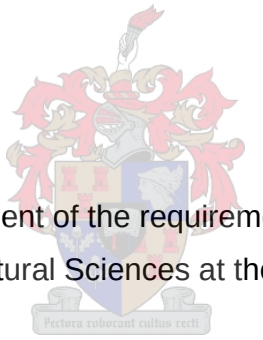
Expression and characterization of an intracellular cellobiose phosphorylase in *Saccharomyces cerevisiae.*

by

Christa J. Sadie



Thesis presented in partial fulfilment of the requirements for thr degree of Masters of Science in the Faculty of Natural Sciences at the University of Stellenbosch

Supervisor: Prof. W.H. van Zyl

March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

_____ _____

C. J. Sadie Date

Acknowledgements

The following people are very dear to me and without who's help this thesis and my studies at Stellenbosch University would have not been possible:

Prof van Zyl, thank you for the opportunity for being part of your team and for your guidance, help and financial support in accomplishing the goals I have set for myself.

Dr. Riaan den Haan, Tania de Villiers and the rest of the van Zyl-lab. Thank you for your support, ideas and most of all, friendship. Riaan, I deeply appreciate the effort and time you spent proof reading this thesis and making this one of my biggest accomplishments.

To my mother and father, Denise and Danie Sadie, thank you for your encouragement, love and confidence that I can achieve anything I set my mind to. I am extremely grateful for all the sacrifices you made, both personally and financially.

To my aunt and uncle, Maretha and Frikkie Basson. Words seem to be insufficient to express how thankful I am for everything you have done for me. Thank you for your support in every aspect and your willingness to help wherever you can.

Arnold, thank you for your love, patience and encouragement.

And lastly to the rest of my friends and dearest family, thank you for phone calls, tea-times, social gatherings, comic relief and companionship that gave me strength and balance to accomplish my studies.

SUMMARY

Cellulose, a glucose polymer, is considered the most abundant fermentable polymer on earth. Agricultural waste is rich in cellulose and exploiting these renewable sources as a substrate for ethanol production can assist in producing enough bioethanol as a cost-effective replacement for currently used decreasing fossil fuels. *Saccharomyces cerevisiae* is an excellent fermentative organism of hexoses; however the inability of the yeast to utilize cellulose as a carbon source is a major obstruction to overcome for its use in the production of bio-ethanol. Cellobiose, the major-end product of cellulose hydrolysis, is hydrolyzed by β-glucosidase or cellobiose phosphorylase, the latter having a possible metabolic advantage over β-glucosidase. Recently, it has been showed that *S. cerevisiae* is able to transport cellobiose. The construction of a cellulolytic yeast that can transport cellobiose has the advantage that end-product inhibition of the extracellular cellulases by glucose and cellobiose is relieved. Furthermore, the extracellular glucose concentration remains low and the possibility of contamination is decreased.

In this study the cellobiose phosphorylase gene, *cepA,* of *Clostridium stercorarium* was cloned and expressed under transcriptional control of the constitutive *PGK1* promoter and terminator of *S. cerevisiae* on a multicopy episomal plasmid. The enzyme was expressed intracellulary and thus required the transport of cellobiose into the cell. The *fur1* gene was disrupted for growth of the recombinant strain on complex media without the loss of the plasmid. The recombinant strain, *S. cerevisiae*[yCEPA], was able to sustain aerobic growth on cellobiose as sole carbon source at 30°C with V_{max} = 0.07 h^{-1} and yielded 0.05 g biomass per gram cellobiose consumed. The recombinant enzyme had activity optima of 60℃ and pH 6-7. Using Michaelis-Menten kinetics, the K_m values for the colorimetric substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and cellobiose was estimated to be 1.69 and 92.85 mM respectively. Enzyme activity assays revealed that the recombinant protein was localized in the membrane fraction and no activity was present in the intracellular fraction. Due to an unfavourable codon bias in *S. cerevisiae*, CepA activity was very low. Permeabilized *S. cerevisiae*[yCEPA] cells had much higher CepA activity than whole cells indicating that the transport of cellobiose was inadequate even after one year of selection. Low activity and insufficient cellobiose

transport led to an inadequate glucose supply for the yeast resulting in low biomass formation. Cellobiose utilization increased when combined with other sugars (glucose, galactose, raffinose, maltose), as compared to using cellobiose alone. This is possibly due to more ATP being available for the cell for cellobiose transport. However, no cellobiose was utilized when grown with fructose indicating catabolite repression by this sugar.

To our knowledge this is the first report of a heterologously expressed cellobiose phosphorylase in yeast that conferred growth on cellobiose. Furthermore, this report also reaffirms previous data that cellobiose can be utilized intracellularly in *S. cerevisiae.*

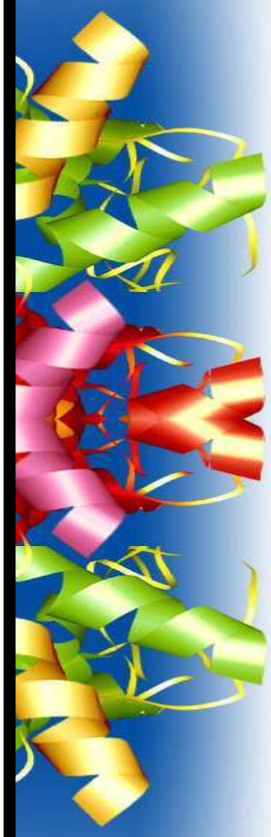
OPSOMMING

Sellulose, ‘n homopolimeer van glukose eenhede, word beskou as die volopste suiker polimeer op aarde. Landbou afval produkte het ‘n hoë sellulose inhoud en benutting van diè substraat vir bio-etanol produksie kan dien as ‘n koste-effektiewe aanvulling en/of vervanging van dalende fossielbrandstof wat tans gebruik word. Die gis, *Saccharomyces cerevisiae*, is ‘n uitmuntende organisme vir die fermentasie van heksose suikers, maar die onvermoë van die gis om sellulose as koolstofbron te benut is ‘n groot struikelblok in sy gebruik vir die produksie van bio-etanol. Sellobiose, die hoof eindproduk van ensiematiese hidrolise van sellulose, word afgebreek deur β-glukosidase of sellobiose fosforilase. Laasgenoemde het ‘n moontlike metaboliese voordeel bo die gebruik van β-glukosidase vir sellobiose hidrolise. Daar was onlangs gevind dat *S. cerevisiae* in staat is om sellobiose op te neem. Die konstruksie van ‘n sellulolitiese gis wat sellobiose intrasellulêr kan benut, het die voordeel dat eindproduk inhibisie van die ekstrasellulêre sellulases deur sellobiose en glukose verlig word. Verder, wanneer die omsetting van glukose vanaf sellobiose intrasellulêr plaasvind, word die ekstrasellulêre glukose konsentrasie laag gehou en die moontlikheid van kontaminasie beperk.

In hierdie studie was die sellobiose fosforilase geen, *cepA*, van *Clostridium stercorarium* gekloneer en uitgedruk onder transkripsionele beheer van die konstitutiewe *PGK1* promoter en termineerder van *S. cerevisiae* op ‘n multikopie episomale plasmied. Die ensiem is as ‘n intrasellulêre proteïen uitgedruk en het dus die opneem van die sellobiose molekuul benodig. Die disrupsie van die *fur1* geen het toegelaat dat die rekombinante ras op komplekse media kon groei sonder die verlies van die plasmied. Die rekombinante ras, *S. cerevisiae*[yCEPA], het aërobiese groei by 30℃ op sellobiose as enigste koolstofbron onderhou met μ_{max} = 0.07 h^{-1} en ‘n opbrengs van 0.05 gram selle droë gewig per gram sellobiose. Die rekombinante ensiem het optima van 60℃ en pH 6-7 gehad. Die K_m waardes vir die kolorimetriese substraat *p*NPG en sellobiose was 1.69 en 92.85 mM onderskeidelik. Ondersoek van die ensiem aktiwiteit het getoon dat die rekombinante proteïen gelokaliseer was in die membraan fraksie en geen aktiwiteit was teenwoordig in die intrasellulêre fraksie nie. CepA aktiwiteit was laag as gevolg van ‘n lae kodon voorkeur in *S. cerevisiae*. Verder het geperforeerde *S. cerevisiae*[yCEPA] selle aansienlik beter

CepA aktiwiteit getoon as intakte selle. Hierdie aanduiding van onvoldoende transport van sellobiose na binne in die sel tesame met die lae aktiwiteit van die CepA ensiem het gelei tot onvoldoende glukose voorraad vir die sel en min biomassa vorming. Sellobiose verbruik het toegeneem wanneer dit tesame met ander suikers (glukose, galaktose, raffinose, maltose) gemeng was, heelwaarskynlik deur die vorming van ekstra ATP's vir die sel wat 'n toename in sellobiose transport teweeg gebring het. Fruktose het egter kataboliet onderdrukking veroorsaak en sellobiose was nie benut nie.

Sover ons kennis strek, is hierdie die eerste verslag van 'n heteroloë sellobiose fosforilase wat in *S. cerevisiae* uitgedruk is en groei op sellobiose toegelaat het. Verder, bewys die studie weereens dat *S. cerevisiae* wel sellobiose kan opneem.



General Introduction and Project Aims

Chapter 1

1 INTRODUCTION

Saccharomyces cerevisiae has contributed to both fundamental research as well as biotechnological application, including the fermentation industry, because of (1) its success as an expression host for recombinant enzymes, (2) its ability to withstand high ethanol concentrations (3) and an optimized ethanol yield on glucose [Yu, *et al*., 2004; Ryabova *et al*., 2003; Van Rensburg *et al*., 1998]. A major constraint of this versatile organism is its inability to grow on the glucose polymer, cellulose, the most abundant fermentable polymer on earth that could provide a cost-effective substrate for the fermentation industry [Demain *et al*., 2005; Yoon *et al*., 2003; Lynd *et al*., 1999; Van Rensburg *et al*., 1998]. The production of ethanol from waste products or other lignocellulosic biomass could improve energy security; reduce trade insufficiency, urban air pollution and dependence on imported liquid fuel [Rajoka *et al*., 2003; Lin and Tanaka, 2006; Gray *et al*., 2006].

Since *S. cerevisiae* does not produce the enzymes needed for cellulose degradation, considerable research is dedicated to the expression of cellulases in this organism. Cellulolytic organisms typically produce endoglucanase and/or cellobiohydrolase that result in the formation of the disaccharide cellobiose, the most common degradation product of cellulose hydrolysis [Lynd *et al*., 2002]. A wide variety of cellulases have already been expressed in *S. cerevisiae* including cellobiohydrolases, endoglucanases and β-glucosidases, the latter being extensively investigated [Freer S.N., 1993; Fujita *et al*., 2004; McBride *et al*., 2005; van Rensburg *et al*., 1998]. β-Glucosidases are responsible for cleavage of the β-1,4-linkage in the cellobiose molecule to release two glucose molecules [Bhatia *et al*., 2002]. Organisms such as anaerobic, gram positive *Clostridiium* species also make use of an intracellular cellobiose phosphorylase and cellodextrin phosphorylase [Alexander J.K, 1961]. These enzymes are responsible for cleaving and simultaneously phosphorylating cellobiose and longer cellodextrins respectively. Since one of the glucose molecules is already phosphorylated prior to entering the glycolytic pathway, the expression of a cellobiose phosphorylase in yeast could be energetically advantageous and ultimately lead to an increase in ethanol production [Zhang and Lynd, 2004].

Only a few yeast species, including *Clavispora lusitaniae*, are able to transport

cellobiose for intracellular utilization [Freer and Greene, 1990; Freer S.N., 1991; Gonde *et al*., 1984; Kaplan J.G., 1965]. Recently, it has been shown that *S. cerevisiae* is able to transport cellobiose when an intracellular β-glucosidase was expressed in this yeast [van Rooyen *et al*., unpublished data]. *S. cerevisiae* is known only to transport two disaccharides, namely maltose and sucrose [Stambuk *et al*., 2000]. These disaccharides are transported by the general α-glucoside transporter AGT1 and recently van Rooyen *et al*. revealed that the AGT1 transporter together with the MAL61 transporter of *S. cerevisiae* was activated when grown in cellobiose as sole carbon source [unpublished data].

The construction of a cellulolytic yeast that can transport cellobiose has the advantage that end-product inhibition of the extracellular cellulases by glucose and cellobiose is relieved [Lynd *et al*., 1999]. Furthermore, by internalising the formation of glucose from cellobiose, the extracellular glucose concentration remains low and the possibility of contamination is decreased.

1.1 AIMS OF THE STUDY

In this study we report the first successful expression of an intracellular cellobiose phosphorylase from *Clostridium stercorarium* in *S. cerevisiae* to confirm that this yeast is able to transport and utilize cellobiose intracellularly. The specific aims of the study were:

1. Cloning of the cellobiose phosphorylase gene from *C. stercorarium* on an episomal plasmid under control of the *S. cerevisiae* phosphoglycerate kinase gene (*PGK1*) promotor and terminator.
2. Selection of the recombinant strain for enhanced cellobiose transport.
3. The characterization of the recombinant enzyme activity produced by *S. cerevisiae.*
4. The characterization of the growth and cellobiose utilization of the recombinant strain on cellobiose as sole carbon source.
5. The characterization of the growth and cellobiose utilization of the recombinant strain in combination with other sugars.

1.2 LITERATURE CITED

Alexander , J. K. 1961. Characteristics of cellobiose phosphorylase. J Bacteriol **81**:903-910.

Bhatia, Y., S. Mishra, and V. S. Bisaria. 2002. Microbial beta-glucosidases: cloning, properties, and applications. Crit Rev. Biotechnol **22**:375-407.

Demain, A. L., M. Newcomb, and J. H. Wu. 2005. Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev **69**:124-154.

Freer, S. N. and R. V. Greene. 1990. Transport of glucose and cellobiose by *Candida wickerhamii* and *Clavispora lusitaniae*. J Biol Chem **265**:12864-12868.

Freer, S. N. 1991. Fermentation and aerobic metabolism of cellodextrins by yeasts. Appl Environ Microbiol **57**:655-659.

Freer, S. N. 1993. Kinetic characterization of a beta-glucosidase from a yeast, *Candida wickerhamii*. J Biol Chem **268**:9337-9342.

Fujita, Y., J. Ito, M. Ueda, H. Fukuda, and A. Kondo. 2004. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol **70**:1207-1212.

Gonde, P., B. Blondin, M. Leclerc, R. Ratomahenina, A. Arnaud, and P. Galzy. 1984. Fermentation of cellodextrins by different yeast strains. Appl Environ. Microbiol **48**:265-269.

Gray, K. A., L. Zhao, and M. Emptage. 2006. Bioethanol. Curr Opin Chem Biol **10**:141-146.

Kaplan, J. G. 1965. An inducible system for the hydrolysis and transport of beta-glucosides in yeast. 1. Characteristics of the beta-glucosidase activity of intact and of lysed cells. J Gen Physiol **48**:873-886.

Lin, Y. and S. Tanaka. 2006. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol **69**:627-642.

Lynd, L. R., C. E. Wyman, and T. U. Gerngross. 1999. Biocommodity engineering Biotechnol Prog **15**:777-793.

Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev **66**:506-77

McBride, J. E., J. J. Zietsman, W. H. van Zyl, and L. R. Lynd. 2005. Utilization of cellobiose by recombinant [beta]-glucosidase-expressing strains of *Saccharomyces cerevisiae*: characterization and evaluation of the sufficiency of expression. Enz Microb Technol

Rajoka, M. I., S. Khan, F. Latif, and R. Shahid. 2004. Influence of carbon and nitrogen sources and temperature on hyperproduction of a thermotolerant beta-glucosidase from synthetic medium by *Kluyveromyces marxianus*. Appl Biochem Biotechnol **117**:75-92.

Ryabova, O. B., O. M. Chmil, and A. A. Sibirny. 2003. Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res. **4**:157-164.

Stambuk, B. U., A. S. Batista, and P. S. de Araujo. 2000. Kinetics of active sucrose transport in Saccharomyces cerevisiae. J Biosci Bioeng **89**:212-214.

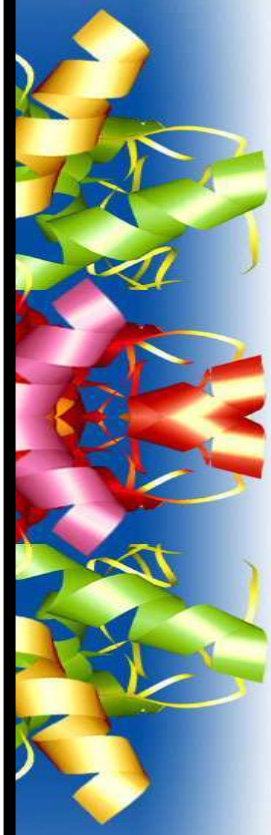
Van Rensburg, P., W. H. van Zyl, and I. S. Pretorius. 1998. Engineering yeast for efficient cellulose degradation. Yeast **14**:67-76.

Yoon, S. H., R. Mukerjea, and J. F. Robyt. 2003. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. Carbohydr Res **338**:1127-1132.

Yu, Z. and H. Zhang. 2004. Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with *Saccharomyces cerevisiae*. Bioresour Technol **93**:199-204.

Zhang, Y. H. and L. R. Lynd. 2004. Kinetics and relative importance of phosphorolytic and hydrolytic cleavage of cellodextrins and cellobiose in cell extracts of *Clostridium thermocellum*. Appl Environ Microbiol **70**:1563-1569.

Literature Review



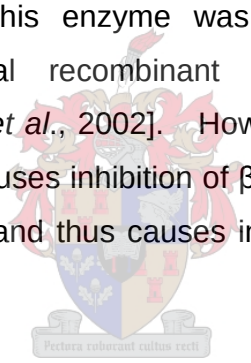
Cellobiose utilization of cellulolytic and recombinant organisms for the conversion to bioethanol

Chapter 2

2.1 INTRODUCTION

As a structural biopolymer found in all plant cell walls, cellulose is considered the most abundant, stable and resistant hexose polymer on earth [Demain *et al*., 2005; Desvaux, 2005]. The exploitation of this molecule as a carbon and energy source for microbial utilization have led scientists to the belief that the production of ethanol through microbial conversion may be a feasible and sustainable replacement for the decreasing fossil fuels that are currently used.

Cellobiose, a glucose dimer linked with a β-1,4-bond, is the major cellulose hydrolysis product of free enzyme cellulolytic systems [Mcbride *et al*., 2005]. β-glucosidase (E.C. 3.2.1.21) hydrolyses cellobiose to produce two glucose moieties available for fermentation [Kaplan, 1965]. This enzyme was found to be expressed as an extracellular enzyme in several recombinant organisms including the yeast *Saccharomyces cerevisiae* [Lynd *et al*., 2002]. However, the accumulation of glucose in the extracellular environment causes inhibition of β-glucosidases which in turn results in the accumulation of cellobiose and thus causes inhibition of cellulases [Freer *et al*., 1990].



S. cerevisiae is the most efficient microorganism for fermenting glucose to ethanol and has proven to be ideal for industrial fermentation processes as well as being a model recombinant organism [Van Rensburg *et al*., 1998; Hahn-Hägerdal *et al*., 2001; Kosaric *et al*., 2001; Ryabova *et al*., 2003; Yu and Zhang, 2004; Gray *et al*., 2006]. The inability of *S. cerevisiae* to grow on the complex sugars present in lignocellulosic materials is an obstacle that needs to be overcome. A range of enzymes including hemicellulases and cellulases have been expressed in *S. cerevisiae* that enable growth on lignocellulosic materials and/or their component sugars although at rates that are not yet efficient for the economic production of ethanol [Lynd *et al*., 2002]. Recently it was found that *S. cerevisiae* is indeed able to transport cellobiose across its membrane although the transport mechanism is still unclear [van Rooyen *et al*., unpublished work]. The ability of this organism to transport cellobiose into the cell allows for the intracellular expression of

a β-glucosidase that could relieve end-product inhibition of the other cellulases, notably endoglucanases and cellobiohydrolases [McBride *et al*., 2005].

Anaerobic bacteria are able to sustain growth on crystalline cellulose despite of low amounts of ATP available as a result of the efficiency of oligosaccharide transport combined with intracellular phosphorolytic cleavage of β-glycosidic bonds [Demain *et al*., 2005; Lynd *et al*., 2005]. Generally, cellulolytic anaerobic bacteria prefer phosphorolytic cleavage of cellodextrins above hydrolysis [Zhang and Lynd, 2005]. Cellobiose phosphorylase (2.4.1.20) and cellodextrin phosphorylase (2.4.1.49) are preferentially used by these organisms for the intracellular cleavage of cellobiose and longer cellodextrins that ultimately leads to the generation of more ATP.

Here we describe the expression of an intracellular cellobiose phosphorylase (cepA) from *Clostridium stercorarium* in *S. cerevisiae* for potential energetic advantage, and we investigate the possible transport mechanism for cellobiose. To our knowledge this is the first study in which a phosphorolytic cellulase was expressed in a yeast. The remainder of this chapter will provide a summary of published literature relative to this field of study.

2.2 LIGNOCELLULOSE AS SOURCE OF FERMENTABLE SUGARS

Plant biomass is plentiful and rich in carbohydrates [Demain *et al*., 2005]. These carbohydrates together with other structural molecules (such as lignin) are collectively known as lignocellulose. These molecules can be exploited as an energy source for desired industries and unlike other currently used sources, it is renewable. The global production of plant biomass amounts to 200 x 10^9 tons per year and about 180 million ton of this biomass is accessible for alternative employment [Demain *et al*., 2005; Lin and Tanaka, 2006]. It is therefore possible to produce large quantities of ethanol from available cellulosic biomass [Yu and Zhang, 2004]. During photosynthesis, carbon that is released during energy consumption (in the form of CO_2) is reintroduced into plant

material. Using cellulosic biomass for biotechnological purposes therefore aids in the conservation of the environment by means of maintaining a closed carbon cycle.

2.2.1 Availability of lignocellulose resources

Cellulose is the most abundant sugar polymer of plant biomass and the most abundant, fermentable homopolymer found on earth [Lynd *et al*., 2002]. Cellulose is seldom found in nature in its pure form (one of which is cotton balls) but mainly serves a structural role in plant biomass as cellulose fibres embedded in a matrix of other sugar-polymers. This mixture of sugars is referred to as lignocellulose and consists of a highly ordered and tightly packed structure of cellulose fibres (38% – 50%), hemicellulose (23% - 32%) and lignin (15% - 25%) [Hahn-Hägerdal *et al*., 2001].

Any material that contains lignocellulosic sugars can theoretically be fermented to ethanol [Sun and Cheng, 2002]. Currently large amounts of plant matter are treated as waste such as grasses, sawdust, crop residues, wood deposits, fast-growing invading trees and municipal waste. This supply of cheap raw materials led research to explore the opportunities to convert it to useful products. In some countries, where the bio-ethanol industry are already in an advanced state, dedicated energy crops provide the raw material needed for ethanol production [Lin and Tanaka, 2006]. At present, sugar cane and corn are the dominant feedstocks for ethanol production but the increasing demand for fuel indicates that alternative substrates are needed to supplement or possibly replace currently used materials [Palmarola-Adrados *et al*., 2004].

2.2.2 Structure of lignocellulose

The cellulose molecules in plant cell walls are surrounded by hemicellulose and lignin, forming a matrix which imparts strength [Mosier *et al*., 2005]. Because these molecules are structurally intertwined, it is important to consider the organization of these molecules in the plant cell wall for a better understanding of the underlying processes involved in their degradation.

2.2.2.1 Cellulose

Cellulose is synthesized as linear molecules of β-1,4-linked β–D-glucopyranose units [Harjunpää, 1998]. Two successive glucose residues are rotated by 180° relative to each other forming the disaccharide cellobiose (see Figure 1) which is the repeating unit of the cellulose chain.

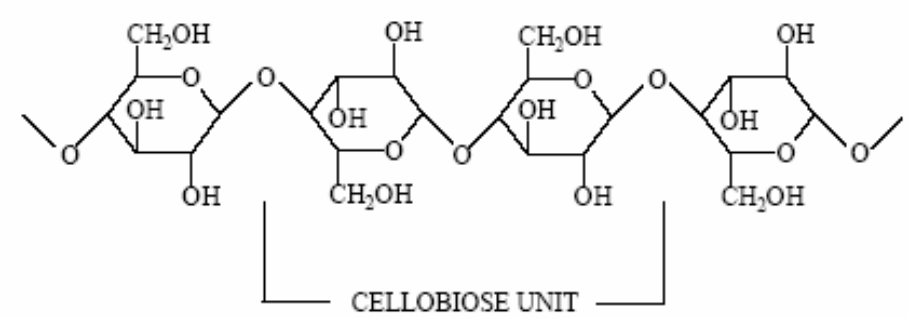


Figure 1. Cellobiose, the repeating unit of the cellulose chain linked with a β-1,4-linkage [Harjunpää, 1998]

Longer cello-oligosaccharides are formed by different degrees of glucopyranose polymerisation of up to 15 000 units [van Rensburg *et al*., 1998]. Approximately 30 of the linear cello-oligosaccharides chains assemble to form units known as protofibrils, which are packed into larger units known as microfibrils (see Figure 2).

Cellulose fibres consist of bundles of these microfibrils and are strengthened by intrachain and interchain hydrogen bonds [Lynd *et al*., 2002]. Van der Waals forces keep overlaying sheets of the cellulose molecules in place and together all of these forces create a fixed matrix of atoms that are impermeable to water molecules, as well as enzymes needed for its degradation.

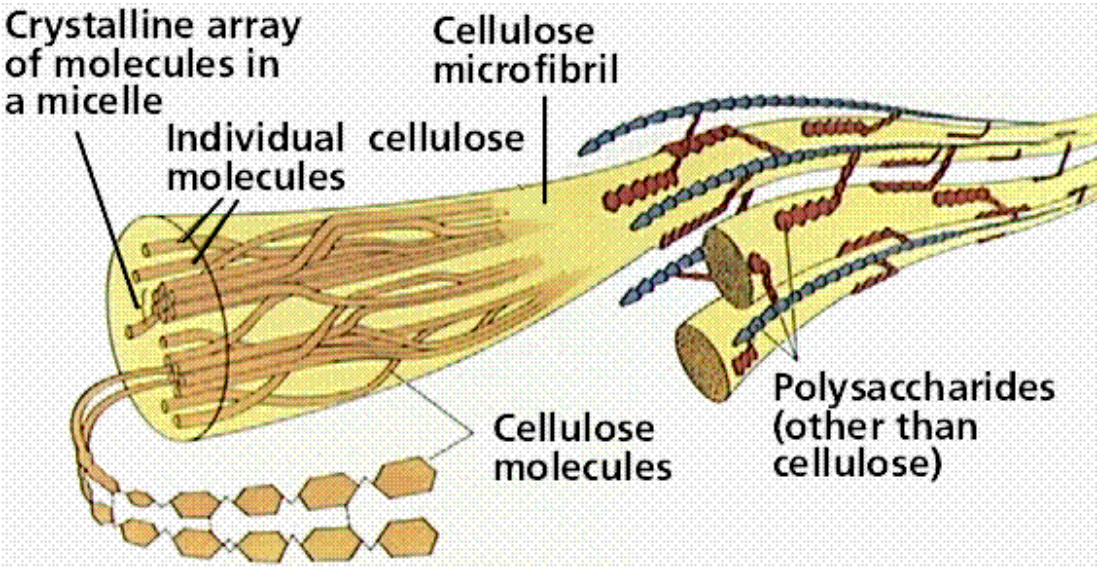


Figure 2. The organization of the cellulose chains into compact structures [http://www.emc.maricopa.edu/faculty/farabee/biobk/cellulose.gif]

The dense crystalline structure of cellulose is not maintained in nature but exists as different forms that range from purely crystalline to completely amorphous [Hildèn and Johansson, 2004]. The crystalline structure can constitute between 40% and 90% of the cellulose while the rest is amorphous. This is as a result of the presence of other hemicellulose sugars such as mannan and xylan that penetrate the microfibrils [Vincent, 1999]. Water molecules are able to penetrate the amorphous areas, making it partially soluble and also allowing cellulose degrading enzymes (cellulases) to disperse the cellulose chain [Lynd *et al*., 2002]. However, the presence of hemicellulose and lignin prevent cellulases from completely degrading the cellulose while it is still part of this matrix.

2.2.2.2. Hemicellulose

Hemicelluloses are a amorphous heteropolymers and usually consists of a mixture of (1) the pentoses xylose and arabinose, (2) the hexoses glucose, mannose and galactose, and (3) uronic acids, linked with β-1,4-, β-1,3-, α-1,2 and ester bonds [Hahn-Hägerdal *et al*., 2001; Pèrez *et al*., 2002]. Hemicellulose are a very diverse group of molecules and it's composition tends to vary depending on the type of plant [Mosier *et al*., 2005; Gray

et al., 2006]. The major hemicelluloses are glucuronoxylan and glucomannan that are found in hardwoods and arabinoglucuronoxylan and galactoglucomannan that are found in softwoods [Harjunpää, 1998]. These two groups have different side groups substituting the xylan backbone such as acetyl and 4-O-methylglucuronic acid in the hardwoods and L-rhamnose and galacturonic acid in the softwoods.

Hemicelluloses are low molecular weight polysaccharides and the backbone usually only reaches a degree of polymerization of about 200. The backbone of the hemicellulose molecules is non-covalently linked via hydrogen bonds to the cellulose fibres and form bridges to other cellulose fibres by means of the side-chain molecules (see Figure 3) [Vincent, 1999; Mosier *et al*., 2005].

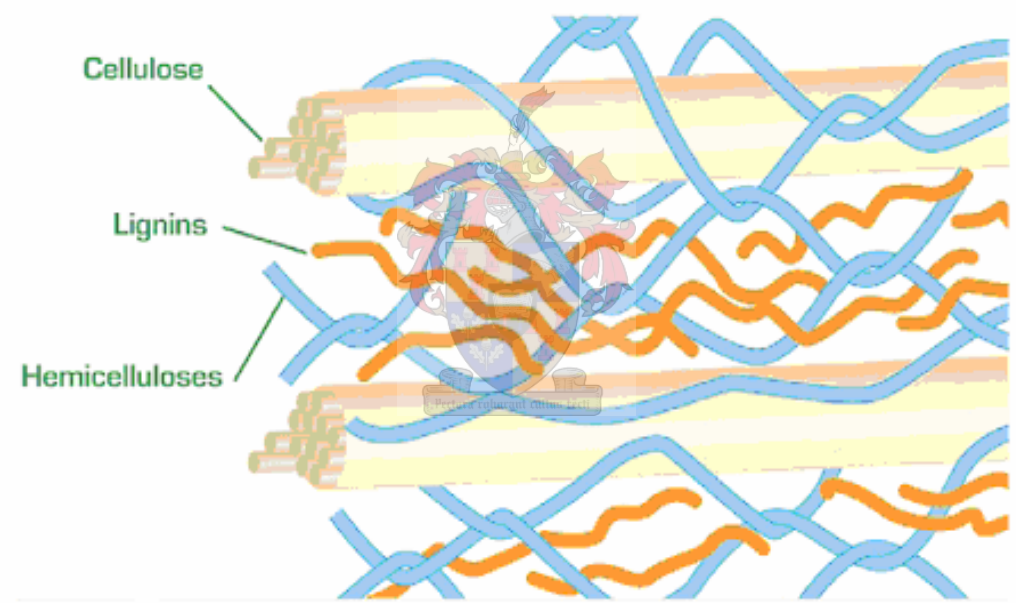


Figure 3. Cellulose is embedded in a matrix of hemicellulose and lignin molecules within the plant cell wall [Boudet *et al*., 2003].

2.2.2.3. Lignin

Lignin is the most abundant non-fermentable polymer found in nature and is synthesized from aromatic phenylpropanoid precursors [Pèrez *et al*., 2002; Mai *et al*., 2004]. The three-dimensional structure is held together by carbon-carbon and aryl-ether linkages and forms covalent cross-linkages with the cellulose and hemicellulose molecules. The

complex lignin structure forms a sheath surrounding the carbohydrate moieties of the plant cell wall and adding to this is its high molecular weight and its insolubility that impedes enzymatic degradation of the sugar polymers [Pèrez *et al*., 2002; Mosier *et al*., 2005]. It has also been shown that lignin actually inhibits the enzymes involved in cellulose and hemicellulose degradation, having the least effect on β-glucosidases [Berlin *et al*., 2006]. In the plant cell it imparts strength and provides resistance against diseases and pests. Lignin's building blocks are non-fermentable, but its degradation (which goes beyond the scope of this study) does release large amounts of energy that can be used in the electrical industry and for heating [Mosier *et al*., 2005; Potera, 2005].

Other molecules present in plant materials can be classified as extractive and non-extractive materials by non-polar solvents, that are not found in the plant cell wall [Klinke *et al*., 2004]. The non-extractives are mostly silica and alkali salts, pectin, proteins and starch, while the extractives are resins, terpenes, phenols, quinones and tannins.

2.3 ENZYMATIC DEGRADATION OF LIGNOCELLULOSE MATERIALS

The degradation barriers that are set by the complex arrangement of lignocellulosic material in plants contrast the low cost and availability of the substrate for the production of fermentable sugars. Recently, ethanol production from cellulosic biomass was found to be the most promising technology in renewable energy research that is currently investigated [Lin and Tanaka, 2006]. The industrial application of this process is still however mired by technological issues such as the cost of enzymatic treatment of the biomass. Research has focussed on the chemical treatment and the different enzymes involved in the degradation and hydrolysis of the lignocellulose material.

There are three types of processes to produce ethanol from lignocellulose: (1) acid or alkaline hydrolysis; (2) thermochemical hydrolysis and (3) enzymatic hydrolysis [Lynd *et al*., 1999]. Although the first two processes have been extensively researched, enzymatic treatment of the substrate has the potential to lower processing cost as technology improves. Furthermore, enzymatic hydrolysis should not produce as many

toxic compounds or compounds that inhibit the growth of cellulolytic and fermentative organisms as is the case with chemical and thermochemical hydrolysis [Freer, 1990]. In this review we will focus on the enzymatic treatment of sugar polymers, particularly cellulose, for ethanol production. Both of the groups of carbohydrates found in lignocellulose, requires different enzymes for complete degradation and hydrolysis. These enzymes have been subjected to extensive research and classified according to mode of action and DNA sequence similarity [Schulein, 2000; Lynd *et al*., 2002; Zhang and Lynd, 2004].

2.3.1 Hemicellulose degradation enzymes

Hydrolysis of hemicellulose proceeds by the synergistic action of a range of enzymes to release simple sugars [Perez *et al*., 2002]. The major enzyme activity required for the depolymerization of hemicellulose is xylanase, for degradation of the xylan backbone, the most common sugar polymer found in hemicellulosic material [Collins *et al*., 2005]. β-Mannanases are responsible for the hydrolysis of the hemicellulose molecules with a mannose backbone [Harjunpää, 1998; Perez *et al*., 2002]. These endoenzymes randomly attack at internal sites of the xylan and mannan molecules, releasing shorter polymers with substituted side-chains (see Figure 4). The exo-enzymes, β-xylosidase, β-mannosidase and β-glucosidase, are responsible for hydrolysing these shorter chains and subsequently releasing the component pentose and hexose sugars. Complete degradation is only accomplished with the additional activity of α-arabinofuranosidases, α-galactosidase, α-glucoronidase and esterases, named according to their substrate specificity, to release the substituted groups and the monomeric sugars [Perez *et al*., 2002; Collins *et al*., 2005].

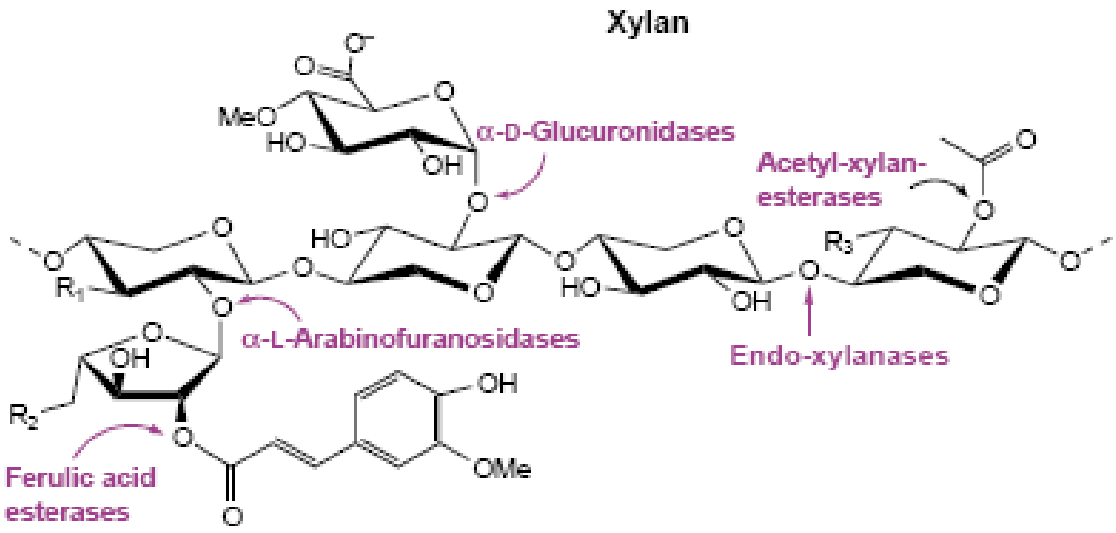


Figure 4. The most common hemicellulose, xylan is arranged as D-xylopyranosyl units linked by β-1,4-glycosidic bonds. The xylan backbone is modified with various substitutions, including 4-O-methyl-D-glucuronic acid, acetic acid, uronic acids and L-arabinofuranose residues. These side-chains vary in abundance and linkage types between xylans from different sources [Shallom and Shoham, 2003].

2.3.2 Cellulose degradation enzymes

Cellulolytic organisms are spread over all the kingdoms but are predominantly found in the prokaryotes and fungal eucaryotes [Hilden and Johansson, 2004]. Cellulose molecules cannot be transported across the cell membrane by cellulolytic organisms due to its insoluble, complex nature and therefore most cellulases are secreted extracellulary (free enzyme system) with the exception of some cellodextrinases [Demain *et al*., 2005]. Some of the cellulolytic organisms are able to form hyphal extensions enabling them to reach otherwise inaccessible cellulose molecules [Lynd *et al*., 2002].

Most anaerobic cellulolytic bacteria on the other hand have maximised their potential to exploit the energy available in cellulose molecules by developing a multifaceted complexed cellulase system known as a cellulosome [Demain *et al*., 2005]. Cellulosomes are found on the cell walls of cellulolytic bacteria when grown on

cellulolytic material and consist of catalytic domains that are joined to non-catalytic domains by protein linkers onto the cell wall [Schwarz, 2001; Lynd *et al*., 2002]. These structures allow for enzyme activity close to the cell that permits optimum intramolecular synergism to take place. Cellulose hydrolysis products are also in closer proximity to the cell for rapid transportation of these cellodextrins into the cell.

As with hemicellulases, cellulases act synergistically to degrade the cellulose chain efficiently. Cellulases are all able to hydrolyse β-1,4-glycosidic linkages but differ in their ability to hydrolyse oligosaccharides of different lengths, their sites of attack and their processivity [Mielienz, 2001]. These enzymes are relatively slow catalysts and optimum activity requires synergistic action from a range of related enzymes to efficiently hydrolyse the cellulose chain.

2.3.2.1 Enzymes involved in the hydrolysis of cellulose

Two main groups of enzymes are responsible for the release of shorter cello-oligosaccharides from the cellulose chain [Lynd *et al*., 2002]:

1.) Endoglucanases (1,4-β-D-glucan-4-glucanohydrolases, E.C. 3.2.1.4) attack randomly inside the amorphous cellulose chain (Figure 5) creating cello-oligosaccharides with different lengths and newly produced free ends.
2.) Exoglucanases (cellobiohydrolases) (1,4-β-D-glucan cellobiohydrolase, E. C. 3.2.1.91) hydrolyse cellulose from the reducing and non-reducing ends of the cellulose chain and also acts on the free ends generated by endoglucanases.

Some enzyme functions seem to overlap since it has been found that certain endoglucanases possess the ability to attack the free ends of the cellulose chain while exoglucanases may have the ability to aid in the function of the endoglucanases [Hildèn and Johansson, 2004]. *Clostridium stercorarium* produces two enzymes, Avicelase I and Avicelase II that have been shown to possess a combination of endoglucanase and exoglucanase activity and cellodextrinohydrolase activity, respectively [Riedel *et al*., 1997].

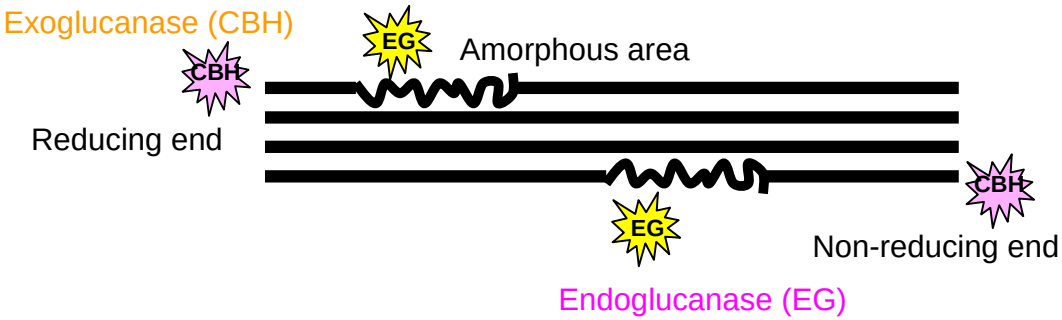


Figure 5. Actions of the enzymes involved in hydrolysing the cellulose chain to shorter cello-oligosaccharides

Exoglucanases, including cellobiohydrolases, are able to degrade crystalline cellulose and their efficiency is enhanced by their ability to remain bound to the substrate while the products from the cellulose chains are released sequentially [Fujita *et al*., 2002; Hildèn and Johansson, 2004]. Cellobiohydrolases have been the focus for cellulases engineering since they constitute 60 – 80 % of natural cellulase systems [Gray *et al*., 2006]. Endoglucanases function at internal sites of the cellulose chains where they can cut amorphous and substituted celluloses randomly. Both of these enzymes release glucose, cellobiose and longer cello-oligosaccharides that are available for the organism to utilize or subject to further degradation.

Different combinations of these groups of cellulases have been described to have a higher cumulative activity than the individual enzymes on their own. This phenomena is referred to as synergism and can be found between (i) endoglucanases and exoglucanases, (ii) different acting exoglucanases and (iii) exoglucanases and β-glucosidases [Lynd *et al*., 2002; Zhang and Lynd, 2004]. These enzymes can also form a complex with the substrate that enables the enzymes to be more stable and thus function optimally [Maheshwari *et al*., 2000].

2.3.2.2. Enzymes involved in the hydrolysis of shorter cello-oligosaccharides

A variety of cellodextrinases have been identified that hydrolyse cellobiose and longer soluble cello-oligosaccharides to form glucose [Maheshwari *et al*., 2000]. These enzymes are considered part of the cellulase system because they stimulate cellulose degradation although they have no direct effect on the cellulose molecule itself. It has been found that cellulolytic organisms are diverse in their action to metabolise cellobiose and cellodextrins [Lynd *et al*., 2002]. The presence of intracellular cellobiose and cellodextrin phosphorylase together with extracellular cellodextrinase and intracellular β-glucosidase suggest that the diversity of enzymes are important for degradation of cellodextrins (see section 2.4 Bioenergetics of cellodextrin degradation).

The best studied cellodextrinases are the β-glucosidases (β-glucoside glucohydrolyase, E. C. 3.2.1.21) [Lynd *et al*., 2002]. These enzymes are able to cleave the β-glucosidic linkages in several glycoconjugates and are important in a wide range of biological processes including fruit ripening, cell wall synthesis etc. [Roy *et al*., 2005]. In industry, β-glucosidases are used for aroma enhancement (by releasing volatile terpenes) during the production of wine and for the hydrolysis of bitter compounds in juice as well as juice clarification [Hernandez *et al*., 2003; Spagna *et al*., 2002; Rajoka *et al*., 2004].

Cellulolytic microorganisms use this enzyme to cleave the β-1,4-glycosidic linkages in shorter cello-oligosaccharides to release glucose [Freer and Greene, 1990]. The simple sugars released from cellulose degradation are used as carbon and energy sources by the organisms expressing the cellulases as well as other organisms present in the environment [Pèrez *et al*., 2002]. In the case where glucose is not immediately used, it results in product inhibition of the β-glucosidase while ethanol can cause activation of the enzyme [Freer and Greene, 1990; Spagna *et al* 2002]. Inhibition of β-glucosidase activity results in the accumulation of cellobiose which in turn inhibits the exoglucanase activity. Therefore apart from its ability to form glucose from cellobiose, β-glucosidase also reduces cellobiose inhibition, enabling the other cellulases to perform more efficiently.

2.3.2.3 Phosphorolytic cellulose degradation enzymes

In anaerobic bacteria, the breakdown products of cellulose are predominantly cellobiose and cellodextrins [Demain *et al*., 2005]. Cellodextrins can only be utilized by a limited number of organisms ensuring the availability of these sugars for intracellular consumption by these cellulolytic organisms [Liu *et al*., 1998]. The cello-oligosaccharides can either be split by cellodextrin phosphorylase (2.4.1.49), cellobiose phosphorylase (2.4.1.20) or β-glucosidase [Tanaka *et al*., 1994]. Whereas the previously described cellulases are all hydrolytic enzymes that lead to the release of simple sugars and water, the phosphorolytic enzymes release the sugars and simultaneously phosphorylate one of the sugars produced [Kitaoka and Hayashi, 2002]. Cellulolytic species that produce a cellobiose phosphorylase prefer cellobiose to glucose as an energy source [Ng and Zeikus, 1982]. Cellodextrin and cellobiose phosphorylase are part of the family 36 glycosyl transferase enzyme family [Nidetzky *et al*., 2000]. They are also capable of catalysing the inverse reaction where cellodextrins are synthesized from glucose and cellobiose (see Formula 1 and 2) [Alexander, 1961].

Figure 6 shows the addition of an inorganic phosphate group to one of the glucose molecules released from the reaction mechanism of the cellobiose phosphorylase. This reaction could also be written as follow:

$$G_2 + P_i \xleftrightarrow{CbP} G + G\text{-}1\text{-}P \qquad \text{(Formula 1)}$$

In the case of a cellodextrinase the reaction can be written as:

$$G_n + P_i \xleftrightarrow{CdP} G_{n-1} + G1P \qquad \text{(Formula 2)}$$

G_n refers to the cellodextrin with n amount of glucose residues, P_i denotes inorganic phosphate and G-1-P is the phosphorylated product [Zhang and Lynd, 2004].

2.3.2.4 Reaction sequence of cellobiose phosphorylase

Cellobiose phosphorylases are very specific with regards to cleaving and synthesizing glycosidic bonds but their specificity towards the reducing sugar that acts as a glucocyl receptor in the inverse reaction are not as strict [Nidetzky *et al*., 2000].

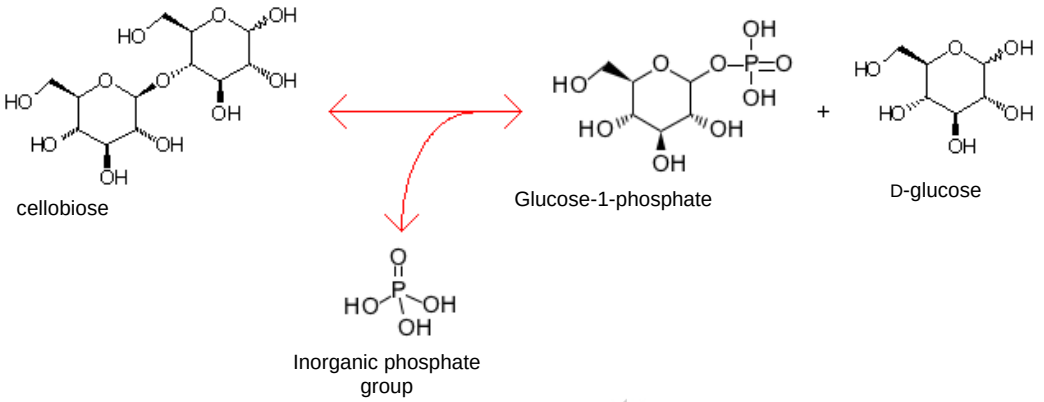


Figure 6. The reaction sequence of cellobiose phosphorylase for the release of glucose and glucose-1-phosphate from cellobiose [http://www.genome.jp/ dbget-bin/www_bget?rn+R00952]

Other substrates such as D-mannose, D-arabinose, D-xylose, L-galactose, isomaltose and melibiose can act as glucocyl receptor and allows for the synthesis of other compounds apart from cello-oligosaccharides [Alexander, 1968; Hidaka *et al*., 2006].

2.3.2.5 Cellobiose phosphorylase from *Clostridium stercorarium*

The cellobiose phosphorylase (cepA) from *C. stercorarium* was produced heterologously in *S. cerevisiae* in this study (Chapter 3) and has a theoretical molecular mass of 93 kDa [Reichenbecher *et al*., 1997]. It is proposed to exist monomerically and phosphorylate cellobiose exclusively. Maximum activity of this enzyme was observed at 65°C (see Table 1) and pH 6-7, and the enzyme was stable for 42 h at 60°C. It was found that the enzyme functioned optimally in the presence of 20 mM inorganic phosphate.

Table 1. Organisms reported to produce a cellobiose phosphorylase that shares significant protein homology with the cellobiose phosphorylase from *C. stercorarium*

Organism	Percentage protein homology to CepA from *C. stercorarium*	Optimum temperature	Reference
C. thermocellum	72 %	60°C	Alexander, 1961; Tanaka *et al*., 1994
Thermotoga maritima	72 %	80°C	Rajashekhara *et al*., 2002
Thermotoga neapolitana	71 %	85°C	Yernool *et al*., 2000
Saccharophagus degradans	66 %	unknown	Taylor *et al*., 2006
Cellulomonas uda	62 %	30°C	Nidetzky *et al*., 2004
Cellvibrio gilvus	61 %	37°C	Liu *et al*., 1998

2.4 BIOENERGETICS OF CELLODEXTRIN UTILIZATION

Anaerobic cellulolytic organisms are specifically challenged because basic cellular functions as well as cellulase production need to be maintained with the limited number of ATP's available from anaerobic catabolism [Zhang and Lynd, 2005]. Large amounts of cellulases need to be produced to make up for the slow reaction rates of these enzymes and hence large amounts of ATP are needed for their production. The phosphorolytic cellulases are thought to contribute in the energy efficient catabolism of cellobiose and longer cellodextrins in the cytoplasm.

2.4.1 Hydrolytic cleavage vs. phosphorolytic cleavage

Theoretically, there is a greater bioenergetic advantage from phosphorolytic cleavage than from hydrolytic cleavage [Lynd *et al*., 2002]. In the case of β-glucosidase, where two glucose molecules are released from cellobiose, both of these glucose units join the first step of glycolysis where they are phosphorylated to glucose-6-phophate using an ATP molecule by means of the enzyme hexokinase [Lodish *et al*., 2000]. This is done to ensure that glucose resides inside the cell and is not transported back to the extracellular environment. Conversely, cellobiose phosphorylase leads to the release of a glucose molecule as well as a glucose-1-phosphate molecule (see Figure 5). Glucose-1-phosphate is converted into glucose-6-phosphate and this shift in the phosphoryl group is catalysed by phosphoglucomutase (PGM) and does not require energy in the form of ATP [Berg *et al*., 2002]. It has been reported that PGM activity becomes limiting when carbon flow increases and the accumulation of G-1-P can lead to rerouting of metabolism, such as exopolysaccharide biosynthesis and glycogen production as seen in *C. thermocellum* [Desvaux, 2005].

During glycolysis, four ADP's are converted to ATP's during the conversion of one glucose molecule to two pyruvate molecules [Lodish *et al*., 2000]. The net energy yield is however only two ATP's since two of the ATP's formed are consumed during synthesis of fructose-1,6-diphosphate of this pathway, one of which is the first step of glucose activation.

In a fermentative organism the products from cellobiose hydrolysed with a β-glucosidase will be:

$$C_{12}H_{22}O_{11} \longrightarrow 2C_2H_6O + 2CO_2 + \mathbf{4}ATP \quad \text{(Formula 3)}$$

Thus from every cellobiose molecule a total of 4 ATP's are formed. In a fermentative organism harbouring a cellobiose phosphorylase, cellobiose will be converted to:

$$C_{12}H_{22}O_{11} \longrightarrow 2C_2H_6O + 2CO_2 + \mathbf{5}ATP \quad \text{(Formula 4)}$$

In this case a theoretical yield of 5 ATP's is expected since one of the glucose molecules entering the glycolysis pathway is already phosphorylated and consequently there are more ATP's available for other cellular functions such as biomass production. In the ruminal bacterium, *Ruminococcus albus*, it was found that the rate of cellobiose phosphorolysis exceeded the rate of hydrolysis by nine- fold [Lou *et al*., 1997].

2.4.2 C. thermocellum *as model organism for cellodextrin hydrolysis*

The thermophilic, anaerobic bacteria *C. thermocellum* was first found to produce a cellobiose phosphorylase [Alexander, 1961]. Numerous organisms have since been identified that express this enzyme (see Table 1). *C. thermocellum* produces an intracellular β-glucosidase as well as an intracellular cellobiose phosphorylase and cellodextrin phosphorylase [Zhang and Lynd, 2004]. Zhang and Lynd (2004) showed that phosphorolytic cleavage rates exceed hydrolytic cleavage rates by more than twenty-fold. By measuring the G-1-P formation relative to the total amount of glucose formed, they concluded that β-glucosidase has a limited contribution to glucose formation from cellobiose and that it may be associated with non-fermentative functions. This differentiation in favour of phosphorolysis in an energetically challenged environment confirms the fact that phosphorolytic cleavage is energetically more advantageous for the organism than hydrolytic cleavage.

In comparison with aerobic cellulolytic organisms such as *Trichoderma reesei,* whose primary product after cellulose hydrolysis is cellobiose, *C. thermocellum* assimilates cellodextrins with a polymerisation degree of four or more [Zhang and Lynd, 2005; Demain *et al*., 2005]. Cellodextrin transport in anaerobic cellulolytic bacteria occurs by means of the adenosine-binding cassette (ABC) transport system that requires one ATP for every molecule transported. For an anaerobic organism, the transport of cellobiose could become an energetically expensive way of living and they avoid this situation by transporting cellodextrins with a higher degree of polymerisation [Zhang and Lynd, 2005]. This net gain in ATP synthesis was seen in the cell yields of *C. thermocellum*

increasing in correlation with the increasing degree of polymerisation of the soluble cellodextrin on which it was grown [Strobel *et al*., 1995].

2.5 ETHANOL PRODUCTION FROM LIGNOCELLULOSIC MATERIAL

2.5.1 Ethanol as fuel replacement

With the unavoidable depletion of the earth's petroleum supply, there is an urgent need to exploit alternative sources of energy to decrease the world's dependence on non-renewable resources [Gray *et al*., 2006]. Bioethanol has a number of environmental advantages over currently used fossil fuels, including the recirculation of carbon in the atmosphere and lower gas emissions [Golias *et al*., 2002; Galbe and Zacchi, 2002]. Ethanol has a higher octane rating than gasoline and is more effective during burning in the engine although its fuel value is lower than that of hydrocarbons [Demain *et al*., 2005]. Furthermore the production of domestically produced transport fuels is important to become less dependent on the Oil Producing and Exporting Countries (OPEC) [Mielenz, 2001].

Pure ethanol is a clear, volatile liquid which is flammable, toxic, boils at 78.4℃ and is soluble in water and most organic liquids [Kosaric *et al*., 2001]. The major use of ethanol is as an oxygenated fuel additive and this mixture is known as gasohol [Kosaric *et al*., 2001; Galbe and Zacchi, 2002]. All cars with a catalyst can make use of this blend and ethanol can also replace diesel although an emulsifier is needed [Galbe *et al*., 2002]. Other uses for ethanol includes acting as a solvent, extractant, antifreeze and as a intermediate feedstock for the production of numerous organic chemicals [Kosaric *et al*., 2001]

In the US, the Energy Policy Act of 2005 states that by 2012 the oil industry is required to blend 28.4 billion L of renewable fuels into gasoline [Gray *et al*., 2006]. In South Africa a commission has been launched that stated by the year 2010 1.1 billion L of ethanol from multiple feedstocks should be produced [Nassiep, K.M., 2006]. To reach the goals set in the US and also in other countries, dedicated feedstocks are needed for

ethanol production [Gray *et al*., 2006]. To produce economically viable ethanol for commercial use in the required quantities, the cost of the product should dramatically decrease. This will be achieved by using a cost-effective and abundant substrate, reducing the cost of the enzymes by a combination of protein engineering process development and the exploitation of by-products formed during the process (collectively known as biocommodity engineering) [Lynd *et al*., 1999; Mosier *et al*., 2005; Gray *et al*., 2006].

2.5.2 Currently used substrates for ethanol production

During the past two decades fuel ethanol has been produced from corn and sugarcane while current technologies work towards the production of ethanol from promising non-food-plant resources, also referred to as biomass or lignocellulosic material [Mielenz, 2001; Palmarola-Adrados *et al*., 2005]. The production of ethanol from corn starch may not be practical because of the vast amount of agricultural land needed for dedicated crops [Sun and Cheng, 2002]. Molasses is the most widely used sugar for ethanol fermentation and is produced during the refinement of sugarcane [Lin and Tanaka, 2006]. However, molasses needs to be sterilized beforehand to stop naturally occurring microorganisms from interfering with the fermentation process.

Plant biomass is the only viable sustainable source for fuel alternatives and other compounds [Lynd *et al*., 1999]. Furthermore, the products are biodegradable and non-hazardous. It has been estimated that the theoretical amount of ethanol that can be produced from cellulose is an order of magnitude larger than from corn [Demain *et al* 2005]. Figure 7 shows the proposed ethanol yield that could be obtained when all the sugars present in typical plant biomass are fermented.

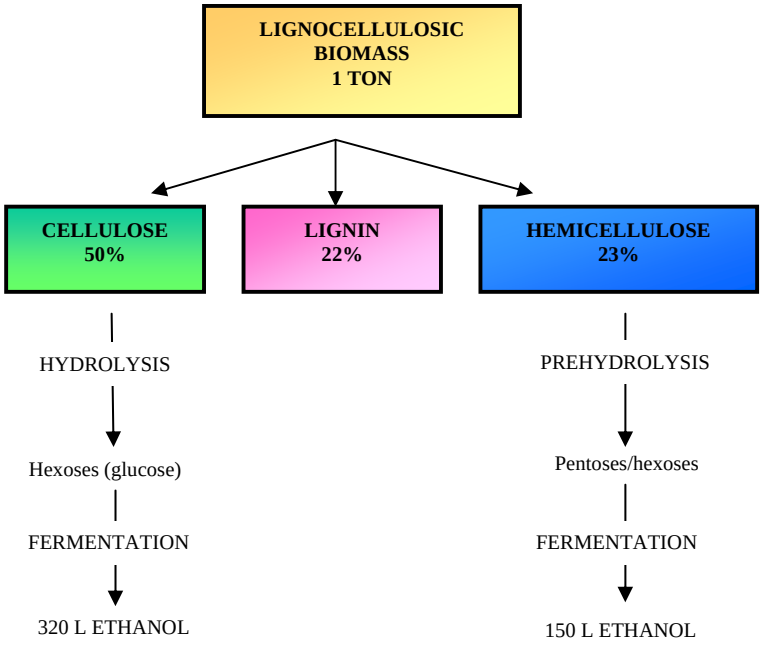


Figure 7. The different components of lignocellulose and the proposed ethanol yield from cellulose and hemicellulose [Kosaric *et al*., 2001].

Corn (maize) kernels, consists mainly out of starch (~70%), a homopolymer comprising of glucose linked with α-1,4 and α-1,6 glycosidic linkages whereas cellulose is composed exclusively of β-1,4 glycosidic linkages [Gray *et al*., 2006]. The structure of starch and its ability to be gelatinized during high-temperature processing makes it easier to degrade enzymatically by amylases and therefore the process cost is less expensive than ethanol production from cellulose [Kosaric *et al*., 2001]. Inexpensive, efficient cellulases are needed to hydrolyse cellulosic biomass to its component sugars and significant progress has been made in the past 50 years with the cellulases of *Trichoderma reesei* where a 20-fold cost reduction was reported recently [Gray *et al*., 2006]. During fermentation, the net reaction from one glucose molecule involves the production of two mol of ethanol, carbon dioxide and ATP respectively (reaction nr. 3 and 4, p. 16) [Kosaric *et al*., 2001]. The theoretical ethanol yield is 0.51 g per gram of glucose but due to cell maintenance and other products formed, only 90 – 95% of this value is obtained in practice.

2.5.3 Pretreatment of biomass substrates

As mentioned earlier, the presence of lignin greatly hinders the ability of the cellulases and hemicellulases to attack their substrates and pretreatment of the substrate is thus required to alter the structure and make it more accessible to the enzymes for rapid hydrolysis and greater yields [Mosier *et al*. 2005]. This step has been viewed as one of the most expensive processing steps in the conversion of biomass to ethanol. Effective pretreatment are measured by the following criteria [Mosier *et al* 2005]:

1.) Avoiding the need for reducing the size of the substrate molecules.
2.) Maintaining the pentose portion of the substrate.
3.) Limiting formation of inhibitory by-products.
4.) Minimizing energy load and cost.

Pretreatment methods can be classified as physical, chemical or a combination of these two methods. Mechanical blending, steam explosion and hydrothermolysis are used in the physical pretreatment process to make material handling easier [Mosier *et al*., 2005]. Chemical treatment involves acids, bases and other cellulose solvents that promote hydrolysis and improve the yield of glucose recovery. The highest yield of cellulose and hemicellulose obtained after one-step pretreatment was 75% acquired with dilute acid (H_2SO_4) and high temperature treatment [Galbe and Zacchi, 2002].

The formation of degradation products such as phenols, furans and carboxylic acids have an inhibitory effect on fermentation that needs to be reduced for this process to be economically feasible [Klinke *et al*., 2004]. Removal of inhibitors can be done by extraction, ion exchange, active coal, overliming (addition of $Ca(OH)_2$) or laccase and peroxidase treatment. Effective enzymatic degradation may decrease the need for pretreatment of the lignocellulosic materials and subsequent problems arising with the removal of inhibitory compounds [Galbe and Zacchi, 2002]. Specific pretreatment methodology is beyond the scope of this review but was recently reviewed by Mosier *et al*., [2005] and Sun and Cheng, [2002].

2.5.4 Saccharomyces cerevisiae *as an ideal ethanol producer*

Organisms such as *Escherichia coli, Zymomonas mobilis* and C*lostridium* species have been used in the production of ethanol with each organism having its own characteristics and advantages for sustained growth and ethanol production during fermentation [Kosaric *et al*., 2001; Demain *et al*., 2005]. Some fermenting bacteria display high ethanol productivity; however their inability to perform under high ethanol concentrations as well as the need to sterilize the culture medium complicates their use in the fermentation industry [Kosaric *et al*., 2001].

The production of ethanol from sugar substrates has been commercially dominated by the yeast *S. cerevisiae* based on its ease of handling and advantages in terms of:

1.) growth at higher temperatures (up to 35℃);
2.) high ethanol yield per unit substrate;
3.) ethanol tolerance;
4.) stability under fermentation conditions;
5.) tolerance to low pH.

S. cerevisiae compares favourably with other fermentative organisms regarding these conditions [Kosaric *et al*., 2001; Klinke *et al*., 2004; Gray *et al*., 2006]. This organism has proven to be robust and suitable for the fermentation of pre-hydrolysed lignocellulosic biomass although its inability to ferment pentoses is an obstacle yet to be fully overcome [Galbe and Zacchi, 2002]. *S. cerevisiae* is also able to ferment a variety of hexoses and efficiently produce ethanol at low pH values and temperatures ranging from 28 – 35℃ [Kosaric *et al*., 2001].

2.5.5 Ethanol production processes

In current processes, lignin needs to be dissociated from the biomass materials before hydrolysis of the cellulosic and hemicellulosic sugar polymers can take place [Lin and Tanaka, 2006]. After pretreatment, four biologically mediated events occur during the process of enzymatic degradation of the lignocellulosic substrate: enzyme production, substrate hydrolysis, hexose fermentation and pentose fermentation [Lynd *et al*., 1999].

The different strategies that are presented in Figure 8 are currently being used in the industries or are proposed as alternatives to existing processes.

Separate **h**ydrolysis and **f**ermentation (SHF) involves four distinct steps and enzymatic hydrolysis is performed separately from the fermentation step [Mosier *et al*., 2005]. As depicted in Figure 8, **S**imultaneous **s**accharification and **f**ermentation (SSF) involves the hydrolysis of the substrate (cellulose or hemicellulose) and simultaneous fermentation of the hexoses that is carried out in the presence of a fermentative organism [Sun *et al*., 2002]. The microorganisms used in this case are usually *T. reesei* for the production of cellulases and *S. cerevisiae* for the fermentation of the hexose sugars leading to limited end-product inhibition. The major disadvantage of such a system are the inability of *T. reesei* to grow in the anaerobic environment that is needed for effective ethanol production by *S. cerevisiae* [Lin and Tanaka, 2006]. Furthermore the use of two different organisms leads to incompatible temperatures for hydrolysis and fermentation as well as decreased microbial viability in the presence of another organism.

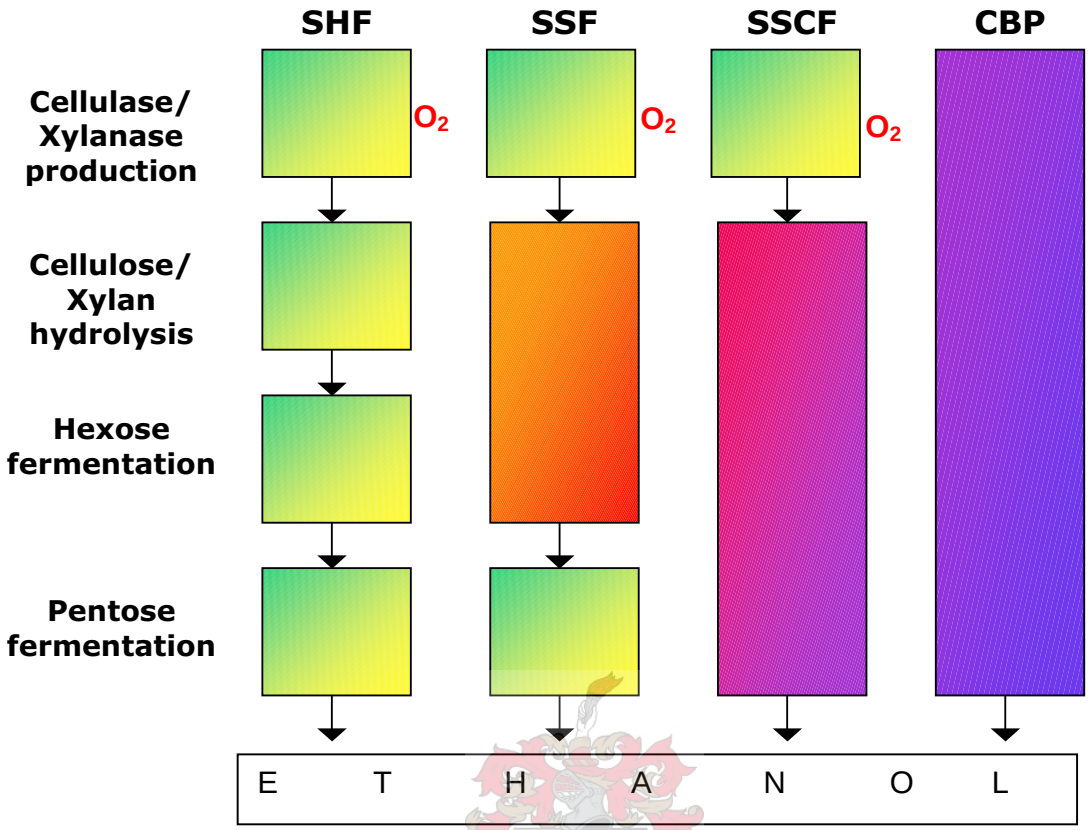


Figure 8. Ethanol production in current processing plants as well as proposed strategies for the enhancement of existing processes. The different processing methods refers to separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP). Note that currently, cellulose and hemicellulose hydrolysis and fermentation takes place separately but the processes stay the same for both substrates. Each box in the diagram represents a different bioreactor.

SSCF (**s**imultaneous **s**accharification and **c**o-**f**ermentation) refers to simultaneous hydrolysis of cellulose and hemicellulose and the subsequent fermentation of the released hexoses and pentoses in one bioreactor [Mosier *et al*., 2005]. SSF and SSCF are preferred above SHF, since the reactions are done in the same bioreactor, resulting in lower costs. A disadvantage for SSF and SSCF is that it will be difficult to recycle the microorganisms since it will be mixed with the residues left behind after hydrolysis of the substrate [Galbe and Zacchi., 2002].

These three processes (SHF, SSF and SSCF) depend on the production of enzymes in a separate unit under aerobic conditions while the rest of the process is anaerobic [Lynd *et al*., 1999]. Aerobic conditions are preferable because of the higher ATP yields and therefore potentially higher enzyme yields, though the dedicated production of these enzymes are costly and require high enzyme yields and specificity [Lynd *et al*., 2002].

The last process, CBP (**c**onsolidated **b**io**p**rocessing) is proposed as a logical alternative to SSCF. It differs from all of the other processes mentioned because it does not have a separate step where enzyme production takes place [Lynd *et al*., 2005]. Instead, it proposes that enzyme production takes place in the same bioreactor as substrate hydrolysis and fermentation and that all of this is carried out by a single organism to reduce the cost that arises from the use of pure enzymes. The challenge is to develop a cellulolytic organism that also efficiently ferments pentoses (and other sugars apart from glucose) and enhance the fermentation of lignocellulosic biomass to ethanol in one step under anaerobic conditions [Lynd *et al*., 1999]. Once these obstacles are overcome, CBP presents the potential for reduced cost and higher efficiency than any of the other processes.

One approach is to enhance native cellulolytic organisms for improved ethanol production and tolerance so that industrial requirements are met [Lynd *et al*., 2005]. These organisms' (including many anaerobic species) cellulase systems are thoroughly developed but they are often difficult to culture and research are limited due to inadequate gene transferring methods.

2.6 S*. CEREVISIAE* AS A RECOMBINANT HOST FOR CELLULOLYTIC ENZYMES

S. cerevisiae has contributed to both fundamental research as well as biotechnological application especially in the fermentation industry, because of (1) its success as an expression host for recombinant enzymes, (2) its ability to withstand high ethanol concentrations (3) near-theoretical ethanol yield on glucose (4) larger cell size that simplifies their separation after fermentation and (5) their resistance to viral infections

[Van Rensburg *et al*., 1998; Hahn-Hägerdal *et al*., 2001; Ryabova *et al*., 2003; Yu and Zhang, 2004]. Advantages of using *S. cerevisiae* as an expression host includes characteristics such as ease of genetic manipulation and successful production of heterologous proteins [Kauffman *et al*., 2002]. However, this yeast cannot utilize cellulose or shorter cellodextrins and needs to be genetically engineered to use this glucose-rich substrate. In the following sections some factors influencing recombinant protein expression in *S. cerevisiae* and the expression of native and recombinant β-glucosidases in this yeast will be discussed as they pertain to this study.

2.6.1 Factors influencing the expression of recombinant proteins in yeasts

The expression of a recombinant protein in a host is related to several genetic and physiological factors that will determine the successful production of such an enzyme. Limiting factors include promoter strength for efficient transcription, gene copy number, the codon bias in the host, processing and correct folding of the recombinant protein in the endoplasmic reticulum (ER), protein stability and protein allocation [Bennetzen and Hall, 1982; Cudna and Dickson, 2002; Hohenblum *et al*., 2004; Mattanovich *et al*., 2004]. A sensible manner to optimise gene expression would be to identify the major problem because all of these factors are interdependent.

2.6.1.1 Codon bias

The first of two very important factors during heterologous protein expression is that mRNA-coding genes show a statistically significant bias towards the choice of codons used to code for a particular amino acid [Bennetzen and Hall, 1982]. Though these preferences can differ from one gene to the other, it has been found that genes within the same genome also have related nucleotide-triplet preferences. Bennetzen and Hall (1982) first suggested the use of the codon bias index (CBI) that measures the extent to which a gene uses particular codons which appear to be most favourable in *S. cerevisiae.* In addition to that, Carbone *et al*. (2003) designed an algorithm to predict the codon adaptation index (CAI) that detect the most dominant codon bias in the

genome based on the “household” genes that are highly expressed. The CAI of a given gene shows a positive correlation with gene expression in the recombinant host as does codon usage frequencies against mRNA expression [Friberg *et al*., 2004].

There have been diverse hypotheses on why different organisms prefer different codons. These include mutation-selection between different synonymous codons in each organism, reduction in the amount of tRNA available to limit metabolical load, protein amino acid composition, protein structure and GC composition [Gustafsson *et al*., 2004; Wan *et al*., 2004]. However, it remains a fact that if a gene contains codons that do not correlate with the codons used in the expression host, it is unlikely that the heterologous protein will be expressed at high levels.

2.6.1.2 Protein folding and processing in ER

The metabolic burden that is placed on the cell by the overexpression of a heterologous protein leads to cell stress [Smith *et al*., 2002; Mattanovich *et al*., 2004]. Folding of proteins takes place in the ER lumen and when high levels of extra heterologous proteins are produced, misfolded or unfolded proteins often accumulate in the ER leading to the unfolded protein response (UPR) and the endoplasmic reticulum overload response (EOR) [Cudna and Dickson, 2003]. The UPR up-regulates chaperone and foldase expression to reduce misfolded proteins [Smith *et al*., 2005]. It also acts to decongest the ER by inhibition of protein synthesis and may furthermore influence the cell as a whole and lead to programmed cell death (apoptosis). Ultimately, the inhibition of protein synthesis results in the absence of necessary proteins for cell cycle progression that leads to cell arrest or eventually cause cell death. Kauffman *et al*. (2002) showed that the expression of a heterologous protein lead to an increase in intracellular protein as well as upregulation of the proteins involved in the UPR and a reduction of the specific growth rate. Smith *et al*. (2005) proposed an increase in the expression temperature for a thermophilic β-glucosidase enabling the enzyme to be less rigid and more susceptible to folding at higher temperatures. It was shown that secreted protein levels improved drastically at higher temperatures.

2.6.2 Endogenous β-glucosidase activity of S. cerevisiae *strains*

S. cerevisiae can grow on a wide variety of monosaccharides, disaccharides and even some trisaccharides, but it is generally known that this yeast cannot utilise lactose, melibiose and cellobiose [Yoon *et al*., 2003]. It has been reported that some *S. cerevisiae* strains do exhibit a β-glucosidase activity, especially those active on grapes for wine fermentation [Hernàndez *et al*., 2003; Spagna *et al*., 2002]. These enzymes are active on the chromogenic substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) but they function most likely in the release of volatile compounds for the enhancement of aromas during grape fermentation. Furthermore, even if present, the enzyme occurs in very low amounts and does not enable growth on cellobiose as carbon substrate.

However, one strain, *S. cerevisiae C,* has been identified that could sustain growth on cellobiose as sole carbon source [Kaplan, 1965]. It was concluded that there was an active β-glucosidase present on the yeast cell wall that could hydrolyse cellobiose (at very low rates) as well as an inactive intracellular β-glucosidase incapable of recognising cellobiose as a substrate [Kaplan and Tacreiter, 1966]. However, no literature was found following these two publications and no reports have since been published of a *S. cerevisiae* strain capable of utilising cellobiose as its sole carbon source.

2.6.3 Heterologous β-glucosidase expression in S. cerevisiae

Towards the development of CBP, it is important to create a strain that can efficiently ferment cellobiose (and longer cellodextrins) since it is the major soluble by-product of cellulose hydrolysis and greatly hinders the effectiveness of the cellulases if not removed from the culture broth. To achieve this goal β-glucosidases from various organisms have been expressed in *S. cerevisiae* and other yeasts and fermenting bacteria to hydrolyse cellobiose and thus relieve end-product inhibition [McBride *et al*., 2005; van Rooyen *et al*., 2005].

S. cerevisiae generally cannot utilize cellobiose as a carbon source and it was assumed that it therefore cannot transport this sugar across its membrane. Hence all of the research focussed on expressing β-glucosidases as extracellular proteins. Expression

of a β-glucosidase from *Cellulomonas biazotea* in *S. cerevisiae* showed that the yeast was able to produce the recombinant enzyme at levels 16X higher than supported by the native organism [Rajoka *et al*., 2003]. Skory *et al*. (1996) reported expression of the extracellular β-glucosidase of *Candida wickerhamii* in *S. cerevisiae* both extracellulary and intracellulary. The latter was found as mRNA transcripts but no protein could be detected and it was concluded that the intracellular expression of this gene led to rapid protein degradation.

Recently the expression of a β-glucosidase from *Saccharomycopsis fibuligera* that supported growth of *S. cerevisiae* on cellobiose that could be compared to growth on glucose under aerobic and anaerobic conditions was reported [McBride *et al*., 2005; van Rooyen *et al*., 2005]. These studies also showed that anchoring a β-glucosidase to the yeast cell wall instead of secreting it to the extracellular environment, could lead to better activity of the enzyme on cellobiose if the enzyme yields are not satisfactory. Recently, van Rooyen *et al*. expressed the β-glucosidase from *S. fibilugera* without a secretion signal that led to the intracellular expression of this enzyme in *S. cerevisiae* [Unpublished data]. Results have shown that this strain was able to sustain growth on cellobiose with rates similar to the strain secreting the β-glucosidase and that *S. cerevisiae* must therefore be able to transport cellobiose for intracellular consumption. As the present study deal with a cytoplasmic cellobiose phosphorylase expressed in *S. cerevisiae* and thus requires cellobiose to be internalised, the following section will explore sugar transport in yeast, focussing on disaccharide transport.

2.7 SUGAR TRANSPORT IN YEASTS

2.7.1 Cellobiose transport and utilization in yeasts

Little is known concerning the mechanism by which yeasts transport and metabolize cellobiose and longer cellodextrins. The presence of β-glucosidases in yeasts urged the exploration of the possibility of cellobiose utilization and transport. A list of all yeasts that have been investigated as such are presented in Table 2. The β-glucosidases of yeasts are generally located in the cytoplasm but no specific transporter has as yet been identified for cellobiose transport.

Freer *et al* (1990) have reported the first evidence of a yeast, *Clavispora lusitaniae*, that could transport cellobiose across its membrane and found that the transporter was glucose-repressed under aerobic conditions. The transport rate also appeared to be higher under anaerobic conditions than aerobic conditions. These authors suggested that the transport functioned by proton symport and they could deduce from the kinetic data that the yeast appeared to produce at least two cellobiose transporters. Furthermore, cellobiose transport was investigated in the filamentous fungi *T. reesei* using radiolabelled cellobiose and the transporter was found to be permeable for cellobiose, laminaribiose, sophorose, maltose, sucrose, xylobiose and longer cellodextrins (see Figure 9 for the structure of some of these sugars) [Kubicek *et al*., 1993]. The highest transport rates were observed at pH 5 and the data obtained suggested that

Table 2. Yeast species displaying β-glucosidase activity and their ability to utilize and transport cellobiose.

Yeast species	β-glucosidase location			Ability to hydrolyse cellobiose	Ability to transport cellobiose	Utilization of cellooligo-saccharides	Reference
	Extra-cellular	Cellwall bound	Intra-cellular				
Kluyveromyces dobzhanskii			√	√	unknown	X	Freer (1991)
Kluyveromyces lactis Y-1118			√	√	unknown	X	Freer (1991)
Kluyveromyces marxianus		√		√	X	Unknown	Rajoka *et al*. (2004)
Candida sake			√	√	unknown	Unknown	Gueguen *et al*. (2001)
Candida wickerhamii	√			√	X	G6	Skory and Freer (1995), Skory *et al*. (1996), Freer *et al*., (1993)
		√		√		unknown	
			√	X		unknown	
Candida peltata	√			√	X	G6	Saha *et al*. (1996
Candida pelliculosa		√		√	X	Unknown	Kochi and Toh-e (1986)
Candida guilliermondii			√	√	unknown	G6	Roth (1978)
Candida molischiana	unknown			√	unknown	G6	Freer (1991)
Clavispora lusitaniae			√	√	√	√	Freer and Greene (1990)
Saccharomycopsis fibuligera	√			√	X	G4	Machida *et al*. (1988)
	√			X		G4	
Saccharomyces cerevisiae C		√		√	X	X	Kaplan (1965)
			√	X			
Hansenula polymorpha	Unknown			√	unknown	unknown	Ryabova *et al*. (2003)
Rhodotorula minuta IFO879		√		√	X	G4	Onishi and Tanaka (1996) Freer (1991)
Dekkera polumorpha			√	√	unknown	G3	Freer (1991)
Dekkera intermedia			√	√	unknown	X	Gondè *et al*. (1984)
Brettanomyces anomalus			√	√	unknown	X	Freer (1991)
Brettanomyces claussenii			√	√	unknown	X	Freer (1991)
Debaryomyces castellii CBS 2923			√	√	unknown	X	Barnett (1992)
Debaryomyces polymorphus	unknown			√	unknown	Unknown	Freer (1991)
Pichia etchellsii		√		√	X	Unknown	Wallecha and Mishra (2003)
		√		√		Unknown	
Pichia guilliermondii			√	√	√	G3	Freer (1991)
Trichosporon cutaneum		√		√	unknown	unknown	Mörtberg and Neujahr (1986)
Torulopsis molischiana	√			√	unknown	G6	Gondè *et al*. (1984)

√ = positive; X = negative ; unknown = no information available

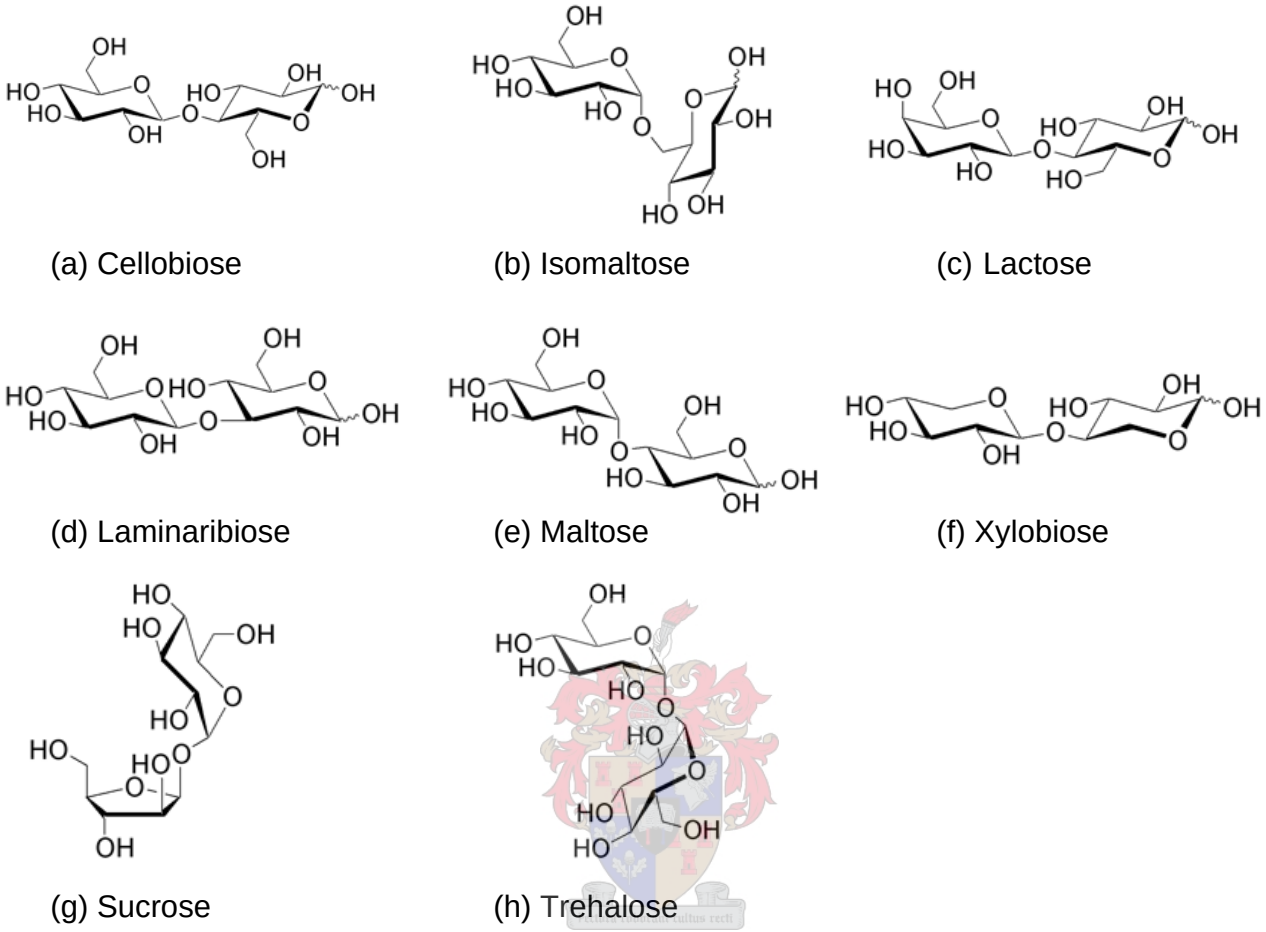


Figure. 9 Structures of sugars found to be transported by some species that also transported cellobiose. The arrangement of these sugars suggests that the transport mechanism is non-specific in its identification of the sugar that is being transported, although disaccharides with a glycosidic bond seem to be preferred [http://www.faculty.virginia.edu/mcgarveylab/Carbsyn/Carblist/html/disacch.html].

cellobiose transport was directly coupled to a proton gradient across the plasma membrane that required ATP hydrolysis.

In *Trichosporon cutaneum* it was shown that there was some interaction between the transport mechanisms of lactose, cellobiose and glucose since these molecules interfered with each other's transport [Mörtberg and Neujahr, 1986].

2.7.2 Disaccharide sugar transport and utilisation in S. cerevisiae

2.7.2.1 General disaccharide utilization of *S. cerevisiae* and other yeasts

A variety of sugars are utilised by yeasts and they can be characterised based on the sugars they prefer [Flores *et al*., 2000]. They all have the common theme of the conversion of the sugar to glucose-6-phosphate and then to pyruvate through the glycolytic pathway. Depending on the yeast species and the structure of the sugar, disaccharides are hydrolysed outside the cell, in the periplasmic space or intracellularly after transport of the disaccharide. Of the ~6000 genes found in *S. cerevisiae*, 271 encode for membrane transporters or are deemed putative transporters because of a high sequence similarity [Day *et al*., 2002].

Sucrose is hydrolysed by the enzyme invertase, which is responsible for the external hydrolysis of this sugar to release the glucose and fructose for subsequent uptake [Flores *et al*., 2000]. However, it was discovered that sucrose can also enter the cell by making use of the general α-glucoside transporter, AGT1 and the MAL2 transporter also responsible for transporting maltose across the *S. cerevisiae* cell membrane. [Batista *et al*., 2004; Stambuk *et al*., 2000]. Furthermore melibiose is a disaccharide that is utilised by some strains of *S. cerevisiae* that harbour the melibiase enzyme, though is generally absent in laboratory strains [Vincent, 1999]. The disaccharides are hydrolyzed outside the cell and the products are transported inside the cell and no transporters exist for the transfer of these disaccharides inside the yeast cell.

An interesting phenomenon that occurs in yeasts utilising carbohydrates is called the Kluyver effect [Fukuhara, 2003]. This involves the assimilation of certain mono- and oligosaccharides aerobically but not anaerobically. A possible explanation of this phenomenon is that optimal transport of the sugars does not occur because of the lack of ATP in anaerobic conditions and the inability of the ATPase enzyme to function optimally (section 2.7.2.2). *S. cerevisiae* is Kluyver effect negative for most sugars except for trehalose that is mainly transported by the AGT1 transporter (general α-

glucoside transporter). This raises the question if all sugars transported by AGT1 would be subject to the Kluyver effect in *S. cerevisiae.*

2.7.2.2 Transporters in *S. cerevisiae*

S. cerevisiae uses several mechanisms to transport metabolites through the plasma membrane (Figure 10).

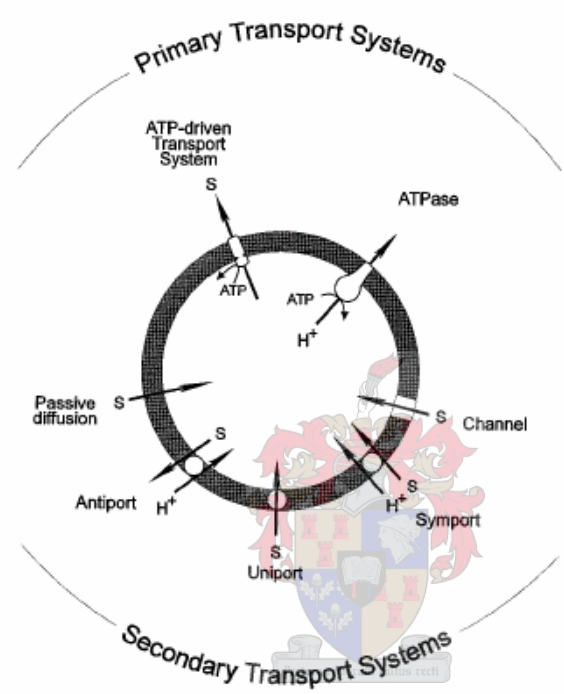


Figure 10. Transport mechanisms present in *S. cerevisiae.* Disaccharide transport are mainly allowed using the H+-symport system by the proton gradient established by the ATPase [van der Rest *et al*., 1995].

S. cerevisiae mainly transports its monomeric sugars using facilitated diffusion, while disaccharide transport primarily occurs via the proton-symport mechanism [van der Rest *et al*., 1995]. The proton is symported via the plasma membrane with an ATPase and thus the transport of the sugar molecule costs the cell one ATP. ATPase is estimated to consume about 15% of the ATP produced during yeast growth to create the proton gradient.

The plasma-membrane H^+-ATPase is essential for maintenance of intracellular pH and transport of essential nutrients [Flores *et al*., 2000]. This membrane protein is activated by the presence of glucose, likely due to a phosphorylation of the glucose molecule. The proton-gradient transporters of *S. cerevisiae* have been found to be unidirectional and a possible explanation for this is that accumulation of solute intracellulary inhibits further uptake (*trans*-inhibition) [Harma *et al*., 2001].

2.7.2.3 Maltose utilisation and transport systems

Maltose is the preferred disaccharide of yeasts and is consumed after hexoses such as glucose and fructose [Dietvorst *et al*., 2005; Hazell and Attfield, 1999]. Maltose is transported via a maltose-proton symport mechanism and is the rate-limiting step during maltose fermentation [Cheng and Michels, 1989; Jansen *et al*., 2004].

Several maltose permeases have been identified in *S. cerevisiae* namely *MAL11* to *MAL41, MAL61, AGT1* and two putative transporters *MPH2* and *MPH3* [Stambuk and Araujo, 2001; Day *et al*., 2002]. The first three have been thoroughly investigated [Cheng and Michels, 1989; Harma *et al*., 2001] Glucose acts as a catabolite repressor since the addition of this sugar leads to the termination of synthesis as well as loss of existing maltose permeases [Cheng and Michels., 1989].

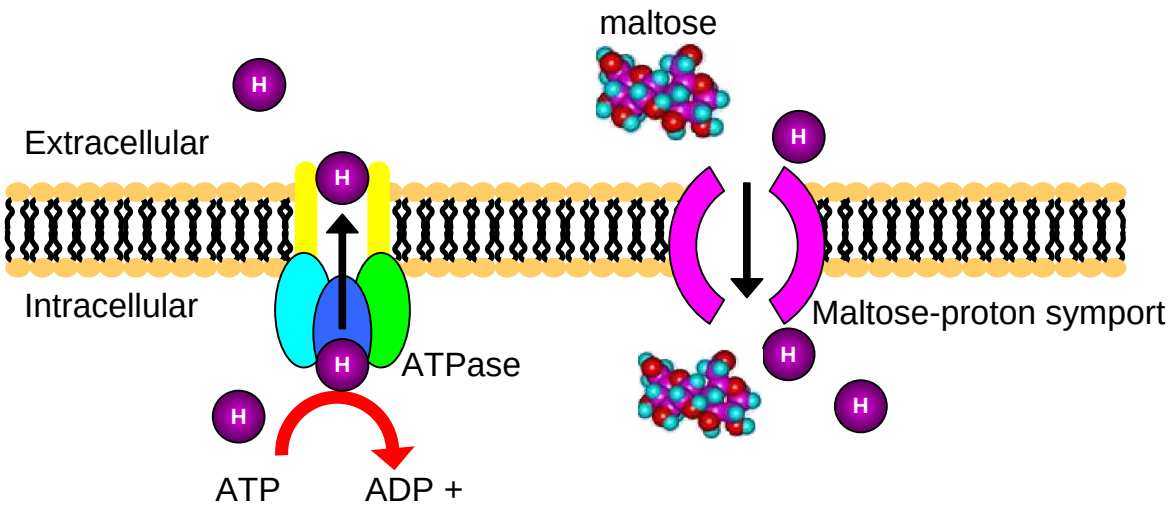


Figure 11. Transport mechanism of the yeast *S. cerevisiae* for maltose. An ATPase enzyme, located in the membrane, transports a proton to the extracellular environment at the cost of one ATP and this proton gradient that are created enables maltose to enter the cell via a proton symport mechanism.

AGT1 is a general α-glucoside transporter that has a high affinity for trehalose, sucrose and the chromogenic substrate *4*-nitrophenyl α-D-glucopyranoside, a low affinity for maltose and maltotriose and a very low affinity for α-methyl-glucoside [Plourde-Owobi *et al*., 1999; Stambuk and Araujo, 2001; Jules *et al*., 2004; Dietvorst *et al*., 2005]. Isomaltose, melezitose, palatinose and turanose have also been found to be transported by this general α-glucoside transporter [Day *et al*., 2002].

2.7.2.4 Cellobiose transport via the maltose permeases?

Apart from the α-glucoside transporters, little is known about other specific transport mechanisms existing for disaccharides in S*. cerevisiae.* However, a review of all literature suggest that the process used for cellobiose transport in yeast is similar than that of maltose. In unpublished results, van Rooyen *et al*. have shown that the transport of cellobiose of a *S. cerevisiae* strain harbouring an intracellular β-glucosidase was enhanced with the addition of maltose. Furthermore, RNA slot blots confirmed that the

mRNA levels of the AGT1 transporter as well as the MAL61 transporter of this strain was upregulated when grown on cellobiose [Unpublished data].

The resemblance of these two transport processes can be found in [Freer and Greene, 1990; Kubicek *et al*., 1993]:

1.) the use of a proton-symport mechanism for intracellular transport
2.) glucose repression of the transporters [Freer and Greene, 1990]
3.) Optimum transport activity at pH 5
4.) several transporters required that act as high-affinity and low-affinity transporters
5.) AGT1's affinity for a wide variety of sugars

2.8 Literature Cited

Alexander, J. K. 1961. Characteristics of cellobiose phosphorylase. J Bacteriol **81**:903-910.

Alexander, J. K. 1968. Purification and specificity of cellobiose phosphorylase from *Clostridium thermocellum*. J Biol Chem **243**:2899-2904.

Barnett, J. A. 1992. Some controls on oligosaccharide utilization by yeasts: the physiological basis of the Kluyver effect. FEMS Microbiol Lett **79**:371-378.

Bennetzen, J. L. and B. D. Hall . 1982. Codon selection in yeast. J Biol Chem **257**:3026-3031.

Berlin, A., M. Balakshin, N. Gilkes, J. Kadla, V. Maximenko, S. Kubo, and J. Saddler. 2006. Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol.

Boudet, A. M., S. Kajita, J. Grima-Pettenati, and D. Goffner. 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci **8**:576-581.

Carbone, A., A. Zinovyev, and F. Kepes. 2003. Codon adaptation index as a measure of dominating codon bias. Bioinformatics **19**:2005-2015.

Cheng, Q. and C. A. Michels. 1989. The maltose permease encoded by the MAL61 gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics **123**:477-484.

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

Cudna, R. E. and A. J. Dickson. 2003. Endoplasmic reticulum signalling as a determinant of recombinant protein expression. Biotechnol Bioeng **81**:56-65.

Day, R. E., P. J. Rogers, I. W. Dawes, and V. J. Higgins. 2002. Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. Appl Environ Microbiol **68**:5326-5335.

Day, R. E., V. J. Higgins, P. J. Rogers, and I. W. Dawes. 2002. Characterization of the putative maltose transporters encoded by YDL247w and YJR160c. Yeast **19**:1015-1027.

Demain, A. L., M. Newcomb, and J. H. Wu. 2005. Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev **69**:124-154.

Desvaux, M. 2005. *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic clostridia. FEMS Microbiol Rev **29**:741-764.

Dietvorst, J., J. Londesborough, and H. Y. Steensma. 2005. Maltotriose utilization in lager yeast strains: MTT1 encodes a maltotriose transporter. Yeast **22**:775-788.

Flores, C. L., C. Rodriguez, T. Petit, and C. Gancedo. 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol Rev **24**:507-529.

Freer, S. N. and R. V. Greene. 1990. Transport of glucose and cellobiose by *Candida wickerhamii* and *Clavispora lusitaniae*. J Biol Chem **265**:12864-12868.

Freer, S. N. 1991. Fermentation and aerobic metabolism of cellodextrins by yeasts. Appl Environ. Microbiol **57**:655-659.

Freer, S. N. 1993. Kinetic characterization of a beta-glucosidase from a yeast, *Candida wickerhamii*. J Biol Chem **268**:9337-9342.

Friberg, M., P. von Rohr, and G. Gonnet. 2004. Limitations of codon adaptation index and other coding DNA-based features for prediction of protein expression in *Saccharomyces cerevisiae*. Yeast **21**:1083-1093.

Fujita, Y., S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda, and A. Kondo. 2002. Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Appl Environ Microbiol **68**:5136-5141.

Fukuhara, H. 2003. The Kluyver effect revisited. FEMS Yeast Res **3**:327-331.

Galbe, M. and G. Zacchi. 2002. A review of the production of ethanol from softwood. Appl Microbiol Biotechnol **59**:618-628.

Golias, H., G. J. Dumsday, G. A. Stanley, and N. B. Pamment. 2002. Evaluation of a recombinant *Klebsiella oxytoca* strain for ethanol production from cellulose by simultaneous saccharification and fermentation: comparison with native cellobiose-utilising yeast strains and performance in co-culture with thermotolerant yeast and *Zymomonas mobilis*. J Biotechnol **96**:155-168.

Gonde, P., B. Blondin, M. Leclerc, R. Ratomahenina, A. Arnaud, and P. Galzy. 1984. Fermentation of cellodextrins by different Yeast Strains. Appl Environ Microbiol **48**:265-269.

Gray, K. A., L. Zhao, and M. Emptage. 2006. Bioethanol. Curr Opin Chem Biol **10**:141-146.

Gueguen, Y., P. Chemardin, and A. Arnaud. 2001. Purification and characterization of an intracellular beta-glucosidase from a *Candida sake* strain isolated from fruit juices. Appl Biochem Biotechnol **95**:151-162.

Gustafsson, C., S. Govindarajan, and J. Minshull. 2004. Codon bias and heterologous protein expression. Trends Biotechnol **22**:346-353.

Hahn-Hagerdal, B., C. F. Wahlbom, M. Gardonyi, W. H. van Zyl, R. R. Cordero Otero, and L. J. Jonsson. 2001. Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. Adv Biochem Eng Biotechnol **73**:53-84.

Harjunpää, V. 1998. Enzymes hydrolysing wood polysaccharides. A progress curve study of oligosaccharide hydrolysis by two cellobiohydrolases and three beta-mannanases. 1998. Technical Research centre of Finland. Ref Type: Thesis/Dissertation

Harma, T., T. H. Brondijk, W. N. Konings, and B. Poolman. 2001. Regulation of maltose transport in *Saccharomyces cerevisiae*. Arch Microbiol **176**:96-105.

Hazell, B. and P. Attfield. 1999. Enhancement of maltose utilisation by *Saccharomyces cerevisiae* in medium containing fermentable hexoses. J Ind Microbiol Biotechnol **22**:627-632.

Hernandez, L. F., J. C. Espinosa, M. Fernandez-Gonzalez, and A. Briones. 2003. Beta-glucosidase activity in a *Saccharomyces cerevisiae* wine strain. Int J Food Microbiol **80**:171-176.

Hidaka, M., M. Kitaoka, K. Hayashi, T. Wakagi, H. Shoun, and S. Fushinobu. 2006. Structural dissection of the reaction mechanism of cellobiose phosphorylase. Biochem J **398**:37-43.

Hilden, L. and G. Johansson. 2004. Recent developments on cellulases and carbohydrate-binding modules with cellulose affinity. Biotechnol Lett **26**:1683-1693.

Hohenblum, H., B. Gasser, M. Maurer, N. Borth, and D. Mattanovich. 2004. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol Bioeng **85**:367-375.

Jansen, M. L., P. Daran-Lapujade, J. H. de Winde, M. D. Piper, and J. T. Pronk. 2004. Prolonged maltose-limited cultivation of *Saccharomyces cerevisiae* selects for cells with improved maltose affinity and hypersensitivity. Appl Environ Microbiol **70**:1956-1963.

Jules, M., V. Guillou, J. Francois, and J. L. Parrou. 2004. Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae*. Appl Environ. Microbiol **70**:2771-2778.

Kaplan, J. G. 1965. An inducible system for the hydrolysis and transport of beta-glucosides in yeast. 1. Characteristics of the beta-glucosidase activity of intact and of lysed cells. J Gen Physiol **48**:873-886.

Kaplan, J. G. and W. Tacreiter . 1966. The beta-glucosidase of the yeast cell surface. J Gen Physiol **50**:9-24.

Kauffman, K. J., E. M. Pridgen, F. J. Doyle, III, P. S. Dhurjati, and A. S. Robinson. 2002. Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in *Saccharomyces cerevisiae*. Biotechnol Prog **18**:942-950.

Kitaoka, M. and K. Hayashi. 2002. Carbohydrate-processing phosphorolytic enzymes. Trends Glycosc Glygotech **14**:35-50.

Klinke, H. B., A. B. Thomsen, and B. K. Ahring. 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol **66**:10-26.

Kohchi, C. and Toh-e A. 1986. Cloning of *Candida pelliculosa* beta-glucosidase gene and its expression in *Saccharomyces cerevisiae*. Mol Gen Genet **203**:89-94.

Kosaric, N., H. J. Pieper, and F. Vardar-Sukan. 2001. The Biotechnology of Ethanol. Classical and Future Applications. Wiley-VCH, Weinheim.

Kubicek, C. P., R. Messner, F. Gruber, M. Mandels, and E. M. Kubicek-Pranz. 1993. Triggering of cellulase biosynthesis by cellulose in *Trichoderma reesei*. Involvement of a constitutive, sophorose-inducible, glucose-inhibited beta-diglucoside permease. J Biol Chem **268**:19364-19368.

Lin, Y. and S. Tanaka. 2006. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol **69**:627-642.

Liu, A., H. Tomita, H. Li, H. Miyaki, C. Aoyagi, S. Kaneko, and K. Hayashi. 1998. Cloning, sequencing and expression of the cellobiose phosphorylase gene of *Cellvibrio gilvus*. Jf Ferm Bioeng **85**:511-513.

Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. E. Darnell. 2000. Molecular Cell Biology. WH Freeman and Company, New York.

Lou, J., K. A. Dawson, and H. J. Strobel. 1997. Cellobiose and cellodextrin metabolism by the ruminal bacterium *Ruminococcus albus*. Curr. Microbiol **35**:221-227.

Lynd, L. R., C. E. Wyman, and T. U. Gerngross. 1999. Biocommodity engineering. Biotechnol. Prog. **15**:777-793.

Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev **66**:506-77, table.

Lynd, L. R., W. H. van Zyl, J. E. McBride, and M. Laser. 2005. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. **16**:577-583.

Machida, M., I. Ohtsuki, S. Fukui, and I. Yamashita. 1988. Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular beta-glucosidases as expressed in *Saccharomyces cerevisiae*. Appl Environ Microbiol **54**:3147-3155.

Maheshwari, R., G. Bharadwaj, and M. K. Bhat. 2000. Thermophilic fungi: their physiology and enzymes. Microbiol Mol Biol Rev. **64**:461-488.

Mai, C., U. Kues, and H. Militz. 2004. Biotechnology in the wood industry. Appl Microbiol Biotechnol **63**:477-494.

Mattanovich, D., B. Gasser, H. Hohenblum, and M. Sauer. 2004. Stress in recombinant protein producing yeasts. J Biotech **113**:121-135.

McBride, J. E., J. J. Zietsman, W. H. van Zyl, and L. R. Lynd. 2005. Utilization of cellobiose by recombinant [beta]-glucosidase-expressing strains of *Saccharomyces cerevisiae*: characterization and evaluation of the sufficiency of expression. Enz Microb Tech **In Press, Corrected Proof**.

Mielenz, J. R. 2001. Ethanol production from biomass: technology and commercialization status. Curr Opin Microbiol **4**:324-329.

Mortberg, M. and H. Y. Neujahr . 1986. Transport and hydrolysis of disaccharides by *Trichosporon cutaneum*. J Bacteriol **168**:734-738.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol **96**:673-686.

Nassiep, K. M. 2006. Personal communication. Biomass Energy and Africa: Perspectives (workshops) 7 June 2006; South African Biofuels Strategy Development.

Ng, T. K. and J. G. Zeikus. 1982. Differential metabolism of cellobiose and glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. J Bacteriol **150**:1391-1399.

Nidetzky, B., C. Eis, and M. Albert. 2000. Role of non-covalent enzyme-substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*. Biochem J **351 Pt 3**:649-659.

Onishi, N. and T. Tanaka. 1996. Purification and properties of a galacto- and gluco-oligosaccharide-producing [beta]-glycosidase from *Rhodotorula minuta* IFO879. J Ferm Bioeng **82**:439-443.

Palmarola-Adrados, B., P. Choteborska, M. Galbe, and G. Zacchi. 2005. Ethanol production from non-starch carbohydrates of wheat bran. Bioresour Technol **96**:843-850.

Perez, J., J. Munoz-Dorado, R. T. de la, and J. Martinez. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol **5**:53-63.

Plourde-Owobi, L., S. Durner, J. L. Parrou, R. Wieczorke, G. Goma, and J. Francois. 1999. AGT1, encoding an alpha-glucoside transporter involved in uptake and intracellular accumulation of trehalose in *Saccharomyces cerevisiae*. J Bacteriol **181**:3830-3832.

Potera, C. 2005. Souped-up yeast. Environ Health Perspect **113**:A231.

Rajashekhara, E., M. Kitaoka, Y. K. Kim, and K. Hayashi. 2002. Characterization of a cellobiose phosphorylase from a hyperthermophilic eubacterium, *Thermotoga maritima* MSB8. Biosci Biotechnol Biochem **66**:2578-2586.

Rajoka, M. I., F. Shaukat, M. T. Ghauri, and R. Shahid. 2003. Kinetics of beta-glucosidase production by *Saccharomyces cerevisiae* recombinants harboring heterologous bgl genes. Biotechnol Lett **25**:945-948.

Rajoka, M. I., S. Khan, F. Latif, and R. Shahid. 2004. Influence of carbon and nitrogen sources and temperature on hyperproduction of a thermotolerant beta-glucosidase from synthetic medium by *Kluyveromyces marxianus*. Appl Biochem Biotechnol **117**:75-92.

Reichenbecher, M., F. Lottspeich, and K. Bronnenmeier. 1997. Purification and properties of a cellobiose phosphorylase (CepA) and a cellodextrin phosphorylase (CepB) from the cellulolytic thermophile *Clostridium stercorarium*. Eur J Biochem **247**:262-267.

Riedel, K., J. Ritter, and K. Bronnenmeier. 1997. Synergistic interaction of the *Clostridium stercorarium* cellulases Avicelase I (CelZ) and Avicelase II (CelY) in the degradation of microcrystalline cellulose. FEMS Microbiol Lett **147**:239-244.

Roth, W. W. and V. R. Srinivasan. 1978. Affinity chromatographic purification of beta-glucosidase of *Candida guilliermondii*. Prep Biochem **8**:57-71.

Roy, P., S. Mishra, and T. K. Chaudhuri. 2005. Cloning, sequence analysis, and characterization of a novel beta-glucosidase-like activity from *Pichia etchellsii*. Biochem Biophys. Res Commun. **336**:299-308.

Ryabova, O. B., O. M. Chmil, and A. A. Sibirny. 2003. Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res. **4**:157-164.

Saha, B. C. and R. J. Bothast. 1996. Production, purification, and characterization of a highly glucose-tolerant novel beta-glucosidase from Candida peltata. Appl Environ Microbiol **62**:3165-3170.

Schulein, M. 2000. Protein engineering of cellulases. Biochimica et Biophysica Acta (BBA) - Protein Struc and Mole Enzymol **1543**:239-252.

Schwarz, W. H. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol **56**:634-649.

Shallom, D. and Y. Shoham. 2003. Microbial hemicellulases. Curr Opinion Microbiol **6**:219-228.

Skory, C. D. and S. N. Freer. 1995. Cloning and characterization of a gene encoding a cell-bound, extracellular beta-glucosidase in the yeast *Candida wickerhamii*. Appl Environ Microbiol **61**:518-525.

Skory, C. D., S. N. Freer, and R. J. Bothast. 1996. Expression and secretion of the *Candida wickerhamii* extracellular beta-glucosidase gene, *bglB*, in *Saccharomyces cerevisiae*. Curr Genet. **30**:417-422.

Skory, C. D., S. N. Freer, and R. J. Bothast. 1996. Properties of an intracellular beta-glucosidase purified from the cellobiose-fermenting yeast *Candida wickerhamii*. Appl Microbiol. Biotechnol **46**:353-359.

Smith, J. D. and A. S. Robinson. 2002. Overexpression of an archaeal protein in yeast: secretion bottleneck at the ER. Biotechnol Bioeng **79**:713-723.

Smith, J. D., N. E. Richardson, and A. S. Robinson. 2005. Elevated expression temperature in a mesophilic host results in increased secretion of a hyperthermophilic enzyme and decreased cell stress. Biochim Biophys Acta - Proteins & Proteomics **1752**:18-25.

Spagna, G., R. N. Barbagallo, R. Palmeri, C. Restuccia, and P. Giudici. 2002. Properties of endogenous [beta]-glucosidase of a *Saccharomyces cerevisiae* strain isolated from Sicilian musts and wines. Enz Microb Technol **31**:1030-1035.

Stambuk, B. U. 2000. A simple laboratory exercise illustrating active transport in yeast cells. Biochemi Molec Biol Edu **28**:313-317.

Stambuk, B. U. and P. S. de Araujo. 2001. Kinetics of active alpha-glucoside transport in *Saccharomyces cerevisiae*. FEMS Yeast Res **1**:73-78.

Strobel, H. J., F. C. Caldwell, and K. A. Dawson. 1995. Carbohydrate transport by the anaerobic thermophile *Clostridium thermocellum* LQRI. Appl Environ Microbiol **61**:4012-4015.

Sun, Y. and J. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. **83**:1-11.

Tanaka, K., T. Kawaguchi, Y. Imada, T. Ooi, and M. Arai. 1994. Purification and properties of cellobiose phosphorylase from *Clostridium thermocellum*. J Ferm and Bioeng **79**:212-216.

Taylor, L. E., B. Henrissat, P. M. Coutinho, N. A. Ekborg, S. W. Hutcheson, and R. M. Weiner. 2006. Complete cellulase system in the marine bacterium *Saccharophagus degradans* strain 2-40T. J Bacteriol **188**:3849-3861.

van der Rest, M. E., A. H. Kamminga, A. Nakano, Y. Anraku, B. Poolman, and W. N. Konings. 1995. The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis. Microbiol Rev **59**:304-322.

Van Rensburg, P., W. H. van Zyl, and I. S. Pretorius. 1998. Engineering yeast for efficient cellulose degradation. Yeast **14**:67-76.

van Rooyen, R., B. Hahn-Hagerdal, D. C. la Grange, and W. H. van Zyl. 2005. Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains. J Biotech **120**:284-295.

Vincent, J. F. 1999. From cellulose to cell. J Exp Biol **202**:3263-3268.

Vincent, S. F., P. J. Bell, P. Bissinger, and K. M. Nevalainen. 1999. Comparison of melibiose utilizing baker's yeast strains produced by genetic engineering and classical breeding. Lett Appl Microbiol **28**:148-152.

Wallecha, A. and S. Mishra. 2003. Purification and characterization of two beta-glucosidases from a thermo-tolerant yeast *Pichia etchellsii*. Biochim Biophys Acta **1649**:74-84.

Wan, X. F., D. Xu, A. Kleinhofs, and J. Zhou. 2004. Quantitative relationship between synonymous codon usage bias and GC composition across unicellular genomes. BMC Evol Biol **4**:19.

Yernool, D. A., J. K. McCarthy, D. E. Eveleigh, and J. D. Bok. 2000. Cloning and characterization of the glucooligosaccharide catabolic pathway beta-glucan glucohydrolase and cellobiose phosphorylase in the marine hyperthermophile *Thermotoga neapolitana*. J Bacteriol **182**:5172-5179.

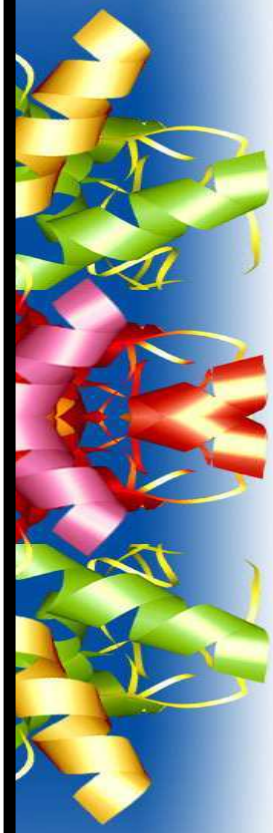
Yoon, S. H., R. Mukerjea, and J. F. Robyt. 2003. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. Carbohydr Res **338**:1127-1132.

Yu, Z. and H. Zhang. 2004. Ethanol fermentation of acid-hydrolyzed cellulosic-pyrolysate with *Saccharomyces cerevisiae*. Bioresour Technol **93**:199-204.

Zhang, Y. H. and L. R. Lynd. 2004. Kinetics and relative importance of phosphorolytic and hydrolytic cleavage of cellodextrins and cellobiose in cell extracts of *Clostridium thermocellum*. Appl Environ Microbiol **70**:1563-1569.

Zhang, Y. H. and L. R. Lynd. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng **88**:797-824.

Zhang, Y. H. and L. R. Lynd. 2005. Cellulose utilization by Clostridium thermocellum: bioenergetics and hydrolysis product assimilation. Proc Natl Acad Sci U. S. A **102**:7321-7325.



Article

Characterization of a recombinant cellobiose phosphorylase in *Saccharomyces cerevisiae* for the intracellular conversion of cellobiose to ethanol

Chapter 3

3 Expression and characterization of an intracellular cellobiose phosphorylase in *Saccharomyces cerevisiae*

C.J. Sadie and W.H. van Zyl

Department of Microbiology, University of Stellenbosch, Private bag X1, 7600 Stellenbosch, South Africa

3.1 ABSTRACT

The cellobiose phosphorylase (*cepA*) gene from *Clostridium stercorarium* encoding a 93 kDa intracellular protein, was cloned and successfully expressed under transcriptional control of the phosphoglycerate kinase gene (*PGK1)* promoter and terminator on an episomal plasmid in *S. cerevisiae* CEN.PK 21-C. The recombinant enzyme had activity optima of 60℃ and pH 6 - 7. Enzyme activity was tested on the chromogenic substrate *p*NPG (Km = 1.69 mM) and a maximum specific activity of 0.21 U/mg cell dry weight was achieved. The recombinant enzyme's K_m value for cellobiose was 92.85 mM. The recombinant strain, *S. cerevisiae*[yCEPA], sustained growth on cellobiose as sole carbon source with μ_{max} = 0.07 h^{-1} and yielded 0.05 g biomass per gram cellobiose consumed. To our knowledge this is the first report of a heterologously expressed cellobiose phosphorylase in yeast that conferred growth on cellobiose. Furthermore, this report also reaffirms previous data that cellobiose can be utilized intracellularly in *S. cerevisiae.*

3.2 INTRODUCTION

A worldwide decline in crude oil production has initiated the exploration of alternative energy sources such as bioethanol that is already extensively used as a partial gasoline replacement [Sun *and* Cheng, 2002]. Optimisation of yeasts for efficient cellulose degradation from cellulosic biomass could improve the high cost associated with the current enzymatic hydrolysis and fermentation processes of cellulose to ethanol [Lynd *et al.,* 2002]. Developing *Saccharomyces cerevisiae* for cellulose degradation requires the successful expression of cellulases, including the three key enzymes: cellobiohydrolase, endoglucanase and β-glucosidase [van Rensburg *et al.,* 1998]. The employment of a single organism for degradation of cellulose as well

as the fermentation of the resulting hexoses (known as consolidated bioprocessing) can bring about a significant reduction in costs by simplifying the production process [Lynd *et al.,* 1999].

β-Glucosidases are essential for the efficient utilization of cellobiose, the main end product of cellobiohydrolase activity and are also capable of degrading longer cello-oligosaccharides [Gonde *et al.,* 1984; Lynd *et al*,. 2002]. The products that are formed from cellobiose are two glucose molecules available for the microorganism. Organisms such as anaerobic *Clostridium* species also use another enzyme, cellobiose phosphorylase, that is very specific in its ability to hydrolyse cellobiose and at the same time phosphorylate one of the glucose molecules with an inorganic phosphate group yielding a glucose molecule and a glucose-1-phosphate molecule [Tanaka *et al.,* 1994]. Because one of the glucose molecules are already phosphorylated prior to entering the glycolytic pathway, the expression of a cellobiose phosphorylase in yeast could be energetically advantageous and ultimately lead to an overall increase in ethanol production [Fujita *et al*., 2002, Reichenbecher *et al*., 1997, Skory *et al*., 1996]. In organisms where cellobiose cleavage occurs via hydrolysis and phosphorolysis, the rate of phosphorylation greatly exceeds the rate of hydrolysis, indicating the possible metabolic advantage of this reaction [Zhang and Lynd, 2004].

Cellobiose phosphorylase is an intracellular enzyme and thus requires the transport of the cellobiose molecule inside the cell [Alexander, 1961]. Several bacterial and some yeast species (including the yeasts *Clavispora lusitaniae* and *Pichia guillermondii*) have the ability of transporting cellobiose across the plasma membrane [Freer and Greene, 1990; Freer, 1991]. However, the transport mechanism in yeast has not yet been characterized. *S. cerevisiae* is known to transport the disaccharides maltose and sucrose, both via the maltose proton symport system [van der Rest *et al.,* 1995; Stambuk *et al.,* 2000]. Until recently, *S. cerevisiae* was not known to transport cellobiose across its cell wall. Unpublished data from our laboratory have shown that a recombinant *S. cerevisiae* was able to utilize cellobiose when an intracellular β-glucosidase was expressed in this yeast, indicating the presence of a transport mechanism for cellobiose in *S. cerevisiae.* In a consolidated bioprocessing configuration of converting cellulose to ethanol, transporting cellobiose

into the cell has the advantage that end-product inhibition of the cellulases by glucose and cellobiose can be relieved. Furthermore, the possibility of contamination is decreased since the available glucose concentration is kept very low.

Here we report for the first time the cloning and successful expression of a cellobiose phosphorylase (*cepA*) gene from *Clostridium stercorarium* in *S. cerevisiae.* We also confirm that *S. cerevisiae* is able to transport cellobiose across its cell wall.

3.3 MATERIALS AND METHODS

3.3.1 Microbial Strains and Plasmids

The relevant genotypes and corresponding sources of the microbial strains, as well as the plasmids used in this study are summarized in Table 1. Genomic DNA of *C. stercorarium* was obtained from the Institute for Microbiology, Technical University Munich, Germany [Reichenbecher *et al*., 1997].

3.3.2 Media and Culture conditions

S. cerevisiae CEN.PK strains were cultivated at 30°C in YP mediu m (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) or selective synthetic complete (SC^{-ura}) medium (1.7 g L^{-1} yeast nitrogen base (Difco) containing amino acid supplements except uracil) with either glucose (10.52 g L^{-1}) or cellobiose (concentration depending on the experiment) as its sole carbon source.

Table 1. Microbial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference
Strains		
S. cerevisiae CEN.PK 21-C	*ura leu trp his*	Eliasson *et al*., 2000
S. cerevisiae CEN.PK[pJC1]	*ura leu trp his fur1::LEU2 URA3*	This study
S. cerevisiae CEN.PK[yCEPA]	*ura leu fur1::LEU2 URA3 PGK_P-cepA-PGK_T*	This study
E. coli XL^{-1} Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F'.proAB lac^q ZΔM15 Tn10 (tet)]*	Stratagene
Plasmids		
pGEM-T-Easy®	*bla*	Promega
pJC1	*bla URA3 $PGK1_{PT}$*	Crous *et al*., 1995
yCEPA	*bla URA3 $PGK1_{PT}$ CEPA*	This study

For growth on cellobiose in combination with other sugars, SC media was supplemented with the seven amino acids suggested by Görgens *et al*. [2001] for enhanced heterologous enzyme production, as well as 2.9 g L^{-1} K_2HPO_4 and 1.5 g L^{-1} KH_2PO_4 [Johnson *et al.,* 1981]. The yeasts were routinely cultured aerobically at 30℃ on a rotary shaker at and inoculated from the pre-culture to an absorbance value of 0.02 at 600 nm (OD_{600} = 0.02) unless otherwise stated. All pre-cultures were grown in YPC media containing 10 g L^{-1} cellobiose.

Recombinant plasmids were amplified in *Escherichia coli* XL^{-1} Blue and cultivated at 37℃ in Luria-Bertani liquid medium [Sambrook *et al*., 1989]. Ampicilin (100 µg mL^{-1}) was added for selecting recombinant bacteria. All solid media contained 20 g L^{-1} agar.

3.3.3 DNA manipulation and plasmid construction

Standard protocols were followed for DNA manipulations [Sambrook *et al*., 1989]. Plasmid isolation was performed using the CTAB-method described by Hoffman and Winston [1987]. Restriction endonucleases and T4 DNA ligase were purchased from Fermentas and used as recommended by the supplier. Restriction endonuclease-digested DNA was removed from agarose gels by the method of Tautz and Renz [1984].

The cellobiose phosphorylase gene (*cepA*) was isolated form *C. stercorarium* genomic DNA using PCR amplification (Table 2). The sequence-specific primers (5'-3' and 5'- 3') were designed from the *cepA* sequence from Genbank (accession number **U56242**) [Reichenbecher *et al.,* 1997]. Restriction sites are underlined on the primer sequences. DNA was amplified using the Perkin-Elmer GeneAmp® PCR System 2400 (The Perkin-Elmer Corporation, 761 main Avenue, Norwalk, Connecticut 06859). The PCR reaction mixture (50 µl) was as follows: 200 ng template, 400 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate, 3.5 U of Taq® DNA polymerase (Roche Molecular Biochemicals) and 5 µl of reaction buffer (Roche Molecular Biochemicals). The temperature profiles were as follow: 94˚C – 5 min, 94˚C – 0.30 min, 55˚C – 0.30 min, 72˚C - 2.26 min, 72˚C – 7 min. The resulting 2436-bp PCR product obtained from the reaction was cloned into a commercial vector, pGEM-Teasy® (Promega) as recommended by the manufacturer, to yield pGEM-Teasy®-cepA.

The 2436-bp *cepA* open reading frame was digested with *Xho*I and *Bgl*II and ligated into the corresponding sites of plasmid pJC1, a multi-copy yeast expression vector constructed previously in this laboratory [Crous *et al*., 1995]. The recombinant plasmid was designated yCEPA.

3.3.4 DNA sequencing

The 2436-bp *cepA* PCR product fragment was partially sequenced by the dideoxy chain termination method, with an ABI PRISMTM 3100 Genetic Analyzer with AmpliTag DNA polymerase F5 (Applied Biosystems kit) using fluorescent labelled nucleotides. The data were analysed with DNAMAN (version 4.1, Lynnon Biosoft). The sequences obtained were BLASTED at National Centre for Biotechnology

Information (http://www.ncbi.nlm.nih.gov/ BLAST/) to confirm the gene sequence.

Table 2. PCR primers used for gene isolation and identification in this study. Restriction sites are underlined

Primer Name	Sequence (5'→3')	Reference
cepA isolation		Reichenbecher *et al.*, 1997
cepA-L	GACTAGATCTATGAAGTTCGGTTATTTTGAC	
cepA-R	CAGTCTCGAGCAGCCCATTATAACAATTACT	
FUR1 disruption		La Grange *et al*., 1996
FUR1-L	TCCGTCTGGCATATCCTA	
FUR1-R	TTGGCTAGAGGACATGTA	
ADH PT		This laboratory
ADH1-L	GGATCCGCTACCAGTATAAATAGACAGG	
ADH1-R	AAGCTTCTAGAATTAATGCAGCTGGCAC	

3.3.5 Yeast transformation

DNA transformation of *S. cerevisiae* was performed using the lithium acetate dimethyl-sulfoxide (DMSO) method described by Hill *et al*., (1991). Disruption of the uracil phosphoribosyltranferase (*FUR1*) gene in *S. cerevisiae*[yCEPA] with the *LEU2* gene was performed to ensure auto-selection of recombinant plasmids containing the *URA3* gene in non-selective medium [La Grange *et al*., 1996]. A strain containing the pJC1 plasmid with no expression cassette was also created to act as reference strain. Total DNA isolated from *S. cerevisiae*[yCEPA] was used as template for PCR to confirm the presence of the *cepA* gene as well as the *fur1::LEU2* disruption. Furthermore, the identity of the *S. cerevisiae* strain was confirmed by isolating a fragment of the *ADH1* gene using the primers as described in Table 2. The primers used for the confirmation of the *fur1::LEU2* disruption correspond to those described by La Grange *et al,* [1996].

3.3.6 Selection of strain on cellobiose

S. cerevisiae[yCEPA] was plated onto SC^{-URA} medium containing cellobiose as sole carbon source. Colonies were transferred on fresh plates every two weeks. To avoid bacterial contamination, streptomycin was added to a final concentration of 2 mg L^{-1} to the solid medium. The reference strain, *S. cerevisiae*[pJC1] was also plated onto selective medium containing cellobiose.

3.3.7 Measurement of growth

Growth of the recombinant yeast was measured after dilution to OD_{600} < 0.8 [Pharmacia Biotech Ultrospech III]. The dry cell weight was measured by filtering 5 mL culture through 0.45 μm polycarbonate filters (Millipore), washing with distilled water and drying in a microwave [Plűddeman and van Zyl, 2003]. All growth curves and dry weight estimations were done in triplicate.

3.3.8 Substrate consumption

Analysis of the media and sugars consumed was performed using a high performance liquid chromatography system (model Dionex DX 500) consisting of an anion-exchange column (Carbopoc PA-100, 4x250 and Carbopac PA-100, guard) and a pulsed amperometric detector (ED40). Sulphuric acid (5 mM) in milli-Q water served as a mobile phase at 1.0 $mL.min^{-1}$. Data were analyzed using the Dionex Peaknet software package. All samples and standards were properly diluted with milli-Q water and filtered (0.22 μm, Millipore). Sugar standards were obtained by diluting known amounts of sugar to be analysed chromatographically and setting up a standard curve.

3.3.9 Purification of recombinant enzyme

3.3.9.1 For Enzyme assays

Whole yeast cells were prepared by centrifuging early stationary phase cultures (OD_{600} = 0.6) at 2.795 x *g* for 10 minutes. The cell pellets were washed twice with ice cold distilled water and resuspended in citric acid/phosphate buffer (0.05M, pH 5.0). Intracellular proteins were isolated using the method described by La Grange *et al* [1996]. β-Mercaptoethanol was excluded from any of the solutions or buffers, and the supernatants containing the proteins were used without precipitation with acetone as these may affect the activity of the recombinant cellobiose phosphorylase. Isolated

proteins were stored at -20℃ and kept for fast pro tein liquid chromatography (FPLC) analysis.

Permeabilized whole cells were obtained by adding toluene (with a final concentration of 0.01% v/v) and phenylmethylsulphonyl fluoride (PMSF) (1 µM) to resuspended whole cells and incubating for 1 h. Cells were also lysed by treatment with 20 U of Zymolyase™ per gram cells. Cell lysis was followed microscopically and the yeast membrane suspension was centrifuged at 1,006 x *g* for 15 min, resuspended in distilled water and used in the assay.

3.3.9.2 For SDS-PAGE analysis

For protein analysis on SDS-PAGE, intracellular and membrane protein fractions were obtained as follows. A 100 ml culture was grown until stationary phase, centrifuged at 1,006 x *g* for 10 minutes and the pellet was washed in breaking buffer (BB). The BB included 50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 5% v/v glycerol and 1 mM PMSF. Sodium hydroxide was used to adjust the pH and the buffer was stored at 4ºC. PMSF was added immediately before use. The yeast cells were collected at 1,957 x *g* at 4ºC and resuspended in BB to an OD_{600} of ~60 in Eppendorf tubes. An equal volume of acid-washed beads (~0.45 µm) was added and vortexed 8 times for 30s. The vortexing was alternated with incubating the mixture on ice for 30s. The samples were centrifuged at 4ºC for 10 minutes at 11,269 x *g* and the supernatant were transferred to a new eppendorf tube for protein analysis.

The pellet (with glass beads) was treated with 1% Triton-X100, mixed for 30s and centrifuged for 10 minutes at 11,269 x *g*. The supernatant (containing the insoluble proteins) was kept for protein analysis. Protein concentrations were determined using the Bradford protein assay method (Biolab) with bovine serum albumin (BSA) as standard and properly diluting the samples to the desired concentration [Bradford, 1976].

3.3.10 Fast protein liquid chromatography

Total intracellular protein was appropriately diluted with milli-Q water and analysed using FPLC in a Superdex 75-gelfiltration-column (10/30 column, 300 x 100 cm) with

an AKTA purifier (Pharmacia Biotech Company) at 0.2 mL.min^{-1} and separated according to size. Samples (200 µL) were collected and stored at -20℃.

3.3.11 SDS-PAGE

For SDS-page analysis 10 µg protein samples were mixed with a 5X loading buffer containing 2% sodium dodecyl sulfate, 25% glycerol, 14 mM β-mercaptoethanol, 0.1% bromophenol blue dye and 60 mM Tris-Cl, pH 6.8, followed by heating at 80ºC for 5 minutes. The molecular marker (PageRuler ™ Prestained Protein Ladder, Fermentas) was used to estimate protein size. Electrophoresis was conducted by the method of Laemmli [1970] on an 8% polyacrylamide gel. Proteins were visualised using the silver staining method [Otsuka *et al.,* 1988].

3.3.12 Enzyme assays

β-D-glucosidase activity of cellobiose phosphorylase was determined using the chromogenic substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma). Different cell fractions (depending on the experiment) were appropriately diluted and incubated with 5 mM *p*NPG in 50 mM citrate-phosphate buffer (pH 6.5 for cell extracts and pH 5 for whole cells) at 30℃ and 60℃ for 15 minutes. Equal volumes of sodium carbonate (1 M) were added to the reaction mixture to raise the pH and terminate the enzyme reaction. Release of the *p*-nitrophenyl group was measured as absorbance at 405 nm. One unit of activity (U) was defined as the amount of enzyme catalyzing the release of 1 µmol *p*-nitrophenyl per minute.

Enzyme activity on cellobiose was determined by incubating the cell membrane extract with different concentrations of cellobiose and 50 mM citrate/phosphate buffer (pH 6.5) at 60℃. Samples were taken at time inter vals and boiled for 5 minutes after which cellobiose consumption was determined with HPLC. All assays were done in triplicate. One unit of activity is defined as the amount of enzyme catalysing the hydrolysis of 1 µmol cellobiose per minute.

3.3.13 Data analysis

The codon bias index of the *cepA* sequence when expressed in *S. cerevisiae* was determined by the method of Carbone *et al.,* [2003]. Specific growth rates of the

strains were calculated using the slope of the exponential part of the growth curves. The yield of the recombinant strain on glucose and cellobiose was estimated by measuring the dry weight of cells formed per gram sugar consumed. S-values and all relevant parameters were calculated as described by McBride *et al*. [2005]. Enzyme kinetic data were analysed using Michaelis Menten equations and graphs.

3.4 RESULTS

3.4.1 Cloning of the cellobiose phosphorylase gene

After amplification of the putative *cepA* fragment from the genomic DNA of *C. stercorarium,* the resulting 2436-bp fragment was cloned in the pGEM-Teasy® plasmid. The fragment was partially sequenced and corresponded with the *cepA* sequence from Genbank (accession number **U56424**) after which the gene was cloned in the pJC1 vector and designated yCEPA (Figure 1).

S. cerevisiae CEN.PK 21-C was transformed with the multicopy yeast expression vector, yCEPA, expressing the *cepA* gene under the transcriptional control of the constitutive phosphoglycerate kinase gene (*PGK1)* promoter and terminator. The codon bias index of the *cepA* gene sequence was calculated for expression in *S. cerevisiae* and estimated to be 0.058.

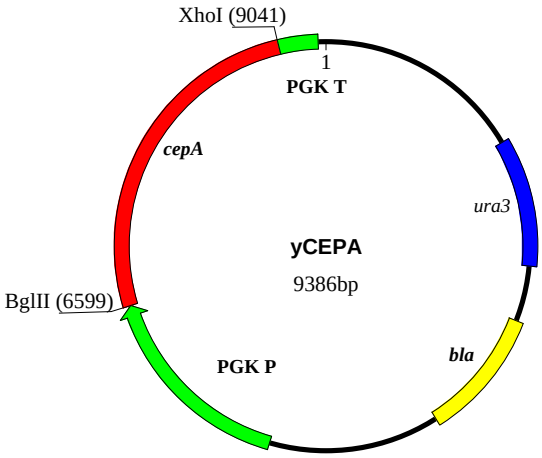
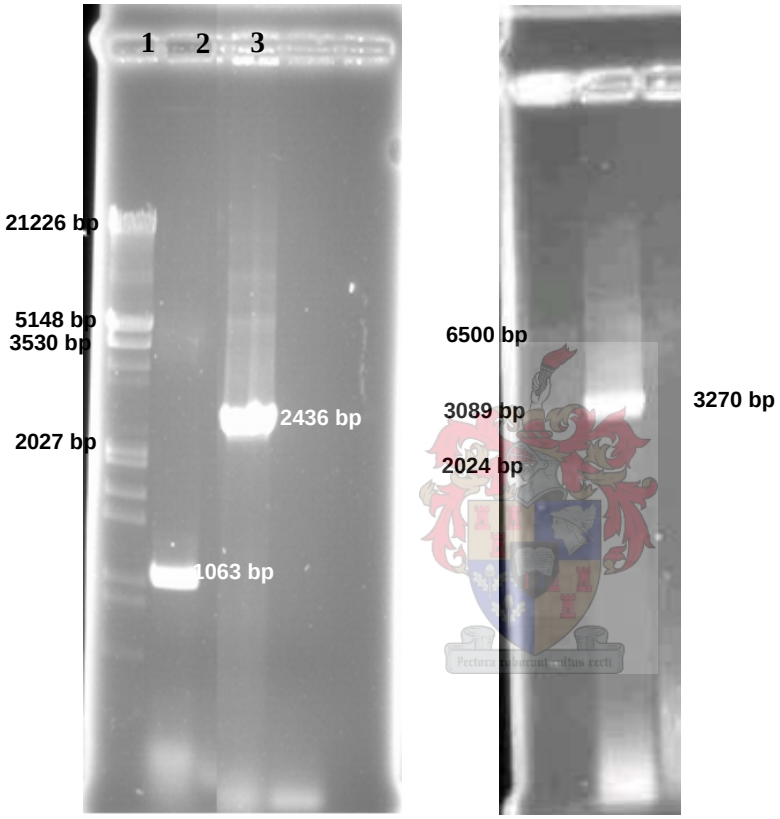


Figure 1. The episomal plasmid yCEPA containing the cepA expression cassette (red), the ampicilin resistance gene, *bla* (yellow), and the *ura3* (blue) selection marker are indicated. The gene was under transcriptional control of the *PGK1* promoter and terminator (green).

The *FUR1* gene of the recombinant *S. cerevisiae* CEN.PK[yCEPA] strain was disrupted with the *fur1::LEU2* allele to ensure auto-selection of the plasmid in non-selective growth medium. The constructs were confirmed with PCR using total DNA isolated from the recombinant strains as template. Sequence specific primers for a fragment of the ADH1 gene in *S. cerevisiae* was used to confirm the host strain. Results are shown in Figure 2.



(a) (b)

FIGURE 2. Confirmation of cellobiose phosphorylase (*cepA*) transformants as well as the *fur1::LEU2* disruption in the genome of *S. cerevisiae* CEN.PK [*y*CEPA]. Total DNA from the recombinant strain was used as template for the PCR reaction. In lane 1 λ-marker was used to estimate the sizes of the PCR-fragments. Lane 2 contains the amplificated *ADH1* fragments to confirm the identity of the *S. cerevisiae* strain. Lane 3 verifies the presence of the *cepA* gene (2436 *bp*s). Figure 2 (b) shows the presence of the *fur1::LEU2* allele that confirms the *FUR1* disruption.

3.4.2 Selection on cellobiose

Confirmed *S. cerevisiae*[yCEPA] transformants were plated on rich medium agar containing cellobiose (10 g L^{-1}) as its only carbohydrate source. After 10 days very small colonies appeared that were transferred onto fresh plates. This routine was followed every two weeks for one year and all experiments were conducted with the selected strain unless otherwise stated. The strain was refrained from growing on glucose throughout the selection process.

3.4.3 Enzyme activity

The intracellular crude extract of the recombinant strain was assayed with the chromogenic substrate *p*NPG to test the hydrolytic activity of the cellobiose phosphorylase protein. No activity could be found in the intracellular protein extract even after two hours of incubation. *S. cerevisiae*[yCEPA] whole cells incubated with *p*NPG did result in yellow colour development, indicating hydrolytic activity. The yeast cell walls were removed with Zymolyase and the cell membrane fractions and supernatant were subsequently collected. The supernatant did not show any activity, however the membrane fraction displayed a relatively high hydrolytic activity (Figure 3). The control strain *S. cerevisiae*[pJC1] did not show hydrolytic activity in any of the fractions (results not shown).

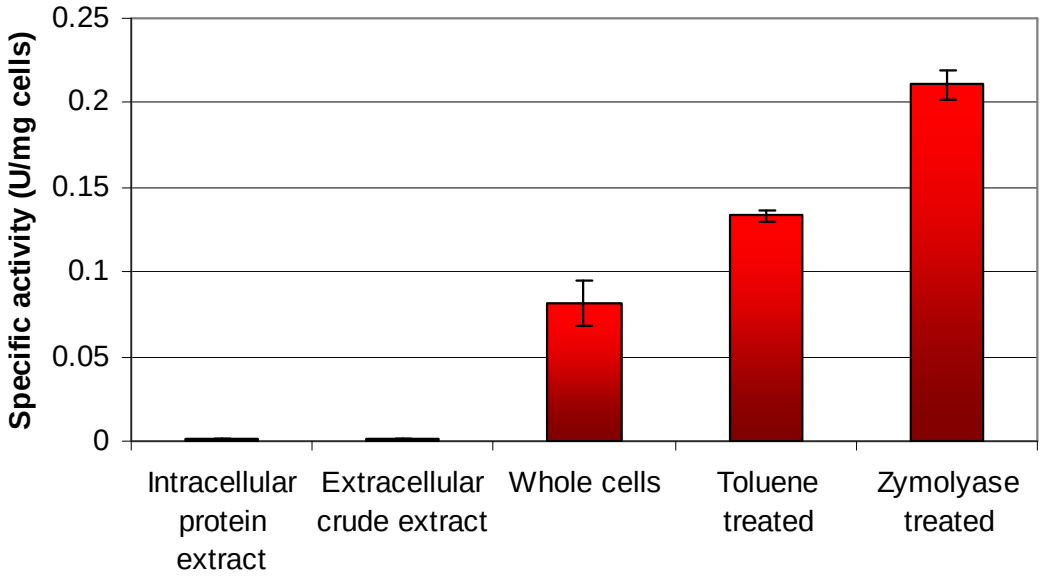


Figure 3. Comparison of the different cell associated fractions of *S. cerevisiae*[yCEPA] and their hydrolytic activity on the chromogenic substrate *p*NPG at 30℃. Error bars show the standard deviation between 3 different samples.

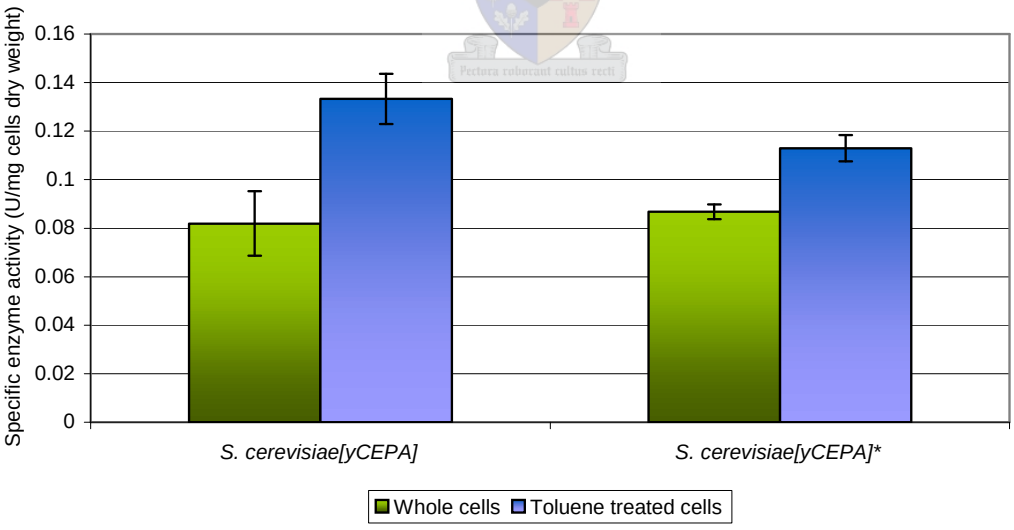


Figure 4. Comparison of the hydrolytic activity of the cellobiose phosphorylase activity of the selected *(S. cerevisiae*[yCEPA]) and unselected (*S. cerevisiae*[yCEPA]*) recombinant strains grown on cellobiose as tested on the chromogenic substrate *p*NPG. The reference strain *S. cerevisiae*[pJC1] was treated similarly and no activity was detected (results not shown). Error bars show the standard deviation between 3 different samples.

Treatment of the *S. cerevisiae*[yCEPA] whole cells with toluene to perforate the cells, led to an increased activity of 69 % in the selected strain and 35 % in the unselected strain (Figure 4). Selection of the recombinant strain did not seem to have an effect on the activity of whole cells, though perforated selected cells showed a small increase in hydrolytic activity. Statistical analysis using the Student's T-test, verified the statistical significance between these two values (p <0.05).

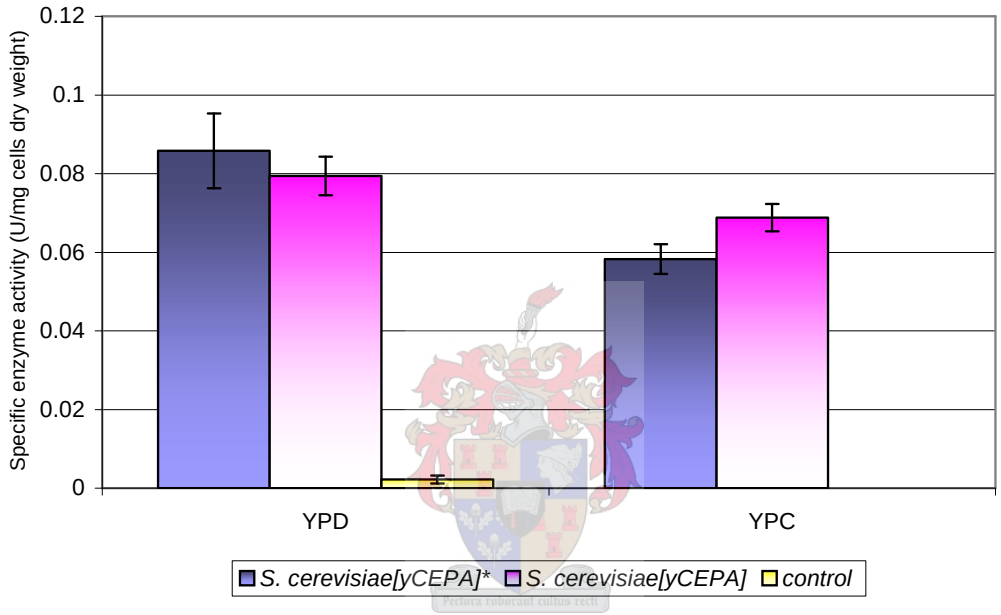


Figure 5. Comparison of the hydrolytic activity of the selected and unselected (marked with *) recombinant strains of *S. cerevisiae*[yCEPA] grown on glucose (YPD) and cellobiose (YPC) on the chromogenic substrate, *p*NPG, at 30℃. The reference strain *S. cerevisiae*[pJC1] was included in the experiment but did not show any activity. Error bars show the standard deviation between 3 different samples.

The recombinant enzyme activity of cells grown in glucose and cellobiose was compared and showed in figure 5. When grown in glucose, both the selected and unselected recombinant strain had a higher specific activity than cells grown in cellobiose (p <0.05), while no statistical difference could be seen between the selected and unselected strain when grown in either glucose or cellobiose (p >0.05). The reference strain *S. cerevisiae*[pJC1] did not grow in YPC and did not show any hydrolytic activity when grown in YPD.

The optimum temperature of the hydrolytic activity of the cellobiose phosphorylase harbouring *S. cerevisiae* strain was estimated to be at 60℃ (Figure 6). At 30℃ (optimum temperature for yeast growth) the enzyme had only 14% of its activity, while only 53% of the maximum activity still remained at 65℃. Although the enzyme is able to function over a wide range of temperatures, the activity remains sub optimal at the ideal growth temperature (30ºC) of *S. cerevisiae*.

Using cell membrane fractions, the optimum pH for the recombinant CepA protein was estimated to be between pH 6 and 7, while whole cells revealed a maximum activity at pH 5 (results not shown).

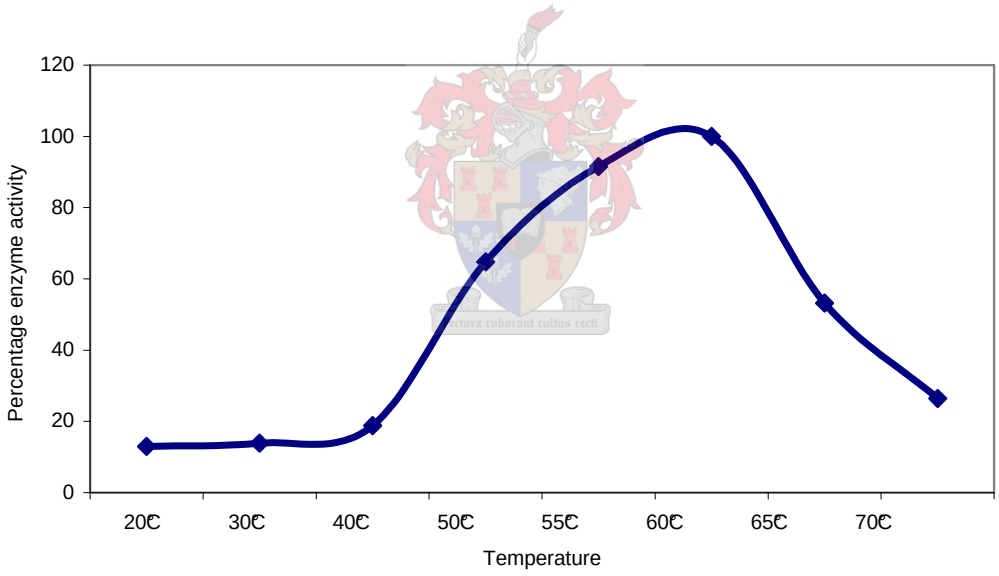
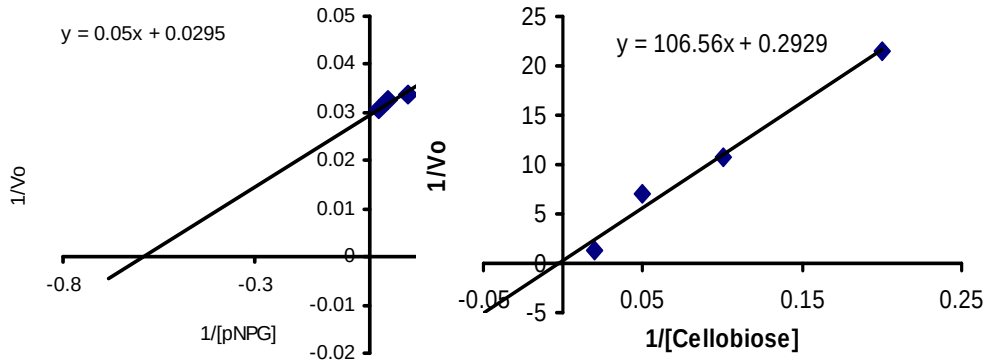


Figure 6. The activity of *S. cerevisiae*[yCEPA] whole cells on *p*NPG (pH 5) at different temperatures



(a) **(b)**

Figure 7. Lineweaver Burke plots for determination of K_m and V_{max} values of the recombinant cellobiose phosphorylase for the chromogenic substrate *p*NPG and cellobiose. Cell membrane fractions were incubated with different concentrations (5 to 50 mM) of each substrate. The initial velocity of the enzyme with each substrate was calculated and the reciprocal values of the substrate concentration plotted against the inverse of the initial velocity of each.

The recombinant enzyme's affinity constant and maximum velocity was estimated using Michaelis-Menten kinetics. The enzyme activities on different cellobiose and *p*NPG concentrations were determined and used to draw a Lineweaver Burke plot (Figure 7). The value where the graphs cross the y-axis represents the inverse of the V_{max} value and K_m values were estimated by determining the negative inverse of the value where the graph crosses the X-axis. For *p*NPG, the K_m of the enzyme was determined to be 1.69 mM and the V_{max} was 33.89 $U.min^{-1}$. For cellobiose the K_m was 363.81 mM and the V_{max} 3.41 $U.min^{-1}$. These differences in affinity for cellobiose and *p*NPG were also observed for other β-glucosidases and cellobiose phosphorylases (Table 3).

Table 3. Comparison of the affinity constants (K_m) for different cellobiose phosphorylases and β-glucosidases for cellobiose and *p*NPG

Cellobiose phosphorylase (CbP) or β-glucosidase (Bgl):	K_m cellobiose (mM)	K_m pNPG(mM)	Reference
S. cerevisiae[yCEPA] (CbP)	363.8	3.41	This study
C. stercorarium (CbP)	6.2	unknown	Reichenbecher *et al*., 1997
Thermotoga neapolitana (CbP)	28.6	0.28	Yernool *et al*., 2000
*Thermotoga maritima (*CbP)	0.29	unknown	Rajashekhara *et al*., 2002
Kluyveromyces fragilis (Bgl)	79.0	0.52	Leclerc *et al*., 1987
Candida wickerhamii (Bgl)	210.7	3.13	Freer, 1993

3.4.4 Protein fractionation and localization

3.4.4.1 Fast Protein Liquid Chromatography

The intracellular proteins of the recombinant cellobiose utilizing yeast strain were analysed on an AKTA purifier using fast protein liquid chromatography. The samples were properly diluted and equal amounts of samples were applied and analysed using fast protein liquid chromatography (FPLC). The proteins were separated on the basis of molecular weight and the results are seen in Figures 8. The recombinant strain had an increased protein concentration in the range of 90 kDa in comparison with the protein concentration of the control strain *S. cerevisiae*[pJC1]. The individual samples obtained from FPLC were tested for *p*NPG-hydrolysing activity. Assays were performed at different incubation temperatures, as well as different protein concentrations. However no enzymatic activity was detected even after 2 hours of incubation of the extract with the substrate.

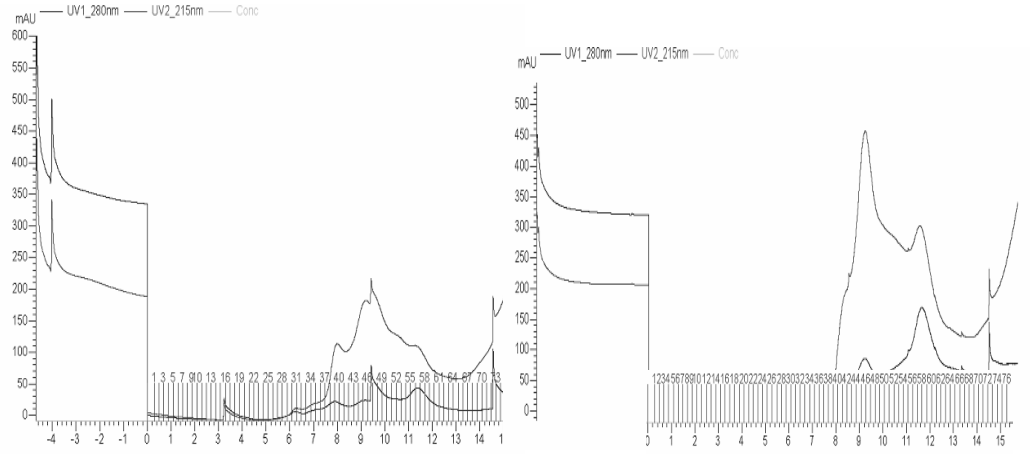


FIGURE 8. Intracellular proteins extracted from (a) the control strain *S. cerevisiae*[pJC1] and (b) the cellobiose utilizing strain *S. cerevisiae*[yCEPA]. All proteins were separated on the basis of molecular weight. Samples collected between 8 and 10 ml constitute all proteins between molecular masses 130 and 60 kDa. The highest peak observed in (b) represents a molecular mass of ~90 kDa.

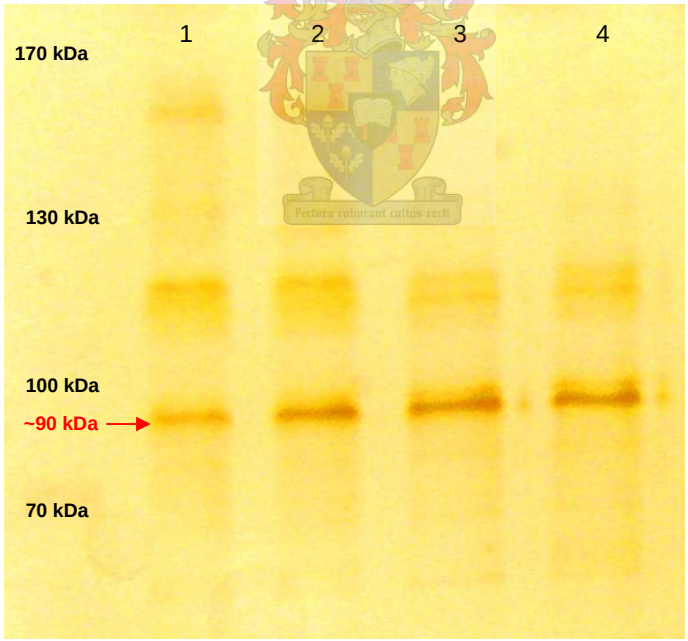


Figure 9. SDS-PAGE analysis of the membrane protein fraction from the control *S. cerevisiae*[pJC1] (lane 1), the membrane protein fraction of *S. cerevisiae*[yCEPA] (lane 2), intracellular protein extract from the control *S. cerevisiae*[pJC1] (lane 3) and *S. cerevisiae*[yCEPA] (lane 4).

3.4.4.2 SDS-PAGE

S. cerevisiae[yCEPA] and *S. cerevisiae*[pJC1] were grown on YPD until stationary phase and the intracellular proteins extracted. The cell membrane fractions were treated with 1% Triton-X100 to release the proteins trapped in the membrane. The released fractions were analyzed with SDS-PAGE and visualized using the silver staining method (Figure 9).

There was no considerable difference between the intracellular cell fractions of the two strains (lane 3 and 4). Lane 1 represents the membrane associated proteins of the control strain and lane 2 the same fraction of the recombinant strain. Although there appeared to be an increase in protein quantity in the membrane protein fraction of the recombinant strain in the area that represents the fraction of proteins with a relative size of 90 kDa, the result remains inconclusive. Western blot analysis could confirm the presence of the recombinant CepA protein if an appropriate antibody can be obtained.

3.4.5 Growth of strain on cellobiose

The *S. cerevisiae*[yCEPA] strain that was subjected to multiple rounds of selection on cellobiose-containing plates and liquid medium was evaluated for improved transport and utilisation of cellobiose. After two months' selection on SC^{-ura} (10 g L^{-1} cellobiose) or YPC medium (10 g L^{-1} cellobiose), aerobic growth of *S. cerevisiae*[yCEPA] was compared on glucose and cellobiose, respectively.

The strain was grown in a rich YP medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) with glucose (10.52 g L^{-1}), cellobiose (10 g L^{-1}) or no carbohydrate carbon source (Figure 10). The maximum specific growth rate (μ_{max}) for *S. cerevisiae*[yCEPA] was 0.33 h^{-1} and 0.063 h^{-1} on glucose and cellobiose, respectively. After sugar consumption was confirmed with HPLC, the yield ($Y_{X/S}$) on each of these carbon sources was determined as 0.117 g cells / g glucose and 0.05 g cells/g cellobiose consumed. Figure 11 shows the growth of *S. cerevisiae*[yCEPA] on different cellobiose concentrations and the maximum growth rate (0.069 h^{-1}) was observed in 20 g L^{-1} cellobiose while a growth rate of 0.063 h^{-1} was obtained in both 5 and 10 g L^{-1} cellobiose.

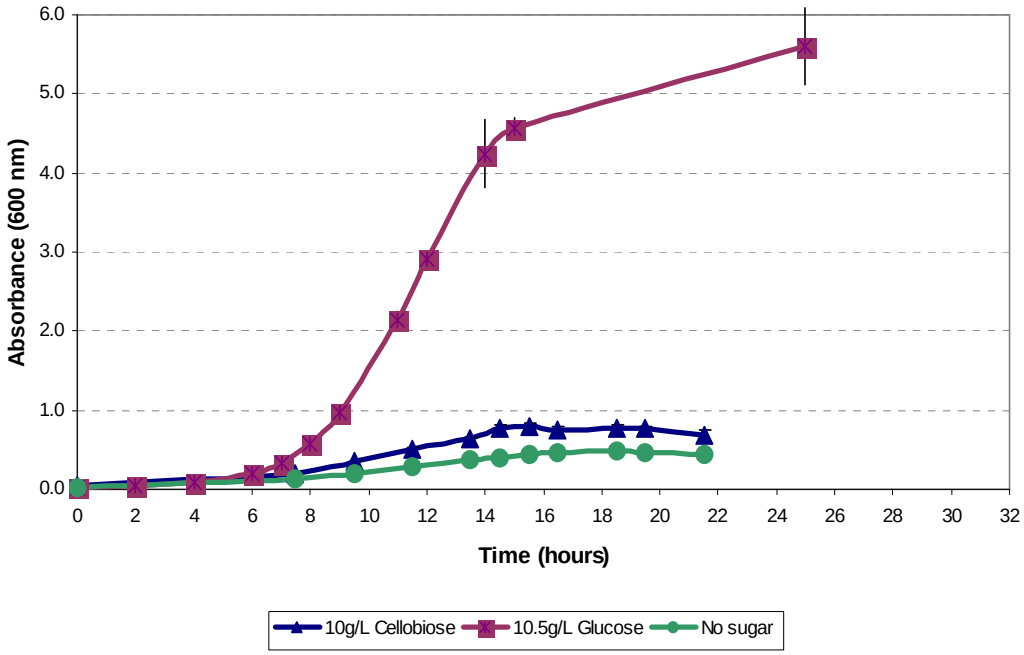


Figure 10. Comparative growth of *S. cerevisiae*[yCEPA] on (■) YPD (10.52 g L^{-1} glucose), (▲) YPC (10 g L^{-1} cellobiose) and (●) YP (no carbohydrate source). A small amount of growth occurred on the media without any sugar with a slight increase of growth seen in the media containing cellobiose as main carbon source. Error bars indicate the standard deviation seen between 3 different cultures.

The control strain, *S. cerevisiae*[pJC1], was also inoculated in YPC as well as SC medium (cellobiose 10 g L^{-1}) (results not shown). Very little growth occurred in YPC media, that was similar to growth of *S. cerevisiae*[yCEPA] on YP media with no sugar. No growth could be detected in SC media after 5 days (results not shown). When *S. cerevisiae*[yCEPA] was grown in rich media with an initial cellobiose concentration of 10 g L^{-1}, the maximum cellobiose consumption was estimated to be 2.34 g L^{-1} (Figure 11 and 12). After 20 hours, cellobiose consumption ceased, however 10 hours later a sharp reduction of cellobiose in the medium was observed in all cultures. During this time cells entered stationary phase and cell numbers decreased.

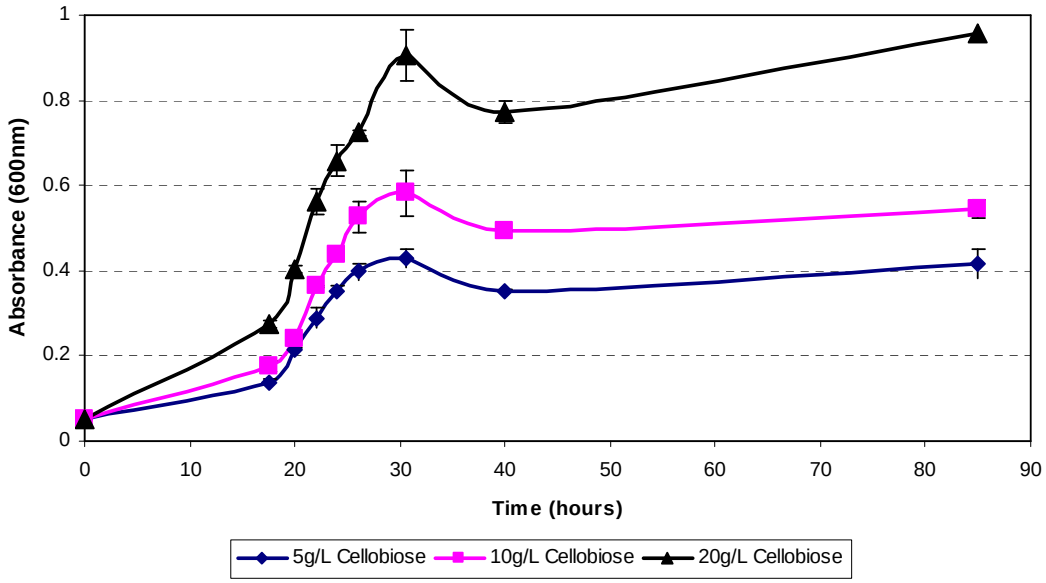


Figure 11. Growth of recombinant *S. cerevisiae*[yCEPA] in YP media with different cellobiose concentrations. The maximum growth rate (0.069 h^{-1}) on cellobiose was seen on 20 g L^{-1} cellobiose and 0.063 h^{-1} was reached both in 10 g L^{-1} and 5 g L^{-1} initial concentrations. Three replicate cultures were used and the error bars an indication of the standard deviation

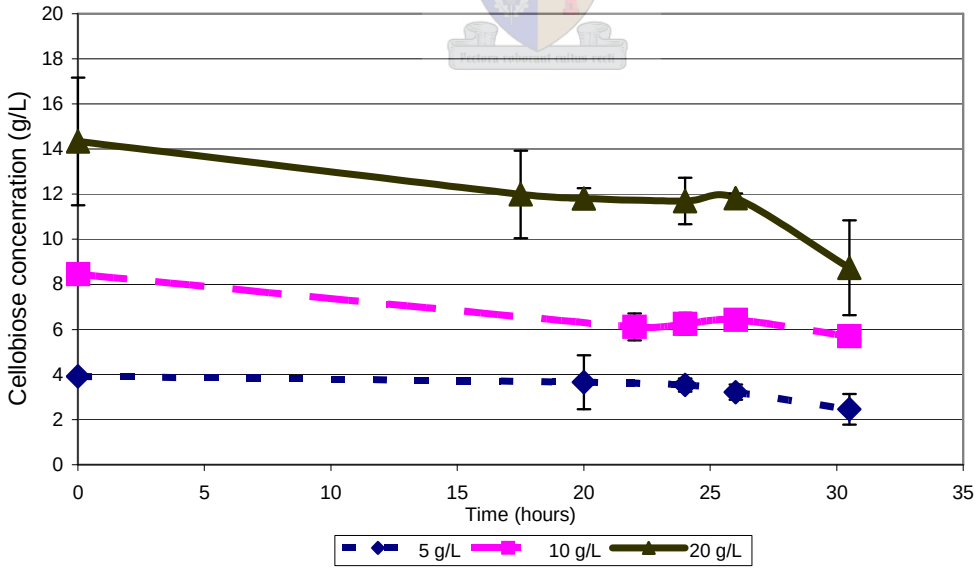


Figure 12. Cellobiose consumption during growth of the strain on different concentrations of cellobiose. The sugar concentration was determined by HPLC and all values are the average of three different cultures with standard deviations indicated.

3.4.6 Sufficiency

To determine whether the heterologous enzyme is efficient in its supply of glucose to the cell, the sufficiency parameter (S) as described by McBride *et al.* [2005] were used.

$$\mathbf{S} = \frac{a}{q_{\max}} \qquad \text{and} \qquad q_{\max} = \frac{\mu_{max}}{Y_{X/S}}$$

where a is the cell-specific activity of the heterologous enzyme and $q_{\max}$ the maximum cell-specific substrate consumption rate that can be calculated using the maximum growth rate ($\mu_{\max}$) and the cell yield ($Y_{X/S}$) on glucose. The S-value for the recombinant cellobiose phosphorylase was estimated to be 0.07.

3.4.7 Growth of S. cerevisiae*[yCEPA*] *on different sugars*

S. cerevisiae[yCEPA] was investigated for its growth on cellobiose in conjunction with other sugars in synthetic complete (SC) and yeast peptone (YP) media. Sugar concentrations of cultures grown in SC media were determined with HPLC. Growth of *S. cerevisiae*[yCEPA] on cellobiose (10 g L^{-1}) with additional maltose (3 g L^{-1}), fructose (3 g L^{-1}) or glucose (1 g L^{-1}) in SC media had no effect on the growth pattern or cell density in comparison with the growth of the strain on these sugars alone (Figure 13 a, b, e). HPLC results showed that glucose and maltose were utilized in SC media within the first 24 hours and more cellobiose was also consumed in this time than that was seen when the strain was grown on cellobiose alone (Table 2). In contrast with galactose that was simultaneously utilized with cellobiose, cellobiose was not simultaneously consumed with fructose. With only raffinose (3 g L^{-1}) in the medium, the recombinant strain grew poorly at first but growth increased after 100 hours (results not shown). The mixed medium with cellobiose plus raffinose resulted in immediate enhanced growth and sugar analysis revealed that after 24 hours all the raffinose was utilized and in this time cellobiose was also consumed up to a concentration of 1.9 g L^{-1}.

In rich YP media (Figure 14), the combination of cellobiose with some of the sugars resulted in different growth characteristics than in SC media. The presence of cellobiose hindered growth on galactose and fructose (14. b, e) while additional maltose (Figure 14 c) resulted in more biomass than theoretically expected. YP

media with cellobiose and additional raffinose or glucose had the same growth trend as in SC media.

TABLE 2. Sugar utilization of *S. cerevisiae*[yCEPA] grown on SC media with cellobiose (10 g L^{-1}) supplemented with different sugars.

Additional sugar	g L^{-1} cellobiose utilized		g L^{-1} additional sugar utilized	
	24 h	48 h	24 h	48 h
None (only cellobiose [a])	0.4	0.4	-	-
Glucose [b]	1.8	2.4	1	1
Galactose [c]	1.0	2.3	1.1	2.9
Fructose [c]	0	0.1	2.6	2.97
Raffinose [c]	1.9	1.9	3	3
Maltose [c]	1.5	2.4	3	3

[a] 10 g L^{-1} [b] 1 g L^{-1} [c] 3 g L^{-1}

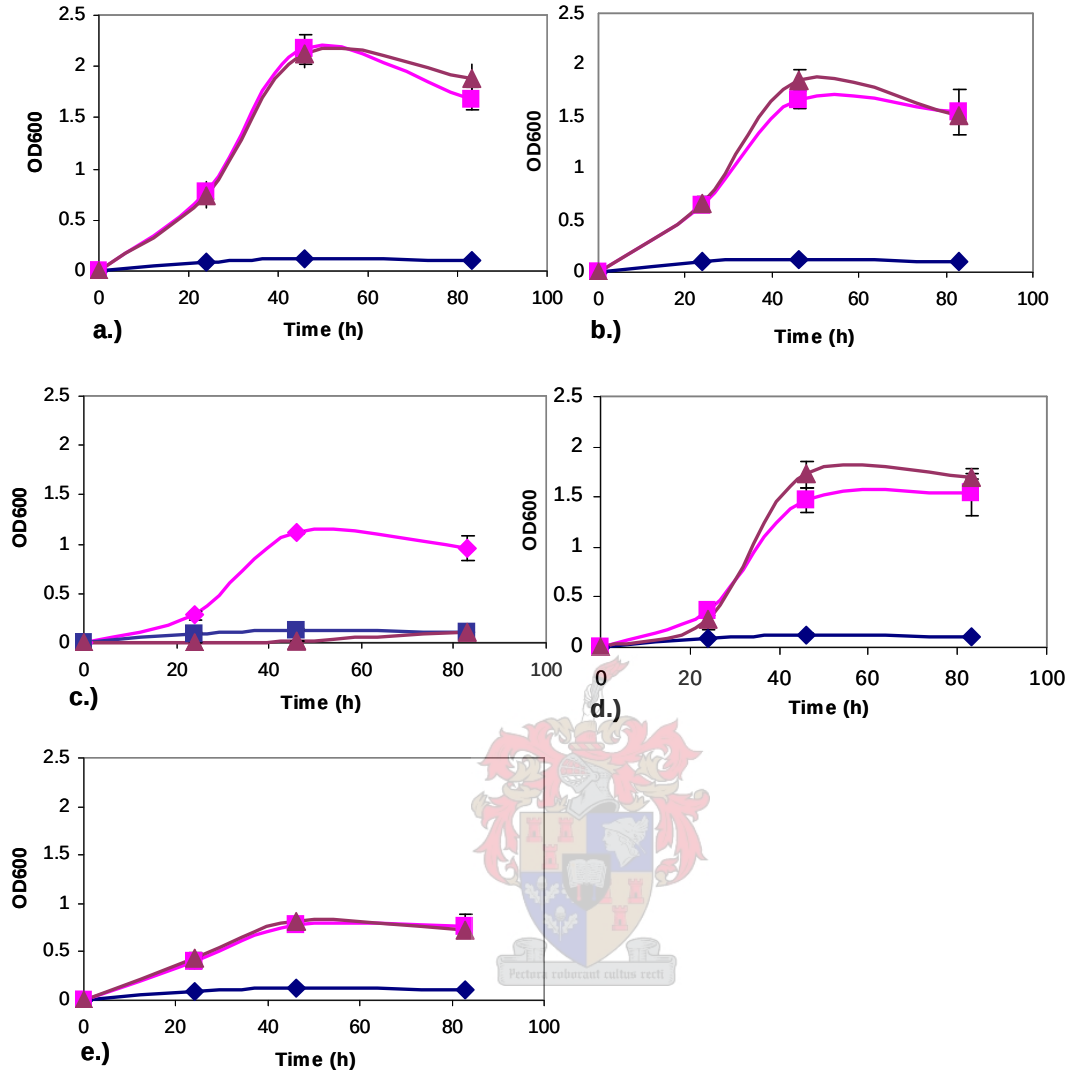


Figure 13. Growth of *S. cerevisiae*[yCEPA] on synthetic complete medium with 10 g L^{-1} cellobiose (♦) and supplemented with an additional sugar (■) that was 3 g L^{-1} maltose (a), fructose (b), raffinose (c), galactose (d) and 1 g L^{-1} glucose (e) as well as the growth on the additional sugar alone (▲). All growth curves were done in triplicate and the standard deviation indicated with error bars on the graphs. The control strain *S. cerevisiae*[pJC1] did not show any growth in medium containing cellobiose (results not shown).

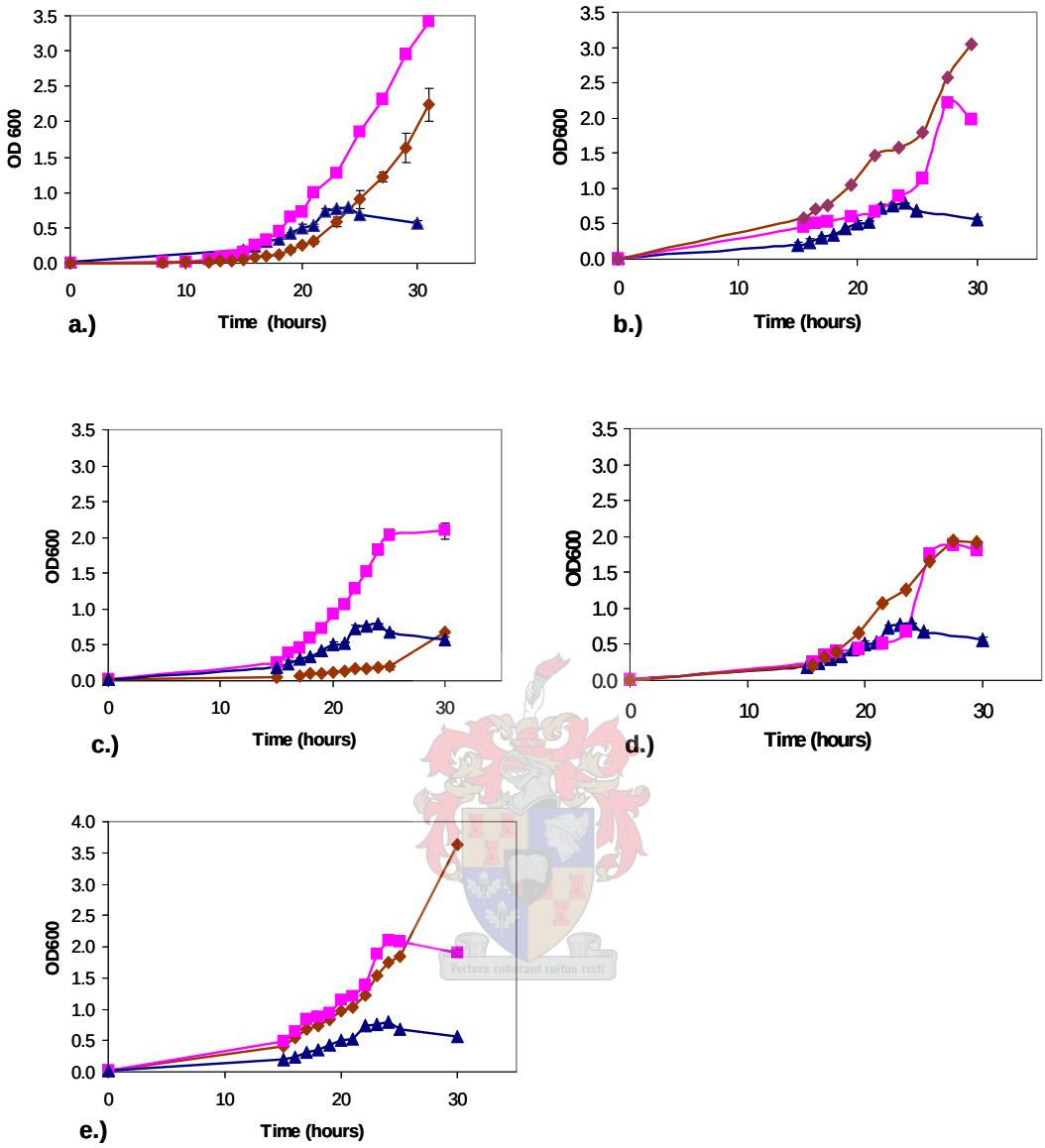


Figure 14. Growth of *S. cerevisiae*[yCEPA] on complex yeast-peptone medium with 10 g L^{-1} cellobiose (▲) and supplemented with an additional sugar (■) that was 3 g L^{-1} maltose (a), fructose (b), raffinose (c), galactose (d) and 1 g L^{-1} glucose (e) as well as the growth on the additional sugar alone (♦). All growth curves were done in triplicate and the standard deviation indicated with error bars on the graphs.

3.5 DISCUSSION

Cellulose is a viable and potentially cost-effective substrate for the production of bioethanol as substitute for the decreasing fossil fuels [Kosaric *et al.,* 2001]. Using organisms that are efficient in utilizing this substrate and subsequent fermentation to ethanol are critical for the commercialisation of such a process [Lynd *et al.,* 2002]. β-Glucosidase is the commonly used enzyme for recombinant organisms to utilize cellobiose, the main product of cellobiohydrolase activity [McBride *et al*., 2005]. However, anaerobic cellulolytic organisms make use of a second enzyme, cellobiose phosphorylase, that provides a metabolic advantage in ATP saving instead of an intracellular β-glucosidases [Alexander, 1961; Zhang and Lynd, 2004]. The aim of this study was to express and characterize a cellobiose phosphorylase (CepA) from the thermophilic, anaerobic bacterium *C. stercorarium* in the yeast *S. cerevisiae* and determine the effect that different sugars has on cellobiose consumption.

The *cepA* open reading frame was cloned from *C. stercorarium* genome DNA and expressed from a multicopy yeast expression vector under transcriptional control of the constitutive *PGK1* promoter and terminator in *S. cerevisiae.* Disruption of the *FUR1* gene of the *S. cerevisiae* transformants enabled the ability of growth in undefined media for enhanced biomass formation without the loss of the episomal plasmid containing the *URA3* marker [La Grange *et al.,* 1996]. Activity assays showed that the enzyme was indeed constitutively expressed under control of the *PGK1* promoter and terminator and had a higher cell specific activity when the cells were grown in a rich medium containing glucose than when grown in cellobiose (Figure 4). This is likely due to the metabolic burden that is placed on the cell when grown on the foreign substrate cellobiose [Hahn-Hägerdal *et al.,* 2001].

The CepA protein is natively found as a monomeric protein with a molecular mass of 93 kDa [Reichenbecher *et al*., 1997]. The commercial lack of antibodies against the CepA protein hinders the proper identification of this protein in cell extracts. However, SDS-PAGE results suggest an increase in membrane bound proteins with size ~90 kDa of the *S. cerevisiae*[yCEPA] strain versus the *S. cerevisiae*[pJC1] control strain (Figure 6), indicating trapping of the overexpressed protein in the membrane fraction. However, no conclusive remarks could be made after comparative visualization of the intracellular proteins of the recombinant and the

control strains. Conversely, after molecular weight fractionation of the intracellular proteins with FPLC, a peak could be seen in Figure 8 (b) that corresponds to proteins with a size of approximately 90 kDa. The significant increase in protein concentration at this point can be an indication of the presence of the CepA protein, however this was not conclusive. The inability to detect enzyme activity in the intracellular fractions is possibly due to unstable protein structures in the cytosol, method of isolation, degradation by proteases, instability of the heterologous protein in the cytosol or protein trapping in the membranes [Smith and Robinson, 2002; Hohenblum *et al.,* 2004; Mattanovich *et al.,* 2004]. The latter was confirmed by locating enzyme activity in the cell membrane fraction after lysis of yeast protoplasts. The trapped enzyme was not released from the membrane fraction since there was no enzyme activity detected in the supernatant of the lysed fractions.

The hydrolytic activity of CepA was measured using the substrate *p*NPG since the ability of β-glucosidase to hydrolyse *p*NPG correlates with its ability to hydrolyse cellobiose [Freer and Greene, 1990; Reichenbecher *et al*., 1997]. Activity was tested in a citrate/phosphate buffer, since omission of phosphate in previous studies led to inactivation of the enzyme's phosphorylation activity although the hydrolytic activity remained. The optimal pH range of the recombinant cellobiose phosphorylase was identical to its native host (pH 6 - 7) and concurred with other intracellular proteins [Alexander, 1961; Rajashekhara *et al*., 2002; Tanaka *et al*., 1994]. Maximum activity using *S. cerevisiae*[yCEPA] whole cells was achieved at pH 5, typically the pH where maximum transfer of sugars take place using the proton symport mechanism [Kubicek *et al.,* 1993].

Since CepA is produced intracellularly, cellobiose has to be transported across the cell membrane of *S. cerevisiae* in order to be hydrolysed. Previous results from this laboratory showed that *S. cerevisiae* was able to enhance cellobiose transport when selected on plates containing cellobiose as sole carbohydrate carbon source for prolonged periods (van Rooyen *et al*., unpublished data). The results obtained from the current work support this data and proved that the recombinant strain producing the cellobiose phosphorylase was also able to internalise and utilize cellobiose. Treating the cells with toluene, that perforate the cells and allowed effortless solute access to the cytosol, did result in increased activity in comparison with whole cell

activity [Figure 4]. This signified the importance of an effective transport mechanism for cellobiose (and *p*NPG) and that the transport of cellobiose across the cell wall of *S. cerevisiae* in this study seemed to be insufficient for maximal use of the recombinant enzyme. These results have also shown that selection of *S. cerevisiae*[yCEPA] did result in an increase of the specific activity of the enzyme but cellobiose transport did not improve since comparison of selected and unselected whole cells yielded the same specific activity (Figure 4). Compared to β-glucosidase enzymes, the specific activity of the recombinant CepA enzyme is relatively low [Skory *et al.,* 1996; Rajoka *et al.,* 2003; van Rooyen *et al.,* 2005]. Van Rooyen *et al.,* [2005] reported a recombinant *S. cerevisiae* strain harbouring a β-glucosidase from *S. fibilugera* with a specific activity of 0.8 U mg^{-1} cells that was able to sustain growth on cellobiose with a growth rate similar to that on glucose. *S. cerevisiae*[yCEPA] had a maximum specific activity of 0.22 U mg^{-1} cells and the growth rate on cellobiose was much less than that on glucose. The codon bias value is an indication of the ability of an organism to express a heterologous gene and given the low value of the *cepA* sequence for translation in *S. cerevisiae,* the inadequate enzyme activity is possibly due to insufficient heterologous protein expression in the host [Bennetzen and Hall., 1982; Gustafsson *et al.,* 2004].

Because the cellobiose phosphorylase requires inorganic phosphate for the enzyme to have an energetic advantage, it was thought that phosphate starvation could explain inferior growth on cellobiose compared to growth on glucose [Alexander, 1961]. *S. cerevisiae* is known to acquire phosphate by active transport through the *PHO* transporters and when phosphate is limited, a series of events are activated to scavenge for phosphate, including increased transport [Lenburg and O'Shea, 1996; Persson *et al*., 1999; Wykoff and O'Shea, 2001]. Reichenbecher *et al.*, [1997] has reported that the cellobiose phosphorylase from *C. stercorarium* required 20 mM phosphate for optimum phosphorylase activity although in the absence of phosphate the hydrolytic activity still remained intact. The addition of extra phosphate as described by Johnson *et al*. [1981] for *Clostridia* species growing on cellulose did not result in enhanced growth of the *S. cerevisiae*[yCEPA] strain on cellobiose (Figure 13). This suggests that phosphate was not the limiting factor and that there are other restricting factors involved in the growth arrest of *S. cerevisiae*[yCEPA].

Functional expression of the *cepA* gene in *S. cerevisiae* did enable the recombinant yeast to utilize cellobiose as sole carbon source. When *S. cerevisiae*[yCEPA] was grown aerobically in a rich YP medium containing 10 g L^{-1} cellobiose or no carbohydrate carbon source (Figure 10), an increase in biomass was detected in all the grown cultures. Limited growth was noticed in the medium without any carbohydrate source, therefore suggesting that the amino acids and other components of the yeast-peptone medium can sustain a small amount of growth. Enhanced growth in YPC media suggested that the yeast was able to utilize cellobiose. Results obtained from HPLC analysis of the remaining sugars in the medium confirmed this assumption. Results presented in Figure 11 illustrated that increasing cellobiose concentrations resulted in better growth rates and higher cell densities; however, the yield of this strain remained identical. The recombinant strain had a relatively short growth period with cellobiose as sole carbon source as the yeast entered exponential phase and quickly reached stationary phase even though cellobiose utilization was incomplete (Figures 8 and 9). When grown on cellobiose in combination with other sugars the recombinant yeast was indeed able to utilize more cellobiose than grown on this sugar alone. This could be explained by more ATP available for the cell to transport cellobiose across the membrane and thus result in better cellobiose consumption. Since all active disaccharide transport observed in yeast uses proton symport (using 15% of all ATP generated), ATP is critical for the proper functioning of the transport process [van der Rest *et al.,* 1995]. The sufficiency parameter (S) as described by McBride *et al.,* [2003] is an indication of the adequacy of a recombinant enzyme to enable growth on a non-native substrate. When S = 1 the recombinant enzyme provides sufficient product (a sugar such as glucose) for the recombinant cell to utilize without inhibiting the growth rate. For values less than 1 the recombinant enzyme provides too little product (glucose) and the growth rate is negatively affected. In the case of the *S. cerevisiae*[yCEPA] strain, S was determined to be 0.07 and growth was thus greatly inhibited due to insufficient fermentable sugar availability due to low levels of recombinant enzyme present. An inadequate amount of ATP production causes vital cellular functions to cease and ultimately leads to cell death. Furthermore, the inability of *S. cerevisiae* to recognize the substrate as a fermentable carbon source could also lead to a series of responses typically found after cell starvation such as apoptosis [Ashe *et al.,* 2000; Holsbeeks *et al.,* 2004]. The insufficient ATP supply as well as the “starvation” of the

cells explains the growth arrest of the culture even though cellobiose consumption was incomplete.

Van Rooyen and van Zyl (unpublished work) have shown that a recombinant *S. cerevisiae* strain harbouring an intracellular β-glucosidase gave higher biomass yield when grown on medium containing both cellobiose and maltose than the combined yields on these two sugars separately. Furthermore it was confirmed that the transporters *MAL61* and *AGT1* are activated in the presence of cellobiose in this strain and that the induction of the maltose transporters in the presence of maltose also had the effect of enhanced cellobiose transport via the same transporters since these sugars are structurally very similar [Kubicek *et al.,* 1993]. In this study, growth of *S. cerevisiae*[yCEPA] on mixtures of cellobiose and maltose had the same effect in rich YP media, however this was not the case in synthetic media. It is postulated that the extra amino acids and other compounds in the rich media possibly enhanced this phenomenon, while activity of the recombinant cellobiose phosphorylase in synthetic media was not optimal. Cellobiose utilization, as seen with maltose and glucose supplements (Table 2), probably occurred after maltose and glucose were depleted since *S. cerevisiae* preferred these substrates (catabolite repression) [Gancedo, 1998]. Utilization of these sugars offers the cell enough energy to transport cellobiose and utilize this sugar until ATP production become limited after which the cells go in arrest.

Catabolite repression by fructose was evident since only a small amount of cellobiose was consumed after fructose was depleted from the medium. Raffinose utilization of the recombinant strain was restricted at first in both complex and synthetic media. The addition of cellobiose in synthetic media caused raffinose to be consumed within the first 24 hours (Table 2). Raffinose is hydrolysed extracellulary with invertase to yield fructose and melibiose, of which *S. cerevisiae* CEN.PK strains can only consume fructose [Yoon *et al.,* 2003]. Invertase is also responsible for sucrose hydrolysis and sucrose can be transported inside the cell via the maltose transporter *AGT1* [Stambuk *et al.,* 2000]. The cellobiose utilization that was observed with the mixture of raffinose and cellobiose may be due to the indirect induction of the active maltose transporters.

3.6 FUTURE RESEARCH AND RECOMMENDATIONS

For the application of a cellobiose phosphorylase in the industry, enzyme activity needs to be enhanced for efficient cellobiose hydrolysis in *S. cerevisiae.* Since the codon usage pattern of *S. cerevisiae* and *C. stercorarium* is very diverse, enhanced gene expression may possibly be achieved by codon optimisation of the *cepA* sequence for expression in *S. cerevisiae.* Codon optimisation have proved to be successful for increased heterologous protein expression [Carbone *et al*., 2003; Gustafsson *et al.*, 2004]. A more economic alternative for enhanced heterologous protein expression would be to address other factors such as chaperone co-expression as well as suppressing the unfolded protein response (UPR) that are up-regulated when heterologous proteins are expressed [Cudna and Dickson, 2003; Gasch and Werner-Washburne, 2002; Hohenblaum *et al*., 2004; Kaufman *et al*., 2004].

Because transport of cellobiose across the cytoplasmic membrane of *S. cerevisiae* is not clearly understood, it would be valuable to investigate the *AGT1* and other possible transporters involved in the selected strains for its affinity for cellobiose and rate of transport. Over-expression of the *AGT1* and *MAL61* transporters of *S. cerevisiae* may show an increase in cellobiose transport and could be valuable in a strain expressing an intracellular β-glucosidase or cellobiose phosphorylase. Furthermore, by expressing an intracellular cellodextrin phosphorylase in *S. cerevisiae*, longer cello-oligosaccharide transport can be investigated after a selection process that can have an added metabolic advantage for the yeast. Valuable information, regarding the possible metabolic advantage of cellobiose phosphorylase and cellodextrin phosphorylase versus β-glucosidase, could be obtained from a comparative growth study of recombinant strains expressing the relevant enzymes.

3.7 LITERATURE CITED

Alexander, J. K. 1961. Characteristics of cellobiose phosphorylase. J Bacteriol **81**:903-910.

Ashe, M. P., S. K. De Long, and A. B. Sachs. 2000. Glucose depletion rapidly inhibits translation initiation in yeast. Mol Biol Cell **11**:833-848.

Bennetzen, J. L. and B. D. Hall. 1982. Codon selection in yeast. J Biol Chem **257**:3026-3031.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**:248-254.

Carbone, A., A. Zinovyev, and F. Kepes. 2003. Codon adaptation index as a measure of dominating codon bias. Bioinformatics **19**:2005-2015.

Crous, J. M., I. S. Pretorius, and W. H. van Zyl. 1995. Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiae*. Curr Genet **28**:467-473.

Cudna, R. E. and A. J. Dickson. 2003. Endoplasmic reticulum signaling as a determinant of recombinant protein expression. Biotechnol Bioeng **81**:56-65.

Eliasson, A., E. Boles, B. Johansson, M. Osterberg, J. M. Thevelein, I. Spencer-Martins, H. Juhnke, and B. Hahn-Hagerdal. 2000. Xylulose fermentation by mutant and wild-type strains of *Zygosaccharomyces* and *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol **53**:376-382.

Freer, S. N. and R. V. Greene. 1990. Transport of glucose and cellobiose by *Candida wickerhamii* and *Clavispora lusitaniae*. J Biol Chem **265**:12864-12868.

Freer, S. N. 1991. Fermentation and aerobic metabolism of cellodextrins by yeasts. Appl Environ. Microbiol **57**:655-659.

Freer, S. N. 1993. Kinetic characterization of a beta-glucosidase from a yeast, *Candida wickerhamii*. J Biol Chem **268**:9337-9342.

Fujita, Y., S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda, and A. Kondo. 2002. Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Appl Environ Microbiol **68** :5136-5141.

Gancedo, J. M. 1998. Yeast carbon catabolite repression. Microbiol Mol Biol Rev **62**:334-361.

Gasch, A. P. and M. Werner-Washburne. 2002. The genomics of yeast responses to environmental stress and starvation. Funct Integr Genomics **2**:181-192.

Gonde, P., B. Blondin, M. Leclerc, R. Ratomahenina, A. Arnaud, and P. Galzy. 1984. Fermentation of cellodextrins by different yeast strains. Appl Environ. Microbiol **48**:265-269.

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

Gustafsson, C., S. Govindarajan, and J. Minshull. 2004. Codon bias and heterologous protein expression. Trends Biotechnol **22**:346-353.

Hahn-Hagerdal, B., C. F. Wahlbom, M. Gardonyi, W. H. van Zyl, R. R. Cordero Otero, and L. J. Jonsson. 2001. Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. Adv Biochem Eng Biotechnol **73**:53-84.

Hill, J., K. A. Donald, D. E. Griffiths, and G. Donald. 1991. DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res **19**:5791.

Hoffman, C. S. and F. Winston. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57**:267-272.

Hohenblum, H., B. Gasser, M. Maurer, N. Borth, and D. Mattanovich. 2004. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol Bioeng **85**:367-375.

Holsbeeks, I., O. Lagatie, A. Van Nuland, d. Van, V, and J. M. Thevelein. 2004. The eukaryotic plasma membrane as a nutrient-sensing device. Trends Biochem Sci **29**:556-564.

Johnson, E. A., A. Madia, and A. L. Demain. 1981. Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum.* Appl Environ. Microbiol **41**:1060-1062.

Kauffman, K. J., E. M. Pridgen, F. J. Doyle, III, P. S. Dhurjati, and A. S. Robinson. 2002. Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in *Saccharomyces cerevisiae*. Biotechnol Prog **18**:942-950.

Kosaric, N., H. J. Pieper, and F. Vardar-Sukan. 2001. The Biotechnology of Ethanol. Classical and Future Applications. Wiley-VCH, Weinheim.

Kubicek, C. P., R. Messner, F. Gruber, M. Mandels, and E. M. Kubicek-Pranz. 1993. Triggering of cellulase biosynthesis by cellulose in *Trichoderma reesei*. Involvement of a constitutive, sophorose-inducible, glucose-inhibited beta-diglucoside permease. J Biol Chem **268**:19364-19368.

la Grange, D. C., I. S. Pretorius, and W. H. van Zyl. 1996. Expression of a *Trichoderma reese*i beta-xylanase gene (XYN2) in *Saccharomyces cerevisiae*. Appl Environ Microbiol **62**:1036-1044.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Leclerc, M., P. Chemardin, A. Arnaud, R. Ratomahenina, P. Galzy, C. Gerbaud, A. Raynal, and M. Guerineau. 1987. Comparison of the properties of the purified beta-glucosidase from the transformed strain of *Saccharomyces cerevisiae* TYKF2 with that of the donor strain *Kluyveromyces fragilis* Y610. Biotechnol Appl Biochem **9**:410-422.

Lenburg, M. E. and E. K. O'Shea. 1996. Signaling phosphate starvation. Trends Biochem Sci **21**:383-387.

Lynd, L. R., C. E. Wyman, and T. U. Gerngross. 1999. Biocommodity Engineering. Biotechnol. Prog. **15**:777-793.

Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev. **66**:506-77, table.

Mattanovich, D., B. Gasser, H. Hohenblum, and M. Sauer. 2004. Stress in recombinant protein producing yeasts. J. of Biotechnol **113**:121-135.

McBride, J. E., J. J. Zietsman, W. H. van Zyl, and L. R. Lynd. 2005. Utilization of cellobiose by recombinant [beta]-glucosidase-expressing strains of *Saccharomyces cerevisiae*: characterization and evaluation of the sufficiency of expression. Enzy Microb Techno

Otsuka, F., S. Koizumi, M. Kimura, and M. Ohsawa. 1988. Silver staining for carboxymethylated metallothioneins in polyacrylamide gels. Analytical Biochemistry **168**:184-192.

Persson, B. L., J. Petersson, U. Fristedt, R. Weinander, A. Berhe, and J. Pattison. 1999. Phosphate permeases of *Saccharomyces cerevisiae*: structure, function and regulation. Biochimica et Biophysica Acta (BBA) - Rev on Biomembr **1422**:255-272.

Pluddemann, A. and W. H. van Zyl. 2003. Evaluation of *Aspergillus niger* as host for virus-like particle production, using the hepatitis B surface antigen as a model. Curr Genet. **43**:439-446.

Rajashekhara, E., M. Kitaoka, Y. K. Kim, and K. Hayashi. 2002. Characterization of a cellobiose phosphorylase from a hyperthermophilic eubacterium, *Thermotoga maritima* MSB8. Biosci. Biotechnol Biochem **66**:2578-2586.

Rajoka, M. I., F. Shaukat, M. T. Ghauri, and R. Shahid. 2003. Kinetics of beta-glucosidase production by *Saccharomyces cerevisiae* recombinants harboring heterologous bgl genes. Biotechnol Lett. **25**:945-948.

Reichenbecher, M., F. Lottspeich, and K. Bronnenmeier. 1997. Purification and properties of a cellobiose phosphorylase (CepA) and a cellodextrin phosphorylase (CepB) from the cellulolytic thermophile *Clostridium stercorarium*. Eur. J Biochem. **247**:262-267.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Skory, C. D., S. N. Freer, and R. J. Bothast. 1996. Expression and secretion of the *Candida wickerhamii* extracellular beta-glucosidase gene, *bglB*, in *Saccharomyces cerevisiae*. Curr Genet **30**:417-422.

Smith, J. D. and A. S. Robinson. 2002. Overexpression of an archaeal protein in yeast: secretion bottleneck at the ER. Biotechnol Bioeng **79**:713-723.

Stambuk, B. U. 2000. A simple laboratory exercise illustrating active transport in yeast cells. Biochem Mol Biol Education **28**:313-317.

Stambuk, B. U., A. S. Batista, and P. S. de Araujo. 2000. Kinetics of active sucrose transport in *Saccharomyces cerevisiae*. J Biosci. Bioeng. **89**:212-214.

Sun, Y. and J. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol **83**:1-11.

Tanaka, K., T. Kawaguchi, Y. Imada, T. Ooi, and M. Arai. 1994. Purification and properties of cellobiose phosphorylase from *Clostridium thermocellum*. J Ferm Bioeng **79**:212-216.

Tautz, D. and M. Renz. 1983. An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal Biochem **132**:14-19.

van der Rest, M. E., A. H. Kamminga, A. Nakano, Y. Anraku, B. Poolman, and W. N. Konings. 1995. The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis. Microbiol Rev **59**:304-322.

Van Rensburg, P., W. H. van Zyl, and I. S. Pretorius. 1998. Engineering yeast for efficient cellulose degradation. Yeast **14**:67-76.

van Rooyen, R., B. Hahn-Hagerdal, D. C. la Grange, and W. H. van Zyl. 2005. Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains. J Biotech **120**:284-295.

Wykoff, D. D. and E. K. O'Shea. 2001. Phosphate transport and sensing in *Saccharomyces cerevisiae*. Genetics **159**:1491-1499.

Yernool, D. A., J. K. McCarthy, D. E. Eveleigh, and J. D. Bok. 2000. Cloning and characterization of the glucooligosaccharide catabolic pathway beta-glucan glucohydrolase and cellobiose phosphorylase in the marine hyperthermophile *Thermotoga neapolitana*. J Bacteriol **182**:5172-5179.

Yoon, S. H., R. Mukerjea, and J. F. Robyt. 2003. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. Carbohydr Res **338**:1127-1132.

Zhang, Y. H. and L. R. Lynd. 2004. Kinetics and relative importance of phosphorolytic and hydrolytic cleavage of cellodextrins and cellobiose in cell extracts of *Clostridium thermocellum*. Appl Environ Microbiol **70**:1563-1569.