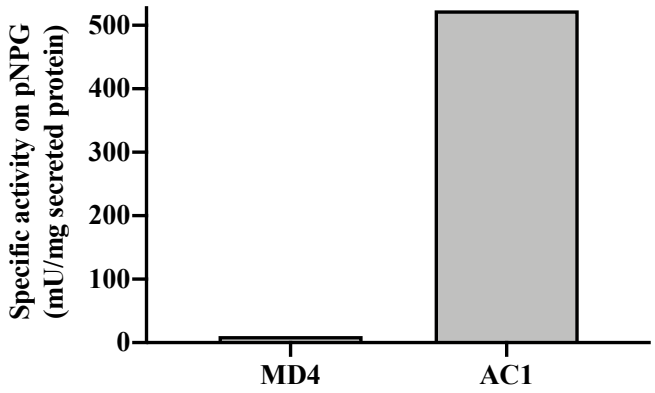


Figure A2.1. BGL activity of strains MD4 and AC1. Specific activity of secreted BGL in mU/mg secreted protein in cell-free supernatant of MD4 and AC1 on pNPG substrate. Average of three technical repeats is shown.

Appendix Chapter 3

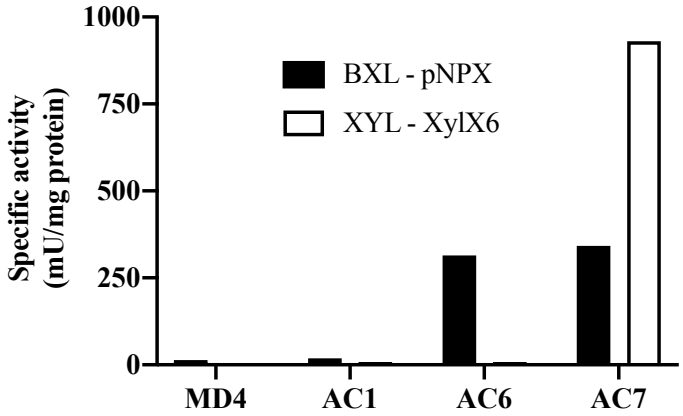


Figure A3.1. BXL and XYL activity of strain AC7 compared to MD4, AC1 and AC6. Specific activity of secreted BXL and XYL with pNPX and XylX6 as substrate, respectively, in mU/mg secreted protein in cell-free supernatant of MD4, AC1, AC6 (containing integrated *AnxlnD*, encoding BXL) and AC7 (containing integrated *AnxlnB*, encoding XYL). Average of three technical repeats is shown.

Appendix Chapter 4

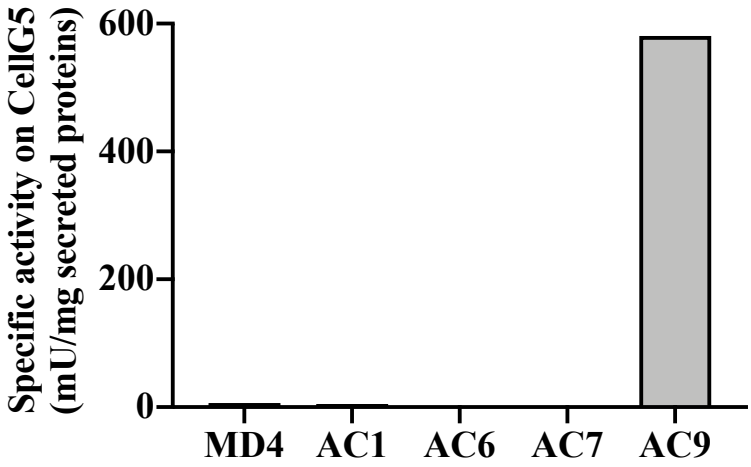


Figure A4.1. Evaluation of secreted EG activity in mU/mg secreted proteins upon genomic integration of *AocelB* (35°C, pH5.5, CellG5). Specific activity of secreted EG in strain AC9 with CellG5 as substrate after genomic integration of *AocelB* compared to strains MD4, AC1, AC6 and AC7, which lack EG activity. Average of three technical repeats is shown.

Appendix Chapter 5

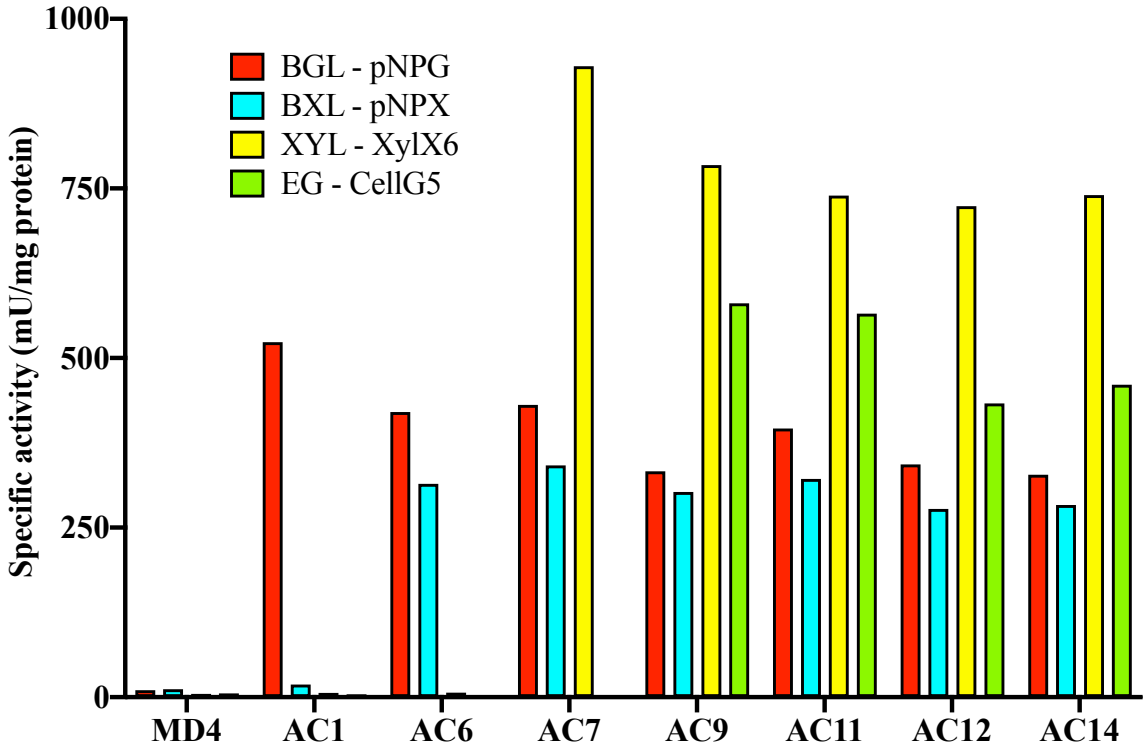


Figure A5.1. Overview of the specific activity of secreted lignocellulolytic enzymes in mU/mg secreted protein in cell-free supernatant of the different strains constructed from MD4 till AC14 (35°C, pH5.5). BGL, BXL, XYL and EG activity with pNPG, pNPX, XylX6 and CellG5 as substrates, respectively. Average of three technical repeats is shown.

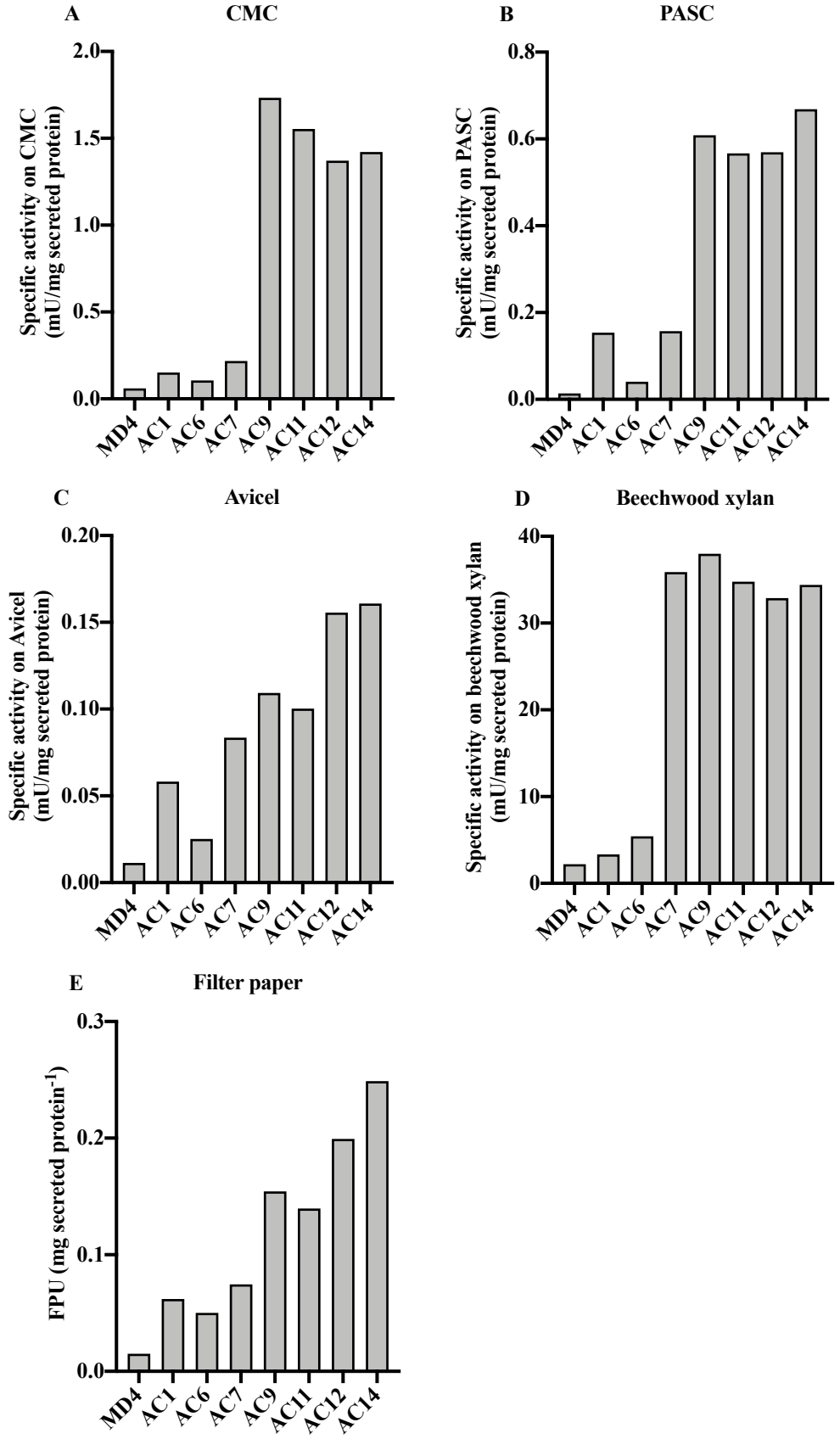


Figure A5.2. Combined secreted hydrolytic activity measured with multiple polymeric substrates in mU/mg secreted protein in cell-free supernatant of all constructed strains from MD4 till AC14 (A-D: 35°C, E: 50°C, A-E: pH 5.5). (A) CMC. (B) PASC. (C) Avicel. (D) Beechwood xylan. (E) Filter paper units. Average of three technical repeats is shown.

2. Tables

Appendix Chapter 2

Table A2.1. Codon-optimized nucleotide sequence of *Trbgl1* and *Sfbgl1*

Gene	Codon-optimized nucleotide sequence
Trbgl1	ATGAGATACCGTACTGCTGCCGCCTTAGCTTTAGCTACCGGTCCATTTGCCAGAGCCGACTCTCAC TCCACTTCTGGTGCTTCCGCTGAAGCTGTTGTTCCACCAGCCGGTACTCCATGGGGTACTGCTTAC GATAAGGCCAAAGCCGCCTTAGCTAAGTTAAACTTACAAGACAAGGTCGGTATCGTTTCTGGTGTT GGTTGGAATGGTGGTCCATGTGTCGGTAACACCTCCCCAGCCTCCAAGATTTCTTACCCATCTTTG TGTTTGCAAGACGGTCCATTGGGTGTTAGATATTCTACTGGTTCCACCGCTTTCACTCCAGGTGTTC AAGCTGCTTCTACCTGGGACGTCAATTTAATTAGAGAAAGAGGTCAATTTATTGGTGAAGAAGTC AAGGCTTCTGGTATTCATGTCATCTTGGGTCCAGTTGCTGGTCCATTGGGTAAGACTCCACAAGGT GGTAGAAATTGGGAAGGTTTCGGTGTCGATCCATACTTGACTGGTATTGCTATGGGTCAAACTATT AACGGTATCCAATCTGTCGGTGTTCAAGCCACTGCTAAGCACTACATTTTGAACGAACAAGAATTG AACAGAGAGACTATCTCTTCCAATCCAGACGACAGAACTTTGCACGAATTATACACCTGGCCATTC GCCGATGCTGTTCAAGCCAACGTCGCTTCTGTTATGTGTTCCTACAACAAGGTCAATACTACTTGG GCTTGTGAAGATCAATACACTTTGCAAACTGTTTTGAAGGACCAATTGGGTTTCCCAGGTTACGTC ATGACTGACTGGAATGCTCAACACACTACCGTCCAATCCGCCAACTCCGGTTTGGATATGTCCATG CCAGGTACTGATTTCAATGGTAACAACAGATTGTGGGGTCCAGCTTTGACCAACGCTGTTAACTCT AACCAAGTCCCAACCTCTAGAGTCGACGATATGGTCACTAGAATCTTGGCTGCCTGGTACTTAACT GGTCAAGATCAAGCTGGTTACCCATCTTTTAACATTTCTAGAAACGTCCAAGGTAACCACAAGACT AACGTTAGAGCCATCGCTCGTGATGGTATTGTTTTATTGAAGAACGATGCCAACATTTTGCCATTA AAGAAGCCAGCTTCTATTGCTGTTGTCGGTTCCGCTGCCATTATCGGTAACCATGCTCGTAACTCT CCTTCCTGTAACGATAAGGGTTGTGATGATGGTGCCTTGGGTATGGGTTGGGGTTCTGGTGCCGTC AATTACCCATACTTCGTCGCTCCATACGATGCTATTAACACCAGAGCCTCTTCCCAAGGTACCCAA GTCACTTTATCCAACACCGATAACACTTCTTCCGGTGCTTCTGCTGCTAGAGGTAAAGATGTTGCT ATCGTTTTCATCACCGCTGACTCCGGTGAAGGTTACATCACTGTTGAAGGTAACGCCGGTGATAGA AACAATTTGGACCCATGGCACAATGGTAACGCCTTAGTCCAAGCTGTCGCTGGTGCTAACTCTAAT GTTATCGTCGTTGTTCACTCCGTCGGTGCTATCATTTTGGAACAAATCTTAGCTTTGCCACAAGTTA AGGCCGTTGTCTGGGCTGGTTTGCCATCTCAAGAATCTGGTAATGCTTTGGTTGACGTTTTGTGGG GTGACGTTTCCCCATCCGGTAAGTTGGTTTACACCATTGCTAAGTCCCCAAATGACTACAACACTA GAATCGTTTCTGGTGGTTCCGATTCCTTTTCTGAAGGTTTGTTCATTGACTACAAACATTTCGACGA TGCTAACATTACCCCTAGATATGAATTTGGTTACGGTTTGTCTTACACCAAATTTAACTACTCTCGT TTGTCTGTTTTGTCCACCGCTAAATCTGGTCCAGCCACCGGTGCTGTCGTTCCTGGTGGTCCTTCTG ACTTGTTCCAAAACGTTGCCACCGTCACTGTTGACATCGCTAATTCTGGTCAAGTCACTGGTGCTG AAGTCGCTCAATTGTACATTACCTACCCATCTTCCGCTCCAAGAACTCCACCAAAACAATTGAGAG GTTTCGCCAAATTGAACTTAACTCCAGGTCAATCCGGTACCGCTACCTTTAACATTAGAAGAAGAG ACTTATCTTACTGGGACACTGCTTCCCAAAAGTGGGTCGTCCCATCTGGTTCTTTCGGTATCTCTGT CGGTGCTTCTTCCAGAGACATCAGATTGACCTCTACTTTGTCTGTTGCCTAA
Sfbgl1	ATGTTGATGATTGTTCAACTATTGGTTTTTGCTCTGGGCCTAGCAGTTGCCGTTCCTATCCAGAATT ATACGCAATCCCCATCTCAAAGGGACGAGAGCTCTCAATGGGTGAGCCCCCATTATTATCCGACA CCTCAAGGCGGAAGGCTACAGGACGTCTGGCAAGAAGCATATGCAAGAGCAAAGGCAATTGTCG GGCAAATGACCATAGTCGAAAAAGTGAATTTAACTACAGGTACCGGTTGGCAATTAGATCCGTGT GTTGGAAATACTGGTAGTGTTCCAAGGTTCGGCATTCCCAATTTATGTCTACAAGACGGCCCACTA GGCGTTAGGTTCGCAGATTTTGTTACTGGATATCCTAGCGGTCTTGCAACTGGCGCCACCTTTAAT AAAGACCTATTTCTGCAAAGGGGACAAGCCTTGGGACACGAATTTAACTCTAAAGGAGTGCACAT CGCCTTAGGACCAGCAGTTGGCCCCTTGGGAGTTAAAGCCCGTGGCGGTCGTAACTTTGAGGCCTT TGGTAGTGATCCCTATTTGCAGGGTACTGCCGCCGCCGCGACGATCAAGGGTTTGCAGGAGAATA ACGTTATGGCTTGCGTGAAACATTTTATTGGTAACGAGCAAGAAAAATATAGACAACCTGATGAT ATTAATCCGGCCACTAATCAAACCACGAAAGAAGCAATCAGTGCTAATATCCCTGATAGGGCCAT GCACGAATTATACTTATGGCCATTTGCTGATTCAGTCAGAGCTGGCGTTGGCTCAGTAATGTGCTC ATATAACAGGGTGAACAATACTTATGCTTGCGAAAACAGTTATATGATGAATCATTTGTTAAAGG AAGAGCTGGGCTTTCAAGGATTTGTTGTTTCCGATTGGGGTGCGCAATTAAGTGGTGTGTACTCAG CTATTTCTGGACTGGATATGTCAATGCCGGGCGAAGTATACGGTGAATGGAACACGGGTACAAGT TTTTGGGGTCAAAATTTAACTAAAGCCATCTATAACGAAACCGTCCCTATAGAGAGACTTGATGAC ATGGCAACTAGGATTTTGGCTGCTCTGTACGCAACTAACTCATTTCCCACCGAAGACCATTTGCCC AACTTTAGCTCTTGGACAACTAAGGAATATGGAAATAAGTACTATGCAGATAATACTACTGAAAT CGTTAAGGTAAATTACAATGTAGACCCCTCTAACGATTTTACAGAGGATACGGCACTAAAGGTTG CAGAAGAAAGTATTGTGTTGTTAAAGAATGAAAATAATACGCTTCCGATCAGCCCCGAGAAGGCT AAAAGATTGTTGTTAAGTGGCATCGCTGCTGGCCCTGATCCTATTGGCTATCAATGCGAGGACCAG

	TCTTGTACCAATGGGGCTCTTTTCCAGGGCTGGGGTTCTGGTAGCGTTGGTTCCCCAAAATATCAG GTTACGCCTTTTGAAGAGATTAGCTACTTGGCTCGTAAGAATAAAATGCAATTTGATTACATCAGG GAGAGCTACGATTTGGCTCAAGTCACTAAGGTGGCCTCAGATGCACATTTATCAATCGTTGTCGTT TCCGCCGCGTCAGGTGAAGGCTATATCACGGTTGATGGAAACCAGGGAGATAGAAAAAACCTTAC CTTATGGAATAATGGAGATAAATTGATTGAAACCGTCGCAGAAAACTGTGCTAATACAGTCGTAG TGGTGACATCAACGGGTCAGATAAATTTTGAAGGGTTTGCTGATCATCCAAACGTAACAGCTATTG TTTGGGCCGGTCCTTTAGGAGATAGAAGCGGAACTGCAATTGCGAATATATTATTTGGTAAGGCA AATCCAAGCGGACATTTGCCATTCACTATAGCCAAGACAGATGATGATTACATCCCTATTGAGAC GTACAGCCCATCATCCGGTGAACCAGAGGACAATCATCTGGTGGAGAATGATTTACTAGTTGATT ATAGATATTTTGAGGAAAAAAATATCGAACCTAGATATGCATTTGGTTATGGACTGTCTTACAATG AGTATGAAGTATCTAACGCGAAGGTCAGTGCAGCTAAAAAAGTAGATGAAGAGTTACCTGAACCA GCTACTTATCTTTCTGAATTCTCCTATCAAAACGCCAAGGATTCCAAGAACCCTAGTGATGCATTT GCTCCTGCTGATCTGAACAGAGTCAATGAATATCTATATCCTTATCTGGATAGTAATGTCACTTTG AAAGATGGCAATTACGAATACCCAGATGGTTACAGTACAGAACAACGTACTACTCCTATTCAACC TGGTGGCGGCTTAGGCGGCAATGACGCGTTGTGGGAGGTCGCTTATAAGGTCGAAGTTGATGTTC AAAACCTTGGTAACTCTACGGATAAGTTCGTACCCCAGCTGTATCTGAAACATCCGGAGGATGGT AAGTTCGAAACCCCAATACAATTACGTGGCTTCGAAAAGGTTGAGTTGTCACCAGGGGAAAAAAA GACCGTTGAGTTCGAACTGCTTAGAAGAGATTTGAGTGTATGGGATACTACTAGACAAAGTTGGA TTGTTGAATCTGGAACATATGAAGCCTTAATAGGTGTCGCTGTCAATGACATTAAAACTTCCGTGC TGTTTACGATCTAA

Appendix Chapter 3

Table A3.1. Codon-optimized nucleotide sequence of *Trxyn2* and *AnxlnD*

Gene	Codon-optimized nucleotide sequence
Trxyn2	ATGGTTGCTTTCTCCTCCTTGATCTGTGCTTTGACTTCCATTGCTTCTACTTTGGCTATGCCAACTG GTTTAGAACCAGAATCTTCTGTTAACGTCACTGAACGTGGTATGTACGACTTCGTCTTGGGTGCTC ATAATGACCATAGAAGAAGAGCCTCTATTAACTACGATCAAAACTATCAAACTGGTGGTCAAGTT TCTTACTCTCCATCCAACACTGGTTTTTCCGTTAACTGGAACACCCAAGACGACTTCGTTGTTGGT GTCGGTTGGACCACTGGTTCCTCCGCTCCAATTAACTTTGGTGGTTCCTTCTCCGTTAATTCTGGTA CTGGTTTATTGTCCGTTTATGGTTGGTCCACTAATCCATTGGTTGAATATTACATCATGGAAGATA ACCACAATTACCCAGCTCAAGGTACCGTCAAGGGTACTGTTACCTCCGACGGTGCTACTTACACT ATCTGGGAAAACACCAGAGTTAACGAACCATCTATCCAAGGTACTGCTACCTTTAACCAATACAT TTCTGTTAGAAACTCTCCTAGAACCTCTGGTACCGTCACTGTCCAAAACCACTTCAACGCTTGGGC CTCTTTAGGTTTGCATTTGGGTCAAATGAATTATCAAGTTGTTGCTGTCGAAGGTTGGGGTGGTTC TGGTTCCGCCTCTCAATCTGTTTCTAACTAA
AnxlnD	ATGGCTCACTCTATGTCCAGACCAGTTGCCGCTACCGCCGCTGCTTTATTAGCTTTAGCTTTGCCTC AAGCCTTGGCCCAAGCTAATACCTCTTACGTTGATTACAACGTCGAAGCCAACCCTGACTTGTATC CATTGTGTATCGAAACTATCCCATTGTCTTTCCCAGATTGTCAAAACGGTCCATTAAGATCTCACT TGATCTGTGACGAGTCTGCTACTCCTTACGATAGAGCCGCTTCCTTGATTTCCTTGTTCACTTTGGA TGAATTGATTGCTAACACCGGTAACACTGGTTTGGGTGTTTCCAGATTGGGTTTACCAGCTTACCA AGTCTGGTCTGAAGCCTTGCACGGTTTGGACCGTGCTAATTTTTCCGATTCTGGTTCTTACAATTG GGCTACCTCTTTCCCACAACCAATCTTGACTACCGCTGCTTTGAACAGAACCTTGATTCATCAAAT CGCTTCTATTATTTCCACCCAAGGTAGAGCTTTCAACAATGCTGGTCGTTACGGTTTAGACGTTTA CGCCCCAAACATTAACACCTTTAGACACCCTGTTTGGGGTAGAGGTCAAGAAACTCCAGGTGAAG ACGTTTCTTTGGCTGCTGTCTATGCTTACGAATACATCACTGGTATCCAAGGTCCAGATCCTGACT CTAACTTGAAATTGGCTGCCACTGCTAAGCATTATGCTGGTTACGACATTGAAAACTGGCACAAC CACTCCAGATTGGGTAACGACATGAATATTACTCAACAAGATTTATCTGAATACTACACCCCTCA ATTCCACGTTGCCGCTAGAGACGCCAAAGTTCACTCTGTCATGTGTGCTTACAACGCTGTCGATGG TGTTCCAGCTTGTGCTGACTCTTATTTTTTGCAAACTTTGTTGCGTGACACTTTCGGTTTTGTTGAC CACGGTTACGTTTCTTCTGATTGTGATGCTGCCTACAATATTTACAATCCACACGGTTATGCCTCTT CTCAAGCTGCTGCTGCTGCTGAAGCTATCTTGGCTGGTACCGACATCGACTGTGGTACTACCTACC AATGGCACTTGAACGAATCTATCACCGCTGGTGACTTGTCTCGTGATGATATTGAGAAGGGTGTT ATTAGATTGTACACCACTTTGGTTCAAGCTGGTTACTTCGACTCCAACACCACTAAAGCTAACAAC CCATACCGTGACTTGACCTGGTCTGACGTTTTGGAAACTGATGCCTGGAATATCTCTTACCAAGCT GCCACCCAAGGTATCGTCTTGTTAAAAAACTCCAACAACGTTTTGCCATTGACCGAAAAAGCTTA CCCACCATCTAACACCACTGTCGCTTTAATCGGTCCATGGGCTAACGCTACTACCCAATTGTTGGG TAATTACTATGGTAACGCCCCATACATGATTTCTCCAAGAGCTGCCTTCGAAGAAGCTGGTTACA AAGTCAACTTCGCTGAAGGTACTGGTATCTCCTCCACTTCTACCTCCGGTTTTGCTGCTGCTTTGTC TGCTGCTCGTTCCGCTGATGTCATCATTTATGCTGGTGGTATTGATAATACTTTGGAAGCCGAAGC TTTGGACAGAGAATCTATCGCTTGGCCAGGTAACCAATTAGACTTGATTCAAAAATTGGCCTCTTC CGCTGGTTCCAAACCATTGATTGTCTTGCAAATGGGTGGTGGTCAAGTCGATTCTTCTTCCTTAAA AAATAACACTAACGTTACTGCCTTGTTGTGGGGTGGTTATCCAGGTCAATCTGGTGGTTTCGCTTT GAGAGATATCATTACTGGTAAAAAGAATCCAGCCGGTAGATTAGTTACTACCCAATATCCAGCTT CCTACGCCGAAGAATTTCCAGCCACTGACATGAACTTGCGTCCAGAAGGTGACAACCCAGGTCAA ACTTACAAGTGGTACACTGGTGAGGCTGTTTATGAGTTTGGTCATGGTTTGTTTTATACTACTTTTG CCGAATCTTCCTCCAATACTACTACTAAGGAAGTCAAGTTAAATATTCAAGACATTTTGTCTCAAA CTCACGAAGAATTGGCTTCTATCACCCAATTGCCAGTCTTGAACTTCACTGCTAACATCAAGAACA CTGGTAAGTTGGAATCCGATTACACCGCTATGGTCTTCGCTAACACCTCCGACGCTGGTCCAGCTC CTTACCCAGTCAAATGGTTGGTTGGTTGGGATAGATTGGGTGACGTTAAGGTCGGTGAAACCAGA GAATTGAGAGTTCCAGTCGAAGTTGGTTCTTTCGCCAGAGTTAACGAAGATGGTGATTGGGTTTT ATTCCCAGGTACCTTCGAATTAGCTTTGAACTTGGAAAGAAAAGTCCGTGTTAAGGTTGTCTTGGA GGGTGAAGAAGAAGTTGTTTTGAAGTGGCCAGGTAAAGAGTAA

Table A3.2. Codon-optimized nucleotide sequence of three evaluated genes encoding xylanases

Gene	Codon-optimized nucleotide sequence
XYL1 ***Trxyn2***	ATGGTTGCTTTCTCCTCCTTGATCTGTGCTTTGACTTCCATTGCTTCTACTTTGGCTATGCCAACTG GTTTAGAACCAGAATCTTCTGTTAACGTCACTGAACGTGGTATGTACGACTTCGTCTTGGGTGCTC ATAATGACCATAGAAGAAGAGCCTCTATTAACTACGATCAAAACTATCAAACTGGTGGTCAAGTT TCTTACTCTCCATCCAACACTGGTTTTTCCGTTAACTGGAACACCCAAGACGACTTCGTTGTTGGT

	GTCGGTTGGACCACTGGTTCCTCCGCTCCAATTAACTTTGGTGGTTCCTTCTCCGTTAATTCTGGTA CTGGTTTATTGTCCGTTTATGGTTGGTCCACTAATCCATTGGTTGAATATTACATCATGGAAGATA ACCACAATTACCCAGCTCAAGGTACCGTCAAGGGTACTGTTACCTCCGACGGTGCTACTTACACT ATCTGGGAAAACACCAGAGTTAACGAACCATCTATCCAAGGTACTGCTACCTTTAACCAATACAT TTCTGTTAGAAACTCTCCTAGAACCTCTGGTACCGTCACTGTCCAAAACCACTTCAACGCTTGGGC CTCTTTAGGTTTGCATTTGGGTCAAATGAATTATCAAGTTGTTGCTGTCGAAGGTTGGGGTGGTTC TGGTTCCGCCTCTCAATCTGTTTCTAACTAA
XYL2 ***AnxlnB***	ATGCTAACTAAAAACTTGCTTTTATGTTTTGCCGCTGCCAAAGCAGCGTTAGCTGTTCCGCACGAT TCTGTTGCCCAGCGTAGCGATGCGCTGCACATGCTGTCTGAAAGGTCCACGCCTTCTTCAACCGGG GAGAACAACGGTTTTTACTATTCCTTCTGGACTGACGGAGGGGGAGATGTCACGTATACCAACGG GGATGCGGGGGCTTATACGGTTGAGTGGAGTAATGTCGGAAACTTCGTAGGCGGAAAAGGATGG AACCCGGGTAGCGCCCAAGACATCACTTATTCTGGAACTTTCACGCCATCTGGAAATGGCTATTT GTCAGTCTATGGCTGGACTACCGATCCCTTGATTGAGTATTACATCGTTGAGAGTTATGGGGACTA CAATCCGGGTTCAGGTGGAACCTATAAAGGGACGGTGACCAGTGACGGGTCCGTGTATGACATAT ACACTGCAACTCGTACAAACGCCGCCTCAATTCAAGGCACGGCTACCTTTACTCAATACTGGTCT GTCAGACAGAATAAGAGAGTAGGTGGAACCGTGACTACCAGCAACCATTTTAATGCGTGGGCAA AACTAGGAATGAACCTTGGAACTCACAATTATCAGATCGTAGCTACCGAAGGATACCAATCCAGC GGGAGTAGTTCAATTACGGTCCAGTAA
XYL3 ***TfxynD***	ATGACATTGGTGAAGAGCATCCTATTGGCACTGGCGGCCGGGCATGTTGCACAGGCGCAACTGAA TACGGCCGCAAAAGCAGCCGGTCTGCTGTATTTTGGAACCGCCGTTGACAACCCCGATCTATCTG ATAGCAAGTATCTGGTAAATCTTGAAACTGCCGATTTCGGACAAATAACCCCCGCTAATGCTATG AAGTGGCAACCAACGGAGCCCAGCCAAGGGTCATATACATTCACACAAGGAGATCAGATTGCCT CTCTTGCAAAGTCTAACAATGATTATTTAAGATGTCACAATTTAGTGTGGTACAATCAATTACCCA GCTACATCACGAGTGGTAGTTGGACTAACGCGACGCTAATCGCGGCCCTTAAAGAACACATTAAC GGTGTGGTAACCCATTATAAGGGACAGTGTTATGCATGGGACGTGGTTAATGAGGCACTGAATGA GGATGGGACCTATAGACAAAACGTTTTCTACCAACATATAGGGGAAGCTTACATACCTATAGCTT TCGCGGCTGCTGCAGCCGCCGATCCTAATGCAAAACTATACTACAACGACTACAACATAGAATAT GCCGGTGCCAAGGCTACCGGGGCTCAGGGCATAGTAAAGTTGATACAAGCCGCCGGAGGTAGAA TTGACGGGGTTGGTCTGCAATCTCACTTCATTGTAGGTCAAACGCCCTCCTTAGCGACTCAAAAAG CGAACATGGCTGCATTTACCGCATTGGGTGTCGATGTCGCTATAACGGAACTTGACATCAGGATG ACCCTGCCTGACACCTCCGCTTTGCAAACGCAGCAGTCTACAGATTACCAGACGACAACAACAGC GTGTGTGCAGACCAAAGGCTGTGTTGGAATAACTCTTTGGGACTACACAGACAAATATAGCTGGG TCCCTGGCACTTTTAGTGGACAAGGGGATGCTTGCCCCTGGGACTCTAACTACAACAAGAAACCC GCATACTATGGTATTTTGGCTGGTTTACAATCCGGTAGTGGCTCAAGTAGTTCTACGAGCTCCACT ACTTTAATAACTACTACTACGCCCACAGCTAGTAGCAGCACGACGTCTGCAACTACTACGTCAGC CACTTCCGGTGCGGCGCATTGGGGCCAGTGTGGTGGAATAGGTTGGTCTGGTCCAACTATATGCG TCTCCCCGTACACTTGCCAGGTACTGAACCCCTACTATTCTCAATGTTTATAA

Appendix Chapter 4

Table A4.1. Codon-optimized nucleotide sequence of six evaluated genes encoding endoglucanases

Gene	Codon-optimized nucleotide sequence
EG1 ***PbcelA***	ATGACCAAGACCTTTAAGAAATTTAGCATCGCAGGATTAGCTCTTTTATTCATGGCCACAGC AGCATTCGCGGGATGGTCTACCAAAGCGAGTGCTGCCGACATGCGTTCCTTGACTGCGGCC CAGATAACGGCGGAAATGGGTGCTGGTTGGAACCTAGGAAACCAACTTGAGGCTACAGTC AACGGTACTCCAAATGAGACCTCCTGGGGGAACCCTACCATAACCCCGGAATTGATTAAGA AAGTCAAAGCAGCGGGCTTTAAGACTATTCGTATTCCCGTCAGCTACCTAAACTACATAGG ATCCGCACCTAACTACACCGTGAATGCCAGTTGGCTGAACAGGATACAGCAGGTCGTAGAT TATGCATACAATGAGGGCTTATACGTTGTCATAAATATGCATGGCGACGGGTTCCATAGCA TACCTGGCTCATGGTTGCATGTGAACAGTAGCAATCAAAATGTCATCAGGGATAAGTACCA AAAAGTTTGGCAACAAGTCGCCACGAGGTTTAGTGCTTACAACGAGCGTCTAATTTTCGAG TCCATGAATGAGGTTTTTGATGGGAACTATAACAATCCTAACACTTCATACTATGGGAACCT AAATGCTTACAATCAAATATTCGTGGATACTGTTAGAAAAACGGGTGGCAATAATAACGCT CGTTGGTTATTAGTACCCGGGTGGAATACAAATATTGACTATACAGTGGGGAACTATGGCT TCGTGGTACCAACCGACAACTTCCGTAGTTCTGCTATCCCCAGTAGTCAGAAAAGGATAAT GATTTCCGCCCACTACTATTCACCTTGGGACTTCGCGGGTGAGGAGAACGGTAACATCACG CAATGGGGCGCAACTGCAACTAATCCCGCTAAGAGAAGTACTTGGGGACAGGAAGACTAT CTTGATAGTCAGTTTAAAAGTATGTACGACAAGTTCGTAACCCAAGGATACCCTGTAGTAA TGGGGGAGTTTGGTAGCATTGATAAATCCAGCTATGACTCCTCTAACAACAACTATAGAGC CGTTTATGCGAAGGCTGTCACGGCCACTGCAAAAAAATATAAACTTGTACCCGTATATTGG GACAACGGATTCAATGGCCAACACGGCTTCGCTTTATTCAACAGATTCAACAACACTGTCA CACAACAAAATATAATCAACGCAATTATGCAAGGTATGCAATAA
EG2 ***Bfend1***	ATGCATAAATCAAAGTGCATAAAGCGTGTATTTACGTTTCTACTAGCGCTGTTTGTGTTCGT GATGGCGATCCCTGCTACCAAGGTCTCAGCCGCCGGCGGAACTGACCGTTCCGCGACACAA GTAGTAAGTGACATGCGTGTAGGTTGGAATATTGGTAACAGTTTGGATTCCTTCGGACAATC CTATAACTTCCCTTATACGTCACTTAATGAAACCTATTGGGGAAATCCTGCTACCACAAAAG CGCTGATTGATGAGGTGGCCAAAGCCGGTTTCAATACTATCAGGATTCCCGTCAGTTGGGG ACAATATACCACCGGCTCTGATTACCAGATACCTGACTTTGTAATGAATAGGGTAAAAGAA GTCGTTGATTACTGTATTGTGAACGATATGTACGTGATCCTTAACAGTCATCACGATATCAA TTCCGACTACTGTTTTTATGTTCCAAACAATGCGAATAAAGATAGATCTGAGAAATATTTTA AGTCAATTTGGACCCAAATAGCTAAGGAGTTCAAAAATTATGACTATCACCTTGTATTTGAG ACCATGAATGAGCCTAGACTTGTAGGCCACGGAGAGGAGTGGTGGTTCCCCAGGAATAACC CAAGTAACGATATAAGAGAAGCTGTAGCCTGTATAAATGACTACAATCAGGTTGCTTTGGA CGCAATTAGAGCAACCGGCGGCAACAACGCGACGAGGTGTGTAATGGTCCCGGGATATGA CGCAAGCATCGAAGGCTGCATGACCGACGGCTTCAAGATGCCAAACGACACAGCTAGCGG ACGTCTAATCCTTTCAGTTCACGCTTACATTCCATACTACTTTGCTCTAGCTAGCGACACGTA CGTGACCAGATTTGATGATAATCTGAAATATGACATAGACAGCTTCTTCAATGATCTTAACA GCAAGTTCCTTTCTAGGAACATCCCAGTAGTCGTCGGTGAGACTAGCGCGACAAACCGTAA CAATACCGCTGAACGTGTCAAATGGGCCGATTACTACTGGGGGAGAGCGGCAAGATACTCC AATGTGGCGATGGTCCTTTGGGACAATAATATATATCAGAATAACTCTGCCGGATCTGATG GGGAATGCCATATGTATATTGACCGTAACTCATTACAGTGGAAAGATCCGGAAATCATTTC AACAATTATGAAACATGTTGATGGTACGCCAGCTACTATAAACGGGAAAGAAATCCCGTCC ACAGAACAACCCGATCCTACCCCTGTAGATCCTGACCCGACGCCCGTTGATCCAGATCCTA CCCCCGTGGACCCTGATCCTACCCCAGTTGATCCCGACCCGCAGCCAGTTGACCCAACGCCT GTTTCCGGCGCGCTAAAAGCCGAATACACGATTAACAACTGGGGCTCTGGATATCAAGTTC TGATTAAAGTGAAGAACGACTCTGCATCTAGGGTGGATGGGTGGACTTTAAAGATCAGCAA GTCCGAAGTCAAAATTGATTCTTCCTGGTGCGTCAACATCGCCGAAGAAGGGGGTTACTAT GTAATCACCCCTATGTCATGGAATTCAAGTCTTGAGCCTTCCGCAAGTGTTGACTTCGGTAT CCAGGGTTCAGGTTCTATAGGAACATCTGTCAACATCTCCGTGCAGTAG
EG3 ***Tregl1***	ATGGCACCTAGTGTTACTCTTCCCTTAACGACGGCTATTCTAGCAATCGCGAGGCTTGTTGC CGCCCAACAGCCTGGTACTTCAACACCGGAAGTTCATCCCAAGCTGACGACATATAAATGC ACGAAATCTGGCGGTTGCGTTGCTCAGGACACATCAGTCGTGTTGGATTGGAATTATAGAT GGATGCATGACGCCAATTACAACTCCTGTACCGTTAATGGAGGGGTTAATACTACTTTGTGC CCAGACGAAGCGACGTGCGGGAAGAACTGTTTCATTGAAGGTGTAGATTATGCTGCTTCCG GCGTAACGACATCTGGGAGCTCTCTGACTATGAATCAGTATATGCCCTCAAGCAGCGGAGG CTACAGTAGTGTATCTCCTAGATTGTACCTTTTGGATAGTGATGGCGAGTACGTGATGCTGA AGCTGAACGGGCAGGAACTGAGCTTTGACGTAGATCTTAGTGCGCTTCCATGCGGTGAAAA

	CGGGTCTTTATATTTATCACAGATGGACGAGAATGGTGGAGCGAACCAATACAACACGGCA GGAGCGAATTATGGGTCAGGGTACTGCGACGCGCAGTGTCCTGTGCAGACATGGAGAAAC GGTACACTAAATACCTCTCATCAGGGTTTCTGTTGCAATGAAATGGATATTTTAGAGGGTAA TTCACGTGCTAACGCACTTACGCCGCACAGTTGTACCGCGACTGCTTGTGACTCCGCCGGGT GCGGATTTAATCCATATGGATCAGGCTACAAAAGCTATTATGGTCCCGGCGACACAGTCGA CACTTCAAAAACATTTACAATCATTACTCAATTCAACACAGATAACGGAAGCCCTAGCGGA AACTTGGTTTCCATTACAAGGAAATACCAGCAGAATGGAGTTGACATTCCAAGCGCGCAAC CTGGAGGCGATACAATTAGCTCTTGTCCAAGTGCATCCGCTTACGGGGGTCTAGCTACGAT GGGCAAAGCACTATCCTCCGGTATGGTGTTAGTGTTCAGCATCTGGAATGACAATTCTCAAT ATATGAATTGGCTAGACTCTGGTAACGCGGGCCCCTGTTCTAGTACGGAAGGCAACCCCAG TAATATACTTGCGAACAACCCTAACACACATGTTGTTTTTTCCAACATTAGGTGGGGGGATA TCGGCAGCACCACAAACAGTACTGCCCCACCACCTCCTCCCGCTAGTAGTACTACCTTTAGC ACCACAAGGAGGAGTAGTACCACTAGTAGCAGTCCATCATGCACCCAAACTCATTGGGGGC AATGTGGAGGAATCGGCTACTCCGGTTGTAAAACATGCACATCAGGAACAACTTGCCAGTA TTCTAACGATTATTATTCACAGTGTTTATGA
EG4 ***Tregl2***	ATGAACAAATCAGTTGCACCTCTTCTGTTAGCTGCAAGCATTCTATATGGAGGCGCTGTGGC GCAACAAACGGTCTGGGGACAGTGCGGAGGTATCGGCTGGTCAGGTCCGACTAATTGTGCT CCGGGCTCTGCCTGTTCCACTCTAAATCCATATTATGCGCAATGCATACCAGGGGCAACGAC AATTACCACTTCAACGCGTCCACCATCTGGTCCAACCACGACGACAAGAGCAACATCAACA TCCTCTTCAACTCCTCCCACGAGTAGTGGGGTCCGTTTCGCCGGGGTTAATATTGCAGGCTT TGATTTTGGGTGCACGACGGATGGTACATGCGTAACTAGTAAGGTGTATCCGCCGTTGAAG AATTTCACAGGGTCAAATAATTATCCTGATGGGATCGGGCAAATGCAGCACTTTGTAAACG AGGACGGAATGACGATCTTCCGTCTGCCGGTTGGATGGCAGTACCTTGTGAATAACAACCT GGGTGGCAATCTGGACTCTACCTCCATTAGTAAGTATGATCAACTTGTTCAGGGGTGTTTAT CTTTGGGCGCTTACTGCATTGTCGACATTCACAATTATGCGAGGTGGAATGGGGGTATCATT GGACAGGGTGGTCCTACTAATGCTCAGTTTACTTCATTATGGTCTCAGTTGGCATCTAAGTA TGCGTCTCAATCAAGAGTCTGGTTCGGCATAATGAACGAACCGCATGACGTGAATATAAAC ACATGGGCCGCAACAGTACAAGAAGTGGTCACCGCTATCAGAAATGCGGGGGCGACTTCCC AATTTATCAGTTTACCCGGAAACGACTGGCAATCTGCCGGGGCGTTCATTAGTGATGGATC AGCCGCCGCATTGAGTCAGGTGACAAATCCCGATGGTAGCACCACGAATCTGATTTTCGAT GTCCATAAGTATTTAGACAGCGATAATTCCGGAACCCACGCAGAGTGCACAACCAACAACA TAGATGGAGCGTTTTCTCCCTTAGCGACGTGGCTAAGGCAGAACAATAGACAAGCCATTCT GACCGAGACGGGAGGAGGAAACGTGCAAAGTTGTATACAAGATATGTGCCAGCAAATTCA GTACTTAAACCAAAACTCAGACGTCTACTTAGGTTATGTCGGGTGGGGAGCCGGCAGTTTC GATTCCACCTATGTCCTGACAGAGACTCCAACCTCATCCGGAAATTCATGGACCGACACGT CCTTAGTTAGCTCCTGTTTAGCTAGAAAATAA
EG5 ***Pceg5A***	ATGTTAAAATATGCGTCTATAGCTCTGGCTTTGGCTACGCTGGGCGTCGCTCAGCAACAACA GTGGGGACAATGTGGTGGCATCGGCTGGACCGGGGCTACCACATGCGTTGCTGGCAGTGTT TGCAGCGTGTTGAACCCATATTACAGCCAGTGCATTCCAGGCGCCGCGACTGTGACGTCCTC CTCCGCTCCCTCAACGCCTACACCGCCAGCGGGAGCCTTGCCGAGACTAGGTGGAGTGAAT ACTGCGGGGTATGACTTCAGCGTGGCCACTGATGGCTCTTTCACAGGCACAGGCGTTAGTC CGCCCGTAAGCCAATTCTCTCACTTTTCCAGCCAAGGGGCTAATCTTTATAGGATCCCGTTC GCCTGGCAGCTAATGACACCTACACTTGGTGGAACAATTTCCCAATCTTTCCTTAGCCGTTA TGACCAAACTGTTCAGGCGGCTCTTAATAGCGGGCCAAATGTTTTTGTAATAATCGATCTAC ATAACTATGCCCGTTGGAACGGGGGCATAATCGCGCAAGGAGGTCCAACGGATGCCCAGTT TCAGTCAATTTGGACACAATTAGCTCAAAAGTATGGCTCAAACCAGCGTGTAATTTTTGGTA TTATGAACGAACCACACGACATTCCTAGCATCTCTACCTGGGTAAATTCTGTTCAGGGAGCA GTGAATGCGATCAGAGCGGCGGGTGCAACAAATTATCTACTGCTACCTGGATCATCCTGGT CTAGTGCACAGGCGTTTCCAACGGAAGCCGGACCCTTGCTGGTCAAAGTTACAGACCCACT TGGGGGGACCTCCAAGTTGATATTTGATGTGCACAAGTACTTAGATAGTGATAACTCTGGG ACGCACCCGGACTGCACTACCGACAATGTTCAGGTCTTACAGACTCTTGTGCAATTTTTGCA GGCCAATGGCAATAGGCAAGCCATTTTAAGCGAAACGGGAGGGGGTAATACCAGTAGCTG TGAGAGCCTGCTGGCCAACGAGTTGGCATACGTTAAGAGCGCTTATCCCACTCTAGCTGGC TTTTCCGTTTGGGCCGCAGGTGCGTTTGACACTACATATGTACTGACGGTCACGCCGAACGC AGACGGCTCCGACCAGCCATTATGGGTAGATGCTGTAAAACCTAATCTTCCGTAA
EG6 ***AocelB***	ATGATATGGACCTTAGCTCCCTTTGTCGCCTTGCTACCTCTTGTAACGGCTCAGCAAGTAGG AACGACCGCGGATGCCCATCCCAGACTTACCACCTACAAGTGTACTTCCCAGAACGGCTGT ACTCGTCAAAATACAAGCGTGGTATTGGATGCCGCCACCCATTTCATCCACAAAAAAGGGA CTCAAACCAGCTGTACGAACTCCAACGGACTAGATACCGCAATTTGTCCTGACAAGCAGAC CTGCGCGGACAACTGTGTGGTAGATGGAATCACTGATTACGCTAGTTATGGAGTGCAAACA

	AAAAATGACACACTAACGTTACAACAGTATTTACAGACCGGTAATGCCACGAAATCATTGA GCCCAAGAGTATATTTGCTTGCAGAGGACGGGGAGAATTACTCAATGCTGAAATTATTAAA CCAAGAGTTCACCTTTGATGTGGACGCGTCAACACTTGTCTGTGGTATGAATGGTGCATTGT ACCTAAGCGAGATGGAAGCTAGTGGCGGCAAGAGTAGCCTAAATCAAGCTGGTGCTAAGT ACGGAACCGGATATTGCGATGCTCAATGCTACACTACGCCTTGGATCAACGGAGAGGGGAA TACTGAATCAGTAGGGAGCTGCTGCCAGGAGATGGACATCTGGGAGGCGAATGCACGTGC AACGGGCCTTACGCCCCACCCTTGCAACACGACGGGTTTGTATGAGTGTTCAGGGTCCGGG TGTGGAGATTCTGGCGTTTGTGATAAAGCTGGATGTGGTTTTAACCCTTACGGATTGGGTGC AAAAGATTACTACGGGTACGGCTTAAAAGTAAACACCAACGAAACCTTTACCGTCGTCACC CAATTCCTAACAAACGATAACACCACAAGTGGGCAGCTAAGTGAAATCAGGAGACTATATA TACAAAACGGACAGGTCATTCAAAATGCAGCGGTGACCTCCGGTGGAAAGACAGTGGACT CAATTACGAAGGACTTCTGTTCAGGAGAGGGCTCCGCGTTTAACAGATTGGGTGGGCTTGA AGAAATGGGTCACGCTTTGGGTAGGGGGATGGTTTTAGCGTTGTCCATTTGGAACGATGCT GGGAGCTTCATGCAATGGCTTGACGGTGGTAGTGCCGGACCCTGCAACGCGACTGAGGGCA ATCCGGCCTTAATAGAAAAACTGTATCCGGATACACACGTAAAATTCAGTAAAATTAGATG GGGAGATATAGGCTCCACTTATAGGCACTGA

Table A4.2. Codon-optimized nucleotide sequence of six evaluated genes encoding cellobiohydrolase II

Gene	Codon-optimized nucleotide sequence
CBHII1 ***Trcbh2***	ATGATCGTTGGAATTCTAACCACGTTAGCGACGCTTGCCACTCTTGCCGCATCCGTGCCTTT GGAAGAAAGACAGGCTTGCTCCTCAGTGTGGGGCCAATGTGGAGGACAAAATTGGTCAGG GCCCACATGCTGTGCATCAGGGTCAACTTGTGTCTACTCAAATGACTACTATTCCCAGTGCT TACCCGGCGCAGCATCCTCCAGCTCATCCACTCGTGCCGCATCCACGACGAGTAGGGTGAG CCCAACTACAAGCAGAAGTAGCTCCGCGACTCCCCCGCCTGGCTCTACCACTACTCGTGTGC CGCCAGTGGGAAGTGGGACGGCGACGTACTCCGGTAACCCGTTTGTAGGTGTAACACCGTG GGCCAATGCATATTACGCAAGTGAAGTTAGTAGTCTAGCGATTCCATCATTGACTGGTGCG ATGGCAACTGCCGCAGCGGCAGTAGCAAAGGTACCCAGCTTTATGTGGTTAGACACGCTAG ATAAGACCCCACTGATGGAACAGACTTTAGCTGATATTAGAACAGCTAATAAGAATGGGGG GAATTACGCCGGTCAGTTCGTCGTGTATGACTTACCTGACAGGGATTGCGCTGCGCTTGCGA GTAACGGTGAATATAGTATCGCTGATGGCGGCGTGGCTAAGTATAAGAATTACATTGATAC CATACGTCAGATCGTGGTCGAGTATTCAGACATCAGAACGTTGCTAGTGATTGAGCCTGAC TCACTTGCCAATCTAGTCACGAATCTGGGCACACCGAAGTGTGCGAACGCACAGTCCGCTT ATCTGGAGTGCATCAACTATGCCGTGACCCAGTTGAACCTACCTAATGTTGCAATGTACCTT GATGCTGGCCACGCGGGCTGGTTAGGGTGGCCTGCAAATCAAGATCCAGCGGCACAATTGT TTGCAAATGTGTATAAAAATGCGAGTTCCCCAAGGGCGCTACGTGGGTTGGCTACGAACGT AGCAAATTATAATGGGTGGAATATCACTTCACCGCCATCCTACACTCAGGGGAATGCCGTC TACAACGAAAAACTGTATATACATGCAATTGGCCCCTTGCTAGCCAATCATGGATGGAGTA ACGCTTTCTTTATTACAGATCAGGGCAGATCTGGGAAACAACCGACCGGGCAGCAACAATG GGGTGATTGGTGCAATGTTATCGGGACGGGCTTTGGTATCAGGCCCTCTGCTAATACCGGTG ACTCCTTACTGGACTCCTTCGTATGGGTCAAGCCGGGCGGGGAGTGCGATGGAACGTCTGA TAGCAGTGCCCCCAGGTTTGACTCACATTGCGCACTGCCTGATGCATTGCAACCAGCGCCTC AAGCGGGCGCGTGGTTCCAAGCGTACTTCGTACAACTACTAACGAACGCGAATCCTTCCTT CCTATGA
CBHII2 ***Clcbh2b***	ATGGCTAAAAAACTATTCATCACGGCAGCGTTGGCAGCCGCAGTTTTAGCCGCGCCGGTGA TCGAAGAAAGGCAGAATTGCGGCGCGGTATGGACACAGTGTGGCGGCAATGGATGGCAAG GGCCGACGTGTTGTGCCAGCGGCTCAACCTGTGTTGCGCAAAACGAGTGGTATTCCCAATG TTTGCCAAACTCACAGGTAACTAGTTCAACTACACCTTCCAGTACGTCCACGTCACAAAGGT CTACCTCTACCTCATCAAGCACCACGCGTAGCGGGTCCTCTAGCAGCTCATCTACCACTCCC CCGCCGGTAAGCAGTCCTGTCACGTCAATCCCAGGAGGAGCTACCTCTACTGCGTCATACA GTGGGAATCCATTTAGCGGAGTGAGATTATTCGCCAACGACTATTACAGGAGTGAGGTGCA CAACCTAGCTATACCTTCTATGACTGGGACTTTGGCAGCAAAGGCTTCAGCAGTAGCCGAA GTCCCGTCTTTCCAGTGGCTAGACCGTAACGTCACAATAGATACTTTAATGGTACAGACGTT GAGTCAAGTTCGTGCACTTAATAAAGCAGGGGCTAACCCCCCATACGCAGCTCAGTTGGTA GTCTATGATCTGCCCGACCGTGACTGCGCGGCCGCCGCGTCTAACGGGGAATTTTCAATCGC AAACGGCGGCGCTGCAAACTATAGGAGCTATATCGACGCAATCAGGAAGCATATTATAGA GTATTCAGATATTAGGATTATCCTAGTGATTGAGCCCGATTCTATGGCCAACATGGTCACTA ACATGAACGTCGCTAAGTGTTCAAATGCGGCGTCTACGTACCATGAATTGACAGTATATGC GCTAAAGCAGCTAAATCTACCAAACGTAGCAATGTACTTAGACGCCGGCCATGCTGGCTGG

	TTAGGATGGCCTGCCAACATTCAGCCCGCCGCTGAGCTGTTTGCTGGGATTTACAACGATGC AGGAAAACCCGCAGCAGTACGTGGCCTTGCTACAAATGTGGCCAATTACAATGCGTGGTCA ATTGCATCTGCTCCCAGCTACACGAGCCCCAATCCTAATTATGACGAAAAACATTATATCGA AGCTTTCAGTCCGCTTTTGAACTCTGCGGGGTTTCCTGCTAGATTTATAGTCGACACAGGCA GAAACGGTAAACAGCCGACCGGCCAACAGCAATGGGGCGACTGGTGTAATGTCAAGGGGA CAGGCTTCGGCGTGCGTCCTACTGCGAACACGGGCCACGAATTGGTAGACGCCTTCGTGTG GGTGAAACCTGGCGGCGAGTCTGATGGCACGTCTGACACCTCAGCCGCGAGGTATGATTAT CATTGCGGACTTTCCGACGCACTGCAGCCCGCGCCTGAAGCAGGCCAGTGGTTTCAAGCTT ATTTTGAACAGCTGTTGACAAACGCAAACCCCCCTTTCTGA
CBHII3 ***Chcel7***	ATGCTTAGCAATGTCTTTCTTACAGCTGCTTTAGCTGCAGGTCTGGCGCAAGCTCTGCCACA AGCCACTCCTACGCCGACTGCGGCACCCAGCGGGAATCCTTTCGCAGGAAAAAACTTTTAC GCTAATCCATACTACAGTTCTGAGGTACATACTCTTGCAATGCCAAGTCTACCCGCCTCACT TAAGCCTGCTGCAACGGCGGTAGCCAAAGTTGGTAGTTTTGTTTGGATGGATACGATGGCT AAGGTGCCCCTTATGGACACTTATCTTGCGGATATAAAGGCGAAAAACGCGGCCGGGGCCA ATCTAATGGGGACTTTCGTTGTGTACGACTTGCCCGATAGAGACTGTGCGGCCTTGGCTAGT AATGGGGAACTGAAGATCGACGAGGGGGGTGTGGAAAAGTACAAAACCCAGTACATTGAC AAAATCGCTGCGATAATTAAGAAATATCCCGATGTCAAGATCAATCTTGCCATCGAACCGG ACTCTCTTGCAAATATGGTGACCAATATGGGTGTGCAGAAATGCTCAAGGGCCGCTCCCTA TTATAAGGAACTGACAGCTTACGCACTTAAAACGTTAAATTTTAATAATGTAGATATGTATA TGGACGGCGGCCATGCAGGATGGCTGGGTTGGGACGCCAACATTGGTCCCACCGCTAAGTT ATTTGCCGAAGTTTACAAGGCTGCAGGTAGCCCAAGAGGCGTCAGGGGTATAGTAACGAAC GTCTCCAACTACAATGCCCTTAGAGTGAGTTCTTGTCCTAGCATCACTCAAGGCAACAAGA ATTGTGACGAAGAGAGGTATATTAACGCTTTGGCGCCACTTCTGAAGAACGAAGGGTTCCC GGCTCATTTCATCGTAGACCAGGGAAGAAGCGGCAAAGTTCCCACAAACCAGCAAGAGTG GGGCGATTGGTGCAATGTTAGTGGGGCAGGATTCGGGACCCGTCCGACCACTAATACAGGC AATGCCTTAATTGATGCGATTGTTTGGGTAAAACCTGGTGGAGAAAGCGACGGGACCTCCG ATACCTCTGCTGCGCGTTACGATGCCCACTGCGGACGTAACTCTGCGTTTAAGCCAGCACCG GAAGCAGGGACGTGGTTCCAAGCATATTTTGAAATGCTGCTAAAGAACGCTAATCCTGCAT TGGCCTAA
CBHII4 ***Ctcbh2***	ATGGCCAAGCAGTTACTGCTTACTGCTGCACTTGCAGCGATATCATTGGCCGCGCCGTTACT AGAGGAGAGGCAAAGCTGCTCAAGTGTTTGGGGCCAATGTGGTGGAATCAATTACAATGG GCCAACTTGCTGCCAATCAGGTTCCGTTTGTGCCTACCTGAATGACTGGTATTCACAATGTA TACCTGGTCAGGCTCAACCCGGCACGACTTCCACTACAGCAAGGACAACGAGTACAAGTAC CACGAGTACGTCTAGCGTCAGACCTACCACCAGTAATACACCCGTAACTACAGCCCCGCCC ACCACCACGATACCTGGCGGCGCGTCAAGCACCGCTAGTTATAACGGGAATCCCTTTAGCG GCGTTCAACTATGGGCGAACACGTACTATTCCTCTGAAGTGCACACTCTGGCCATACCTTCC TTGTCTCCCGAACTTGCCGCTAAGGCGGCGAAGGTCGCGGAAGTACCATCTTTCCAGTGGCT GGATCGTAATGTAACCGTGGATACACTGTTCAGCGGGACACTAGCTGAGATAAGAGCAGCG AACCAGAGGGGGGCCAACCCGCCTTACGCCGGGATCTTCGTAGTGTATGATCTACCAGACA GGGATTGTGCAGCGGCCGCCAGTAATGGCGAGTGGTCAATCGCGAATAATGGCGCGAATA ACTTGCAAAGATACATCGATCGTATACGTGAGTTGTTGATACAATATAGCGATATCAGAAC TATTCTGGTCATTGAACCCGACAGTCTAGCCAACATGGTGACGAATATGAACGTACAGAAA TGCTCTAACGCTGCCTCCACCTATAAGGAGTTAACCGTGTATGCCTTAAAGCAACTAAATTT ACCGCATGTCGCGATGTATATGGACGCAGGTCACGCTGGCTGGTTGGGATGGCCTGCGAAT ATACAACCGGCAGCGGAGCTGTTTGCTCAAATATATAGGGATGCGGGGAGACCAGCTGCAG TGCGTGGTCTAGCCACCAATGTGGCCAACTACAACGCCTGGAGTATCGCTAGCCCACCGTC TTACACCTCACCTAACCCTAACTATGATGAAAAACATTATATTGAGGCTTTTGCCCCTTTAT TGAGGAATCAGGGGTTTGATGCAAAATTCATAGTTGATACTGGAAGAAACGGTAAACAACC CACCGGTCAGTTGGAGTGGGGTCACTGGTGCAATGTGAAGGGTACGGGATTCGGCGTCCGT CCTACTGCCAACACAGGTCATGAGCTTGTTGATGCCTTTGTATGGGTCAAACCTGGTGGTGA ATCAGATGGGACTTCAGACACTTCCGCCGCTAGGTACGATTATCATTGCGGACTGTCCGAC GCATTAACCCCCGCCCCCGAGGCCGGACAATGGTTTCAAGCCTACTTCGAGCAGTTGCTAA TTAATGCTAATCCCCCGTTCTGA
CBHII5 ***Abcel3***	ATGTTCAAGTTTGCAGCCCTTTTGGCACTTGCCTCATTGGTGCCTGGTTTTGTTCAGGCCCAA TCCCCAGTTTGGGGACAATGTGGAGGGAATGGGTGGACGGGTCCTACGACTTGTGCTTCAG GTTCCACATGCGTGAAGCAGAATGACTTCTACTCCCAATGCTTGCCCAACAACCAAGCTCCT CCCTCAACAACCACACAACCCGGTACTACACCACCGGCAACAACGACCTCTGGCGGGACGG GGCCGACAAGCGGCGCGGGGAATCCCTATACCGGGAAGACGGTCTGGTTGTCACCTTTTTA CGCAGATGAAGTCGCCCAGGCCGCTGCTGACATCTCAAATCCCAGCCTGGCAACTAAGGCA GCCTCCGTTGCCAAAATCCCCACGTTCGTATGGTTTGATACGGTCGCCAAAGTCCCGGATTT

	AGGGGGCTATCTGGCCGATGCAAGGTCTAAGAATCAGCTGGTCCAAATTGTAGTTTACGAT CTTCCTGATCGTGATTGCGCCGCTTTAGCCTCTAATGGCGAATTCAGCCTTGCCAACGACGG ATTAAACAAGTACAAGAACTATGTCGATCAGATTGCTGCGCAGATAAAACAGTTTCCCGAC GTTTCAGTAGTAGCTGTGATCGAGCCAGATAGCTTAGCTAACCTAGTAACCAATCTGAATGT TCAGAAGTGTGCGAATGCGCAGTCAGCCTATAAAGAAGGGGTTATCTATGCTGTCCAGAAG CTGAATGCGGTGGGGGTGACTATGTACATAGATGCCGGACATGCAGGCTGGCTGGGGTGGC CTGCAAACTTATCCCCAGCAGCGCAACTTTTTGCGCAAATATATAGGGACGCAGGGTCACC CAGGAACTTGCGTGGGATCGCAACCAATGTAGCAAACTTTAACGCCCTTAGGGCTAGTTCC CCTGACCCAATTACACAAGGGAATAGTAACTATGACGAGATACACTACATAGAAGCGCTAG CTCCAATGCTGTCTAATGCCGGGTTCCCGGCACATTTTATCGTCGACCAAGGGAGGTCAGG GGTTCAGAACATAAGGGACCAGTGGGGGGATTGGTGTAATGTCAAAGGCGCTGGTTTTGGC CAAAGACCGACTACTAACACGGGGTCATCCCTAATCGATGCCATCGTTTGGGTCAAGCCTG GCGGAGAATGCGATGGCACTTCAGATAACTCTAGCCCTCGTTTCGACTCTCACTGTAGTCTG TCTGATGCACACCAACCAGCCCCTGAGGCCGGCACATGGTTCCAGGCTTATTTCGAGACCCT AGTAGCTAATGCCAACCCCGCGCTGTAA
CBHII6 ***Mocel6A***	ATGGCTAGCAAACTTTTCCTTGCTGCCGCCTTGCTGCAGGGAGCACTAAGTTCACCACTAGC AGTGGAAGAAAGGCAGGCATGCGCTGCTCAGTGGGGCCAGTGTGGGGGACAAGACTATAC TGGACCAACCTGTTGCCAGTCTGGGAGTACGTGCGTCGTGTCAAACCAATGGTACTCTCAAT GCTTGCCCGGCTCCTCAAATCCAACCACTACCAGTAGAACATCTACCTCCAGCAGTAGTTCA ACGTCCAGAACTTCCTCCTCTACAAGTCGTCCACCCAGTTCTGTTCCCACGACGCCTACCAG CGTCCCCCCAACTATTACCACTACTCCCACGACTACACCGACTGGCGGAAGTGGTCCCGGT ACGACGGCATCCTTTACCGGAAATCCATTCGCGGGAGTTAACCTTTTCCCAAATAAATTTTA TTCTTCTGAGGTGCATACCTTGGCAATTCCAAGCCTAACAGGGTCCTTGGTAGCGAAGGCTT CTGCTGTTGCGCAGGTTCCTTCATTCCAATGGCTTGACATTGCCGCCAAAGTAGAGACGTTA ATGCCGGGAGCTCTGGCCGACGTTCGTGCAGCTAACGCGGCGGGTGGTAATTATGCGGCTC AGCTAGTGGTATATGACTTACCTGACCGTGATTGTGCCGCCGCCGCAAGTAACGGTGAATT CTCTATTGCTGATGGTGGCGTTGTAAAGTATAAGGCGTACATAGATGCGATCAGAAAGCAA TTATTAGCGTATAGCGACGTTAGGACGATTCTAGTTATAGAACCCGATAGTTTGGCTAATAT GGTGACTAACATGGGAGTTCCAAAGTGTGCTGGCGCTAAAGACGCCTATCTGGAGTGTACA ATCTACGCAGTAAAGCAATTGAACCTTCCACACGTTGCCATGTATTTGGATGGAGGCCACG CTGGGTGGTTGGGGTGGCCTGCGAACTTGCAGCCCGCGGCGGATTTGTTCGGGAAACTGTA CGCTGACGCTGGCAAGCCCAGTCAACTAAGAGGCATGGCAACAAACGTCGCTAACTACAAC GCATGGGACCTAACGACTGCGCCGTCATATACAACACCCAACCCGAATTTTGACGAAAAAA AGTACATATCTGCTTTCGCCCCTCTACTTGCCGCTAAAGGTTGGTCTGCACACTTCATAATC GACCAGGGACGTTCTGGAAAACAACCGACGGGCCAGAAAGAATGGGGCCACTGGTGTAAT CAACAAGGCGTAGGTTTCGGCAGGCGTCCCAGTGCGAACACCGGGAGTGAATTAGCTGATG CATTCGTGTGGATTAAGCCAGGGGGCGAGTGCGATGGAGTGTCAGACCCAACCGCCCCACG TTTTGACCATTTTTGCGGCACGGATTATGGAGCAATGTCTGATGCACCCCAAGCAGGACAGT GGTTCCAAAAGTATTTTGAGATGTTACTAACAAACGCGAATCCACCTCTTTAA

Table A4.3. Codon-optimized nucleotide sequence of six evaluated genes encoding cellobiohydrolase I

Gene	Codon-optimized nucleotide sequence
CBHI1 ***Aacbh1***	ATGGTAGATAGTTTCTCTATATATAAGACAGCCTTGCTTTTATCTATGCTGGCGACGTCTAA CGCACAGCAGGTCGGTACATACACCGCCGAGACACACCCCAGCTTGACCTGGCAGACATGC AGTGGCTCCGGAAGTTGCACTACTACGTCAGGCTCAGTGGTAATCGACGCCAATTGGCGTT GGGTCCATGAGGTCGGTGGTTATACTAACTGTTATTCAGGAAACACCTGGGATAGTTCAAT ATGCAGTACAGATACGACTTGTGCGTCTGAATGTGCACTTGAGGGAGCAACGTACGAGAGT ACCTATGGTGTTACCACGTCCGGCTCAAGTTTAAGGTTGAATTTTGTGACGACCGCATCACA AAAAAACATCGGGAGCCGTCTATACTTGCTAGCAGATGACAGTACATACGAAACATTTAAG CTATTCAACCGTGAGTTTACATTCGACGTAGATGTATCAAACTTACCTTGTGGTCTGAATGG GGCGTTGTATTTCGTATCCATGGACGCTGACGGTGGAGTAAGTAGGTTTCCGACTAACAAA GCTGGTGCCAAATACGGGACGGGATATTGTGATAGTCAGTGCCCCAGGGATTTGAAATTCA TCGACGGACAGGCTAATATCGAAGGCTGGGAACCATCTAGCACGGACGTCAACGCGGGCA CCGGTAACCATGGCTCCTGTTGCCCAGAAATGGACATCTGGGAAGCAAACAGCATTTCCTC TGCGTTCACTGCCCATCCTTGCGACAGTGTTCAACAAACCATGTGCACGGGAGATACGTGC GGGGGTACCTATAGTGATACTACCGACAGATACAGCGGAACCTGCGATCCCGATGGCTGCG ACTTTAATCCGTATCGTTTTGGGAACACAAATTTCTATGGCCCTGGGAAAACTGTTGACAAT TCAAAACCCTTCACCGTAGTGACACAATTTATTACCCACGATGGCACCGACACCGGGACGT

	TAACGGAAATCCGTAGGCTTTATGTGCAAAACGGTGTAGTAATAGGGAACGGACCGTCAAC ATACACCGCTGCAAGCGGAAACAGTATTACTGAATCATTCTGCAAAGCTGAGAAGACGCTT TTTGGCGACACGAACGTTTTTGAGACGCATGGCGGCCTATCCGCGATGGGTGATGCGTTGG GTGACGGCATGGTTCTGGTACTTAGCCTTTGGGACGATCATGCTGCGGACATGCTATGGTTA GACAGTGATTACCCGACCACATCTTGTGCTTCCAGTCCCGGCGTAGCGAGGGGGACGTGTC CTACCACTACAGGGAACGCTACCTACGTCGAGGCGAATTACCCAAATTCCTACGTCACTTAT AGTAACATAAAATTCGGAACGTTAAACTCCACATATTCCGGTACTTCCAGCGGCGGTTCCA GCTCAAGCTCAACCACCCTGACAACCAAGGCGTCCACGTCTACCACGAGTTCTAAAACAAC AACTACGACGTCCAAAACAAGTACGACATCCAGCTCTTCCACAAATGTGGCACAGTTGTAT GGACAGTGTGGCGGGCAGGGATGGACAGGGCCGACAACGTGTGCGTCAGGTACGTGTACT AAACAAAATGACTACTATTCACAGTGTTTGTGA
CBHI2 ***Trcbh1***	ATGTATCGTAAACTTGCTGTAATCTCTGCATTTCTGGCGACGGCCAGGGCTCAATCAGCCTG TACATTACAATCCGAGACCCATCCCCCGCTAACGTGGCAGAAATGTAGTAGTGGCGGAACT TGTACTCAGCAGACTGGTTCAGTCGTGATAGACGCAAACTGGAGATGGACCCACGCTACAA ACTCATCAACGAACTGTTATGACGGAAACACATGGAGTTCCACGCTATGCCCCGATAACGA AACCTGCGCTAAGAATTGCTGTTTGGATGGTGCGGCCTATGCCTCTACCTACGGTGTTACCA CATCTGGTAATAGCCTATCAATAGGCTTTGTGACCCAATCAGCGCAGAAAAATGTTGGTGC TCGTCTGTACCTAATGGCGAGCGACACCACTTACCAAGAGTTCACCCTTTTGGGGAACGAG TTTTCCTTCGATGTCGATGTGAGTCAACTACCCTGCGGGCTAAACGGAGCCCTTTACTTTGT TAGCATGGACGCAGATGGCGGAGTCTCTAAATATCCAACAAACACAGCAGGAGCTAAGTAT GGAACTGGTTACTGCGATTCACAATGTCCAAGGGATTTGAAGTTCATAAACGGGCAAGCCA ATGTCGAAGGATGGGAGCCTAGCTCTAACAATGCAAATACCGGGATCGGCGGTCACGGCTC ATGTTGTAGCGAGATGGATATCTGGGAGGCCAACAGCATTAGTGAGGCCCTTACTCCGCAT CCTTGCACAACGGTTGGGCAAGAGATCTGCGAGGGTGATGGATGTGGAGGAACGTACAGT GACAACAGATATGGCGGAACGTGTGACCCAGACGGTTGCGACTGGAATCCCTATAGGTTGG GCAATACATCTTTCTACGGCCCGGGCTCCTCATTCACATTAGACACCACCAAGAAACTAAC GGTCGTGACCCAGTTCGAAACGAGTGGGGCCATTAATAGGTACTATGTCCAAAACGGTGTG ACTTTTCAACAGCCAAACGCAGAATTGGGCAGCTATTCTGGTAATGAACTTAATGATGATT ACTGTACGGCGGAGGAGGCCGAATTTGGTGGCTCTTCTTTCAGTGACAAAGGGGGCCTGAC GCAGTTTAAAAAGGCAACTAGTGGCGGGATGGTGCTAGTGATGTCTCTTTGGGACGACTAC TATGCGAACATGCTTTGGTTAGATTCCACTTACCCCACTAATGAAACCTCCTCAACCCCTGG TGCCGTCAGAGGCAGTTGTTCCACCTCTTCTGGGGTTCCGGCCCAGGTAGAAAGCCAAAGC CCTAATGCGAAAGTCACATTCTCTAATATTAAATTTGGTCCGATAGGCTCCACAGGTAACCC GTCCGGAGGGAACCCTCCAGGCGGAAATAGAGGAACAACAACCACCAGGAGACCTGCAAC CACAACAGGGAGCAGTCCAGGTCCTACTCAATCTCACTATGGTCAGTGCGGGGGTATTGGT TACTCCGGACCAACGGTGTGCGCTAGTGGGACAACGTGCCAAGTCTTAAACCCGTATTATT CTCAATGTTTGTAA
CBHI3 ***Tecbh1***	ATGCTACGTAGAGCCTTACTGTTGTCTAGCTCTGCAATACTTGCCGTTAAGGCTCAACAAGC CGGGACAGCGACCGCGGAAAATCACCCGCCACTGACTTGGCAAGAATGCACGGCTCCAGG ATCCTGTACTACGCAGAATGGCGCCGTCGTGTTAGATGCGAATTGGAGATGGGTTCACGAT GTAAATGGATACACTAATTGTTACACTGGGAACACTTGGGATCCAACGTATTGCCCAGACG ACGAGACATGCGCCCAGAATTGCGCCTTGGACGGCGCAGATTACGAAGGGACATACGGAG TGACTTCCAGTGGTTCTTCTCTGAAACTTAACTTTGTCACTGGAAGTAATGTCGGTTCCAGA CTATATTTGTTACAAGACGACTCTACGTATCAGATTTTCAAGCTTTTAAACCGTGAGTTCTC ATTCGACGTTGATGTTTCTAACTTGCCATGTGGATTAAATGGAGCTCTATATTTCGTCGCTAT GGATGCCGATGGAGGCGTCAGTAAATATCCGAACAACAAGGCCGGTGCGAAATATGGCAC AGGTTACTGCGACAGTCAATGTCCCCGTGATCTTAAATTCATAGACGGTGAAGCCAATGTG GAGGGCTGGCAGCCCAGTTCCAACAACGCCAATACGGGCATCGGCGATCACGGAAGTTGTT GCGCTGAGATGGATGTTTGGGAAGCGAACTCCATATCCAACGCAGTCACTCCACACCCATG CGACACCCCTGGTCAGACAATGTGCTCAGGTGACGATTGTGGTGGCACTTACTCCAATGAC AGATACGCGGGTACCTGTGACCCTGATGGTTGCGACTTCAATCCATATAGGATGGGTAACA CATCATTCTACGGCCCTGGAAAGATCATAGACACCACCAAGCCCTTCACGGTTGTCACCCA GTTTCTTACTGATGATGGCACGGACACTGGCACCTTATCCGAAATTAAAAGATTTTATATCC AAAACAGCAACGTTATTCCGCAGCCTAACTCTGACATCTCCGGGGTCACAGGAAATAGTAT AACGACCGAGTTTTGTACTGCACAGAAGCAGGCGTTCGGCGATACGGACGACTTTTCACAG CATGGCGGACTAGCGAAGATGGGTGCCGCGATGCAACAGGGAATGGTGTTGGTAATGAGC CTATGGGACGATTATGCCGCTCAAATGCTATGGCTGGATTCTGACTATCCAACTGATGCGGA TCCAACTACCCCCGGGATTGCAAGAGGGACCTGCCCCACAGATAGCGGGGTCCCATCAGAC GTGGAATCTCAATCTCCGAACAGTTACGTAACTTACAGCAACATTAAATTTGGACCCATTAA CAGCACTTTCACGGCAAGTTAA

CBHI4 ***Hgcbh1***	ATGAGGACAGCCAAATTTGCGACCTTAGCCGCGCTGGTCGCGTCAGCGGCGGCTCAACAGG CCTGCAGCCTGACCACCGAACGTCACCCGAGTCTATCTTGGAACAAGTGTACCGCCGGAGG ACAGTGCCAAACAGTTCAAGCCTCTATTACACTTGACAGTAACTGGAGGTGGACTCATCAA GTTAGCGGCAGCACAAACTGTTACACGGGGAATAAATGGGATACGTCCATATGCACAGATG CAAAGTCCTGTGCGCAGAATTGTTGTGTGGACGGCGCAGATTATACTTCCACATATGGAAT AACGACTAATGGGGATTCCCTATCTCTGAAATTCGTAACTAAGGGACAACATTCTACAAAT GTAGGAAGTCGTACGTATCTGATGGACGGTGAAGACAAATACCAGACATTTGAACTATTAG GGAATGAATTCACTTTCGATGTCGACGTATCCAATATAGGGTGCGGACTAAACGGTGCACT TTATTTTGTGAGCATGGATGCAGATGGGGGACTTAGTAGATACCCTGGTAACAAGGCGGGG GCAAAATATGGCACCGGGTATTGTGATGCTCAGTGTCCAAGGGACATCAAGTTCATTAACG GGGAAGCGAATATCGAGGGCTGGACAGGATCCACCAATGACCCTAATGCAGGAGCAGGTA GATATGGGACGTGCTGTAGTGAAATGGACATATGGGAGGCAAACAATATGGCCACGGCTTT TACTCCCCACCCTTGCACGATAATTGGACAATCACGTTGCGAAGGCGACTCTTGCGGAGGT ACTTATAGCAACGAGAGATATGCGGGAGTTTGCGATCCTGACGGTTGCGACTTTAACAGTT ATAGACAGGGTAATAAAACCTTCTATGGAAAAGGGATGACGGTCGACACCACTAAGAAGA TAACCGTTGTTACGCAGTTTTTAAAAGACGCCAACGGGGACCTTGGGGAGATCAAAAGGTT TTATGTACAGGACGGCAAGATTATACCTAACAGTGAGTCTACGATCCCAGGAGTCGAAGGG AATTCCATTACGCAGGACTGGTGTGATCGTCAGAAGGTCGCATTTGGGGATATTGATGACTT TAACCGTAAGGGTGGGATGAAGCAAATGGGAAAAGCGCTTGCTGGGCCCATGGTCCTTGTT ATGTCTATATGGGACGATCACGCCAGTAACATGTTATGGCTTGACTCCACTTTCCCGGTTGA TGCTGCCGGCAAGCCGGGCGCAGAGAGGGGTGCTTGTCCGACTACCTCCGGGGTTCCTGCT GAAGTCGAGGCTGAAGCACCGAATTCAAATGTCGTATTCAGCAACATACGTTTCGGGCCGA TAGGGTCAACCGTTGCGGGCCTTCCAGGGGCTGGCAATGGGGGGAACAACGGAGGAAATC CCCCGCCTCCAACGACAACCACGTCATCAGCGCCTGCAACAACCACTACGGCTTCTGCAGG GCCTAAAGCAGGTCGTTGGCAGCAATGCGGCGGAATTGGCTTTACCGGCCCTACCCAGTGT GAGGAACCGTATATCTGTACTAAACTTAATGACTGGTATTCACAATGCCTGTAG
CBHI5 ***AncbhB***	ATGTCCTCATTTCAGATCTACAGGGCCGCTCTGCTTCTAAGTATTCTTGCAACTGCGAACGC ACAACAGGTGGGCACATATACTACTGAAACTCATCCGTCACTTACTTGGCAGACCTGTACG TCTGACGGAAGTTGCACAACTAATGATGGAGAAGTTGTAATAGACGCCAATTGGAGATGGG TACATTCAACGTCCAGTGCTACTAACTGTTACACAGGCAACGAATGGGATACTAGTATTTGT ACGGACGATGTGACTTGTGCGGCTAACTGTGCGCTAGACGGTGCCACATACGAAGCTACAT ATGGAGTTACCACTAGTGGGTCAGAGTTAAGGCTGAATTTTGTAACCCAAGGTTCATCCAA AAACATAGGTAGCCGTCTGTATCTAATGAGTGACGACAGTAACTATGAGTTATTCAAATTA CTAGGACAAGAGTTCACATTTGATGTTGACGTCTCTAACTTGCCTTGTGGTCTTAATGGGGC ACTGTATTTCGTAGCAATGGATGCAGACGGGGGAACCTCAGAGTACTCTGGAAACAAGGCC GGTGCTAAGTATGGCACTGGATACTGTGACAGTCAATGCCCGCGTGATCTTAAATTCATCA ACGGTGAAGCTAACTGTGATGGATGGGAGCCCAGCAGTAATAATGTAAACACTGGGGTTGG TGACCACGGTTCATGTTGTGCCGAAATGGACGTCTGGGAGGCGAACAGTATATCCAATGCG TTTACTGCACATCCGTGCGATTCAGTTTCACAAACTATGTGCGATGGTGATAGCTGCGGTGG GACATATTCAGCTTCCGGGGACAGGTACTCAGGCACATGTGATCCCGATGGGTGTGATTAC AATCCGTATAGATTAGGGAATACCGACTTTTACGGCCCAGGTCTTACCGTTGACACTAACA GCCCCTTTACTGTGGTAACGCAGTTTATCACAGATGACGGAACCTCCTCTGGAACGCTTACG GAGATTAAAAGGCTGTATGTCCAAAACGGAGAAGTTATAGCGAACGGGGCTTCTACCTACT CCTCAGTGAATGGATCTAGCATAACTTCCGCCTTCTGCGAGAGCGAGAAGACACTGTTTGG AGATGAAAACGTCTTTGATAAACACGGCGGTTTAGAAGGGATGGGCGAAGCAATGGCGAA AGGGATGGTCCTAGTTCTTTCCCTGTGGGATGACTATGCTGCAGATATGTTATGGTTGGACT CAGACTATCCTGTAAACAGCAGTGCATCAACACCAGGTGTTGCCCGTGGTACGTGTTCCAC CGATAGCGGCGTCCCAGCAACAGTTGAGGCGGAGTCTCCGAATGCATACGTGACTTACAGC AACATAAAGTTCGGCCCAATTGGGTCCACGTATAGTAGTGGTAGCTCCAGTGGGTCCGGTT CCTCCAGTTCATCCAGCTCTACTACTACTAAAGCGACAAGTACGACGTTAAAGACGACGTC AACAACCAGCAGTGGTTCAAGTTCTACGTCTGCGGCCCAAGCATATGGGCAATGTGGCGGT CAGGGGTGGACGGGACCAACTACATGTGTTAGCGGGTACACGTGTACGTACGAGAATGCCT ATTACTCTCAGTGTTTATAA
CBHI6 ***PfxynA***	ATGAGCGCACTTAACAGCTTTAATATGTACAAAAGTGCTCTTATCCTAGGCAGCCTGTTGGC CACTGCCGGTGCTCAACAGATCGGTACATATACTGCGGAGACCCATCCCTCTCTAAGCTGGT CAACGTGCAAATCCGGCGGCTCCTGCACGACTAACTCAGGCGCGATAACTCTAGACGCTAA TTGGAGATGGGTCCACGGTGTTAACACGTCAACAAACTGTTATACTGGTAACACCTGGAAT TCCGCAATATGTGATACCGATGCCTCCTGTGCCCAGGACTGCGCACTGGACGGGGCGGATT ATAGTGGGACCTACGGCATTACTACCTCAGGAAACTCTTTGCGTCTAAACTTCGTGACGGGC TCCAATGTCGGGTCCAGAACCTATTTGATGGCTGACAATACGCACTACCAGATATTCGACTT

	ATTGAATCAAGAGTTCACCTTCACGGTCGATGTGAGCCATCTTCCCTGTGGCTTAAACGGAG CTCTATATTTTGTTACGATGGACGCAGACGGTGGTGTCTCAAAGTATCCAAATAACAAGGC CGGTGCGCAGTATGGTGTAGGTTATTGTGATTCTCAATGTCCTAGGGACCTGAAGTTTATTG CAGGTCAGGCTAACGTAGAAGGATGGACACCCAGTGCTAACAACGCCAATACAGGTATTG GTAACCACGGAGCTTGCTGTGCTGAATTGGATATATGGGAAGCGAATTCAATCTCTGAAGC ACTGACGCCACATCCTTGTGATACACCGGGACTATCCGTGTGTACTACAGATGCTTGCGGA GGAACTTATTCTAGCGATAGGTACGCAGGAACGTGTGACCCAGATGGGTGCGACTTCAACC CCTACAGACTTGGCGTTACCGATTTCTACGGTTCCGGAAAAACGGTAGATACGACCAAACC CTTCACCGTCGTAACACAGTTCGTAACAAATGATGGCACTAGCACCGGGAGTTTAAGCGAG ATAAGAAGATATTATGTGCAGAATGGGGTCGTTATCCCTCAACCGTCTTCTAAAATTTCTGG CATATCAGGAAACGTGATTAATTCAGACTACTGCGCGGCAGAAATTTCCACGTTTGGCGGA ACGGCGAGCTTTAATAAACATGGGGGCCTGACCAACATGGCTGCGGGGATGGAGGCAGGG ATGGTATTGGTAATGTCTTTGTGGGACGACTATGCCGTAAACATGCTTTGGTTAGATAGCAC GTATCCGACGAATGCCACTGGTACTCCTGGGGCGGCTCGTGGGACTTGCGCAACAACATCT GGTGATCCAAAGACTGTTGAGTCCCAATCCGGAAGCTCCTACGTCACGTTTTCAGATATCCG TGTAGGACCGTTTAATTCTACGTTTAGCGGTGGGAGTAGCACCGGGGGTTCTACTACAACA ACGGCGAGTAGGACTACGACGACCTCAGCCTCTTCAACATCAACCAGCAGCACAAGTACTG GGACGGGAGTAGCAGGTCATTGGGGACAGTGTGGTGGGCAGGGTTGGACCGGCCCAACCA CTTGTGTTTCAGGGACCACATGCACTGTTGTTAACCCTTATTATAGCCAGTGTTTGTAA

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Table A5.1. Codon-optimized nucleotide sequence of evaluated genes of which three encoding acetylxylan esterases and four encoding bifunctional acetylxylan esterase/xylanases

Gene	Codon-optimized nucleotide sequence
AXE1 ***TpaxeA***	ATGAAGTCTCTAAGTTTTAGTTTTCTTGTCACGCTATTTCTTTACTTAACTCTTTCTTCTGC CAGAACTTTAGGAAAAGACGTAAATAAGCGTGTAACGGCGGGGAGTTTGCAACAGGTA ACAGGTTTTGGCGACAATGCTTCTGGGACGCTGATGTACATTTATGTGCCCAAGAATCTA GCGACGAATCCGGGAATTGTAGTCGCGATTCATTACTGCACCGGCACCGCTCAGGCCTA TTATACAGGGTCACCGTACGCGCAGCTTGCGGAACAATATGGTTTCATAGTGATTTATCC TCAGAGTCCTTACAGCGGTACGTGTTGGGATGTGTCTTCACAGGCAGCACTGACGCATA ATGGAGGGGGCGACTCAAACTCCATCGCGAATATGGTGACCTGGACCATTTCTCAATAC AACGCTAACACAGCAAAGGTGTTTGTCACGGGAAGTTCTTCTGGCGCGATGATGACAAA TGTTATGGCTGCAACATACCCAGAGTTATTTGCTGCTGCCACAGTATATAGCGGCGTCGG GGCAGGCTGTTTCTATTCCAGTAGTAACCAAGCGGATGCGTGGAATAGCTCTTGTGCGA CCGGGTCAGTGATTAGTACACCAGCGGTATGGGGTGGTATCGCAAAAAATATGTATTCT GGGTATTCCGGCTCTAGGCCGAGGATGCAGATCTATCACGGCAGTGCTGACACGACTTT GTACCCTCAGAATTACTATGAGACATGTAAACAGTGGGCGGGGGTTTTCGGATACAACT ACGACAGCCCGCAGAGCACGTTAGCCAATACACCAGACGCTAATTATCAGACTACAAAC TGGGGCCCCAATTTACAGGGTATATACGCCACTGGGGTTGGTCATACTGTTCCCATTCAT GGCGCGAAAGATATGGAATGGTTTGGGTTCTCCGGAAGCGGGAGTTCTTCAACGACAAC GGCGAGTGCAACGAAGACAAGCACCACCAGCACTACGTCTACTAAAACAACCTCCAGC ACAAGTAGTACCACTACTAGTTCTACAGGAGTCGCGGCTCATTGGGGCCAATGTGGAGG TAGTGGTTGGACCGGGCCCACCGTCTGCGAGTCAGGCTACACGTGTACATATTCTAATG CGTGGTATTCCCAATGTCTGTAG
AXE2 ***PraxeA1***	ATGAACCGTAAATTGTTCATGACGGGGTTGCTTATGCTAGCGATGACTATGCAGGCTCA GACGGCCAAAAAGTTTACACTAAATTTATCAGATGACGGCAAGGCTCAGATGGTATGCT TTCTGCCAGAGAACCCCTCCGGTAGAGCGATCGTTGGCGTACCTGGGGGAGGCTATTCT ATGCTATCAAACACCCACGAGGGTTACCAGGCAAGCGATTGGCTGAACAAGCAGGGCA TCGCATATTTCGTCGTAAATTACAGATTGCCGCACGGGGACAGGACTATCCCCGTAGGT GACGTGGAGCAGGGTTTTCGTATCGTCCGTGACTCTGCAAAAGTCTGGAATATCAACCC GAATGATGTCGGGATCATGGGATTTAGTGCTGGTGGTCATCTGAGCTCTGTGATATCCAC TATGTCACCTTACGAAGTGAGACCGAATTTTTCCATCCTTTTCTACCCTGTCATAAGTAT GGACGAGAGAGTTTCCCACAAGTGGTCTTGTATAAACTTTCTGGGAAAAGAGGGGTACA AGGATCCCAAGCTTATAGGACAATACTCTACACAGAATGCGGTAAGAAGCCATTTAACG CCCCCGGCCTGCATTATAAGTGCAAACGATGACAGGCTAGTTCCGGTAGTCACTAATGG CATTCAATATTACAGTGCCATGCGTAACGCCGGAAACGAGTGCAGCTTGTTCATCTATCC TAGCGGGGACCATGGATTCGGTTTTGGTACTTGGTTCAAGTACCACGATCAGTTGTTGCA GGATCTTGGCAATTGGCTTAAGTCCATTCCCGCTCCCAAGGAAGACGCAATACGTGTTG CGTGTATTGGGAACAGCATAACAGATGGATTCGGTATAGACATGAGAGCTAAATACGGA TACCCGGCCCAACTACAAGGTATTCTAGGGGATGGGTATTGGGTTAAAAACTTCGGTGT TTCCGCACGTACAATGCTAAACAAGGGGGACTTCCCATATATGAATGAGATGGCTTGGA AGGACGCACTGGCTTTCAAGCCAGATGTAGTGGTCATTAAATTAGGCACAAATGATTCC AAGCCTGAAAATTGGCAATATGGCTCCGAGTTTAGGCAGGATCTAGAACAAATGATCAA AGCGTTAAGACCTGATCTTGCCCAGCCCGCCAAAAAGGGAAAGAAAAAAGCCAAAGCA GCGGCGCAACCCGCGGGCCCTAAAATCCTGCTGTGCACGCCTATTCCCGCCTTTAAGCC AAGTTGGAATATTAACGACAAAGTTATAACGGATGAGATCATTCCGATACAGCAGGAGG TGGCCAAACAGTACGGGTTGCAAATAATTGACCTGCATGCCTTGATGTTGAATGATGGG GATAAAGTCGTGGACGACGGGATACACCCCAACGAGAAAGGCGCTAAAAAAATGGCAG AAATAATAGCGGCGGCCATTAAGTAA
AXE3 ***Traxe1***	ATGCCATCTGTCAAAGAAACTCTAACATTATTGTTGAGCCAGGCCTTCCTAGCCACTGGC AGTCCAGTAGACGGAGAGACAGTTGTAAAAAGGCAATGTCCAGCCATTCACGTATTCGG AGCTCGTGAGACTACAGTAAGCCAAGGTTATGGTAGCTCTGCTACAGTAGTCAATCTGG TGATCCAAGCCCACCCCGGGACGACGTCAGAAGCAATCGTGTACCCTGCGTGCGGCGGA CAGGCCTCATGCGGTGGAATCTCTTATGCCAACAGTGTCGTGAACGGCACTAATGCCGC CGCCGCGGCTATCAACAATTTTCACAATAGTTGTCCAGACACCCAATTAGTCTTGGTAGG CTACTCCCAGGGTGCTCAGATCTTCGATAATGCACTGTGCGGTGGGGGGGATCCTGGAG AAGGGATCACGAATACTGCGGTGCCGCTTACCGCTGGGGCAGTGAGTGCTGTCAAAGCC GCGATTTTCATGGGTGATCCCAGAAACATCCATGGTTTGCCCTATAACGTTGGGACTTGC

	ACAACGCAAGGTTTCGATGCAAGGCCAGCAGGCTTCGTCTGCCCTAGCGCTTCTAAGAT TAAGTCCTATTGTGATGCCGCTGATCCATACTGCTGTACTGGTAATGATCCAAATGTTCA TCAGGGTTATGGACAAGAGTACGGCCAACAGGCCTTGGCTTTCATAAACAGTCAACTTT CTTCTGGTGGATCTCAGCCTCCTGGTGGAGGACCCACGAGCACCTCAAGGCCTACCAGC ACGCGTACAGGTTCCTCACCCGGACCTACCCAAACACATTGGGGTCAGTGCGGGGGTCA GGGGTGGACAGGTCCGACGCAGTGTGAAAGTGGTACAACTTGTCAGGTCATATCTCAGT GGTATAGCCAGTGCCTGTAG
AXEXYL1 ***NpxynS20E***	ATGAGGCTTGGAGTCGCTCTATCCACCATCGCCGTCCTGCTTACTGCTACGTCAGCAAGG AACTTAGACAAAAGGCAATGGGGATGGCCGAACTTCGGTGGCGGTAATGGGGGGAACG GTGGCAATGGGGGAAAGACTATAAATGACTACAAGCGTGAGCAGGGAGCGGGGAGAGA CATTCACGTTTACGCCCCGAGTAATTTAGCACCAAATAGCCCCTTGTTGCTTTCATTACA CGGAATGGATCAAGACCCTAACTATCAACAATCCAATACCCATTGGGAAACCCTAGCTG ATTCTGAAGGTTTCGTGGTAGTATATCCTCGTGGCGGAACCGGCATGTCAACCTGGGAC ATTCAGGGAACGAAAGACACGCAGTGGGTCAGCCAGATTATTGACCAGATGAAGAAGG AGTATAACATCGATACCAAGAGAGTATATCTTTCTGGGTTCTCCATGGGAGGGATGTTTA CCTACCATGCGATGTCCCAAATAGCTAACAAGATTGCCGCGTTCGCGCCTTGTTCTGGTC CTAACGTCTTTGGTGCTTCTAAGGCCCAAAGGCCGGTGCCCATCTTCCACGTTCACGGCA CAAATGACGACGTTCTAAATTACCAACAGGTAGAAGGCTTTCTAAAGAATTACCGTGAC CAATTCCACTGTCCCTCTCAAGCTGACACTAAGACAAATTACCCGAATAGAGAGAATCC CAACGCTACCTTGTACACTTGGGGGCCATGTGATAAAGGAGTCTATATCAAACACTTAA AATTGCAAGGGAGAGGCCATAGCCCATCATCTGCCGACATACAAGACATTTGGGACTTT GTCAGCCAGTGGACCGTAGACGGTCCGGTCTCTGCTTCTGGAAATGGAGGAGGAAACAC AACCCCAACTAATCCGTCCACCGGCGGTAACGGTAACGGAAATGGGGGTGGCAATACTA CACCGACGAATCCCTCAACGGGCGGAAATGGCAATGGGAATGGAGGGTCTACCGATAA ATGTTCTTCAAATATAACAAAACAGGGGTACAAGTGCTGTGCTTCTAATTGCGAAGTAG TTTACACGGATTCTGATGGAGATTGGGGTGTAGAGAACGACCAATGGTGTGGCTGCGGC AATCGTGTTACCGTGGGAAGCGGTACCTGTAGCGCTAAGATTCTGCAACAAGGGTATAA GTGTTGTCCAAGTGGATGCATAATTTATTATACGGACGAGGACGGAACATGGGGAGTCA ACGGCGAAGAGTGGTGTGGATGCGGCAGTGGGAGTTCATCAACAGGCGGTGGCAACGA TGCGCCGAGTTCTGGCTCCGGTTATCAAGGCGCAAATGGGACCAATTTTTGCAACAATG CAAAACATAGCGGAGAATCAGTCACCGTCACTTCAAACAAAGTGGGAGACATCAATGG TATTGGTTACGAACTGTGGGCAGACAGTGGGAATAACTCAGCTACTTTTTACGACGATG GGTCTTTTTCCTGCTCATTTCAGAGAGCTAAAGACTATCTGTGCAGATCAGGTCTTTCTTT TGACTCCACCAAGACTCACAAGCAGATTGGCCACATCTACGCTGAGTTTAAGCTGGTTA AGCAGAATATCCAAAATGTTGACTACTCTTACGTTGGTATCTATGGTTGGACCCGTAACC CGTTAGTGGAATTCTACGTGGTAGACAACTGGCTATCACAGTGGAGGCCTGGCGATTGG GTTGGGAATAAGAAACATGGCGACTTCACGATTGGCGGGGCTCAATATACGGTCTATGA GAATACACGTTACGGGCCTAGTATTGATGGCGACACGAATTTTAAGCAGTATTTTAGTAT TAGACAGCAACCGAGGGACTGCGGAACCATTGACATTACAGCCCATTTCGAACAATGGG AGAAGTTAGGAATGACGATGGGAAAAATGCATGAAGCCAAGGTTCTAGGAGAAGCGGG ATCAAACAATGGCGGAACTAGCGGCACCGCAGACTTCCCCTTCGCGAAAGTGTACGTTA AGAATTAA
AXEXYL2 ***CcxynA***	ATGAAACAAAAAATGAGGATTGTGTTTAGTATGTTAATGTGTATCACGATACTGCTGGC TTCTTCTGCAGTCGGAGTTCTAGCGGCTACCAAGACCATAACCAGCAACGAGACGGGCA ACTTTGAGGGATACGATTACGAATATTGGAAAGACAATGGTACCGGCACGATGACCCTG ACAGGGGGTGGGACCTTTTCTTGCAGTTGGAGCAACATCAATAATATCTTATTCAGGAC GGGGAAAAAATTAGGCAGCACAAAGACTTATAAAGACTATGGTAAAATATCAATCAAC TACGATTGCAATTACCAGCCCAATGGGAACAGCTATATGGCAGTGTACGGATGGACGGA AGACCCCCTAGTAGAGTATTATATAATCGATAGTTACGGAACTTGGAATCCAGGCTCAA ACAGCACGCTAAAGGGTACGACGACTGTCGATGGGCGTACATACCAGATCTATCAAAAC TCCAGAACGGGACCCTCTATTAAGGGGAATAATACGACCTTTCAGCAATACTGGAGTAT TTGCACAAGCAAAAGGACTAGCGGTACGATAACTGTCAGTGACCACTTCAAAGCCTGGG AGAACCTGGGTATGAAAATGGGAAAAATGTATGAGGTATCAATGGTTGTTGAAGGCTAC CAATCTTCAGGTAAAGCAGACATGACGAAAATGGATCTAATTATGGGAGACACACCTCC TGTTATCGTCGACCAACCCACACCAGAGAAGCTGTTGGGAGACGTCGACAACAACGGGA CAGTTAATGCCCTGGACTTGGCAATATATAAGAAATTCTTGTTAGGGATGATCCCCAGTC TTCCGGCGACCGGTGACGTCGACCAGAACGGACGTATGAACGCGATTGACTTTGCCATG ATTAAACAGCACCTGCTAGGTATTATTAAGCTTGGGAAATTACCAACAACAAGTGAAGA TCCCAATAAGAAATTGGTGGCATTGACTTTTGATGATGGTCCGTCATCACAGACTGGCCT TGTCTTGGACAAGTTAAAGAAGTACAATGCAAAGGCTACGTTTATGGTTATCGGCAACA

	AAATTTCTAGCGCGGACGCCATCATGAAACGTATAGTTTCAGAGGGGCACGAGATAGGT AATCATGGGTGGAGCTATGATTCAATGGCGAATATGTCAGCCTCCCAGATTACACAGGA AATAAACCAATGCAACAGCGCCATCAAACAATACACAGGATCAGATCCGAAATTTTTTA GGCCGGCTAACCTTGCCACCTCTCAGACGTTATTCCAAACCGTCAAGCTACCATTTGTTC AGGGGGTTATTGCGCAAGACTGGAATGGAGCCTCAGCGACTACAGCAGAAGCTAGGGC GCAACTGGTGTTGAACGGCACACAGGACGGGAGTATCGTGTTAATGCACTGCACTCAGC CTGGGTATCATCCTACTCCGGAGGCGTTAGATATTATTATACCCCAATTGCAGCAACAAG GATACTCTTTTGTCACCTTGTCCGACTTGTTCAAGCTAAAAGGTAAGACCCCGCAGCTGG GGACGATGCTAAACGGCGCATTTTAA
AXEXYL3 ***Pxxyn11A***	ATGTCTGCAACACTACTTGTTCCTTCTATGACAGTTAAAGCGGCCGATACAATCTATAAC AATAAAACAGGTAATCAAGACGGATACGACTACGAATTATGGAAGGATACAGGTAACA CTTCTATGACCTTGAATGCGGGAGGTACTTTCGATTGTAGTTGGTCCAACATCAATAACG CACTTTTCAGGAAGGGTAAGAAGTTTGACTCCACTCAAACTTATCAGCAGATCGGAAAT ATAACCTTCGATTACGGTTGTGACTACAGGCCAAATGGAAATTCCTATCTATGTGTTTAC GGTTGGACAGTTGACCCGCTGGTTGAGTACTACATTGTCGACTCATGGGGGACATGGAG GCCCCCTGGGGGGACGCCAAAGGGACAGATCCAAGTCGACGGAGGGACATATGACGTA TATGAAACAACTAGATATAACGCGCCTAGTATCCAAGGAGACACCACATTCAAACAATA TTTCAGCGTAAGGACGAGCAAAAGGACTTCTGGGACAATAAGTGTCTCAGAACATTTCA AAGCGTGGGAGAGGATGGGAATGCGTTGCGGTAACTTTATGAAACCAGCACTAAACATT GAAGGTTATCAGTCAAGTGGATCTGCCTCCGTCTACAAGAATAATATGACTATAGGTGG CTCCTCTAGTAGTAGCGGTAACCAGGGTGGCAACCAGGGCGGGAACACAGGAAATGAA AACGCCGGTAATAATTTAGTCACTGTGGCCGACGCGGACAAGATTCAGTGTGAAACGAT GACAAAAAGTGGGCAGTATACGGGTAACATCAGCTCTCCGTTTAATGGTGTTGCGTTAT ATGCCAACAACGACGCAGTGAAATATACCCAATACTTCGCCTCCGGGACCCACGATTTT ACGCTAAGAGGATGTAGCAACAATAACAAAATGGCCAGAGTGGATTTAAAAATTGGGG GCCAAAATAAGGGAACCTTTTACTATGGAGATTCCTATCCGGCCGAATACACAATAAAG AATGTGAGTCATGGGACCGGCAATCAGACTATTGAGCTAGTTGTAACAGCCGACGACGG GCAATGGGATGCCTACCTTGACTACTTCAATAACAGCGTGGAGCCCGGGTGTTCTCTTGT ACCGGGGGCGGTCGTTGTATTGGTGGCTTTAGGGAGCAGTAGTAATACTGGCAATAACT CAGGGACTAACACCCAAAATCAGAAGCTAATCGCGCTAACTTTTGACGATGGTCCGTCC AGCACCACTTCACAAGTACTGGATATGTTAGAGAAATATAATGTAAAGGCGACTTTTTT CCTTATCGGACAAAATGTCAATAGTAACACCGCTAGCATAGTACAGAGGCAGGTGAAAA TGGGATGTGAATTAGCCTGCCACTCTTACACTCATGAAGACATGACCAAGATGAACGCT TCTCAAATTAGAAACCAAATCGACTGGACCGCGTCAGCGATCAAAAATACAGCCGGTGT AGACGTAAAATTTTTCAGGCCACCATACATCAGTGTCAACAACACTATGTATCAGAACA TTGACTTGCCATTCATACAGGGTTCTATGCACAATGATTGGGAATCCTCCACCAGCGCGT CTCAACGTGTTAACAGTGTACTATCTAGCGCAAAAGATGGCGACATCATACTACTTCAC GACTTTCAGGGCAACTCCCAAACAGTATCAGCCTTACCTCAAATCATCGAAGGTTTGAA GAATCAAGGTTATACTTTCGTGACCGTTTCCGAACTGTTTGAAATGAAGGGAGTTAATCC TAATGTTGAGTACAAAATTTGGTCAAATGTGAAGTGA
AXEXYL4 ***CfxynD***	ATGTCTGATTCATTTGAAGCTACGAGAACCACGAGAAGAAGACGTCCTCTTCAAGCTCT TACAGGACTATTAGCAGCGGGTGCGCTTGTTGCAGGGGCATTAGCGGCTGCATCTCCCG CGGCTGCTGCAGTCACGTCAAATACGACCGGAACCCATGATGGATATTTCTACTCTTTCT GGACTGATTCTCCGGGATCTGTAAGCATGGACCTGAACAGTGGTGGCGGATATACTAGG TGGAGTAATACTGGAAATTTCGTAGCGGGGAAAGGCTGGTCAACAGGGGGGCGTAAAA CTGTGAGTTATAGTGGGCAATTTAACCCGAGCAGAAACGCATACCTGACCCTGTATGGG TGGACCCAAAGTCCACTTGTTGAGTATTATATAGTGGATTCCTGGGGCACATACAGGCC AACGGGAACTTTCATGGGCACCGTTACCTCAGACGGGGGCACTTATGATATATACAGAA CTCAGCGTGTGAACAAACCAAGTATTGAAGGTGACAGCTCAACGTTCTATCAATACTGG TCAGTTCGTCAGCAGAAGAGGACTGGAGGAACCATTACATCCGGCAATCATTTTGACGC ATGGGCGTCCAAGGGCATGAACCTAGGGAGACACAACTACATGATCATGGCTACGGAA GGGTACCAATCAAGTGGGAGCTCATCCATCACCGTAAGTGAGGGTAGTGGAGGGGGTG GAGGTGGGGACACTGGTGGAGGCGGTGGTAGTACCGGATGTTCAGTAACAGCGACCCG TGCGGAGGAATGGAGCGATAGGTTTAATGTGACCTATTCTGTGAGTGGGTCTTCTGCGT GGACAGTCAACTTAGCGTTGAATGGCTCCCAAACCATACAGGCTTCATGGAACGCCAAT GTGACCGGATCCGGATCCACACGTACAGTAACACCCAATGGTTCCGGAAACACGTTTGG AGTAACTGTGATGAAGAATGGTTCCAGCACCACGCCAGCAGCGACTTGTGCGGGCTCCG GGGGAGGAACGGCTACTCCGACACCCACACCCACCCCGACACCTACACCCCAGAGTTGC TCAGCAGGGTACGTAGGCTTGACTTTTGATGATGGACCGAATACAGGTACAACAAACCA GATCCTTTCCACTTTAACTCAGTACGGTGCCACAGCCACAGTGTTTCCTACAGGCCAAAA

	TGCGCAGGGGAATCCTTCACTTATGCAGGCTTATAAAAATGCTGGGGTCCAGATCGGAA ACCACTCATGGGACCACCCCCATTTGGTGAATATGAGTCAATCAGACATGCAGTCTCAG TTGACAAGAACGCAGCAGGCAATACAACAGACGGCCGGAGTTACACCGACCCTATTCCG TCCGCCATACGGCGAGTCCAATGCGACCCTACGTCAGGTGGAATCATCTCTAGGCTTAA GGGAAATTATCTGGGATGTAGATTCCCAGGACTGGAACAACGCTTCTGCTAGTCAAATC AGGCAAGCAGCGAGCAGATTAACCAATGGGCAAATTATTTTGATGCACGACTGGCCTGC CGCAACTGTGCAGGCTTTACCGGGCATCTTGCAAGATCTGAGGAGTAGAAACTTATGTA CGGGGCACATCTCCAGTTCAACTGGCAGGGCAGTCGCGCCTAGCTCCGCTGGTGGCGGG GGAGGAGGCGGGGGAGGCACGGGATCCTGTTCAGTGTCCGCTGTACGTGGCGAAGAAT GGGCCGATAGATTTAATGTGACCTATTCTGTTTCCGGTTCATCCTCCTGGGTTGTGACCC TGGGCCTGAACGGAGGTCAGAGCGTACAGTCTTCTTGGAACGCTGCTTTGACTGGAAGT TCCGGTACGGTCACAGCGAGACCTAACGGCAGCGGCAACAGTTTTGGCGTAACCTTCTA TAAGAACGGGTCAAGCGCTACCCCAGGTGCAACATGTGCCACAGGTTAG

Table A5.2. Conversion rates of cell-free medium from all strains compared for all substrates tested in Fig. 5.7. The value shown is the mean of three replicates with standard deviation.

	Conversion rate (% . mL cell-free supernatant^{-1} . h^{-1})				
Strain	**CMC**	**PASC**	**Avicel**	**Beechwood xylan**	**Filter paper**
MD4	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.65 ± 0.18	0.02 ± 0.02
AC1	0.05 ± 0.00	0.05 ± 0.00	0.02 ± 0.01	0.93 ± 0.13	0.09 ± 0.03
AC6	0.04 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	1.71 ± 0.31	0.09 ± 0.02
AC7	0.07 ± 0.00	0.05 ± 0.01	0.03 ± 0.00	9.89 ± 0.38	0.11 ± 0.04
AC9	0.53 ± 0.01	0.18 ± 0.01	0.03 ± 0.01	9.60 ± 0.32	0.18 ± 0.04
AC11	0.53 ± 0.00	0.19 ± 0.01	0.03 ± 0.00	9.82 ± 0.37	0.21 ± 0.04
AC12	0.51 ± 0.01	0.21 ± 0.01	0.06 ± 0.02	10.26 ± 0.26	0.36 ± 0.02
AC14	0.49 ± 0.00	0.23 ± 0.01	0.06 ± 0.01	9.98 ± 0.68	0.39 ± 0.07

References

Abedinifar, S., Karimi, K., Khanahmadi, M., Taherzadeh, M. J., 2009. Ethanol production by *Mucor indicus* and *Rhizopus oryzae* from rice straw by separate hydrolysis and fermentation. Biomass and Bioenergy. 33**,** 828-833.

Adam, A. C., Rubio-Texeira, M., Polaina, J., 1995. Induced expression of bacterial beta-glucosidase activity in *Saccharomyces*. Yeast. 11**,** 395-406.

Adeboye, P. T., Bettiga, M., Aldaeus, F., Larsson, P. T., Olsson, L., 2015. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. Microb Cell Fact. 14**,** 149.

Adeboye, P. T., Bettiga, M., Olsson, L., 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express. 4**,** 46-55.

Aden, A., Foust, T., 2009. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. Cellulose. 16**,** 535-545.

Aden, A., Ruth, M., Ibsen, K., Jechura, J., Neeves, K., Sheehan, J., Wallace, B., 2002. Lignocellulosic biomass to ethanol process design and economics utilizing co-current dilute acid prehydrolysis and enzymatic hydrolysis for corn stover. National Renewable Energy Laboratory.

Aditiya, H. B., Mahlia, T. M. I., Chong, W. T., Nur, H., Sebayang, A. H., 2016. Second generation bioethanol production: A critical review. Renewable and Sustainable Energy Reviews. 66**,** 631-653.

Aditiya, H. B., Sing, K. P., Hanif, M., Mahlia, T. M. I., 2015. Effect of acid pretreatment on enzymatic hydrolysis in bioethanol production from rice straw. International Journal of Technology. 6.

Adney, B., Baker, J., 2008. Measurement of cellulase activities. National Renewable Energy Laboratory (NREL).

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., Levin, D. B., 2011. Biomass pretreatment: fundamentals toward application. Biotechnol Adv. 29**,** 675-85.

Ahmad, M., Hirz, M., Pichler, H., Schwab, H., 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol. 98**,** 5301-17.

Ahmed, S., Riaz, S., Jamil, A., 2009. Molecular cloning of fungal xylanases: an overview. Appl Microbiol Biotechnol. 84**,** 19-35.

Ajanovic, A., 2011. Biofuels versus food production: Does biofuels production increase food prices? Energy. 36**,** 2070-2076.

Alexandre, H., Ansanay-Galeote, V., Dequin, S., Blondin, B., 2001. Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Letters. 498**,** 98-103.

Alizadeh, H., Teymouri, F., Gilbert, T. I., Dale, B. E., 2005. Pretreatment of switchgrass by ammonia fiber explosion (AFEX). Applied Biochemistry and Biotechnology. 124**,** 1133-1141.

Alper, H., Fischer, C., Nevoigt, E., Stephanopoulos, G., 2005. Tuning genetic control through promoter engineering. Proc Natl Acad Sci U S A. 102**,** 12678-83.

Alper, H., Moxley, J., Nevoigt, E., Fink, G. R., Stephanopoulos, G., 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science. 314**,** 1565-1568.

Álvarez-Chávez, C. R., Edwards, S., Moure-Eraso, R., Geiser, K., 2012. Sustainability of bio-based plastics: general comparative analysis and recommendations for improvement. Journal of Cleaner Production. 23**,** 47-56.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M. J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology. 101**,** 4851-4861.

Andric, P., Meyer, A. S., Jensen, P. A., Dam-Johansen, K., 2010. Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. Biotechnol Adv. 28**,** 308-24.

Aranda, A., del Olmo, M. l., 2004. Exposure of *Saccharomyces cerevisiae* to acetaldehyde induces sulfur amino acid metabolism and polyamine transporter genes, which depend on Met4p and Haa1p transcription factors, respectively. Appl Environ Microbiol. 70**,** 1913-1922.

Arantes, V., Saddler, J. N., 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels. 3**,** 4.

Arfi, Y., Shamshoum, M., Rogachev, I., Peleg, Y., Bayer, E. A., 2014. Integration of bacterial lytic polysaccharide monooxygenases into designer cellulosomes promotes enhanced cellulose degradation. Proc Natl Acad Sci U S A. 111**,** 9109-14.

Aristidou, A., Penttilä, M., 2000. Metabolic engineering applications to renewable resource utilization. Current Opinion in Biotechnology. 11**,** 187-198.

Arnling Baath, J., Martinez-Abad, A., Berglund, J., Larsbrink, J., Vilaplana, F., Olsson, L., 2018. Mannanase hydrolysis of spruce galactoglucomannan focusing on the influence of acetylation on enzymatic mannan degradation. Biotechnol Biofuels. 11**,** 114.

Ask, M., Olofsson, K., Di Felice, T., Ruohonen, L., Penttilä, M., Lidén, G., Olsson, L., 2012. Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF. Process Biochemistry. 47**,** 1452-1459.

Astolfi, P., Brandi, P., Galli, C., Gentili, P., Gerini, M. F., Greci, L., Lanzalunga, O., 2005. New mediators for the enzyme laccase: mechanistic features and selectivity in the oxidation of non-phenolic substrates. New Journal of Chemistry. 29**,** 1308-1317.

Avanthi, A., Kumar, S., Sherpa, K. C., Banerjee, R., 2016. Bioconversion of hemicelluloses of lignocellulosic biomass to ethanol: an attempt to utilize pentose sugars. Biofuels. 8**,** 431-444.

Bachmann, S. L., McCarthy, A. J., 1991. Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Appl Environ Microbiol. 57**,** 2121-2130.

Bae, J., Kuroda, K., Ueda, M., 2015. Proximity effect among cellulose-degrading enzymes displayed on the S*accharomyces cerevisiae* cell surface. Appl Environ Microbiol. 81**,** 59-66.

Bae, S. J., Kim, S., Hahn, J. S., 2016. Efficient production of acetoin in *Saccharomyces cerevisiae* by disruption of 2,3-butanediol dehydrogenase and expression of NADH oxidase. Sci Rep. 6**,** 27667.

Bae, Y. H., Kang, K. H., Jin, Y. S., Seo, J. H., 2014. Molecular cloning and expression of fungal cellobiose transporters and ß-glucosidases conferring efficient cellobiose fermentation in *Saccharomyces cerevisiae*. J Biotechnol. 169**,** 34-41.

Baerends, R. J., Qiu, J. L., Rasmussen, S., Nielsen, H. B., Brandt, A., 2009. Impaired uptake and/or utilization of leucine by *Saccharomyces cerevisiae* is suppressed by the SPT15-300 allele of the TATA-binding protein gene. Appl Environ Microbiol. 75**,** 6055-61.

Bajpai, P., 2016. Structure of lignocellulosic biomass. Pretreatment of Lignocellulosic Biomass for Biofuel Production. Springer Singapore, Singapore, pp. 7-12.

Balat, M., 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management. 52**,** 858-875.

Balat, M., Balat, H., 2009. Recent trends in global production and utilization of bio-ethanol fuel. Applied Energy. 86**,** 2273-2282.

Balat, M., Balat, H., Öz, C., 2008. Progress in bioethanol processing. Progress in Energy and Combustion Science. 34**,** 551-573.

Ball, A. S., McCarthy, A. J., 1989. Production and properties of xylanases from actinomycetes. Journal of Applied Bacteriology. 66**,** 439-444.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochemistry. 39**,** 1843-1848.

Banerjee, G., Car, S., Scott-Craig, J. S., Borrusch, M. S., Bongers, M., Walton, J. D., 2010a. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. Bioresource Technology. 101**,** 9097-9105.

Banerjee, G., Scott-Craig, J. S., Walton, J. D., 2010b. Improving enzymes for biomass conversion: A basic research perspective. BioEnergy Research. 3**,** 82-92.

Banka, A. L., Albayrak Guralp, S., Gulari, E., 2014. Secretory expression and characterization of two hemicellulases, xylanase, and β-xylosidase, isolated from *Bacillus subtilis* M015. Applied Biochemistry and Biotechnology. 174**,** 2702-2710.

Bao, Z., Xiao, H., Liang, J., Zhang, L., Xiong, X., Sun, N., Si, T., Zhao, H., 2015. Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. ACS Synth Biol. 4**,** 585-94.

Barnett, C. C., Berka, R. M., Fowler, T., 1991. Cloning and amplification of the gene encoding an extracellular β-glucosidase from *Trichoderma reesei*: evidence for improved rates of saccharification of cellulosic substrates. Bio/Technology. 9**,** 562-567.

Basso, L. C., de Amorim, H. V., de Oliveira, A. J., Lopes, M. L., 2008. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 8**,** 1155-1163.

Bastawde, K. B., 1992. Xylan structure, microbial xylanases, and their mode of action. World Journal of Microbiology and Biotechnology. 8**,** 353-368.

Bause, E., 1983. Structural requirements of *N*-glycosylation of proteins. Studies with proline peptides as conformational probes. Biochem J. 209**,** 331-6.

Becker, J., Boles, E., 2003. A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. Appl Environ Microbiol. 69**,** 4144-50.

Bednarek, S. Y., Ravazzola, M., Hosobuchi, M., Amherdt, M., Perrelet, A., Schekman, R., Orci, L., 1995. COPI- and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. Cell. 83**,** 1183-1196.

Beg, Q. K., Kapoor, M., Mahajan, L., Hoondal, G. S., 2001. Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol. 56**,** 326-38.

Behera, B. C., Sethi, B. K., Mishra, R. R., Dutta, S. K., Thatoi, H. N., 2017. Microbial cellulases - Diversity & biotechnology with reference to mangrove environment: A review. J Genet Eng Biotechnol. 15**,** 197-210.

Bellissimi, E., van Dijken, J. P., Pronk, J. T., van Maris, A. J., 2009. Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based *Saccharomyces cerevisiae* strain. FEMS Yeast Res. 9**,** 358-64.

Bertrand, E., Vandenberghe, L. P. S., Soccol, C. R., Sigollot, J.-C., Faulds, C., 2016. First generation bioethanol. Green Fuels Technology. Springer International Publishing, Switzerland, pp. 175-212.

Besada-Lombana, P. B., Da Silva, N. A., 2019. Engineering the early secretory pathway for increased protein secretion in *Saccharomyces cerevisiae*. Metab Eng. 55**,** 142-151.

Bettiga, M., Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M. F., 2009. Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. Microbial cell factories. 8**,** 40-40.

Beurdeley, M., Bietz, F., Li, J., Thomas, S., Stoddard, T., Juillerat, A., Zhang, F., Voytas, D. F., Duchateau, P., Silva, G. H., 2013. Compact designer TALENs for efficient genome engineering. Nature Communications. 4**,** 1762.

Bezerra, R. M., Dias, A. A., 2005. Enzymatic kinetic of cellulose hydrolysis: inhibition by ethanol and cellobiose. Appl Biochem Biotechnol. 126**,** 49-59.

Bhat, M. K., Hazlewood, G. P., 2001. Enzymology and other characteristics of cellulases and xylanases. In: Bedford, M. R., Partridge, G. G., Eds.), Enzymes in farm animal nutrition. CABI Publishing, Wallingford, pp. 11-60.

Bhattacharya, A. S., Bhattacharya, A., Pletschke, B. I., 2015. Synergism of fungal and bacterial cellulases and hemicellulases: a novel perspective for enhanced bio-ethanol production. Biotechnol Lett. 37**,** 1117-29.

Biely, P., MacKenzie, C. R., Puls, J., Schneider, H., 1986. Cooperativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. Nature Biotechnology. 4**,** 731-733.

Biely, P., Puls, J., Schneider, H., 1985. Acetyl xylan esterases in fungal cellulolytic systems. FEBS Letters. 186**,** 80-84.

Binod, P., Gnansounou, E., Sindhu, R., Pandey, A., 2019. Enzymes for second generation biofuels: Recent developments and future perspectives. Bioresource Technology Reports. 5**,** 317-325.

Binod, P., Sindhu, R., Singhania, R. R., Vikram, S., Devi, L., Nagalakshmi, S., Kurien, N., Sukumaran, R. K., Pandey, A., 2010. Bioethanol production from rice straw: An overview. Bioresour Technol. 101**,** 4767-74.

Blobel, G., Sabatini, D. D., 1971. Ribosome-membrane interaction in eukaryotic cells. In: Manson, L. A., (Ed.), Biomembranes: Volume 2. Springer US, Boston, MA, pp. 193-195.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu Rev Plant Biol. 54**,** 519-46.

Boisset, C., Fraschini, C., Schulein, M., Henrissat, B., Chanzy, H., 2000. Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. Appl Environ Microbiol. 66**,** 1444-52.

Boisset, C., Pétrequin, C., Chanzy, H., Henrissat, B., Schülein, M., 2001. Optimized mixtures of recombinant *Humicola insolens* cellulases for the biodegradation of crystalline cellulose. Biotechnology and Bioengineering. 72**,** 339-345.

Bolognesi, B., Lehner, B., 2018. Reaching the limit. eLife. 7**,** e39804.

Boraston, Alisdair B., Bolam, David N., Gilbert, Harry J., Davies, Gideon J., 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemical Journal. 382**,** 769-781.

Brat, D., Boles, E., Wiedemann, B., 2009. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 75**,** 2304-11.

Brethauer, S., Studer, M. H., 2014. Consolidated bioprocessing of lignocellulose by a microbial consortium. Energy & Environmental Science. 7.

Brigham, J., Adney, W. S., Himmel, M. E., 2018. Hemicellulases: Diversity and applications. In: Wyman, C., (Ed.), Handbook on bioethanol: Production and utilization. Taylor & Francis, Washington, pp. 119-141.

Brijwani, K., Oberoi, H. S., Vadlani, P. V., 2010. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Process Biochemistry. 45**,** 120-128.

Brownell, H. H., Yu, E. K. C., Saddler, J. N., 1986. Steam-explosion pretreatment of wood: Effect of chip size, acid, moisture content and pressure drop. Biotechnology and Bioengineering. 28**,** 792-801.

Bruinenberg, P. M., Jonker, R., van Dijken, J. P., Scheffers, W. A., 1985. Utilization of formate as an additional energy source by glucose-limited chemostat cultures of *Candida utilis* CBS 621 and *Saccharomyces cerevisiae* CBS 8066. Archives of Microbiology. 142**,** 302-306.

Brule, C. E., Grayhack, E. J., 2017. Synonymous codons: choose wisely for expression. Trends Genet. 33**,** 283-297.

Bule, P., Pires, V. M., Fontes, C. M., Alves, V. D., 2018. Cellulosome assembly: paradigms are meant to be broken! Curr Opin Struct Biol. 49**,** 154-161.

Bura, R., Chandra, R., Saddler, J., 2009. Influence of xylan on the enzymatic hydrolysis of steam-pretreated corn stover and hybrid poplar. Biotechnol Prog. 25**,** 315-22.

Burda, P., Aebi, M., 1998. The *ALG10* locus of *Saccharomyces cerevisiae* encodes the alpha-1,2 glucosyltransferase of the endoplasmic reticulum: the terminal glucose of the lipid-linked oligosaccharide is required for efficient *N*-linked glycosylation. Glycobiology. 8**,** 455-62.

Cai, Z., Zhang, B., Li, Y., 2012. Engineering *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: reflections and perspectives. Biotechnol J. 7**,** 34-46.

Cakar, Z. P., Seker, U. O., Tamerler, C., Sonderegger, M., Sauer, U., 2005. Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res. 5**,** 569-78.

Campbell, M. M., Sederoff, R. R., 1996. Variation in lignin content and composition. Plant Physiol. 110**,** 3-13.

Carvalheiro, F., Duarte, L. C., Gírio, F. M., 2008. Hemicellulose biorefineries: a review on biomass pretreatments. Journal of Scientific & Industrial Research. 849-864.

Casey, E., Mosier, N. S., Adamec, J., Stockdale, Z., Ho, N., Sedlak, M., 2013. Effect of salts on the co-fermentation of glucose and xylose by a genetically engineered strain of *Saccharomyces cerevisiae*. Biotechnol Biofuels. 6**,** 83-92.

Caspeta, L., Buijs, N. A. A., Nielsen, J., 2013. The role of biofuels in the future energy supply. Energy & Environmental Science. 6.

Cassman, K. G., Liska, A. J., 2007. Food and fuel for all: realistic or foolish? Biofuels, Bioproducts and Biorefining: Innovation for a sustainable economy. 1**,** 18-23.

Cepeljnik, T., Rincon, M. T., Flint, H. J., Marinsek-Logar, R., 2006. Xyn11A, a multidomain multicatalytic enzyme from *Pseudobutyrivibrio xylanivorans* Mz5T. Folia Microbiol (Praha). 51**,** 263-7.

Chandra Rajak, R., Banerjee, R., 2015. Enzymatic delignification: an attempt for lignin degradation from lignocellulosic feedstock. RSC Advances. 5**,** 75281-75291.

Change, I. C., 2014. Mitigation of climate change. Contribution of working group III to the fifth assessment report of the intergovernmental panel on climate change. 1454.

Chauhan, P. S., Puri, N., Sharma, P., Gupta, N., 2012. Mannanases: microbial sources, production, properties and potential biotechnological applications. Appl Microbiol Biotechnol. 93**,** 1817-30.

Chauve, M., Mathis, H., Huc, D., Casanave, D., Monot, F., Lopes Ferreira, N., 2010. Comparative kinetic analysis of two fungal ß-glucosidases. Biotechnol Biofuels. 3.

Chen, A. H., Silver, P. A., 2012. Designing biological compartmentalization. Trends Cell Biol. 22**,** 662-70.

Chen, B., Ling, H., Chang, M. W., 2013. Transporter engineering for improved tolerance against alkane biofuels in *Saccharomyces cerevisiae*. Biotechnology for Biofuels. 6.

Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., Chang, J. S., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresour Technol. 102**,** 71-81.

Chen, H., Hayn, M., Esterbauer, H., 1992. Purification and characterization of two extracellular β-glucosidases from *Trichoderma reesei*. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology. 1121**,** 54-60.

Chen, H., Jin, S., 2006. Effect of ethanol and yeast on cellulase activity and hydrolysis of crystalline cellulose. Enzyme and Microbial Technology. 39**,** 1430-1432.

Chen, Q., Marshall, M. N., Geib, S. M., Tien, M., Richard, T. L., 2012a. Effects of laccase on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover. Bioresource Technology. 117**,** 186-192.

Chen, R., Dou, J., 2016. Biofuels and bio-based chemicals from lignocellulose: metabolic engineering strategies in strain development. Biotechnol Lett. 38**,** 213-21.

Chen, Z., Jia, H., Yang, Y., Yan, Q., Jiang, Z., Teng, C., 2012b. Secretory expression of a ß-xylosidase gene from *Thermomyces lanuginosus* in *Escherichia coli* and characterization of its recombinant enzyme. Lett Appl Microbiol. 55**,** 330-7.

Cherry, J. R., Fidantsef, A. L., 2003. Directed evolution of industrial enzymes: an update. Curr Opin Biotechnol. 14**,** 438-43.

Chompoosri, J., Fraser, T., Rongsriyam, Y., Komalamisra, N., Siriyasatien, P., Thavara, U., Tawatsin, A., Fraser, M. J., Jr., 2009. Intramolecular integration assay validates integrase phi C31 and R4 potential in a variety of insect cells. Southeast Asian J Trop Med Public Health. 40**,** 1235-53.

Choudhary, J., Singh, S., Nain, L., 2016. Thermotolerant fermenting yeasts for simultaneous saccharification fermentation of lignocellulosic biomass. Electronic Journal of Biotechnology. 21**,** 82-92.

Chundawat, S. P., Balan, V., Dale, B. E., 2008. High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass. Biotechnol Bioeng. 99**,** 1281-94.

Chung, H.-J., Park, S.-M., Kim, H.-R., Yang, M.-S., Kim, D.-H., 2002. Cloning the gene encoding acetyl xylan esterase from *Aspergillus ficuum* and its expression in *Pichia pastoris*. Enzyme and Microbial Technology. 31**,** 384-391.

Collins, T., Gerday, C., Feller, G., 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev. 29**,** 3-23.

Cooper, A., Bussey, H., 1989. Characterization of the yeast *KEX1* gene product: a carboxypeptidase involved in processing secreted precursor proteins. Mol Cell Biol. 9**,** 2706-2714.

Coughlan, M., Hazlewood, G., 1993. ß-1,4-D-xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnology and Applied Biochemistry. 17**,** 259-289.

Cowles, C. R., Snyder, W. B., Burd, C. G., Emr, S. D., 1997. Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. The EMBO journal. 16**,** 2769-2782.

Cripwell, R. A., Rose, S. H., Viljoen-Bloom, M., van Zyl, W. H., 2019. Improved raw starch amylase production by *Saccharomyces cerevisiae* using codon optimisation strategies. FEMS Yeast Res. 19.

Cui, Y., Xu, J., Cheng, M., Liao, X., Peng, S., 2018. Review of CRISPR/Cas9 sgRNA design tools. Interdiscip Sci. 10**,** 455-465.

Cummings, C., Fowler, T., 1996. Secretion of *Trichoderma reesei* β-glucosidase by *Saccharomyces cerevisiae*. Current Genetics. 29**,** 227-233.

Cunha, J. T., Soares, P. O., Romani, A., Thevelein, J. M., Domingues, L., 2019. Xylose fermentation efficiency of industrial *Saccharomyces cerevisiae* yeast with separate or combined xylose reductase/xylitol dehydrogenase and xylose isomerase pathways. Biotechnol Biofuels. 12**,** 20.

Cybinski, D. H., Layton, I., Lowry, J. B., Dalrymple, B. P., 1999. An acetylxylan esterase and a xylanase expressed from genes cloned from the ruminal fungus *Neocallimastix patriciarum* act synergistically to degrade acetylated xylans. Appl Microbiol Biotechnol. 52**,** 221-5.

David, F., Siewers, V., 2015. Advances in yeast genome engineering. FEMS Yeast Res. 15**,** 1-14.

Davison, S. A., den Haan, R., van Zyl, W. H., 2020. Exploiting strain diversity and rational engineering strategies to enhance recombinant cellulase secretion by *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 104**,** 5163-5184.

de Vries, R. P., Visser, J., 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Mol Biol Rev. 65**,** 497-522.

Dekker, R. F. H., Richards, G. N., 1976. Hemicellulases: their occurrence, purification, properties, and mode of action. Advances in Carbohydrate Chemistry and Biochemistry Volume 32. pp. 277-352.

Del Pozo, M. V., Fernández-Arrojo, L., Gil-Martínez, J., Montesinos, A., Chernikova, T. N., Nechitaylo, T. Y., Waliszek, A., Tortajada, M., Rojas, A., Huws, S. A., Golyshina, O. V., Newbold, C. J., Polaina, J., Ferrer, M., Golyshin, P. N., 2012. Microbial β-glucosidases from cow rumen metagenome enhance the saccharification of lignocellulose in combination with commercial cellulase cocktail. Biotechnol Biofuels. 5.

Delneri, D., Tomlin, G. C., Wixon, J. L., Hutter, A., Sefton, M., Louis, E. J., Oliver, S. G., 2000. Exploring redundancy in the yeast genome: an improved strategy for use of the cre–*loxP* system. Gene. 252**,** 127-135.

Demain, A. L., Vaishnav, P., 2009. Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv. 27**,** 297-306.

Demeke, M., Engineering efficient D-xylose fermentation capacity in industrial Saccharomyces cerevisiae for advanced bioethanol production. Departement of Biology. KU Leuven, Heverlee, 2013, pp. 161.

Demeke, M. M., Dietz, H., Li, Y., Foulquie-Moreno, M. R., Mutturi, S., Deprez, S., Den Abt, T., Bonini, B. M., Liden, G., Dumortier, F., Verplaetse, A., Boles, E., Thevelein, J. M., 2013a. Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol Biofuels. 6**,** 89.

Demeke, M. M., Dumortier, F., Li, Y., Broeckx, T., Foulquie-Moreno, M. R., Thevelein, J. M., 2013b. Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. Biotechnol Biofuels. 6**,** 120.

Demirbas, A., 2008. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energy Conversion and Management. 49**,** 2106-2116.

Demirbas, A., 2009. Political, economic and environmental impacts of biofuels: A review. Applied Energy. 86**,** S108-S117.

Demirbaş, A., 2005. Bioethanol from cellulosic materials: A renewable motor fuel from biomass. Energy Sources. 27**,** 327-337.

den Haan, R., Kroukamp, H., Mert, M., Bloom, M., Görgens, J. F., van Zyl, W. H., 2013a. Engineering *Saccharomyces cerevisiae* for next generation ethanol production. Journal of Chemical Technology & Biotechnology. 88**,** 983-991.

den Haan, R., Rose, S. H., Lynd, L. R., van Zyl, W. H., 2007. Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab Eng. 9**,** 87-94.

den Haan, R., van Rensburg, E., Rose, S. H., Gorgens, J. F., van Zyl, W. H., 2015. Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. Curr Opin Biotechnol. 33**,** 32-8.

den Haan, R., van Zyl, J. M., Harms, T. M., van Zyl, W. H., 2013b. Modeling the minimum enzymatic requirements for optimal cellulose conversion. Environ. Res. Lett. 8.

Deng, P., Li, D., Cao, Y., Lu, W., Wang, C., 2006. Cloning of a gene encoding an acidophilic endo-β-1,4-xylanase obtained from *Aspergillus niger* CGMCC1067 and constitutive expression in *Pichia pastoris*. Enzyme and Microbial Technology. 39**,** 1096-1102.

Deparis, Q., Claes, A., Foulquie-Moreno, M. R., Thevelein, J. M., 2017. Engineering tolerance to industrially relevant stress factors in yeast cell factories. FEMS Yeast Res. 17.

Deutschmann, R., Dekker, R. F., 2012. From plant biomass to bio-based chemicals: latest developments in xylan research. Biotechnol Adv. 30**,** 1627-40.

Diao, L., Liu, Y., Qian, F., Yang, J., Jiang, Y., Yang, S., 2013. Construction of fast xylose-fermenting yeast based on industrial ethanol-producing diploid *Saccharomyces cerevisiae* by rational design and adaptive evolution. BMC Biotechnology. 13.

DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., Church, G. M., 2013. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. Nucleic Acids Res. 41**,** 4336-43.

Dimarogona, M., Topakas, E., Christakopoulos, P., 2013. Recalcitrant polysaccharide degradation by novel oxidative biocatalysts. Appl Microbiol Biotechnol. 97**,** 8455-65.

Ding, J., Huang, X., Zhang, L., Zhao, N., Yang, D., Zhang, K., 2009. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. 85**,** 253-63.

Dinh, T. N., Nagahisa, K., Yoshikawa, K., Hirasawa, T., Furusawa, C., Shimizu, H., 2009. Analysis of adaptation to high ethanol concentration in *Saccharomyces cerevisiae* using DNA microarray. Bioprocess Biosyst Eng. 32**,** 681-8.

Dodd, D., Cann, I. K., 2009. Enzymatic deconstruction of xylan for biofuel production. Glob Change Biol Bioenergy. 1**,** 2-17.

Doi, R. H., Tamaru, Y., 2001. The *Clostridium cellulovorans* cellulosome: An enzyme complex with plant cell wall degrading activity. The Chemical Record. 1**,** 24-32.

dos Santos, L. V., Carazzolle, M. F., Nagamatsu, S. T., Sampaio, N. M., Almeida, L. D., Pirolla, R. A., Borelli, G., Correa, T. L., Argueso, J. L., Pereira, G. A., 2016. Unraveling the genetic basis of xylose consumption in engineered *Saccharomyces cerevisiae* strains. Sci Rep. 6**,** 38676.

Doudna, J. A., Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. Science. 346**,** 1258096.

Du, B., Sharma, L. N., Becker, C., Chen, S. F., Mowery, R. A., van Walsum, G. P., Chambliss, C. K., 2010a. Effect of varying feedstock-pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. Biotechnol Bioeng. 107**,** 430-40.

Du, F., Wolger, E., Wallace, L., Liu, A., Kaper, T., Kelemen, B., 2010b. Determination of product inhibition of CBH1, CBH2, and EG1 using a novel cellulase activity assay. Appl Biochem Biotechnol. 161**,** 313-7.

Duitama, J., Quintero, J. C., Cruz, D. F., Quintero, C., Hubmann, G., Foulquié-Moreno, M. R., Verstrepen, K. J., Thevelein, J. M., Tohme, J., 2014. An integrated framework for discovery and genotyping of genomic variants from high-throughput sequencing experiments. Nucleic Acids Res. 42**,** e44-e44.

Dykhuizen, D. E., Hartl, D. L., 1983. Selection in chemostats. Microbiology Reviews. 47**,** 150-168.

Ebringerová, A., 2005. Structural diversity and application potential of hemicelluloses. Macromolecular Symposia. 232**,** 1-12.

Ecker, M., Mrsa, V., Hagen, I., Deutzmann, R., Strahl, S., Tanner, W., 2003. O-mannosylation precedes and potentially controls the N-glycosylation of a yeast cell wall glycoprotein. EMBO Rep. 4**,** 628-632.

Eggert, C., Temp, U., Dean, J. F., Eriksson, K. E., 1996. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett. 391**,** 144-8.

Eguchi, Y., Makanae, K., Hasunuma, T., Ishibashi, Y., Kito, K., Moriya, H., 2018. Estimating the protein burden limit of yeast cells by measuring the expression limits of glycolytic proteins. eLife. 7**,** e34595.

Eklund, R., Zacchi, G., 1995. Simultaneous saccharification and fermentation of steam-pretreated willow. Enzyme and Microbial Technology. 17**,** 255-259.

Emilsson, V., Kurland, C. G., 1990. Growth rate dependence of transfer RNA abundance in *Escherichia coli*. The EMBO Journal. 9**,** 4359-4366.

Eriksen, D. T., Hsieh, P. C. H., Lynn, P., Zhao, H., 2013. Directed evolution of a cellobiose utilization pathway in *Saccharomyces cerevisiae* by simultaneously engineering multiple proteins. Microbial Cell Factories. 12.

Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme and Microbial Technology. 31**,** 353-364.

EUParliament, 2009. Regulation (EC) No 443/2009 of the European Parliament and of the Council of 23 April 2009 setting emission performance standards for new passenger cars as part of the Community's integrated approach to reduce CO2 emissions from light-duty vehicles. Official Journal of the European Union. 52**,** L140.

Fan, L. H., Zhang, Z. J., Yu, X. Y., Xue, Y. X., Tan, T. W., 2012. Self-surface assembly of cellulosomes with two miniscaffoldins on *Saccharomyces cerevisiae* for cellulosic ethanol production. Proc Natl Acad Sci U S A. 109**,** 13260-5.

Fan, Z., South, C., Lyford, K., Munsie, J., van Walsum, P., Lynd, L. R., 2003. Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor. Bioprocess Biosyst Eng. 26**,** 93-101.

Fang, X., Yano, S., Inoue, H., Sawayama, S., 2009. Strain improvement of *Acremonium cellulolyticus* for cellulase production by mutation. Journal of Bioscience and Bioengineering. 107**,** 256-261.

Fernandes, A. R., Mira, N. P., Vargas, R. C., Canelhas, I., Sa-Correia, I., 2005. *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem Biophys Res Commun. 337**,** 95-103.

Ferreira, V., Faber, M. d. O., Mesquita, S. d. S., Pereira Jr, N., 2010. Simultaneous saccharification and fermentation process of different cellulosic substrates using a recombinant *Saccharomyces cerevisiae* harbouring the β-glucosidase gene. Electronic Journal of Biotechnology. 13.

Fierobe, H. P., Bayer, E. A., Tardif, C., Czjzek, M., Mechaly, A., Belaich, A., Lamed, R., Shoham, Y., Belaich, J. P., 2002. Degradation of cellulose substrates by cellulosome chimeras. Substrate targeting versus proximity of enzyme components. J Biol Chem. 277**,** 49621-30.

Fontes, C. M., Gilbert, H. J., 2010. Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. Annu Rev Biochem. 79**,** 655-81.

Fraczek, M. G., Naseeb, S., Delneri, D., 2018. History of genome editing in yeast. Yeast. 35**,** 361-368.

Frandsen, K. E. H., Simmons, T. J., Dupree, P., Poulsen, J.-C. N., Hemsworth, G. R., Ciano, L., Johnston, E. M., Tovborg, M., Johansen, K. S., von Freiesleben, P., Marmuse, L., Fort, S., Cottaz, S., Driguez, H., Henrissat, B., Lenfant, N., Tuna, F., Baldansuren, A., Davies, G. J., Lo Leggio, L., Walton, P. H., 2016. The molecular basis of polysaccharide cleavage by lytic polysaccharide monooxygenases. Nature Chemical Biology. 12**,** 298-303.

Fujita, Y., Ito, J., Ueda, M., Fukuda, H., Kondo, A., 2004. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol. 70**,** 1207-12.

Fujita, Y., Takahashi, S., Ueda, M., Tanaka, A., Okada, H., Morikawa, Y., Kawaguchi, T., Arai, M., Fukuda, H., Kondo, A., 2002. Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Appl Environ Microbiol. 68**,** 5136-41.

Galazka, J. M., Tian, C., Beeson, W. T., Martinez, B., Glass, N. L., Cate, J. H. D., 2010. Cellodextrin transport in yeast for improved biofuel production. Science. 330**,** 84-86.

Gan, Y., Lin, Y., Guo, Y., Qi, X., Wang, Q., 2018. Metabolic and genomic characterisation of stress-tolerant industrial *Saccharomyces cerevisiae* strains from TALENs-assisted multiplex editing. FEMS Yeast Research. 18.

Gao, D., Uppugundla, N., Chundawat, S. P., Yu, X., Hermanson, S., Gowda, K., Brumm, P., Mead, D., Balan, V., Dale, B. E., 2011. Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. Biotechnol Biofuels. 4**,** 5.

Garcia Sanchez, R., Karhumaa, K., Fonseca, C., Sànchez Nogué, V., Almeida, J. R. M., Larsson, C. U., Bengtsson, O., Bettiga, M., Hahn-Hägerdal, B., Gorwa-Grauslund, M. F., 2010. Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. Biotechnology for Biofuels. 3**,** 13.

Garcia-Aparicio, M. P., Ballesteros, I., Gonzalez, A., Oliva, J. M., Ballesteros, M., Negro, M. J., 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl Biochem Biotechnol. 129-132**,** 278-88.

García-Aparicio, M. P., Ballesteros, M., Manzanares, P., Ballesteros, I., González, A., José Negro, M., 2007. Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. Applied Biochemistry and Biotechnology. 137**,** 353-365.

Ghazali, K. A., Salleh, S. F., Riayatsyah, T. M. I., Aditiya, H. B., Mahlia, T. M. I., 2016. The effect of dilute acid pre-treatment process in bioethanol production from durian (*Durio zibethinus*) seeds waste. IOP Conf Series: Earth and Environmental Science. 32.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., Smith, H. O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods. 6**,** 343-345.

Giersch, R. M., Finnigan, G. C., 2017. Yeast still a beast: Diverse applications of CRISPR/Cas editing technology in *S. cerevisiae*. Yale J Biol Med. 90**,** 643-651.

Gietz, R. D., Schiestl, R. H., 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc. 2**,** 31-4.

Girio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., Bogel-Lukasik, R., 2010. Hemicelluloses for fuel ethanol: A review. Bioresour Technol. 101**,** 4775-800.

Giudici, P., Solieri, L., Pulvirenti, A. M., Cassanelli, S., 2005. Strategies and perspectives for genetic improvement of wine yeasts. Appl Microbiol Biotechnol. 66**,** 622-8.

Gold, M. H., Patteson, T. E., Green, G. I., 1976. Cellulase bezoar injection: a new endoscopic technique. Gastrointestinal Endoscopy. 22**,** 200-202.

Gong, Y., Tang, G., Wang, M., Li, J., Xiao, W., Lin, J., Liu, Z., 2014. Direct fermentation of amorphous cellulose to ethanol by engineered *Saccharomyces cerevisiae* coexpressing *Trichoderma viride* EG3 and BGL1. J Gen Appl Microbiol. 60**,** 198-206.

Gopal, C. V., Broad, D., Lloyd, D., 1989. Bioenergetic consequences of protein overexpression in *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 30**,** 160-165.

Görgens, J. F., van Zyl, W. H., Knoetze, J. H., Hahn-Hägerdal, B., 2001. The metabolic burden of the *PGK1* and *ADH2* promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium. Biotechnology and Bioengineering. 73**,** 238-245.

Goyal, G., Tsai, S.-L., Madan, B., DaSilva, N. A., Chen, W., 2011. Simultaneous cell growth and ethanol production from cellulose by an engineered yeast consortium displaying a functional mini-cellulosome. Microbial Cell Factories. 10**,** 89.

Graham-Rowe, D., 2011. Agriculture: Beyond food versus fuel. Nature. 474**,** S6.

Grassick, A., Murray, P. G., Thompson, R., Collins, C. M., Byrnes, L., Birrane, G., Higgins, T. M., Tuohy, M. G., 2004. Three-dimensional structure of a thermostable native cellobiohydrolase, CBH IB, and molecular characterization of the cel7 gene from the filamentous fungus, *Talaromyces emersonii*. European Journal of Biochemistry. 271**,** 4495-4506.

Graves, T., Narendranath, N. V., Dawson, K., Power, R., 2006. Effect of pH and lactic or acetic acid on ethanol productivity by *Saccharomyces cerevisiae* in corn mash. J Ind Microbiol Biotechnol. 33**,** 469-74.

Grous, W. R., Converse, A. O., Grethlein, H. E., 1986. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. Enzyme and Microbial Technology. 8**,** 274-280.

Guerfal, M., Ryckaert, S., Jacobs, P. P., Ameloot, P., Van Craenenbroeck, K., Derycke, R., Callewaert, N., 2010. The *HAC1* gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. Microbial Cell Factories. 9**,** 49.

Guimaraes, P. M., Francois, J., Parrou, J. L., Teixeira, J. A., Domingues, L., 2008. Adaptive evolution of a lactose-consuming *Saccharomyces cerevisiae* recombinant. Appl Environ Microbiol. 74**,** 1748-56.

Gundllapalli, S. B., Pretorius, I. S., Cordero Otero, R. R., 2007. Effect of the cellulose-binding domain on the catalytic activity of a ß-glucosidase from *Saccharomycopsis fibuligera*. J Ind Microbiol Biotechnol. 34**,** 413-21.

Guo, G. L., Chen, W. H., Chen, W. H., Men, L. C., Hwang, W. S., 2008. Characterization of dilute acid pretreatment of silvergrass for ethanol production. Bioresour Technol. 99**,** 6046-53.

Gupta, A., Verma, J. P., 2015. Sustainable bio-ethanol production from agro-residues: A review. Renewable and Sustainable Energy Reviews. 41**,** 550-567.

Gupta, R., Sharma, K. K., Kuhad, R. C., 2009. Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresource Technology. 100**,** 1214-1220.

Gurunathan, S., David, D., Gerst, J. E., 2002. Dynamin and clathrin are required for the biogenesis of a distinct class of secretory vesicles in yeast. The EMBO journal. 21**,** 602-614.

Gusakov, A. V., 2011. Alternatives to *Trichoderma reesei* in biofuel production. Trends Biotechnol. 29**,** 419-25.

Gusakov, A. V., Sinitsyn, A. P., 1992. A theoretical analysis of cellulase product inhibition: effect of cellulase binding constant, enzyme/substrate ratio, and beta-glucosidase activity on the inhibition pattern. Biotechnol Bioeng. 40**,** 663-71.

Gutierrez, A., Rencoret, J., Cadena, E. M., Rico, A., Barth, D., del Rio, J. C., Martinez, A. T., 2012. Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks. Bioresour Technol. 119**,** 114-22.

Ha, S. J., Galazka, J. M., Kim, S. R., Choi, J. H., Yang, X., Seo, J. H., Glass, N. L., Cate, J. H., Jin, Y. S., 2011. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. Proc Natl Acad Sci U S A. 108**,** 504-9.

Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G. H., Gholami, M., Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renewable and Sustainable Energy Reviews. 27**,** 77-93.

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M. F., 2007. Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol. 74**,** 937-53.

Hamelinck, C. N., Hooijdonk, G. v., Faaij, A. P. C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy. 28**,** 384-410.

Hann, B. C., Walter, P., 1991. The signal recognition particle in *S. cerevisiae*. Cell. 67**,** 131-144.

Hanson, G., Coller, J., 2018. Codon optimality, bias and usage in translation and mRNA decay. Nat Rev Mol Cell Biol. 19**,** 20-30.

Hari Krishna, S., Janardhan Reddy, T., Chowdary, G. V., 2001. Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast. Bioresource Technology. 77**,** 193-196.

Harsay, E., Bretscher, A., 1995. Parallel secretory pathways to the cell surface in yeast. J Cell Biol. 131**,** 297-310.

Hasunuma, T., Kondo, A., 2012. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. Process Biochemistry. 47**,** 1287-1294.

Hasunuma, T., Okazaki, F., Okai, N., Hara, K. Y., Ishii, J., Kondo, A., 2013. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. Bioresour Technol. 135**,** 513-22.

Hauf, J., Zimmermann, F. K., Müller, S., 2000. Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. Enzyme and Microbial Technology. 26**,** 688-698.

Hemsworth, G. R., Johnston, E. M., Davies, G. J., Walton, P. H., 2015. Lytic polysaccharide monooxygenases in biomass conversion. Trends Biotechnol. 33**,** 747-761.

Henne, William M., Buchkovich, Nicholas J., Emr, Scott D., 2011. The ESCRT pathway. Developmental Cell. 21**,** 77-91.

Hernandez, N. V., Lopez-Ramirez, L. A., Diaz-Jimenez, D. F., Mellado-Mojica, E., Martinez-Duncker, I., Lopez, M. G., Mora-Montes, H. M., 2017. *Saccharomyces cerevisiae* KTR4, KTR5 and KTR7 encode mannosyltransferases differentially involved in the N- and O-linked glycosylation pathways. Res Microbiol. 168**,** 740-750.

Hickman, M. A., Paulson, C., Dudley, A., Berman, J., 2015. Parasexual ploidy reduction drives population heterogeneity through random and transient aneuploidy in *Candida albicans*. Genetics. 200**,** 781-94.

Hirasawa, T., Yoshikawa, K., Nakakura, Y., Nagahisa, K., Furusawa, C., Katakura, Y., Shimizu, H., Shioya, S., 2007. Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. J Biotechnol. 131**,** 34-44.

Hoffman, C. S., Winston, F., 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene. 57**,** 267-72.

Hohmann, S., 2002. Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev. 66**,** 300-372.

Hohmann, S., Krantz, M., Nordlander, B., 2007. Yeast osmoregulation. Methods in Enzymology. 428**,** 29-45.

Holt, S., Kankipati, H., De Graeve, S., Van Zeebroeck, G., Foulquie-Moreno, M. R., Lindgreen, S., Thevelein, J. M., 2017. Major sulfonate transporter Soa1 in *Saccharomyces cerevisiae* and considerable substrate diversity in its fungal family. Nat Commun. 8**,** 14247.

Holtzapple, M. T., Lundeen, J. E., Sturgis, R., Lewis, J. E., Dale, B. E., 1992. Pretreatment of lignocellulosic municipal solid waste by ammonia fiber explosion (AFEX). Applied Biochemistry and Biotechnology. 34**,** 5.

Hong, J., Wang, Y., Kumagai, H., Tamaki, H., 2007. Construction of thermotolerant yeast expressing thermostable cellulase genes. J Biotechnol. 130**,** 114-23.

Hong, J., Yang, H., Zhang, K., Liu, C., Zou, S., Zhang, M., 2014. Development of a cellulolytic *Saccharomyces cerevisiae* strain with enhanced cellobiohydrolase activity. World J Microbiol Biotechnol. 30**,** 2985-93.

Horn, S. J., Vaaje-Kolstad, G., Westereng, B., Eijsink, V., 2012. Novel enzymes for the degradation of cellulose. Biotechnology for Biofuels. 5**,** 45.

Horvat, J., Klaic, B., Metelko, B., Sunjic, V., 1985. Mechanism of levulinic acid formation. Tetrahedron Letters. 26**,** 2111-2114.

Horwitz, A. A., Walter, J. M., Schubert, M. G., Kung, S. H., Hawkins, K., Platt, D. M., Hernday, A. D., Mahatdejkul-Meadows, T., Szeto, W., Chandran, S. S., Newman, J. D., 2015. Efficient multiplexed integration of synergistic alleles and metabolic pathways in yeasts via CRISPR-Cas. Cell Syst. 1**,** 88-96.

Hou, J., Tyo, K. E., Liu, Z., Petranovic, D., Nielsen, J., 2012. Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. FEMS Yeast Res. 12**,** 491-510.

Hou, L., 2009. Novel methods of genome shuffling in *Saccharomyces cerevisiae*. Biotechnol Lett. 31**,** 671-7.

Hou, L., 2010. Improved production of ethanol by novel genome shuffling in *Saccharomyces cerevisiae*. Appl Biochem Biotechnol. 160**,** 1084-93.

Houghton, J. T., Jenkins, G. J., Ephraums, J. J., 1991. Climate change.

Hsieh, C. W., Cannella, D., Jorgensen, H., Felby, C., Thygesen, L. G., 2014. Cellulase inhibition by high concentrations of monosaccharides. J Agric Food Chem. 62**,** 3800-5.

Hu, J., Arantes, V., Pribowo, A., Saddler, J. N., 2013. The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific. Biotechnology for biofuels. 6**,** 112-112.

Hu, J., Gourlay, K., Arantes, V., Van Dyk, J. S., Pribowo, A., Saddler, J. N., 2015. The accessible cellulose surface influences cellulase synergism during the hydrolysis of lignocellulosic substrates. ChemSusChem. 8**,** 901-7.

Huang, D., Shusta, E. V., 2005. Secretion and surface display of green fluorescent protein using the yeast *Saccharomyces cerevisiae*. Biotechnology Progress. 21**,** 349-357.

Hyeon, J.-e., Yu, K.-O., Suh, D. J., Suh, Y.-W., Lee, S. E., Lee, J., Han, S. O., 2010. Production of minicellulosomes from *Clostridium cellulovorans* for the fermentation of cellulosic ethanol using engineered recombinant *Saccharomyces cerevisiae*. FEMS Microbiology Letters. 310**,** 39-47.

Idiris, A., Tohda, H., Kumagai, H., Takegawa, K., 2010. Engineering of protein secretion in yeast: strategies and impact on protein production. Appl Microbiol Biotechnol. 86**,** 403-17.

Igarashi, K., Uchihashi, T., Koivula, A., Wada, M., Kimura, S., Okamoto, T., Penttila, M., Ando, T., Samejima, M., 2011. Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. Science. 333**,** 1279-82.

Ilmén, M., den Haan, R., Brevnova, E., McBride, J., Wiswall, E., Froehlich, A., Koivula, A., Voutilainen, S. P., Siika-aho, M., la Grange, D. C., Thorngren, N., Ahlgren, S., Mellon, M., Deleault, K., Rajgarhia, V., van Zyl, W. H., Penttilä, M., 2011. High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. Biotechnology for Biofuels. 4**,** 30.

Inokuma, K., Bamba, T., Ishii, J., Ito, Y., Hasunuma, T., Kondo, A., 2016. Enhanced cell-surface display and secretory production of cellulolytic enzymes with *Saccharomyces cerevisiae* Sed1 signal peptide. Biotechnol Bioeng. 113**,** 2358-66.

Inokuma, K., Hasunuma, T., Kondo, A., 2014. Efficient yeast cell-surface display of exo- and endo-cellulase using the *SED1* anchoring region and its original promoter. Biotechnology for Biofuels. 7**,** 8.

Isaksen, T., Westereng, B., Aachmann, F. L., Agger, J. W., Kracher, D., Kittl, R., Ludwig, R., Haltrich, D., Eijsink, V. G. H., Horn, S. J., 2014. A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. Journal of Biological Chemistry. 289**,** 2632-2642.

Ishii, J., Okazaki, F., Djohan, A. C., Hara, K. Y., Asai-Nakashima, N., Teramura, H., Andriani, A., Tominaga, M., Wakai, S., Kahar, P., Yopi, Prasetya, B., Ogino, C., Kondo, A., 2016. From mannan to bioethanol: cell surface co-display of ß-mannanase and ß-mannosidase on yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels. 9**,** 188.

Isikgor, F. H., Becer, C. R., 2015. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polymer Chemistry. 6**,** 4497-4559.

Islam, T., 2019. CRISPR-Cas technology in modifying food crops. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 14.

Jaafar, Z., Mazeau, K., Boissière, A., Le Gall, S., Villares, A., Vigouroux, J., Beury, N., Moreau, C., Lahaye, M., Cathala, B., 2019. Meaning of xylan acetylation on xylan-cellulose interactions: A quartz crystal microbalance with dissipation (QCM-D) and molecular dynamic study. Carbohydrate Polymers. 226**,** 115315.

Jakob, C. A., Burda, P., Roth, J., Aebi, M., 1998. Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure. J Cell Biol. 142**,** 1223-33.

Jakociunas, T., Bonde, I., Herrgard, M., Harrison, S. J., Kristensen, M., Pedersen, L. E., Jensen, M. K., Keasling, J. D., 2015a. Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*. Metab Eng. 28**,** 213-22.

Jakociunas, T., Jensen, M. K., Keasling, J. D., 2016. CRISPR/Cas9 advances engineering of microbial cell factories. Metab Eng. 34**,** 44-59.

Jakociunas, T., Rajkumar, A. S., Zhang, J., Arsovska, D., Rodriguez, A., Jendresen, C. B., Skjodt, M. L., Nielsen, A. T., Borodina, I., Jensen, M. K., Keasling, J. D., 2015b. CasEMBLR: Cas9-facilitated multiloci genomic integration of in vivo assembled DNA parts in *Saccharomyces cerevisiae*. ACS Synth Biol. 4**,** 1226-34.

Jalak, J., Kurasin, M., Teugjas, H., Valjamae, P., 2012. Endo-exo synergism in cellulose hydrolysis revisited. J Biol Chem. 287**,** 28802-15.

Janes, M., Meyhack, B., Zimmermann, W., Hinnen, A., 1990. The influence of *GAP* promoter variants on hirudin production, average plasmid copy number and cell growth in *Saccharomyces cerevisiae*. Current Genetics. 18**,** 97-103.

Jayasekara, S., Ratnayake, R., 2019. Microbial cellulases: An overview and applications Cellulose.

Jeffries, T. W., 2006. Engineering yeasts for xylose metabolism. Curr Opin Biotechnol. 17**,** 320-6.

Jeffries, T. W., Jin, Y. S., 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol. 63**,** 495-509.

Jeoh, T., Ishizawa, C. I., Davis, M. F., Himmel, M. E., Adney, W. S., Johnson, D. K., 2007. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnology and Bioengineering. 98**,** 112-122.

Jeon, E., Hyeon, J., Eun, L. S., Park, B. S., Kim, S. W., Lee, J., Han, S. O., 2009a. Cellulosic alcoholic fermentation using recombinant *Saccharomyces cerevisiae* engineered for the production of *Clostridium cellulovorans* endoglucanase and *Saccharomycopsis fibuligera* beta-glucosidase. FEMS Microbiol Lett. 301**,** 130-6.

Jeon, E., Hyeon, J. E., Suh, D. J., Suh, Y. W., Kim, S. W., Song, K. H., Han, S. O., 2009b. Production of cellulosic ethanol in *Saccharomyces cerevisiae* heterologous expressing *Clostridium thermocellum* endoglucanase and *Saccharomycopsis fibuligera* ß-glucosidase genes. Mol Cells. 28**,** 369-73.

Jeppsson, M., Bengtsson, O., Franke, K., Lee, H., Hahn-Hagerdal, B., Gorwa-Grauslund, M. F., 2006. The expression of a *Pichia stipitis* xylose reductase mutant with higher K(M) for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng. 93**,** 665-73.

Jiang, T. T., Liang, Y., Zhou, X., Shi, Z. W., Xin, Z. J., 2019. Optimization of a pretreatment and hydrolysis process for the efficient recovery of recycled sugars and unknown compounds from agricultural sweet sorghum bagasse stem pith solid waste. PeerJ. 6**,** e6186.

Jin, M., Gunawan, C., Balan, V., Lau, M. W., Dale, B. E., 2012. Simultaneous saccharification and co-fermentation (SSCF) of AFEXTM pretreated corn stover for ethanol production using commercial enzymes and *Saccharomyces cerevisiae* 424A(LNH-ST). Bioresource Technology. 110**,** 587-594.

Johannes, C., Majcherczyk, A., 2000. Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol. 66**,** 524-528.

Johansen, K. S., 2016. Lytic polysaccharide monooxygenases: The microbial power tool for lignocellulose degradation. Trends Plant Sci. 21**,** 926-936.

Johnson, E., 2016. Integrated enzyme production lowers the cost of cellulosic ethanol. Biofuels, Bioproducts and Biorefining. 10**,** 164-174.

Johnson, K. G., Fontana, J. D., MacKenzie, C. R., 1988. Measurement of acetylxylan esterase in *Streptomyces*. Methods in Enzymology. vol. 160. Academic Press, pp. 551-560.

Jørgensen, H., Pinelo, M., 2017. Enzyme recycling in lignocellulosic biorefineries. Biofuels, Bioproducts and Biorefining. 11**,** 150-167.

Jorgensen, K., Rasmussen, A. V., Morant, M., Nielsen, A. H., Bjarnholt, N., Zagrobelny, M., Bak, S., Moller, B. L., 2005. Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr Opin Plant Biol. 8**,** 280-91.

Julius, D., Blair, L., Brake, A., Sprague, G., Thorner, J., 1983. Yeast alpha factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. Cell. 32**,** 839-52.

Jullesson, D., David, F., Pfleger, B., Nielsen, J., 2015. Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. Biotechnol Adv. 33**,** 1395-402.

Jung, G., Ueno, H., Hayashi, R., 1999. Carboxypeptidase Y: structural basis for protein sorting and catalytic triad. J Biochem. 126**,** 1-6.

Juturu, V., Wu, J. C., 2013. Insight into microbial hemicellulases other than xylanases: a review. Journal of Chemical Technology & Biotechnology. 88**,** 353-363.

Kaar, W. E., Gutierrez, C. V., Kinoshita, C. M., 1998. Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. Biomass and Bioenergy. 14**,** 277-287.

Kabel, M. A., Carvalheiro, F., Garrote, G., Avgerinos, E., Koukios, E., Parajó, J. C., Gírio, F. M., Schols, H. A., Voragen, A. G. J., 2002. Hydrothermally treated xylan rich by-products yield different classes of xylo-oligosaccharides. Carbohydrate Polymers. 50**,** 47-56.

Kafri, M., Metzl-Raz, E., Jona, G., Barkai, N., 2016. The cost of protein production. Cell Rep. 14**,** 22-31.

Kam, D. K., Jun, H. S., Ha, J. K., Inglis, G. D., Forsberg, C. W., 2005. Characteristics of adjacent family 6 acetylxylan esterases from *Fibrobacter succinogenes* and the interaction with the Xyn10E xylanase in hydrolysis of acetylated xylan. Can J Microbiol. 51**,** 821-32.

Karhumaa, K., Wiedemann, B., Hahn-Hägerdal, B., Boles, E., Gorwa-Grauslund, M.-F., 2006. Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. Microbial Cell Factories. 5**,** 18.

Kasana, R. C., Gulati, A., 2011. Cellulases from psychrophilic microorganisms: a review. J Basic Microbiol. 51**,** 572-9.

Katahira, S., Fujita, Y., Mizuike, A., Fukuda, H., Kondo, A., 2004. Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells. Appl Environ Microbiol. 70**,** 5407-14.

Katz, L., Brown, D. P., Donadio, S., 1991. Site-specific recombination in *Escherichia coli* between the att sites of plasmid pSE211 from *Saccharopolyspora erythraea*. Molecular and General Genetics MGG. 227**,** 155-159.

Kaushal, J. R., Kakkar, V. K., Malik, N. S., Makkar, G. S., 1981. Chemical composition of sugarcane bagasse and filter-mud and the effect of source and alkali treatment on the in vitro dry matter digestibility of bagasse Indian Journal of Dairy Science v. 34**,** 458-462.

Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. Curr Opin Biotechnol. 42**,** 30-39.

Kawahata, M., Masaki, K., Fujii, T., Iefuji, H., 2006. Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yeast Res. 6**,** 924-36.

Kawamoto, H., Nakatsubo, F., Murakami, K., 1992. Protein-adsorbing capacities of lignin samples. Journal of the Japan Wood Research Society. 38**,** 81-84.

Keasling, J. D., 2010. Manufacturing molecules through metabolic engineering. Science. 330**,** 1355-1358.

Kell, D. B., Swainston, N., Pir, P., Oliver, S. G., 2015. Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. Trends Biotechnol. 33**,** 237-46.

Kerppola, T. K., Serwold-Davis, T., Gross, D. C., Kahn, M. L., 1987. Effect of increased ß-glucosidase activity on virulence of *Erwinia amylovora*. Appl Environ Microbiol. 53**,** 677-682.

Khan, A. W., Lamb, K. A., Overend, R. P., 1990. Comparison of natural hemicellulose and chemically acetylated xylan as substrates for the determination of acetyl-xylan esterase activity in *Aspergilli*. Enzyme and Microbial Technology. 12**,** 127-131.

Khramtsov, N., McDade, L., Amerik, A., Yu, E., Divatia, K., Tikhonov, A., Minto, M., Kabongo-Mubalamate, G., Markovic, Z., Ruiz-Martinez, M., Henck, S., 2011. Industrial yeast strain engineered to ferment ethanol from lignocellulosic biomass. Bioresour Technol. 102**,** 8310-3.

Kim, D.-M., Nakazawa, H., Umetsu, M., Matsuyama, T., Ishida, N., Ikeuchi, A., Takahashi, H., Asano, R., Kumagai, I., 2012. A nanocluster design for the construction of artificial cellulosomes. Catalysis Science & Technology. 2.

Kim, H., Lee, W. H., Galazka, J. M., Cate, J. H., Jin, Y. S., 2014. Analysis of cellodextrin transporters from *Neurospora crassa* in *Saccharomyces cerevisiae* for cellobiose fermentation. Appl Microbiol Biotechnol. 98**,** 1087-94.

Kim, J. K., Oh, B. R., Shin, H.-J., Eom, C.-Y., Kim, S. W., 2008. Statistical optimization of enzymatic saccharification and ethanol fermentation using food waste. Process Biochemistry. 43**,** 1308-1312.

Kim, J. W., Kim, J., Seo, S. O., Kim, K. H., Jin, Y. S., Seo, J. H., 2016. Enhanced production of 2,3-butanediol by engineered *Saccharomyces cerevisiae* through fine-tuning of pyruvate decarboxylase and NADH oxidase activities. Biotechnol Biofuels. 9**,** 265.

Kim, J. W., Seo, S. O., Zhang, G. C., Jin, Y. S., Seo, J. H., 2015. Expression of *Lactococcus lactis* NADH oxidase increases 2,3-butanediol production in Pdc-deficient *Saccharomyces cerevisiae*. Bioresour Technol. 191**,** 512-9.

Kim, S., Hahn, J. S., 2015. Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing. Metab Eng. 31**,** 94-101.

Kim, S. J., Seo, S. O., Jin, Y. S., Seo, J. H., 2013. Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*. Bioresour Technol. 146**,** 274-81.

Kim, T. H., Lee, Y. Y., Sunwoo, C., Kim, J. S., 2006. Pretreatment of corn stover by low-liquid ammonia recycle percolation process. Applied Biochemistry and Biotechnology. 133**,** 41-57.

Kitamoto, N., Go, M., Shibayama, T., Kimura, T., Kito, Y., Ohmiya, K., Tsukagoshi, N., 1996. Molecular cloning, purification and characterization of two endo-1,4-beta-glucanases from *Aspergillus oryzae* KBN616. Appl Microbiol Biotechnol. 46**,** 538-44.

Klein, T., Lange, S., Wilhelm, N., Bureik, M., Yang, T. H., Heinzle, E., Schneider, K., 2014. Overcoming the metabolic burden of protein secretion in *Schizosaccharomyces pombe*-a quantitative approach using 13C-based metabolic flux analysis. Metab Eng. 21**,** 34-45.

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., Blanch, H. W., 2010. Technoeconomic analysis of biofuels: A wiki-based platform for lignocellulosic biorefineries. Biomass and Bioenergy. 34**,** 1914-1921.

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., Blanch, H. W., 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng. 109**,** 1083-7.

Kohchi, C., Toh-e, A., 1986. Cloning of *Candida pelliculosa* β-glucosidase gene and its expression in *Saccharomyces cerevisiae*. Molecular and General Genetics MGG. 203**,** 89-94.

Komor, R. S., Romero, P. A., Xie, C. B., Arnold, F. H., 2012. Highly thermostable fungal cellobiohydrolase I (Cel7A) engineered using predictive methods. Protein Engineering, Design and Selection. 25**,** 827-833.

Kont, R., Kurašin, M., Teugjas, H., Väljamäe, P., 2013. Strong cellulase inhibitors from the hydrothermal pretreatment of wheat straw. Biotechnology for biofuels. 6**,** 135-135.

Koppram, R., Albers, E., Olsson, L., 2012. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. Biotechnology for Biofuels. 5.

Kormelink, F. J. M., Lefebvre, B., Strozyk, F., Voragen, A. G. J., 1993. Purification and characterization of an acetyl xylan esterase from *Aspergillus niger*. Journal of Biotechnology. 27**,** 267-282.

Korona, B., Korona, D., Bielecki, S., 2006. Efficient expression and secretion of two co-produced xylanases from *Aspergillus niger* in *Pichia pastoris* directed by their native signal peptides and the *Saccharomyces cerevisiae* α-mating factor. Enzyme and Microbial Technology. 39**,** 683-689.

Koseki, T., Miwa, Y., Akao, T., Akita, O., Hashizume, K., 2006. An *Aspergillus oryzae* acetyl xylan esterase: molecular cloning and characteristics of recombinant enzyme expressed in *Pichia pastoris*. J Biotechnol. 121**,** 381-9.

Koseki, T., Miwa, Y., Fushinobu, S., Hashizume, K., 2005. Biochemical characterization of recombinant acetyl xylan esterase from *Aspergillus awamori* expressed in *Pichia pastoris*: mutational analysis of catalytic residues. Biochim Biophys Acta. 1749**,** 7-13.

Koskela, S., Wang, S., Xu, D., Yang, X., Li, K., Berglund, L. A., McKee, L. S., Bulone, V., Zhou, Q., 2019. Lytic polysaccharide monooxygenase (LPMO) mediated production of ultra-fine cellulose nanofibres from delignified softwood fibres. Green Chemistry. 21**,** 5924-5933.

Kostylev, M., Wilson, D., 2014. Synergistic interactions in cellulose hydrolysis. Biofuels. 3**,** 61-70.

Kosugi, A., Murashima, K., Doi, R. H., 2001. Characterization of xylanolytic enzymes in *Clostridium cellulovorans*: expression of xylanase activity dependent on growth substrates. J Bacteriol. 183**,** 7037-43.

Kosugi, A., Murashima, K., Doi, R. H., 2002. Xylanase and acetyl xylan esterase activities of XynA, a key subunit of the *Clostridium cellulovorans* cellulosome for xylan degradation. Appl Environ Microbiol. 68**,** 6399-402.

Kotaka, A., Bando, H., Kaya, M., Kato-Murai, M., Kuroda, K., Sahara, H., Hata, Y., Kondo, A., Ueda, M., 2008. Direct ethanol production from barley β-glucan by sake yeast displaying *Aspergillus oryzae* β-glucosidase and endoglucanase. Journal of Bioscience and Bioengineering. 105**,** 622-627.

Kötter, P., Ciriacy, M., 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 38**,** 776-783.

Kramer, S. J., Pochapin, M. B., 2012. Gastric phytobezoar dissolution with ingestion of diet coke and cellulase. Gastroenterol Hepatol (N Y). 8**,** 770-772.

Kristensen, J. B., Felby, C., Jorgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels. 2**,** 11.

Kroukamp, H., den Haan, R., van Wyk, N., van Zyl, W. H., 2013. Overexpression of native *PSE1* and *SOD1* in *Saccharomyces cerevisiae* improved heterologous cellulase secretion. Applied Energy. 102**,** 150-156.

Kroukamp, H., den Haan, R., van Zyl, J. H., van Zyl, W. H., 2017. Rational strain engineering interventions to enhance cellulase secretion by *Saccharomyces cerevisiae*. Biofuels, Bioproducts and Biorefining. 12**,** 108-124.

Kulkarni, N., Shendye, A., Rao, M., 1999. Molecular and biotechnological aspects of xylanases. FEMS Microbiology Reviews. 23**,** 411-456.

Kumar, L., Arantes, V., Chandra, R., Saddler, J., 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. Bioresour Technol. 103**,** 201-8.

Kumar, R., Singh, S., Singh, O. V., 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol. 35**,** 377-391.

Kumar, R., Wyman, C. E., 2009. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnology Progress. 25**,** 302-314.

Kumaran, R., Yang, S.-Y., Leu, J.-Y., 2013. Characterization of chromosome stability in diploid, polyploid and hybrid yeast cells. PLOS ONE. 8**,** e68094.

Kurland, C. G., 1992. Translational accuracy and the fitness of bacteria. Annu Rev Genet. 26**,** 29-50.

Kuyper, M., Hartog, M. M., Toirkens, M. J., Almering, M. J., Winkler, A. A., van Dijken, J. P., Pronk, J. T., 2005a. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res. 5**,** 399-409.

Kuyper, M., Toirkens, M. J., Diderich, J. A., Winkler, A. A., van Dijken, J. P., Pronk, J. T., 2005b. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res. 5**,** 925-34.

Kwak, S., Jin, Y. S., 2017. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. Microb Cell Fact. 16**,** 82.

La Grange, D. C., Pretorius, I. S., Claeyssens, M., van Zyl, W. H., 2001. Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* ß-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl Environ Microbiol. 67**,** 5512-5519.

La Grange, D. C., Pretorius, I. S., van Zyl, W. H., 1996. Expression of a *Trichoderma reesei* ß-xylanase gene (*XYN2*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 62**,** 1036-1044.

Laca, A., Laca, A., Díaz, M., 2019. Hydrolysis: From cellulose and hemicellulose to simple sugars. In: Basile, A., Dalena, F., Eds.), Second and Third Generation of Feedstocks. Elsevier, pp. 213-240.

Lang, G. I., Murray, A. W., Botstein, D., 2009. The cost of gene expression underlies a fitness trade-off in yeast. PNAS. 106**,** 5755-5760.

Lanza, A. M., Curran, K. A., Rey, L. G., Alper, H. S., 2014. A condition-specific codon optimization approach for improved heterologous gene expression in *Saccharomyces cerevisiae*. BMC Systems Biology. 8**,** 33.

Larsson, S., Nilvebrant, N. O., Jönsson, L., 2001. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Applied Microbiology and Biotechnology. 57**,** 167-174.

Lebbink, J. H. G., Kaper, T., Bron, P., van der Oost, J., de Vos, W. M., 2000. Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* ß-glucosidase CelB by directed evolution. Biochemistry. 39**,** 3656-3665.

Lee, C. R., Sung, B. H., Lim, K. M., Kim, M. J., Sohn, M. J., Bae, J. H., Sohn, J. H., 2017. Co-fermentation using recombinant *Saccharomyces cerevisiae* yeast strains hyper-secreting different cellulases for the production of cellulosic bioethanol. Sci Rep. 7**,** 4428.

Lee, J., 1997. Biological conversion of lignocellulosic biomass to ethanol. Journal of Biotechnology. 56**,** 1-24.

Lee, J., Saddler, J. N., Um, Y., Woo, H. M., 2016. Adaptive evolution and metabolic engineering of a cellobiose- and xylose-negative *Corynebacterium glutamicum* that co-utilizes cellobiose and xylose. Microb Cell Fact. 15**,** 20.

Lee, J. W., Gwak, K. S., Park, J. Y., Park, M. J., Choi, D. H., Kwon, M., Choi, I. G., 2007. Biological pretreatment of softwood *Pinus densiflora* by three white rot fungi. J Microbiol. 45**,** 485-91.

Lee, R. A., Lavoie, J.-M., 2013. From first- to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity. Animal Frontiers. 3**,** 6-11.

Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., Keasling, J. D., 2008. Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Curr Opin Biotechnol. 19**,** 556-63.

Lee, W. H., Nan, H., Kim, H. J., Jin, Y. S., 2013. Simultaneous saccharification and fermentation by engineered *Saccharomyces cerevisiae* without supplementing extracellular ß-glucosidase. J Biotechnol. 167**,** 316-22.

Lennartsson, P. R., Erlandsson, P., Taherzadeh, M. J., 2014. Integration of the first and second generation bioethanol processes and the importance of by-products. Bioresour Technol. 165**,** 3-8.

Levasseur, A., Asther, M., Record, E., 2005. Overproduction and characterization of xylanase B from *Aspergillus niger*. Can J Microbiol. 51**,** 177-83.

Lewis, J. A., Elkon, I. M., McGee, M. A., Higbee, A. J., Gasch, A. P., 2010. Exploiting natural variation in *Saccharomyces cerevisiae* to identify genes for increased ethanol resistance. Genetics. 186**,** 1197-205.

Li, S., Du, J., Sun, J., Galazka, J. M., Glass, N. L., Cate, J. H., Yang, X., Zhao, H., 2010. Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a ß-glucosidase in *Saccharomyces cerevisiae*. Mol Biosyst. 6**,** 2129-32.

Li, Y., Liu, N., Yang, H., Zhao, F., Yu, Y., Tian, Y., Lu, X., 2014. Cloning and characterization of a new β-glucosidase from a metagenomic library of rumen of cattle feeding with *Miscanthus sinensis*. BMC Biotechnology. 14.

Liang, Y., Sarkany, N., Cui, Y., 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett. 31**,** 1043-9.

Liang, Y., Si, T., Ang, E. L., Zhao, H., 2014. Engineered pentafunctional minicellulosome for simultaneous saccharification and ethanol fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 80**,** 6677-84.

Lieberman, R. L., Wustman, B. A., Huertas, P., Powe, A. C., Jr., Pine, C. W., Khanna, R., Schlossmacher, M. G., Ringe, D., Petsko, G. A., 2007. Structure of acid ß-glucosidase with pharmacological chaperone provides insight into Gaucher disease. Nat Chem Biol. 3**,** 101-7.

Lilly, M., Fierobe, H. P., van Zyl, W. H., Volschenk, H., 2009. Heterologous expression of a *Clostridium* minicellulosome in *Saccharomyces cerevisiae*. FEMS Yeast Res. 9**,** 1236-49.

Limayem, A., Ricke, S. C., 2012. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science. 38**,** 449-467.

Lin, X. Q., Liang, S. L., Han, S. Y., Zheng, S. P., Ye, Y. R., Lin, Y., 2013. Quantitative iTRAQ LC-MS/MS proteomics reveals the cellular response to heterologous protein overexpression and the regulation of *HAC1* in *Pichia pastoris*. J Proteomics. 91**,** 58-72.

Lin, Y., Zhang, W., Li, C., Sakakibara, K., Tanaka, S., Kong, H., 2012. Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. Biomass and Bioenergy. 47**,** 395-401.

Ling, H., Chen, B., Kang, A., Lee, J., Chang, M. W., 2013. Transcriptome response to alkane biofuels in *Saccharomyces cerevisiae* - Identification of efflux pumps involved in alkane tolerance. Biotechnology for Biofuels. 6.

Liu, E., Hu, Y., 2010. Construction of a xylose-fermenting *Saccharomyces cerevisiae* strain by combined approaches of genetic engineering, chemical mutagenesis and evolutionary adaptation. Biochemical Engineering Journal. 48**,** 204-210.

Liu, H., Liu, K., Yan, M., Xu, L., Ouyang, P., 2011. gTME for improved adaptation of *Saccharomyces cerevisiae* to corn cob acid hydrolysate. Appl Biochem Biotechnol. 164**,** 1150-9.

Liu, H., Sun, J., Chang, J. S., Shukla, P., 2018a. Engineering microbes for direct fermentation of cellulose to bioethanol. Crit Rev Biotechnol. 38**,** 1089-1105.

Liu, H., Xu, L., Yan, M., Lai, C., Ouyang, P., 2008. gTME for construction of recombinant yeast co-fermenting xylose and glucose. Chinese Journal of Biotechnology. 24**,** 1010-1015.

Liu, H., Yan, M., Lai, C., Xu, L., Ouyang, P., 2010. gTME for improved xylose fermentation of *Saccharomyces cerevisiae*. Appl Biochem Biotechnol. 160**,** 574-82.

Liu, J., Basit, A., Miao, T., Zheng, F., Yu, H., Wang, Y., Jiang, W., Cao, Y., 2018b. Secretory expression of ß-mannanase in *Saccharomyces cerevisiae* and its high efficiency for hydrolysis of mannans to mannooligosaccharides. Appl Microbiol Biotechnol. 102**,** 10027-10041.

Liu, L., Zhang, Z., Wang, J., Fan, Y., Shi, W., Liu, X., Shun, Q., 2019. Simultaneous saccharification and co-fermentation of corn stover pretreated by H2O2 oxidative degradation for ethanol production. Energy. 168**,** 946-952.

Liu, Z., Ho, S. H., Sasaki, K., den Haan, R., Inokuma, K., Ogino, C., van Zyl, W. H., Hasunuma, T., Kondo, A., 2016. Engineering of a novel cellulose-adherent cellulolytic *Saccharomyces cerevisiae* for cellulosic biofuel production. Sci Rep. 6**,** 24550.

Liu, Z., Inokuma, K., Ho, S.-H., den Haan, R., Hasunuma, T., van Zyl, W. H., Kondo, A., 2015. Combined cell-surface display- and secretion-based strategies for production of cellulosic ethanol with *Saccharomyces cerevisiae*. Biotechnology for Biofuels. 8**,** 162.

Liu, Z., Inokuma, K., Ho, S.-H., den Haan, R., van Zyl, W. H., Hasunuma, T., Kondo, A., 2017. Improvement of ethanol production from crystalline cellulose via optimizing cellulase ratios in cellulolytic *Saccharomyces cerevisiae*. Biotechnology and Bioengineering. 114**,** 1201-1207.

Liu, Z., Tyo, K. E., Martinez, J. L., Petranovic, D., Nielsen, J., 2012a. Different expression systems for production of recombinant proteins in *Saccharomyces cerevisiae*. Biotechnol Bioeng. 109**,** 1259-68.

Liu, Z.-H., Chen, H.-Z., 2016. Simultaneous saccharification and co-fermentation for improving the xylose utilization of steam exploded corn stover at high solid loading. Bioresource Technology. 201**,** 15-26.

Liu, Z. L., Li, H. N., Song, H. T., Xiao, W. J., Xia, W. C., Liu, X. P., Jiang, Z. B., 2018c. Construction of a trifunctional cellulase and expression in *Saccharomyces cerevisiae* using a fusion protein. BMC Biotechnol. 18**,** 43.

Liu, Z. L., Weber, S. A., Cotta, M. A., Li, S. Z., 2012b. A new ß-glucosidase producing yeast for lower-cost cellulosic ethanol production from xylose-extracted corncob residues by simultaneous saccharification and fermentation. Bioresour Technol. 104**,** 410-6.

Loibl, M., Strahl, S., 2013. Protein *O*-mannosylation: what we have learned from baker's yeast. Biochim Biophys Acta. 1833**,** 2438-46.

Lu, Y., Yang, B., Gregg, D., Saddler, J. N., Mansfield, S. D., 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Applied Biochemistry and Biotechnology. 98**,** 641-654.

Lynd, L. R., 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, economics, the environment, and policy. Annual Review of Energy and the Environment. 21**,** 403-465.

Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J. D., Sheehan, J., Wyman, C. E., 2008. How biotech can transform biofuels. Nature Biotechnology. 26**,** 169-172.

Lynd, L. R., Liang, X., Biddy, M. J., Allee, A., Cai, H., Foust, T., Himmel, M. E., Laser, M. S., Wang, M., Wyman, C. E., 2017. Cellulosic ethanol: status and innovation. Curr Opin Biotechnol. 45**,** 202-211.

Lynd, L. R., van Zyl, W. H., McBride, J. E., Laser, M., 2005. Consolidated bioprocessing of cellulosic biomass: an update. Current Opinion in Biotechnology. 16**,** 577-583.

Ma, L., Zhang, J., Zou, G., Wang, C., Zhou, Z., 2011. Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. Enzyme Microb Technol. 49**,** 366-71.

Mach, R. L., Seiboth, B., Myasnikov, A., Gonzalez, R., Strauss, J., Harkki, A. M., Kubicek, C. P., 1995. The bgl1 gene of *Trichoderma reesei* QM 9414 encodes an extracellular, cellulose-inducible β-glucosidase involved in cellulase induction by sophorose. Molecular Microbiology. 16**,** 687-697.

Machida, M., Ohtsuki, I., Fukui, S., Yamashita, I., 1988. Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular ß-glucosidases as expressed in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 54**,** 3147-3155.

Madson, P. W., Lococo, D. B., 2000. Recovery of volatile products from dilute high-fouling process streams. Applied Biochemistry and Biotechnology. 84**,** 1049-1061.

Magrey, A., Sahay, S., Gothalwal, R., 2018. Cellulases for biofuel: A review. International Journal of Recent Trends in Science And Technology. 17-25.

Makanae, K., Kintaka, R., Makino, T., Kitano, H., Moriya, H., 2013. Identification of dosage-sensitive genes in *Saccharomyces cerevisiae* using the genetic tug-of-war method. Genome Res. 23**,** 300-11.

Malgas, S., van Dyk, J. S., Pletschke, B. I., 2015. A review of the enzymatic hydrolysis of mannans and synergistic interactions between ß-mannanase, ß-mannosidase and alpha-galactosidase. World J Microbiol Biotechnol. 31**,** 1167-75.

Maly, R. R., Degen, W., 2001. Trends in future fuels for mobile applications.

Mandels, M., Reese, E. T., 1965. Inhibition of cellulases. Annual Review of Phytopathology. 3**,** 85-102.

Mans, R., van Rossum, H. M., Wijsman, M., Backx, A., Kuijpers, N. G., van den Broek, M., Daran-Lapujade, P., Pronk, J. T., van Maris, A. J., Daran, J. M., 2015. CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15.

Martinez, A. T., Speranza, M., Ruiz-Duenas, F. J., Ferreira, P., Camarero, S., Guillen, F., Martinez, M. J., Gutierrez, A., del Rio, J. C., 2005. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol. 8**,** 195-204.

Martinez-Martinez, I., Montoro-Garcia, S., Lozada-Ramirez, J. D., Sanchez-Ferrer, A., Garcia-Carmona, F., 2007. A colorimetric assay for the determination of acetyl xylan esterase or cephalosporin C acetyl esterase activities using 7-amino cephalosporanic acid, cephalosporin C, or acetylated xylan as substrate. Anal Biochem. 369**,** 210-7.

Matano, Y., Hasunuma, T., Kondo, A., 2012. Display of cellulases on the cell surface of *Saccharomyces cerevisiae* for high yield ethanol production from high-solid lignocellulosic biomass. Bioresour Technol. 108**,** 128-33.

Matano, Y., Hasunuma, T., Kondo, A., 2013. Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing. Bioresour Technol. 135**,** 403-9.

Mathew, A. K., Parameshwaran, B., Sukumaran, R. K., Pandey, A., 2016. An evaluation of dilute acid and ammonia fiber explosion pretreatment for cellulosic ethanol production. Bioresource Technology. 199**,** 13-20.

McClendon, S. D., Mao, Z., Shin, H.-D., Wagschal, K., Chen, R. R., 2012. Designer xylanosomes: Protein nanostructures for enhanced xylan hydrolysis. Applied Biochemistry and Biotechnology. 167**,** 395-411.

McMillan, J. D., Newman, M. M., Templeton, D. W., Mohagheghi, A., 1999. Simultaneous saccharification and cofermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. Applied Biochemistry and Biotechnology. 79**,** 649-665.

Meijnen, J. P., Randazzo, P., Foulquie-Moreno, M. R., van den Brink, J., Vandecruys, P., Stojiljkovic, M., Dumortier, F., Zalar, P., Boekhout, T., Gunde-Cimerman, N., Kokosar, J., Stajdohar, M., Curk, T., Petrovic, U., Thevelein, J. M., 2016. Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels. 9**,** 5.

Mellitzer, A., Glieder, A., Weis, R., Reisinger, C., Flicker, K., 2012. Sensitive high-throughput screening for the detection of reducing sugars. Biotechnol J. 7**,** 155-62.

Meng, X., Ragauskas, A. J., 2014. Recent advances in understanding the role of cellulose accessibility in enzymatic hydrolysis of lignocellulosic substrates. Curr Opin Biotechnol. 27**,** 150-8.

Meyer, H. A., Hartmann, E., 1997. The yeast SPC22/23 homolog Spc3p is essential for signal peptidase activity. J Biol Chem. 272**,** 13159-64.

Michele, P. C., 2006. The phiC31 integrase system for gene therapy. Current Gene Therapy. 6**,** 633-645.

Millati, R., Niklasson, C., Taherzadeh, J., 2002. Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by *Saccharomyces cerevisiae*. Process Biochemistry. 38**,** 515-522.

Millward-Sadler, S. J., Poole, D. M., Henrissat, B., Hazlewood, G. P., Clarke, J. H., Gilbert, H. J., 1994. Evidence for a general role for high-affinity non-catalytic cellulose binding domains in microbial plant cell wall hydrolases. Mol Microbiol. 11**,** 375-82.

Mira, N. P., Becker, J. D., Sa-Correia, I., 2010a. Genomic expression program involving the Haa1p-regulon in *Saccharomyces cerevisiae* response to acetic acid. OMICS. 14**,** 587-601.
Mira, N. P., Palma, M., Guerreiro, J. F., Sa-Correia, I., 2010b. Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microbial Cell Factories. 9.
Mira, N. P., Teixeira, M. C., Sa-Correia, I., 2010c. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. OMICS. 14**,** 525-40.
Mitchell, D. J., Grohmann, K., Himmel, M. E., Dale, B. E., Schroeder, H. A., 1990. Effect of the degree of acetylation on the enzymatic digestion of acetylated xylans. Journal of Wood Chemistry and Technology. 10**,** 111-121.
Modenbach, A. A., Nokes, S. E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings – A review. Biomass and Bioenergy. 56**,** 526-544.
Mohagheghi, A., Evans, K., Chou, Y. C., Zhang, M., 2002. Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of *Zymomonas mobilis* AX101. Appl Biochem Biotechnol. 98-100**,** 885-98.
Mohr, A., Raman, S., 2013. Lessons from first generation biofuels and implications for the sustainability appraisal of second generation biofuels. Energy Policy. 63**,** 114-122.
Morales-Rodriguez, R., Gernaey, K. V., Meyer, A. S., Sin, G., 2011. A mathematical model for simultaneous saccharification and co-fermentation (SSCF) of C6 and C5 sugars. Chinese Journal of Chemical Engineering. 19**,** 185-191.
Moreira, L. R., Filho, E. X., 2008. An overview of mannan structure and mannan-degrading enzyme systems. Appl Microbiol Biotechnol. 79**,** 165-78.
Mori, A., Hara, S., Sugahara, T., Kojima, T., Iwasaki, Y., Kawarasaki, Y., Sahara, T., Ohgiya, S., Nakano, H., 2015. Signal peptide optimization tool for the secretion of recombinant protein from *Saccharomyces cerevisiae*. J Biosci Bioeng. 120**,** 518-25.
Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol. 96**,** 673-86.
Motta, F. L., Andrade, C. C. P., Santana, M. H. A., 2013. A review of xylanase production by the fermentation of xylan: classification, characterization and applications. Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization. pp. 251-275.
Moyses, D. N., Reis, V. C., de Almeida, J. R., de Moraes, L. M., Torres, F. A., 2016. Xylose fermentation by *Saccharomyces cerevisiae*: Challenges and prospects. Int J Mol Sci. 17**,** 207.
Mukai, N., Masaki, K., Fujii, T., Kawamukai, M., Iefuji, H., 2010. *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. J Biosci Bioeng. 109**,** 564-9.
Murai, T., Ueda, M., Atomi, H., Shibasaki, Y., Kamasawa, N., Osumi, M., Kawaguchi, T., Arai, M., Tanaka, A., 1997. Genetic immobilization of cellulase on the cell surface of *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 48**,** 499-503.
Murphy, L., Bohlin, C., Baumann, M. J., Olsen, S. N., Sørensen, T. H., Anderson, L., Borch, K., Westh, P., 2013. Product inhibition of five *Hypocrea jecorina* cellulases. Enzyme and Microbial Technology. 52**,** 163-169.
Musunuru, K., 2017. The hope and hype of CRISPR-Cas9 genome editing: A review. JAMA Cardiol. 2**,** 914-919.
Naik, S. N., Goud, V. V., Rout, P. K., Dalai, A. K., 2010. Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews. 14**,** 578-597.
Nakatani, Y., Yamada, R., Ogino, C., Kondo, A., 2013. Synergetic effect of yeast cell-surface expression of cellulase and expansin-like protein on direct ethanol production from cellulose. Microbial Cell Factories. 12**,** 66.
Narendranath, N. V., Thomas, K. C., Ingledew, W. M., 2001. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in minimal medium. Journal of Industrial Microbiology and Biotechnology. 26**,** 171-177.
Nevalainen, K. M., Te'o, V. S., Bergquist, P. L., 2005. Heterologous protein expression in filamentous fungi. Trends Biotechnol. 23**,** 468-74.
Neves, M., Kimura, T., Shimizu, N., Nakajima, M., 2007. State of the art and future trends of bioethanol production, dynamic biochemistry, process biotechnology and molecular biology. Global Science Books. 1-13.
Nevoigt, E., 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev. 72**,** 379-412.
Ng, C. Y., Jung, M., Lee, J., Oh, M., 2012. Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. Microbial Cell Factories. 11.
Nicolaou, S. A., Gaida, S. M., Papoutsakis, E. T., 2010. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng. 12**,** 307-31.

Nidetzky, B., Steiner, W., Hayn, M., Claeyssens, M., 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. Biochem J. 298 Pt 3**,** 705-710.

Nissen, T. L., Anderlund, M., Nielsen, J., Villadsen, J., Kielland-Brandt, M. C., 2001. Expression of a cytoplasmic transhydrogenase in *Saccharomyces cerevisiae* results in formation of 2-oxoglutarate due to depletion of the NADPH pool. Yeast. 18**,** 19-32.

Njokweni, A. P., Rose, S. H., van Zyl, W. H., 2012. Fungal ß-glucosidase expression in *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol. 39**,** 1445-52.

Obeng, E. M., Adam, S. N. N., Budiman, C., Ongkudon, C. M., Maas, R., Jose, J., 2017. Lignocellulases: a review of emerging and developing enzymes, systems, and practices. Bioresources and Bioprocessing. 4.

Öhgren, K., Bengtsson, O., Gorwa-Grauslund, M. F., Galbe, M., Hahn-Hägerdal, B., Zacchi, G., 2006. Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. Journal of Biotechnology. 126**,** 488-498.

Okamura, K., Kimata, Y., Higashio, H., Tsuru, A., Kohno, K., 2000. Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. Biochemical and Biophysical Research Communications. 279**,** 445-450.

Olson, D. G., McBride, J. E., Shaw, A. J., Lynd, L. R., 2012. Recent progress in consolidated bioprocessing. Curr Opin Biotechnol. 23**,** 396-405.

Olsson, L., Soerensen, H. R., Dam, B. P., Christensen, H., Krogh, K. M., Meyer, A. S., 2006. Separate and simultaneous enzymatic hydrolysis and fermentation of wheat hemicellulose with recombinant xylose utilizing *Saccharomyces cerevisiae*. Appl Biochem Biotechnol. 129-132**,** 117-29.

Orr-Weaver, T. L., Szostak, J. W., Rothstein, R. J., 1981. Yeast transformation: a model system for the study of recombination. Proceedings of the National Academy of Sciences of the United States of America. 78**,** 6354-6358.

Pai, C. K., Wu, Z. Y., Chen, M. J., Zeng, Y. F., Chen, J. W., Duan, C. H., Li, M. L., Liu, J. R., 2010. Molecular cloning and characterization of a bifunctional xylanolytic enzyme from *Neocallimastix patriciarum*. Appl Microbiol Biotechnol. 85**,** 1451-62.

Palmqvist, E., Grage, H., Meinander, N. Q., Hahn-Haegerdal, B., 1999. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnology and Bioengineering. Medium: X; Size: pp. 146-155.

Palmqvist, E., Hahn-Hägerdal, B., 2000a. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technology. 74**,** 17-24.

Palmqvist, E., Hahn-Hägerdal, B., 2000b. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology. 74**,** 25-33.

Panda, T., Gowrishankar, B. S., 2005. Production and applications of esterases. Appl Microbiol Biotechnol. 67**,** 160-9.

Parajó, J. C., Domínguez, H., Domínguez, J. M., 1998. Biotechnological production of xylitol. Part 1: interest of xylitol and fundamentals of its biosynthesis. Bioresour Technol. 65**,** 191-201.

Pareek, N., Gillgren, T., Jönsson, L. J., 2013. Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses. Bioresource Technology. 148**,** 70-77.

Parisutham, V., Kim, T. H., Lee, S. K., 2014. Feasibilities of consolidated bioprocessing microbes: From pretreatment to biofuel production. Bioresource Technology. 161**,** 431-440.

Patel, S. J., Onkarappa, R., Shobha, K. S., 2007. Fungal pretreatment studies on rice husk and bagasse for ethanol production. Electronic Journal of Environmental, Agricultural and Food Chemistry. 6**,** 1921-1926.

Patil, C., Walter, P., 2001. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Current Opinion in Cell Biology. 13**,** 349-355.

Pawar, P. M.-A., Derba-Maceluch, M., Chong, S. L., Gomez, L. D., Miedes, E., Banasiak, A., Ratke, C., Gaertner, C., Mouille, G., McQueen-Mason, S. J., Molina, A., Sellstedt, A., Tenkanen, M., Mellerowicz, E. J., 2016. Expression of fungal acetyl xylan esterase in *Arabidopsis thaliana* improves saccharification of stem lignocellulose. Plant Biotechnol J. 14**,** 387-97.

Payne, T., Finnis, C., Evans, L. R., Mead, D. J., Avery, S. V., Archer, D. B., Sleep, D., 2008. Modulation of chaperone gene expression in mutagenized *Saccharomyces cerevisiae* strains developed for recombinant human albumin production results in increased production of multiple heterologous proteins. Appl Environ Microbiol. 74**,** 7759-66.

Peng, L., Chen, Y., 2011. Conversion of paper sludge to ethanol by separate hydrolysis and fermentation (SHF) using *Saccharomyces cerevisiae*. Biomass and Bioenergy. 35**,** 1600-1606.

Penttilä, M., 1998. Heterologous protein production in *Trichoderma*. Trichoderma & Gliocladium. vol. 2. CRC Press, pp. 365-382.

Penttilä, M. E., André, L., Lehtovaara, P., Bailey, M., Teeri, T. T., Knowles, J. K. C., 1988. Efficient secretion of two fungal cellobiohydrolases by *Saccharomyces cerevisiae*. Gene. 63**,** 103-112.

Perez, J., Munoz-Dorado, J., de la Rubia, T., Martinez, J., 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol. 5**,** 53-63.

Pérez-González, J. A., van Peij, N. N. M. E., Bezoen, A., Maccabe, A. P., Ramón, D., de Graaff, L. H., 1998. Molecular cloning and transcriptional regulation of the *Aspergillus nidulans* xlnD gene encoding a β-xylosidase. Appl Environ Microbiol. 64**,** 1412-1419.

Philippidis, G. P., Smith, T. K., 1995. Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol. Applied Biochemistry and Biotechnology. 51**,** 117-124.

Pinel, D., D'Aoust, F., del Cardayre, S. B., Bajwa, P. K., Lee, H., Martin, V. J., 2011. *Saccharomyces cerevisiae* genome shuffling through recursive population mating leads to improved tolerance to spent sulfite liquor. Appl Environ Microbiol. 77**,** 4736-43.

Piper, R. C., Bryant, N. J., Stevens, T. H., 1997. The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. The Journal of cell biology. 138**,** 531-545.

Pool, M. R., 2005. Signal recognition particles in chloroplasts, bacteria, yeast and mammals (Review). Molecular Membrane Biology. 22**,** 3-15.

Poutanen, K., Puls, J., 1989. The xylanolytic enzyme system of *Trichoderma reesei*. Plant Cell Wall Polymers. vol. 399. American Chemical Society, pp. 630-640.

Poutanen, K., Sundberg, M., Korte, H., Puls, J., 1990. Deacetylation of xylans by acetyl esterases of *Trichoderma reesei*. Applied Microbiology and Biotechnology. 33**,** 506-510.

Poutanen, K., Tenkanen, M., Korte, H., Puls, J., 1991. Accessory enzymes involved in the hydrolysis of xylans. Enzymes in Biomass Conversion. vol. 460. American Chemical Society, pp. 426-436.

Pouvreau, L., Jonathan, M. C., Kabel, M. A., Hinz, S. W. A., Gruppen, H., Schols, H. A., 2011. Characterization and mode of action of two acetyl xylan esterases from *Chrysosporium lucknowense* C1 active towards acetylated xylans. Enzyme and Microbial Technology. 49**,** 312-320.

Preuss, D., Mulholland, J., Franzusoff, A., Segev, N., Botstein, D., 1992. Characterization of the *Saccharomyces* Golgi complex through the cell cycle by immunoelectron microscopy. Mol Biol Cell. 3**,** 789-803.

Prima, A., Hara, K. Y., Djohan, A. C., Kashiwagi, N., Kahar, P., Ishii, J., Nakayama, H., Okazaki, F., Prasetya, B., Kondo, A., Yopi, Ogino, C., 2017. Glutathione production from mannan-based bioresource by mannanase/mannosidase expressing *Saccharomyces cerevisiae*. Bioresour Technol. 245**,** 1400-1406.

Puls, J., 1997. Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis. Macromolecular Symposia. 120**,** 183-196.

Puls, J., Tenkanen, M., Korte, H., Poutanen, K., 1991. Products of hydrolysis of beechwood acetyl-4-O-methylglucuronoxylan by a xylanase and an acetyl xylan esterase. Enzyme and Microbial Technology. 13**,** 483-486.

Qi, M., Jun, H. S., Forsberg, C. W., 2007. Characterization and synergistic interactions of *Fibrobacter succinogenes* glycoside hydrolases. Appl Environ Microbiol. 73**,** 6098-105.

Qing, Q., Yang, B., Wyman, C. E., 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresour Technol. 101**,** 9624-30.

Quinlan, R. J., Sweeney, M. D., Lo Leggio, L., Otten, H., Poulsen, J. C., Johansen, K. S., Krogh, K. B., Jorgensen, C. I., Tovborg, M., Anthonsen, A., Tryfona, T., Walter, C. P., Dupree, P., Xu, F., Davies, G. J., Walton, P. H., 2011. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci U S A. 108**,** 15079-84.

Ragauskas, A. J., Beckham, G. T., Biddy, M. J., Chandra, R., Chen, F., Davis, M. F., Davison, B. H., Dixon, R. A., Gilna, P., Keller, M., Langan, P., Naskar, A. K., Saddler, J. N., Tschaplinski, T. J., Tuskan, G. A., Wyman, C. E., 2014. Lignin valorization: improving lignin processing in the biorefinery. Science. 344**,** 1246843.

Rajoka, M. I., Bashir, A., Hussain, S. R. A., Ghauri, M. T., Parvez, R., Malik, K. A., 1998. Cloning and expression of β-glucosidase genes in *Escherichia coli* and *Saccharomyces cerevisiae* using shuttle vector pYES 2.0. Folia Microbiologica. 43**,** 129-135.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., Zhang, F., 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 8**,** 2281-2308.

Ranatunga, T. D., Jervis, J., Helm, R. F., McMillan, J. D., Wooley, R. J., 2000. The effect of overliming on the toxicity of dilute acid pretreated lignocellulosics- the role of inorganics, uronic acids and ether-soluble organics. Enzyme and Microbial Technology. 27**,** 240-247.

Rani, V., S., M., Tiwari, R., Nain, L., Arora, A., 2015. Beta-glucosidase: Key enzyme in determining efficiency of cellulase and biomass hydrolysis. Journal of Bioprocessing & Biotechniques. 05.

Rasmussen, H., Sorensen, H. R., Meyer, A. S., 2014. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms. Carbohydr Res. 385**,** 45-57.

Ratanakhanokchai, K., Waeonukul, R., Pason, P., Tachaapaikoon, C., Lay, K., Sakka, K., Kosugi, A., Mori, Y., 2013. *Paenibacillus curdlanolyticus* strain B-6 multienzyme complex: A novel system for biomass utilization. Biomass Now - Cultivation and Utilization.

Rausch, H., Lehmann, M., 1991. Structural analysis of the actinophage phiC31 attachment site. Nucleic Acids Res. 19**,** 5187-5189.

Raynal, A., Guerineau, M., 1984. Cloning and expression of the structural gene for β-glucosidase of *Kluyveromyces fragilis* in *Escherichia coli* and *Saccharomyces cerevisiae*. Molecular and General Genetics MGG. 195**,** 108-115.

Remize, F., Roustan, J. L., Sablayrolles, J. M., Barre, P., Dequin, S., 1999. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. Appl Environ Microbiol. 65**,** 143-149.

Rennie, E. A., Scheller, H. V., 2014. Xylan biosynthesis. Curr Opin Biotechnol. 26**,** 100-7.

Resch, M. G., Donohoe, B. S., Baker, J. O., Decker, S. R., Bayer, E. A., Beckham, G. T., Himmel, M. E., 2013. Fungal cellulases and complexed cellulosomal enzymes exhibit synergistic mechanisms in cellulose deconstruction. Energy & Environmental Science. 6**,** 1858-1867.

Richard, P., Verho, R., Putkonen, M., Londesborough, J., Penttila, M., 2003. Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway. FEMS Yeast Research. 3**,** 185-189.

Rico, A., Rencoret, J., del Río, J. C., Martínez, A. T., Gutiérrez, A., 2014. Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of *Eucalyptus* feedstock. Biotechnology for Biofuels. 7**,** 6.

Rizzi, M., Harwart, K., Erlemann, P., Bui-Thanh, N., Dellweg, H., 1989. Purification and properties of the NAD+-xylitol-dehydrogenase from the yeast *Pichia stipitis*. Journal of Fermentation and Bioengineering. 67**,** 20-24.

Robak, K., Balcerek, M., 2018. Review of second generation bioethanol production from residual biomass. Food Technol Biotechnol. 56**,** 174-187.

Roberfroid, M. B., 1997. Health benefits of non-digestible oligosaccharides. In: Kritchevsky, D., Bonfield, C., Eds.), Dietary Fiber in Health and Disease. Springer US, Boston, MA, pp. 211-219.

Rocha, G. J. M., Gonçalves, A. R., Oliveira, B. R., Olivares, E. G., Rossell, C. E. V., 2012. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. Industrial Crops and Products. 35**,** 274-279.

Rockwell, N. C., Krysan, D. J., Komiyama, T., Fuller, R. S., 2002. Precursor processing by kex2/furin proteases. Chem Rev. 102**,** 4525-48.

Roth, J., Zuber, C., Park, S., Jang, I., Lee, Y., Kysela, K. G., Le Fourn, V., Santimaria, R., Guhl, B., Cho, J. W., 2010. Protein N-glycosylation, protein folding, and protein quality control. Mol Cells. 30**,** 497-506.

Roze, L. V., Chanda, A., Linz, J. E., 2011. Compartmentalization and molecular traffic in secondary metabolism: a new understanding of established cellular processes. Fungal Genet Biol. 48**,** 35-48.

Ryan, O. W., Skerker, J. M., Maurer, M. J., Li, X., Tsai, J. C., Poddar, S., Lee, M. E., DeLoache, W., Dueber, J. E., Arkin, A. P., Cate, J. H., 2014. Selection of chromosomal DNA libraries using a multiplex CRISPR system. Elife. 3.

Sadie, C. J., Rose, S. H., den Haan, R., van Zyl, W. H., 2011. Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. 90**,** 1373-80.

Saha, B. C., 2003. Hemicellulose bioconversion. J Ind Microbiol Biotechnol. 30**,** 279-91.

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V., 2005. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol Prog. 21**,** 816-22.

Saini, J. K., Saini, R., Tewari, L., 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech. 5**,** 337-353.

Sakamoto, T., Hasunuma, T., Hori, Y., Yamada, R., Kondo, A., 2012. Direct ethanol production from hemicellulosic materials of rice straw by use of an engineered yeast strain codisplaying three types of hemicellulolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells. J Biotechnol. 158**,** 203-10.

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez, C., 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv. 27**,** 185-94.

Sanchez, O. J., Cardona, C. A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol. 99**,** 5270-95.

Sardonini, C. A., DiBiasio, D., 1987. A model for growth of *Saccharomyces cerevisiae* containing a recombinant plasmid in selective media. Biotechnology and Bioengineering. 29**,** 469-475.

Saritha, M., Arora, A., Lata, 2012. Biological pretreatment of lignocellulosic substrates for enhanced delignification and enzymatic digestibility. Indian J Microbiol. 52**,** 122-30.
Sarkar, N., Ghosh, S. K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: An overview. Renewable Energy. 37**,** 19-27.
Sauer, B., 1987. Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol. 7**,** 2087-2096.
Sauer, U., 2001. Evolutionary engineering of industrially important microbial phenotypes. In: Nielsen, J., Eggeling, L., Dynesen, J., Gárdonyi, M., Gill, R. T., de Graaf, A. A., Hahn-Hägerdal, B., Jönsson, L. J., Khosla, C., Licari, R., McDaniel, R., McIntyre, M., Miiller, C., Nielsen, J., Cordero Otero, R. R., Sahm, H., Sauer, U., Stafford, D. E., Stephanopoulos, G., Wahlbom, C. E., Yanagimachi, K. S., van Zyl, W. H., Eds.), Metabolic Engineering. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 129-169.
Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., Fischer, E., 2004. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. J Biol Chem. 279**,** 6613-9.
Scheller, H. V., Ulvskov, P., 2010. Hemicelluloses. Annu Rev Plant Biol. 61**,** 263-89.
Schubert, C., 2006. Can biofuels finally take center stage? Nature Biotechnology. 24**,** 777-784.
Sclimenti, C. R., Thyagarajan, B., Calos, M. P., 2001. Directed evolution of a recombinase for improved genomic integration at a native human sequence. Nucleic Acids Res. 29**,** 5044-51.
Scott, M., Gunderson, C. W., Mateescu, E. M., Zhang, Z., Hwa, T., 2010. Interdependence of cell growth and gene expression - Origins and consequences. Science. 330**,** 1099-1102.
Scott, M., Hwa, T., 2011. Bacterial growth laws and their applications. Curr Opin Biotechnol. 22**,** 559-65.
Scott, M., Klumpp, S., Mateescu, E. M., Hwa, T., 2014. Emergence of robust growth laws from optimal regulation of ribosome synthesis. Mol Syst Biol. 10**,** 747.
Selig, M. J., Adney, W. S., Himmel, M. E., Decker, S. R., 2009. The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. Cellulose. 16**,** 711-722.
Selig, M. J., Knoshaug, E. P., Adney, W. S., Himmel, M. E., Decker, S. R., 2008a. Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Bioresource Technology. 99**,** 4997-5005.
Selig, M. J., Knoshaug, E. P., Decker, S. R., Baker, J. O., Himmel, M. E., Adney, W. S., 2008b. Heterologous expression of *Aspergillus niger* β-d-Xylosidase (XlnD): characterization on lignocellulosic substrates. Applied Biochemistry and Biotechnology. 146**,** 57-68.
Selmecki, A. M., Maruvka, Y. E., Richmond, P. A., Guillet, M., Shoresh, N., Sorenson, A. L., De, S., Kishony, R., Michor, F., Dowell, R., Pellman, D., 2015. Polyploidy can drive rapid adaptation in yeast. Nature. 519**,** 349-352.
Shamu, C. E., Cox, J. S., Walter, P., 1994. The unfolded-protein-response pathway in yeast. Trends in Cell Biology. 4**,** 56-60.
Shapouri, H., Gallagher, P., USDA's 2002 Ethanol cost-of-production survey. Vol. Agriculture Economic Report Number 841. United States Department of Agriculture, 2005.
Sharma, H. K., Xu, C., Qin, W., 2019. Biological pretreatment of lignocellulosic biomass for biofuels and bioproducts: An overview. Waste and Biomass Valorization. 10**,** 235-251.
Sharma, R., Sharma, P. C., Rana, J. C., Joshi, V. K., 2015. Improving the olive oil yield and quality through enzyme-assisted mechanical extraction, antioxidants and packaging. Journal of Food Processing and Preservation. 39**,** 157-166.
Shen, Y., Zhang, Y., Ma, T., Bao, X., Du, F., Zhuang, G., Qu, Y., 2008. Simultaneous saccharification and fermentation of acid-pretreated corncobs with a recombinant *Saccharomyces cerevisiae* expressing ß-glucosidase. Bioresour Technol. 99**,** 5099-103.
Sheng, J., Feng, X., 2015. Metabolic engineering of yeast to produce fatty acid-derived biofuels: bottlenecks and solutions. Front Microbiol. 6**,** 554.
Shi, D. J., Wang, C. L., Wang, K. M., 2009. Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol. 36**,** 139-47.
Shi, S., Liang, Y., Zhang, M. M., Ang, E. L., Zhao, H., 2016. A highly efficient single-step, markerless strategy for multi-copy chromosomal integration of large biochemical pathways in *Saccharomyces cerevisiae*. Metab Eng. 33**,** 19-27.
Siddiqui, M. S., Thodey, K., Trenchard, I., Smolke, C. D., 2012. Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. FEMS Yeast Res. 12**,** 144-70.
Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D., Osborne, J., 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour Technol. 98**,** 3000-11.
Sims, R. E., Mabee, W., Saddler, J. N., Taylor, M., 2010. An overview of second generation biofuel technologies. Bioresour Technol. 101**,** 1570-80.

Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass – An overview. Bioresource Technology. 199**,** 76-82.

Singh, P., Suman, A., Tiwari, P., Arya, N., Gaur, A., Shrivastava, A. K., 2008. Biological pretreatment of sugarcane trash for its conversion to fermentable sugars. World Journal of Microbiology and Biotechnology. 24**,** 667-673.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., Pandey, A., 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol. 127**,** 500-7.

Singleton, C., Howard, T. P., Smirnoff, N., 2014. Synthetic metabolons for metabolic engineering. J Exp Bot. 65**,** 1947-54.

Skory, C. D., Freer, S. N., Bothast, R. J., 1996. Expression and secretion of the *Candida wickerhamii* extracellular β-glucosidase gene, *bglB*, in *Saccharomyces cerevisiae*. Current Genetics. 30**,** 417-422.

Smith, J., van Rensburg, E., Görgens, J. F., 2014. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. BMC Biotechnology. 14.

Sonderegger, M., Sauer, U., 2003. Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl Environ Microbiol. 69**,** 1990-1998.

Spiro, R. G., 2002. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology. 12**,** 43r-56r.

Srere, P. A., 1987. Complexes of sequential metabolic enzymes. Ann. Rev. Biochem. 56**,** 89-124.

Srienc, F., Campbell, J. L., Bailey, J. E., 1986. Analysis of unstable recombinant *Saccharomyces cerevisiae* population growth in selective medium. Biotechnology and Bioengineering. 28**,** 996-1006.

Srikrishnan, S., Chen, W., Da Silva, N. A., 2013. Functional assembly and characterization of a modular xylanosome for hemicellulose hydrolysis in yeast. Biotechnol Bioeng. 110**,** 275-85.

Stampe, E., Alcock, R., Westby, C., Chisholm, T., 1983. Energy consumption of a farm-scale ethanol distillation system. Energy in Agriculture. 2**,** 355-368.

Stanley, D., Fraser, S., Chambers, P. J., Rogers, P., Stanley, G. A., 2010. Generation and characterisation of stable ethanol-tolerant mutants of *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol. 37**,** 139-49.

Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, M., Voordeckers, K., Verstrepen, K. J., 2014. Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev. 38**,** 947-95.

Stephanopoulos, G., 2007. Challenges in engineering microbes for biofuels production. Science. 315**,** 801-804.

Stephen, J. D., Mabee, W. E., Saddler, J. N., 2012. Will second-generation ethanol be able to compete with first-generation ethanol? Opportunities for cost reduction. Biofuels, Bioproducts and Biorefining. vol. 6, pp. 159-176.

Stovicek, V., Borodina, I., Forster, J., 2015. CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains. Metabolic Engineering Communications. 2**,** 13-22.

Stovicek, V., Holkenbrink, C., Borodina, I., 2017. CRISPR/Cas system for yeast genome engineering: advances and applications. FEMS Yeast Research. 17.

Strahl-Bolsinger, S., Gentzsch, M., Tanner, W., 1999. Protein *O*-mannosylation. Biochimica et Biophysica Acta (BBA) - General Subjects. 1426**,** 297-307.

Suga, H., Matsuda, F., Hasunuma, T., Ishii, J., Kondo, A., 2013. Implementation of a transhydrogenase-like shunt to counter redox imbalance during xylose fermentation in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. 97**,** 1669-78.

Sukumaran, R. K., Singhania, R. R., Mathew, G. M., Pandey, A., 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Renewable Energy. 34**,** 421-424.

Sun, J., Wen, F., Si, T., Xu, J. H., Zhao, H., 2012. Direct conversion of xylan to ethanol by recombinant *Saccharomyces cerevisiae* strains displaying an engineered minihemicellulosome. Appl Environ Microbiol. 78**,** 3837-45.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology. 83**,** 1-11.

Sun, Y., Cheng, J. J., 2005. Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. Bioresource Technology. 96**,** 1599-1606.

Sunna, A., Antranikian, G., 1997. Xylanolytic enzymes from fungi and bacteria. Crit Rev Biotechnol. 17**,** 39-67.

Suriyachai, N., Weerasaia, K., Laosiripojana, N., Champreda, V., Unrean, P., 2013. Optimized simultaneous saccharification and co-fermentation of rice straw for ethanol production by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture using design of experiments. Bioresource Technology. 142**,** 171-178.

Szczodrak, J., Fiedurek, J., 1996. Technology for conversion of lignocellulosic biomass to ethanol. Biomass and Bioenergy. 10**,** 367-375.

Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J., Stahl, F. W., 1983. The double-strand-break repair model for recombination. Cell. 33**,** 25-35.

Taha, M., Foda, M., Shahsavari, E., Aburto-Medina, A., Adetutu, E., Ball, A., 2016. Commercial feasibility of lignocellulose biodegradation: possibilities and challenges. Curr Opin Biotechnol. 38**,** 190-7.

Taherzadeh, M. J., Gustafsson, L., Niklasson, C., Liden, G., 2000. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. 53**,** 701-708.

Taherzadeh, M. J., Karimi, K., 2007. Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials : A review. BioResources. 2**,** 707-738.

Taiz, L., Honigman, W. A., 1976. Production of cell wall hydrolyzing enzymes by barley aleurone layers in response to gibberellic acid. Plant Physiol. 58**,** 380-386.

Takada, G., Kawaguchi, T., Sumitani, J., Arai, M., 1998. Expression of *Aspergillus aculeatus* No. F-50 cellobiohydrolase I (*cbhI*) and ß-glucosidase 1 (*bgl1*) genes by *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem. 62**,** 1615-8.

Takors, R., 2012. Scale-up of microbial processes: impacts, tools and open questions. J Biotechnol. 160**,** 3-9.

Tang, H., Hou, J., Shen, Y., Xu, L., Yang, H., Fang, X., Bao, X., 2013. High ß-glucosidase secretion in *Saccharomyces cerevisiae* improves the efficiency of cellulase hydrolysis and ethanol production in simultaneous saccharification and fermentation. J Microbiol Biotechnol. 23**,** 1577-85.

Tang, H., Song, M., He, Y., Wang, J., Wang, S., Shen, Y., Hou, J., Bao, X., 2017. Engineering vesicle trafficking improves the extracellular activity and surface display efficiency of cellulases in *Saccharomyces cerevisiae*. Biotechnol Biofuels. 10**,** 53.

Tang, H., Wang, S., Wang, J., Song, M., Xu, M., Zhang, M., Shen, Y., Hou, J., Bao, X., 2016. N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in *Saccharomyces cerevisiae*. Sci Rep. 6**,** 25654.

Teixeira, L. C., Linden, J. C., Schroeder, H. A., 2000. Simultaneous saccharification and cofermentation of peracetic acid-pretreated biomass. Applied Biochemistry and Biotechnology. 84**,** 111-127.

Teleman, A., Tenkanen, M., Jacobs, A., Dahlman, O., 2002. Characterization of *O*-acetyl-(4-*O*-methylglucurono)xylan isolated from birch and beech. Carbohydrate Research. 337**,** 373-377.

Tenenbaum, D. J., 2008. Food vs. fuel: Diversion of crops could cause more hunger. Environmental Health Perspectives. 116.

Tengborg, C., Bioethanol production: Pretreatment and enzymatic hydrolysis of softwood. Departement of Chemical Engineering. Lund University, Lund, 2000, pp. 82.

Tenkanen, M., 1998. Action of *Trichoderma reesei* and *Aspergillus oryzae* esterases in the deacetylation of hemicelluloses. Biotechnology and Applied Biochemistry. 27**,** 19-24.

Tenkanen, M., Puls, J., Poutanen, K., 1992. Two major xylanases of *Trichoderma reesei*. Enzyme and Microbial Technology. 14**,** 566-574.

Teugjas, H., Väljamäe, P., 2013. Product inhibition of cellulases studied with 14C-labeled cellulose substrates. Biotechnology for Biofuels. 6.

Teunissen, A., Dumortier, F., Gorwa, M. F., Bauer, J., Tanghe, A., Loïez, A., Smet, P., Van Dijck, P., Thevelein, J. M., 2002. Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs. Appl Environ Microbiol. 68**,** 4780-4787.

Teymouri, F., Laureano-Pérez, L., Alizadeh, H., Dale, B. E., Ammonia fiber explosion treatment of corn stover. Humana Press, Totowa, NJ, 2004, pp. 951-963.

Thomason, L. C., Calendar, R., Ow, D. W., 2001. Gene insertion and replacement in *Schizosaccharomyces pombe* mediated by the *Streptomyces* bacteriophage phiC31 site-specific recombination system. Mol Genet Genomics. 265**,** 1031-8.

Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C., Williams, R., 2009. Beneficial biofuels—The food, energy, and environment trilemma. Science. 325**,** 270-271.

Timell, T. E., 1967. Recent progress in the chemistry of wood hemicelluloses. Wood Science and Technology. 1**,** 45-70.

Tomas-Pejo, E., Ballesteros, M., Oliva, J. M., Olsson, L., 2010. Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. J Ind Microbiol Biotechnol. 37**,** 1211-20.

Torronen, A., Mach, R. L., Messner, R., Gonzalez, R., Kalkkinen, N., Harkki, A., Kubicek, C. P., 1992. The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Biotechnology (N Y). 10**,** 1461-5.

Trantas, E., Panopoulos, N., Ververidis, F., 2009. Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. Metab Eng. 11**,** 355-366.

Tsai, C. S., Kong, II, Lesmana, A., Million, G., Zhang, G. C., Kim, S. R., Jin, Y. S., 2015. Rapid and marker-free refactoring of xylose-fermenting yeast strains with Cas9/CRISPR. Biotechnol Bioeng. 112**,** 2406-11.

Tsai, S. L., Goyal, G., Chen, W., 2010. Surface display of a functional minicellulosome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production. Appl Environ Microbiol. 76**,** 7514-20.

Tsai, S. L., Oh, J., Singh, S., Chen, R., Chen, W., 2009. Functional assembly of minicellulosomes on the *Saccharomyces cerevisiae* cell surface for cellulose hydrolysis and ethanol production. Appl Environ Microbiol. 75**,** 6087-93.

Uzuner, S., Cekmecelioglu, D., 2019. Enzymes in the Beverage Industry. Enzymes in food biotechnology: Production, applications, and future prospects. pp. 29-43.

Valkonen, M., Penttilä, M., Saloheimo, M., 2003. Effects of inactivation and constitutive expression of the unfolded- protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol. 69**,** 2065.

Van Dongen, F. E. M., Van Eylen, D., Kabel, M. A., 2011. Characterization of substituents in xylans from corn cobs and stover. Carbohydrate Polymers. 86**,** 722-731.

Van Dyk, J. S., Pletschke, B. I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes--factors affecting enzymes, conversion and synergy. Biotechnol Adv. 30**,** 1458-80.

van Maris, A. J. A., Winkler, A. A., Kuyper, M., de Laat, W. T. A. M., van Dijken, J. P., Pronk, J. T., 2007. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: Xylose isomerase as a key component. In: Olsson, L., (Ed.), Biofuels. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 179-204.

van Peij, N. N. M. E., Brinkmann, J., Vršanská, M., Visser, J., de Graaff, L. H., 1997. β-Xylosidase activity, encoded by xlnD, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. European Journal of Biochemistry. 245**,** 164-173.

van Rensburg, E., den Haan, R., Smith, J., van Zyl, W. H., Gorgens, J. F., 2012. The metabolic burden of cellulase expression by recombinant *Saccharomyces cerevisiae* Y294 in aerobic batch culture. Appl Microbiol Biotechnol. 96**,** 197-209.

van Rooyen, R., Hahn-Hagerdal, B., La Grange, D. C., van Zyl, W. H., 2005. Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains. J Biotechnol. 120**,** 284-95.

van Zyl, J. H., den Haan, R., van Zyl, W. H., 2014. Over-expression of native *Saccharomyces cerevisiae* exocytic SNARE genes increased heterologous cellulase secretion. Appl Microbiol Biotechnol. 98**,** 5567-78.

Van Zyl, J. H., Den Haan, R., Van Zyl, W. H., 2016. Overexpression of native *Saccharomyces cerevisiae* ER-to-Golgi SNARE genes increased heterologous cellulase secretion. Appl Microbiol Biotechnol. 100**,** 505-18.

van Zyl, W. H., den Haan, R., Rose, S. H., la Grange, D. C., 2015. Expression of fungal hydrolases in *Saccharomyces cerevisiae*. Direct Microbial Conversion of Biomass to Advanced Biofuels. pp. 153-175.

van Zyl, W. H., Lynd, L. R., den Haan, R., McBride, J. E., 2007. Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. In: Olsson, L., (Ed.), Biofuels. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 205-235.

van Zyl, W. H., Rose, S. H., Trollope, K., Görgens, J. F., 2010. Fungal β-mannanases: Mannan hydrolysis, heterologous production and biotechnological applications. Process Biochemistry. 45**,** 1203-1213.

Vanholme, R., Morreel, K., Ralph, J., Boerjan, W., 2008. Lignin engineering. Curr Opin Plant Biol. 11**,** 278-85.

Vardakou, M., Katapodis, P., Topakas, E., Kekos, D., Macris, B. J., Christakopoulos, P., 2004. Synergy between enzymes involved in the degradation of insoluble wheat flour arabinoxylan. Innovative Food Science & Emerging Technologies. 5**,** 107-112.

Varki, A., 1993. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology. 3**,** 97-130.

Varki, A., Freeze, H. H., Manzi, A. E., 2009. Overview of glycoconjugate analysis. Curr Protoc Protein Sci. Chapter 12**,** Unit-12.1.8.

Varnai, A., Huikko, L., Pere, J., Siika-Aho, M., Viikari, L., 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresour Technol. 102**,** 9096-104.

Vázquez, M. J., Alonso, J. L., Domínguez, H., Parajó, J. C., 2000. Xylooligosaccharides: manufacture and applications. Trends in Food Science & Technology. 11**,** 387-393.

Verduyn, C., Van Kleef, R., Frank, J., Schreuder, H., Van Dijken, J. P., Scheffers, W. A., 1985. Properties of the NAD(P)H-dependent xylose reductase from the xylose fermenting yeast *Pichia stipitis*. Biochem. J. 226**,** 669-677.

Vermaas, J. V., Petridis, L., Qi, X., Schulz, R., Lindner, B., Smith, J. C., 2015. Mechanism of lignin inhibition of enzymatic biomass deconstruction. Biotechnol Biofuels. 8**,** 217.

Vieira Gomes, A. M., Souza Carmo, T., Silva Carvalho, L., Mendonca Bahia, F., Parachin, N. S., 2018. Comparison of yeasts as hosts for recombinant protein production. Microorganisms. 6.

Viikari, L., Vehmaanperä, J., Koivula, A., 2012. Lignocellulosic ethanol: From science to industry. Biomass and Bioenergy. 46**,** 13-24.

Vilela, L. d. F., de Araujo, V. P., Paredes, R. d. S., Bon, E. P., Torres, F. A., Neves, B. C., Eleutherio, E. C., 2015. Enhanced xylose fermentation and ethanol production by engineered *Saccharomyces cerevisiae* strain. AMB Express. 5**,** 16.

Villares, A., Moreau, C., Bennati-Granier, C., Garajova, S., Foucat, L., Falourd, X., Saake, B., Berrin, J. G., Cathala, B., 2017. Lytic polysaccharide monooxygenases disrupt the cellulose fibers structure. Sci Rep. 7**,** 40262.

Vind, J., Sørensen, M. A., Rasmussen, M. D., Pedersen, S., 1993. Synthesis of proteins in *Escherichia coli* is limited by the concentration of free ribosomes. Journal of Molecular Biology. 231**,** 678-688.

von Freiesleben, P., Spodsberg, N., Stenbaek, A., Stalbrand, H., Krogh, K., Meyer, A. S., 2018. Boosting of enzymatic softwood saccharification by fungal GH5 and GH26 endomannanases. Biotechnol Biofuels. 11**,** 194.

Wallace-Salinas, V., Signori, L., Li, Y.-Y., Ask, M., Bettiga, M., Porro, D., Thevelein, J. M., Branduardi, P., Foulquié-Moreno, M. R., Gorwa-Grauslund, M., 2014. Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate. AMB Express. 4**,** 56.

Wang, H., Ma, R., Shi, P., Huang, H., Yang, P., Wang, Y., Fan, Y., Yao, B., 2015. Insights into the substrate specificity and synergy with mannanase of family 27 alpha-galactosidases from *Neosartorya fischeri* P1. Appl Microbiol Biotechnol. 99**,** 1261-72.

Watanabe, M., Tamura, K., Magbanua, J. P., Takano, K., Kitamoto, K., Kitagaki, H., Akao, T., Shimoi, H., 2007a. Elevated expression of genes under the control of stress response element (STRE) and Msn2p in an ethanol-tolerance sake yeast Kyokai no. 11. J Biosci Bioeng. 104**,** 163-70.

Watanabe, S., Abu Saleh, A., Pack, S. P., Annaluru, N., Kodaki, T., Makino, K., 2007b. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. Microbiology. 153**,** 3044-54.

Watanabe, S., Saleh, A. A., Pack, S. P., Annaluru, N., Kodaki, T., Makino, K., 2007c. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein engineered NADP+-dependent xylitol dehydrogenase. J Biotechnol. 130**,** 316-9.

Wei, N., Quarterman, J., Jin, Y. S., 2013. Marine macroalgae: an untapped resource for producing fuels and chemicals. Trends Biotechnol. 31**,** 70-7.

Wen, F., Sun, J., Zhao, H., 2010. Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Environ Microbiol. 76**,** 1251-60.

Wheals, A. E., Basso, L. C., Alves, D. M. G., Amorim, H. V., 1999. Fuel ethanol after 25 years. Trends Biotechnol. 17**,** 482-487.

Wilcox, C. A., Fuller, R. S., 1991. Posttranslational processing of the prohormone-cleaving Kex2 protease in the *Saccharomyces cerevisiae* secretory pathway. Journal of Cell Biology. 115**,** 297-307.

Wingren, A., Galbe, M., Zacchi, G., 2003. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. Biotechnology Progress. 19**,** 1109-1117.

Wisselink, H. W., Toirkens, M. J., del Rosario Franco Berriel, M., Winkler, A. A., van Dijken, J. P., Pronk, J. T., van Maris, A. J., 2007. Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ Microbiol. 73**,** 4881-91.

Wong, W. K. R., Curry, C., Parekh, R. S., Parekh, S. R., Wayman, M., Davies, R. W., Kilburn, D. G., Skipper, N., 1988. Wood hydrolysis by *Cellulomonas fimi* endoglucanase and exogiucanase coexpressed as secreted enzymes in *Saccharomyces cerevisiae*. Bio/Technology. 6**,** 713-719.

Woodward, J., Wiseman, A., 1982. Fungal and other β-d-glucosidases - Their properties and applications. Enzyme Microb. Technol. 4**,** 73-79.

Wright, J., Bellissimi, E., de Hulster, E., Wagner, A., Pronk, J. T., van Maris, A. J., 2011. Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*. FEMS Yeast Res. 11**,** 299-306.

Wu, Z., Lee, Y. Y., 1997. Inhibition of the enzymatic hydrolysis of cellulose by ethanol. Biotechnology Letters. 19**,** 977-979.

Wyman, C., Decker, S., Himmel, M., Brady, J., Skopec, C., Viikari, L., 2005a. Hydrolysis of cellulose and hemicellulose. Polysaccharides: Structural Diversity and Functional Versatility.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., Lee, Y. Y., 2005b. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology. 96**,** 1959-1966.

Wythes, J. R., Wainwright, D. H., Blight, G. W., 1978. Nutrient composition of Queensland molasses. Australian Journal of Experimental Agriculture and Animal Husbandry. v. 18**,** 629-634.

Xiao, Z., Zhang, X., Gregg, D. J., Saddler, J. N., 2004. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Applied Biochemistry and Biotechnology. 113-116**,** 1115-1126.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M., 2010. Inhibition of cellulases by phenols. Enzyme and Microbial Technology. 46**,** 170-176.

Xu, Q., Knoshaug, E. P., Wang, W., Alahuhta, M., Baker, J. O., Yang, S., Vander Wall, T., Decker, S. R., Himmel, M. E., Zhang, M., Wei, H., 2017. Expression and secretion of fungal endoglucanase II and chimeric cellobiohydrolase I in the oleaginous yeast *Lipomyces starkeyi*. Microbial cell factories. 16**,** 126-126.

Xu, Q., Singh, A., Himmel, M. E., 2009. Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. Curr Opin Biotechnol. 20**,** 364-71.

Xu, X., Kanbara, K., Azakami, H., Kato, A., 2004. Expression and characterization of *Saccharomyces cerevisiae* Cne1p, a calnexin homologue. J Biochem. 135**,** 615-8.

YaDeau, J. T., Klein, C., Blobel, G., 1991. Yeast signal peptidase contains a glycoprotein and the Sec11 gene product. Proceedings of the National Academy of Sciences. 88**,** 517.

Yamada, R., Hasunuma, T., Kondo, A., 2013a. Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. Biotechnology Advances. 31**,** 754-763.

Yamada, R., Nakatani, Y., Ogino, C., Kondo, A., 2013b. Efficient direct ethanol production from cellulose by cellulase- and cellodextrin transporter-co-expressing *Saccharomyces cerevisiae*. AMB Express. 3.

Yamane, Y.-I., Fujita, J., Izuwa, S., Fukuchi, K., Shimizu, R.-I., Hiyoshi, A., Fukuda, H., Mikami, S., Kizaki, Y., Wakabayashi, S., 2002. Properties of cellulose-degrading enzymes from *Aspergillus oryzae* and their contribution to material utilization and alcohol yield in sake mash fermentation. Journal of Bioscience and Bioengineering. 93**,** 479-484.

Yan, Q. J., Wang, L., Jiang, Z. Q., Yang, S. Q., Zhu, H. F., Li, L. T., 2008. A xylose-tolerant ß-xylosidase from *Paecilomyces thermophila*: characterization and its co-action with the endogenous xylanase. Bioresour Technol. 99**,** 5402-10.

Yanase, S., Yamada, R., Kaneko, S., Noda, H., Hasunuma, T., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2010. Ethanol production from cellulosic materials using cellulase-expressing yeast. Biotechnol J. 5**,** 449-55.

Yang, B., Wyman, C. E., 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnology and Bioengineering. 94**,** 611-617.

Yang, P., Zhang, H., Jiang, S., 2016. Construction of recombinant *sestc Saccharomyces cerevisiae* for consolidated bioprocessing, cellulase characterization, and ethanol production by in situ fermentation. 3 Biotech. 6**,** 192.

Yeasmin, S., Kim, C. H., Park, H. J., Sheikh, M. I., Lee, J. Y., Kim, J. W., Back, K. K., Kim, S. H., 2011. Cell surface display of cellulase activity–free xylanase enzyme on *Saccharomyces cerevisiae* EBY100. Applied Biochemistry and Biotechnology. 164**,** 294-304.

Yu, A. Q., Juwono, N. K., Foo, J. L., Leong, S. S., Chang, M. W., 2016. Metabolic engineering of *Saccharomyces cerevisiae* for the overproduction of short branched-chain fatty acids. Metab Eng. 34**,** 36-43.

Yu, J., Zhang, J., He, J., Liu, Z., Yu, Z., 2009. Combinations of mild physical or chemical pretreatment with biological pretreatment for enzymatic hydrolysis of rice hull. Bioresource Technology. 100**,** 903-908.

Yu, Y., Tong, Q., Li, Z., Tian, J., Wang, Y., Su, F., Wang, Y., Liu, J., Zhang, Y., 2014. Improved site-specific recombinase-based method to produce selectable marker- and vector-backbone-free transgenic cells. Sci Rep. 4**,** 4240.

Yuan, Y., Zhai, R., Li, Y., Chen, X., Jin, M., 2018. Developing fast enzyme recycling strategy through elucidating enzyme adsorption kinetics on alkali and acid pretreated corn stover. Biotechnol Biofuels. 11**,** 316.

Yuldashev, B. T., Rabinovich, M. L., Rakhimov, M. M., 1993. Comparative study of cellulase behavior on the cellulose and lignocellulose surface during enzymic hydrolysis. Prikl. Biokhim. Mikrobiol. 29**,** 233-243.

Zabed, H., Sahu, J. N., Suely, A., Boyce, A. N., Faruq, G., 2017. Bioethanol production from renewable sources: Current perspectives and technological progress. Renewable and Sustainable Energy Reviews. 71**,** 475-501.

Zacchi, G., Axelsson, A., 1989. Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions. Biotechnol Bioeng. 34**,** 223-233.

Zha, Y., Muilwijk, B., Coulier, L., 2012. Inhibitory compounds in lignocellulosic biomass hydrolysates during hydrolysate fermentation processes. Journal of Bioprocessing & Biotechniques. 02.

Zhang, J., Lynd, L. R., 2010. Ethanol production from paper sludge by simultaneous saccharification and co-fermentation using recombinant xylose-fermenting microorganisms. Biotechnology and Bioengineering. 107**,** 235-244.

Zhang, J., Siika-aho, M., Tenkanen, M., Viikari, L., 2011. The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. Biotechnology for Biofuels. 4**,** 60.

Zhang, J., Tang, M., Viikari, L., 2012. Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. Bioresour Technol. 121**,** 8-12.

Zhang, L., Fu, Q., Li, W., Wang, B., Yin, X., Liu, S., Xu, Z., Niu, Q., 2017. Identification and characterization of a novel ß-glucosidase via metagenomic analysis of *Bursaphelenchus xylophilus* and its microbial flora. Sci Rep. 7**,** 14850.

Zhang, W., Geng, A., 2012. Improved ethanol production by a xylosefermenting recombinant yeast strain constructed through a modified genome shuffling method. Biotechnology for Biofuels. 5.

Zhang, X.-Z., Zhang, Y.-H. P., 2013. Cellulases: Characteristics, Sources, Production, and Applications. Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and Polymers. pp. 131-146.

Zhang, X. Z., Sathitsuksanoh, N., Zhang, Y. H., 2010a. Glycoside hydrolase family 9 processive endoglucanase from *Clostridium phytofermentans*: heterologous expression, characterization, and synergy with family 48 cellobiohydrolase. Bioresour Technol. 101**,** 5534-8.

Zhang, Y., Li, S., Li, L., Pan, X., Cahoon, R. E., Jaworski, J. G., Wang, X., Jez, J., Chen, F., Yu, O., 2006. Using unnatural protein fusions to engineer resveratrol biosynthesis in yeast and mammalian cells. J. Am. Chen. Soc. 128**,** 13030-13031.

Zhang, Y.-H. P., 2008. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. J Ind Microbiol Biotechnol. 35**,** 367-375.

Zhang, Y.-H. P., Lynd, L. R., 2006. A functionally based model for hydrolysis of cellulose by fungal cellulase. Biotechnol Bioeng. 94**,** 888-98.

Zhang, Y. H., Lynd, L. R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng. 88**,** 797-824.

Zhang, Z., Lohr, L., Escalante, C., Wetzstein, M., 2010b. Food versus fuel: What do prices tell us? Energy Policy. 38**,** 445-451.

Zhao, X., Zhang, L., Liu, D., 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels, Bioproducts and Biorefining. 6**,** 465-482.

Zheng, D. Q., Wu, X. C., Tao, X. L., Wang, P. M., Li, P., Chi, X. Q., Li, Y. D., Yan, Q. F., Zhao, Y. H., 2011a. Screening and construction of *Saccharomyces cerevisiae* strains with improved multi-tolerance and bioethanol fermentation performance. Bioresour Technol. 102**,** 3020-7.

Zheng, D. Q., Wu, X. C., Wang, P. M., Chi, X. Q., Tao, X. L., Li, P., Jiang, X. H., Zhao, Y. H., 2011b. Drug resistance marker-aided genome shuffling to improve acetic acid tolerance in *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol. 38**,** 415-22.

Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. Int J Agric & Biol Eng. 2**,** 51-68.

Zhou, H., Cheng, J. S., Wang, B. L., Fink, G. R., Stephanopoulos, G., 2012. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. Metab Eng. 14**,** 611-22.

Zhou, S., Ingram, L. O., 2000. Synergistic hydrolysis of carboxymethyl cellulose and acid-swollen cellulose by two endoglucanases (CelZ and CelY) from *Erwinia chrysanthemi*. J Bacteriol. 182**,** 5676-5682.

Zhu, J., Pavelka, N., Bradford, W. D., Rancati, G., Li, R., 2012. Karyotypic determinants of chromosome instability in aneuploid budding yeast. PLOS Genetics. 8**,** e1002719.

Zhu, J.-Q., Qin, L., Li, B.-Z., Yuan, Y.-J., 2014a. Simultaneous saccharification and co-fermentation of aqueous ammonia pretreated corn stover with an engineered *Saccharomyces cerevisiae* SyBE005. Bioresource Technology. 169**,** 9-18.

Zhu, Y. O., Sherlock, G., Petrov, D. A., 2016. Whole genome analysis of 132 clinical *Saccharomyces cerevisiae* strains reveals extensive ploidy variation. G3 (Bethesda). 6**,** 2421-34.

Zhu, Y. O., Siegal, M. L., Hall, D. W., Petrov, D. A., 2014b. Precise estimates of mutation rate and spectrum in yeast. Proc Natl Acad Sci U S A. 111**,** E2310-8.

Zilberman, D., Hochman, G., Rajagopal, D., Sexton, S., Timilsina, G., 2012. The impact of biofuels on commodity food prices: Assessment of findings. American Journal of Agricultural Economics. 95**,** 275-281.