EVALUATION OF HIGH RECOMBINANT PROTEIN SECRETION PHENOTYPE OF *SACCHAROMYCES CEREVISIAE* SEGREGANTS

by

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RESEARCH DISSERTATION

Submitted in fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

at the

UNIVERSITY OF LIMPOPO

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2016

DECLARATION

I hereby declare that the dissertation submitted to the University of Limpopo for the degree of Master of Science in Biochemistry has not previously been submitted by me for a degree at this or any other University and that it is my own work and all the material contained therein has been properly acknowledged.

Signature: _____

Date: _____

DEDICATIONS

I dedicate this dissertation to my parents. They have supported and encouraged me throughout my academic career.

Most importantly to my son, he has been the driving force within me.

ACKNOWLEDGEMENTS

I send my deepest gratitude and appreciation to the following persons and institutions:

The Almighty God for providing constant strength and protection.

My supervisor, Dr. D.C la Grange for guidance, support and the interest he has shown in making the research a success.

Heinrich Kroukamp for mentorship, supervision and guidance throughout the study.

Zimasa Sithole for her help with the SDS-PAGE experiments.

Dr. K. Lucas, for providing insightful inputs and feedback.

The National Research Foundation - Renewable Energy Scholarship (NRF-RSES) for financial support.

Stellenbosch University – Van Zyl laboratory for collaborating with us when undertaking this study.

University of Limpopo – Biotechnology Unit for providing their facilities, equipment and research inputs.

University of Limpopo – Biofuel Laboratoty – for providing their facilities and equipment.

ABSTRACT

The ever increasing cost of fossil-based fuels and the accompanying concerns about their impact on the environment is driving research towards clean and renewable sources of energy. Bioethanol has the potential to be a replacement for liquid transportation fuels. In addition to its near zero nett carbon dioxide emissions, bio-ethanol has a high energy to weight ratio and can easily be stored in high volumes. To produce bioethanol at economically competitive prices, the major cost in the production process needs to be addressed. The addition of enzymes to hydrolyse the lignocellulosic fraction of the agricultural waste to simple sugars is considered to be the major contributor to high production cost. A consolidated bioprocess (CBP) which ideally combines all the steps that are currently accomplished in different reactors by different microorganisms into a single process step would be a more economically feasible solution. In this study the potential of yeast hybridization with a CBP approach was used. In order to evaluate the reduction or elimination of the addition of cellulolytic and hemi-cellulolytic enzymes to the ethanol production process.

High cellobiohydrolase I secreting progeny from hybridization of an industrial bioethanol yeast strain, *S. cerevisiae* M0341, and a laboratory strain *S. cerevisiae* Y294 were isolated. In order to determine if this characteristic was specific to cellobiohydrolase I secretion, these strains were evaluated for their ability to secrete other relevant recombinant hydrolase enzymes for CBP-based ethanol production.

A total of seven *S. cerevisiae* strains were chosen from a progeny pool of 28 supersecreting hybrids and reconstructed to create two parental strains; *S. cerevisiae* M0341 and *S. cerevisiae* Y294, together with their hybrid segregants strains H3M1, H3M28, H3H29, H3K27 and H3O23. Three episomal plasmids namely pNS201, pNS202 and pNS203 were constructed; these plasmids together with two already available plasmids, namely pRDH166 and pRDH182 contained genes for different reporter enzymes, namely β-glucosidase I, xylanase II, endoglucanase lll, cellobiohydrolase l and α-glucuronidase. To allow for selection of the episomal plasmids, homologous recombination was used to replace the functional *URA3* gene of selected strains, with the non-functional *ura3* allele from the Y294 strain. Enzyme activity was used as an indicator of the amount of enzyme secreted. Fermentation studies in a bioreactor were used to determine the metabolic burden imposed on the segregants expressing the cellobiohydrolase at high levels. In addition all segregants were tested for resistance to inhibitors commonly found in pre-treated lignocellulosic

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Keywords: yeast hybridization**,** enzyme secretion**,** β-glucosidase I, xylanase II, endoglucanase III, cellobiohydrolase I, and α-glucuronidase

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ABBREVIATIONS

AA$^+$	Extended family of ATPase associated with a variety of cellular activities
AFEX	Ammonia Fibre Explosion
BiP	Binding Immunoglobulin Protein
BGL	β-1,4-glucosidase
CAZy	Carbohydrate Active Enzymes
CBH	exo-β-1,4-glucanase cellobiohydrolase
CBMs	Carbohydrate Binding Modules
CBP	Consolidated Bioprocessing
CCT	Cytosolic chaperonin containing the peptide tcp1 (tall-less complex polypeptide1)
CEs	Carbohydrate Esters
CMC	carboxymethyl-cellulose
CNX	Calnexin homologue
CRT	Calreticulum
Dol-*P*-Man	dolichol monophosphate mannose
EG	endo-β-1,4-glucanase
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
Ero1	Endoplasmic Reticulum Oxidoreductin 1
FAD	Flavin Adenine Dinucleotide
FDA	Food and Drug Association
GH	Glycoside Hydrolase
GRAS	Generally Regarded as Safe
HDEL	His-Asp-Glu-Leu
hGH	Human Growth Hormone
HSP	Heat Shock Protein

Kar2	Karyogamy
LiOAc/DMSO	Lithium Acetate/Dimethyl Sulphoxide
Ln	Natural Logarithm ($\log_e x$)
Man	Mannose
NSF	N-ethylmaleimide sensitive factor
OST	Oligosaccharyltransferase complex
OT	Oligosaccharyltransferase
PAGE	Polyacrylamide gel electrophoresis
PASC	Phosphoric Acid-swollen Cellulose
PDI	Protein Disulphide Isomerase
***p*NPC**	*p*-Nitrophenyl-β-D-cellobioside
***p*NPG**	*p*-Nitrophenyl-β-D-glucopyranoside
PSWGSA	Pooled-Segregant Whole Genome Sequence Analysis
QC	Quality Control
QTL	Quantitative Trait Loci
ROS	Reactive Oxygen Species
SGD	*Saccharomyces* Genome Database
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SHF	Separate Hydrolysis and Fermentation
SM	Sec1/Munc18
SNARE	Soluble N-ethylmaleimide-sensitive-factor Attachment Protein Receptor
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
TEMED	*N*-tetramethylethylene diamine
Tcp1	Tall-less Complex Polypeptide 1

UPR	Unfolded Protein Response
μ_{max}	Maximum Specific Growth Rate
5-FOA	5-Fluoroorotic Acid
5-FU	5-Fluorouracil
5-HMF	5-Hydroxymethyl-2-furaldehyde

CHAPTER 1. INTRODUCTION

1.1 Background Information

Lignocellulosic biomass is the most abundant renewable feedstock in nature (Stephanopoulos, 2007). Its use in the production of renewable biofuel could be economically feasible provided the recalcitrance of lignocellulose can be overcome. Second generation biofuels have the potential to replace liquid fossil fuels, as a renewable and clean alternative, if the supply and bioprocessing of lignocellulosic biomass can be optimized (Van Zyl *et al.*, 2007).

Consolidated Bioprocessing (CBP) is an attractive one-step process for the economically feasible conversion of lignocellulosic biomass to bioethanol (Den Haan *et al.*, 2007a). It integrates several process steps including, enzyme production, hydrolysis of lignocellulosic biomass, and fermentation of the resulting hexose and pentose sugars. All these steps are normally performed in different reactors by different organisms where required (Olsen *et al.*, 2012). Currently, no naturally occurring organism possesses all the desired properties for CBP; hence numerous groups around the world are working on engineering an organism capable of such a process (Fujita *et al.*, 2002; Ilmen *et al.*, 2011).

The complete breakdown of cellulosic biomass necessitates the action of a number of different enzymes namely, cellulases and hemicellulases. The three main types of cellulases, required for the hydrolysis of crystalline cellulose, are endo-1,4-β-D-glucanases (EGs), exo-1,4-β-D-glucanase cellobiohydrolases (CBHs) and β-glucosidases (BGLs). Natural cellulose contains highly ordered crystalline structures and the hydrolysis of crystalline cellulose is more challenging than the hydrolysis of amorphous cellulose. A mixture of the three different types of cellulase enzymes are required for the complete hydrolysis of β-1,4-glycosidic bonds in cellulose. Cellobiohydrolases act from the ends of the cellulose chains releasing cellobiose. Endo-glucanases hydrolyse cellulose chains internally, usually in amorphous regions. CBHs and EGs act synergistically to release small oligosaccharides from cellulose. Lastly, β-glucosidases act on these small cellooligosaccharides, to yield glucose and thereby, allowing complete hydrolysis of cellulose to glucose (Sukumaran *et al.*, 2008).

The yeast *Saccharomyces cerevisiae* has been widely studied and is the first eukaryote to have its entire genome sequenced and the maintenance and annotation of the genome sequence have long been provided by the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/). Protein expression in *S. cerevisiae* has been extensively studied and is well documented (Hahn-Hagerdal *et al.*, 2001; Fujita *et al.*, 2004; Den Haan *et al.*, 2007b; Hasunuma *et al.*, 2011). *S. cerevisiae* is capable of post-translational processing and secretion of heterologous eukaryotic proteins in their native, biologically active form, making it an important organism for use in both medicine and industry (Porro *et al.*, 2005).

However, *S. cerevisiae* is not without shortcomings; for instance it is incapable of producing and secreting proteins, especially heterologous proteins, at levels high enough to be used in a consolidated bioprocessing process for the production of ethanol from lignocellulosic biomass. The focus of this study was to evaluate cellobiohydrolase I supersecreting segregants of *S. cerevisiae,* for their ability to also secrete an endo-glucanase, an endo-xylanase, a β-glucosidase, and an α-glucuronidase from multicopy plasmids. These *S. cerevisiae* segregant strains have been found to secrete cellobiohydrolase I from a single integrated copy, at high levels (Kroukamp *et al*., 2012).

1.2 Research problem

Although *S. cerevisiae* is the preferred industrial ethanologenic organism, the ability to engineer cellulolytic capability into this yeast is limited by its relative low protein secretion capacity (Den Haan *et al.*, 2007b), which has limited its suitability for use as an organism for consolidated bioprocessing.

The following three strategies are available to increase total extracellular enzyme activity:

i. Increase overall protein secretion by *S. cerevisiae.*
ii. Improve the specific activity of recombinant cellulolytic enzymes produced in *S. cerevisiae* to enable complete hydrolysis of cellulose using low levels of very efficient enzymes.
iii. Increase the temperature at which the fermentation process is conducted since enzymes are more active at elevated temperatures.

This study will concentrate on the first strategy which aims to improve the secretion ability of *S. cerevisiae*. In a study by Kroukamp *et al.* (2012) several hybrid yeast strains capable of secreting higher levels of CBHI compared to the original parent strains, from a single integrated copy of the gene, were created. From this study, it was unclear whether this high secretion phenotype was copy number dependent and/or reporter cellulase specific. This study therefore aimed at answering this question.

1.3 Motivation of Study

Many types of high value Food and Drug Administration (FDA)-approved therapeutic proteins are secreted at very low levels by *S. cerevisiae* (Rader, 2007). In monetary terms, cellulases are the third highest of the industrial enzymes. Hydrolytic enzymes especially cellulases, have a diversified application in industry. This is one of the reasons for the increased demand in cellulases. Globally, Genencor and Novozymes are the major manufacturers of industrial enzymes, with Novozymes holding an estimated 47% of the global enzyme market (Jonathan, 2010; Singhania *et al.*, 2010). The applications of hydrolytic enzymes include the textile, pulp and paper, detergent, food, pharmaceuticals and biofuels industries.

In addition to the biotechnological applications of yeast protein secretion, gaining insight in the protein secretion process could enhance our understanding of several human diseases such as Parkinson's, Alzheimer's, Diabetes Mellitus, Ischemia, Atherosclerosis, and diseases related to the liver and heart, which are all related to protein folding and secretion (Yoshida, 2007). Yeasts such as *S. cerevisiae* can be used as a eukaryotic model, to gain insight into the molecular background of such diseases (Coughlan and Brodsky, 2005) due to the conservation of the eukaryotic secretory pathway.

This study is essential to aid in understanding the processes of protein secretion by *S. cerevisiae* and aims in achieving high cellulase secretion. This will ultimately aid in the construction of *S. cerevisiae* strains capable of secreting high levels of heterologous proteins and subsequently improve the economics of a number of industrial processes. This will also shed some light on basic mechanisms influencing secretion of recombinant proteins.

1.4 Purpose of the study

1.4.1 Aim of the study

The aim of the current project was to evaluate the general protein secretion ability of *S. cerevisiae* segregants created by Kroukamp *et al*. (2012) that were shown to secrete a cellobiohydrolase I, from a single integrated copy, at higher levels than the parental strains.

1.4.2 Objectives of the study

The objectives of the study were to:

i. Construct three episomal plasmids namely pNS201, pNS202 and pNS203 that would facilitate the high copy expression of a β-glucosidase, cellobiohydrolase l and and α-glucuronidase.

ii. Transform the above mentioned three plasmids as well as plasmids pRDH166 and pRDH182 containing an endo-glucanase and an endo-xylanase, respectively, into five different high cellobiohydrolase secreting segregants of *S. cerevisiae.*

iii. Evaluate the secreted enzyme activity of these episomal plasmids containing strains as an indication of the amount of protein secreted.

iv. Use microbial growth studies as an indicator to determine the metabolic burden imposed on the yeast by the high level secretion of the expressed cellobiohydrolase I.

v. Determine the sensitivity of the high secreting segregants towards inhibitors commonly found in pre-treated feedstocks.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

A large portion of forestry residues, grasses, woody materials and agricultural waste consist of lignocellulosic biomass and can be characterized as such. Lignocellulose is the most abundant renewable feedstock in nature, and is composed of three main structural components which include 40% - 50% cellulose, 20% - 30% hemicellulose and 10% - 20% lignin (Saha, 2003; Gnansounou, 2008). The exact content of these polysaccharides varies among the different plant sources. These polysaccharide components have complex structures, and together produce biomass that has a complex internal structure (Hamelinck *et al.*, 2005; Hahn-Hagerdal *et al.*, 2007).

The chemical composition of lignocellulosic biomass is mostly carbon, hydrogen and oxygen. Biomass however, does not include organic materials such as fossil fuels which have been transformed by geological processes into substances such as coal or crude oil. Therefore fossil fuels are not considered biomass by definition. This is due to the fact that they contain carbon that has been displaced out of the carbon cycle for a very long time. Unlike biomass, the combustion of fossil fuels adds to the nett amount of carbon dioxide in the atmosphere. Biomass is a feedstock which can be converted to fuels and has the ability to power everything that requires energy such as cars, trucks, aeroplanes and conventional power stations. There are a number of ways to convert biomass into fuel including direct combustion, gasification, combined heat and power, as well as anaerobic and aerobic digestion (Biofuels Association of Australia, 2014).

2.2 Lignocellulose

2.2.1 Cellulose

The most abundant carbohydrate polymer in nature which is responsible for the mechanical strength of plants is cellulose. Moreover, it is the major crystalline polysaccharide in plant cell walls, which is insoluble and usually fibrous in nature (Klemm *et al.*, 2005).

Cellulose forms a crystalline structure due to repeating units of β-(1,4)-linked-D-glucose condensed by hydrogen bonds formed between the individual fibres. These glucose units which yield repeating units of cellobiose, and the adjacent D-glucose

units are inverted 180° (Klemm *et al.*, 2005; Himmel *et al.*, 2007) – (Figure 2.1). Cellulose fibres naturally aggregate to form microfibrils. Microfibres have both crystalline and amorphous regions (Zhang and Lynd, 2004; Iqbal *et al.*, 2011). Cellulose is an ordered structure and ensures rigidity of the plant cell wall (Klemm *et al.*, 2005).

2.2.2 Hemicellulose

The second most abundant polymer in nature is hemicellulose. This polymer is heterogeneous, often branched and its composition can differ greatly depending on its origin. It is composed mostly of β-1,4-linked xylose monomers (Saha, 2003; Wyman *et al.*, 2005). Hemicellulose is usually shorter than cellulose, with short chains containing other sugars, acetyl groups and phenolic groups and a high degree of structural heterogeneity (Figure 2.1), (Ebringerova and Heinze, 2000). The high level of branching and the presence of acetyl groups which are connected to the polymer chain are responsible for the lack of crystallinity resulting in an amorphous structure. This makes hemicellulose more amenable to enzymatic hydrolysis (Hamelinck *et al.*, 2005).

Hemicelluloses keep fibres from aggregating and add a certain degree of flexibility to the cell wall (Templeton *et al.*, 2010). They usually bind to the surface of cellulose microfibrils and form a matrix between the fibres by non-covalent hydrogen bonding. Hemicellulose can be extracted by alkaline solution from the plant cell wall, and can be classified according to composition into four major groups. (a) Mannans that contain galactomannan and glucomannan. (b) Mixed-linkage glucans comprised of a backbone of D-glucose residues having both β-1,3 and β-1,4 linkages. (c) Xylans that have β-1,4-linked D-xylose backbones and may include arabinose and/or glucuronic acid side chains. (d) Xyloglucan that has a β-1,4-glucan backbone with xylose-containing branches that may contain other monosaccharide substitutions, such as galactose, arabinose and fucose (Smole *et al.*, 2013).

The complete breakdown of cellulosic biomass requires the action of a number of different cellulases and hemicellulases (Grabber, 2005). The enzymatic hydrolysis of lignocellulose is limited by several factors, including: crystallinity of cellulose, degree of polymerization, moisture, available surface area, and lignin content (Laureano-Perez *et al.*, 2005).

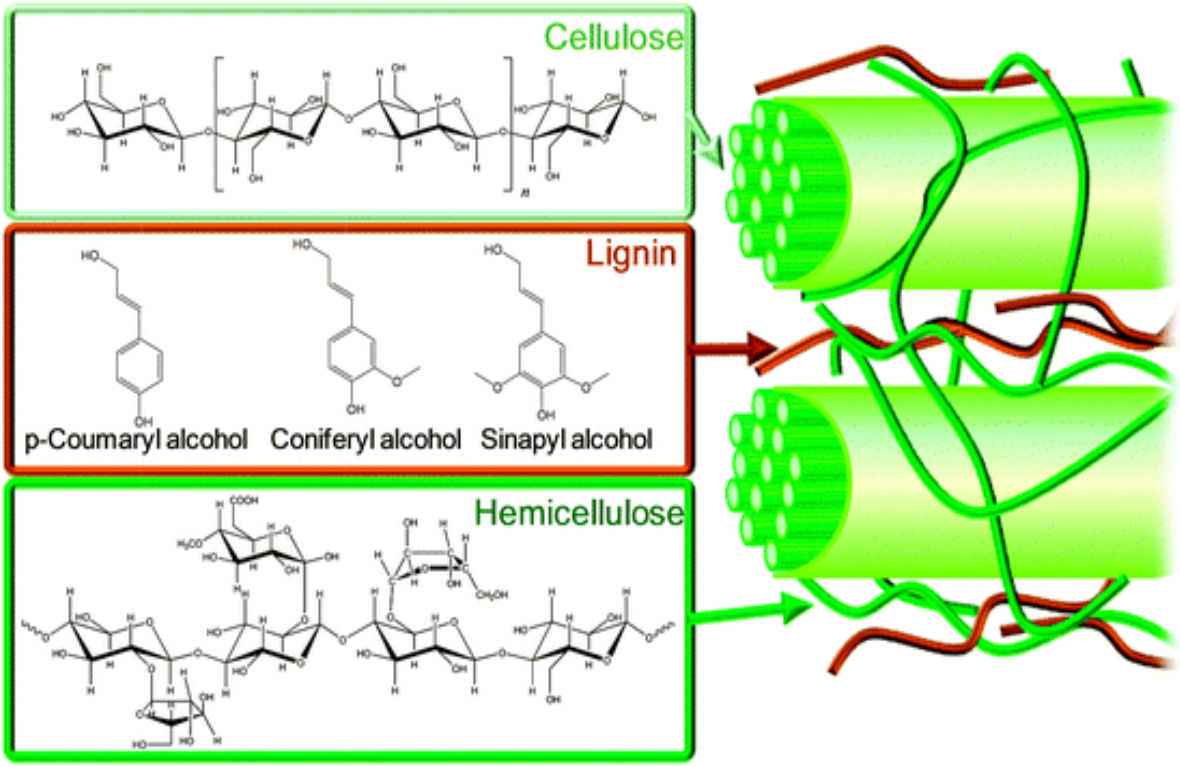


Figure 2.1. Schematic diagram of the lignocellulosic biomass, displaying the positions and structures of cellulose, lignin and hemicellulose (Pixgood, 2013).

2.3 Lignocellulases

2.3.1 Cellulases

Multiple sequential enzymatic activities in a synergistic system facilitate the hydrolysis of cellulose to glucose. Cellulose hydrolysis in most cellulolytic organisms is accomplished by three classes of enzymes, namely (i) EGs, (ii) CBHs and (iii) BGLs. The endo-glucanases and exo-glucanases act synergistically on cellulose yielding small cellooligosaccharides. Endo-glucanases act randomly on amorphous regions while exo-glucanases act sequentially from both the reducing and non-reducing ends. These cellooligosaccharides, mainly cellobiose, are subsequently broken down to glucose by β-glucosidases (Figure 2.2) (Cummings and Fowler, 1996).

Cellulases are glycoside hydrolase (GH) enzymes which are made up of an extensive group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety such as glycosides, glycans and glycoconjugates (Wilson, 2009). These enzymes use an acid-base catalysis mechanism which inverts and retains the glucose anomeric

configuration. Two common types of active sites are present within the GH enzymes. The first is the open groove which tends to bind anywhere along the length of cellulose to hydrolyse the β-1,4-linkages, which is typical of endo-cellulases. The second is the tunnel-like active site, which binds the end of cellulose to yield short oligo-saccharides. This is typical of CBHs which are processive enzymes, meaning that they do not detach from a cellulose chain until it is completely hydrolysed (Gray *et al.*, 2006; Prasad *et al.*, 2007; Balat *et al.*, 2008; Sukharinikov *et al.*, 2011). The nomenclature of GHs is based on amino acid sequence similarities; primarily because of the direct relationship between sequence and folding similarities, allows the prediction of catalytic mechanisms and important catalytic residues which are conserved in most GH families (Cantarel *et al.*, 2009). The GHs are classified into 133 families on the frequently updated database Carbohydrate Active EnZYmes (CAZy) (www.cazy.org).

Most cellulases and some hemicellulases are modular proteins and usually include functional modules such as catalytic domains, carbohydrate binding modules (CBMs) and dockerins. Primarily, CBMs assist cellulases to bind cellulose; however they can also play a role in the initial disruption of cellulose fibres. The disruption of cellulose fibres is usually carried out by endo-cellulases along with CBMs, making it possible for the CBHs to degrade cellulose by removing the di- and oligosaccharides (2 – 4 residues) from the disrupted ends of cellulose (Sukharinikov *et al.*, 2011). The targeting of enzymes to insoluble polysaccharides is facilitated by CBMs. Dockerins are modules which are present in enzymes from organisms that produce cellulosomes. Dockerin modules mediate the binding of catalytic domains to the cellulosome using cohesion-dockerin interactions.

The GH enzymes have catalytic residues that are found close to each other within the consensus motif Glu-X-Asp-X-X-Glu, where the first Glu acts as a catalytic nucleophile and the last Glu as a general acid or base (Divne *et al.*, 1994; Stahlberg *et al.*, 1996; Ducros *et al.*, 2003).

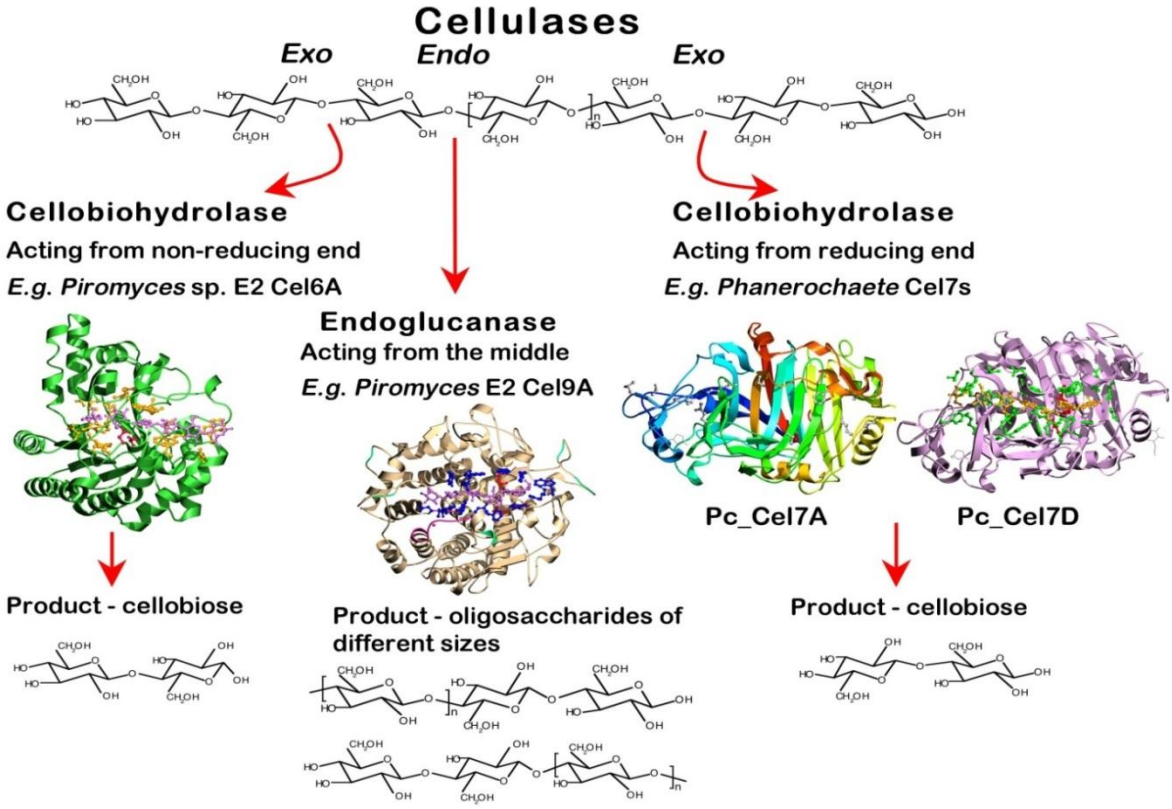


Figure 2.2. The action of cellulases on a cellulose polymer and the resulting products from hydrolytic reactions (Ubhayasekera, 2001).

2.3.1.1 Endo-β-1,4-glucanases

Endo-glucanases act randomly on the amorphous regions of cellulose producing cellodextrins with reducing and non-reducing ends. The degradation of cellulose is facilitated by the endo-exo synergism between EGs and CBHs. This is a phenomenon where EGs act randomly to hydrolyse the long cellulose chain, yielding free cellulose ends that can be attacked by the CBHs (Olsson *et al.*, 2004). Furthermore, exo-exo synergism between CBHs has been reported (Woodward, 1991; Teeri, 1997; Medve *et al.*, 1998; Zhang and Lynd, 2004). EGs are classified in the GH families 45, 131 and 124 (www.cazy.org).

The *Neosartorya fischerii* EG3, used in this study, is a relatively small protein with molecular mass of 25 kDa and contains no *N*-glycosylation sites.

2.3.1.2 Exo-β-1,4-glucanases

The exo-glucanases include CBHs that hydrolyze crystalline cellulose from the chain free ends; reducing and non-reducing ends.

The *Talaromyces emersonii* CBH I, used in this study, is a 55 kDa protein with 3 *N*-glycosylation sites and has been expressed in *S. cerevisiae* previously (Ilmen *et al.*, 2011). It consists of two β-sheets that are packed face-to-face to form a β-sandwich with long loops that cover the cellulose binding tunnels as shown in Figure 2.3 (Grassick *et al.*, 2004). The active site is found inside the tunnel. It is 50 Å long containing 10 binding sites for glycosyl units. The role of the tunnel is to position the substrate correctly in the active site (Divne *et al.*, 1998). The tunnel is structured in such a way that it is slightly open and straight, allowing better access of short oligosaccharides to the active site (Figure 2.3). This was postulated by Tuohy *et al*. (2002) after noticing a higher catalytic rate and efficiency on *p*-nitrophenyl-lactopyranoside than on cellobiose. CBH I hydrolyses the cellulose chain in a processive manner, because the binding site is responsible for retaining the cellulose chain in the tunnel at the end of each catalytic event (Divne *et al.*, 1998).

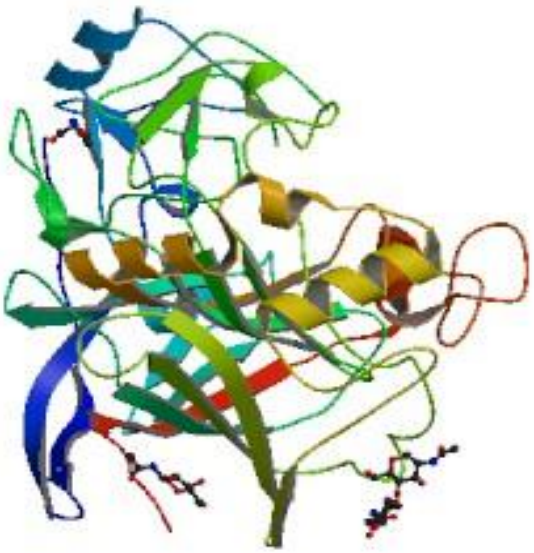


Figure 2.3. A schematic representation of the 3-dimensional structure of native *Cel7A* (CBH I) from *Talaromyces emersonii* [(Grassick *et al.*, 2004), PDBID: 1Q9H, RCSB Protein Data Bank].

2.3.1.3 β-1,4-glucosidases

β-1,4-Glucosidases are a key enzyme component in cellulases, because it is the final step in cellulose degradation, where BGLs hydrolyze cellobiose and cellooligosaccharides to glucose (Zhang and Lynd, 2004). Although BGL activity is inhibited by glucose, the activity of BGL in the hydrolysis of cellulose is very

important as cellobiose is a greater inhibitor of CBH activity than glucose (Holtzapple *et al.*, 1990).

The *Saccharomycopsis fibuligera* β-glucosidase I enzyme, used in this study, is a fairly large protein with a size of 120 kDa. Measuring β-glucosidase activity is fairly simple using *p*-nitrophenyl-β-D-glucopyranoside as substrate. It has been expressed in *S. cerevisiae* previously (Van Rooyen *et al.*, 2005).

2.3.2 Hemicellulases

Hemicellulose is highly variable in structure and organization; therefore it requires the synergistic action of a consortium of enzymes such as xylanases, xylosidases, glucuronidases, and ferulic acids, collectively referred to as hemicellulases, in order to ensure its complete degradation. The catalytic modules can be GHs which either hydrolyse glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety or carbohydrate esters (CEs) that hydrolyse ester linkages of acetate or ferulic acid esterase side chains (Polizeli *et al.*, 2005).

2.3.2.1 Xylanases

Xylanases hydrolyse the β-1,4-bond in the xylan backbone and yield short xylo-oligosaccharides. Xylan from grass usually contain 4-*O*-methyl-glucuronic acid and arabinofuranase side chains, which can hinder the binding and hydrolysis of xylan by xylanases (Bailey *et al.*, 1999; Hulbert and Preston, 2001; Natesh *et al.*, 2003). There are a large number of gene sequences of xylanases that belong to GH families 10 and 11, with some also distributed between families 5 and 8 (Collins *et al.*, 2002; Van Petegem *et al.*, 2003). Xylanases are also found in GH families 51 and 43 (www.cazy.org).

The *Trichoderma reesei* xylanase 2 (*XYN2*) reporter enzyme used in this study is a small protein of 21 kDa and has been expressed in *S. cerevisiae* previously (La Grange *et al.*, 1996). It has been shown that Xyn2 can be secreted at high levels by lab strains of *S. cerevisiae* and is simple to assay (Bailey *et al.*, 1992).

2.3.2.2 β-D-xylosidases

β-D-Xylosidases are important for the complete hydrolysis of the major component of hemicellulose, β-D-xylan. They act by attacking and cleaving the non-reducing ends of xylooligosaccharides to release xylose (Lama *et al.*, 2004). β-Xylosidases are

important in the hydrolysis of hemicellulose as they relieve the rate-limiting step in xylan hydrolysis caused by product inhibition of endo-xylanases (Saha, 2003). These enzymes are found in families 52, 43, 51, 116 and 120 of the glycoside hydrolases (www.cazy.org).

2.3.2.3 α-L-Arabinofuranosidase

The α-L-Arabinofuranosides are classified in GH families 2,3 10, 43, 51, 54 and 62 with different substrate specificities (www.cazy.org). Xylan is substituted with arabinosyl, acetyl, uronyl, mannosyl and glucosyl side chains. The α-L-Arabinofuranosidases fall in a class of enzymes together with uronidases, mannosidases and acetyl esterases; that are responsible for the removal of side-chain substituents (Utt *et al.*, 1991). A number of enzymes of this class are bifunctional for the release of xylose and glucose or arabinose (Mai *et al.*, 2000; Whitehead and Cotta, 2001; Sorensen *et al.*, 2003). The α-L-arabinosides have synergy with other xylanolytic enzymes such as α-D-glucuronidases which result in either an enhancing or inhibitory effect on their activity depending on the order in which the enzymes are allowed to act (Siika-aho *et al.*, 1994).

2.3.2.4 α-D-glucuronidases

Cleavage of the α-1,2-glycosidic bond from the 4-*O*-methyl-glucuronic acid side chain of the xylan backbone is completed by the α-D-glucuronidases (Nurizzo *et al.*, 2002) which are accessory enzymes. These enzymes are found in GH family 67 and 115 (www.cazy.org), and contain three domains with the central one which is a $(\beta/\alpha)_8$ barrel, containing the active site (Zaide *et al.*, 2001).

The *Scheffersomyces stipitis* α-D-glucuronidases (*GLU)* used in this study is a large protein; 113 kDa in size with 16 *N*-glycosylation sites. It has been expressed in *S. cerevisiae* although its expression levels were very poor (Gomes, 2012).

2.4 Protein secretion in *Saccharomyces cerevisiae*

Protein secretion is an essential process for living organisms such as yeasts that use an absorptive nutrition mode where enzymes are secreted in order to degrade the nutrient source before it is absorbed by the cell (Burgoyne, 1988). It involves a number of steps, including translocation of newly synthesized polypeptides through the endoplasmatic reticulum (ER), protein folding, glycosylation, protein quality control (QC) and vesicle-mediated secretion. In order for protein secretion to take

place, a secretion signal is required at the N-terminal side of the protein. This sequence is predicted by the SGD for 543 proteins accounting for 9.2% of the entire proteome (http://www.yeastgenome.org/).

Protein secretion is important because in the bigger scheme of improving volumetric protein yield, it can be a limiting factor caused by a decrease in cell specific productivity (Nevoight, 2008). Cell specific productivity is a function of efficient transcription and translation of the relevant genes producing the protein of interest (Porro *et al.*, 2005).

S. cerevisiae is an attractive host organism for the production of bioethanol by means of CBP. *S. cerevisiae* has become very popular in molecular biology as a eukaryotic protein expression system; and as such it has been extensively studied and used in the production of foreign proteins (Park and Ramirez, 1988). It is generally regarded as safe (GRAS) making it safe to use. Moreover it has well defined and accessible genetic tools. Furthermore, it grows rapidly using simple and inexpensive culturing conditions. *In vivo* genetic modifications of *S. cerevisiae* is a simple process (Olsson and Hahn-Hagerdal, 1993; Nevoight, 2008). Therefore it has been used widely in industry for several applications such as commercial enzyme production. It is also tolerant to inhibitors commonly produced from plant material at concentrations commonly encountered during pre-treatment of biotechnological processes (Jeffries and Jin, 2000). Compared to other yeasts often used for heterologous protein expression, *S. cerevisiae* is not efficient with regard to the secretion of proteins at high levels into the extracellular medium. Several studies have been done to improve the expression and secretion level of heterologous proteins (Ilmen *et al.*, 2011; Kroukamp *et al.*, 2012).

The yeast *S. cerevisiae* has an established protein expression and secretion pathway (Muller *et al.*, 1998). Protein secretion involves several post-translational modification and translocation steps in the ER lumen. The ER-resident protein-folding machinery folds the proteins into their native structure under strict quality control (QC) (Anelli and Satia, 2008). A number of modifications are achieved in the ER including, signal sequence processing, disulphide bond formation, *N*- and *O*-glycosylation, glycosyl-phosphatidyl-inositol addition, sorting and degradation of faulty proteins. This is followed by translocation of only the properly folded and assembled proteins into the Golgi apparatus for further modification. In the Golgi

apparatus further sorting takes place. Finally the fully assembled proteins are transported to the extracellular space, vacuoles or other organelles (Klausner, 1989).

The QC system is extensively stringent and any mis-folded or aggregated proteins are recognized and also led to bind to the karyogamy protein (Kar2) complex which leads to ER-associated protein degradation (ERAD) in the cytosol (Yoshida, 2007). The unfolded protein response (UPR) stimulates proteolysis by ERAD and inhibits the transcription and translocation of proteins. This response is induced by the prolonged binding of the BiP complex to partially mis-folded or aggregated proteins. During heterologous protein secretion the complexity and stringency of the QC system in the ER, tends to distinguish protein folding as rate-limiting (Gasser *et al.*, 2007).

2.4.1 Protein folding in *Saccharomyces cerevisiae*

In a review by Tu and Weissman (2004) the authors state that disulphide bond formation is essential for protein secretion for the following reasons. Firstly, disulphide bonds ensure proper maturation and function of a protein. Secondly, the stability of a protein can be accredited among other things, to the number and position of disulphide bonds present. And lastly, cysteine residues, when mispaired can lead to misfolding of proteins, because the native conformation has not been reached.

Disulphide bond formation relies on redox reactions that require electron acceptors and hence it is a slow assisted process *in vivo* (Bardwell *et al.*, 1991). The electron acceptors are endoplasmic reticulum oxidoreductin 1 (Ero1) and protein disulphide isomerases (PDIs), however these enzymes also act as electron donors. Disulphide bond formation is facilitated by the ER-resident enzymes such as PDI which donates electrons to the ER-membrane associated enzyme Ero1 causing it to bind to flavin adenine dinucleotide (FAD). The FAD-bound Ero1 further donates its electrons to molecular oxygen, resulting in oxygen anions; therefore an increased production of reactive oxygen species (ROS). It can be noted that an increased level of ROS during protein folding can cause an increase in cellular oxidative stress, which can be detrimental to the ER-membrane.

2.4.2 Association of proteins with chaperones

Molecular chaperones are proteins that are involved in both the folding or unfolding and the assembling or disassembling of macromolecular structures. (AlbanÃ¨se *et al.*, 2006). Chaperones are highly conserved and divided into different families according to size, cellular distribution and most importantly sequence similarity which is responsible for their function (Gong *et al.*, 2009). Bukau *et al.* (2006) report that, the major function of chaperones is to prevent aggregation of newly synthesized polypeptides or any newly assembled subunits. Moreover, most chaperones are heat-shock proteins (HSP), due to the fact that conformational stress causes proteins to denature and thus increasing the tendency of aggregation. Chaperones are also involved in a number of other cellular processes, including protein translocation across membranes, ribosomal RNA processing, and ERAD.

The association of resident ER chaperones with newly synthesized proteins is a primary QC mechanism. ER chaperones and folding enzymes such as the Kar2 complex, calnexin homologue (Cne1), PDI, ERp57 and ERp72, assists not only the folding and assembly process, but are also retention anchors for immature proteins. Therefore, binding to any of these resident ER proteins prevents reverse transport of newly synthesized proteins. These chaperones and folding enzymes are localized and retained in the ER by retention and retrieval signals. Most of these luminal-ER proteins contain COOH-terminal His-Asp-Glu-Leu (HDEL) sequences. These HDEL sequences ensure the retrieval of the lumenal-ER proteins from vesiculotubular clusters and the Golgi complex, if they happen to escape (Nilsson and Warren, 1994).

Saccharomyces cerevisiae contains seven heat shock proteins in the ER, three chaperones of the AAA^+ family which is the extended family of ATPase associated with a variety of cellular activities, six proteins of the prefoldin/GinC complex, eight proteins of the CCT/TRiC complex which is the cytosolic chaperonin containing the peptide tcp1 (tall-less complex polypeptide 1), twenty two Hsp40s, one Hsp60, fourteen Hsp70s and two Hsp90 proteins (Gong *et al.*, 2009).

2.4.3 Protein glycosylation

The most complex covalent modification of proteins is glycosylation. *S. cerevisiae* is capable of both *O*- and *N*-glycosylation. *N*-glycosylated proteins contain *N*-linked

oligo- and polymannose chains while *O*-glycosylated proteins contain only short *O*-linked oligomannose chains (Lehle and Basoue, 1984).

2.4.3.1 *O*-glycosylation in *S. cerevisiae*

O-glycosylation takes place in the ER, and requires dolichol monophosphate mannose (Dol-*P*-Man) as a donor. However the extension of the *O*-linked-saccharides occurs in the Golgi apparatus (Trimble and Atkinson, 1986).

2.4.3.2 *N*-glycosylation in *S. cerevisiae*

N-glycosylation is a highly conserved metabolic process, which requires at least 15 enzymatic reactions. *N*-glycosylation is initiated in the ER by the addition of a preassembled oligosaccharyl moiety to a protein by the oligosaccaryltransferase complex (OST) and matured in the Golgi apparatus. The Asparagine-linked glycosylation pathway involves the transfer of an oligosaccharyl moiety namely $Glc_3Man_9GlcNAc_2$, to an asparagine in the consensus sequence Asn-X-Thr/Ser, where X can be any amino acid residue except for proline. The oligosaccharyl moiety is usually transferred from a dolichol-linked pyrophosphate donor. This process takes place during translocation of the newly synthesized protein across the ER membrane into the lumen of the ER (Tu and Weissman, 2004).

The major roles of *N*-glycosylation include the folding, stabilizing and production of mannoproteins that are required in cell wall function and structure; and the processing of proteins that are intended for secretion (Sagt *et al.*, 2000).

The importance of *N*-glycosylation in protein secretion as summarized by Knauer and Lehle (1994) is marked by the function of the enzyme oligosaccharyltransferase (OT). OT plays a role at a point on the secretory pathway where convergence of the following processes takes place, namely, protein translocation into the ER, protein folding and the biosynthesis of the oligosaccharide precursor.

2.4.4 Quality control in the secretory pathway

A primary QC mechanism that is applicable to newly synthesized proteins, irrespective of whether they are endogenous, heterologous or viral in origin, is due to association with the ER chaperones and folding factors (Scheckman, 1982). Primary QC is retention based and dependent upon general biophysical properties shared by incompletely folded proteins, which include the presence of hydrophobic surface patches, mobile loops, and a lack of compactness. This could mean any structural

feature that can be recognized by the molecular chaperones and folding enzymes that are present in the ER (Ferreira *et al.*, 2002). A secondary QC mechanism available entails the rerouting of proteins from the Golgi complex to the vacuole for degradation as detailed in Section 2.4.4.1.

2.4.4.1 Proteolytic degradation

- *Endoplasmic reticulum associated protein degradation*

Degradation of proteins in the ER can be due to prolonged retention of improperly or incompletely folded proteins and this defines the ERAD. The 26S proteosome found in the cytosol is responsible for ERAD. The process takes place in a number of steps. Firstly ER chaperones like CNX, BiP, or protein-specific factors recognize misfolded proteins that are unassembled and possibly deglycosylated and or polyubiquitinated. This is followed by retrotranslocation and lastly proteosomal degradation through Sec61 to the cytosol. There is however a gap in knowledge on how exactly proteins targeted for translocation and degradation are identified. It is however likely that the machinery that is responsible for protein folding could also have a role in selecting and preparing proteins for disposal (Su *et al.*, 1993; Helenius, 1994; Knop *et al.*, 1996; Liu *et al.*, 1999).

The degradation of glycoproteins includes glucosidases I and II, glucosyltransferase and ER mannosidases I and II. A properly folded glycoprotein that contains trimmed mannoses is free to leave the ER due to the lack of mannose residues that could facilitate its binding to CNX. Moreover, the glycoprotein comes to be marked for degradation by the action of mannosidase I given that it is unfolded. This subsequently generates the $Man_8GlcNAc_2$ form of the oligosaccharide. At last the misfolded glycoprotein is retrotranslocated and degraded by the proteosome (Liu *et al.*, 1999).

- *Unfolded Protein Response*

Proteins that are found not to proceed properly along the secretory pathway are degraded. When protein folding is inhibited in the ER a series of signal transduction steps takes place, and these define the unfolded protein response. These steps include the increment of the protein folding capacity by inducing the ER resident molecular chaperones and the phospholipid synthesis, to expand the ER volume. The attenuation of general translation and the up regulation of ERAD, in order to decrease the unfolded protein load incurred in the ER, are also steps that make up

the UPR. The UPR maintains homeostasis in a cell in that the protein folding demand is balanced with cellular protein folding capacity, and if maintaining homeostasis proves impossible, the UPR induces cell death with the aid of the ERAD system in the cytosol (Schroder, 2006).

- *Rerouting from the Golgi complex*

Having escaped ER retention, misfolded and incompletely assembled proteins are sent to the vacuole from the Golgi complex for degradation. This is a secondary QC mechanism that is prominent in *S. cerevisiae*, where Vps10p a transmembrane protein which cycles between the late Golgi and the endosome assists in the rerouting of certain unfolded proteins (Hong *et al.*, 1996; Jorgensen *et al.*, 1999; Li *et al.*, 1999). There is a possibility that Vps10p is a folding sensor that can seize a receptor for transport to the vacuole from the Golgi complex. It also carries misfolded proteins as well as vacuolar proteins such as carboxypeptidase Y and proteinase A to the vacuole (Marcusson *et al.*, 1994; Cooper and Stevens, 1996).

2.4.5 Limitations of *Saccharomyces cerevisiae* in protein secretion

Heterologous protein secretion has prospective value in the large scale production of recombinant proteins as reported by Gasser and Mattanovich (2007) and Idris *et al.* (2010a). The level of secreted proteins in *S. cerevisiae* is low when compared to other yeast expression systems such as *S. stipitis*, *Yarrowia lipolytica*, and *Hansenula polymorpha* (Muller *et al.*, 1998). The study conducted by Den Haan *et al*. (2007b) is a good indication of the poor levels of protein secretion, especially when expressing cellulase-encoding genes. Proteins need to enter the secretory pathway where they are properly folded and processed. This is a major requirement for the production of proteins and can be largely limited by cellular stress reactions, thus influencing production of the proteins (Mattanovich *et al.*, 2004).

2.4.6 Methods for improving protein secretion in *Saccharomyces cerevisiae*

From the discussion above it is clear that protein secretion is a very complicated process involving many proteins. There are a number of strategies reported in literature to improve protein secretion in yeast. These include:

- Vesicle Trafficking engineering
- Pooled-Segregant Whole Genome Sequence Analysis (PSWGSA)

Vesicle trafficking is a targeted approach requiring a detailed understanding of the secretion process whereas PSWGSA is not targeted, rather it is an indirect method used to identify gene targets which could enhance secretion with the aid of quantitative trait loci (QTL) mapping. Good enzyme secreting strains obtained with the latter process need further investigation to determine the source of the improvement (Parts *et al*., 2011).

2.4.6.1 Vesicle Trafficking engineering

Although some heterologous proteins may fold properly, they can at times be retained intracellularly due to poor secretion. Idris *et al*. (2010b) reported that the recombinant growth hormone (hGH) in fission yeast, *Schizosaccharomyces pombe*, was retained intracellularly and subsequently mis-sorted from the Golgi apparatus to the vacuole. This means that there are certain limits found in some of the steps of the secretory pathway, for instance steps taking place from the ER to the Golgi apparatus, or the steps of the post-Golgi secretion. These limitations can be counteracted by secretory pathway engineering which includes vesicle trafficking. Hou *et al*. (2012) reported that the over-expression of the Sec1/Munc18 protein (SM protein) encoding genes *SEC1* and *SLY1*, improves protein secretion in *S. cerevisiae*. SM proteins are essential components of the membrane fusion apparatus which may act with SNARE proteins before and after vesicle attachment. These proteins regulate the assembly of the SNARE complex, and they also work together with SNAREs to stimulate membrane fusion (Carr and Rizo, 2010). Improved secretion of the heterologous proteins suchas the human insulin precursor and alpha-amylase and the secretion of an endogenous invertase was accomplished by engineering of Sec1p, the SM protein that is involved in vesicle trafficking from the Golgi apparatus to the cell membrane. Over-expressing the genes encoding the yeast syntaxin proteins, *SSO1 or SSO2*, which are responsible for the Golgi-derived vesicles fusion to the plasma membrane could ultimately increase the secretion of invertase or *Bacillus* alpha-amylase in *S. cerevisiae* (Ruohen *et al.*, 1997). These studies validated engineering the secretory pathway to improve vesicle trafficking and can improve heterologous protein production.

However, there are several rate-limiting steps in the secretory pathway caused by the lack of strategies that improves protein secretion for a broad range of proteins. The protein secretion pathway in *S. cerevisiae* is responsible for protein trafficking to the extracellular space, vacuole or cell membrane. This secretory pathway spans

several compartments (Ellgard and Helenius, 2003), and includes the protein trafficking through the ER, Golgi apparatus, trans-Golgi network, endosome and either to the cell membrane or vacuole, using vesicles to transport the proteins (Figure 2.4). The secretion vesicle fuses to the target membrane and then delivers the transported proteins. The process of vesicle trafficking requires soluble *N*-ethylmaleimide-sensitive factor (NSF) receptor complex formation which involves the SM proteins (Malsam *et al.*, 2008). The SNARE complex is a large super-family of proteins made up of more than 60 members in both yeast and mammalian cells. Its primary function is to drive vesicle fusion such as exocytosis (Gerald, 2002).

SNAREs are small, abundant proteins that are bound to the plasma membrane. They vary substantially in size and structure, and all share a *SNARE* motif which is a segment in the cytosolic domain. The *SNARE* motif is made up of 60-70 amino acids which are capable of reversible assembly into tight, four helix bundles called *trans-SNARE* complexes. Membrane fusion is accomplished by the SNARE proteins anchored to both target and vesicle membranes and assembled into a helix, *SNARE* complex (Sudhof and Rothman, 2009). There are four SM proteins in *S. cerevisiae* namely, Sec1p, which interacts between the Golgi apparatus to the cell membrane; Sly1p, which regulates the ER-Golgi trafficking; Vps45p interacting in the Golgi-late endosomal trafficking and lastly Vps33p regulating the endosome and vacuole trafficking (Furgason *et al.*, 2009).

The assignment of some SNAREs to certain trafficking steps, in particular the R-SNAREs, is still debated. *S. cerevisiae* has two endosomal syntaxins, Pep12 and Vam3p that are thought to be involved in consecutive trafficking steps towards the vacuole, whereas other fungi only have one.

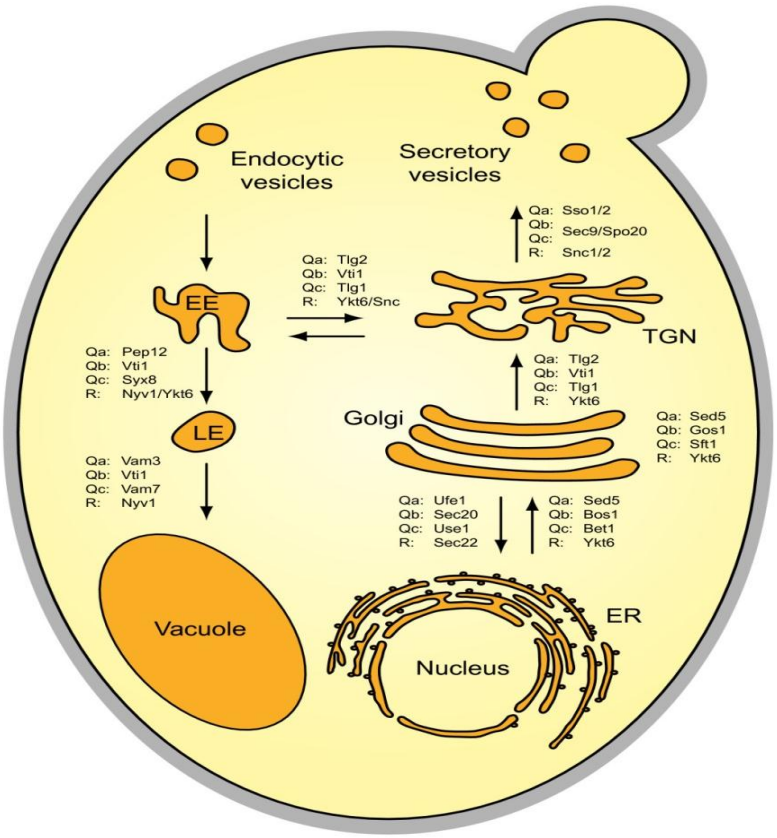


Figure 2.4. Schematic outline of the vesicle trafficking pathways and tentative assignment of the involved sets of SNARE proteins of *S. cerevisiae* (Kienle *et al.*, 2009).

2.4.6.2 Pooled Segregant Whole Genome Sequence Analysis

Kroukamp *et al.* (2012) created super-secreting strains of *S. cerevisiae* through successive mating of two haploid strains (α and a) selected based on their secretory yield of the recombinant *Talaromyces emersonii Cel7*A (*CBH I*) (Figure 2.5). One of the haploid parents had intermediate secretion ability (*S. cerevisiae* Y294) and the other high (*S. cerevisiae* M0341).

After sporulation of the diploids, the resulting haploid progeny (F1) were screened for extracellular cellobiohydrolase activity and a number of these strains had higher enzyme activity than the parental strains. The 28 progeny (out of ±530) with the highest secreted CBH I enzyme activity were selected and re-evaluated with assays done in duplicate (Figure 2.6).

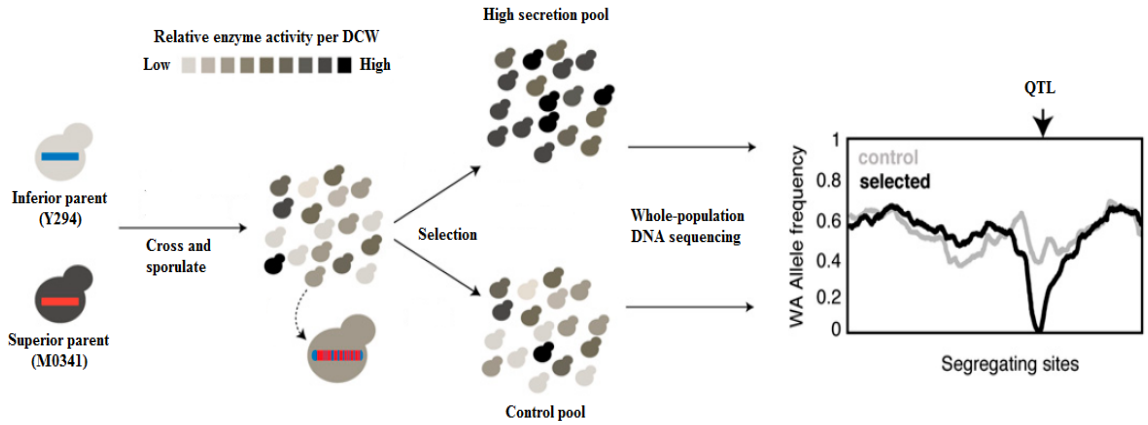


Figure 2.5. Schematic representation of the overall strategy of polygenic analysis used in the construction of hybrid segregant strains evaluated in this study. This is a three-step QTL mapping strategy which starts by crossing two phenotypically different strains, (in this case an *S. cerevisiae* inferior parent Y294 and a superior parent M0341). This is followed by sporulation to generate a large segregating pool of individuals of various fitness, and growing the pool under restrictive conditions that enriches for beneficial alleles that can be detected by sequencing the total DNA from the pool (Parts *et al.*, 2011; Kroukamp *et al.*, 2016).

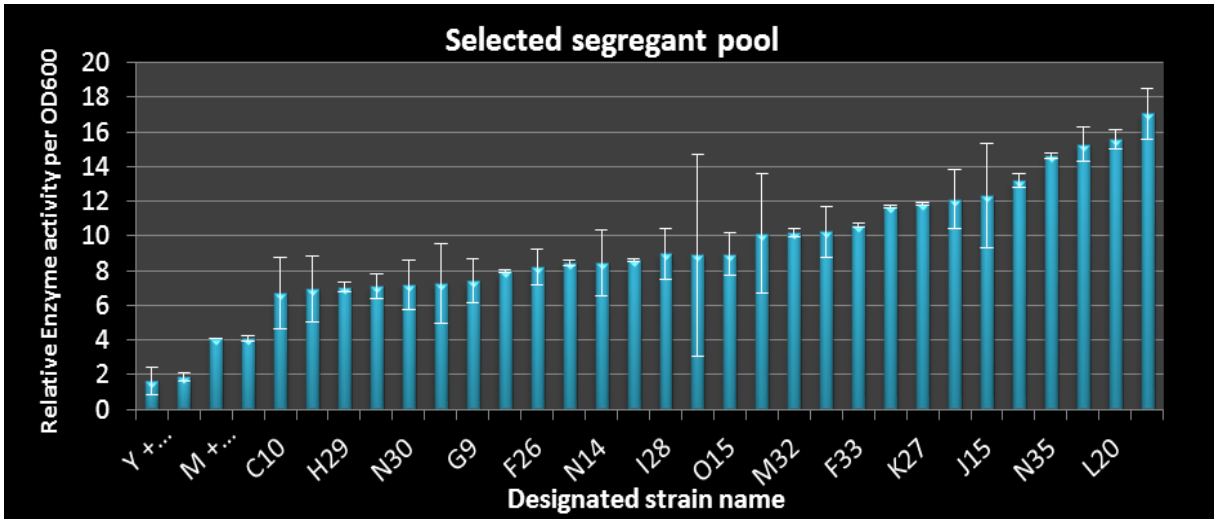


Figure 2.6. Extracellular cellobiohydrolase activity of 28 segregants obtained after mating and sporulating of a high and an intermediate secreting parental strain. Y represents the parent with intermediate secretion and M the parent with high secretion of CBH I (Kroukamp *et al.*, 2016).

The studies by (Kroukamp *et al.*, 2012; Kroukamp *et al.*, 2016) are part of a genome sequencing project that aims to identify genes that confer or can potentially confer high secretion in *S. cerevisiae*. The over-expression of native genes/alleles involved

in protein modification in *S. cerevisiae* was investigated and also found to have a positive influence on improving the secretion of cellulases, specifically cellobiohydrolase (Kroukamp *et al*., 2012).

High secretion ability has many applications in industry. Hydrolysing lignocellulosic biomass to produce bioethanol require the synergistic action of a number of different enzymes.

2.5 Hydrolase secretion for the biofuel industry

There is a global interest in the production of biofuel resulting from governmental incentives to establish greater energy independence. This is mainly due to the belief in reduced cost of production compared to fossil fuels. Environmental concerns and the depletion of oil reserves are further reasons for the exploration of alternative fuel sources. Biofuel production relies mostly on the provision that the bioconversion technologies are optimized. These conversion technologies are centred on the use of lignocellulosic biomass found in many low value agricultural or wood pulping wastes. These wastes are abundant, low in cost and environmentally friendly (Tsai *et al.*, 2009). Biofuels have the potential to displace liquid fossil fuels (Van Zyl *et al.*, 2007). The major obstacle in the large-scale production of biofuels is the lack of a low-cost bioconversion technology that is able to overcome the recalcitrant nature of lignocellulose (Lynd *et al.*, 2002; Olsen *et al.*, 2012).

2.5.1 Recombinant enzyme production for consolidated bioprocessing

CBP is the combination of four biological events required for the conversion of lignocellulose to bioethanol in one reactor. The four process steps include, production of saccharolytic enzymes, hydrolysis of the polysaccharides present in pre-treated biomass, fermentation of hexose sugars and lastly fermentation of pentose sugars (Van Zyl *et al.*, 2007).

As illustrated in Figure 2.7, current conversion technologies include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and lastly CBP. CBP is the most cost effective bioconversion process relative to the other technologies which at times require expensive thermochemical processes such as combustion, pyrolysis and gasification (Lynd *et al.*, 2005). Therefore, recombinant

microorganisms such as *S. cerevisiae* are being engineered to achieve maximum productivity of the desired end-product.

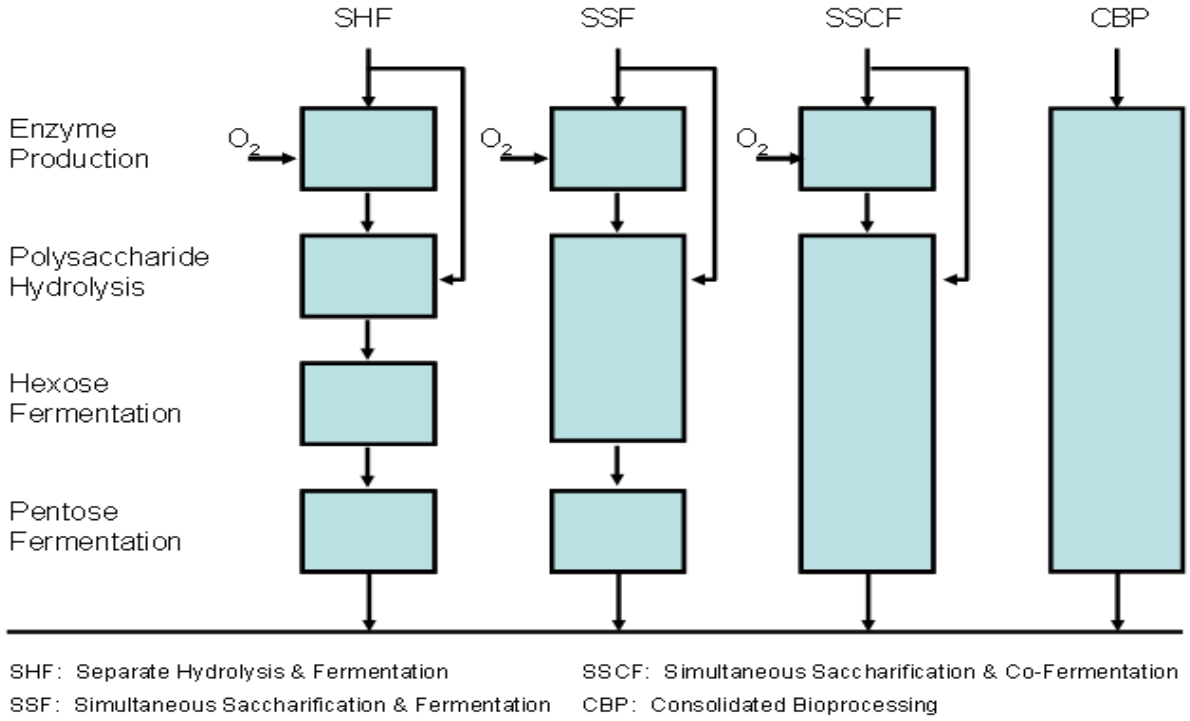


Figure 2.7. Schematic diagram outlining four bioconversion technologies that can be used for the production of recombinant bioethanol from lignocellulose (Adapted from Lynd, 1996).

2.5.2 Engineering *Saccharomyces cerevisiae* for CBP

Engineering *S. cerevisiae* for CBP requires the development of a strain that can utilize pentose sugar and produce all the enzymes required to hydrolyse cellulose, hemicellulose or starch (Den Haan *et al.*, 2013).

There have been several approaches to engineer *S. cerevisiae* to produce all the hydrolases required for CBP, and these include:

- extracellular secretion of multiple hydrolases
- cell surface engineering of hydrolases
- minicellulosome display of hydrolases on the cell surface

2.5.2.1 Secretion of multiple hydrolases by *Saccharomyces cerevisiae*

In the quest for the construction of an ideal organism for CBP, several advances have been made for the expression and secretion of hydrolytic enzymes (Van Rooyen *et al.*, 2005; Den Haan *et al.*, 2007a; Ilmen *et al.*, 2011). There have been

reports of relatively high protein production levels by *S. cerevisiae* (Schimdt, 2004). Enhancing the capacity of *S. cerevisiae* is significant because the ideal yeast should be able to express different amylases, cellulases, hemicellulases and all accessory enzymes.

The maximisation of the production of heterologous proteins is reliant upon the gene copy number, codon usage and the proper choice of promoters in order to obtain sufficient transcript levels in *S. cerevisiae*. Moreover, the transcripts produced by the host have to be stable for the maintenance of high levels of translation of the heterologous genes (Mattanovich *et al.*, 2004). In order to produce extracellular proteins, it is required that the secreted proteins possess the ability to enter in to the secretory pathway, where they are correctly folded and processed and subsequently secreted in an active form into the extracellular medium (Mattanovich *et al.*, 2004; Schroder, 2006).

Illmen *et al*. (2011) reported a significant increase in the secretion of various CBHs expressed by *S. cerevisiae* for potential use in CBP. Furthermore, they demonstrated that the UPR was activated as a result of the production of CBH I. High levels of secretion during the co-expression of hydrolases is possible and has been successfully reported (Cho *et al.*, 1999; Yamada *et al.*, 2010; Ilmen *et al.*, 2011).

The successful co-expression and secretion of endo-glucanase and β-glucosidase by *S. cerevisiae* was reported by den Haan *et al*. (2007a) indicating that *S. cerevisiae* has potential as a CBP strain, provided that heterologous protein secretion is improved. This study validates the importance of not only secreting CBH I, but also a number of other cellulases and hemicellulases.

2.5.2.2 Cell surface engineering of hydrolases in *S. cerevisiae*

Another promising alternative in the attempt to improve cellulolytic enzyme secretion is the display of these enzymes on the yeast cell surface. As shown by Kondo and Ueda (2004), this method requires for example the glycosylphosphatidylinositol anchoring system which enables the display of various kinds of functional proteins on the surface of the cell without loss of activity. This method has allowed the construction of efficient whole cell biocatalysts for SSF (Fujita *et al.*, 2002; Fujita *et al.*, 2004; Kotaka *et al.*, 2008; Yamada *et al.*, 2010). The cellulolytic enzymes displayed on the yeast cell surface are genetically self-immobilized on the yeast

allowing the enzymes to retain activity as long as the yeast continues to grow. This is a major advantage because it is challenging to maintain the activities of enzymes for a long reaction period in the conventional direct fermentation system where cellulolytic enzymes are secreted into the medium (Tanaka and Ueda, 2000). An additional advantage of the cell surface display of cellulolytic enzymes method is the ability to separate the biocatalyst from the product with ease. This makes it possible to reduce the cost of propagating the yeast or the addition of more enzymes by re-utilizing the yeast cells and thus enabling the reuse of the enzymes that are displayed on the cell surface without having to reproduce the yeast cells and cellulolytic enzymes (Kondo *et al.*, 2002).

A good example of the use of cell surface engineering was reported by Fujita *et al*. (2004) where SSF of phosphoric acid-swollen cellulose (PASC) to ethanol using a recombinant yeast strain co-displaying three cellulolytic enzymes, *T. reesei* EGII and CBHII, and *A. aculeatus* BGLI was accomplished. This strain yielded 0.45 g/g ethanol which is 88.5% of the theoretical maximum, whilst consuming PASC as the sole carbon source. The complete hydrolysis of cellulose can be achieved through the display of multiple enzymes on the yeast surface. The cellobiose produced by the action of EGII and CBHII was further cleaved into glucose which the cell can metabolise through glycolysis. The advantages of whole cell biocatalysts to degrade cellulose include the conversion of cellobiose to glucose, which can potentially inhibit cellulase activities, through the action of BGLs. Ethanol is produced simultaneously as glucose is taken up, thus reducing the level of sterility required. Lastly, the use of a single bioreactor saves on capital costs.

This system also allows for the elimination of additional exogenous enzymes in the production of ethanol from agricultural waste (Yamada *et al.*, 2011).

2.5.2.3 Minicellulosome display on the cell surface of *Saccharomyces cerevisiae*

A bifunctional cell surface display system was constructed by Ito *et al.* (2009). This system employs the Z domain of protein A and the Fc domain of human immunoglobulin G as well as the cohesin and dockerin domain from *Clostridium cellulovorans* to construct a minicellulosome on the *S. cerevisiae* cell surface. Several similar strategies to display a minicellulosome on the surface of *S. cerevisiae* have been reported in a quest to increase cellulolytic capability (Ito *et al.*, 2009; Lilly *et al.*, 2009; Tsai *et al.*, 2009; Tsai *et al.*, 2010; Wen *et al.*, 2010).

A tri-functional minicellulosome which consisted of three types of cellulases; EGll, CBHll and BGLI; that each had a C-terminal dockerin and a mini-scaffoldin which contained a CBM and three cohesion modules were anchored to the yeast cell surface by Wen *et al*. (2010). This system yielded 1.8 g/l of ethanol produced from the simultaneous degradation and fermentation of PASC which was enhanced by the close proximity and ordering of the EGll and CBHll on the miniscaffoldin.

2.5.3 Inhibitors produced during lignocellulosic pre-treatment

Due to the composition of lignocellulose, enzymatic digestion of cellulose is problematic. Pretreatment is required as an initial step in the bioconversion of lignocellulosic material. The structural recalcitrance necessitates the need for chemical and or physicochemical pre-treatments such as steam explosion, ammonia fibre explosion (AFEX), liquid hot water, lime, or acid treatment. Pre-treatment facilitates the breakdown of lignin thus separating it from cellulose and in turn disrupting the crystalline structure of cellulose allowing enzymes access to hydrolyse cellulose (da Costa Sousa *et al.*, 2009; Hendriks and Zeeman, 2009). A range of degradation products of lignin and sugar are produced due to the harsh conditions used during pre-treatment of lignocellulose. These products inhibit ethanol fermentation by *S. cerevisiae* as discussed in reviews by Palmqvist and Hahn-Hagerdal (2000); Almeida *et al*. (2007) and Liu and Blascheck (2009). The most common inhibitors are: i) weak organic acids such as acetic, levulinic and formic acids which are formed by the de-acetylation of hemicelluloses; ii) furan derivatives such as 5-hydroxymethyl-2-furaldehyde (5-HMF) and 2-furaldehyde (furfural) which are formed by the dehydration of hexoses and pentoses, respectively and; iii) phenolic compounds such as vanillin, and ferulic acids generated when lignin breaks down or as a result of carbohydrate degradation.

In this study, the effects of acetic acid, furfural and vanillin were evaluated.

2.6 Conclusion

Protein secretion is a very important step in the production of extracellular proteins (Tan and Ding, 2002). It is a complex process that is influenced by a number of factors. Many of these factors have been studied in detail and a large number of super-secreting strains have been constructed using different strategies. Mating and sporulation of recombinant yeasts is a powerful method for creating yeast strains with

desired properties (Parts *et al.*, 2011). This study will evaluate whether the high secretion phenotype of a number of different *S. cerevisiae* segregants results from a general increase in secretion ability or if it is specific to a single enzyme.

CHAPTER 3. MATERIALS AND METHODS

3.1 Microbial strains and plasmids

The relevant genotypes and corresponding sources of the yeast and bacterial strains, that were constructed and used in this study, are summarized in Table 1.

Table 3.1. Microbial strains and plasmids.

Strain/ plasmid	Genotype	Source/reference
Plasmids		
pMU1531	*ENO_P-ENO_T*	Illmen *et al.*, 2011
pNS201	*ENO_P-cel3A -ENO_T*	This work
pNS202	*$ENO1_P$-cel7A-$ENO1_T$*	This work
pNS203	*$ENO1_P$-glu-$ENO1_T$*	This work
pRDH166	*$ENO1_P$-cel12A-$ENO1_T$*	Illmen *et al.*, 2011
pRDH182	*$ENO1_P$-Xyn2-$ENO1_T$*	Illmen *et al.*, 2011
Parental yeast strains		
S. cerevisiae Y294	α *leu*2-3112 *ura*3-52 his3 *trp*1-289	ATCC 201160
S. cerevisiae M0341	*Wild type*	Illmen *et al.*, 2011
Hybrid yeast strains		
S. cerevisiae H3O23	*leu*2-3112 *ura*3-52 *his*3 *trp*1-289	Kroukamp *et al*., 2016
S. cerevisiae H3M1	*leu*2-3112 *ura*3-52 *his*3 *trp*1-289	Kroukamp *et al*., 2016
S. cerevisiae H3M28	*his*3 *trp*1-298	Kroukamp *et al*., 2016
S. cerevisiae H3H29	*leu*2-3112 *his*3 *trp*1-289	Kroukamp *et al*., 2016
S. cerevisiae H3K27	*leu*2-3112 *his*3 *trp*1-289	Kroukamp *et al*., 2016
Bacterial strain		
Escherichia coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	GIBCO/Bethesda Research Laboratories, Life Technologies Ltd.

Escherichia coli DH5α strain (GIBCO/Bethesda Research Laboratories, Life Technologies Ltd.) was used as the bacterial cloning host. *Saccharomyces cerevisiae* Y294 (*α leu-3112, ura3-52, his-3, trp1-289; ATCC201160*) and M0341 were used as expression hosts. Plasmid pMU1531, containing the constitutive enolase (*ENO1*) promoter and terminator and the *URA3* selectable marker was used as the negative control plasmid and was the backbone used for all yeast expression vectors.

3.2 Media and culture conditions

Bacterial cells were grown in LB medium (0.5% yeast extract, 1 % NaCl, 1% tryptone) supplemented with ampicillin (100 mg/L) at 37 ^{o}C on a rotary shaker at 150 rpm.

Saccharomyces cerevisiae strains were cultured in either synthetic complete (SC) media containing 0.34% yeast nitrogen base w/o amino acids, 0.5% ammonium sulphate, 2% glucose and supplemented with 1.5 g/L amino acids without uracil, the pH was adjusted to pH 6.0 using 1M NaOH, or in yeast peptone dextrose media (YPD) containing 1% yeast extract, 2% peptone and 2% dextrose at 30 ^{o}C on a rotary shaker at 200 rpm. Solid media contained 2% bacteriological agar.

URA3 disrupted strains were grown on 0.2% 5-Flouro-orotic (FOA), 1.2 mg/L uracil, 0.34% yeast nitrogen base w/o amino acids, 1% ammonium sulphate, 2% dextrose, 2% agar and 1.5 g/L amino acids without uracil.

SC medium containing 0.2% of 4-*O*-methyl-D-glucurono-D-xylan–Remazol Brilliant Blue R (RBB)-xylan and 2% glucose as the carbon source and bacteriological agar was used to confirm xylanase activity. RBB-xylan was synthesised according to the methods described by (Biely *et al.*, 1988). Plate activity assays were done for the endo-glucanase (*Cel*12*A)* according to the method described by Van Rensburg *et al*. (2012). Colonies were plated on buffered SC^{-ura} medium containing 1% CMC. The plates were incubated overnight at 30^{o}C, before flooding with 0.1% Congo Red solution.

For enzyme activity assays yeast strains were cultivated on double-strength buffered SC medium containing 0.43% yeast nitrogen base w/o acids, 2% succinate, 1% ammonium sulphate, 2% glucose and 1.5 g/L amino acids without uracil; the pH was adjusted to pH 6.0 with 1M NaOH.

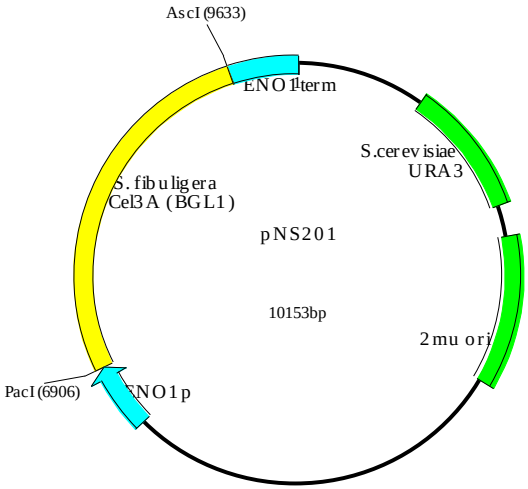
For evaluating the sensitivity of the yeast strains to fermentation inhibitors, cells were spotted on YPD medium containing 2% agar and 0%, 4%, 6% or 8% ethanol, whereas for furfural, acetic acid and vanillin the cells were spotted on SC medium containing 2% agar and 0.2, 0.5, or 1 g/L furfural and 1, 2, or 5 g/L acetic acid and 0.2, or 1 g/L vanillin.

3.3 Plasmid Construction

Standard protocols were used for all DNA manipulations, (Sambrook and Russel, 2001). Restriction endonucleases and T4 DNA ligase were used according to the manufacturer's (Fermentas) directions. For the construction of pNS201 the open reading frame of *S. fibuligera cel*3A β-glucosidase (*BGL1)* was excised with *Asc*I and *Pac*I from plasmid pBKD1_BGL1 and ligated into the corresponding sites of yeast expression vector pMU1531. Subsequently, the codon optimized *T. emersonii cel*7A

(*CBH I)* and *S. stipitis* α-glucuronidase genes were digested with *Asc*I and *Pac*I from plasmids pMI529 and pBKD_Ps.GLU, respectively. These genes were ligated into the corresponding site of pMU1531 to yield pNS202 and pNS203, respectively.

A



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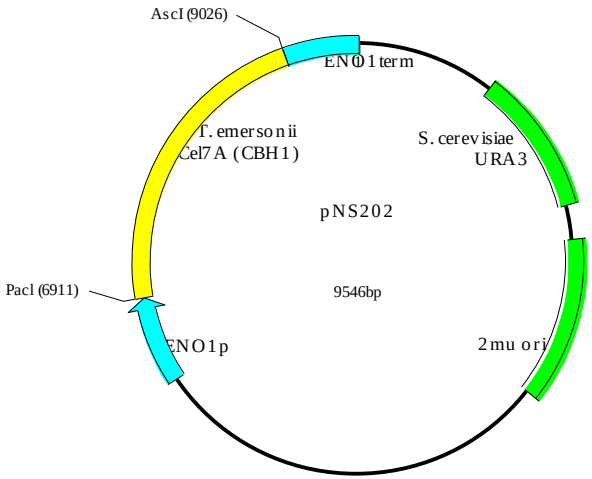

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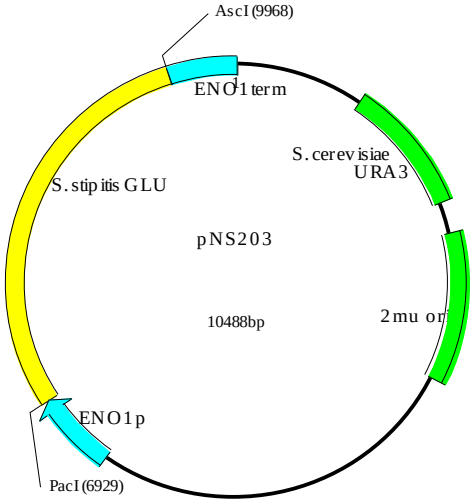

Figure 3.1. Schematic representation of plasmid pNS201, pNS202 and pNS203. (A) shows the *S. fibuligera cel*3A (*BGL1)* gene indicated in yellow, (B) shows the *T. emersonii cel*7A *(CBH I)* gene indicated in yellow, (C) shows the *S. stipitis GLU* gene indicated in yellow; the relevant restriction enzyme positions are also shown. Reporter enzyme expression for all plasmids is under the transcriptional control of the constitutive *ENO1* promoter.

3.4 *Saccharomyces cerevisiae* host strain engineering

3.4.1 Deletion of the *Cel7A (CBH I)* gene

The seven strains of *S. cerevisiae* (Kroukamp *et al*., 2012; 2016) used in this study contained an active *CBH I* gene at the *LEU2* locus (Table 3.3). These *S. cerevisiae* segregant strains have been designed to to secrete CBH I from a single integrated copy, at high levels (Kroukamp *et al*., 2012). Therefore, the single integrated copy *CBHI* had to be deleted in order to evaluate episomal secretionof the reporter enzymes. The non-functional open reading frame of the *leu2* gene of *S. cerevisiae* was amplified using the primer sets LEU2fix-L and LEU2fix-R (Table 3.2). Electrotransformation and homologous recombination was used to replace the *CBH I* with the 1100 bp *LEU2* PCR fragment (Figure 3.2) (Boeke *et al.*, 1984). Total genomic DNA of each transformant was isolated and successful integration was confirmed by PCR using the relevant PCR primer (Table 3.2).

Table 3.2. PCR primers for plasmid and yeast strain construction.

Primer name	Sequence (5'→ 3')
S. cerevisiae LEU2	
LEU2fix – L	GGAGAACTTCTAGTATATCTAC
LEU2fix - R	ATGGTTTCCTCCACAG
S. cerevisiae URA3	
ScURA – L	ATGTCGAAAGCTACATATAAGGAACG
ScURA – R	TTAGTTTTGCTGGCCGCATC
S. cerevisiae	
ENOprom1	GTAACATCTCTCTTGTAATCCCTTATTCCTTCTAGC
ENOterm	GCAACCCTATATAGAATCATAAAACATTCGTGA
S. stipitis	
PSglu-L	TGCAGGATCCAAATGTTGTTTTCATACTTCCCAGC
PSglu-R	GTACTCTAGACTACTTTTTGATGTAAGTTTCTGGTGG
S. fibuligera	
SFBGL1-L	GACTCGCGAGTCCCAATTCAAAACTATACC
SFBGL1-R	CCGCTCGAGCGGTCAAATAGTAAACAGGACAGATG
T. reesei	
sCBH1/2-L	GACTGAATTCATAATGGTCTCCTTCACCTCC
sCBH1-R	GACTCTCGAGTTACAAACATTGAGAGTAGTATGG
N. fischerii	
EG3-3-L	ATCTATAACTACAAAAAACACATACATAAACTAAAATTA ATTAAAATGAAAACCTTCG
EG3-3-R	TCATTAAAAAACTATATCAATTAATTTGAATTAACGGCG CGCCTTAGTTAAC
T. reesei	
nXyn2-L	GATCTTAATTAAAATGGTCTCCTTCACCTCC
nXyn2-R	GTACGGCGCGCCCTCCCTTTAGCTGACGGTG

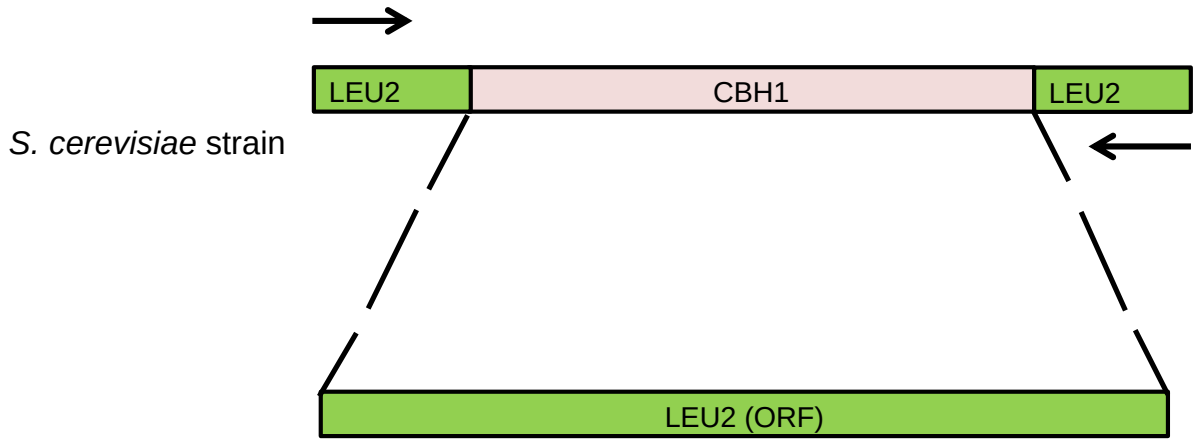

Figure 3.2. Schematic representation of the gene replacement strategy used to remove the *CBH I* gene from all yeast strains, through homologous recombination.

3.4.2 The inactivation of the *URA3*

The reporter genes were expressed from an episomal plasmid using *URA3* selection; however some of the segregants contained an intact *URA3* on the chromosome (Table 3.3). In order to use *URA3* for plasmid selection, the *URA3* had to be deleted from all the hybrids containing an intact *URA3*.

Table 3.3. The URA phenotype of the different *Saccharomyces cerevisiae* strains used in the study.

Yeast Strain	*CBH I*	URA3
Y294	Active	-
M0341	Active	+
H3M1	Active	-
H3M28	Active	+
H3O23	Active	-
H3H29	Active	+
H3K27	Active	+

A mutated non-functional *ura3* was amplified from *S. cerevisiae* Y294 using PCR primers ScURA–L and ScURA-R (Table 3.2). The 1003 bp *ura3* PCR fragment was purified using the Zymoclean extraction kit following electrophoresis on a 1% agarose gel. Electroporation and homologous recombination was used to replace the *URA3* on the chromosome with the *ura3* PCR product as described by Boeke *et al.* (1984). Transformants were plated on 5-flouro-orotic acid (5-FOA) plates, and incubated at 30 ^{o}C for 2 – 3 days. Single colonies that developed were plated on FOA plates again and incubated at 30 ^{o}C for 2 – 3 days. Single colonies from the second round of FOA plates were plated on SC^{-ura} plates and incubated at 30 ^{o}C for 2 days to confirm the *URA3* negative phenotype.

3.5 Reporter gene transformation

Five super secreting *S. cerevisiae* segregants strains and two parents were each transformed with five individual reporter enzyme expressing plasmids. The reporter enzymes used were for *S. fibuligera* β-glucosidase I (Cel3A), the *N. fischerii* endo-glucanase 3 (Cel12A), the *T. emersonii* cellobiohydrolase 1 (Cel7A), the *T. reesei* endo-β-xylanase (Xyn2) and the *S. stipitis* α-glucuronidase (glu). Plasmids were transformed by using the lithium acetate dimethyl sulfoxide (LiOAc/DMSO) protocol described by Hill *et al*. (1991). This resulted in a total of 42 recombinant strains (Table 3.4). Total genomic DNA of each transformant was isolated and a successful

integration was confirmed by PCR analyses using the relevant PCR primer sets (Table 3.2).

Table 3.4: Yeast strains were been constructed for the evaluation of secretion ability of yeast segregants.

Strain/plasmid	Abbreviated name	Genotype
***S. cerevisiae* Y294**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	Y294[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	Y294[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	Y294[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	Y294[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	Y294[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*
***S. cerevisiae* M0341**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	M0341[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	M0341[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	M0341[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	M0341[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	M0341[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*
***S. cerevisiae* H3M1**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	H3M1[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	H3M1[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	H3M1[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	H3M1[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	H3M1[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*
***S. cerevisiae* H3M28**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	H3M28[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	H3M28[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	H3M28[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	H3M28[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	H3M28[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*
***S. cerevisiae* H3O23**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	H3O23[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	H3O23[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	H3O23[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	H3O23[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	H3O23[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*
***S. cerevisiae* H3H29**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	H3H29[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	H3H29[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	H3H29[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	H3H29[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	H3H29[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*
***S. cerevisiae* H3K27**		
pMU1531	control	*ENO1$_P$-ENO1$_T$*
pNS201	H3K27[*cel3A*]	*ura3-52 ENO1$_P$-cel3A-ENO1$_T$*
pNS202	H3K27[*cel7A*]	*ura3-52 ENO1$_P$-cel7A-ENO1$_T$*
pNS203	H3K27[*glu*]	*ura3-52 ENO1$_P$-glu-ENO1$_T$*
pRDH166	H3K27[*cel12A*]	*ura3-52 ENO1$_P$-cel12A-ENO1$_T$*
pRDH182	H3K27[*Xyn2*]	*ura3-52 ENO1$_P$-Xyn2-ENO1$_T$*

3.6 Protein Analyses

3.6.1 Enzyme Activity Assays

All yeast strains were cultured in triplicate in 250 mL Erlenmeyer flasks containing 50 mL double-strength buffered SC^{-ura} for at least 72 hrs on a rotary shaker (150 rpm) at 30 ^{o}C. Endo-glucanase, endo-xylanase, β-glucosidase and cellobiohydrolase activity were determined using carboxymethylcellulose (CMC), beechwood xylan, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and *p*-nitrophenyl-β-D-cellobioside (*p*NPC) as substrates, respectively (La Grange *et al.*, 1996; Van Rooyen *et al.*, 2005; Den Haan *et al.*, 2007a). For endo-glucanase and endo-xylanase assays glucose and xylose were used to prepare calibration curves in the range 0 – 10 g/L. The reducing sugars released through enzyme activity were determined with DNS reagent (Sumner and Somers, 1949) and the absorbance measured at 540 nm. For *p*NPG and *p*NPC assays a *p*NP calibration curve in the range 1.5 – 25 mM was employed. The addition of 1 M Na_2CO_3 stopped the reaction and the liberated *p*-nitrophenol was measured at 400 nm. It is possible to determine α-glucuronidase using birchwood xylan as substrate (Gomes, 2012), however the assay is not very sensitive and requires fairly high concentrations of active enzyme. Therefore, SDS-PAGE was used to quantify the amount of α-glucuronidase produced in this study.

3.6.2 SDS-PAGE

The amount of α-glucuronidase protein secreted was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The proteins were separated on 10% polyacrylamide gels containing 30% acrylamide, 1.5 M Tris (pH 8.8), 10% sodium dodecyl sulphate (SDS), 10% ammonium persulphate and *N*-tetramethylethylene diamine (TEMED). The supernatant (1 mL) from α-glucuronidase producing segregants as concentrated using the ultrafiltration method (Evans *et al.*, 2009) with Amicon Ultra- 0.5 mL Centrifugal filters MWCO = 3000 Da (Merck Millipore) and resuspended in 100 µL distilled water. The loading buffer contained 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14 mM β-mercaptoethanol and 0.1% bromophenol blue. The protein bands were visualised by Coomasie brilliant blue G250 (Sigma) staining. Following detection of the proteins on the gel a digital image was used for densitometric quantification of protein levels, using ImageJ (Abramoff *et al.*, 2004).

3.7 Fermentation Studies

Computer-controlled glass bioreactors (New Brunswick Scientific, Enfield, CT, USA) with a total volume of 2.5 L and a working volume of 1 L were used to conduct fermentations. The fermentation batch culture was inoculated to an OD_{600nm} of 0.1. The bioreactors were equipped with two Rushton impellers, an exhaust gas condenser, a pH electrode (Mettler-Toledo), and a polarographic oxygen probe (Mettler-Toledo). The temperature and pH were maintained at 30 ^{o}C and pH 5.5, respectively, by automatic rotation and 1 M KOH. An aeration rate of 0.5 L/min together with the automatic adjustment of the stirrer speed between 250 and 450 rpm (standard conditions). The oxygen tension was maintained at a minimum of 30% saturation. Silicone antifoam A Emulsion (Sigma) was added to the fermenter to control foaming. All fermentations were done in triplicate in a random order.

3.7.1 Calculations

The maximum specific growth rate (μ_{max}^{-1}) of the cultures was determined from the slope of a curve where the natural logarithm of the biomass concentration was plotted as a function of time, using a minimum of six data points (Van Rensburg *et al*., 2012).

CHAPTER 4.RESULTS

4.1 Plasmid construction

A total of six plasmids were used in this study. Three (pNS201, pNS202 and pNS203) were constructed while three (pRDH166, pRDH182 and pMU1531) were generously donated by WH Van Zyl at the University of Stellenbosch (Table 4.1). The newly constructed expression vectors together with those donated contained the same plasmid backbone.

Table 4.1: Factors considered when choosing the reporter enzymes used in this study.

Gene expressed and reason for selection	*N*-glycosylation sites	Protein size	Plasmid used	Reference
Control plasmid	0	0	pMU1531	Van Zyl lab
Codon optimized *T. emersonii* cel7A (CBH I)	3	55 kDa	pNS202	This study
Codon optimized *N. fischerii cel*12*A* (*EG3)*	0	25 kDa	pRDH166	Van Zyl lab
Native *T. reesei* XYN2	2	21 kDa	pRDH182	Van Zyl lab
Codon optimized *S. fibuligera* cel3A (BGL1)	11	92 kDa	pNS201	This study
Codon optimized *S. stipitis GLU*	16	113 kDa	pNS203	This study

Reporter enzymes were under the transcriptional control of the constitutive *ENO1* promoter and terminator in order to achieve high levels of gene expression. Plasmid pNS201 was used for the expression of the *S. fibuligera* cel3A (BGL), pNS202 for the expression of the *T. emersonii* cel7A (CBH I), and pNS203 the expression of the *S. stipitis* GLU. All the band sizes (Figure 4.1) correlated with the expected gene lengths.

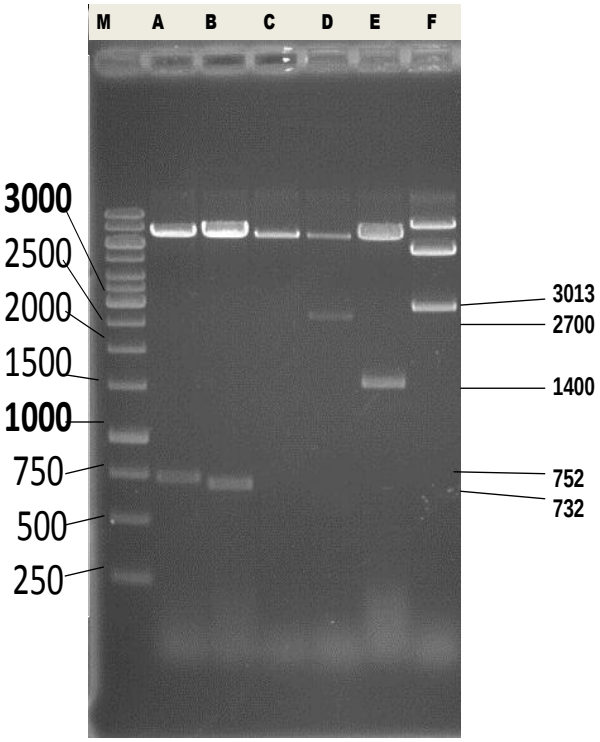

Figure 4.1: Gel separation of all the episomal plasmid DNA used in this study. All plasmids were digested with *Asc*I and *Pac*I to excise the reporter gene. From left to right, the yeast strains were loaded on the gel as follows: [M]: Molecular weight marker (bp), [A]:pRDH166, [B]:pRDH182, [C]:pMU1531, [D]:pNS201, [E]:pNS202, [F]:pNS203.

4.2 *Saccharomyces cerevisiae* host strain engineering

4.2.1 The disruption of the *Cel7A (CBH I)* gene

The *T. emersonii* Cel7A gene was integrated at the *LEU2* locus of the *S. cerevisiae* parental strains, Y294 and M0341 before mating. In order to evaluate the expression of the different reporter enzymes, the Cel7A was removed.

The open reading frame of the *LEU2* gene of *S. cerevisiae* was PCR amplified and transformed into the seven yeast segregant strains ensuring the *CBH I* gene knock-out through homologous recombination. To confirm successful removal of the Cel7A from the *LEU2* locus genomic DNA was isolated using standard DNA isolation methods (Sambrook and Russel, 2001). The genomic DNA was used as template to amplify the *LEU2* open reading frame using primers LEU2fix-L and LEU2fix-R (Table 3.2). The presence of an 1100 bp band (Figure 4.2) confirms the deletion of the *Cel7A* from the chromosome of the segregants.

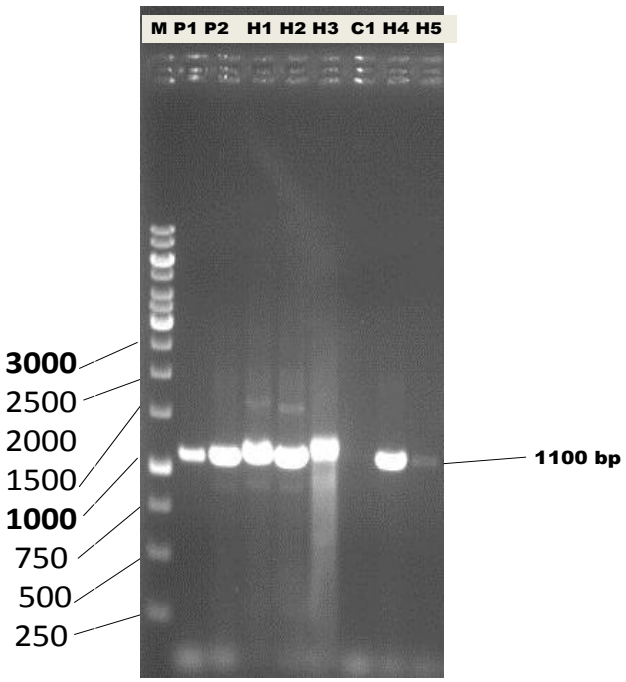

Figure 4.2: Gel electrophoresis to confirm the removal of the *CBHI* from the chromosome of the different super-secreting hybrids. PCR was used to amplify the *LEU2* ORF. With the *Cel7A* present a 4500 bp band is expected. Successful deletion of the *Cel7A*I produced a band of 1100 bp. From left to right, the yeast strains were loaded on the gel as follows: [M]: Molecular weight marker (bp), [P1]:Y294, [P2]:M0341, [H1]:H3M1, [H2]:H3M28, [H3]:H3O23, [C1]: Y294 (negative control), [H4]:H3H29 and [H5]:H3K27.

4.2.2 Disruption of the *URA3* gene

The *S. cerevisiae* Y294 parental strain used during mating is an *ura3* mutant while the M0341 parent has an active *URA3*. After sporulation of the diploid hybrids, super-secreting segregants were identified. Some of these were *ura3* while others contained an intact *URA3* (Table 3.3). In order to enable selection of the *URA3* based episomal expression vectors, all strains had to be *ura3*. The *ura3* ORF was amplified from the *S. cerevisiae* Y294 genome and used to replace the intact *URA3* in strains with *ura3*. Yeast transformants were selected on 5-FOA plates, a common genetic screening method that prevents the growth of strains capable of synthesizing uracil. *URA3* wild-type strains are not able to grow on medium containing the pyrimidine analogue, whereas *ura3* mutants grow normally (Boeke *et al*., 1984). The industrial parent, M0341 and three segregants, H3M28, H3H29 and H3K27 contained a functional *URA3* gene (Table 3.3) which had to be disrupted.

Transformants were plated on buffered SC^{-ura} medium to confirm *URA3* disruption (Figure 4.3). The absence of growth confirms an inactive *ura3*.

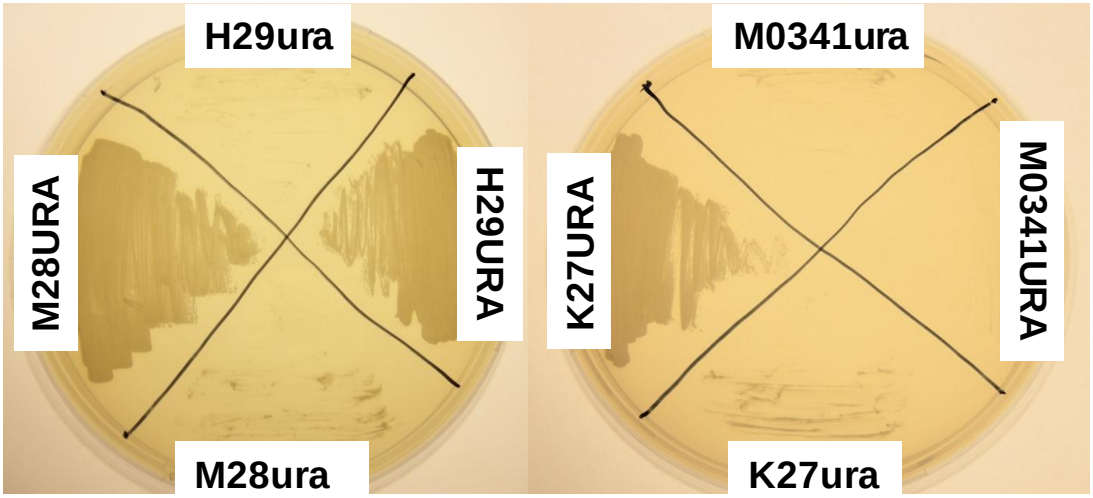

Figure 4.3: *S. cerevisiae* M0341, H3M28, H3H29 and H3K27 colonies on SC^{-ura} medium with *URA3* and *ura3* after disruption, at 30 ºC for 2 days.

4.2.3 Reporter plasmid transformations

The last step in the engineering of *S. cerevisiae* strains expressing different reporter proteins was the transformation with the five expression vectors. Plasmids pMU1531, pRDH182, pRDH166, pNS201, pNS202 and pNS203 were introduced into *S. cerevisiae* Y294, M0341, H3M1, H3M28, H3O23, H3H29 and H3K27. Yeast transformants were selected on SC^{-ura} medium. Genomic DNA from each putative transformant was isolated and used as PCR template to confirm the presence of the different reporter genes. PCR analyses were done using the relevant PCR primers in Table 3.4. The presence of a band, of the predicted size, indicates successful introduction of the reporter enzyme genes (Appendix 7.1, Figures 7.1 – 7.3). The 42 strains created were used to evaluate enzyme secretion by different yeast segregants (Tables 3.4 and 4.2).

Table 4.2: All the recombinant yeast strains constructed and used in this study.

Yeast Hybrid	pMU1531	pNS202	pRDH166	pRDH182	pNS201	pNS203
Reporter	No reporter	Cel7A (CBH I)	Cel12A (EG3)	XYN2	Cel3A (BGL1)	GLU
Y294	Y294_1531	Y294_Cel7A	Y294_Cel12A	Y294_XYN2	Y294_Cel3A	Y294_GLU
M0341	M0341_1531	M0341_Cel7A	M0341_Cel12A	M0341_XYN2	M0341_Cel3A	M0341_GLU
H3M1	H3M1_1531	H3M1_Cel7A	H3M1_Cel12A	H3M1_XYN2	H3M1_Cel3A	H3M1_GLU
H3M28	H3M28_1531	H3M28_Cel7A	H3M28_Cel12A	H3M28_XYN2	H3M28_Cel3A	H3M28_GLU
H3O23	H3O23_1531	H3O23_Cel7A	H3O23_Cel12A	H3O23_XYN2	H3O23_Cel3A	H3O23_GLU
H3H29	H3H29_1531	H3H29_Cel7A	H3H29_Cel12A	H3H29_XYN2	H3H29_Cel3A	H3H29_GLU
H3K27	H3K27_1531	H3K27_Cel7A	H3K27_Cel12A	H3K27_XYN2	H3K27_Cel3A	H3K27_GLU

4.2.4 Cel12A plate activity confirmation

Plate activity assays were conducted in order to confirm that the yeast transformants expressing cel12A secreted the reporter Eg3 enzyme in an active form.

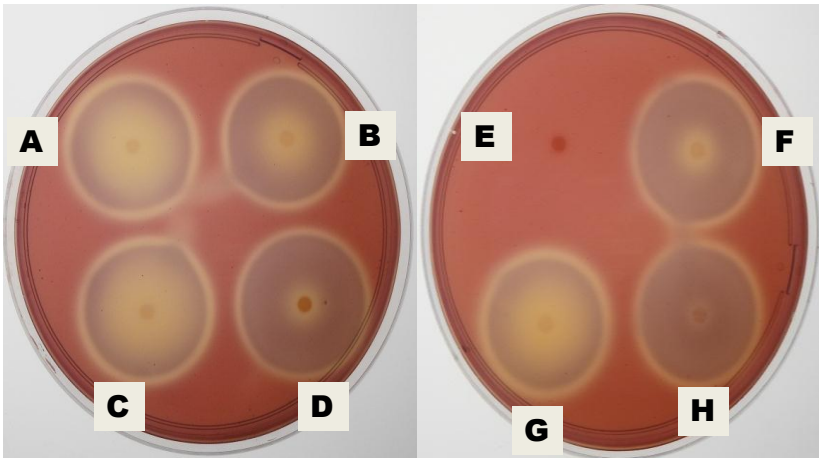

Figure 4.4: Hybrid strains of *S. cerevisiae* containing the episomal plasmid pRDH166 carrying the reporter enzyme *cel*12*A* (EG3). Yeast cells were spotted on SC^{-ura} containing 1% CMC and incubated at 30 ^{o}C for 24 hr. After incubation, colonies were washed off and stained with 0.1% Congo red. The halo-like zone around the yeast indicates cel12A activity. The Y294_1531 is the negative control strain (without a copy of cel12A), and does not exhibit cel12A activity. The supersecreting hybrids were spotted as follows; [A]: H3M28_Cel12A, [B]: H3M1_Cel12A, [C]: M0341_Cel12A, [D]: Y294_Cel12A, [E]: Y294_1531. [F]: H3K27_Cel12A. [G]: H3O23_Cel12A and [F]: H3H29_Cel12A.

4.3 Enzyme activity assays

4.3.1 Cellobiohydrolase secretion

Cel7A (CBH I) was used to create and screen the super-secreting segregants. The single integrated copy on the chromosome was removed and replaced with multiple copies expressed from an episomal plasmid. Cellobiohydrolase 1 activity was determined by using *p*-nitrophenyl-β-D-cellobioside as a substrate. There was an increase in enzyme activity in the hybrid strains compared to their parental strains (Figure 4.5 A). However, the ratio of enzyme secreted changes when expressed from a multicopy plasmid (Figure 4.5 A and B). The highest activity is produced by the hybrid H3M28_Cel7A with Cel7A integrated on the chromosome. It secreted

approximately 9-fold more enzyme than the parental strain Y294. With Cel7A expressed from an episomal plasmid, H3H29_Cel7A displayed the most significant improvement over the Y294 parental strain, with an approximate 3.5-fold increase.

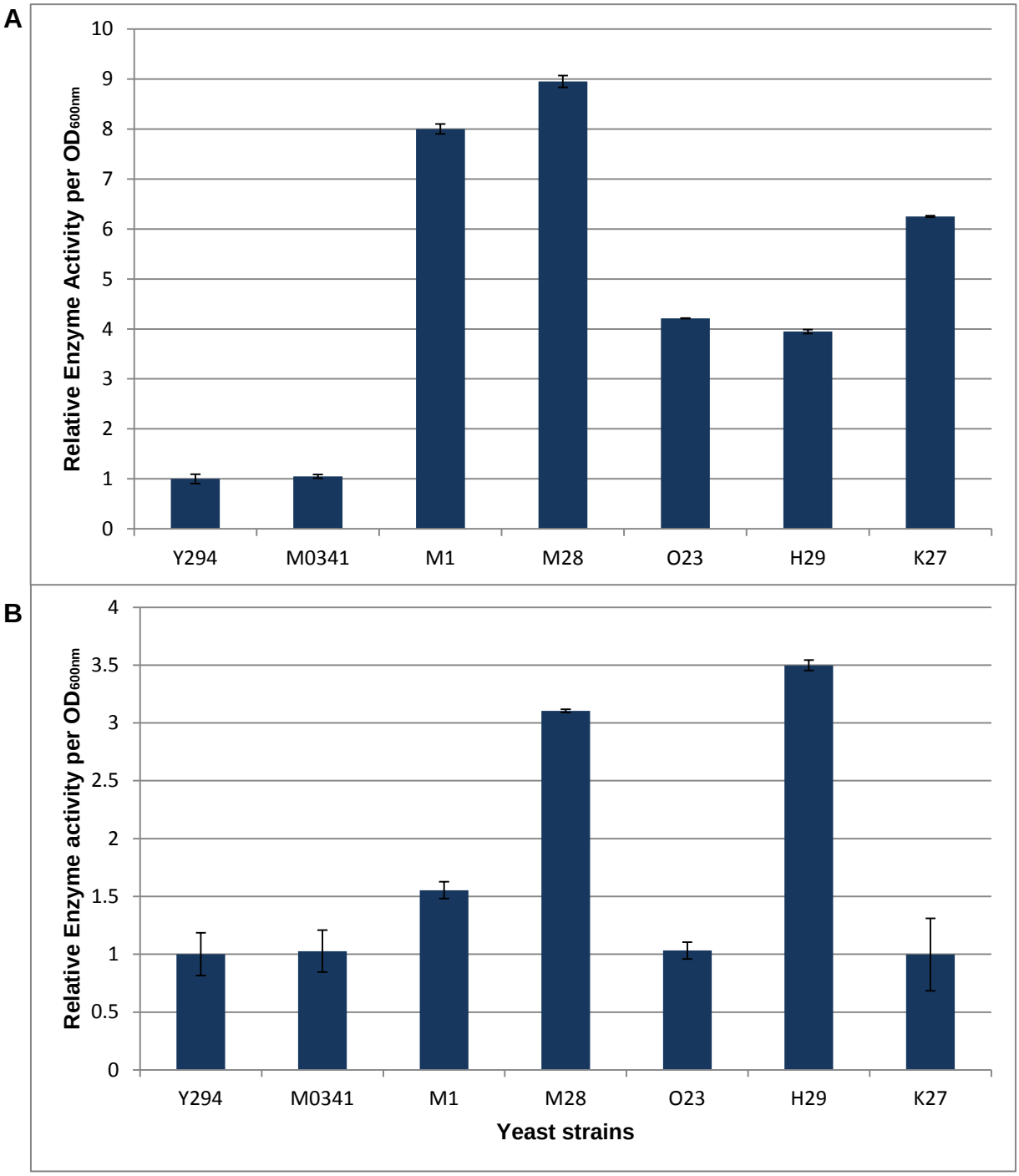

Figure 4.5: Relative enzyme activity depicting secretion levels of *T. emersonii* Cel7A (CBH I) by parental and supersecreting hybrid *S. cerevisiae* segregants after 48 hrs of cultivation on SC^{-ura}. Enzyme activity levels from (A) a single copy of the Cel7A on the chromosome as well as activity from (B) a multi-copy episomal plasmid. All activities were expressed relative to the activities of the *S. cerevisiae* Y294 strain which was normalised to 1. Error bars indicate the standard deviation from the mean value of triplicate samples.

4.3.2 Endo-glucanase secretion

The Eg3 activity was determined by using the DNS assay with 1% CMC as a substrate (Figure 4.6). These sets of yeast hybrids appear not to have an increased ability to express and secrete Eg3. The H3M1 secretes Eg3 at 0.2-fold lower than the *S. cerevisiae* Y294 parental. None of the five hybrids display an increase in secretion ability, because the Y294 parental strain appears to be the best secretor with the highest relative activity at 1. There was no improvement in enzyme secretion.

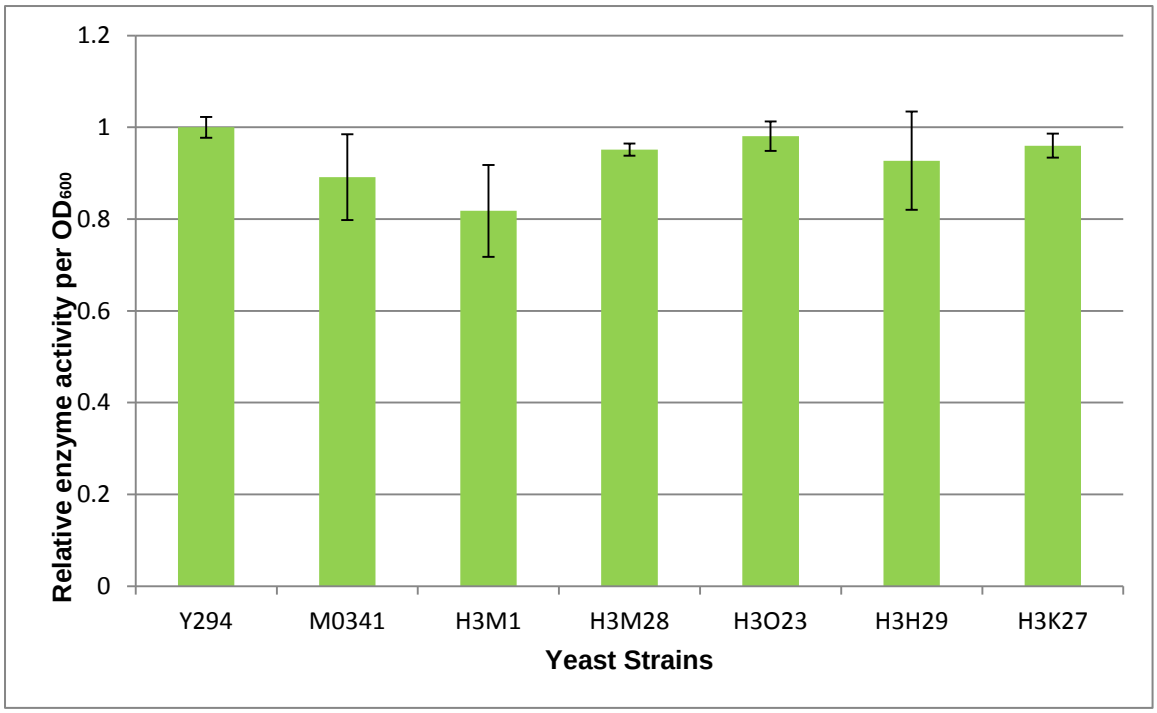

Figure 4.6: Relative enzyme activity depicting secretion levels of *N. fischerii* Cel12A (Eg3) by parental and supersecreting hybrid *S. cerevisiae* segregants after 48 hrs of cultivation on SC^{-ura}. All activities were expressed relative to the activities of the Y294 strain. Error bars indicate the standard deviation from the mean value of triplicate samples.

4.3.3 Endo-xylanase secretion

The Xyn2 activity was determined by using the DNS assay with 1% beechwood xylan as a substrate. This set of hybrid strains also appears to not carry the phenotypic trait exhibited by the hybrid strains engineered by Kroukamp *et al*. (2012), (Figure 4.7). The H3K27 was the best secretor of Xyn2 with only a 0.4-fold increase relative

to the *S. cerevisiae* Y294 parental, resulting in a minor improvement in enzyme secretion.

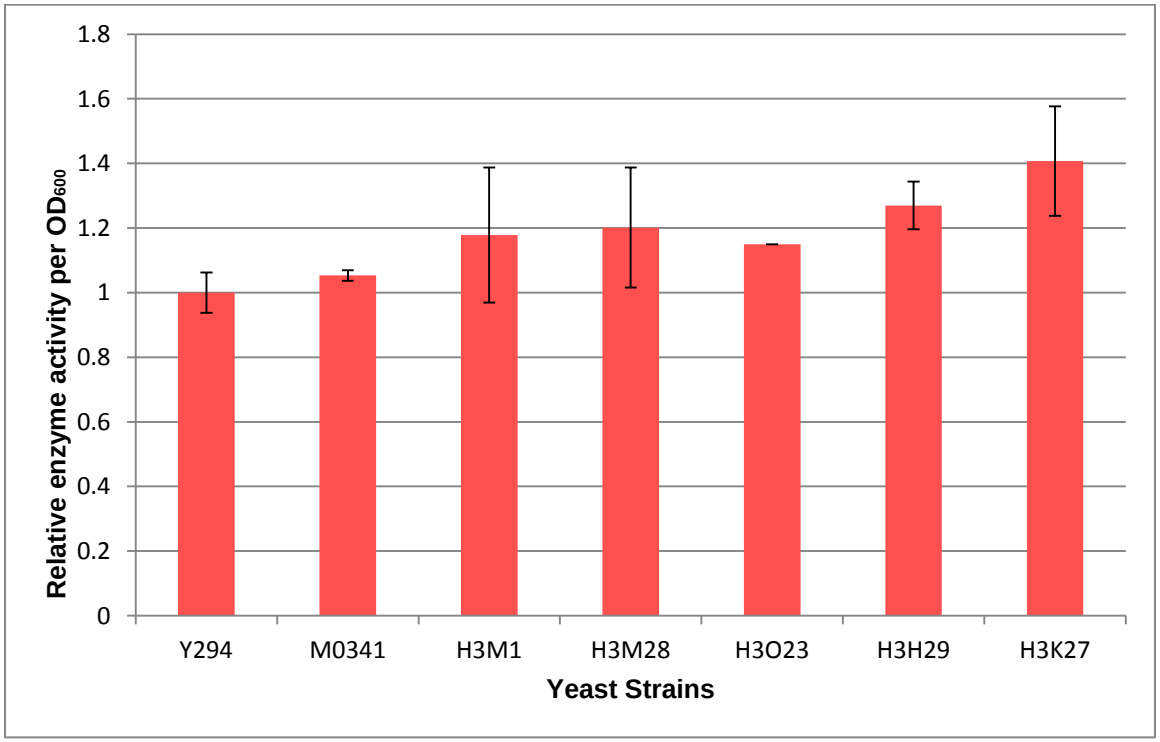

Figure 4.7: Relative enzyme activity depicting secretion levels of *T. reesei* Xyn2 by parental and supersecreting hybrid *S. cerevisiae* segregants after 48 hrs of cultivation on SC^{-ura}. All activities were expressed relative to the activities of the *S. cerevisiae* Y294 strain. Error bars indicate the standard deviation from the mean value of triplicate samples.

4.3.4 β-Glucosidase secretion

The optical density at 400 nm was used to quantify the β-glucosidase enzyme activity. There was no increase in enzyme activity of the hybrid strains when compared to their parental counterparts, in fact most of the segregants secreted less β-glucosidase activity when compared to the parental strains (Figure 4.8). The best hybrid secretor is H3M1. The lowest secretor is H3O23 with half the secretion ability of the *S. cerevisiae* Y294 parent.

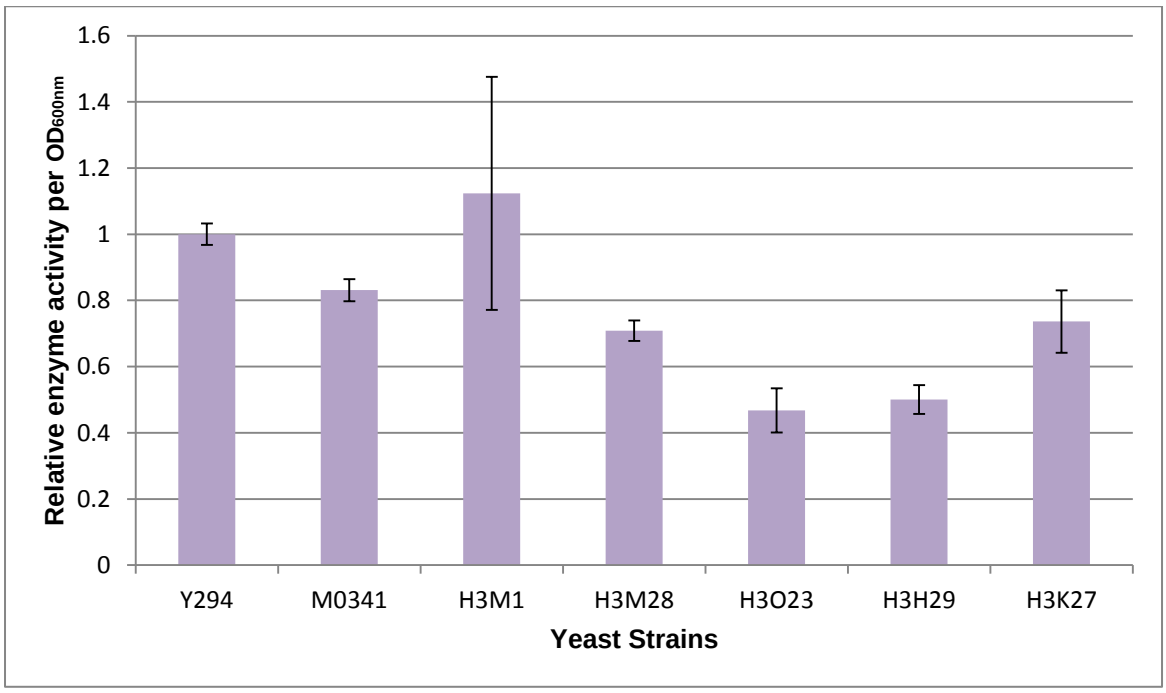

Figure 4.8: Relative enzyme activity depicting secretion levels of *S. fibuligera* Cel3A (BGL1) by parental and super-secreting hybrid *S. cerevisiae* segregants after 48 hrs of cultivation on SC^{-ura}. All activities were expressed relative to the activities of the Y294 strain. Error bars indicate the standard deviation from the mean value of triplicate samples.

4.3.5 α-Glucuronidase secretion

The amount of α-glucuronidase secreted was quantified using SDS-PAGE. This only gives an indication of the amount of protein secreted, but does not give an indication of whether the secreted enzyme is active (Figure 4.9). This ultimately gave an indication of the amount of α-glucuronidase secreted.

The parental strains expressing the four reporter enzymes except for glucuronidase secreted all the reporter enzymes at more or less the same level. However, the M0431_GLU parent secreted α-glucuronidase almost 4 times better than Y294_GLU. Surprisingly, the H3M28_GLU did not produce any enzyme (Figure 4.9) as there was no protein band visible (Lane 6- Figure 4.10). The H3M1_GLU hybrid on the other hand, showed an approximately 2-fold increase in the amount of enzyme secreted as compared to the best parental strain, M0341_GLU. Segregant strains H3K27_GLU and H3O23_GLU, secreted approximately 1.6 times more α-glucuronidase compared to M0341_GLU.

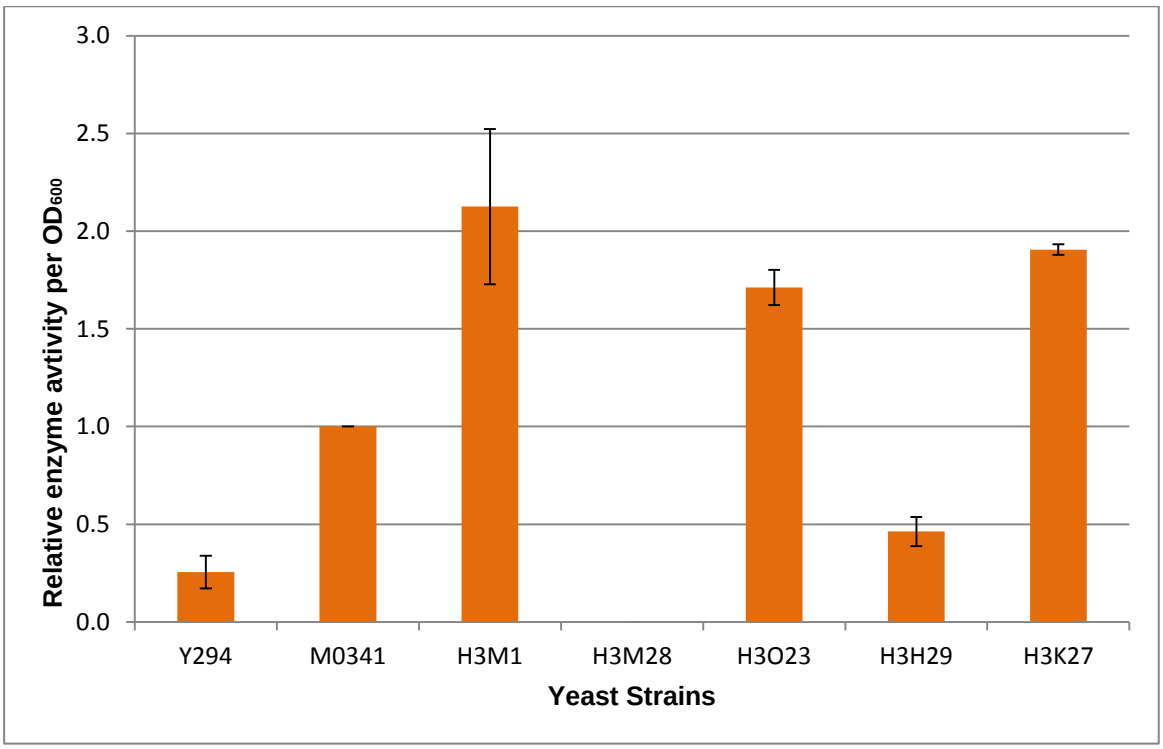

Figure 4.9: Relative secretion levels of *S. stipitis* GLU as calculated using the intensities of the protein bands that were run on a 10% PAGE denaturing gel by parental and supersecreting hybrids after 48 hrs of cultivation in SC^{-ura}. All protein amounts were expressed relative to the amount secreted by the M0341 strain which was normalised to 1. Error bars indicate the standard deviation from the mean value of triplicate samples.

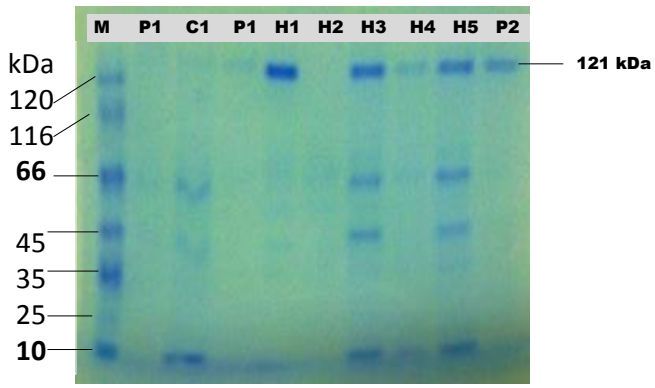

Figure 4.10: SDS-PAGE gel analysis of the *S. cerevisiae* hybrid segregant strains that contained the pNS203 plasmid, in relation to their parental strains Y294_GLU and M0341_GLU. All these strains expressed and secreted the recombinant *S. stipitis* α-glucuronidase enzyme (121 kDa). The concentrated protein was loaded and separated on a 10% polyacrylamide denaturing gel. From left to right, the yeast strains were loaded on the gel as follows: [M]: Molecular weight marker (kDa), [P1]:Y294_GLU, [P2]:M0341_GLU, [C1]: Y294_201 (negative control), [H1]:H3M1_GLU, [H2]:H3M28_GLU, [H3]:H3O23_GLU, and [H4]:H3H29_GLU and [H5]:H3K27_GLU.

4.4 Fermentation Studies

4.4.1 Determintion maximum specific growth rate

The segregant secreting Cel7A at the highest level, H3M28, was used to determine the metabolic burden caused by the Cel7A expression. The control, H3M28_1531 and H3M28_Cel7A were cultured in batch fermentation for 48 hrs and samples were taken at 2 hour intervals to determine biomass concentration. Biomass concentration (OD 600 $_{nm}$) was plotted against time for both strains (Figure 4.11).

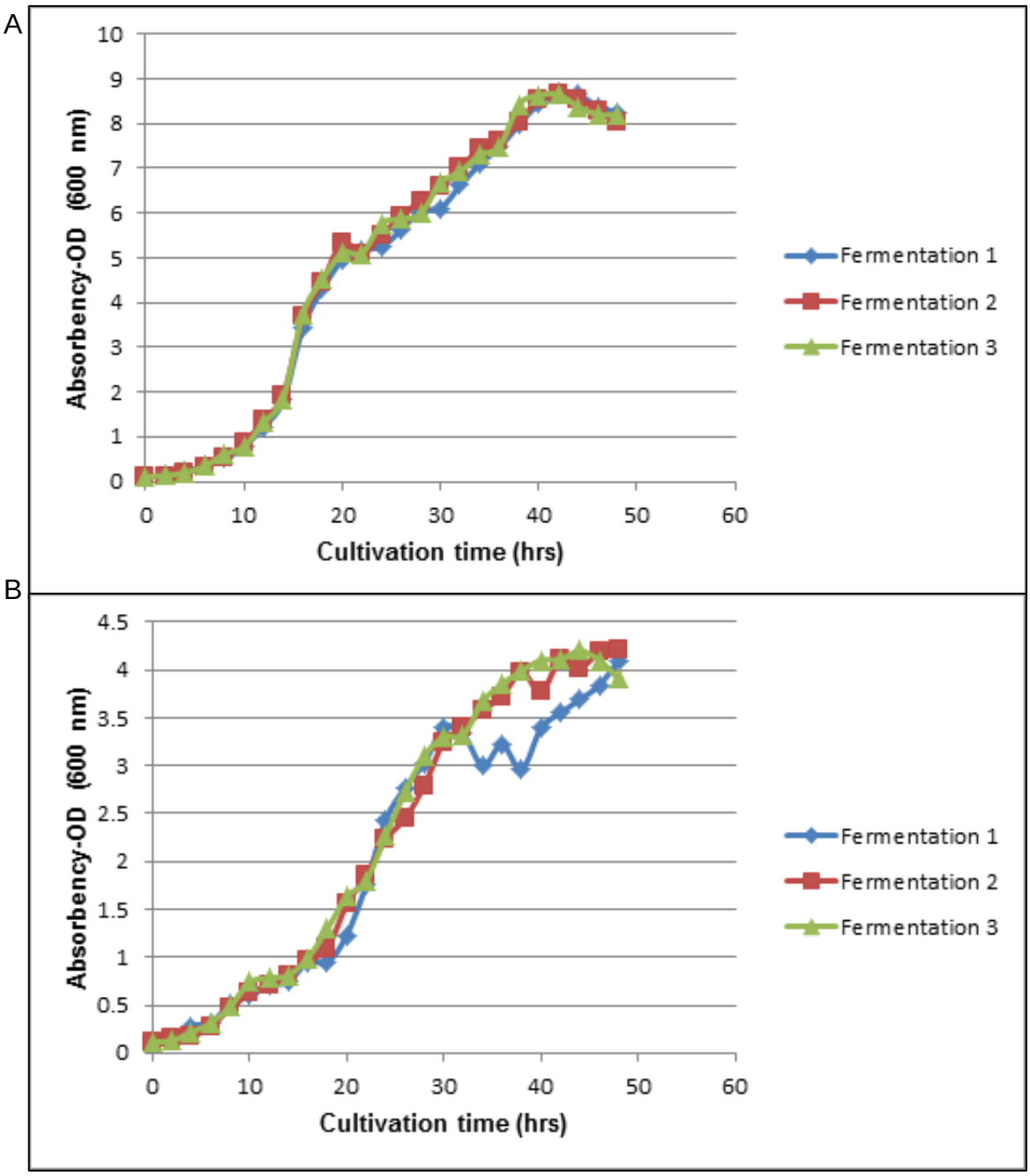

Figure 4.11: The growth analysis of the *S. cerevisiae* (A) control H3M28_1531 and (B) H3M28_Cel7A during batch culture in buffered SC^{-ura}, cultivated over 48 hrs at 30°C. The three plots on each graph indicate three repeats of the fermentation batches conducted randomly.

The data in Figure 4.11 was converted to ln(Biomass) against time (Figure 4.12) in order to calculate the slope during the exponential growth phase to obtain the μ_{max}.

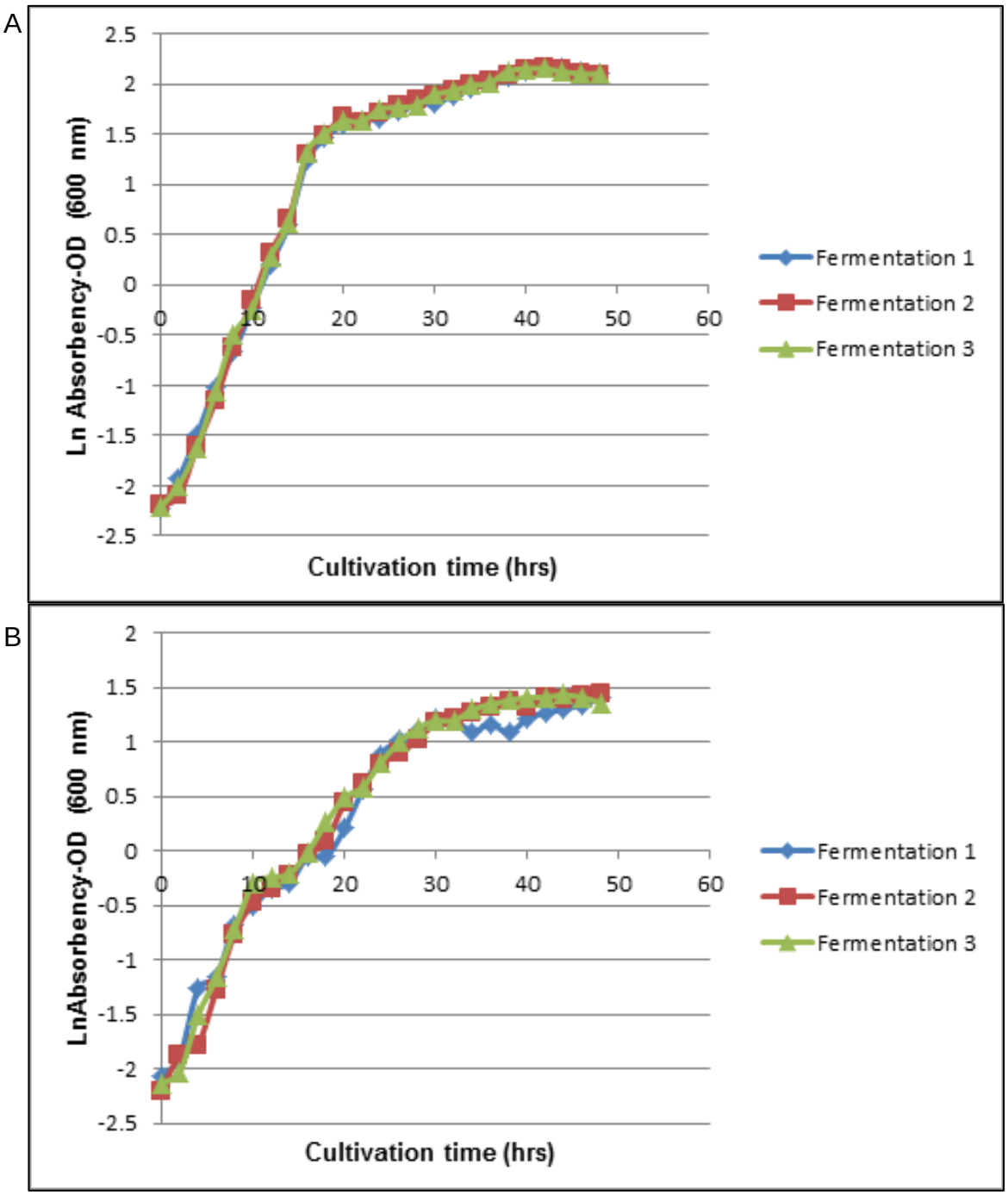

Figure 4.12: The ln(Biomass) of the *S. cerevisiae* (A) control H3M28_1531 and (B) H3M28_Cel7A during batch culture in buffered SC^{-ura}, cultivated over 48 hrs at 30 ^{o}C. The three plots on each graph indicate three repeats of the fermentation batches conducted randomly.

4.4.2 Calculation of maximum specific growth rate

As described previously, the slope of the individual ln(biomass) plots (Figure 4.12), where used to calculate the μ_{max} rates for the yeast hybrid segregant control,

H3M28_1531, as well as H3M28-Cel7A (Appendix, Figure 7.7 and 7.8). The average μ_{max} was then calculated (Table 4.3). The μ_{max} of H3M28_1531 was found to be 0.210 and that of H3M28_Cel7A 0.090.

Table 4.3: Maximum specific growth rates of the H3M28_1531 control as well as H3M28_Cel7A.

	Specific Growth Rate (μ_{max}^{-1})			**Average**	**Standard deviation**	**Co-efficient Variation**
	Batch 1	**Batch 2**	**Batch 3**			
H3M28_1531 Control	0.204	0.214	0.210	0.210	0.005	2.3%
H3M28_Cel7A	0.093	0.089	0.087	0.090	0.003	3.1%

The maximum specific growth rate is decreased by a factor of more than two as a result of Cel7A secretion.

4.5 The effect of growth inhibitors on *Saccharomyces cerevisiae* segregants

4.5.1 Ethanol tolerance

The ethanol tolerance of the *S. cerevisiae* segregant strains was determined by cultivating the yeast host strains (Table 3.3) on YPD plates with different concentrations of ethanol at 0 and 8%. All yeast strains appear to tolerate ethanol up to a concentration of 6% (data not shown) above that inhibition of growth was observed. However, when grown at a concentration of 8% ethanol under the same conditions, the *S. cerevisiae* Y294 and *S. cerevisiae* M0341 parental strains as well as the H3M1 and undiluted H3M28 hybrid segregant showed some growth (Figure 4.13).

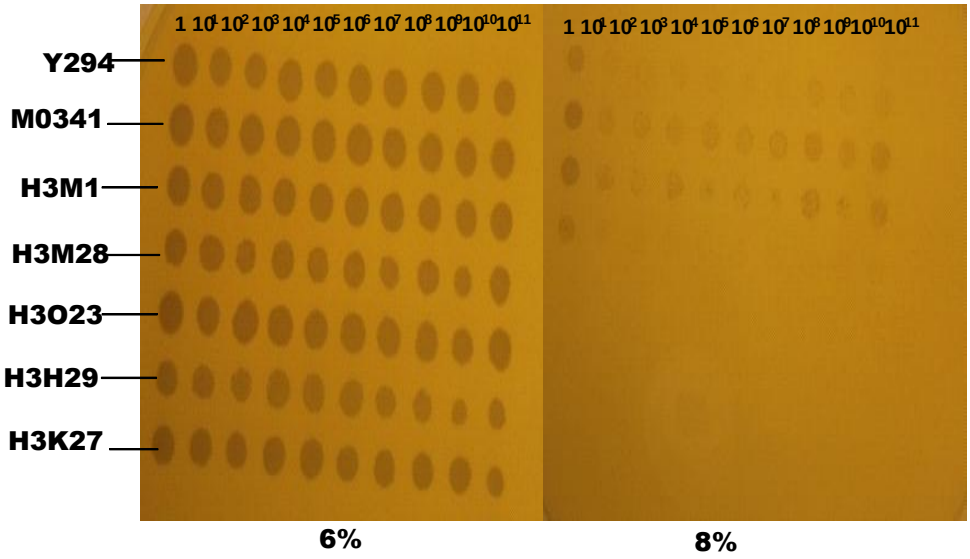

Figure 4.13: Representation of *S. cerevisiae* positive control strains grown on YPD with ethanol at a concentration of 6 and 8% (w/v), after 72 hrs incubation, at 30 ^{o}C. From left to right the yeast strains were spotted as a 10-fold dilutions.

4.5.2 Furfural tolerance

These strains evaluated in this study are highly sensitive to low levels of furfural; this is shown by the lack of growth by any of the strains at 1 g/L of furfural (Figure 4.14). At 0.5 g/L furfural, only the undiluted and 10X diluted series showed growth. With the exception of the H3K27_1531 strain which managed to grow on 0.5 g/L all diluted series at furfural. At 1 g/L furfural there was complete inhibition of growth, and none of the strains were able to grow.

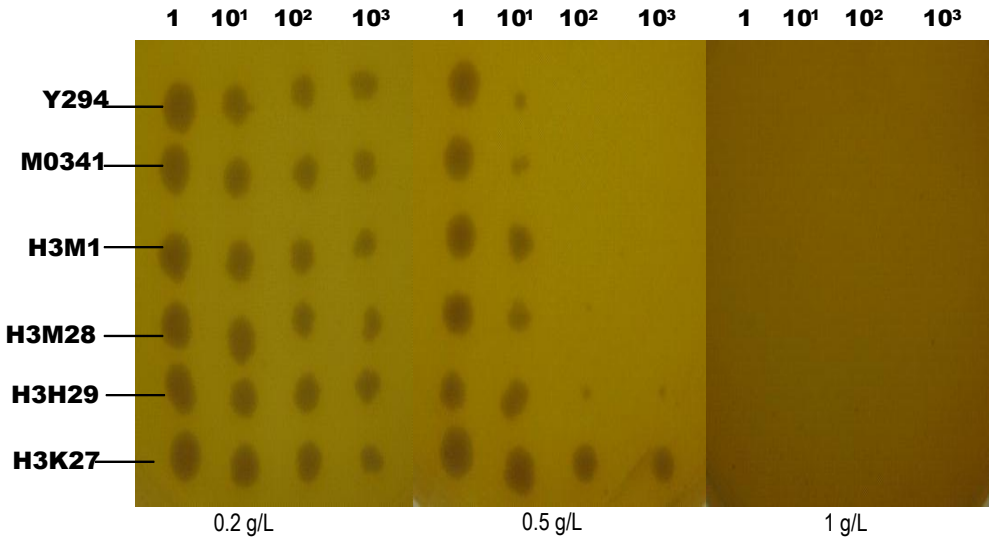

Figure 4.14: Representation of *S. cerevisiae* positive control strains grown on SC^{-ura} with furfural at a concentration range of 0.2 g/L – 1 g/L, after 72 hrs incubation, at 30 ^{o}C. From left to right the yeast strains were spotted as a 10-fold dilutions.

4.5.3 Acetic Acid tolerance

The *S. cerevisiae* segregant strains were evaluated for their ability to grow in the presence of acetic acid. The H3O23_1531 had difficulty growing on 1 g/L acetic acid. Therefore with the exception of H3O23 all the yeast segregant strains can tolerate a maximum of 2 g/L acetic acid. Higher concentrations of acetic acid lowered the pH of the medium such that the medium would not solidify (Figure 4.15).

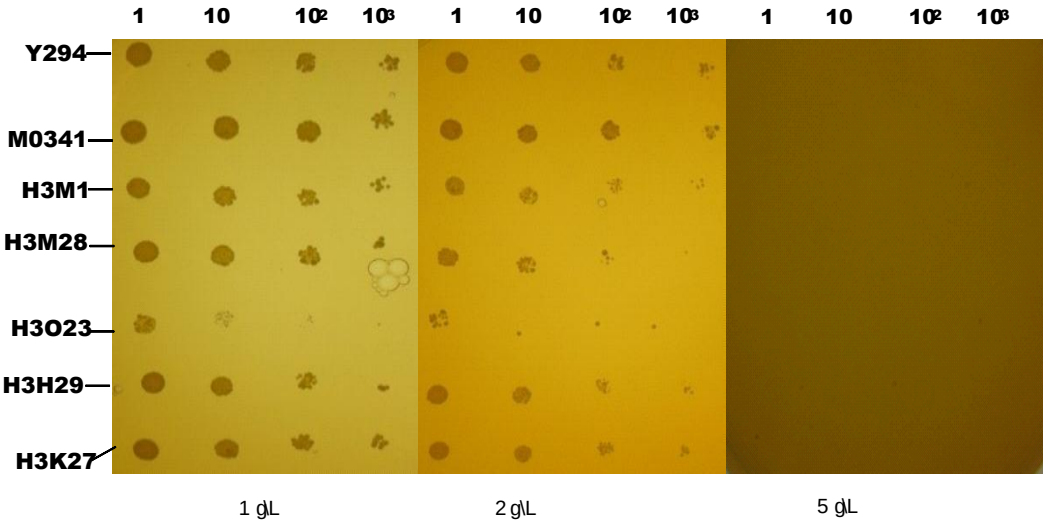

Figure 4.15: Representation of *S. cerevisiae* positive control strains grown on SC^{-ura} with acetic acid at a concentration range of 1 g/L – 5 g/L, after 72 hrs incubation, at 30 ^{o}C. From left to right the yeast strains were spotted as a 10-fold dilutions.

4.5.4 Vanillin tolerance

The last potential inhibitor of growth of the *S. cerevisiae* segregant strains evaluated was vanillin. There was significant inhibition of all the yeast strains at 1 g/L, thus the yeast strains can only grow at concentrations of 0.2 g/L (Figure 4.16).

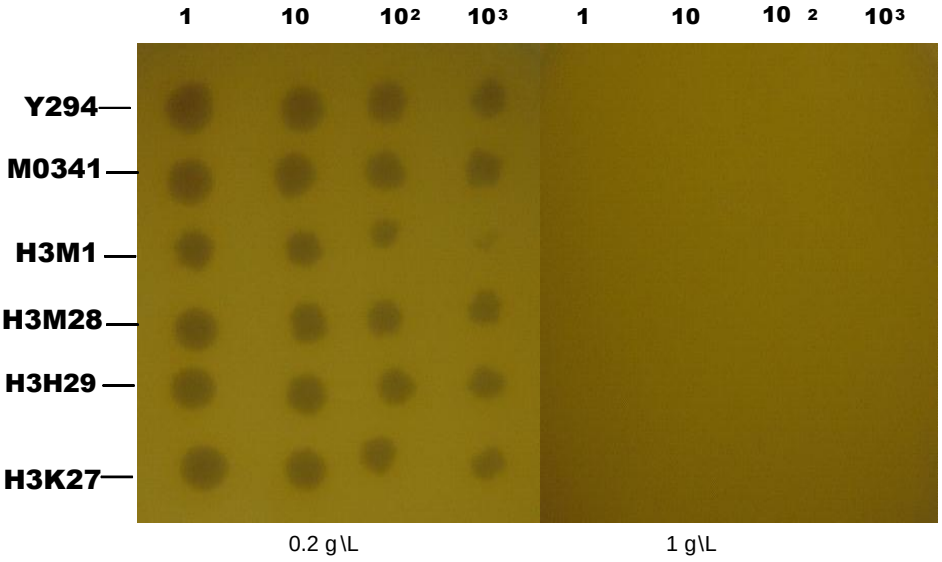

Figure 4.16: Representation of *S. cerevisiae* positive control strains grown on SC^{-ura} with vanillin at a concentration of 0.2 g/L – 1 g/L, after 72 hrs incubation, at 30 ^{o}C. From left to right the yeast strains were spotted as a 10-fold dilutions.

The inability of the yeast segregant strains to grow at working levels of potential inhibitors such as furfural, acetic acid and vanillin is not a good trait considering the harsh conditions encountered in large bioreactors during ethanol fermentation on pre-treated lignocellulosic biomass. The concentration ranges used are those often encountered in industry (Huang *et al.*, 2011).

CHAPTER 5. DISCUSSION

5.1 Plasmid Construction

Five different reporter genes were used to evaluate the secretion ability of five *S. cerevisiae* segregants (Table 4.1). Two of the expression vectors were previously constructed and generously provided by Prof WH van Zyl from the University of Stellenbosch. The remaining three vectors namely pNS201, pNS202 and pNS203 containing genes for a β-glucosidase I (*cel*3*A*), cellobiohydrolase I (*cel*7*A*) and an α-glucuronidase, respectively, were constructed for this study (Section 3.3).

The reporter enzymes used were selected based on their size and number of glycosylation sites as these factors are known to have an impact on secretion. All the reporter enzymes used in this study have been expressed successfully in *S. cerevisiae* before (La Grange *et al.*, 1996; Den Haan *et al.*, 2007b; Gomes, 2012; Kroukamp *et al.*, 2012). Some of the enzymes, however, have not been secreted at satisfactory levels. The size of a heterologous protein can impact on its secretion (Palomares *et al.*, 2004). Naturally *N*- and *O*-glycosylated CBHs have been found to be hyperglycosylated with high mannose glycans in *S. cerevisiae* (Penttila *et al.*, 1988; Hong *et al.*, 2004; Voutilainen *et al.*, 2010). The specific activity of the yeast-produced enzymes appeared to be lower than the native proteins, indicating that overglycosylation may have a detrimental effect on specific activity as previously shown by Penttila *et al*. (1988); Reinikainen *et al.* (1992); Takada *et al*. (1998) and den Haan *et al*. (2007b). Reporter enzymes with varying numbers of *N*-glycosylation sites were chosen in this study in order to evaluate the impact that glycosylation has on heterologous protein secretion. A control plasmid pMU1531, which lacked a reporter enzyme gene, was used as negative control.

5.2 *Saccharomyces cerevisiae* host strain engineering

All the *S. cerevisiae* segregants used for evaluation of secretion contained an integrated *Cel7A* (*CBH I*) gene at the *LEU2* locus. This insertion disrupted the *LEU2* creating *leu2* mutants. Homologous recombination using an intact *LEU2* removed the *Cel7A* and enabled these transformants to grow in the absence of the amino acid leucine.

Furthermore, four of the seven strains also contained an active *URA3* gene, which would make selection of the *URA3* based episomal vector impossible. In *S. cerevisiae* strains with an intact *URA3* (segregants M0341, H3M28, H3H29 and H3K27) coding for an orotidine-5-monophosphate decarboxylase, the gene product of *URA3*; 5-FOA a nontoxic pyrimidine analogue is converted to 5-fluro-uracil (5-FU) which is toxic. During growth on media containing 5-FOA, yeast strains with a functional *URA3* growth will be inhibited while those with a non-functional *ura3* will survive (Ko *et al.*, 2008). A mutant *ura3-52* gene was used to replace the functional *URA*3 gene in strains containing it through homologous recombination (Figure 4.3).

5.3 Yeast Transformations

Six episomal plasmids namely pRDH166, pRDH182, pNS201, pNS202, pNS203 and pMU1531 (control) (Table 4.1) were introduced into seven *S. cerevisiae* segregants, resulting in a total of 42 *S. cerevisiae* segregant hybrid strains (Table 4.2). The six plasmids contained the same *S. cerevisiae ENO*1 promoter and terminator sequences, with different reporter genes. *ENO1* was previously reported as a constitutive promoter (DeRisi *et al.*, 1997) and shown to express some heterologous enzymes at high levels (Den Haan *et al.*, 2007a; Ilmen *et al.*, 2011). Because all the strains used the same promoter and terminator, promoter regulation can be ruled out as the cause of variation in enzyme secretion level.

The five *S. cerevisiae* segregants used were H3M1, H3M28, H3M28, H3O23, H3H29 and H3K27 with the parental strains Y294 and M0341 included as controls.

5.4 Enzyme secretion

It has previously been reported that *S. cerevisiae* is capable of expressing and secreting hydrolases, but at titers that are generally too low for efficient hydrolysis of lignocellulosic material (La Grange *et al.*, 1996; Den Haan *et al.*, 2007a). Improving the heterologous secretion ability of *S. cerevisiae* remains a challenging endeavour.

Cellobiohydrolases I (Cel7A) have been previously secreted by *S. cerevisiae* at relatively high levels, however CBH I continues to be a rate limiting enzyme in lignocellulose hydrolysis (Penttila *et al.*, 1988; Ilmen *et al.*, 2011). Kroukamp *et al.* (2016) mated a lab strain (*S. cerevisiae* Y294) and an industrial strain of *S. cerevisiae* M3041, both expressing the *T. emersonii* Cel7A, from a single integrated copy. The resulting diploids were sporulated to produce haploid segregants. These

were screened and 5 secretors were selected based on several phenotypic differences from the original 28 high secretion segregants. These 5 secretors were used in this study to evaluate their ability to also secrete other hydrolases important in lignocellulosic bioethanol production.

5.4.1 Cellobiohydrolases I secretion

After removal of the single integrated copy of the *T. emersonii* Cel7A from the chromosome of the selected segregants, a multi-copy episomal plasmid with the same expression cassette was introduced. Multi-copy expression changed the expression ratios (Figure 4.5), however H3M28 and H3H29 secreted Cel7A more than 3 times better than the *S. cerevisiae* Y294 parental strain, confirming the ability of the selected segregants to secrete Cel7A at levels higher than the parental strains.

5.4.2 Endo-glucanase secretion

The endoglucanase from *N. fischerii* Cel12A is relatively small in size and does not contain *N*-glycosylation sites. Both parents as well as all the segregants produced approximately 4 nkat/ml of endoglucanase activity (Appendix 7.2.1). The ability of the segregants to secrete Cel7A at high levels does not apply to the secretion of Cel12A, as secreting segregants are similar to the parental strains (Figure 5.1).

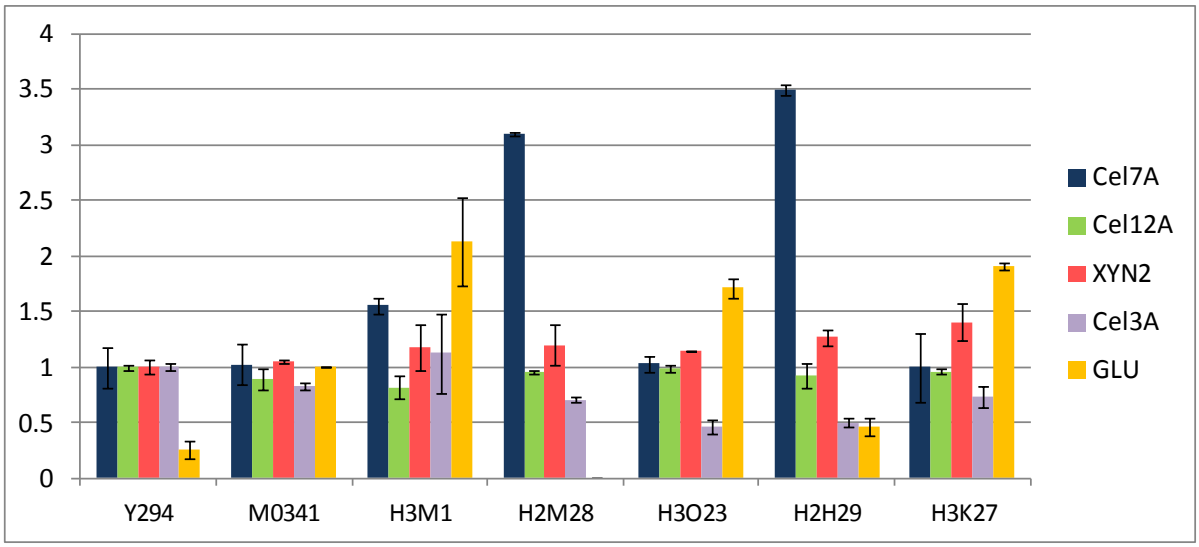

Figure 5.1: Relative enzymatic activity depicting secretion levels of hydrolases by parental and supersecreting segregants. All activities were expressed relative to the activities of the *S. cerevisiae* Y294 strain which was normalised to 1, except for GLU where the M0341 was used. Error bars indicate the standard deviation from the mean value of triplicate samples.

5.4.3 Endo-xylanase secretion

The endo-xylanase (Xyn2) from *T. reesei* is the smallest enzyme tested. It contains two *N*-glycosylation sites and has been expressed at high levels previously (La Grange *et al.*, 1996). Xylanase activity data shows that the segregants secrete the xylanase enzyme at levels similar to the parental strains (Figure 5.1).

5.4.4 β-Glucosidase I secretion

β-glucosidase I (Cel3A) has been previously secreted by *S. cerevisiae* at good levels (Van Rooyen *et al.*, 2005). The codon optimized Cel3A from *S. fibuligera* is relatively large in size at 92 kDa making it the second largest enzyme expressed in this study. Furthermore it contains 11 *N*-glycosylation sites. Cel3A activity data shows that the segregants secrete the Cel3A enzyme at levels that are different from the parental strains (Figure 4.8). The Cel3A expression levels by the hybrids are mostly lower compared to the parental strains.

5.4.5 α-Glucuronidase secretion

The *S. stipitis* α-D-glucuronidase is the largest enzyme expressed in this study. It also contains 16 *N*-glycosylation sites which might complicate post-translational

processing and secretion. The enzyme does not appear to be hyperglycosylated since there is only a small band shift, from 113 to 121 kDa, on SDS-PAGE (Figure 4.9). There is a 2.1-fold increase in extracellular enzyme produced by H3M1_GLU compared to the best parental strain (M0341) (Figure 5.1). Expression levels obtained in this study were higher than the levels reported by Gomes (2012) and Anane *et al.* (2013).

The reporter enzymes used in this study had different numbers of *N*-glycosylation sites (Table 4.1). *N*-glycosylation has the potential to affect secreted protein activities by altering the activity of the secreted protein, the secretion of an extracellular protein could be altered depending upon the degree of glycosylation or the permeability of the cell wall could be affected by glycosylation changes in cell wall proteins (Kroukamp *et al.*, 2016). From the data presented here, it cannot be determined whether the size and the number of *N*-glycosylation sites of an enzyme impacts on the ability of the segregants to secrete it.

5.5 Microbial Growth Studies

The microbial growth in a bioreactor was studied to determine the metabolic burden imposed on the yeast by the high level secretion of the cellobiohydrolase I (Cel7A). The metabolic burden was confirmed by comparing the μ_{max} of the highest secreting yeast hybrid strain namely H3M28_Cel7A with its corresponding negative control strain H3M28_1531.

The maximum specific growth rate for H3M28_Cel7A was found to be 0.090 while μ_{max} of H3M28_1531 was more than double, at 0.210 (Table 4.3). The difference observed between the two growth rates indicate that the metabolic burden imposed upon the hybrid segregant strains is substantial.

Since the H3M28_1531 and H3M28_Cel7A are identical in terms of host strain, and plasmid backbone, the only difference between the strains is the presence of Cel7A. Therefore, the differences in growth rates between the two strains and the final cell density (OD_{600nm} 4.5 versus 1.5 - Figure 4.11) can only be attributed to the expression of the Cel7A. It has been previously reported that cellular stress reactions can be a limitation to the production of heterologous protein production (Mattanovich *et al.*, 2004). Furthermore the accumulation of unfolded proteins in the ER has been shown to cause stress which induces the UPR responsible for coordinating the physiological responses to ER stress (Schroder, 2006). Ilmen *et al*.

(2011) demonstrated that the heterologous expression of Cel7A is responsible for the metabolic burden on yeast cells and that the constraint of heterologous protein secretion appears to be at the ER level. The UPR of *S. cerevisiae* was reported to be induced to different degrees depending on the enzyme expressed (Ilmen *et al.*, 2011). It could therefore be assumed that the expression of *T. emersonii* Cel7A exerts a substantial amount of metabolic stress on *S. cerevisiae*; although the exact point of burden cannot be determined from the data in this study. Taking into account that the yeast was cultivated in synthetic complete media that required amino acid supplementation, a plausible theory supported by Van Rensburg *et al.* (2012) would be that during protein folding and degradation of misfolded proteins, cellular stress has the potential to manifest as a maintenance requirement, thus leading to drainage in energy which is shown by the significant decrease in the μ_{max} of H3M28_Cel7A relative to H3M28_1531.

The supersecreting hybrid H3M28_Cel7A used to evaluate the metabolic burden imposed on the yeast by the expression of Cel7A is a strain that flocculates during growth. The effect of flocculation on Cel7A secretion has been evaluated by Kroukamp *et al*. (2016). They reported a significant difference in secretion levels between the flocculating and non-flocculating strains; however the cause of this phenomenon remains unclear. H3M28_Cel7A can be used to determine the link between flocculation and protein secretion.

5.6 The effect of potential growth inhibitors on *Saccharomyces cerevisiae*

The plasma membrane and cell wall are the final barriers during protein secretion. Altering the secretion ability of an organism sometimes result in changes to the plasma membrane or cell wall (Das and Shultz, 1987; Perlinska-Lenart *et al.*, 2006). Since the plasma membrane and its associated proteins play an important role in protecting cells against inhibitors commonly found in lignocellulosic hydrolysates, such as organic acids, changes in the plasma membrane and acidifying the cytoplasm will also impact on inhibitor sensitivity (Casal *et al.*, 1996; Endo *et al.*, 2009). Furan derivatives have been shown to inhibit at least three enzymes in the central carbon metabolism of *S. cerevisiae* (Palmqvist and Hahn-Hagerdal, 2000; Modig *et al.*, 2002).

There are four potential inhibitors commonly produced during the pretreatment of lignocellulosic biomass and fermentation (Huang *et al*., 2011). These inhibitors can be categorized into the four major groups:

- Ethanol, which is produced during the fermentation process.
- Furan derivatives, such as furfural and 5-HMF,
- Weak organic acids, particularly acetic and formic acid.
- Phenolic compounds, such as vanillin, syringaldehyde and ferulic acid.

Ethanol, furfural, acetic acid and vanillin were chosen for this study as they cover the spectrum of potential inhibitors present after lignocellulosic pre-treatment.

5.6.1 Ethanol tolerance

Naturally the yeast *S. cerevisiae* is capable of tolerating high ethanol levels, and it is this trait that affords it its wide usage in industrial fermentation for various applications.

Swinnen *et al.* (2012) determined ethanol metabolism of *S. cerevisiae* as growth on YP media using ethanol as the only carbon source. Using this method to evaluate ethanol tolerance is tricky because ethanol evaporates very rapidly. However, this method has been used throughout literature and was therefore considered suitable for use in this study.

The supersecreting hybrid yeast strains appeared to be able to grow in the presence of 6% ethanol with little difficulty, however at 8% ethanol only the two parental strains, Y294 and M0341, and the H3M1 and H3M28 strains grew (Figure 4.13). During the production of biofuels, ethanol levels can reach up to 16% in a bioreactor. This shortcoming makes room for further evaluation of ethanol tolerance by these hybrid strains through a more stringent approach of strain engineering, a method used and endorsed by Kroukamp *et al*. (2012) to improve the secretion ability of *S. cerevisiae*.

5.6.2 Furfural tolerance

Furan aldehydes such as furfural prevent yeast cells from growing (Navarro, 1994). The mechanism of growth inhibition in the presence of furfural is unclear, however, Modig *et al*. (2002) showed that alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase were directly inhibited by furfural during ethanol

fermentation. It has also been reported that the accumulation of ROS can be induced by furan aldehydes resulting in damage to the mitochondria and vacuole membranes, cytoskeleton and nuclear chromatin (Allen *et al.*, 2010).

Furan derivatives have been reported (Palmqvist and Hahn-Hagerdal, 2000; Huang *et al.*, 2011) to inhibit growth of *S. cerevisiae* at very low concentrations (0.5 g/L and 1 g/L) (Delgens *et al.*, 1996). These results are consistent with the findings in this study, where the hybrid strains appeared to be highly sensitive to low levels of furfural. This is shown by the lack of growth of all strains at 1 g/L of furfural. At 0.5 g/L furfural, only the undiluted and 10X diluted series of the H3K27_1531 strain showed growth (Figure 4.14). The elevated furfural resistance of this strain should be evaluated further to determine the mechanism of resistance.

5.6.3 Acetic acid tolerance

Russell (1992) ascribed the growth inhibitory effect of weak acids *on S. cerevisiae* to uncoupling and intracellular anion accumulation. Studies have shown that acetic acid in the fermentation medium diffuses through the plasma membrane and dissociates in the cytosol due to the higher intracellular pH, thus lowering the cytosolic pH (Pampulha and Loureiro-Dias, 1990; Guldfeldt and Arneborg, 1998; Mira *et al.*, 2010). This effect inhibits cell metabolic activity and affects the plasma membrane electrochemical gradient. Intracellular acidification results in ATP hydrolysis caused by the proton-translocating ATPase Pma1 which is responsible for pumping protons out of the cell. The depletion of ATP lowers biomass formation and also affects enzymes in the glycolytic pathway that are ATP-dependent such as hexokinase and phosphofructokinase (Pampulha and Loureiro-Dias, 1990; Carmelo *et al.*, 1997). Elevated levels of acetic acid lead to a decrease in biomass formation which result in lower levels of protein secretion (Ferndahl *et al.*, 2010). This observation is in line with Schekman's (1982) report on the secretory pathway, where it was postulated that media components preferential for high cell density correlate with high-secreted protein yields.

All the yeast segregants can tolerate a maximum of 2 g/L acetic acid (Figure 4.15), except for the H3O23_1531 which had difficulty growing on 1 g/L acetic acid, especially when diluted to a concentration of 10^2, with no growth when diluted to a concentration of 10^3. These results are not consistent with findings published by (Maiorella *et al.*, 1983; Larsson *et al.*, 1999; Huang *et al.*, 2011), where acetic acid

appeared to not have an inhibitory effect at levels lower than 4 g/L and only had significant inhibition from 6 g/L and higher.

The yeast segregants used in this study contained the episomal plasmid pMU1531 which did not contain a reporter enzyme gene. As a consequence the impact of the growth inhibitory effect of acetic acid was evaluated, however weak acids can also affect the lipid organization and function of membrane embedded proteins (Piper *et al.*, 1998; Fernandes *et al.*, 2005) which consequently can have a negative effect on protein secretion. This should be investigated in a future study.

5.6.4 Vanillin tolerance

There was significant inhibition of all the supersecreting hybrid yeast strains at 1 g/L vanillin, thus the yeast strains can only thrive at concentrations well below 1 g/L (Figure 4.16).

The mechanism of metabolic inhibition by phenolic compounds is under-studied. It has been suggested however, that phenolic compounds such as vanillin act on biological membranes subsequently causing a loss of integrity. This results in the membranes' ability to serve as selective barriers and enzyme matrices to be compromised (Palmqvist and Hahn-Hagerdal, 2000; Klinke *et al.*, 2004).

These compounds are able to embed themselves into the cell membrane of organisms, causing a loss of membrane integrity. Disruption of the plasma membrane causes proteins, RNAs, ATP and ions to be released in the extracellular medium, resulting in reduced ATP levels, diminished proton motive force and impaired protein function and nutrient transport. Lower-molecular-weight phenolic acids behave like weak acids with respect to disruption of intracellular pH. These inhibitors can be avoided by either a process that removes much of the lignin or leaves the lignin intact (Sutton, 2011; Ibraheem and Ndimba, 2013).

5.7 Conclusion

This study describes the general protein secretion ability or the lack thereof, of *S. cerevisiae* segregants created by Kroukamp *et al*. (2012). These segregants were selected and shown to secrete a cellobiohydrolase I (Cel7A) at higher levels than their parental strains. With the aim of demonstrating a general enhancement in protein secretion by these *S. cerevisiae* segregants, this study employed different reporter enzymes, namely the *S. fibuligera* β-glucosidase I (Cel3A), the *N. fischerii*

endo-glucanase 3 (*cel*12A), the *T. emersonii* cellobiohydrolase 1 (*cel*7A), the *T. reesei* endo-β-xylanase (Xyn2) and the *S. stipitis* α-glucuronidase (glu), to evaluate secretion. These enzymes are between 21 and 113 kDa in size and contain between 0 and 16 *N*-glycosylation sites.

Protein secretion is a complicated process influenced by many factors (Den Haan *et al.*, 2013). Only two enzymes tested (Cel7A and GLU) which were previously reported as being poorly secreted by *S. cerevisiae* (Gomes, 2012; Anane *et al.*, 2013), were secreted at higher levels by some of the segregants compared to the parental strains. The size of the secreted proteins does not appear to influence secretion, since Cel7A and GLU are very different in size (55 kDa and 121 kDa, respectively). The number of glycosylation sites also does not appear to be the deciding factor in determining the secretion level of proteins by the segregants. Cel7A and GLU contain 3 and 16 *N*-glycosylation sites, respectively. Thus inferring that this enhanced secretion might be influenced by other protein properties and warrants further investigation. The changes that occurred during hybridization, QTL mapping and PSWGSA, where PSWGSA is a method developed by Parts *et al*. (2011), Swinnen *et al*. (2012) and used by Kroukamp *et al*. (2016) (Figure 2.5) to create the segregant progeny used in this study, appear to be specific for the secretion of Cel7A. The selected segregants did not display a general enhanced secretion capability; however GLU was also secreted at higher levels. It therefore appears as though the super-secreting segregants are able to secrete enzymes that are normally secreted at low levels, better. The *S. cerevisiae* secretion machinery has the ability to secrete a specific amount of protein to the extracellular medium, before the secretion machinery becomes saturated. La Grange *et al*. (2001) found that secreted xylanase activity fell from 1600 nkat/ml to 800 nkat/ml when co-expressed with a xylosidase. EG3, XYN2 and BGL1 are generally secreted very well and probably take the secretion machinery close to saturation point, therefore the super-secreting segregant were not able to improve on these levels. Cel7A and GLU are normally secreted poorly, leaving room for improvement in the super-secreting segregants.

Since cellobiohydrolase activity is generally the problematic enzyme activity in lignocellulose processing, these segregants are still good candidates for the construction of a CBP yeast. These results indicate the importance of the

compatibility between the individual genes and/or protein specific features in the host, *S. cerevisiae*, required for efficient and high level secretion of proteins.

The inability of these yeast segregant strains to grow at high levels of potential inhibitors such as furfural, acetic acid and vanillin might be linked to their ability to secrete Cel7A at high levels, but it is not a desired trait considering the harsh conditions encountered in large bioreactors during the industrial production of ethanol from biomass. While the mechanism and extent of cytotoxicity of lignocellulose inhibitory compounds generally differ, they all result in physiological and/or metabolic changes in *S. cerevisiae.*

Many strategies have been used successfully to increase protein secretion in *S. cerevisiae*. These include codon optimization, promoter strength optimization, strain selection and variation of secretion signal (Fleer, 1992; Idris *et al.*, 2010b; Kroukamp *et al.*, 2012). PSWGSA proved to be very successful, but the segregant progeny should be further studied in order to determine the factors responsible for the increase in secretion ability. PSWGSA can be used to identify unknown gene targets that are capable of enhancing secretion ability of yeasts (Swinnen *et al.*, 2012). It involves gene shuffling and sequencing in order to identify chromosomal regions that are responsible for specific or desired phenotypes.

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CHAPTER 7. APPENDIX

7.1 Yeast Transformations

Data for pNS201 and pNS202 is not shown.

7.1.1 Transformations of pRDH182.

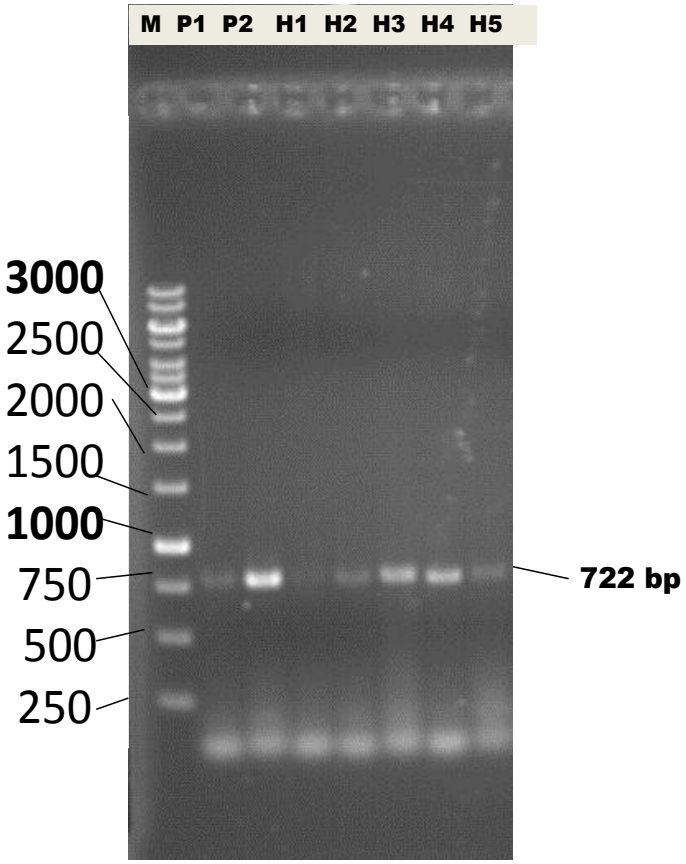


Figure 7.1: Gel separation of the PCR generated *XYN2* gene after yeast transformation for insertion of plasmid pRDH182 into the different super-secreting *S. cerevisiae* host strains. DNA fragments were separated on 1% agarose gel and stained with ethidium bromide.

7.1.2 Plasmid Transformations of pRDH166.

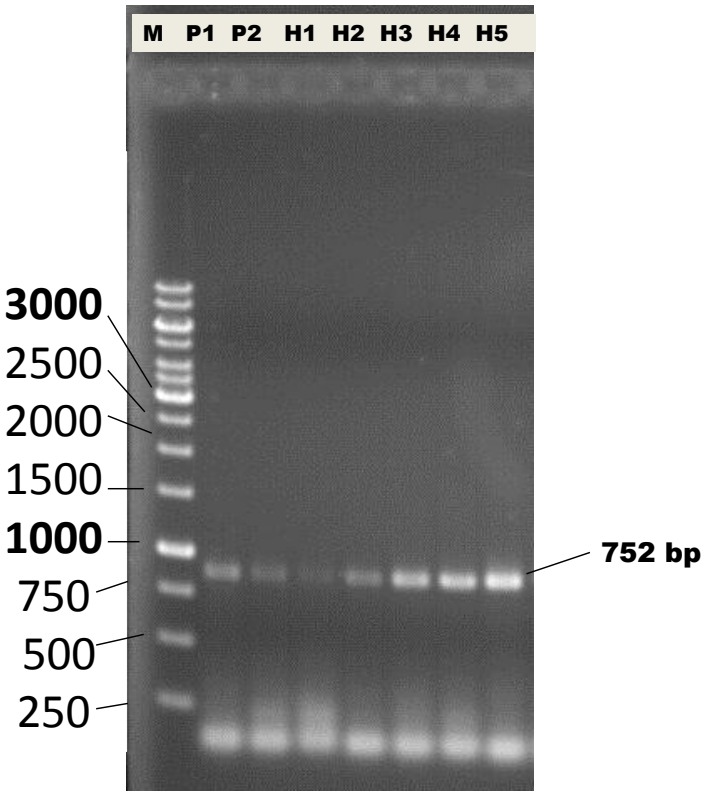


Figure 7.2: Gel separation of the PCR generated *EG3* gene after yeast transformation for insertion of plasmid pRDH166 into the different super-secreting *S. cerevisiae* host strains. DNA fragments were separated on 1% agarose gel and stained with ethidium bromide.

7.1.3 Plasmid Transformations of pNS203.

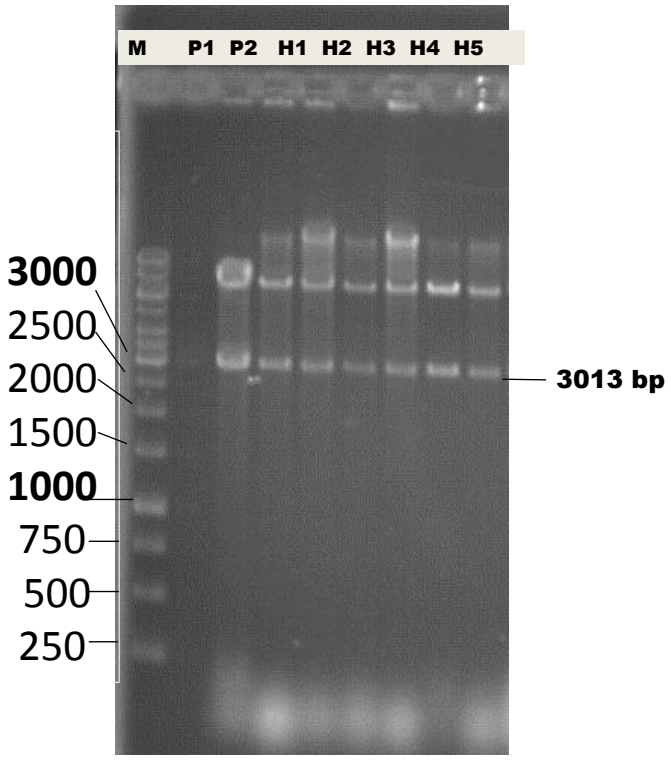
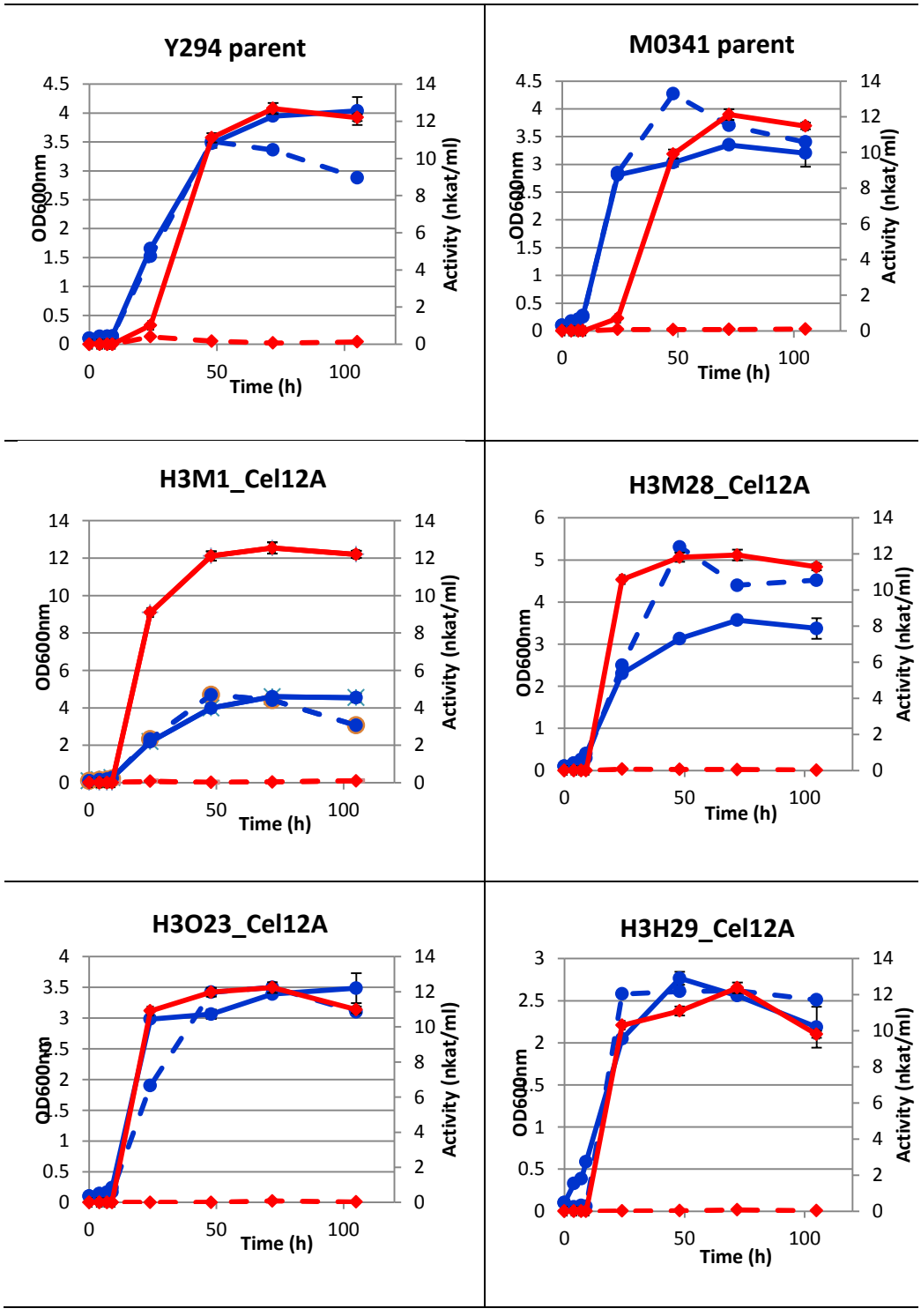


Figure 7.3: Gel separation of the PCR generated *GLU* gene after yeast transformation for insertion of plasmid pNS203 into the different super-secreting *S. cerevisiae* host strains. DNA fragments were separated on 1% agarose gel and stained with ethidium bromide.

7.2 Enzyme Activity Assays

7.2.1 Endo-glucanase secretion

The enzyme activity of the yeast hybrid strains H3M1_Cel12A [C], H3M28_Cel12A [D], H3O23_Cel12A [E], H3H29_Cel12A [F] and H3K27_Cel12A [G] was found to be similar compared to the parental strains *S.cerevisiae* Y294 [A] and *S.cerevisiae* M0341 [B] (Figure 7.7).



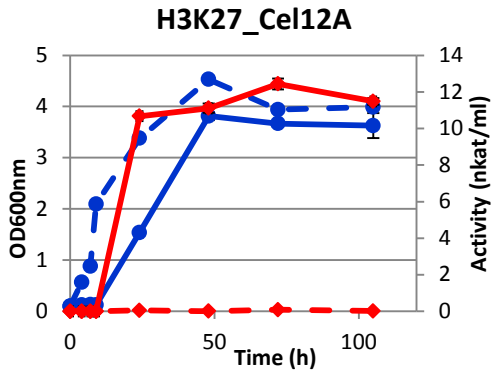
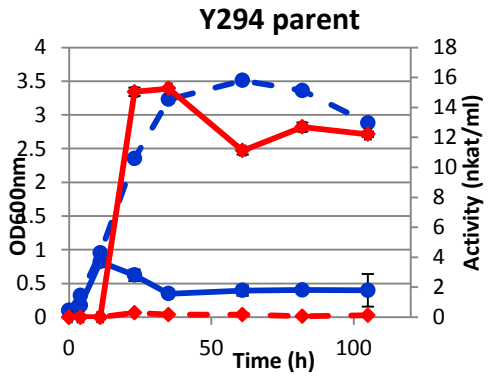
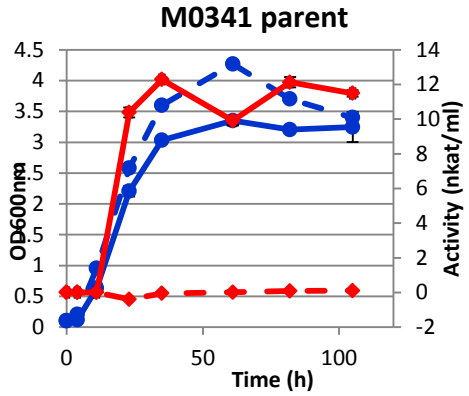


Figure 7.4: Enzyme activity profiles of *S. cerevisiae* hybrid segregant strains H3M1_Cel12A [C], H3M28_Cel12A [D], H3O23_Cel12A [E], and H3H29_Cel12A [F] and H3K27_Cel12A [G] in comparison to the parental hybrid Y294 [A] and M0341 [B] strains represented by the solid line. The red line represents activity and the blue biomass concentration (OD_{600nm}). The dashed line represents the control strains. Each hybrid strain contains the episomal plasmid pRDH166 conferring N. f. Cel12A (EG3) enzyme activity. The dashed lines represent the negative control strains containing the pMU1531. Enzyme activity assays were conducted in triplicate and independently. Error bars indicate the standard deviation from the mean value.

7.2.2 Endo-xylanase secretion

Similarly, the enzyme activity of the yeast hybrid strains H3M1_182 [C], H3M28_XYN2 [D], H3O23_XYN2 [E], H3H29_XYN2 [F] and H3K27_XYN2 [G] was found to be similar compared to the parental strains *S.cerevisiae* Y294 [A] and *S.cerevisiae* M0341 [B] (Figure 7.8).





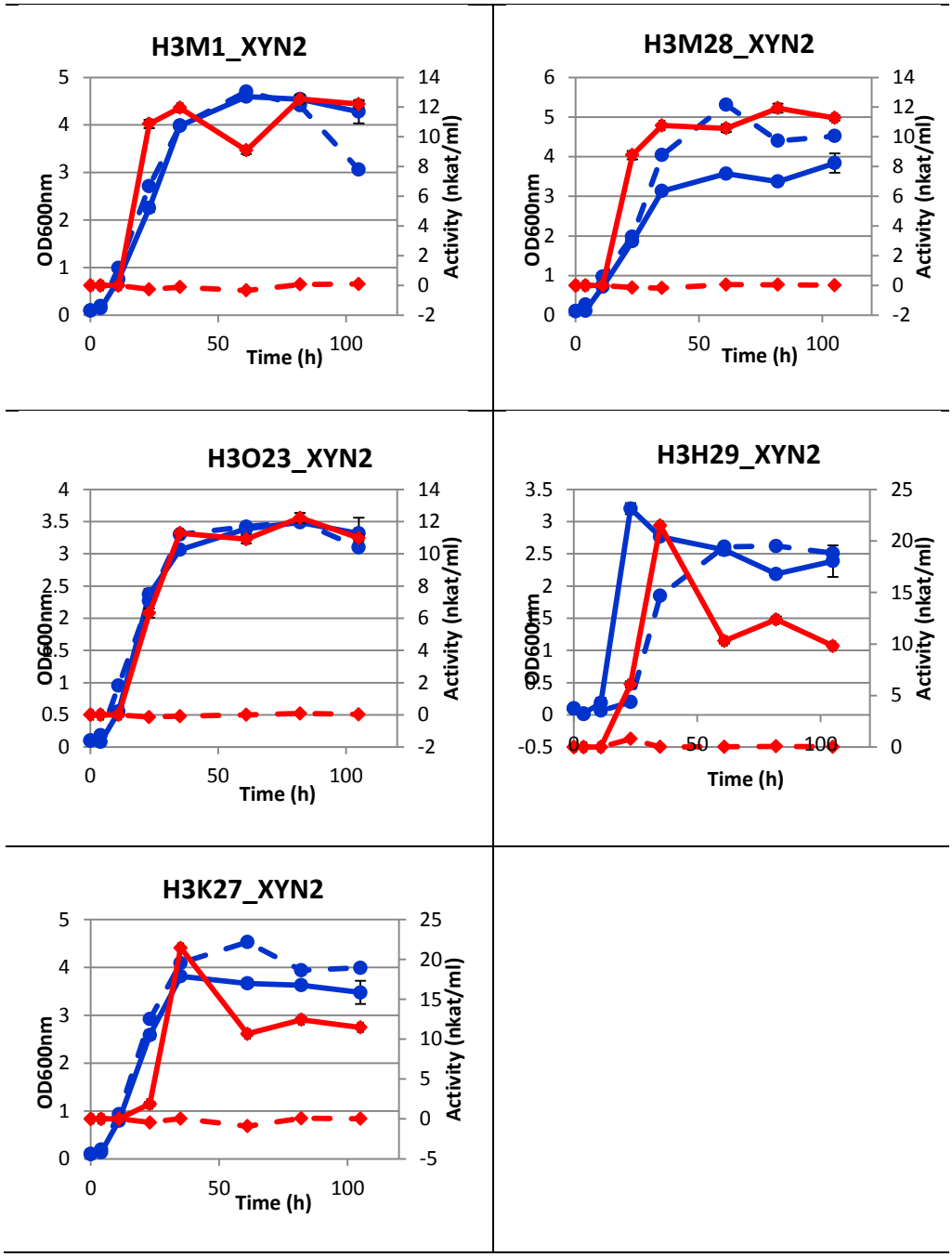


Figure 7.5: Enzyme activity profiles of *S. cerevisiae* hybrid segregant strains H3M1_XYN2 [C], H3M28_XYN2 [D], H3O23_XYN2 [E], and H3H29_XYN2 [F] and H3K27_XYN2 [G] in comparison to the parental hybrid Y294 [A] and M0341 [B] strains represented by the solid line. The red line represents activity and the blue line biomass concentration (OD_{600nm}). The dashed line represents the control strains. Each hybrid strain contains the episomal plasmid pRDH166 conferring T. r. XYN2 enzyme activity. The dashed lines represent the negative control strains containing the pMU1531. Enzyme activity assays were conducted in triplicate and independently. Error bars indicate the standard deviation from the mean value.

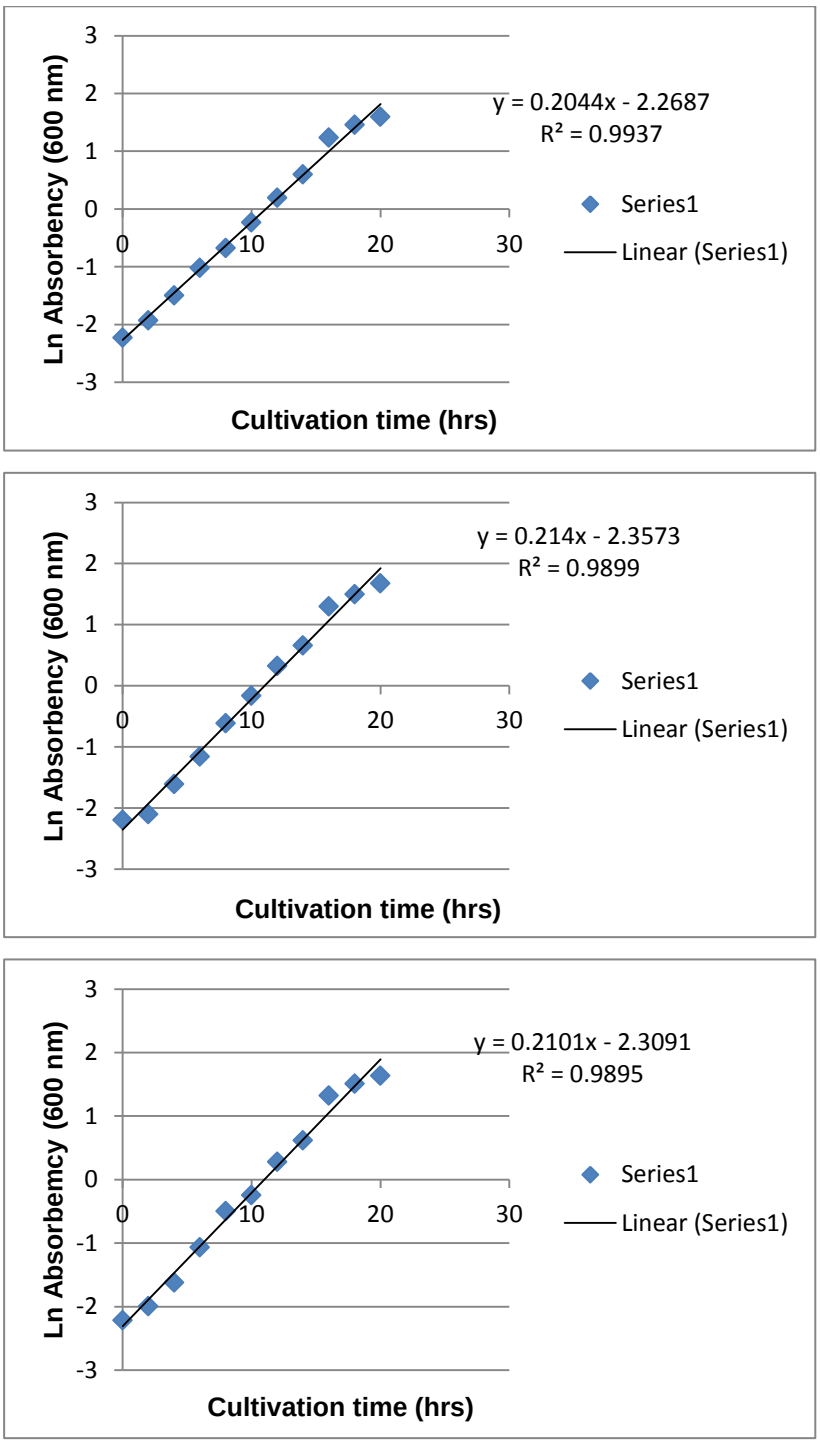


Figure 7.6: Ln growth of *S. cerevisiae* hybrid H3M28_1531 used to calculate the maximum specific growth rate, during batch culturing in buffered SC^{-ura}, cultivated over 48 hrs at 30 ^{o}C. Fermentations 1 – 3 represent the three different fermentation batches conducted randomly.

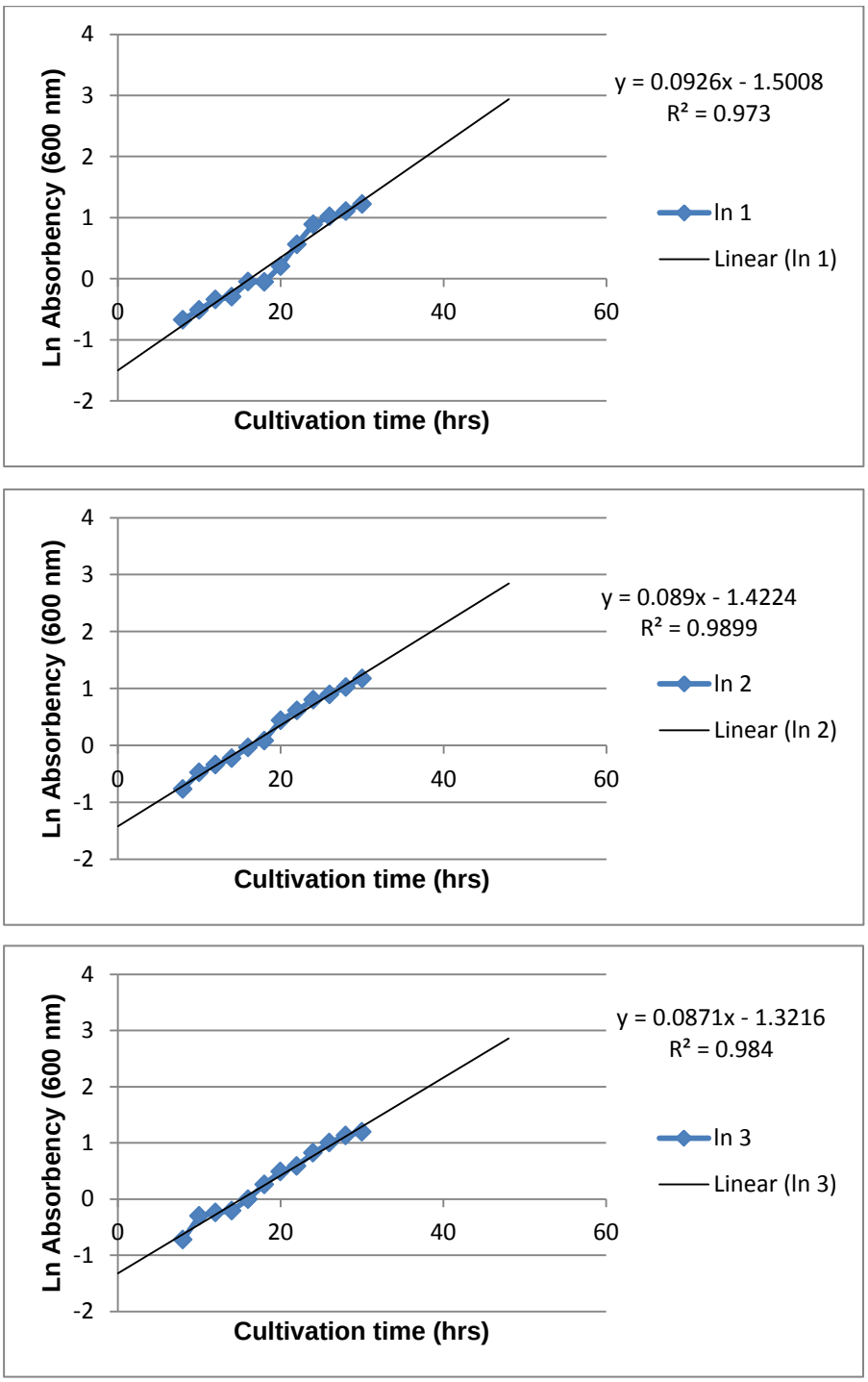


Figure 7.7: Ln growth of *S. cerevisiae* hybrid H3M28_202 used to calculate the maximum specific growth rate, during batch culturing in buffered SC^{-ura}, cultivated over 48 hrs at 30 ^{o}C. Fermentations 1 – 3 represent the three different fermentation batches conducted randomly.