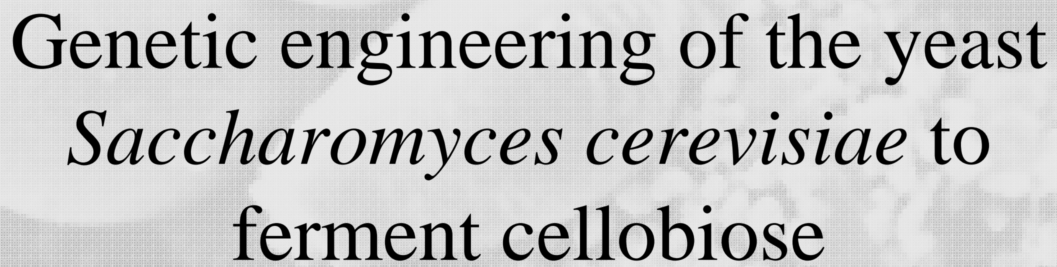

Genetic engineering of the yeast *Saccharomyces cerevisiae* to ferment cellobiose

By:

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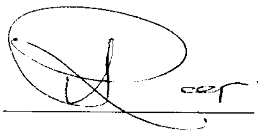
Dissertation presented for the Degree Doctor of Philosophy at the University of Stellenbosch

Supervisor: Prof. W. H. van Zyl

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation 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.



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SUMMARY

The conversion of cellulosic biomass into fuels and chemicals has the potential to positively impact the South African economy, but is reliant on the development of low-cost conversion technology. Perhaps the most important progress to be made is the development of "consolidated bioprocessing" (CBP). CBP refers to the conversion of pretreated biomass into desired product(s) in a single process step with either a single organism or consortium of organisms and without the addition of cellulase enzymes. Among the microbial hosts considered for CBP development, *Saccharomyces cerevisiae* has received significant interest from the biotechnology community as the yeast preferred for ethanol production. The major advantages of *S. cerevisiae* include high ethanol productivity and tolerance, as well as a well-developed gene expression system. Since *S. cerevisiae* is non-cellulolytic, the functional expression of at least three groups of enzymes, namely endoglucanases (EC 3.2.1.4); exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) is a prerequisite for cellulose conversion via CBP. The endo- and exoglucanases act synergistically to efficiently degrade cellulose to soluble cellodextrins and cellobiose, whereas the β-glucosidases catalyze the conversion of the soluble cellulose hydrolysis products to glucose. This study focuses on the efficient utilization of cellobiose by recombinant *S. cerevisiae* strains that can either hydrolyse cellobiose extracellularly or transport and utilize cellobiose intracellularly.

Since it is generally accepted that *S. cerevisiae* do not produce a dedicated cellobiose permease/transporter, the obvious strategy was to produce a secretable β-glucosidase that will catalyze the hydrolysis of cellobiose to glucose extracellularly. β-Glucosidase genes of various fungal origins were isolated and heterologously expressed in *S. cerevisiae*. The mature peptide sequence of the respective β-glucosidases were fused to the secretion signal of the *Trichoderma reesei xyn2* gene and expressed constitutively from a multi-copy yeast expression vector under transcriptional control of the *S. cerevisiae PGK1* promoter and terminator. The resulting recombinant enzymes were characterized with respect to pH and temperature optimum, as well as kinetic properties. The maximum specific growth rates (μ_{max}) of the recombinant strains were compared during batch cultivation in high-performance bioreactors. *S. cerevisiae* secreting the recombinant *Saccharomycopsis fibuligera BGL1* enzyme was identified as the best strain and grew at 0.23 h^{-1} on cellobiose (compared to 0.29 h^{-1} on glucose). More significantly,

was the ability of this strain to anaerobically ferment cellobiose at 0.18 h^{-1} (compared to 0.25 h^{-1} on glucose).

However, extracellular cellobiose hydrolysis has two major disadvantages, namely glucose's inhibitory effect on the activity of cellulase enzymes as well as the increased risk of contamination associated with external glucose release. In an alternative approach, the secretion signal from the *S. fibuligera* β-glucosidase (*BGL1*) was removed and expressed constitutively from the above-mentioned multi-copy yeast expression vector. Consequently, the BGL1 enzyme was functionally produced within the intracellular space of the recombinant *S. cerevisiae* strain. A strategy employing continuous selection pressure was used to adapt the native *S. cerevisiae* disaccharide transport system(s) for cellobiose uptake and subsequent intracellular utilization. RNA Bio-Dot results revealed the induction of the native α-glucoside (AGT1) and maltose (MAL) transporters in the adapted strain, capable of transporting and utilizing cellobiose intracellularly. Aerobic batch cultivation of the strain resulted in a μ_{max} of 0.17 h^{-1} and 0.30 h^{-1} when grown in cellobiose- and cellobiose/maltose-medium, respectively. The addition of maltose significantly improved the uptake of cellobiose, suggesting that cellobiose transport (via the combined action of the maltose permease and α-glucosidase transporter) is the rate-limiting step when the adapted strain is grown on cellobiose as sole carbon source. In agreement with the increased μ_{max} value, the substrate consumption rate also improved significantly from 0.25 g.g $DW^{-1}.h^{-1}$ when grown on cellobiose to 0.37 g.g $DW^{-1}.h^{-1}$ upon addition of maltose to the medium. The adapted strain also displayed several interesting phenotypical characteristics, for example, flocculation, pseudohyphal growth and biofilm-formation. These features resemble some of the properties associated with the highly efficient cellulase enzyme systems of cellulosome-producing anaerobes.

Recombinant *S. cerevisiae* strains that can either hydrolyse cellobiose extracellularly or transport and utilize cellobiose intracellularly. Both recombinant strains are of particular interest when the final goal of industrial-scale ethanol production from cellulosic waste is considered. However, the latter strain's ability to efficiently remove cellobiose from the extracellular space together with its flocculating, pseudohyphae- and biofilm-forming properties can be an additional advantage when the recombinant *S. cerevisiae* strain is considered as a potential host for future CBP technology.

OPSOMMING

Die omskakeling van sellulose-bevattende biomassa na brandstof en chemikalieë beskik oor die potensiaal om die Suid-Afrikaanse ekonomie positief te beïnvloed, indien bekostigbare tegnologie ontwikkel word. Die merkwaardigste vordering tot dusvêr kon in die ontwikkeling van "gekonsolideerde bioprosessering" (CBP) wees. CBP verwys na die eenstap-omskakeling van voorafbehandelde biomassa na gewenste produkte met behulp van 'n enkele organisme of 'n konsortium van organismes sonder die byvoeging van sellulase ensieme. Onder die mikrobiese gashere wat oorweeg word vir CBP-ontwikkeling, het *Saccharomyces cerevisiae* as die voorkeur gis vir etanolproduksie troot belangstelling by die biotegnologie-gemeenskap ontlok. Die voordele van *S. cerevisiae* sluit in hoë etanol-produktiwiteit en toleransie, tesame met 'n goed ontwikkelde geen-uitdrukkingsisteem. Aangesien *S. cerevisiae* nie sellulose kan benut nie, is die funksionele uitdrukking van ten minste drie groepe ensieme, naamlik endoglukanases (EC 3.2.1.4); eksoglukanases (EC 3.2.1.91) en β-glukosidases (EC 3.2.1.21), 'n voorvereiste vir die omskakeling van sellulose via CBP. Die sinergistiese werking van endo- en eksoglukanases word benodig vir die effektiewe afbraak van sellulose tot oplosbare sello-oligosakkariede en sellobiose, waarna β-glukosidases die finale omskakeling van die oplosbare sellulose-afbraak produkte na glukose kataliseer. Hierdie studie fokus op die effektiewe benutting van sellobiose m.b.v. rekombinante *S. cerevisiae*-rasse met die vermoeë om sellobiose ekstrasellulêr af te breek of dit op te neem en intrasellulêr te benut.

Aangesien dit algemeen aanvaar word dat *S. cerevisiae* nie 'n toegewyde sellobiose-permease/transporter produseer nie, was die mees voor-die-hand-liggende strategie die produksie van 'n β-glukosidase wat uitgeskei word om sodoende die ekstrasellulêre hidroliese van sellobiose na glukose te kataliseer. β-Glukosidase gene is vanaf verskeie fungi geïsoleer en daaropvolgend in *S. cerevisiae* uitgedruk. Die geprosesseerde peptiedvolgorde van die onderskeie β-glukosidases is met die sekresiesein van die *Trichoderma reesei xyn2*-geen verenig en konstitutief vanaf 'n multikopie-gisuitdrukkingsvektor onder transkripsionele beheer van die *S. cerevisiae PGK1* promotor en termineerder uitgedruk. Die gevolglike rekombinante ensieme is op grond van hul pH en temperatuur optima, asook kinetiese eienskappe, gekarakteriseer. Die maksimum spesifieke groeitempos (μ_{max}) van die rekombinante rasse is gedurende aankweking in hoë-verrigting bioreaktors vergelyk. Die *S. cerevisiae* ras wat die rekombinante

Saccharomycopsis fibuligera BGL1 ensiem uitskei, was as the beste ras geïdentifiseer en kon teen 0.23 h^{-1} op sellobiose (vergeleke met 0.29 h^{-1} op glukose) groei. Meer noemenswaardig is the ras se vermoë om sellobiose anaërobies teen 0.18 h^{-1} (vergeleke met 0.25 h^{-1} op glukose) te fermenteer.

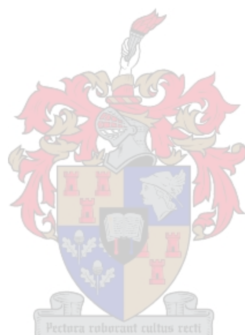
Ekstrasellulêre sellobiose-hidroliese het twee groot nadele, naamlik glukose se onderdrukkende effek op die aktiwiteit van sellulase ensieme, asook die verhoogde risiko van kontaminasie wat gepaard gaan met die glukose wat ekstern vrygestel word. 'n Alternatiewe benadering waarin die sekresiesein van die *S. fibuligera* β-glucosidase (*BGL1*) verwyder en konstitutief uitgedruk is vanaf die bogenoemde multi-kopie gisuitrukkingsvektor, is gevolg. Die funksionele BGL1 ensiem is gevolglik binne-in die intrasellulêre ruimte van die rekombinante *S. cerevisiae* ras geproduseer. Kontinûe selektiewe druk is gebruik om die oorspronklike *S. cerevisiae* disakkaried-transportsisteme vir sellobiose-opname and daaropvolgende intrasellulêre benutting aan te pas. RNA Bio-Dot resultate het gewys dat die oorspronklike α-glukosied (AGT1) en maltose (MAL) transporters in die aangepaste ras, wat in staat is om sellobiose op te neem en intrasellulêr te benut, geïnduseer is. Aërobiese kweking van die geselekteerde ras het gedui dat die ras teen 0.17 h^{-1} en 0.30 h^{-1} groei in onderskeidelik sellobiose en sellobiose/maltose-medium. Die byvoeging van maltose het die opname van sellobiose betekenisvol verbeter, waarna aangeneem is dat sellobiose transport (via die gekombineerde werking van die maltose permease en α-glukosidase transporter) die beperkende stap gedurende groei van die geselekteerde ras op sellobiose as enigste koolstofbron is. In ooreenstemming hiermee, het die substraat-benuttingstempo ook betekenisvol toegeneem van 0.25 $g.g\,DW^{-1}.h^{-1}$, gedurende groei op sellobiose, tot 0.37 $g.g\,DW^{-1}.h^{-1}$ wanneer maltose by die medium gevoeg word. Die geselekteerde ras het ook verskeie interessante fenotipiese kenmerke getoon, byvoorbeeld flokkulasie, pseudohife- en biofilm-vorming. Hierdie eienskappe kom ooreen met sommige van die kenmerke wat met die hoogs effektiewe sellulase ensiem-sisteme van sellulosome-produserende anaerobe geassosieer word.

Hierdie studie beskryf die suksesvolle konstruksie van 'n rekombinante *S. cerevisiae* ras met die vermoë om sellobiose ekstrasellulêr af te breek of om dit op te neem en intrasellulêr te benut. Beide rekombinante rasse is van wesenlike belang indien die einddoel van industriële-skaal etanolproduksie vanaf sellulose afval oorweeg word. Die laasgenoemde ras se vermoë om sellobiose effektief uit die ekstrasellulêre ruimte te verwyder tesame met die flokkulasie,

pseudohife- en biofilm-vormings eienskappe kan ‘n addisionele voordeel inhou, indien die rekombinante *S. cerevisiae* ras as ‘n potensiële gasheer vir toekomstige CBP-tegnologie oorweeg word.

BIOGRAPHICAL SKETCH

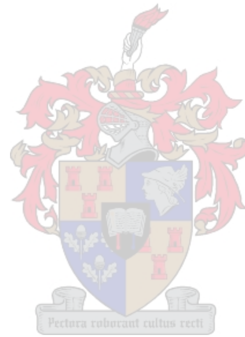
Ronél van Rooyen was born on 2 January 1976 in Lichtenburg, South Africa. She attended the Lichtenburg Primary School and matriculated at the Lichtenburg High School, in 1994. Ronél enrolled at the University of Stellenbosch in 1995 and obtained a B.Sc.Agric degree in Biochemistry, Genetics and Microbiology in 1998. In 2002 she completed a masters degree in Microbiology *cum laude* at the same university. Her masters' thesis was entitled "Cloning of a novel *Bacillus pumilus* cellobiose-utilising system: Functional expression in *Escherichia coli*".



"But it is the spirit in a man, the breath of the Almighty,

that gives him understanding."

Job 32:8



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Jesus Christ, my Lord and Savior, who is my true source of hope, joy and understanding. “Blessing and glory and wisdom, thanksgiving and honor and power and might, be to our God forever and ever. Amen." (Revelation 7:12)

PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the journal to which the manuscript was submitted.

Chapter 3 "Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains" has been published in Journal of Biotechnology 120:284-295.

Chapter 4 "Adaptation and characterization of a recombinant *Saccharomyces cerevisiae* strain that transports and utilizes cellobiose intracellularly" has been submitted to Metabolic Engineering.

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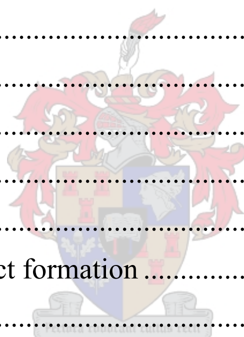

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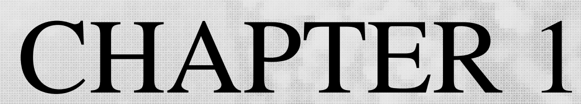

CHAPTER 1

General introduction and project objectives

GENERAL INTRODUCTION

Sustainable development has become the corner stone on which future energy technology is being established. From wind and solar to hydrogen and bioethanol, the major aims of energy production have become cost-effectiveness and sustainability. Cellulose is the most abundant renewable biological resource and therefore an ideal substrate for the production of bio-based products and bioenergy considering the aforementioned criteria.

Cellulose-to-ethanol technology has major challenges. Probably the most important and difficult barrier is to overcome the recalcitrance of natural lignocellulosic biomass [Demain et al., 2005; Mosier et al., 2005; Wyman, 1999]. Currently the effective enzymatic conversion of this recalcitrant lignocellulose to fermentable sugars requires three steps: (i) size reduction; (i) pretreatment; and (iii) enzymatic hydrolysis [Zang and Lynd, 2004; Wyman, 1999]. Cellulase enzymes are expensive and several research groups are dedicated to reducing their production cost and improving their performance and resulting sugar yields [Howard et al., 2003]. Process design is also an important factor that has a significant influence on the cost of this technology. For example, combining the processes of enzymatic saccharification and fermentation of the cellulose-hydrolysis products to ethanol (or chemicals) has shown to result in considerable cost reductions. Simultaneous saccharification and fermentation (SSF) is the term used to describe this particular design [Deshpande et al., 1983]. Based on the correlation between the cost-effectiveness of the technology and the degree to which the process steps are combined (consolidated), Lynd et al. (2002) proposed a design referred to as consolidated bioprocessing (CBP). The goal of CBP is to develop a recombinant microorganism (also called a whole-cell biocatalyst) with the ability to simultaneously hydrolyze the pretreated substrate and ferment the resulting sugars to desired products.

The development of CBP technology has become increasingly popular within the field of ethanol production [Wooley et al., 1999; Wright, 1988; Wright et al., 1988]. Among the microbial hosts considered for CBP development, *Saccharomyces cerevisiae* has received significant interest from the biotechnology community as the yeast preferred for ethanol production. The major advantages of *S. cerevisiae* include high ethanol productivity and tolerance, as well as a well-developed gene expression system [Lynd et al., 2002]. Since *S. cerevisiae* is non-cellulolytic, the functional expression of at least three groups of enzymes, namely endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) is a prerequisite for cellulose conversion via CBP [Henrissat, 1994]. The endo and

exoglucanases act synergistically to efficiently degrade cellulose to soluble cellodextrins and cellobiose. Subsequently, β-glucosidases catalyze the conversion of the soluble cellulose hydrolysis products to glucose [Mansfield and Meder; 2003]. Several groups have obtained a measure of success with the heterologous production of cellulases in *S. cerevisiae* [Fujita et al., 2004; 2002; Cho and Yoo, 1999; Murai et al., 1998; Van Rensburg et al., 1998; Van Rensburg et al., 1996]. The *S. cerevisiae* whole-cell biocatalyst constructed by Fujita et al. (2004) co-displays the *Trichoderma reesei* endoglucanase II (EGII) and cellobiohydrolase II (CBHII) and *Aspergillus aculeatus* β-glucosidase I (BGLI) on its cell surface. High cell densities of the recombinant yeast (~8.7 grams of dry cell weight per liter) were able to convert a significant amount of amorphous cellulose to ethanol (final concentration of 2.9 $g.L^{-1}$ after 40 hours). However, it did not produce sufficient cellulolytic activity to facilitate growth of the yeast on amorphous cellulose.

The hydrolysis of soluble cellodextrins and cellobiose by β-glucosidases has a major influence on the overall rate and extent of cellulose hydrolysis [Yan et al., 1998]. The accumulation of extracellular cellobiose has two disadvantages, namely it causes feedback inhibition of endoglucanases and cellobiohydrolases and the action of β-glucosidases releases glucose in the external environment that increases the risk of contamination. Since *S. cerevisiae* lacks a dedicated cellobiose transporter/permease, previous efforts focused on either secretion [Van Rooyen et al., 2005; Cummings and Fowler, 1996] or attachment of the β-glucosidase enzyme to the yeast cell surface [Van Rooyen et al., 2005; Fujita et al., 2004; 2002]. Engineering *S. cerevisiae* to effectively transport and utilizes cellobiose intracellularly would be of particular interest when the final goal of industrial-scale ethanol production from cellulosic waste is considered.

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OBJECTIVES OF THIS STUDY

The objective of this study was the functional expression of β-glucosidase genes in *S. cerevisiae* and subsequent evaluation of the recombinant cellobiose-fermenting strains in order to obtain a suitable candidate for future CBP technology.

The specific aims of the present study were as follows:

(i) The isolation of β-glucosidases from various fungal origins and their functional expression in the yeast *S. cerevisiae*;

(ii) The characterization of the recombinant β-glucosidase enzymes produced by *S. cerevisiae*;

(iii) The optimization and characterization of growth kinetics of the recombinant *S. cerevisiae* strains in controlled bioreactors on cellobiose as sole carbon source;

(iv) The functional expression of an intracellular β-glucosidase in *S. cerevisiae*;

(v) Adaptation of the *S. cerevisiae* native disaccharide transport system(s) to facilitate cellobiose transport and subsequent intracellular utilization.

(vi) To study the growth kinetics of the recombinant *S. cerevisiae* strain that has been adapted for cellobiose transport and intracellular utilization in a controlled bioreactor.

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CHAPTER 2

Literature Review

Cellulose: Structure, Utilization and Biotechnology

CELLULOSE: STRUCTURE, UTILIZATION AND BIOTECHNOLOGY

Cellulose is the most abundant, renewable bioorganic macromolecule on earth with an annual production of 60 Gt of carbon in terrestrial and 53 Gt in marine ecosystems (1 Gt = 10^{12} kg) [Cox et al., 2000]. Cellulose is the key component of plant cell walls providing structural stability. The seed hairs of cotton plants represent a relatively pure form of cellulose (~95% cellulose), but more commonly, it is combined with lignin and other polysaccharides (so-called hemicelluloses) in the cell wall of woody plants [Sun and Cheng, 2002]. Lignocellulosic material in forests (primarily wood) represents the most important source of cellulose [Krässig, 1993]. Other cellulose-containing materials include agriculture residues, water plants, grasses, and other plant material. Table 1 presents the chemical composition of a number of typical cellulose-containing materials.

Table 1. Chemical composition of cellulose-containing materials [Sun and Cheng, 2002].

Source	% Cellulose	% Hemicellulose	% Lignin
Hardwood	40-55	24-40	18-25
Softwood	45-50	25-35	25-25
Bagasse	40	30	20
Corn Stover	40	30	25
Cotton	95	2	1
Flax (retted)	71	21	2
Hemp	70	22	6
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Switchgrass	45	31.4	12
Nut shells	25-30	25-30	30-40
Waste paper from chemical pulps	60-70	10-20	5-10
Wheat Straw	30	50	15

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CELLULOSE

Structural properties

On a molecular level, cellulose consists of linear polymeric chains of β-1,4-glycosidic linked D-glucopyranose units (Figure 1). The degree of polymerisation (DP), i.e. the number of glucose units included in a cellulose chain, is generally in the range of 8,000–12,000 for plant cellulose [Fan et al., 1980]. The cellulose chains have a strict polarity with one end containing a free C_1 semi-aldehyde group (reducing end) and the other a free OH group at C_4 (non-reducing end). ^{1}HNMR spectroscopy revealed that the β-D-glucopyranose residues adopt the $^4C_1$ chain conformation, which is the lowest free energy conformation of the molecule [Krässig, 1993]. As a result, the hydroxyl groups are positioned in the ring plane (equatorial), while the hydrogen atoms are in the vertical position (axial). These free hydroxyl groups and the oxygen atoms (of both the pyranose ring and the glycosidic bond) play a central role in the formation of intra- and intermolecular hydrogen bonds that establish different hydrogen-bonding networks within the cellulose structure [Sarko and Muggli, 1974].

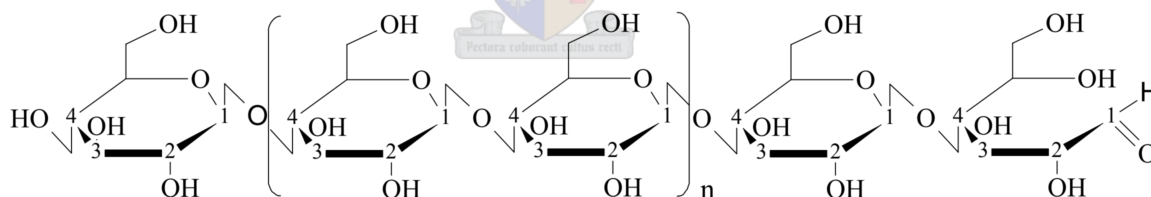


Figure 1. The molecular building blocks of cellulose comprise linear chains of β-1,4-linked anhydroglucose units (AGU). The non-reducing end is indicated at the left and contains a free OH-group at C_4, while the reducing end with its free C_1 semi-aldehyde group is indicated at the right.

Cellulose morphology involves a highly ordered structural design of fibrillar elements (Figure 2). About 36 individual cellulose molecules are assembled into larger units known as elementary fibrils (or protofibrils) [Brown et al., 1996]. The elementary fibril is considered the smallest morphological unit, with a diameter in the range of 3-35 nm (depending on the cellulose source). Elementary fibrils are tightly packed together into rod-like units called microfibrils. These microfibrils are associated though hydrogen and Van der Waals bonds, to form a very rigid macromolecular structure called a macrofibril. A single microfibril consists of about 20

elementary fibrils, while about 200 microfibrils are packed into a single macrofibril with a diameter in the range of micrometers [Krässig, 1993].

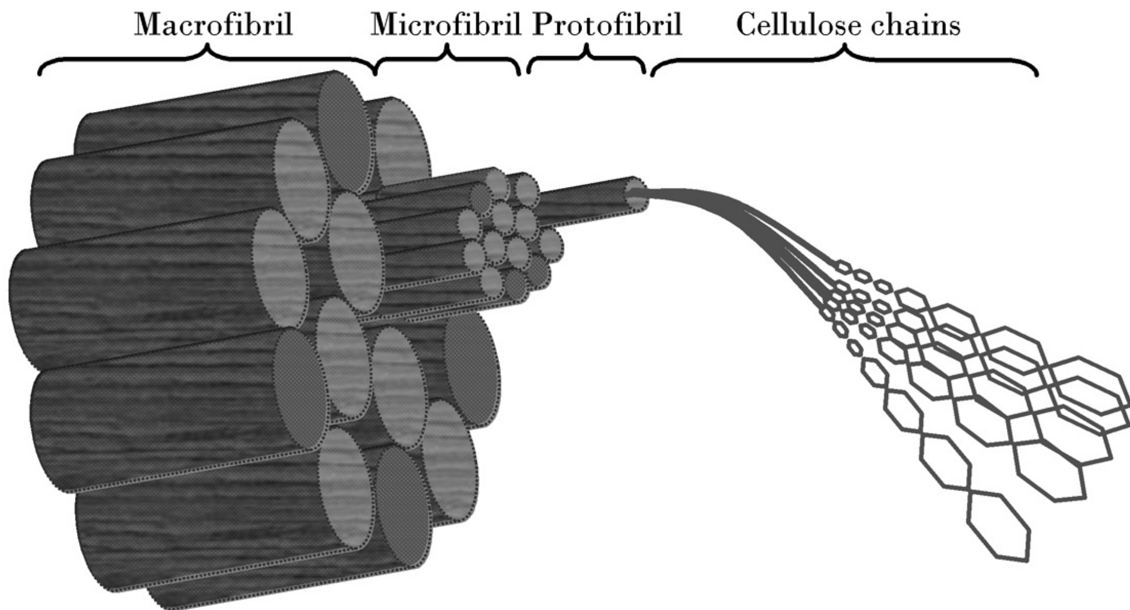


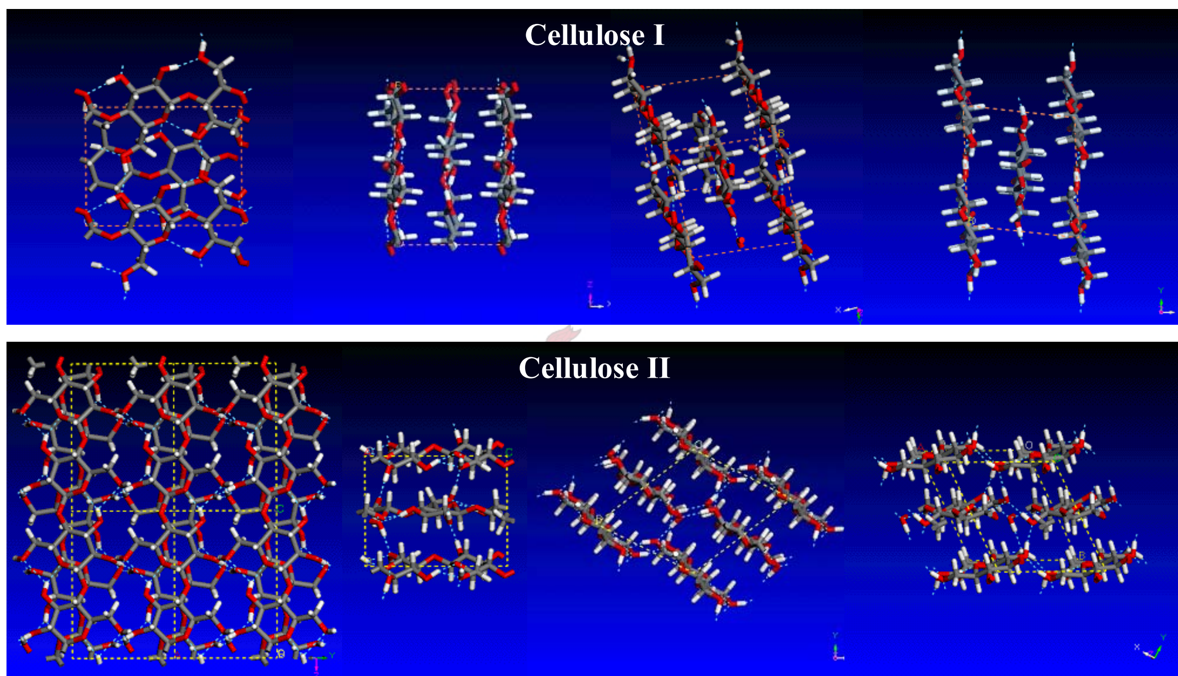
Figure 2. A schematic representation of the macromolecular structure of cellulose. Individual cellulose chains are assembled into larger units known as protofibrils, which are consecutively packed into larger rod-like units called microfibrils. The microfibrils are associated through Van der Waals bonds to form a very rigid macromolecular structure called the macrofibril [Clarke, 1997].

The order of the macrofibrils in a cellulose fibre is not uniform throughout the entire structure. There exist regions of low order (so-called amorphous regions) as well as of very high crystalline order. The current experimental evidence available is satisfactorily explained by a two-phase model, the fringed fibril model (as proposed by Hearle, 1958), presuming low-order (amorphous) and high-order (crystalline) regions and neglecting the relatively small amount of matter with an intermediate state of order [Zugenmaier, 2001].

The crystalline structure of cellulose is an important and relatively unique feature in terms of polysaccharides. Intra- and intermolecular hydrogen bonds reinforce the cellulose molecules and bring about different types of supra-molecular semi-crystalline structures. The degree of crystallinity of cellulose (in the range of 40% to 60%) depends on the origin and pre-treatment of the sample [Schenzel1 et al., 2005]. The entire structure of this hydrogen-bond network is still a

subject of debate. The intramolecular hydrogen bonding plays an important role in determining the single-chain conformation and rigidity [Sarko and Muggli, 1974; Gardner and Blackwell, 1974; Marchessault and Liang, 1960; Liang and Marchessault, 1959], whereas the intermolecular hydrogen bonds contribute to the sheet-like nature of the native polymer [Blackwell et al., 1977].

Figure 3. The simulated structures of the cellulose I and II allomorphs (www.accelrys.com).



Crystalline cellulose has at least two distinct allomorphs, cellulose I and cellulose II, as shown in Figure 3. Both are found in nature, however, cellulose I is considerably more widespread [Zugenmaier, 2001]. Interestingly, a wild-type strain of *Acetobacter xylinum* has been shown to produce cellulose I in liquid media vs. cellulose II when incubated on agar plate medium. It was suggested that cellulose II production is induced by the low mobility of cells in the culture medium due to physical barriers [Shibazaki et al., 1998]. In cellulose I, the adjacent sheets overlie one another and are held together by weak intersheet Van der Waals bonds. Regardless of the weakness of these interactions, their total effect in the overall structure of the elementary fibril is significant [Pizzi and Eaton, 1985]. Due to its high crystallinity, cellulose II is the most thermodynamically stable allomorph of cellulose. In cellulose II the chains have an antiparallel arrangement as well as inter-sheet hydrogen bonding. Cellulose I can be converted directly to cellulose II when treated with alkali (mercerization, a technique used in the textile industry),

whereas the reverse is not possible. During this complex process interdiffusion of the microfibrils result in the parallel arrangement (cellulose I) being converted to an antiparallel arrangement (cellulose II). This conversion poses thermodynamic problems that have not been solved [Zugenmaier, 2001].

In addition to crystalline and amorphous regions, cellulose fibres contain a variety of irregularities, such as bends or twists of the microfibrils, or cavities such as surface micro-pores, large pits, and capillaries [Marchessault and Sundararajan, 1993; Fan et al., 1980; Cowling, 1975; Blouin et al., 1970]. This heterogeneity within the cellulose fibre can result in partial hydration (when immersed in aqueous medium) and some micro-pores and capillaries can even allow the penetration of larger molecules such as cellulolytic enzymes [Stone et al., 1969; 1968].

Finally, cellulose fibres are embedded in a matrix of other structural biopolymers, mainly hemicelluloses and lignin. Although the matrix interactions differ with plant cell type and with maturity [Wilson, 1993], they are a prominent structural feature limiting the rate and extent of utilization of crude biomass.

Biosynthesis

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Electron microscopy of freeze-fractured plasma membranes of various organisms (vascular plants, algae, ferns, mosses, etc.) allowed the identification of organized membrane complexes located at one end of the microfibrils as the sites of cellulose synthesis [Kimura et al. 1999a; Brown 1996]. In vascular plants these complexes have a six-fold symmetry and are called "rosettes" [Delmer, 1999; Brown, 1996]. Structures similar to rosettes have also been identified in other cellulose-synthesizing organisms and are generally referred to as "terminal complexes" (TCs). Interestingly, there is a direct link between the structure of the TC and the size of the cellulose microfibril. For example, the rosettes found in vascular plants (and some green algae) produce microfibrils that consist of 36-90 glucan chains, while the large linear TCs in the green alga *Valonia macrophysa* synthesize microfibrils of up to 1,400 chains [Saxena and Brown, 2005].

TCs or rosettes can either be assembled at the plasma membrane or transported pre-assembled using the ER-Golgi-vesicle pathway [Saxena and Brown, 2005]. On a molecular level, genes encoding cellulose synthases (*CesA*) and cellulose synthase-like (*Csl*) proteins have been identified for more than 170 plant species (http://cellwall.stanford.edu). It seems that a combination of different *CesA* gene products is necessary for the construction of a functional rosette [Doblin et al., 2002]. Recently, the isolation of intact rosettes has revealed valuable information regarding its *in vitro* function as well as multimeric structure. Cross-sections of rosettes showed that the characteristic rosette morphology represents only a fraction of the structural unit that is exposed to the extracellular side of the plasma membrane and that most of the actual complex is deeply embedded in the cytoplasm [Saxena and Brown, 2005]. The revised structural model presented in Figure 4 illustrates the mechanism for cellulose I biosynthesis in plants.

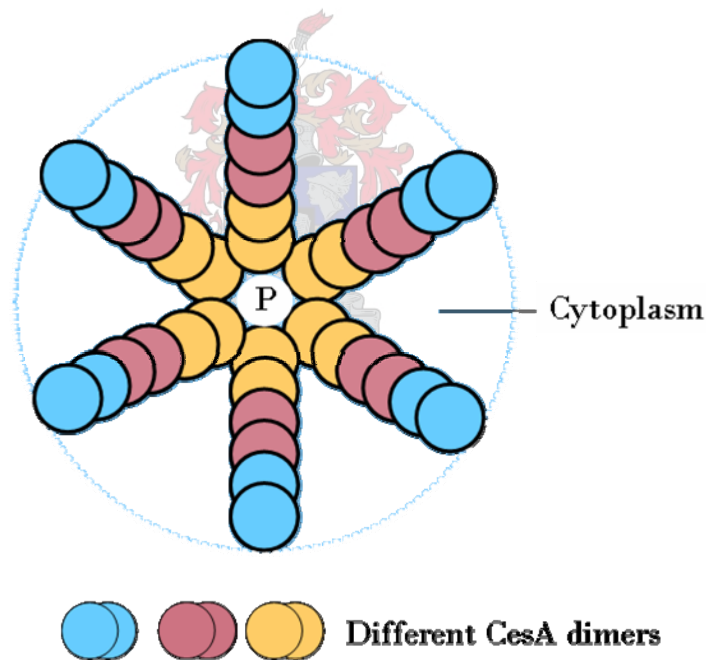


Figure 4. Cytoplasmic view of the structure of a plant rosette. The linear rows of CesA dimers are primarily responsible for the synthesis of individual β-glucan chains from UDP-glucose substrate. The chains from each row associate though van der Waals interactions to generate β-glucan sheets. Finally, the sheets assemble into cellulose microfibrils while being extruded through the pore-like structure (P) in the cell membrane [Saxena and Brown, 2005].

Homodimers of at least three different *CesA* gene products associate to form linear rows of catalytic subunits. The first phase of crystalline cellulose production, namely glucan sheet assembly, is catalyzed by the linear rows comprising of three different cellulose synthase dimers. The CesA dimers utilize uridine diphospho-glucose (UDP-glucose) and is responsible for the polymerization of individual β-(1,4)-glucan chains. The glucan chains resulting from each linear row associate through Van der Waals interactions to produce a glucan chain sheet. The adjacent sheets assemble through hydrogen-bonding to from the crystalline cellulose I microfibril while being extruded from the cell through a pore-like structure [Saxena and Brown, 2005]. Although no proteins are directly implicated in the crystallization process, it was suggested that the proteins involved in the organisation of the CesA dimers as well as the export of the glucan sheets may contribute to this process [Arioli et al., 1998].

In addition to cellulose's general role in providing structural stability to resist turgor pressure in the plant, it is also vital in maintaining the size, shape and differentiation potential of cells. Interestingly, the amount and direction in which the cellulose microfibrils are incorporated into the cell wall is directly linked to the direction of plant cell growth/elongation. It is currently assumed that the process of cell elongation takes place in a direction perpendicular to that of microfibril synthesis [Saxena and Brown, 2005]. It is clear that cellulose synthesis is a complex and carefully regulated process that has to be coordinated with other processes such as growth and differentiation. Identification of the signals and how they are sensed by the cellulose-synthesizing rosette is an exciting challenge for future research.

MICROBIAL DEGRADATION OF CELLULOSE

In nature, cellulose-containing materials are considered a major energy source for microorganisms. Microbial degradation of cellulose can occur in both aerobic and anaerobic environments. The ability to degrade cellulose aerobically is well-known among fungi and specifically among members of the Ascomycota and Basidiomycota groups [Lynd et al., 2002]. Although most of the aerobic cellulose-degrading activity present in soil is associated with fungi, several soil bacterial species in both filamentous (e.g. *Streptomyces*, *Micromonospora*) and non-

filamentous (e.g. *Bacillus*, *Cellulomonas*, *Cytophaga*) genera are able to perform cellulose degradation aerobically [De Boer et al., 2005; Lynd et al., 2002].

Obligately anaerobic bacteria (e.g. *Acetivibrio*, *Clostridium*, *Ruminococcus*) are considered the species responsible for the majority of cellulolytic activity that occurs in most anaerobic environments [Lynd et al., 2002; Leschine, 1995]. However, cellulose-degrading activity has also been found in a number of anaerobic fungi that belong to the phylum Chytridiomycota, a group that colonizes the gastrointestinal tracts of ruminant animals [Teunissen et al., 1991; Bornemann et al., 1989]. Anaerobic bacteria produce highly-effective complexed cellulase systems (termed cellulosomes) that allow the synergistic action of different cellulase enzymes and limit the distance over which the cellulose hydrolysis products have to diffuse to the cells [Doi and Kosugi; 2004; Lynd et al., 2002]. Studies on ruminal bacteria have also indicated that cellulosomes play an important role in positioning the cellulase-producing cells at the site of hydrolysis. In contrast, aerobic cellulolytic fungi and bacteria produce freely diffusible extracellular cellulase enzyme systems comprising of endoglucanases, cellobiohydrolases and β-glucosidases that act in a concerted manner to effectively degrade cellulose [Mansfield and Meder; 2003; Lynd et al., 2002].

The unique properties of a particular cellulose-containing substrate (e.g. association with hemicellulose and lignin, overall accessibility of reactive sites, pH, etc.) are a major cause of niche differentiation among cellulolytic microorganisms [De Boer et al., 2005]. Various studies have aimed to identify and examine the unique features and strategies employed by microorganisms to obtain a selective advantage within a specific niche.

Within the soil environment, the production of hyphae by cellulolytic actinomycetes and fungi seems to be a valuable strategy to penetrate cellulose fibres (via micro-pores in the cell wall material) and secure contact between the cellulases and cellulose polymer [Lynd et al., 2002]. The thin hyphae produced by soft-rot fungi can penetrate the woody cell wall layer in order to get to the cellulose. Figure 5 illustrates the chains of diamond-shaped cavities that form in the direct vicinity of the hyphae as a result of hyphal growth along the microfibrils. Conversely, white-rot fungi gain access to cellulose within lignified plant material through degradation of the lignin polymers [Lynd et al., 2002; Leonowichz et al., 1999; Tuor et al., 1995]. The ability to degrade

lignin is not prevalent, and some fungi, such as brown-rot or soft-rot fungi, access cellulose in woody materials by other processes. Brown-rot fungi can only to a limited extent degrade lignin. However, they have the ability to modify lignin in such a manner that they can access the cellulose within the lignocellulosic complex. The degradation of crystalline cellulose by brown-rot fungi occurs through a strategy that combine enzymatic (mostly endo-acting enzymes, e.g. endoglucanases) and non-enzymatic systems [Goodell, 2003; Bennett and Feibelman, 2001; Green and Highley, 1997]. Non-enzymatic systems include pH reduction (e.g., by secretion of oxalate) and the secretion of iron-containing low molecular weight glycopeptides that produce hydrogen peroxide. This result in the production of free radicals by the Fenton reaction: $Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + .OH + OH^-$. Free radicals can diffuse freely into the woody cell wall layer where they contribute to the degradation of lignocellulose. There are several hypotheses regarding the interaction of low molecular weight metabolites, metals and radicals and their role in the degradation process, but it will not be discussed in further detail [De Boer et al., 2005; Goodell, 2003].

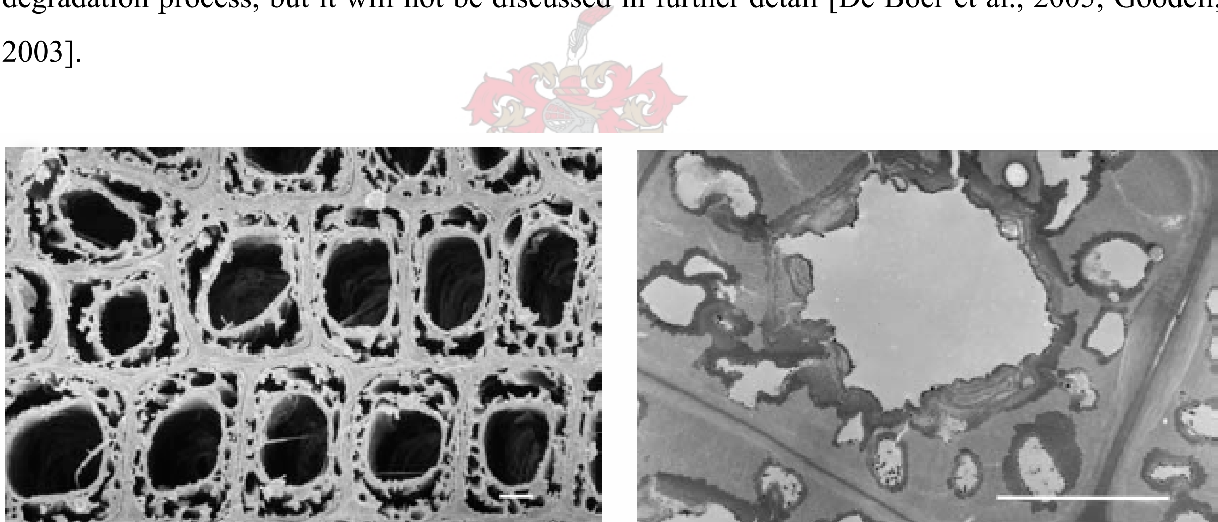
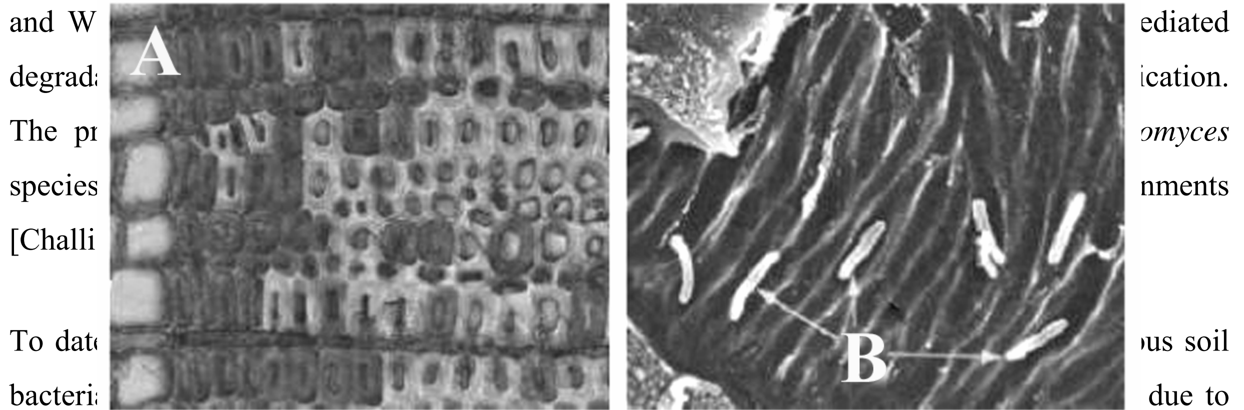


Figure 5. Diamond-shaped cavities in the direct vicinity of the hyphae as a result of hyphal growth along the microfibrils [Blanchette, 2003].

Decomposition of wood is mainly performed by cellulolytic fungi. Interestingly, hyphae-producing actinomycetes are hardly ever implicated in wood decay [Daniel and Nilsson, 1998]. Although they seem to be the most likely direct competitors of cellulolytic fungi, many cellulolytic actinomycetes are incapable of degrading crystalline cellulose [Wirth and Ulrich, 2002; McCarthy and Williams, 1992]. In addition, their cellulase and hemicellulase enzymes have optimal activity at neutral to alkaline pH, whereas fungal enzymes perform best at low pH [McCarthy, 1987]. Both the acidic nature of wood [Stamm, 1961], and the increased

acidification produced by fungal activity, may limit growth of cellulolytic actinomycetes in this particular niche [Goodell, 2003; Tuor et al., 1995]. In contrast, an alkaline environment such as compost (and associated substrates) favours cellulose degradation by actinomycetes [McCarthy and W ediated degrad ication. The pr *omyces* species nments [Challi



To dat us soil bacteri due to their inability to penetrate solids. Interestingly, upon addition of cellulose to agricultural soil there is an initial phase featuring bacterial cellulose degradation, followed by a phase dominated by fungal activity [Hu and Van Bruggen, 1997]. This probably suggests an opportunistic strategy of cellulolytic soil bacteria whereby they react immediately when easily accessible cellulose is present. This may include the production of inhibitory metabolites to "protect" the cellulosic substrate they colonize from attack by actinomycetes and fungi [De Boer et al., 2005]. Cellulose-degrading bacteria, e.g. *Bacillus pumilis*, have the potential to produce antifungal metabolites, however, they have not been investigated in the context of cellulose degradation [Munimbazi and Bullerman, 1998].

Occasionally, non-filamentous cellulolytic bacteria are present in decaying wood. Though, colonization is usually restricted to parts of the wood containing easily accessible cellulose, hemicellulose and pectin, as well as woody environments unfavourable for fungal growth [Daniel and Nilsson, 1998; Clausen, 1996; Rayner, 1988]. Figure 6 indicates the typical patterns associated with activity of these bacteria: (i) tunneling (or cavitation) in the cell wall layer; and (ii) erosion (visible as indents, channels and "honeycomb" patterns) of the cellulose fibres [De Boer et al., 2005; Clausen, 1996]. In addition to the cellulolytic non-filamentous bacteria, a number of soil bacteria produce incomplete cellulolytic systems [Rabinovich et al., 2002]. The cellulases of these bacteria, together with other hydrolytic enzymes, may be involved in the penetration of living plants in either pathogenic or endophytic relationships [De Boer et al., 2005].

Figure 6. Erosion and tunneling of cellulose fibers. (A) is a cross-section through wood showing fresh (lighter) and eroded (darker) cells. (B) is a SEM (scanning electron microscope) picture of bacteria (~2µm) tunneling through the wood structure [Björdal, 2000].

ENZYMES INVOLVED IN DEGRADATION

Cellulases

Cellulose degradation is a phenomenon that is not restricted to microorganisms. Some animal species, including termites and crayfish, produce cellulolytic enzymes distinct from those of their indigenous microflora. Although controversial at first, molecular evidence has confirmed their existence. The role of these enzymes in connection with the nutrition of the animal is still uncertain [Watanabe and Tokuda, 2001]. In plants, cellulolytic enzymes are produced during physiological phases in which a separation of tissues is necessary, e.g. during fruit ripening and the loss of leaves. Generally, there is no cellulolytic activity present in growing plants [Klemm et al., 2002].

Complete hydrolysis of cellulose to glucose involves the cooperative action of three groups of enzymes with different substrate specificities: (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-β-D-glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) β-glucosidases or β-D-glucoside hydrolases (EC 3.2.1.21) [Beguin and Lemaire, 1996; Leschine, 1995; Beguin, 1990]. Cellobiohydrolases remove cellobiose units in a processive manner from the reducing or non-reducing ends of the cellulose chains. They are also active against microcrystalline cellulose, most likely peeling cellulose chains from the microcrystalline structure. The endoglucanases randomly cut the cellulose chains at internal amorphous sites, generating oligosaccharides of various lengths and consequently providing the cellobiohydrolases with further chain ends to act upon [Teeri, 1997]. Finally, β-glucosidases (EC 3.2.1.21) hydrolyze soluble cellodextrins and cellobiose to glucose, thereby providing an easily metabolisable carbon source for the cellulolytic microorganism [Beguin, 1990]. A schematic view on the synergistic action of cellulolytic enzymes is shown in Figure 7.

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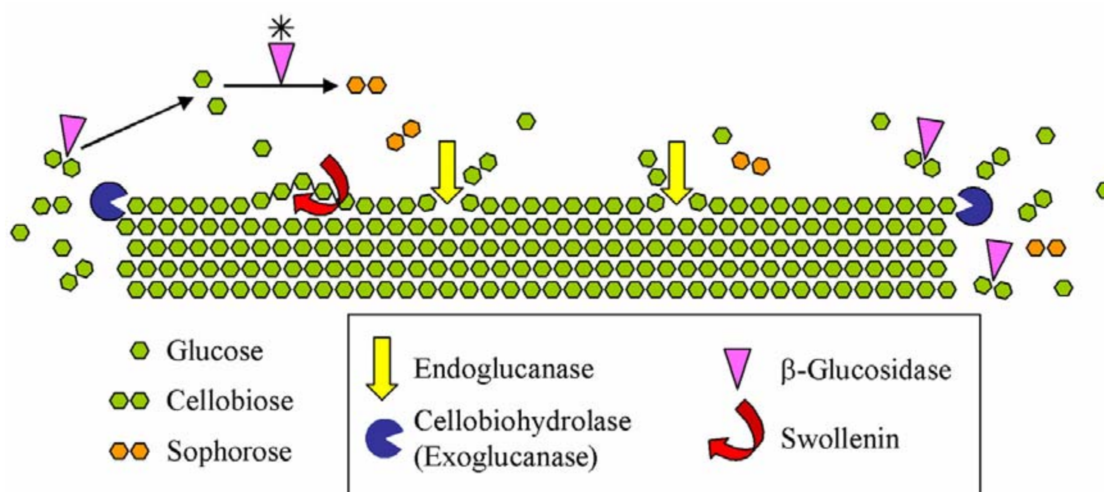
In addition to the more typical cellulases, novel types of cellulases such as the *Trichoderma reesei* swollenin (SWOI) have been identified. SWOI has a high amino acid homology with plant expansins. Expansins disrupt cellulose fibres but lack hydrolytic activity. Therefore, swollenin is probably involved in making cellulose fibres more accessible for cellulases to act upon [Saloheimo et al., 2002].



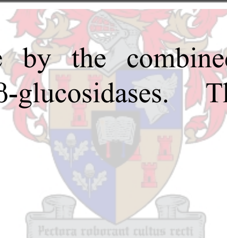


Figure 7. Degradation of cellulose by the combined action of different cellulolytic enzymes: exoglucanases, endoglucanases and β-glucosidases. The biosynthetic activity of β-glucosidase is indicated with an asterisk.

Hemicellulases

In spite of the relatively complex nature of hemicelluloses, their enzymatic degradation is well understood. The hydrolysis of hemicelluloses occurs via the synergistic action of endo-enzymes cleaving within the main chain, exo-enzymes releasing monomeric sugars and additional enzymes cleaving the side chains of the polymers or oligosaccharides to produce various mono- and disaccharides (depending on the type of hemicellulose) [Shallom and Shoham, 2003; Emami and Hack, 2002; Kimura et al., 2000; Gielkens et al., 1997]. For example, the degradation of xylan involves at least endo-1,4-β-D-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) to cleave the major sugar chain and, depending on the type of xylan, side-chain hydrolysing enzymes such as α-glucuronidase (EC 3.2.1.131) and acetyl xylan esterase (3.1.1.72) [De Vries and Visser, 2001]. Figure 8 gives a schematic view of the degradation of arabinoxylan as an example of a hemicellulolytic system.

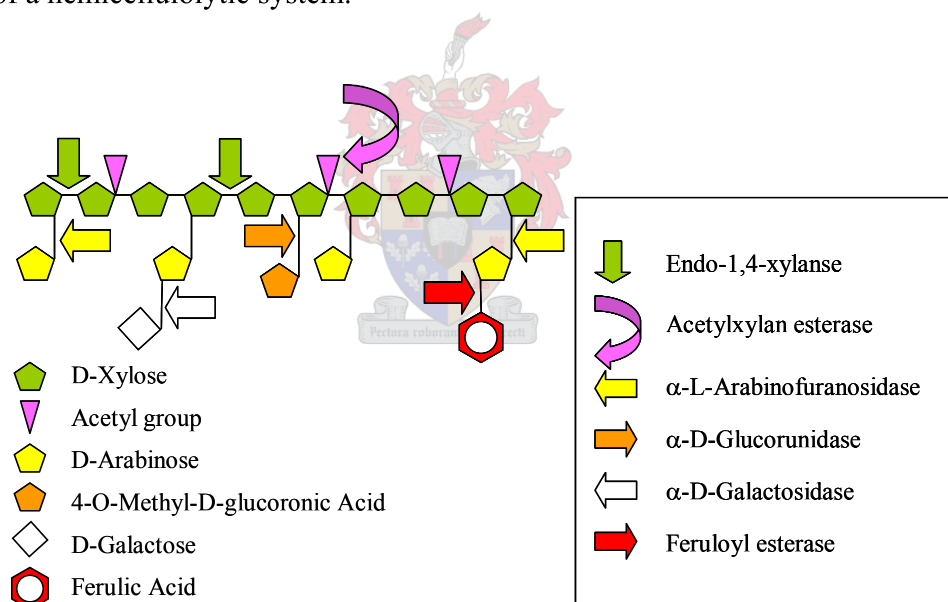


Figure 8. Enzymatic degradation of arabinoxylan by the combined action of different hemicellulases [Aro et al., 2005].

Ligninases

Since cellulose and hemicellulose are surrounded by a lignin matrix in the plant cell wall, its degradation is a prerequisite for hydrolysis of the former polysaccharides. The hydrophobicity and the complex random structure of lignin present a significant barrier for its degradation and

most cellulolytic microorganisms (with the exception of white-rot fungi) are not capable of efficiently hydrolyzing it [Gold and Alic, 1993]. In contrast to cellulases and hemicellulases, the enzymes involved in lignin degradation are oxidative, non-specific and act via non-protein mediators. The major fungal lignin-degrading enzymes are manganese peroxidases (MnP; EC 1.11.1.13) [Li et al., 1999; Lobos et al., 1998; Alic et al., 1997], lignin peroxidases (LiP; EC 1.11.1.14) [Stewart and Cullen, 1999; Zhang et al., 1991] and laccases (EC 1.10.3.1) [Mayer and Staples, 2002; Scheel et al., 2000]. MnP and LiP catalyze a variety of oxidative reactions that require hydrogen peroxide (H_2O_2), whereas laccases oxidize phenolic compounds and reduce molecular oxygen to water. Glyoxal oxidase and glucose-2-oxidase (EC 1.1.3.10) are two extracellular H_2O_2–producing enzymes that are important for peroxidase function and thus essential contributors to the delignification process [Tuor et al., 1995].

Recently, cellobiose dehydrogenase (CDH) [EC 1.1.99.18] has also been implicated in the delignification process. CDH is an extracellular enzyme produced by many cellulolytic fungi [Henriksson et al., 2000a]. CDH's supporting role in cellulose-degradation is highlighted by its ability to bind to cellulose and subsequently oxidizes cellobiose (the major product from cellulose degradation) via a variety of electron acceptors to produce the corresponding lactone [Henriksson et al., 2000b]. Upon oxidation of cellobiose, the hydroxyl radicals generated may convert non-phenolic lignin to phenolic lignin, making it susceptible to attack by other lignin-degrading enzymes, such as MnP and laccase [Stapleton et al., 2004].

CELLULASE ENZYME SYSTEMS

Microorganisms produce multiple enzymes to degrade plant cell polysaccharides, so-called enzyme systems [Warren, 1996]. Although this discussion focuses primarily on the action of cellulase enzyme systems, it is important to note that such systems are also active on hemicellulose and are generally co-produced by cellulolytic microorganisms.

Because of the taxonomic and ecological diversity of cellulolytic microorganisms, it is not surprising that the organization of cellulase systems is equally diverse. In the past, the components of cellulase systems were classified based on their mode of catalytic action, whereas current classification systems are based on the structural properties of the individual components

[Henrissat et al., 1998]. Cellulases differ from other glycoside hydrolases by their ability to cleave β-1,4-glycosidic bonds between glucosyl residues. The enzymatic hydrolysis of the β-1,4-glycosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base. The hydrolysis products can result either in the inversion (single replacement mechanism) or in retention (double replacement mechanism) of the anomeric configuration of C_1 at the reducing end [Withers, 2001; Birsan et al., 1998]. The detailed mechanism of acid hydrolysis is discussed later on. The three major types of enzymatic activities associated with cellulase systems are endoglucanases, exoglucanases, and β-glucosidases [Beguin and Lemaire, 1996; Leschine, 1995; Beguin, 1990]

Cellulose-degrading systems can be divided into two broad categories, i.e., complexed and non-complexed systems [Hazlewood and Gilbert, 1993]. Complexed (or cell associated) systems are generally produced by anaerobic microorganisms, including bacteria and fungi that colonize environments such as the rumen and hindgut of herbivores, composting biomass and sewage [Kajikawa and Masaki, 1999]. In certain anaerobic bacteria, e.g. *Clostridium* spp., the complexed enzymes are contained in distinct high-molecular-weight protein complexes called cellulosomes. Non-complexed systems (or "free enzyme" systems) are representative of aerobic fungi and bacteria and comprise several soluble cellulases and associated polysaccharide depolymerases that are secreted into the extracellular medium. Whereas this distinction between cellulase systems of aerobes and anaerobes is very common, it does not apply to all systems. For instance, facultative anaerobes from the genus *Bacillus* secrete non-complexed systems [Lo et al., 1988], whereas some cellulases in aerobic bacteria may be cell-bound [Schlochtermeier et al., 1992].

A common feature shared by most cellulases is a modular structure that may include catalytic and carbohydrate-binding modules (CBMs). The CBM has a two-fold role (i) binding of the enzyme to the cellulose surface and (ii) facilitating contact between the catalytic domain and insoluble cellulose substrate. The CBMs of exoglucanases play a central role in initiation of hydrolytic activity as well as their processivity [Teeri et al., 1998]. A possible additional (non-catalytic) role for CBMs is the "loosening" of the substrate to render it more susceptible to the action of the catalytic core [Din et al., 1994].

Cellulase systems display higher combined activity than the sum of the individual enzyme activities, a phenomenon known as synergism. Four types of synergism have been described: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases cleaving from the reducing and non-reducing ends of the cellulose chain, (iii) synergy between exoglucanases and β-glucosidases that hydrolyse cellobiose (and cellodextrins) as end products of the former enzymes, thereby relieving feedback inhibition, and (iv) intramolecular synergy between the catalytic domains and the carbohydrate-binding modules (CBM) [Lynd et al., 2002; Teeri, 1997; Din et al., 1994]. Apart from enzymes, the degree of synergism depends on the nature of cellulose [Mansfield et al., 1999]. Synergism is more evident on the semi-crystalline substrates with high DP [e.g. cotton and bacterial cellulose (BC)] than on substrates with very high crystallinity [e.g. *Valonia* cellulose] or lower DP, such as Avicel and bacterial microcrystalline cellulose (BMCC)] [Samejima et al., 1998].

Cellulase systems are not simply an assembly of enzymes representing the three enzyme groups (endoglucanases, exoglucanases, and β-glucosidases, with or without CBMs), but a coordinated interaction between enzymes to efficiently hydrolyze cellulose.

Non-complexed/Free enzyme systems

Pectora roborant cultus recti

Cellulases from aerobic fungi have been studied more intensely than those from any other group of microorganisms. Knowledge of the fungal cellulase system contributed significantly to the understanding of cellulose degradation as well as to the industrial applications of cellulases [Sheehan and Himmel, 1999; Nieves et al., 1998; Gusakov et al., 1991].

The most-studied cellulase system of *T. reesei* has been the focus of research for more than 50 years [Reese, 1956; Reese et al., 1950]. *T. reesei* produces a variety of cellulases that include at least two exoglucanases/cellobiohydrolases (CBH), five endoglucanases (EG), and two β-glucosidases [Nogawa et al., 2001; Takashima et al., 1999]. Interestingly, the two exoglucanases display different directionality on the cellulose chain. Cellobiohydrolase I (CBHI) prefers the reducing end, whereas cellobiohydrolase II (CBHII) acts from the non-reducing end. Thus, the action of the CBHI proceeding from one direction exposes buried chain ends that can be acted on by the CBHII with the opposite directionality [Gilkes et al., 1997]. This is an

illustration of the exo-exo synergy previously discussed for two exo-acting enzymes [Medve et al., 1994; Nidetzky et al., 1994; Chanzy and Henrissat, 1985]. CBHI is regarded as the major enzyme in the degradation of crystalline cellulose. It comprises about 60% of the total cellulolytic proteins, whereas CBHII composes about 20%. CBHI by itself is able to hydrolyze crystalline cellulose significantly, although the rate of degradation is slow [Fägerstam and Pettersson, 1980].

The three-dimensional (3-D) structures of these two enzymes have been determined with crystallography. The catalytic domain of CBHI contains a β-sandwich structure with a 50 Å-long substrate-binding tunnel shaped by the inner β-sheets and four surface loops [Divne et al., 1998; Divne et al., 1994]. Interestingly, the catalytic domain of CBHII is an α/β protein with a fold totally different from that of CBHI. It contains two surface loops that give rise to a tunnel of 20 Å. The tunnel topology of the active site proved to be crucial to the processive character of these enzymes, meaning that a bound enzyme will not disconnect from the cellulose chain before its complete degradation. The surface loops of CBHII can undergo movements, leading to the closing or opening of the tunnel roof [Varrot et al., 1999; Zou et al., 1999]. Apparently, these movements are responsible for the observed endo-activity and lower processivity of CBHII [Boisset et al., 2000]. Hydrolysis proceeds via a double- and single-displacement mechanism for CBHI and CBHII, respectively [Claeyssens et al., 1990]. The 3-D structure of CBHI and CBHII also verified that cellobiose is the major degradation product as the cellulose chain passes through the tunnel. Cellotriose or glucose is sometimes released during early stages of hydrolysis [Divne et al., 1994].

The most abundant of the *T. reesei* endoglucanases, endoglucanase I (EGI), accounts for about 5-10% of the total cellulase enzymes [Bhikabai et al., 1984]. The catalytic domain of EGI is structurally related to CBHI (45% identity), however the existence of shorter loops result in an open groove-shaped active site rather than the long enclosed tunnel of CBHI [Kleywegt et al., 1997]. The groove is consistent with the endo-type mode of action [Teeri, 1997]. EGI acting on cellulose generate nearly equal amounts of cellobiose and glucose together with some cellotriose [Karlsson et al., 2002]. In addition to the activity on cellulose, EGI exhibit considerable xylanolytic activity [Lawoko et al., 2000]. Endoglucanases are predominantly responsible for decreasing DP by randomly cleaving cellulose chains at relatively amorphous regions, thereby

generating new cellulose chain ends susceptible to the action of cellobiohydrolases [Teeri et al., 1998]. The necessity for five endoglucanase species in the *T. reesei* cellulase system is unclear. Especially considering that endoglucanases (with EGI and EGII as main species) represent less than 20% of the total cellulolytic proteins of *T. reesei*. Synergism between endoglucanases and cellobiohydrolases has been shown for EGI [Väljamäe et al., 1998], and EGII [Medve et al., 1998], and EGIII [Nidetzky et al., 1994]. However, synergism between endoglucanases has not been confirmed. Degradation of cellulose and hemicellulose as natural intertwined substrates may explain the diversity of endoglucanases.

The two β-glucosidases produced by *T. reesei* are responsible for the hydrolysis of cellobiose and small oligosaccharides to glucose. The major fraction of both, BGLI and BGLII, are cell wall bound [Messner et al., 1990, Usami et al., 1990]. The close contact between the β-glucosidases and fungal cell wall presumably limit the loss of glucose to the environment during cellulose degradation. *T. reesei* produces β-glucosidases at low concentrations compared to other fungi such as *Aspergillus* species [Reczey et al., 1998]. In addition, the β-glucosidases of *T. reesei* are subject to product (glucose) inhibition [Chen et al., 1992; Gong et al., 1977; Maguire, 1977] whereas those of *Aspergillus* species are more glucose tolerant [Decker et al., 2000; Gunata and Vallier, 1999; Yan and Lin, 1997; Watanabe et al., 1992]. The levels of β-glucosidase produced by *T. reesei* are sufficient for sustaining growth on cellulose, but not adequate for extensive in vitro saccharification of cellulose. The most popular cellulase preparations used for the saccharification of cellulose on industrial scale contain *T. reesei* cellulases supplemented with *Aspergillus* β-glucosidase [Reczey et al., 1998; Sternberg et al., 1977].

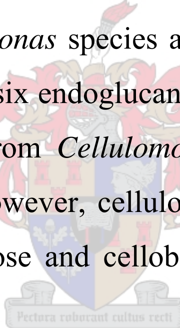
The thermophilic fungus *Humicola insolens* produces a series of enzymes comparable to the *T. reesei* cellulase system. The *H. insolens* system includes two cellobiohydrolases and five endoglucanases [Schülein, 1997]. Both the *H. insolens* EGI and EGIII lack CBMs. Efficient saccharification of crystalline cellulose can be achieved with a mixture of CBHI, CBHII, and EGV [Boisset et al., 2001]. However, optimal saccharification (more than 50% microcrystalline cellulose) occurs when the mixture contains about 70% and 30% of total protein as CBHI and CBHII, respectively. Though endoglucanase EGV is essential for effective crystalline cellulose

hydrolysis by either CBHI or CBHII, only low levels (1-2% of the total protein) are needed for maximum efficiency [Boisset et al., 2001].

The white rot fungus *Phanerochaete chrysosporium* is used as a model organism for lignocellulose degradation [Broda et al., 1994]. Elaborate collections of cellulases, hemicellulases, and lignin-degrading enzymes are produced by *P. chrysosporium* to efficiently hydrolyze the three major components of plant cell walls: cellulose, hemicellulose, and lignin [Broda et al., 1996; Broda et al., 1995; Copa-Patino et al., 1993, Vanden Wymelenberg et al., 1993; Covert et al., 1992]. Cellulose and hemicellulose degradation occur during primary metabolism, while lignin degradation is a secondary metabolic event initiated by carbon, nitrogen, or sulfur limitation [Broda et al., 1996]. *P. chrysosporium* produces a cellulase system with CBHII and six CBHI-like homologues, of which CBHI-4 is the main cellobiohydrolase [Vanden Wymelenberg et al., 1993; Covert et al., 1992]. EG28, an endoglucanase without a CBM, has significant homology with the EGIII of *T. reesei* and *H. insolens*. Synergism between EG28 and the cellobiohydrolases has been confirmed [Henriksson et al., 1997]. Until now, no other typical endoglucanases have been isolated from *P. chrysosporium*. However, it is suggested that differential splicing within the CBM-encoding region of the *cbh*I-like genes of *P. chrysosporium* may yield cellobiohydrolase and/or endoglucanase activity depending on the available substrate [Birch et al., 1995]. *P. chrysosporium* also produces two different extracellular cellobiose-utilizing enzymes, namely β-glucosidase (BGL; EC 3.2.1.21) [Igarashi et al., 2003; Kawai et al, 2003] and cellobiose dehydrogenase (CDH; EC 1.1.99.18) [Yoshida et al., 2001; Li et al., 1996; Raices et al., 1995; Bao et al., 1993]. Cellobiose can either be metabolized through the hydrolytic pathway where BGL cleaves it to glucose or the oxidative pathway where it is converted to cellobionolactone by CDH (Figure 9). Since BGL and CDH are secreted from a single isozyme (enzymes that catalyse the same reaction but are encoded by different genes), it was assumed that cellobiose is first oxidized by CDH and the resulting cellobionolactone hydrolyzed by BGL to gluconolactone and glucose. However, BGL has poor substrate affinity towards cellobionolactone, therefore the two enzymes compete for cellobiose as substrate [Henriksson et al., 1998]. In addition, CDH has the ability to bind microcrystalline cellulose and also positively contribute to it effective degradation. It was therefore suggested that CDH has a prominent role in cellulose hydrolysis [Henriksson et al., 1997; Bao and Renganathan, 1992]. Igarashi et al. (2003) determined that the Michaelis constant for cellobiose is 80 times higher for

BGL than for CDH thereby illustrating that CDH has a significant kinetic advantage over BGL in cellobiose metabolism. Transcriptional analysis also confirmed that *cdh* transcription is initiated by cellobiose, whereas *bgl* transcription is repressed. It is therefore assumed that CDH is the major enzyme responsible for cellobiose metabolism in *P. chrysosporium* during growth on cellulose [Yoshida et al., 2004]. Cellobiose dehydrogenase may also generate hydroxyl radicals that could aid in lignin and cellulose depolymerisation [Henriksson et al., 2000].

Thermobifida and *Cellulomonas* are among the best-studied species of cellulolytic aerobic bacteria. The thermophilic filamentous bacterium *T. fusca* plays an important role in cellulose degradation in soil. *T. fusca* produces two exoglucanases (Cel6B /E3 and Cel48A/E6), three endoglucanases (Cel5B/E1, Cel6A/E2 and Cel5A/E5) and an unusual cellulase (Cel9A/E4), that exhibit both endoglucanase and exoglucanase activity. This latter enzyme acts synergistically with the endoglucanases and exoglucanases to efficiently degrade microcrystalline cellulose [Irwin et al., 1998; 1993]. *Cellulomonas* species are coryneform bacteria that produce at least one exoglucanase (Cel6B) and up to six endoglucanases (Cel 5A, Cel6A, etc.) [Chaudhary et al., 1997]. The individual cellulases from *Cellulomonas* contain CBMs that are similar to the cellulase system of aerobic fungi, however, cellulosome-like structures have been observed on *Cellulomonas* cells grown on cellulose and cellobiose as substrates [Lamed et al., 1987; and Vladut-Talor et al., 1986].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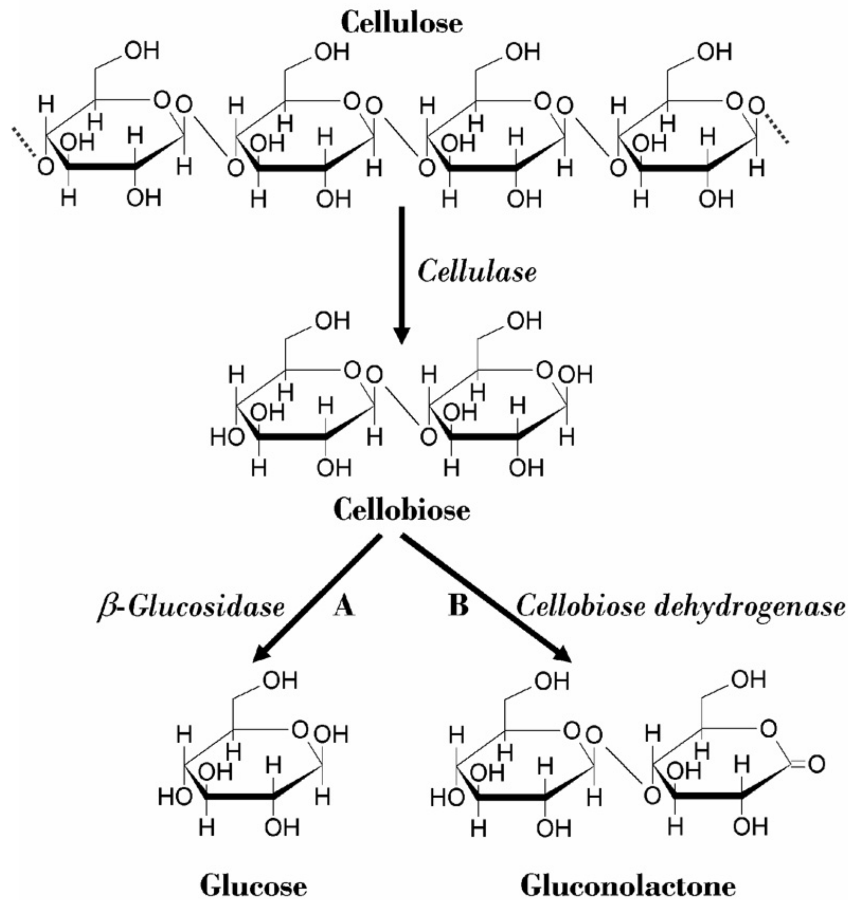

Figure 9. The hydrolytic (A) and oxidative (B) pathways of cellobiose metabolism in *P. chrysosporium*. [Yoshida et al., 2004].

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The thermophilic and hyperthermophilic prokaryotes represent a distinct group of microorganisms that grows at temperatures of 100°C and above. A number of cellulolytic hyperthermophiles have been described in the past decade, for example, *Rhodothermus marinus*, *Caldibacillus cellulovorans*, etc. [Bergquist et al., 1999]. Despite the evidence that archaea can utilize cellobiose and other abundant polysaccharides (such as starch, chitin, and xylan), no cellulolytic thermophilic archaea have been isolated [Driskill et al., 1999, Sunna et al., 1997]. *Acidothermus cellulolyticus* (an actinomycete), *C. cellulovorans* and *Rhodothermus* are the only aerobic thermophilic bacteria in which cellulase production has been confirmed [Halldórsdóttir et al., 1998; Sakon et al., 1996].

Complexed enzyme systems

Caldicellulosiruptor, a genus of highly cellulolytic anaerobic hyperthermophiles, produces hydrolytic enzymes with a unique multifunctional, multi-domain structure [Bergquist et al., 1999]. Many of these "megazymes" contain five or more domains, which include a variety of cellulases, hemicellulases, and CBMs. The multi-functional cellulases contain modules from endoglucanases and cellobiohydrolases in varying pairs, together with mannanases and xylanase units [Gibbs et al., 2000; Zverlov et al., 1998]. The intramolecular synergism acquired within such a "megazyme" produces optimal activity and/or processivity to function at very low enzyme concentrations [Schwarz, 2001].

An even more sophisticated cellulase system found in anaerobic microorganisms involves the formation of large, extracellular enzyme complexes called cellulosomes. The cellulosome consist of a scaffolding protein and several bound enzymes [Bayer et al., 1985]. Initially, cellulosome production was associated exclusively with anaerobic bacteria. Recently, experimental evidence was obtained for the existence of cellulosomes in anaerobic fungi, despite the fact that the scaffoldin gene remains unidentified. A list of cellulosome-producing organisms is presented in Table 2.

Table 2. Cellulosome-producing anaerobic bacteria and fungi [Doi and Kosugi, 2004].

Microorganism	*Growth Temp	Source
ANAEROBIC BACTERIA		
Acetovibrio cellulolyticus	M	Sewage
Bacteriodes cellulosolvens	M	Sewage
Butyrivibrio fibrisolvens	M	Rumen
Clostridium acetobutylicum	M	Soil
Clostridium cellobioparum	M	Rumen
Clostridium cellolyticum	M	Compost
Clostridium cellulovorans	M	Wood
Clostridium josui	M	Compost
Clostridium papyrosolvens	M	Paper mill
Clostridium thermocellum	T	Sewage soil
Ruminococcus albus	M	Rumen
Ruminococcus flavefaciens	M	Rumen

Table 2 (continued). Cellulosome-producing anaerobic bacteria and fungi [Doi and Kosugi, 2004].

AEROBIC FUNGI		
Neocallimastix patriciarum	M	Rumen
Orpinomyces joyonii	M	Rumen
Orpinomyces PC-2	M	Rumen
Piromycs equi	M	Rumen
Piromyces E2	M	Faeces

*M (mesophilic) and T (thermophilic; above 50°C)

Electron-microscopy identified extracellular protuberances on the surface of cellulosome-producing bacteria [Bayer et al., 1998]. These protuberances are assumed to contain cellulosomes. The key components of cellulosomes are (i) the non-catalytic scaffoldin protein with its cohesins (binding sites) for cellulosomal enzymes; and (ii) the catalytic subunits, or cellulosomal enzymes, with their respective cohesin-binding sites, called dockerins [Bayer et al., 1994]. Figure 10 illustrates the different components of the cellulosome.

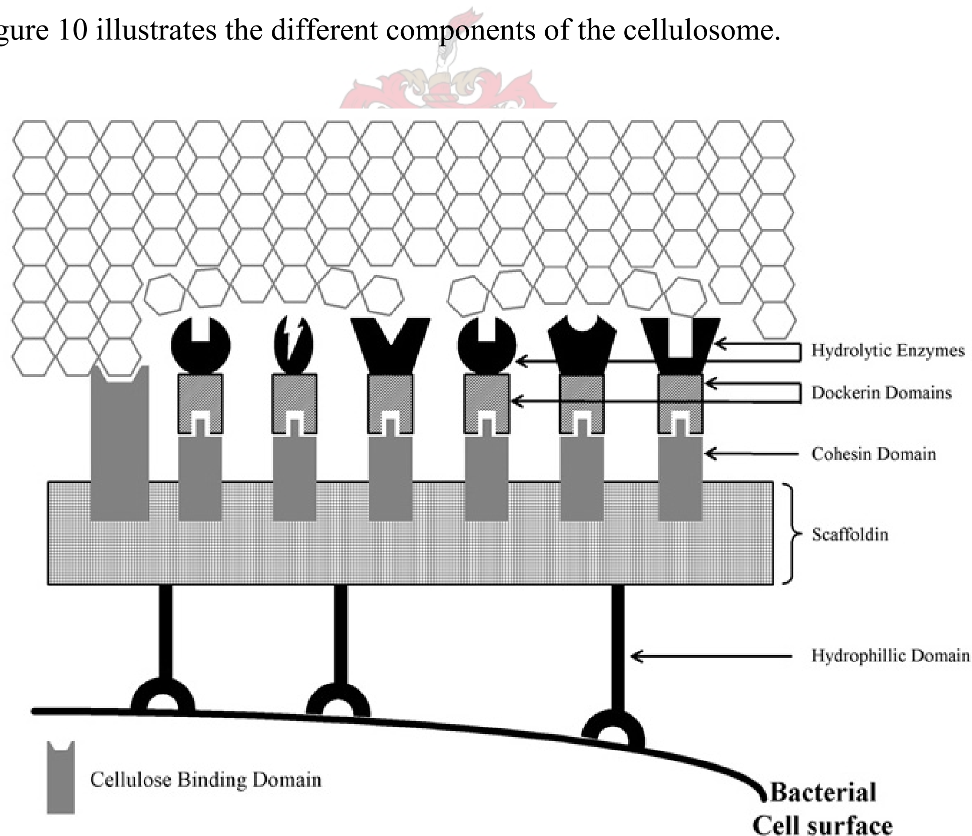


Figure 10. A schematic representation of a *Clostridium cellulovorans* cellulosome. The scaffoldin protein is shown with its cellulose-binding domain (CBD), three hydrophilic domains, seven cohesins, and seven hydrolytic enzymes bound to the scaffoldin through their dockerins [Doi and Kosugi, 2004].

The cohesin-dockerin interaction is crucial for cellulosome assembly. The majority of scaffoldins contain between 6 and 9 different cohesins that can bind up to 26 different cellulosomal enzymes. This is the basis for cellulosome heterogeneity and presents a microorganism with the potential to produce cellulosomes with many different compositions [Doi et al., 2003]. The scaffoldin has an expanded role in the complexed cellulase system that includes binding of the cellulase substrate (via its CBM), as well as attaching the cellulosome to the microorganism through cell-surface-associated proteins [Doi and Kosugi, 2004]. The close association between host cell and substrate limits diffusion losses of hydrolytic products and is considered a major advantage for attached cells [Bayer et al., 1994; Schwarz, 2001].

The most complex and best-investigated cellulosome is that of the thermophilic bacterium *Clostridium thermocellum*. *C. thermocellum* is highly specific for growth on cellulose and cellooligosaccharides, and has minimal growth on glucose and fructose [Nochur et al., 1992]. In some strains, the cellulosomes aggregate to form larger super-complexes, called polycellulosomes, with molecular weights of up to 100 MDa [Park et al., 2001]. The *C. thermocellum* cellulosome comprises a large non-catalytic scaffoldin protein of 197 kDa that is multi-modular and includes 9 cohesins, 4 hydrophilic X-domains, and a CBM. The scaffoldin CBM has high homology with that of other scaffoldins, but clearly differs from the CBM modules of the catalytic components [Bayer et al., 1998]. A total of 26 cellulosomal enzymes have been identified for *C. thermocellum* [Bayer et al., 1998]. The catalytic components include at least 9 endoglucanases, 4 exoglucanases, 5 hemicellulases, a chitinase, and a lichenase. All have dockerin domains that can interact with the cohesins of the scaffoldin protein. The main exoglucanase, CelS, is always present in the cellulosome [Morag et al., 1993]. CelS is a processive cellulase with a high affinity for microcrystalline or amorphous cellulose. Cellobiose is produced as the main soluble byproduct of hydrolysis and has a strong inhibitory effect on CelS [Kruus et al., 1995a; 1995b]. CelA is the main endoglucanase associated with the cellulosome [Schwarz, 2001; Alzari et al., 1996].

The assembly process is one of the most captivating aspects of cellulosome synthesis. A Ca^{2+} ion was shown to play a critical role in the proper folding of the dockerin and thus facilitate tight binding of the catalytic components to the scaffoldin [Lytle et al., 2000]. Knowledge about the

function and regulation of the cellulosomal genes, the organization of the cellulosome, and the secretion and extracellular assembly of the cellulosome is very limited [Doi and Kosugi, 2004].

The cellulosomes of *C. thermocellum* are extensively glycosylated (6 to 13% carbohydrate) with the highest amount associated with the scaffoldin protein. Glycosylation may be involved in protection of the cellulosome against proteases but a role in cohesin-dockerin recognition has also been suggested [Gerwig et al., 1993]. *C. thermocellum* cellulosomal genes are scattered throughout the chromosome, whereas in other clostridia large gene clusters are found, in addition to unlinked genes that encode the cellulosomal enzymes [Schwarz, 2001; Guglielmi and Beguin, 1998].

The high efficiency of microcrystalline cellulose decomposition by *C. thermocellum* has been attributed to (i) the specific ratio between the catalytic components that produce optimized synergism within the complex; (ii) minimal non-productive adsorption due to the optimal spacing and synergism between the individual components; (iii) minimal competitive binding to the available number of binding sites on cellulose is prevented by binding the entire complex to a single site via a strong binding domain with low specificity; and (iv) the diversity of enzymes present in the cellulosome avoid the termination of hydrolysis due to depletion of one structural type of cellulose at the site of adsorption [Lynd et al., 2002; Schwarz, 2001].

Rumen bacteria have for a long time been renowned for their cellulolytic activity. Within the order Clostridiales, *Ruminococcus* and *Butyrivibrio* are members of the family Lachnospiraceae that is phylogenetically related, but taxonomically different to the family Clostridiaceae. Electronmicroscopy, biochemical isolation and characterization have confirmed the existence of cellulosome production in *Ruminococcus albus* and *Ruminococcus. flavefaciens* [Aurilia et al., 2000].

Ruminococcus albus produces a cellulosome of 1.5 MDa, comparable in size to that of *C. thermocellum* [Ohara et al., 2000]. Of the 15 cellulosomal enzymes identified for *R. albus* F-40, 8 have endoglucanase and 9 xylanase activity and thus there are 2 with both activities. Five gene sequences are available for *R. flavefaciens*, of which 3 encode xylanases, one an esterase

and one an endoglucanase. Dockerin domains have been identified in all these genes. The presence of non-cellulosomal endoglucanases has also been reported [Schwarz, 2001].

Fibrobacter succinogenes is another cellulolytic rumen bacterium that produces cellulase enzymes that are cell-associated. However, no cellulosome structures have been identified [Fields et al., 2000]. The *F. succinogenes* endoglucanasses, EG2 and EGF, contain distinct CBMs that may play an important role in its adhesion to cellulose. Scanning electron micrographs identified extracellular protuberances that are involved in the adhesion mechanism of this bacterium (Figure 11). These protuberances may be evidence of either cellulosome complexes or fimbriae [Miron et al., 2001].

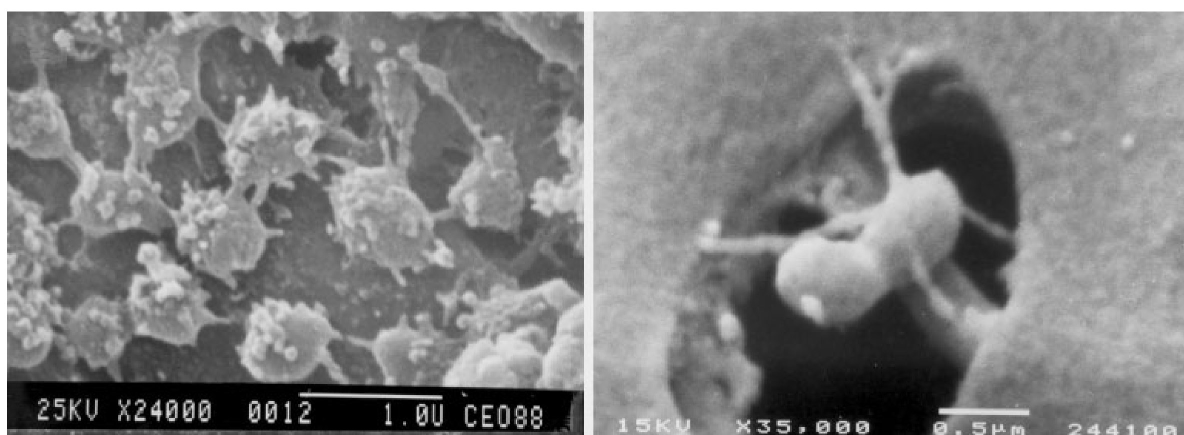


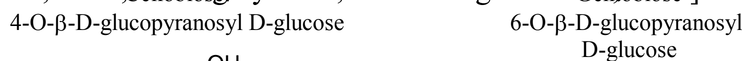
Figure 11. Scanning electron micrographs of the extracellular protuberances on the *F. succinogenes* cell surface that promote adhesion with the cellulose substrate [Miron et al., 2001].

Among the anaerobic fungi found in the rumen, high-molecular-weight complexes with high affinity for microcrystalline cellulose have been isolated from *Piromyces* sp. strain E2 [Steenbakkers et al., 2003], *Neocallimastix frontalis* [Dijkerman et al., 1997; Wilson and Wood, 1992] and *Piromonas communis* P [Wilson and Wood, 1995]. The cellulosomal enzymes from these organisms lack CBMs, but instead possess one, two or three copies of a non-catalytic fungal dockerin domain (NCDD) [Steenbakkers et al., 2001; Fanutti et al., 1995]. Preliminary data indicate the presence of multiple scaffoldins; however, they have not yet been isolated from culture fractions [Steenbakkers et al., 2001]. Although both fungal and bacterial cellulosomes are engaged in crystalline cellulose degradation, it differs substantially with regards to the end-product being produced during hydrolysis [Steenbakkers et al., 2003; 2002a; 2002b]. The action of clostridial cellulosomes produces cellobiose, which is transported into the cell, hydrolyzed to

glucose and fermented [Schwarz, 2001]. In contrast, glucose is the major end-product produced by the cellulosomes of *Piromyces* E2 [Dijkerman et al., 1997], *P. communis* P [Wood and Wilson, 1995] and *N. frontalis* [Wilson and Wood, 1992].

REGULATION OF CELLULASE PRODUCTION

The production of extracellular enzymes in significant quantities is an energy-consuming process for the microbial cell. A common strategy employed by microorganisms to ensure that enzymes are only produced in the presence of the specific carbohydrate (e.g. cellulose, hemicellulose), is carbon source-dependent gene regulation. In addition, synergism between hydrolytic enzymes to efficiently degrade the polymer also requires coordinated regulation of the specific enzymes. There is extensive cross-talk in induction of expression of the genes encoding different classes of enzymes. For example, the same substrate may cause expression of both cellulases and hemicellulases, although to different extents [Margolles-Clark et al., 1997]. Most of the genes studied are induced in the presence of the cellulose (or hemicellulose), and repressed by the presence of easily metabolisable carbon sources (e.g. glucose). Glucose repression is usually very efficient and overrules the induction of genes. Cellulose and hemicellulose are large insoluble polymers that are unable to enter the cells and therefore unlikely to be inducers of gene expression. Instead, a low level of constitutive cellulase activity might degrade cellulose into soluble cellooligosaccharides, thereby allowing these small hydrolysis products of the respective polymers to enter the cell and be converted into inducers that trigger cellulase gene expression [Suto and Tomita, 2001; El-Gogary et al., 1989 Gong and Tsao, 1979].



Regulation of genes encoding non-complexed cellulases

The molecular nature of cellulase gene regulation in *T. reesei* has been studied extensively [Schmoll and Kubicek, 2003; Kubicek et al., 1993]. The major *T. reesei* cellulases, namely CBHI (encoded by *cbhI*) and EGI (encoded by *eglI*) are constitutively produced at low level even in the absence of an inducer. When cellulose is added, however, the expression of these genes is enhanced at least 1,100-fold [Carle-Urioste et al., 1997]. A variety of compounds, namely cellobiose, sophorose, gentiobiose, laminaribiose, lactose and thiocellobiose (shown in Figure 12), have been identified as inducers of cellulase expression in *T. reesei* [Suto and Tomita, 2001].

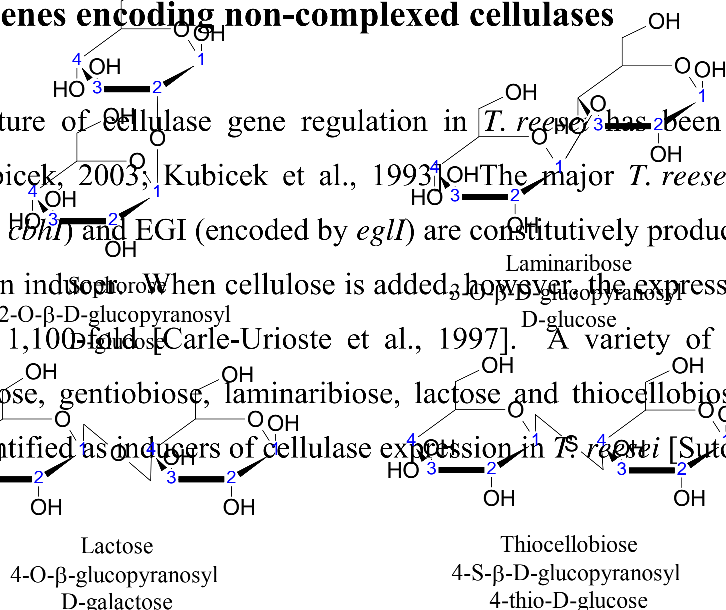


Figure 12. Structures of β-linked disaccharides of glucose that can act as inducers of cellulase production.

Growth in the presence of cellobiose, the end-product of cellobiohydrolase activity [Ilmén et al., 1997, Fritscher et al., 1990; Sternberg and Mandels, 1979], has been linked with the induction of cellulase expression in several fungal species, including all the major cellulases of *T. reesei* [Ilmén et al., 1997], *egl1* of *Volvariella volvacea* [Ding et al., 2001], *egl2* of *P. janthinellum* [Mernitz et al., 1996], and *eglA* of *A. nidulans* [Chikamatsu et al., 1999]. β-Glucosidase enzymes can either hydrolyze cellobiose to glucose, which may cause feedback inhibition of the cellulases, or transglycosylate it into e.g. sophorose, which would lead to induction of cellulase gene transcription. It is speculated that the balance of hydrolysis and transglycosylation, and the subsequent uptake of the generated sugars plays a key role in the intracellular signal transduction pathways being activated in cellobiose-utilizing cells [Aro et al., 2005].

Sophorose (two β-1,2-linked glucose units) is the most effective soluble inducer in *T. reesei* and can induce 2,500-fold more cellulase than cellobiose in this well-known cellulase producer [Mandels et al., 1962]. Early on, studies suggested that sophorose is produced form cellobiose by the transglucosylation activity of β-glucosidases [Gritzali and Brown, 1979; Vaheri et al., 1979]. In agreement with this, deletion of the *bgl1* gene in *T. reesei* encoding the major extracellular β-glucosidase caused a delay in induction of cellulase gene transcription during growth on cellulose [Fowler and Brown, 1992]. However, sophorose is only specific for some fungi, including *T. reesei*, *A. terreus* and *Penicillium purpurogenum*. Sophorose does not induce cellulase expression in *P. janthinellum*, *P. chrysosporium*, *A. nidulans* and *A. niger* [Gielkens et al., 1999; Mernitz et al., 1996; Hrmova et al., 1991; Bisaria and Mishra, 1989]. Although sophorose is a poor substrate for the extracellular β-glucosidase of *T. reesei*, it is readily transported into the mycelium. When sophorose is added to resting cells, an increase in β-linked disaccharide permease activity can be observed. It was therefore concluded that it might be involved in the translocation of sophorose across the plasma membrane [Kubicek et al., 1993]. However, these results have to be confirmed by cloning the gene encoding the permease. The isolation of a *T. reesei* intracellular β-glucosidase (BGLII) with hydrolytic activity towards sophorose also suggested that it might be involved in the regulation of intracellular levels of the inducer [Saloheimo et al., 2002; Suto and Tomita, 200; and Inglin et al., 1980].

The disaccharide lactose (1,4-O-β-D-galactopyranosyl-D-glucose) is currently the only soluble and economically feasible inducer of cellulase gene expression in *T. reesei*. Therefore, it is used in industry to produce homologous enzymes and heterologous proteins under control of the *T. reesei cbh1* promoter [Penttilä, 1998]. The mechanism of lactose induction is not clearly understood. Lactose itself is not found in the plant cell wall. Extracellular β-galactosidase cleaves lactose into glucose and galactose. Results obtained with *T. reesei* in which the galactose utilisation pathway genes, *gal1* or *gal7* (encoding galactokinase and uridyltransferase, respectively), has been deleted, identified intracellular galactose-1-phosphate as the potential signalling molecule [Seiboth et al., 2004; 2002]. Interestingly, galactose metabolism also results in intracellular galactose-1-phosphate production, but galactose itself does not induce cellulase expression in *T. reesei*. Therefore, the formation of galactose-1-phosphate in the intracellular galactose utilisation pathway cannot alone explain cellulase expression on lactose [Margolles-Clark et al., 1997; Seiboth et al., 2004]. New evidence suggests that galactose can be utilized through an alternative route, which involves L-arabinitol-4-dehydrogenase (encoded by *T. reesei lad1* gene) and the subsequent production of galactitol as an intermediate. Lactose induction seems to be mediated, at least partly, by a different mechanism than that of sophorose, since deletion of the *gal1* gene only interferes with lactose signalling [Seiboth et al., 2004]. In contrast, lactose causes repression of cellulase expression in the basidiomycetous fungi *Agaricus bisporus*, *V. volvacea*, and *Irpex lacteus* upon addition to cellulose-induced cultures [Ding et al., 2001; Hamada et al., 1999; Chow et al., 1994; Yague et al., 1994].

Various other oligosaccharides, such as δ-cellobiono-1,5-lactone, laminaribiose, gentiobiose, xylobiose and the monosaccharide L-sorbose, are known to induce cellulase expression in *T. reesei* [Nogawa et al., 2001; Margolles-Clark et al., 1997; Durand et al., 1988; Vaheri et al., 1979]. Interestingly, the intracellular β-glucosidase from *P. purpurogenum* have different characteristics from the extracellular enzyme and it could convert cellobiose to gentiobiose, laminaribiose, and glucose [Suto and Tomita, 2001]. Low levels of soluble cellulose-hydrolysis products are probably transported into the cells where it can be modified by intracellular β-glucosidases to function as inducer molecules of the corresponding cellulases. This may also explain the coordinated manner in which the main cellulases of fungi, e.g. *T. reesei*, are regulated [Ilmén et al., 1997].

The majority of cellulase genes that have been studied in filamentous fungi are repressed during growth on glucose. Since the addition of glucose to induced cultures results in the repression of the cellulase gene expression, it is expected that glucose repression overrules induction [Ilmén et al., 1997; and el-Gogary et al., 1989]. Glucose repression has been shown to be very effective. However, in cultures of *T. reesei* where glucose has been depleted, cellulase transcripts can be detected [Ilmén et al., 1997]. Since the absence of a carbon source does not induce cellulase expression it is suggested that an induction mechanism is involved in the observed derepression of cellulase gene expression in carbon depleted conditions. Inducer molecules can either be generated from carbohydrates released from the fungal cell wall or produced (e.g. by transglycosylation) from the glucose present in the culture [Ilmén et al., 1997; Sternberg and Mandels 1979].

Transcriptional regulation of cellulase genes is coordinated through transcription factors. Two *T. reesei* genes encoding transcription factors, ACEI and ACEII, have been identified based on their ability to bind to the *cbhI* promotor [Aro et al., 2001; Saloheimo et al., 2000]. The mechanism by which inducers, such as sophorose, influence transcription by the transcription factors is not clear. Repression of transcription of cellulase genes in *T. reesei* is mediated by CRE1, the general carbon catabolite repressor protein [Takashima et al., 1996; Ilmén et al., 1995; Strauss et al., 1995]. Although the production of cellulases is repressed by CRE1 in the presence of glucose, basal level expression occurs under conditions of glucose depletion [Carle-Urioste et al., 1997]. A link between catabolite repression and the energy status of the cell has been suggested. A study that involved four filamentous fungi revealed that an intracellular ATP concentration above 10^{-7} $mg.ml^{-1}$ caused repression of extracellular cellulase production. Cyclic AMP (cAMP) was also implicated in the derepression of enzyme production [Wang et al., 1996].

Cellulase gene expression in *T. fusca* is also regulated at the level of induction (by cellobiose) and catabolite repression (in the presence of glucose) [Wilson, 1992]. In the absence of cellulose or cellobiose, cellulase production is repressed by CelR, a transcriptional regulator. However, in the presence of cellobiose (inducer), CelR is inactivated and subsequently dissociates from the cellulase promoters to allow transcription of the genes [Spiridonov and Wilson, 1999]. Studies with *T. curvata* also confirmed catabolite repression of cellulase genes in the presence of glucose

and suggested that cAMP levels play an important role [Wilson and Irwin, 1999; Wood et al., 1984].

Regulation of genes encoding complexed cellulases

The regulation of cellulosomal gene expression has been studied at the microscopic, physiological and transcription levels. The composition of the growth medium is the major factor affecting both subunit structure and function of the cellulosome. Anion-exchange chromatography of fractionated cellulosomes from cells grown on different substrates (such as glucose, cellobiose, xylan, mannan or pectin) indicated distinct subunit compositions and enzymatic activities of the respective cellulosomes [Han et al., 2003a; Ali et al., 1995; Bayer et al., 1985]. This suggests that microorganisms express cellulosomal genes in response to different substrates and the composition of the resulting cellulosomes have activities that are directed towards the available substrate.

The *C. cellulovorans* large cellulosomal gene cluster consists of several operons. Expression of a number of these operons, specifically the operons encoding the cellulase and hemicellulase enzymes, is coordinated [Han et al., 2003b]. The promoters of these genes contain sequences that correspond to the binding site for the Sigma-A (σ^A) component of the RNA polymerases of Gram-positive bacteria. Growth on substrates such as cellulose, xylan, mannan or pectin induces expression of most of the genes within the cellulosomal gene cluster as well as for cellulosomal genes unlinked to the cluster. However, low levels of expression are present when different monosaccharides are the substrates. The *C. cellulovorans* xylanase and pectate lyase genes are specifically induced in the presence of xylan and pectin, respectively. This suggests that cellulase expression is regulated by a catabolite repression-like mechanism and that the presence of hemicelluloses stimulates cellulose utilization by the cell [Han et al., 2003b]. Although the unlinked cellulosomal genes are mostly monocistronic, their expression could also be regulated coordinately with the genes in the large gene cluster [Han et al., 2003a].

C. thermocellum also uses a catabolite-repression-like mechanism to regulate expression of its cellulosomal genes [Mishra et al, 1991]. The CelS exoglucanase is one of the major enzymes present in the *C. thermocellum* cellulosome. It was shown that *CelS* expression is significantly

higher when the organism grows on cellulose compared to cellobiose. Molecular evidence also confirmed that the level of *celS* expression is increased proportionally with increased growth rate. Its transcription is probably regulated at two transcriptional start points (upstream of the translation initiation site) that were identified based on homology with the σ^A and σ^B promoter binding sites in *B. subtilis*. The level of *celS* transcription is controlled by the growth rate under nitrogen and cellobiose-limited conditions. Such conditions are typical of the natural anaerobic cellulose-containing environments where slow growth rates are maintained by the inhabiting thermophiles [Dror et al., 2003a].

The CipA scaffoldin plays a vital role in the *C. thermocellum* cellulosome. Expression of *cipA* together with the tandemly located *olpB* and *orf2* (scaffoldin-related genes) is regulated by the growth rate in a similar way than *celS*. Low growth rate induces high expression of the *cipA*, *olpB* and *orf2* genes, while high growth rate causes lower expression [Dror et al., 2003b]. Two promoter sites upstream of the *cipA* gene share high homology with σ^A and σ^L of *B. subtilis*. Interestingly, transcription from the σ^L-like promoter site is constitutive under all growth conditions, whereas transcription from the σ^A-like promoter site is linked with carbon-limiting conditions [Doi and Kosugi, 2004]. Understanding how the expression of the scaffoldin protein gene is regulated will contribute significantly to the knowledge on cellulosome synthesis and assembly.

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β-GLUCOSIDASES

β-Glucosidases (β-D-glucoside glucohydrolases; EC 3.2.1.21) occur universally in mammals, plants, and microorganisms [Woodward and Wiseman, 1982]. This well-characterized group of enzymes play an important role in biological processes and have many potential biotechnological applications.

Classification

During the late 1950s, the International Union of Biochemistry and Molecular Biology (IUBMB) proposed a classification system based on the grouping of enzymes according to the type of reaction they catalysed. This system for classification also served as a basis for assigning code

numbers to enzymes, generally known as the EC number. The code numbers, prefixed by EC, contain four digits separated by points, with the following meaning: (i) The first digit shows to which of the six main classes the enzyme belongs; (ii) the second digit indicates the subclass; (iii) the third digit gives the sub-subclass; and (iv) the fourth digit is the serial number of the enzyme in its sub-subclass.

Traditionally, β-glucosidases were classified under the more general class of *O*-glycosyl hydrolases (EC 3.2.1.-) that include enzymes that hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and non-carbohydrate moiety. A classification system based on protein motif homology has been established over the last decade [Henrissat and Bairoch, 1996; Henrissat et al., 1995; Henrissat and Bairoch, 1993; Henrissat, 1991]. The identification of glycosyl hydrolases is increasing at an exponential rate as a direct result of the significant amount of genome sequencing projects being completed. Currently, an online database, the carbohydrate-active enzymes (CAZy) database, is available at the Expasy server (http://afmb.cnrs-mrs.fr/~pedro/ CAZY/db.html). CAZy is maintained and updated regularly in order to manage the huge amount of information concerning the classification of glycoside hydrolases [Coutinho and Henrissat, 1999].

Based on their amino acid sequence similarity, β-glucosidases belong to glycoside hydrolase families 1, 3 and 9. Some important properties of these families are listed in Table 3. There is typically a direct relationship between the amino acid sequence and the folding of an enzyme, as confirmed by the 3-D structures included in the database [Henrissat et al., 1995]. Therefore, the family classification system reveals the structural features of the enzymes, which are more informative than substrate specificity alone since the complete range of substrates is only rarely determined for individual enzymes. The 3-D structure, particularly the topology of the active site, dictates the catalytic mechanism, and thus families also contain members whose enzyme mechanism is either inverting or retaining. Some enzymes contain multiple domains that belong to different classes, e.g. the glycoside hydrolases and glycosyl transferases. Classification into families defines the modules of such enzymes and resolves the conflict about substrate specificity for multifunctional enzymes [Henrissat and Bairoch, 1993; Henrissat, 1991].

Recently, a new nomenclature system for plant cell wall-degrading enzymes has been proposed. The nomenclature incorporates substrate specificity, the glycoside hydrolase family, and the order in which the enzymes were first reported, into an enzyme name [Henrissat et al., 1998]. The first 3 digits, indicating the substrate specificity, are according to the names in Table 4. For example, the three enzymes CBHI, CBHII, and EGI of *T. reesei* are designated Cel7A (CBHI), Cel6A (CBHII), and Cel6B (EGI). When more than one catalytic domain is present, it is reflected in the name, such as Cel9A-Cel48A for the two catalytic domains of CelA of *Caldocellulosiruptor saccharolyticus*. Up to now, this classification system has only been applied to a few β-glucosidases, eg. *Piromyces* E2 Cel3A [Steenbakkers et al., 2003] and *T. reesei* Cel1A (BGLII) [Saloheimo et al, 2002].

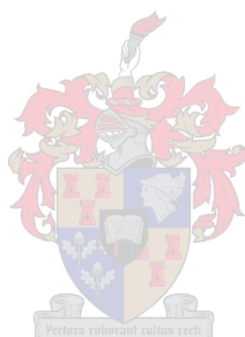
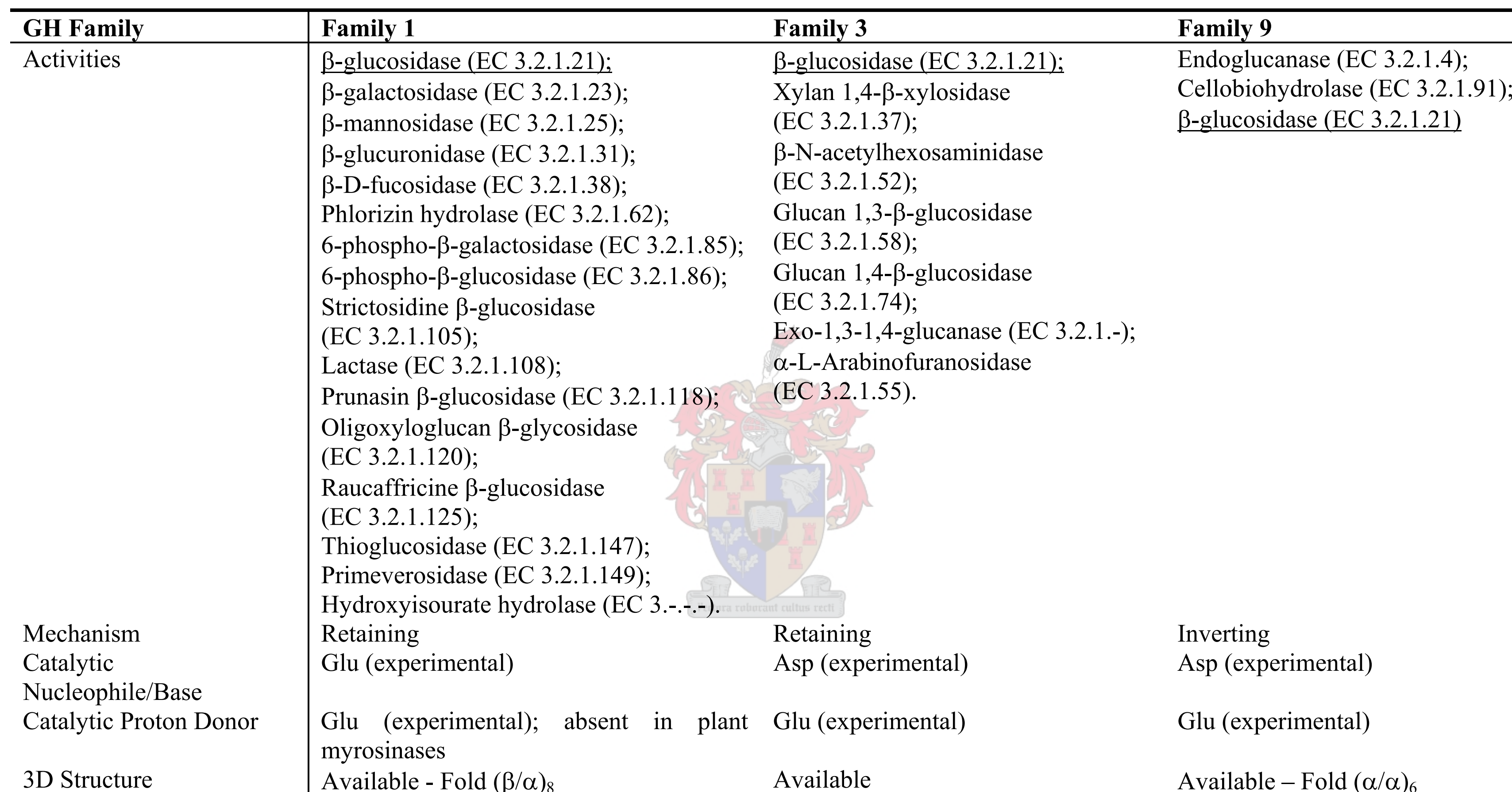


Table 3. Properties of the Glycosyl Hydrolase (GH) families that include the β-glucosidases



GH Family	Family 1	Family 3	Family 9
Activities	β-glucosidase (EC 3.2.1.21); β-galactosidase (EC 3.2.1.23); β-mannosidase (EC 3.2.1.25); β-glucuronidase (EC 3.2.1.31); β-D-fucosidase (EC 3.2.1.38); Phlorizin hydrolase (EC 3.2.1.62); 6-phospho-β-galactosidase (EC 3.2.1.85); 6-phospho-β-glucosidase (EC 3.2.1.86); Strictosidine β-glucosidase (EC 3.2.1.105); Lactase (EC 3.2.1.108); Prunasin β-glucosidase (EC 3.2.1.118); Oligoxyloglucan β-glycosidase (EC 3.2.1.120); Raucaffricine β-glucosidase (EC 3.2.1.125); Thioglucosidase (EC 3.2.1.147); Primeverosidase (EC 3.2.1.149); Hydroxyisourate hydrolase (EC 3.-.-.-).	β-glucosidase (EC 3.2.1.21); Xylan 1,4-β-xylosidase (EC 3.2.1.37); β-N-acetylhexosaminidase (EC 3.2.1.52); Glucan 1,3-β-glucosidase (EC 3.2.1.58); Glucan 1,4-β-glucosidase (EC 3.2.1.74); Exo-1,3-1,4-glucanase (EC 3.2.1.-); α-L-Arabinofuranosidase (EC 3.2.1.55).	Endoglucanase (EC 3.2.1.4); Cellobiohydrolase (EC 3.2.1.91); β-glucosidase (EC 3.2.1.21)
Mechanism	Retaining	Retaining	Inverting
Catalytic Nucleophile/Base	Glu (experimental)	Asp (experimental)	Asp (experimental)
Catalytic Proton Donor	Glu (experimental); absent in plant myrosinases	Glu (experimental)	Glu (experimental)
3D Structure	Available - Fold $(\beta/\alpha)_8$	Available	Available – Fold $(\alpha/\alpha)_6$

Table 4. Acronyms used in the new nomenclature for the cell wall-degrading enzymes according to their substrate specificity [Henrissat et al., 1998].

Enzyme	Gene	Protein
Cellulases	*cel*	Cel
Xylanase	*xyn*	Xyn
Mannanase	*man*	Man
Lichenase	*lic*	Lic
Laminarinase	*lam*	Lam

Microbial β-glucosidases

β-Glucosidase enzymes are prevalent among cellulolytic and non-cellulolytic microorganisms. In cellulose-degrading bacteria and fungi, β-glucosidase's primary role is that of hydrolyzing cello-oligosaccharides and cellobiose to glucose. Generally, β-glucosidase activity decreases with increased glucose chain length [Parry et al. 2001; Kubicek et al., 1993]. Fungal enzymes, such as that of *T. reesei*, display a significant amount of transglycosidic activity under certain conditions. Transglycosidic activity is of specific importance in the production of molecules such as sophorose [Fowler and Brown, 1992; Gritzali and Brown 1979] and gentiobiose [Seidle and Huber, 2005] that act as inducers of cellulase gene expression. In addition, the transglycosidic activity of microbial β-glucosidases may also be important in the production of small oligosaccharides to provide a means of energy storage [Seidle and Huber, 2005]. The two activities of β-glucosidases are discussed in much detail under the heading "Structure and Substrate Specificity of β-glucosidases". In non-cellulolytic yeast and fungi, β-glucosidases have been implicated in β-glucan degradation through the synergistic interaction with β-glucanases [Pitson et al., 1997]. β-Glucosidases have also been isolated from enological yeasts such as *Debaromyces hansenii* [Rosi et al., 1994], *Pichia anomala* and *Hanseniaspora uvarum* [Barbagallo et al., 2004]. These enzymes are usually broad substrate specificity enzymes that play a vital role in the release of a variety of β-linked flavour compounds [Rosi et al., 1994].

Microbial β-glucosidases are extremely diverse with respect to their genetic and enzymatic properties. Several microorganisms produce more than one β-glucosidase, for example, the intracellular, cell wall-bound, and extracellular β-glucosidases produced by both *T. reesei* [Saloheimo et al., 2002; Chirico and Brown, 1987; Umile and Kubicek, 1986; Inglin et al. 1980]

and *C. wickerhamii* [Skory et al., 1996; Himmel et al., 1986; Freer, 1985]. Even more surprising is the phenomenon where three different enzymes are produced from a single gene, for instance the cell wall-bound (CB-1) and 2 extracellular (EX-1 and EX-2) β-glucosidases from *A. kawachii* that are encoded by the *bglA* gene [Iwashita et al., 1999]. Since the enzymes display different degrees of glycosylation, it was suggested that post-translational modifications (e.g. mRNA-processing, differential splicing, partial duplication of transcription etc.) may be involved in generating these protein products with distinct structural and enzymatic features. In addition, the location of the *bglA*-encoded enzyme is significantly influenced by the culture conditions. In liquid cultures 80% of the *bglA*-encoded product is cell wall-bound, while in solid culture medium 80% of the β-glucosidase activity is associated with the extracellular space [Iwashita et al., 1999].

Structurally, β-glucosidases can function as mono-, di-, tri- and tetramers as presented in Table 5. A number of β-glucosidases with novel structural and enzymatic features have been described in the literature. Some of the more recent discoveries include an intracellular enzyme BGL1 from the thermophilic fungus *Talaromyces thermophilus* that exhibits both β-glucosidase and β-galactosidase activity. Although BGL1 prefers glucopyranosides as substrates, its affinity towards galactopyranosides is significantly increased in the presence of mono and divalent cations (i.e. Na^{+}, K^{+} and Mg^{2+}) [Nakkharat and Haltrich, 2006]. Another thermophile, *Thermoascus auranticus*, produces an extracellular β-glucosidase with a pH optimum of 80°C and displays transglycosidic activity in presence of high concentrations of cellobiose and alcohols [Parry et al, 2001]. The intracellular β-glucosidases (G_{I} and G_{II}) from *Penicillium decumbens* are broad substrate specificity enzymes that include a novel deglycosylation activity towards various flavonoid glycosides that are important in both the food and pharmaceutical industries [Mamma et al., 2004]. β-Glucosidase enzymes that can accommodate both β- and α-glucosides as substrates have been isolated from *Botrytis cinerea* [Gueguen et al., 1995] and *A. oryzae* [Riou et al., 1998]. In addition to its extracellular non-complexed β-glucosidase, the cellulosome-producing anaerobic fungus *Piromyces* E2 produces a cellulosomal β-glucosidase (Cel3A). Cel3A is implicated as the key enzyme responsible for the glucose-producing activity of the complex during cellulose hydrolysis whereas the non-

complexed β-glucosidase is probably involved in the induction of the cellulolytic system [Steenbakkers et al., 2003].

Table 5. β-Glucosidases from various microbial origins that function as mono-, di-, tri and tetramers.

Enzyme (origin)	Classification	Location	Reference
Monomeric enzymes:			
BglA (*Bacillus polymyxa*)	Family 1	Intracellular	Painbeni et al., 1992
BglX (*Erwinia chrysanthemi*)	Family 3	Periplasmic space	Vroemen et al., 1995
BglA (*Clostridium thermocellum*)	Family 1	Intracellular	Graebnitz et al., 1991
Bgl (*Acremonium persicinum*)	Family 3	Extracellular	Pitson et al., 1997
Dimeric enzymes:			
BglA (*Thermotoga maritime*)	Family 1	Intracellular	Gabelsberger et al., 1997
BGL1 (*Saccharomycopsis fibuligera*)	Family 3	Extracellular	Machida et al., 1988
BglB (*Clostridium thermocellum*)	Family 3	Intracellular	Romaniec et al., 1993
GI (*Penicillium decumbens*)	Unknown	Intracellular	Mamma et al., 2004
Trimeric enzymes			
Thermoascus aurantiacus	Family 3	Extracellular	Parry et al., 2001
Tetrameric enzymes:			
Pyrococcus horikoshii	Family 1	Membrane bound	Matsui et al., 2000
BGLII (*Pichia etchellsii*)	Family 3	Cell wall bound	Wallecha and Mishra, 2003
Bgl1 (*Kluyveromyces fragilis*)	Family 3	Intracellular	LeClerc et al., 1987
GII (*Penicillium decumbens*)	Unknown	Intracellular	Mamma et al., 2004

Pectora roborant cultus recti

Recombinant enzyme production has become a key technology in various industries - food, textile and pharmaceutical. A great number of microbial β-glucosidases have been cloned and heterologously produced in prokaryotic (*E. coli* [Rajoka et al., 1998; Hashimoto et al, 1998; and Marri et al., 1995]) and eukaryotic (*S. cerevisiae* [Van Rooyen et al., 2005; Saloheimo et al., 2002; Dan et al., 2000; Iwashita et al., 1999; Rajoka et al., 1998; and Machida et al., 1988], *P. pastoris* [Dan et al., 2000], *T. reesei* [Murray et al., 2004; Donzelli et al, 2001; and Barnett et al., 1991], and *Aspergillus* species [Iwashita et al., 1999; and Takashima et al., 1999]) host expression systems.

A strategy employing a shuttle vector for cloning of the β-glucosidase genes (*bglA*, *bglB* and *bglC*) from the well-known cellulolytic bacterium, *Cellulomonas biazotea*, resulted in functional expression of BglA, BglB and BglC enzymes in both *E. coli* and *S. cerevisiae*. The molecular properties (i.e. pH and temperature optima, molar mass, and substrate specificity) of the recombinant products were similar to that of the native *C. biazotea* enzymes and were located in

the periplasmic and extracellular fractions of *E. coli* and *S. cerevisiae*, respectively [Rajoka et al., 1998]. Bacterial β-glucosidases that have been heterologously produced in *E. coli* result in enzymes that are mostly cytosolic and have pH and temperature optima of between 5-7 and 40-60°C. For example, BglA from *Bacillus* sp., pH 6 and 45°C [Hashimoto et al., 1998]; BglA from *Butyrivibrio fibrisolvens*, pH 5 and 42°C [Lin et al., 1990]; Bgl from *Cellovibrio gilvus*, pH 6 and 40°C [Kashiwagi et al., 1991] and BglB from *Microspora bispora*, pH 4.2 and 60°C [Wright et al., 1992]. However, recombinant enzymes from thermophilic bacteria like, *Thermoanaerobacter brockii* [Breves et al., 1997], *Thermotoga neapolitana* [Zverlow et al., 1997], and *Pyrococcus furiosus* [Voorhorst et al., 1995] function optimally at 75, 95 and 105°C, respectively. Feng et al. (2005) applied a directed evolution approach to the β-glucosidase of *Thermus thermophilus* to generate a mutant with significantly improved transglucosidase activity and no hydrolytic activity.

The heterologous production of fungal β-glucosidases is more challenging due to the additional complexity of glycosylation and the existence of introns in the structural genes. β-Glucosidases from fungal origin have been cloned in eukaryotic expression systems such as *S. cerevisiae, P. pastoris T. reesei*, and *Aspergillus* sp. as listed in Table 6. Fungal β-glucosidases that have been heterologously produced in eukaryotic hosts result in enzymes that are mostly extracellular or associated with the periplasmic space and have pH and temperature optima of between 4-5 and 45-65°C [Van Rooyen et al., 2005] with the exception of the *Talaromyces emersonii* enzyme which is optimally active at 71.5 °C [Murray et al., 2004].

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Table 6. Heterologous production of fungal β-glucosidase in various recombinant hosts

Fungal source	β-Glucosidase	Recombinant Host	References
A. niger	BglI	*S. cerevisiae* *P. pastoris*	Dan et al., 2000
A. kawachii	BglA	*S. cerevisiae*	Van Rooyen et al., 2005; Iwashita et al., 1999
T. reesei	Bgl1	*T. reesei* *S. cerevisiae*	Barnett et al., 1991 Cummings and Fowler, 1996
Humicola grisea	Bgl4	*A. oryzae*	Takashima et al., 1999
Talaromyces emersonii	Cel3A	*T. reesei*	Murray et al., 2004

Yeast β-glucosidases are the least investigated. Reports on the functional expression of yeast β-glucosidase genes in *S. cerevisiae* include that of *C. wickerhamii* [Van Rooyen et al., 2005; and

Skory and Freer, 1995], *C. molischiana* [Sànchez-Torres et al., 1998], *C. pelliculosa* [Kohchi and Toh-e, 1986] and *Saccharomycopsis fibuligera* [Van Rooyen et al., 2005; and Machida et al. 1988]. Currently *S. cerevisiae* is the preferred organism for ethanol production and therefore an important recombinant host for the production of cellulose-hydrolyzing enzymes [Lynd et al., 2002]. In addition, it has complete GRAS (Generally Regarded As Safe) status that is of particular interest when producing β-glucosidases for application in the food and wine industries. Skory et al. (1996) compared the levels of gene expression and enzyme activity of the highly glucose-tolerant *C. wickerhamii* β-glucosidase (BglB) when heterologously produced in different host expression systems (*E. coli*, *S. cerevisiae* and *P. pastoris*). Expression of the *bglB* gene in *E. coli* resulted in an intracellular protein that was rapidly degraded. Recombinant *S. cerevisiae* produced a functional BglB enzyme that remained cell-associated possibly as a result of hyper-glycosylation. BglB production in *P. pastoris*, a host that usually does not hyper-glycosylate, resulted in recombinant product that was correctly glycosylated and secreted but expression levels were insufficient to measure activity.

Plant β-glucosidases

Plant β-glucosidases are involved in growth and development via release of phytohormones (auxins, gibberellins, cytokinins) from their inactive glucoconjugated precursors [Duroux et al., 1998], host-parasite interactions [Osbourn, 1996; Stintzi et al., 1993], lignification [Samuels et al., 2002; Dharmawardhana et al., 1995], cell wall degradation in the endosperm during germination [Leah et al., 1995] and circadian rhythm of leaf movements [Ueda and Yamamura, 2000]. Several β-glucosidases have also been implicated in stress-related roles due to their stress-responsive expression [Lee et al., 2006; Thorlby et al., 2004; Chen et al., 2002]. During stress responses, β-glucosidases can release a variety of molecules such as antimicrobials [Sue et al., 2000; Cicek and Esen, 1998], phytohormones [Brzobohaty et al., 1993], and antioxidants [Chong et al., 2002] from their respective precursors.

Mammalian β-glucosidases

Two different β-glucosidases have been identified in mammalian systems: cytosolic and lysosomal β-glucosidases [Beutler, 1992]. Cytosolic β-glucosidases are present in significant

quantities in the liver, kidney and intestines were they are predominantly involved in the detoxification of plant β-glucosides [Gopolan et al., 1992]. A rare genetic disease, namely Gaucher disease, is caused by the accumulation of glycolipids, mainly glucosylceramide and glucosylsphingosine, in the bone marrow, liver, and spleen. The enzyme responsible for hydrolyzing these glycolipids is the lysosomal β-glucosidase (also called glucocerebrosidase) that is defective in Gaucher patients. There are three phenotypes of the disease. In all three types, the enzyme deficiency may cause symptoms such as enlarged spleen and liver, liver malfunction, skeletal disorders and bone lesions, severe neurologic complications, swelling of lymph nodes and (occasionally) the adjacent joints, swolen abdomen, a brownish tint to the skin, anemia, low blood platelets and yellow fatty deposits on the sclera [Beutler and Grabowski, 1995].

Structure and Substrate Specificity of β-glucosidases

The 3-D structures of enzymes from several glycosyl hydrolase families have been determined, in addition to the mechanism of glycosidic bond hydrolysis. The active-site topologies were classified into three categories (irrespective of whether the enzyme is inverting or retaining). The 3 types of active site topologies are (i) the pocket (or crater), (ii) the cleft (or groove) and (iii) the tunnel as illustrated in Figure 13 [Davies and Henrissat, 1995]. The pocket topology is optimal for the recognition of a non-reducing end of a sugar and is characteristic of enzymes such as β-glucosidases, β-galactosidases, glucoamylases, etc. [Jenkins et al., 1995]. This is specifically significant in the case of β-glucosidases that hydrolyse substrates containing β-glycosidic linkages by splitting of the terminal, non-reducing glycone (glucose) or aglycone moieties. Such enzymes are tailored to accommodate substrates having a large number of easily accessible, non-reducing chain ends at the surface and therefore exclude fibrous substrates such as native cellulose that has almost no free chain ends.

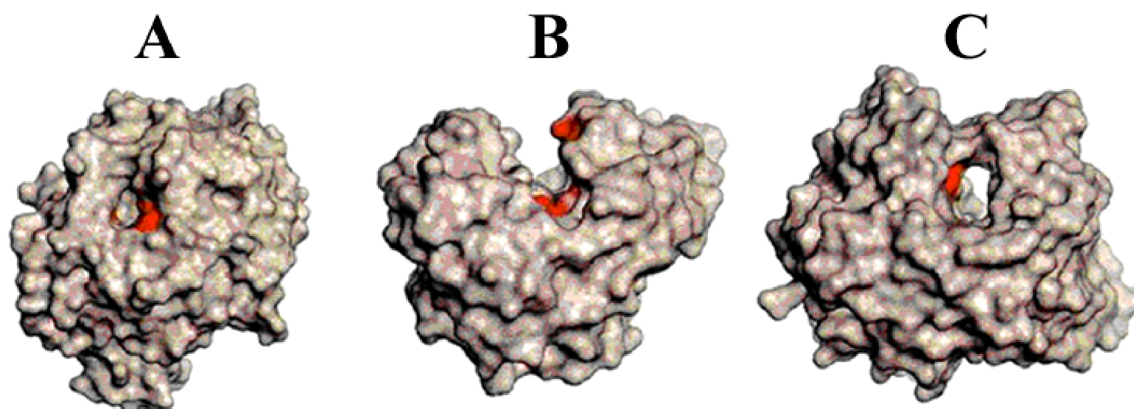

Figure 13. The 3 types of active-sites found in glycosyl-hydrolases. (A) The pocket topology of the active site found in glycosyl hydrolases such as β-glucosidases, β-galactosidases and glucoamylases. The cleft (B) and tunnel (C) topologies of the active site found in xylanases and cellobiohydrolases, respectively. The proposed catalytic residues are shaded in red [Davies and Henrissat, 1995].

β-Glucosidases display broad specificity with regard to both the aglycone and the glycone moieties of their substrates. β-Glucosidases from all sources have similar glycone specificities, however they differ in specificity towards the aglycone part of the glucoside substrate [Poulton, 1993]. The natural substrates of β-glucosidases include steroid β-glucosides and β-glucosyl ceramides of mammals, cyanogenic β-glucosides and glucosinolates of plant secondary metabolism, and oligosaccharide products released from cellulose during plant cell-wall degradation [Clarke et al., 1993]. Artificial substrates such as benzyl, nitrophenyl and methylumbelliferyl-glycosides are commonly used for the identification and characterization of glycosidases. These substrates are important in determining the kinetics (reaction velocity and substrate affinity) of a particular enzyme. However, qualitative and quantitative enzymatic assays on the natural substrates are restricted when compared to artificial substrates.

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Mode of action

Glycosyl hydrolases, including β-glucosidases, hydrolyze glycosidic bonds via the mechanism of general acid catalysis. This mechanism involves two critical residues, namely a proton donor and a nucleophile/base [Sinnott, 1990]. The two amino acid residues commonly implicated for these roles are glutamic and aspartic acids. Binding of the enzyme to the substrate not only positions the proton donor and nucleophile in exact proximity, but also decreases the energy barrier as a

result of substrate deformation and subsequent stabilization of the chemical intermediate [McCarter and Withers, 1994].

Hydrolysis, as implicated by the name, refers to the breaking of bonds by the addition of water. In glycosyl hydrolases, hydrolysis proceeds via two stereochemically-different mechanisms that result in either an overall retention, or an inversion, of the anomeric configuration as depicted in Figure 15. The major difference between retaining and inverting glycosidases is the distance between the catalytic residues. The average distances are 4.8-5.3 Å and 9.0-9.5 Å for retaining and inverting enzymes, respectively. The catalytic residues of inverting glycosidases are more distant since the active site has to accommodate a water molecule in addition to the substrate [McCarter and Withers, 1994].

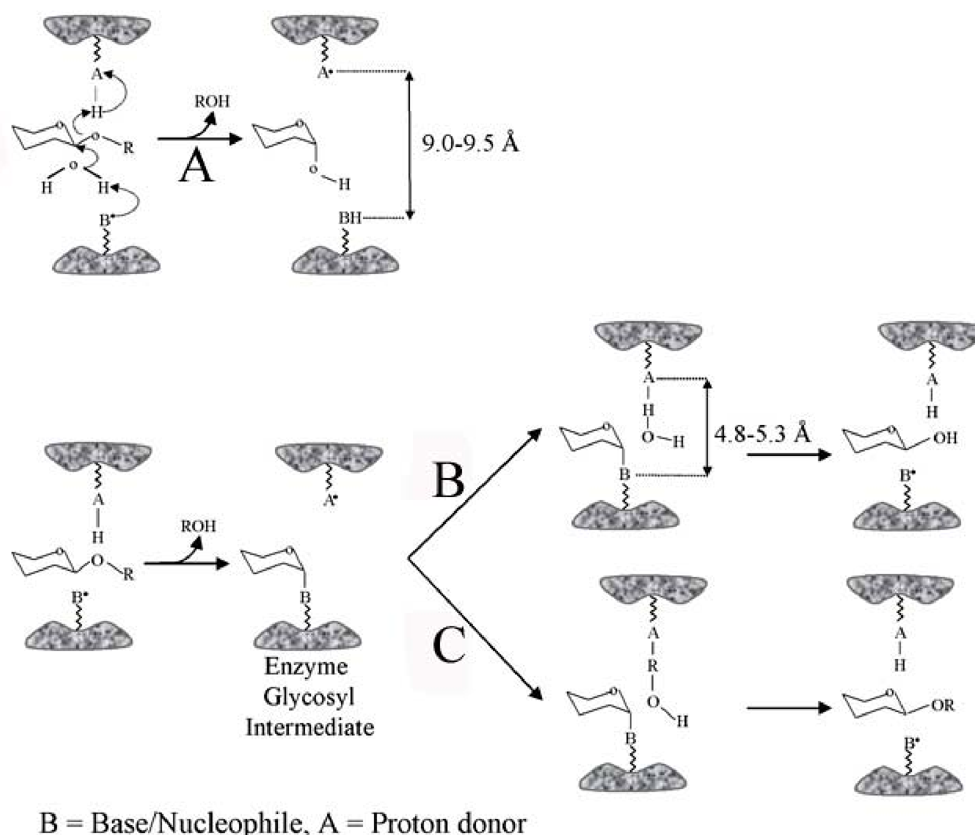


Figure 15. The mechanisms of enzymatic β-glycosidic bond hydrolysis (A and B) and synthesis (C). Hydrolysis can result in either the inversion (A) or retention (B) of the anomeric configuration of the glycosidic bond. Transglycosylation is similar to the retaining mechanism of hydrolysis, but water is replaced by a R'OH group.

Inverting glycosidases utilize the charged environment of the catalytic site to activate a water molecule for nucleophilic attack while an acidic amino acid donates the necessary proton. The charged moiety becomes protonated as a result of the nucleophilic attack of the activated water, while the proton donor becomes the charged moiety that activates the water molecule for the next reaction [Mosier et al., 1999; Davies and Henrissat, 1995]. Since the protonation of the catalytic residues alternates (or invert) at the completion of hydrolysis, the mechanism is designated inverting. This single nucleophilic substitution yields a product with opposite stereochemistry (e.g. α-D-glucopyranoside) to the substrate [Sinnott, 1990].

Retaining glycosidases, such as family 1 and 3 β-glucosidases, utilize an acidic acid residue in the active site for nucleophilic attack on the glycosyl bond of the substrate while an opposing residue donates the necessary proton. This results in the formation of a covalently bound intermediate (oxocarbonium ion). The second nucleophilic attack by an activated water molecule releases the hydrolytic product, which consecutively recharges the catalytic residue that acts as the proton donor. This second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry (i.e. β-D-glucopyranoside) as the substrate [Mosier et al., 1999; Davies and Henrissat, 1995].

An additional feature of retaining β-glucosidases is their biosynthetic ability, i.e. the synthesis of a glycosidic bond. Synthesis of a glycosidic bond occurs via 2 different approaches, reverse hydrolysis and transglycosylation. During reverse hydrolysis, reaction conditions such as low water activity (a_w), trapping of the product or high substrate concentrations result in a shift in the equilibrium of the reaction towards synthesis. This reaction is controlled thermodynamically [Bhatia et al., 2002]. In order to improve yields, the thermodynamic activity of water is often lowered by using high temperatures and organic solvents, therefore thermophilic enzymes with solvent tolerance have obvious advantages over their mesophilic counterparts for the synthesis of glycosidic bonds [Hancock et al., 2005].

During transglycosylation, the initial reaction proceeds via the same mechanism as described for retaining enzymes up to the formation of the covalently bound enzyme-glycosyl intermediate. However, the second nucleophilic attack utilizes glucose, cellobiose, aryl-, amino or alkyl-alcohol instead of water in the final step of the reaction to yield a new elongated product. This

reaction is controlled kinetically [Heather and Huber, 2005; Bhatia et al., 2002]. Transglycosylation generally produces higher yields of glycoconjugates than reverse hydrolysis [Park et al., 2005]. Transglycosylation activity is prevalent among mammalian [Vanderjagt et al. 1995], plant [Kuriyama et al., 1995], fungal [Christakopoulos, 1994], and bacterial [Watt et al., 1998] β-glucosidase enzymes. The biosynthetic properties of these enzymes have gained a lot of interest due to the prospect of using transglycosidic reactions to produce commercially important glycosides.

Applications of β-glucosidases

The two types of activity of β-glucosidases resulting in either cleavage or synthesis of glycosidic bond, is essential in several important biological pathways such as cellular signalling, degradation and biosynthesis of structural and storage polysaccharides, host-pathogen interactions as well as various biotechnological applications. The applications can be grouped into 2 general categories, namely applications based on hydrolytic activity; and applications based on synthetic activity.

Applications based on hydrolytic activity

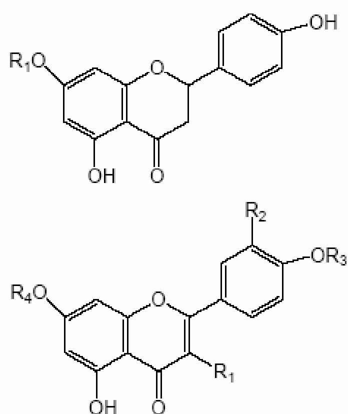
β-Glucosidase enzymes have become of great interest primarily because of their involvement in the biological saccharification of cellulosic material. They work synergistically with endoglucanases and exoglucanases on the degradation of cellulose. They not only catalyse the final step in the saccharification of cellulose, but also stimulate the extent of cellulose hydrolysis by relieving the cellobiose-mediated inhibition of exoglucanase and endoglucanase [Yan et al., 1998].

β-Glucosidases are also included in commercial feed additives, e.g. Barlican (a non-toxic cellulase enzyme mixture), for single-stomached animals such as chickens and pigs. β-Glucosidase supplementation enhances cellulose degradation and thus improves nutrient utilization [Zhang et al., 1996; Coenen et al., 1995].

Flavonoids are a large group of polyphenolic-plant secondary metabolites that contribute to most of the colour and flavour in plant foods such as fruits, vegetables, tea, red wine, and soybeans [Kühnau, 1976]. The flavonoids exist in nature almost exclusively as β-glycosides. The flavanols are mainly 3-O-glycosides, although the 7 and 4-β positions may also be glycosylated in certain plants (e.g. onions). Other classes of flavonoids such as isoflavonoids are generally glycosylated in position 7 [Mamma et al., 2004]. The primary structures of the flavonoids and isoflavonoids, with their respective positions of glycosylation, are shown in Figure 16. Flavonoids in food are generally present as glycosides since industrial and domestic food processing procedures do not cause significant glycosidic bond cleavage [DuPont et al., 2000; Price et al, 1999; 1998; 1997]. Many animal and human clinical studies as well as epidemiological studies have confirmed that isoflavones play an important role in the prevention of diseases such as cancer [Cohen et al., 2000; Messina, 1999; Setchell 1998], cardiovascular disorders [Potter et al., 1998], bone health problems [Messina, 1999; Anderson and Garner, 1998], and postmenopausal symptoms [Kurzer, 2000]. Recently, flavonoids have also attracted considerable interest from both the food industry and consumers due to their anti-oxidative qualities [Yang et al., 2001; Hollman and Katan, 1998] and their ability to inhibit enzymes such as cyclooxygenase and protein kinases involved in cell proliferation and apoptosis [Formica and Regelson, 1995]. β-Glucosidases such as GI and GII from *Penicillium decumbens* display specific activity towards flavonoids glycosylated at the 3 or 7 positions [Mamma et al., 2004]. They provide researchers with valuable tools for altering specific properties that affect the absorption and/or biological activity of (iso)flavonoids in humans [Ismail and Hayes, 2005].

β-Glucosidases also catalyze the hydrolysis of the flavonoid naringin to prunin that is an important step in the removal of the bitterness from citrus fruit juices [Roitner et al., 1984]. Similarly, the hydrolysis of diadzin and genistin to diadzein and genistein, respectively, with a *Lactobacillus casei* subspecies *rhamnosus* β-glucosidase enzyme, eliminates the undesirable bitter and astringent isoflavanoid glucosides from soybean cooked syrup (SCS) [Matsuda et al., 1994].

Flavonoids: Isoflavonoids:



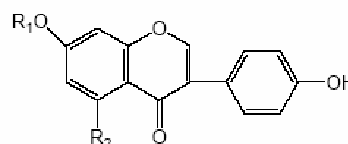


Figure 16. General structure of plant flavonoids and isoflavonoids [Mama et al., 2004].

Recombinant yeasts with the ability to secret β-glucosidases have been of particular interest to the wine industry. The must from red grapes contains high levels of pigments called anthocyanins. These pigments consist of flavylium ions glycosylated with a β-glycosidic bond. Enzymatic cleavage of the glycosidic bond releases the anthocyanidin moiety and causes decolourisation of the juice. Other important roles of β-glucosidases (also termed anthocyanases) in wine-making include the prevention of sediment formation during storage of the bottles and the recovery of free-run juice from red grape cultivars that can be used for the production of white wines with lower red colour [Sánchez-Torres et al., 1998].

In addition to β-glucosidases' function in decolourisation, they also play an important role in releasing flavour compounds, which are present in grape berries as non-volatile glycosides, thereby contributing to the aroma of the wine [Gueguen et al., 1997b]. Volatile terpenols, as well as natural non-odorous and non-volatile glycoside precursors are found in a variety of foods such as apples, grapes, fruit juices, and wines. The aglycone moiety is considered to be the source of volatile phenols such as vanillin, aliphatic, or cyclic alcohols like hexanol, 2-phenylethanoll, benzylalcohol, or terpenols like nerol, linalool, geraniol, and citronelllol [Gunata et al., 1985]. The Japanese brewing industry uses this flavour-releasing property of β-glucosidases during the making of sweet potato shochu. The key enzyme used for this process is the *A. kawachii* β-glucosidase. The enzyme releases several free monoterpene alcohols that contribute to the characteristic flavour of sweet potato shochu [Iwashita et al., 1998].

Gellan gum, an exo-polysaccharide (EPS) produced by *Sphingomonas paucimobilis*, has great potential for the food industry but its application is greatly limited by its high viscosity and low solubility. It was suggested that the hydrolytic activity of β-glucosidase may result in the production of low-viscosity gellan foods. Researchers showed that the intracellular β-glucosidases from *Bacillus* sp. catalyze the cleavage of the trisaccharides, glycosyl rhamnosyl-glucose (produced by the action of gellan lyase and extracellular glycosidases), to release glucose and rhamnosyl-glucose, thereby reducing viscosity. In addition, the structural gene (*bglA*) encoding the β-glucosidase activity in *Bacillus* has been cloned and the subsequent recombinant BglA enzyme exhibits specific hydrolytic activity towards gellan [Hashimoto et al., 1998].

β-Glucosidase activity from the thermophile *Sulfolobus solfataricus* is used in the bioconversion of oleuropein (the component associated with the bitter taste of unripe olives) to a pharmacologically active compound (hydroxytyrosol) useful in the prevention of coronary heart diseases and cancer [Briante et al., 2000; Visioli et al., 2002.]. Similarly, the deglycosylation of betacyanin (betalains) by β-glucosidase from *Beta vulgaris* releases a bioactive cellular metabolite that has anti-tumour properties [Bhatia et al., 2002]. The β-glycosidase from the plant sweet almond has already been used *in vitro* for tumor-specific activation of the naturally occurring cyanogenic glucoside, amygdalin [Syrigos et al., 1998].

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Cassava, the enlarged root of *Manihot esculenta* Crantz, is a staple food for over 500 million people in the developing world [Kostinek et al., 2005]. Depending on the variety, cassava is processed by drying, roasting, boiling, or fermentation. Fermentation is the most popular processing method, particularly for the bitter varieties high in cyanogenic-glucosides. Cyanogenic-glucosides such as linamarin, cyanohydrin and (to a lesser extent) lotaustralin are toxic and can even be fatal when consumed in unprocessed foods. During fermentation, the β-glucosidase activities of lactic acid bacteria (LAB) and yeasts liberate the toxic –CN moiety as well as flavour compounds [Gueguen et al., 1997a; Okafor and Uzuegbu, 1987]. Complete detoxification of cassava, however, requires supplementation with β-glucosidases from microbial sources like *Leuconostoc mesenteroides* [Okafor and Ejiofor, 1990]. In addition, the high starch content of cassava (80% per DW) makes it an inexpensive source of fermentable starch for the production of bioethanol. However, waste processing is a significant problem and enzymatic detoxification with β-glucosidases can be a costly procedure [Lin and Tanaka, 2006].

β-Glucosidases from bacterial sources, such as *Cellovibrio mixtus* [Sakellaris et al., 1997], *Thermoanaerobacter brockii* [Breves et al., 1997], *Thermotoga neopolitana* [Zverlow et al., 1997], also exhibit laminaribiase activity that can be used along with laminarinase (endo β-1,3-glucanase) to hydrolyze β-1,3-glucan and release glucose from laminaridextrins and laminaribiose. This specific activity has an important role in the production of yeast extract and the conversion of algal biomass to fermentable sugars [Bhatia et al., 2002].

Certain plant β-glucosidases play an important role in pigment metabolism and isolation. For example, β-glucosidases are used to isolate precarthamine pigment from dried saffron (*Crocus sativus*) flower florets [Saito, 1993]. These pigments can be used as natural food dyes in confectionery products [Zakharova and Petrova, 2000].

Applications based on synthetic activity

Currently, β-glucosidases are mostly studied for their biosynthetic properties. The transferase activity exhibited by these versatile biocatalysts is used for the production of various products such as different oligosaccharides, glycol-conjugates, alkyl and amino-glucosides. β-Glucosidases are attractive for large-scale application since they are widespread, relatively cost-effective, commercially available, exhibit broad acceptor–substrate specificity and use relatively simple substrates [Moracci, et al., 2001]. Yet, stringent donor specificity is the main feature limiting their use [Hancock et al., 2005]. Enzymatic synthesis's major advantage is its regioselectivity and stereochemistry during bond formation. Both reverse hydrolysis and transglycosylation are used for this purpose. Chemical methods are usually slow and non-specific and require extensive protection routines [Bhatia et al, 2002].

Oligosaccharides have an important role in biological recognition and signalling and receive a lot of attention from the pharmaceutical industry due to their potential as therapeutics [Look et al., 1993]. Recently, a recombinant β-glucosidase enzyme from *Pichia etchellsii* was purified from *E. coli* and subsequently used for the biosynthesis of oligosaccharides. The reverse hydrolysis and transglycosylation mechanisms yielded 14% and 8% oligosaccharides, respectively. Dimethylsulfoxide (DMSO) was found to increase the product yield of transglycosylation by an

additional 10%. The presence of DMSO also significantly increased the V_{max}/K_m of synthesis [Bhatia et al., 2002].

Alkyl-glucosides are new-generation biodegradable, non-ionic surfactants with superior emulsifying and antimicrobial activities. They are used as drug carriers and as solubilizing agents for biological membranes, specifically hexyl-, heptyl-, and octyl-glucosides [Kiwada et al., 1985; Shinoyama et al., 1991]. Butyl-glucoside is important in the production of gemini surfactants and other pharmaceutical products. Gemini surfactants are defined as of particular importance in the manufacturing of liquid crystals [Castro et al., 1997]. In addition, esterification of butyl-glucoside in a coupled β-glucosidase/*Candida* sp. lipase reaction produces an aromatic n-alkyl glucoside ester that is used for the treatment of fever, rheumatism, headache, and other diseases [Otto et al, 1998]. Methyl-glucoside is an important precursor for the synthesis of methyl-laminario oligosaccharides that are used in AIDS medication [Bhatia et al., 2002].

During β-glucosidase-catalyzed biotransformations, various alcohols (e.g. primary, secondary, tertiary, monoterpene, and aryl alcohols) can serve as acceptors of the glucosyl group. For example, the glucosides of organo-silicon alcohols produced by a *Pyrococcus furiosus* β-glucosidase, have potential application in the agrochemical and drug industries [Fischer et al., 1996]. The food industry is particularly interested in the enzymatic synthesis of glucosides and monoterpenyl alcohols that can be marketed as "natural food flavours" [Gunata et al., 1994]. The thermostable β-glucosidase from *Sulfolobus solfataricus* was used to synthesize a number of natural compounds (aryl-glucosidases) that exhibit valuable repellent and anti-feedant properties [Tricone and Pagnotta, 1995].

Recently, the *S. solfataricus* β-glucosidase was mutated in an approach to expand its substrate specificity. The residues implicated in determining glucoside and galactoside specificity, were identified as glutamate (E432) and tryptophan (W433). This specific mutant is of particular value in the synthesis of alkyl glycosides, such as methyl β-mannoside and methyl β-xyloside. The β-glucosidase-catalyzed transglycosylation reactions yielded 53% methyl β-mannoside and 92% methyl β-xyloside [Hancock et al., 2005].

An additional field of study is that of glycosidase inhibitors. Since glycosidases are involved in a variety of metabolic disorders (e.g. diabetes), bacterial infection, viral attachment and cancer development, inhibition of glycosidases are of special interest. Powerful and selective glycosidase inhibitors have potential application in the treatment of AIDS, diabetes, and cancer as well as crop protection. They are also used as probes for determining the active-site topology of β-glucosidases [Bhatia et al., 2002].

ETHANOL PRODUCTION

Ethanol (also termed ethyl alcohol) is described as a clear, colourless, flammable oxygenated hydrocarbon, with the chemical formula C_2H_5OH. Commercially ethanol is produced by two processes: (i) synthetic alcohol from non-renewable sources (eg. crude oil or gas and coal); and (ii) fermentation alcohol from renewable sources (eg. sugar cane, grains, molasses, cellulose etc.). Both synthetic and fermentation alcohol (or bio-ethanol) are chemically identical. Some of the important physical and chemical properties of ethanol are presented in Table 7.

Table 7. Physical and chemical properties of ethanol [NIST Chemistry Web Book: Chemical and Physical Properties of Ethanol; http://webbook.nist.gov/cgi/cbook].

Empirical formula	C_2H_6O
Molar mass	46.07 g/mol
Density (phase)	0.789 g/cm^3 (liquid)
Solubility in water	Fully miscible
Melting point	-114.3°C
Boiling point	78.4°C
Flash point	13°C
Viscosity	1.200 cP at 20°C
Acidity (pKa)	15.9 (H^+ from OH groups)
Energy content	26.7 x 10^9 J/kg
Density	0.789 kg/litre

World ethanol production reached the 45.4 billion-litre mark in 2005, with the U.S. and Brazil contributing 16.3 and 15.0 billion litres to the total, respectively [Ethanol Market Report; www.celunol.com]. Figure 17 illustrates the current market share (%) held by the leading ethanol producing countries. The majority (~80%) of ethanol produced in the world is obtained by fermentation [Lin and Tanaka, 2006]. Alcoholic beverages are certainly the oldest form of use of alcohol. However, the most important market for ethanol as an industrial application is solvents. Solvents are used in the production of paints and coatings, pharmaceuticals, adhesives,

inks, etc. Solvent production and consumption is concentrated in the industrialized countries in the U.S., Europe and Asia and is the only market where synthetic ethanol producers make a significant contribution. Ethanol is also used in the production of perfumes; as a preservative for biological specimens; in the preparation of essences and flavourings; in various medicines and drugs, as a disinfectant; and as a fuel and gasoline additive [Berg, 2004].

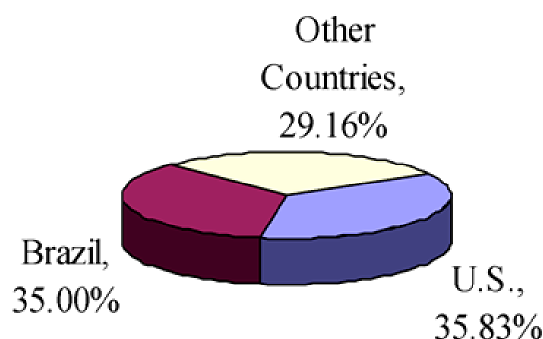

Figure 17. The world market share (%) held by the major ethanol producers, U.S. and Brazil, in 2005 [Ethanol Market Report; www.celunol.com/marketprint.html].

Ethanol as fuel

The utilization of ethanol as fuel dates back to the early 1800s as illustrated on the timeline in Figure 18. Since then it has been widely used by the transportation sector until the discovery of new oil wells in the 1980s and 1990s that provided gasoline as a cheap and abundant alternative. Recently, a renewed interest in ethanol as fuel has been ignited by environmental and health concerns associated with gasoline [Ahmed, 2001]. Complete combustion of ethanol produces only carbon dioxide (CO_2) and water as products and is therefore considered a “cleaner-burning fuel” compared to gasoline. In addition, ethanol produced from renewable sources such as cellulosic biomass results in no net CO_2 being produced and is suggested to have a positive influence on global warming [Von Blottnitz and Curran, 2006].

Fuel ethanol can either be used as a 100% gasoline substitute or in blends. Hydrous ethanol (with purities of up to 96%) is the product used for straight-ethanol combustion. Hydrous ethanol is obtained by the time-honoured distillation process. However, for blending with gasoline, anhydrous ethanol with purities of 99.5 to 99.9% is required. Currently the most

commonly used purification method for the production of anhydrous ethanol is a physical absorption process using molecular sieves [Hu and Xie, 2001; Rakshit et al., 1993].

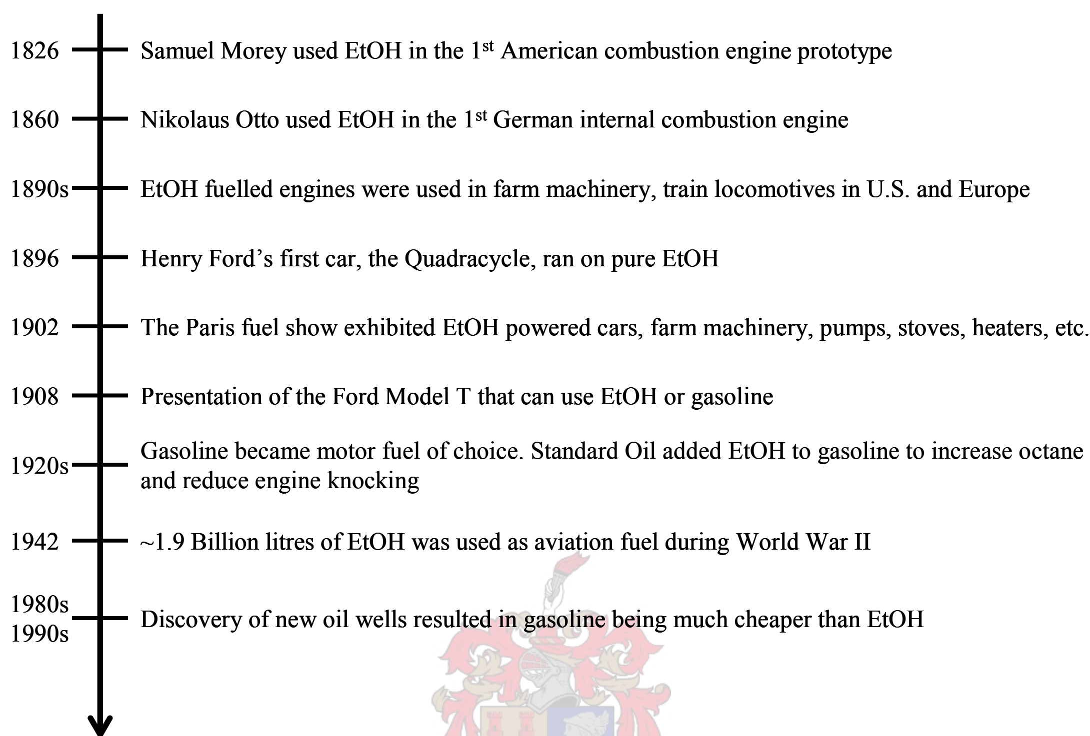

Figure 18. Timeline of ethanol fuel usage [obtained from Energy Information Administraion (EIA), www.eia.doe.gov/oiaf/ethanol3.html].

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Ethanol/gasoline blends are referred to as gasohol. Gasohol with an ethanol content of 5%-25% can be introduced without any modification to vehicle engines or refuelling equipment. Currently, "E10" (containing 10% ethanol and 90% gasoline) is the most common gasohol variant used worldwide (U.S. Department of Energy website, www.eere.energy.gov). Ethanol blending provides a valuable "octane booster". Gasoline/ethanol blends achieve the same octane boosting or anti-knock effect as petroleum derived aromatics like benzene or metallic additives like lead and adds to ethanol's reputation as an environmental friendly alternative [Von Blottnitz and Curran, 2006]. The energy content of ethanol is only 97% compared to that of gasoline. Therefore the 3% lower energy content has to be included in the calculations comparing the cost-competitiveness (fuel economy) of ethanol and gasoline [Kim and Dale, 2003].

Cellulose-to-ethanol technologies

The raw materials currently used for bioethanol production are grouped into 3 categories: (i) sugars; (ii) starches; and (iii) cellulosic biomass. Sugars (from fruit, sugar beets, molasses and sugarcane) can be directly fermented into ethanol. Starches (from corn, cassava, potatoes, etc.) require the action of α-amylase and glucoamylase enzymes to produce fermentable sugars and subsequently ethanol. Cellulose (from wood, agricultural residues, etc.) must also be hydrolysed to fermentable sugars (by either chemical or enzymatic conversion) before microbial ethanol production can proceed [Lin and Tanaka, 2006]. From an economical point of view there are several reasons why cellulosic biomass is considered an attractive feedstock for bioethanol production:

⇨ Low and stable cash costs. Cellulosic feedstock has a much lower and less volatile cost than other conventional starch-based feedstocks.

⇨ Production near demand. Plants can be constructed near end-use markets to reduce transportation costs.

⇨ Cellulosic ethanol plants can make a significant contribution to waste (including agricultural and municipal waste as well as pulp and paper sludge) remediation.

⇨ Environmental benefits. Combustion of cellulosic ethanol will contribute to the reduction in greenhouse gas emissions (CO_2). The "carbon credit" points awarded for the development of technologies that reduce global warming may add an additional financial benefit.

⇨ Energy security. The substitution of gasoline with ethanol will improve energy security by reducing oil imports.

⇨ By-product usability. Lignin is the only major by-product that results from the process and can be burned as fuel to provide steam and electricity for the ethanol production plant in order to reduce energy costs.

The following discussion will focus primarily on the enzymatic hydrolysis and subsequent fermentation of lignocellulose to ethanol. Table 8 presents some of the commercial enzyme mixtures available for the hydrolysis of cellulose. Currently these cocktails are still too expensive to be considered for industrial-scale usage. However, there are several research

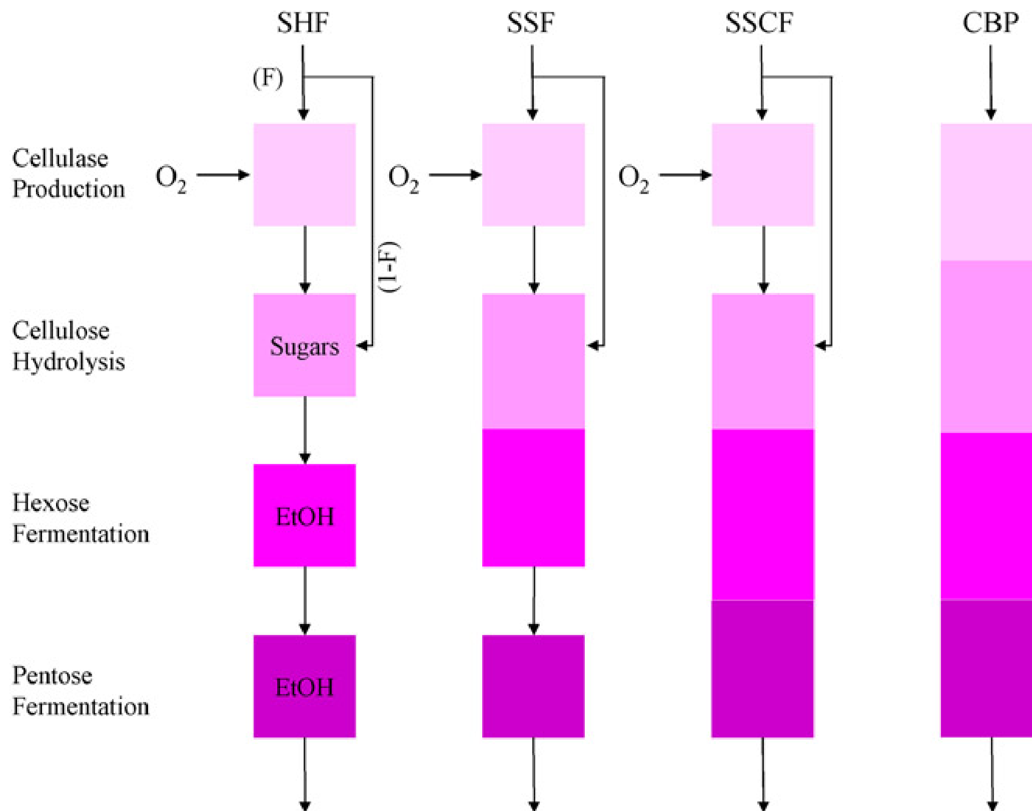
groups dedicated to reduce the cost of cellulase enzyme cocktails. The approaches used to reduce enzyme costs include: (i) screening for novel enzymes; (ii) strain improvement of existing industrial organisms and enzyme engineering; (3) production and operations related aspects such as selection of substrate, culturing conditions, recycling of enzymes and redesigning of processes [Howard et al., 2003].

Table 8. Commercial cellulase enzyme preparations

Preparation	Microbial Source	FPU*/mg	β-Glucosidase U/mg	CMC U/mg
Biocellulase TRI	*T. reesei*	0.24	0.72	5.5
Biocellulase A	*A. niger*	0.01	1.4	3.6
Cellulast 1.5L	*T. reesei*	0.37	0.16	5.1
Cellulase TAP10	*T. viride*	0.13	5.2	14
Cellulase AP30K	*A. niger*	0.03	10	21
Cellulase TRL	*T. reesei*	0.57	1.0	13
Econase CE	*T. reesei*	0.42	0.48	8.5
Multifect CL	*T. reesei*	0.42	0.20	7.1
Multifect GC	*T. reesei*	0.43	0.39	13
Spezyme #1	*T. reesei*	0.54	0.35	15
Spezyme #2	*T. reesei*	0.57	0.42	15
Spezyme #3	*T. reesei*	0.57	0.46	25
Ultra-low Microbial	*T. reesei*	0.48	0.96	ND

*FPU (Filter paper units): 1 FPU = 1 μmole.min^{-1} glucose released.

The complete biological conversion of cellulosic biomass to fuel (or chemicals) involves five successive process steps: (i) cellulase production; (ii) pretreatment of cellulosic biomass to be assessible to enzymes; (iii) hydrolysis of cellulose and (if present) other insoluble polysaccharides; (iv) fermentation of soluble cellulose hydrolysis products; and (v) fermentation of soluble hemicellulose hydrolysis products to ethanol [Lynd et al., 2002]. The term separate hydrolysis and fermentation (SHF) describes the conversion of cellulose to ethanol using four separate process steps. This may implicate as many as four different biocatalysts (enzymes/microorganisms) [Alfani et al., 2000]. The cost-effectiveness of this technology is closely related to the degree to which some of these process steps can be combined (consolidated). For example, during simultaneous saccharification and fermentation (SSF) hydrolysis and fermentation of cellulose hydrolysis products are consolidated into one process step, with cellulase production and fermentation of hemicellulose hydrolysis products occurring in two additional separate process steps [Deshpande et al., 1983]. Simultaneous saccharification and co-fermentation (SSCF) involves two process steps: cellulase production and a second step



that combines cellulose hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products [McMillan et al., 1999]. Ultimately, the goal would be consolidated bioprocessing (CBP), which will combine cellulase production, hydrolysis, and fermentation of products of both cellulose and hemicellulose hydrolysis in a single process step [Lynd et al., 2002]. The trend of consolidating process steps towards CBP is most evident in the field of ethanol production [Wooley et al., 1999; Wright, 1988; Wright et al., 1988]. Figure 19 is a schematic representation of the different processes involved in biomass conversion and the strategies to achieve CBP technology. Cellulase production is a key process in SHF, SSF, and SSCF but not in CBP. Aerobic microorganisms, like *T. reesei*, have been effectively used for the production of cellulolytic enzymes [Sharma, 2000]. The goal of CBP is the construction of a recombinant microorganism that will be able to perform the cellulose-to-ethanol conversion in a single process step. Two strategies have been suggested for the development of a cellulolytic ethanol-producing host: (i) the native cellulolytic strategy; and (ii) the recombinant cellulolytic strategy [Lynd et al., 2002].

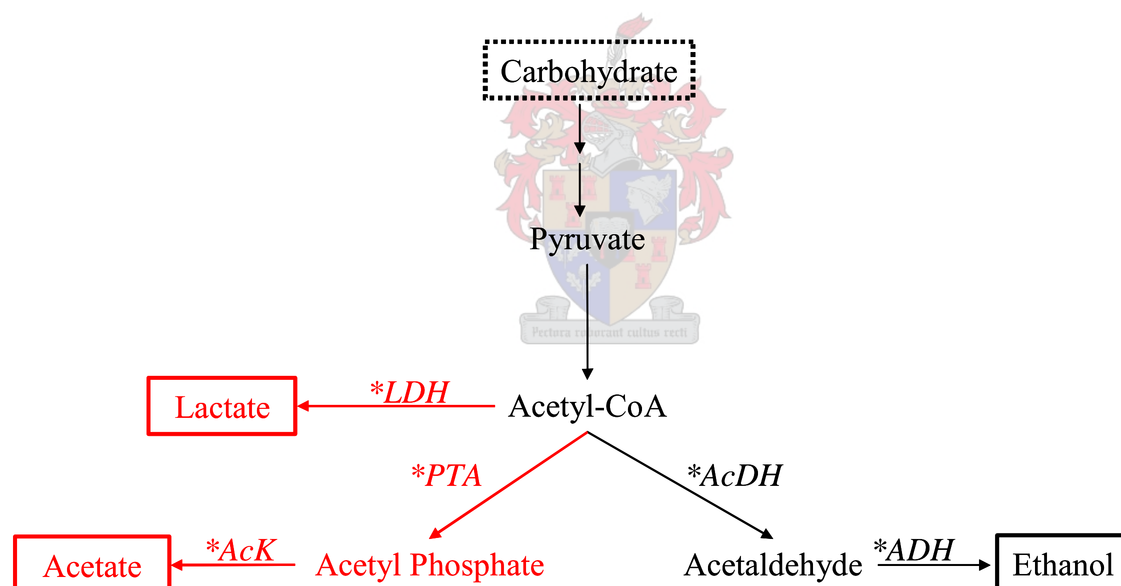
Figure 19. Strategies towards the development of cost-effective biomass-processing technologies. Each box represents a process step or bioreactor in the complete conversion of cellulose to ethanol. A fraction

of the cellulose feedstock (F) is used for cellulase production while the remainder (1-F) is hydrolysed and fermented to ethanol. The abbreviations SHF, SSF, SSCF and CBP are discussed in the text [Lynd et al., 2002].

Native cellulolytic strategy

The native cellulolytic strategy focuses on the metabolic engineering of cellulolytic anaerobes to primarily produce ethanol. Molecular techniques are focussed on identifying potential gene knock-outs that could redirect the carbon flux of the particular microorganism to ethanol as the major end-product [Lynd et al., 2002].

A major step towards the genetic engineering of potential CBP hosts through the native cellulolytic strategy was the successful construction of gene transformation systems for anaerobes such as *C. cellulolyticum* [Tardif et al., 2001; Jennert et al., 2000], *C. thermocellum*



[Tyurin et al., 2004], *C. thermosaccharolyticum* [Klapatch et al., 1996] and *Thermoanaerobacterium* spp. [Mai and Wiegel, 2000; Mai et al., 1997]. The branched catabolism presented in Figure 20 is typical of these ethanol-producing cellulolytic anaerobes. In contrast to a carbon-centred catabolism where a single end-product is produced, the branched catabolic pathway results in the production of several end-products, namely ethanol, lactic acid and acetic acid [Lynd et al., 2002].

Figure 20. A simplified illustration of the branched catabolism that is typical of ethanol-producing cellulolytic bacteria. In addition to ethanol, lactate and acetate are also produced as end-products. The enzymes that catalyses the different reactions are indicated in italics: *LDH* = Lactate Dehydrogenase; *PTA*

= Phosphotransacetylase; *AcK* = Acetate Kinase; *AcDH* = Acetate Dehydrogenase; and *ADH* = Alcohol Dehydrogenase.

The challenge with branched catabolic pathways in ethanol-producing anaerobes is to increase the carbon flux towards ethanol production and away from lactate and acetate. This has been achieved through both metabolic engineering and optimization of culture conditions [Lynd et al., 2002; Murray and Khan, 1983]. Metabolic engineering strategies included classical mutation and selection procedures [Lynd et al., 2002; Mayer et al., 1995; Rothstein, 1986; Wang et al., 1983] as well as molecular based techniques that focused on the deletion and over-expression of specific genes within the "undesirable" and "desirable" pathways, respectively [Harris et al., 2000; Boynton et al., 1996; Green and Bennett, 1996; Green et al., 1996; Biswas et al., 1993]. A significant achievement regarding the deletion (gene knockout) strategy was obtained with a *T. saccharolyticum* strain in which lactic acid [Desai et al., 2004] and acetic acid [Lynd et al., 2005] production were completely eliminated. This resulted in a recombinant strain that was able to produce ethanol as the single end-product. The recent availability of an efficient transformation system for *C. thermocellum* [Tyurin et al., 2004] could allow the construction of similar gene knockouts for this potential CBP host.

Ethanol and organic acid tolerance is an important factor influencing the feasibility of CBP through the current strategy. Most strains of *C. thermocellum* exhibit relatively low ethanol tolerance (50% inhibition at 4-16 g/L) [Sato et al., 1993; Herrero and Gomez, 1980], however, exposure to ethanol have resulted in the isolation of several strains with increased tolerance (50% inhibition at 21-40 g/L) [Baskaran et al., 1995; Klapatch et al., 1994]. Ethanol-induced changes in the cell membrane have been considered a possible mechanism by which ethanol causes end-product inhibition of the glycolytic enzymes [Herrero et al., 1985; 1982]. A recent study aimed at the characterization of ethanol-tolerant strains of *C. thermocellum* has identified a strain that can withstand up to 60 $g.L^{-1}$ added ethanol [Strobel and Lynn, 2004]. In spite of this seemingly high tolerance, ethanol production is currently still limited to ~26 $g.L^{-1}$ by recombinant strains of *T. thermosaccharolyticum* and *C. thermocellum* [Lynd et al., 2001; Sato et al., 1993]. The discrepancy between the apparent tolerance of *C. thermocellum* to added ethanol and the maximum ethanol produced remain unexplained.

Organic acids (e.g. lactate and acetate) and salts have a strong inhibitory effect on the growth of *C. thermocellum* [Ingram et al., 1998]. The level of organic acid production is directly linked to the amount of base added for pH control and consequently the level of salt present in the fermentation broth. Growth of *C. thermocellum* and *T. thermosaccharolyticum* in cellulose medium is strongly inhibited by this increase in salt concentration [Lynd et al., 2002; 2001]. The exact mechanism by which organic acids and salts inhibits cellulose-fermenting cells have not been elucidated. Salt inhibition has been considered a possible factor responsible for the discrepancy between tolerance of *C. thermocellum* (and probably other cellulolytic anaerobes) to added ethanol and maximum ethanol production. If true, increased ethanol production as well as ethanol tolerance could be achieved through metabolic engineering aimed at decreasing/eliminating organic acid production [Lynd et al., 2002].

The methylotrophic yeast, *Hansenula polymorpha*, has also been investigated as a promising organism for the production of ethanol from lignocellulosic sugars. This thermotolerant yeast with optimal growth temperature of 37°C (and maximum growth temperature of 48°C) [Sahm, 1977] has the ability to ferment both xylose and cellobiose to ethanol [Ryabova et al., 2003]. For SSF, the optimum temperature for enzymatic hydrolysis is between 45-50°C and a thermotolerant yeast such as *H. polymorpha* would be considered a highly desirable candidate for fermentation [Chandrakant and Bisaria, 1998]. At 37°C, the rate of xylose fermentation by *H. polymorpha* is comparable with that of *P. stipitis* at 30°C. The highest ethanol yield (after 96 hours at 37°C) was obtained with a strain designated KT2 which produced 3.4 and 5.3 $mg.ml^{-1}$ ethanol from xylose and cellobiose, respectively. Isolation of a riboflavin-deficient mutant of *H. polymorpha* resulted in a significantly increased (40-50%) ethanol productivity from glucose and xylose. It was suggested that under flavin limitation the flux of pyruvate is redistributed to the pyruvate decarboxylase and alcohol dehydrogenase enzymes for subsequent ethanol production [Ryabova et al., 2003]. Although ethanol production by *H. polymorpha* is not yet comparable to that of *S. cerevisiae*, it has significant potential for further improvement through genetic engineering strategies.

Recombinant cellulolytic strategy

The recombinant cellulolytic strategy focuses on the genetic engineering of non-cellulolytic microorganisms with desirable product formation properties. Molecular techniques are directed towards the development of cellulose-utilizing strains by means of heterologous expression of functional cellulase systems [Lynd et al., 2002]. Currently, the major advantage of the recombinant strategy is the availability of advanced tools for genetic engineering of several organisms that have the potential for industrial application. The most important challenge of this strategy is creating recombinant hosts able to utilize pre-treated lignocellulose at a rate comparable with that of cellulolytic anaerobes.

Recombinant cellulase systems (complexed or non-complexed) require a minimum of components/activities to facilitate growth of its host on a specific pre-treated substrate (note that pre-treated substrates are deficient in hemicellulose). Detailed investigations by Lynd et al. (2002) concluded that the following components are minimal requirements for the different cellulase systems. Non-complexed systems need at least (i) a cellobiohydrolase acting on the reducing ends; (ii) a cellobiohydrolase acting on the non-reducing ends; (iii) an endoglucanase; and (iv) a β-glucosidase. For complexed systems (i) a scaffoldin with CBM, minimum 2 cohesins, and cell wall-anchoring domain; (ii) an exoglucanase; (iii) an endoglucanase; and (iv) a β-glucosidase or cellobiose- and cellodextrin phosphorylase, is required. The latter phosphorylases are intracellular enzymes therefore require cellobiose/cello-oligosaccharide permeases. In addition, the cellulases should also contain dockerins that enable binding to the scaffoldin. Recombinant cellulase systems, both non-complexed [Boisset et al., 2001] and complexed [Fiérobe et al., 2001], that meet these requirements have resulted in reasonably good hydrolysis rates on crystalline cellulose.

Bacterial hosts that have been successfully engineered for heterologous cellulase production are *E. coli* [Zhou et al., 1999; Okamoto et al., 1994], *Klebsiella oxytoca* [Zhou and Ingram, 2001; 1999; Wood and Ingram, 1992] and *Zymomonas mobilis* [Okamoto et al., 1994; Wood and Ingram, 1992]. The *Erwinia chrysanthemi* endoglucanases, CelY and CelZ, and *Acetobacter xylinum* CMCase are some of the cellulase enzymes that have been functionally produced in these hosts. Heterologous expression of the *celZ* has been optimised in both *E. coli* and *K. oxytoca*, with total cellulase activity representing 4-6% [Zhou et al., 1999] and 5% [Zhou and Ingram, 1999] of the total cellular protein, respectively. The recombinant CelZ produced in

Z. mobilis displayed specific activity that was similar to that of the native enzyme [Brestic-Goachet et al., 1989]. Heterologous production of the *A. xylinum* CMCase in *Z. mobilis* also resulted in a functional enzyme that was primarily located in the periplasmic space [Okamoto et al., 1994].

Interestingly, Zhou and Ingram (2000) found that the *E. chrysanthemi* endoglucanases (CelY and CelZ) act in a synergistic manner during hydrolysis of CMC and acid-swollen cellulose. Closer investigation concluded that the synergy displayed by these enzymes result in the sequential hydrolysis of CMC. CelY catalyzes the initial hydrolysis of CMC to smaller cello-oligosaccharides, whereas CelZ converts the cello-oligosaccharides to cellotriose and cellobiose. Co-expression of the *celY* and *celZ* genes in a *K. oxytoca* strain that has the native ability to transport and metabolize cellobiose resulted in a 22% increase in ethanol production [Zhou et al., 2001]. This strain was engineered for improved ethanol production by integrating the *Z. mobilis pdc* (pyruvate decarboxylase) and *adhB* (alcohol dehydrogenase) genes on its chromosome. The resulting strain, *K. oxytoca* SZ21, was able to produce 20,000 U of endoglucanase activity per litre and ferment amorphous cellulose to ethanol with yields of 58-76% of the theoretical [Zhou and Ingram, 2001].

With regards to eukaryotic hosts, heterologous cellulase production in the yeast *S. cerevisiae* has been the focus of several research groups (Table 9). Fujita et al., (2002) constructed a recombinant *S. cerevisiae* strain that co-display two types of cellulolytic enzymes on its cell surface. The *T. reesei* endoglucanase II (EGII) and *A. aculeatus* β-glucosidase 1 (BGL1) were anchored to the yeast cell surface by fusing the mature proteins to the α-agglutinin peptide. Functional expression of the EGII and BGL1 enzymes facilitated growth of the recombinant *S. cerevisiae* strain on medium containing β-glucan as sole carbon source. Barley β-glucan is a linear, soluble polymer of β-linked glucose units that consist of about 70% β-1,4- and 30% β-1,3-linkages. During anaerobic fermentation of β-glucan by the recombinant *S. cerevisiae* strain, 0.51 grams of ethanol per grams of carbohydrate was produced which corresponds to 93.3% of the theoretical yield. The ethanol productivity of the strain was 0.53 $g.L^{-1}.h^{-1}$. More recent work by the same group resulted in a recombinant *S cerevisiae* strain that co-displays three types of cellulases on its cell surface [Fujita et al., 2004]. Cell surface display of the *T. reesei* endoglucanase I (EGI) and cellobiohydrolase II (CBHII) as well as the *A. aculeatus*

β-glucosidase I (BGL1) resulted in a strain that could significantly degrade insoluble phosphoric acid-swollen cellulose (10 g/L). The ethanol yield was 0.45 g/g carbohydrate consumed (i.e. 88.5% of theoretical yield) and the ethanol productivity of the strain was 0.25 $g.L^{-1}.h^{-1}$. The current state of whole-cell biocatalysts that can convert amorphous cellulose to ethanol in a single step support the achievability of CBP, however, major improvements need to be achieved in order for it to be recognized as a cost-competitive technology. According to the U.S. Department of Energy's "Biofuels joint roadmap" (June, 2006), the cellulose-to-ethanol process will only become cost-competitive relative to the current cornstarch ethanol process when ethanol yields of greater than 95% (of theoretical yield) and total volumetric productivity of 2-5 g $ethanol.L^{-1}.h^{-1}$ under high-solid conditions are achieved.

Table 9. Recombinant cellulase enzymes produced in the yeast *S. cerevisiae*

Enzyme	Gene	Donor	Reference
ENDOGLUCANASES			
Endo-β-1,3-glucanase	*bglH*	*Bacillus circulans*	Nakajima et al., 1993.
Endo-β-1,3-1,4-glucanase	*end1*	*Bacillus subtilis*	Cantwell et al., 1986; Hinchliffe and Box, 1984; Skipper et al., 1985.
Endo-β-1,4-glucanase	*cenA*	*Butyrivibrio fibrisolvens*	Petersen et al., 1998; Van Rensburg et al, 1994; Van Rensburg et al., 1996.
Endoglucanase		*Cellulomonas fimi*	Skipper et al, 1985.
Endo-β-1,4-glucanase		*Trichoderma longibrachiatum*	Pérez-González et al, 1996; 1993.
Endo-β-1,4-glucanase		*Clostridium thermocellum*	Sacco et al, 1984.
Endo-β-1,3-glucanase	*BGL2*	*Saccharomyces cerevisiae*	Klebl and Tanner, 1989; Mrsa et al., 1993.
Endo-β-1,3-1,4-glucanase I	*egl1*	*Trichoderma reesei*	Aho and Paloheimo, 1990; Bailey et al, 1993; Penttilä et al, 1987a; Van Arsdell et al., 1987.
Endo-β-1,3-1,4-glucanase III	*egl1*, *egl3*, *egl4*, *egl5*	*Trichoderma reesei*	Saloheimo et al., 1997; 1994; Penttilä et al, 1987a; 1987b; Suihko et al., 1991; Zurbriggen et al., 1991.
FI-carboxymethylcellulase		*Aspergillus aculeatus*	Ueda and Tanaka, 2000; Ooi et al., 1994.
Endo-β-1,3-glucanase		*Nicotiana plumbaginifolia*	Demolder et al., 1993.
EXOGLUCANASES			
Exo-β-1,4-glucanase	*cex*	*Cellulomonas fimi*	Curry et al., 1988.
Exo-β-1,3-glucanase I + II	*EXG1*/*BGL1*	*Saccharomyces cerevisiae*	Nebreda et al., 1986.
Exo-β-1,3-glucanase III	*EXG2*	*Saccharomyces cerevisiae*	Klebl and Tanner, 1989.
CELLOBIOHYDROLASES			
Cellobiohydrolase	*CEL3*	*Agaricus bisporus*	Chow et al., 1994.
Cellobiohydrolase	*cbh1*	*Aspergillus aculeatus*	Murai et al, 1998; 1997; Takada et al., 1998.
Cellobiohydrolase		*Clostridium thermocellum*	Uozumi et al., 1993; Saito et al, 1990.
Cellobiohydrolase		*Penicillium janthinellum*	Koch et al., 1993.
Cellobiohydrolase	*CBH1*	*Phanerochaete chrysosporium*	Petersen et al., 1998; Van Rensburg et al, 1998; 1997.

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Table 9 (continued). Recombinant cellulase enzymes produced in the yeast *S. cerevisiae*

Cellobiohydrolase I	*cbh1*	*Trichoderma reesei*	Aho and Paloheimo, 1990; Penttilä et al., 1988; Van Arsdell et al., 1987.
Cellobiohydrolase II	*cbh2*	*Trichoderma reesei*	Bailey et al., 1993; Aho and Paloheimo, 1990; Zurbriggen et al 1990; Penttilä et al., 1987.
ENDO/EXO-GLUCANASES			
Endo/exoglucanase	*cel*	*Bacillus* sp. strain DO4	Cho et al., 1999; Han et al., 1995.
β-GLUCOSIDASES			
β-Glucosidase	*bgl1*	*Aspergillus aculeatus*	Ueda and Tanaka, 2000; Takada et al., 1998.
β-Glucosidase	*bglA*	*Aspergillus kawachii*	Van Rooyen et al., 2005.
β-Glucosidase		*Aspergillus niger*	Penttilä et al., 1984.
β-Glucosidase	*bgl*	*Bacillus circulans*	Cho and Yoo, 1999; Cho et al., 1999.
β-Glucosidase	*bglA*, *bglB*, *bglC*	*Cellulomonas biazotea*	Rajoka and Malik, 1997; Rajoka et al, 1992
β-Glucosidase	*BGLN*	*Candida molischiana*	Sànchez-Torrez et al., 1998
β-Glucosidase		*Candida pelliculosa*	Kohchi and Toh-e, 1986.
β-Glucosidase	*bglB*	*Candida wickerhamii*	Van Rooyen et al., 2005.
β-Glucosidase		*Kluyveromyces lactis*	Raynal et al., 1987; Raynal and Guérineau, 1984.
Cellobiase	*BGL1*	*Saccharomycopsis fibuligera*	Van Rooyen et al., 2005; Van Rensburg et al, 1998; Machida et al., 1988.
β-Glucosidase	*BGL2*	*Saccharomycopsis fibuligera*	Machida et al., 1988.
β-Glucosidase	*Bgl*	*Trichoderma reesei*	Van Rooyen et al., 2005.

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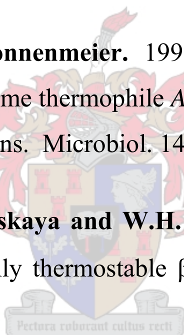

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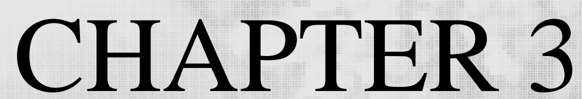

CHAPTER 3

Manuscript 1

Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains

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Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains

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Abstract

β-Glucosidase genes of fungal origins were isolated and heterologously expressed in *Saccharomyces cerevisiae* to enable growth on the disaccharide, cellobiose. To promote secretion of the β-glucosidases, the genes were fused to the secretion signal of the *Trichoderma reesei xyn2* gene and constitutively expressed from a multi-copy yeast expression vector under transcriptional control of the *S. cerevisiae PGK1* promoter and terminator. The resulting recombinant enzymes were characterized with respect to pH and temperature optimum, as well as kinetic properties. The two most promising enzymes, BGL1 from *Saccharomycopsis fibuligera* and BglA from *Aspergillus kawachii*, were anchored to the yeast cell surface by fusing the mature proteins to the α-agglutinin (AGα1) or cell wall protein 2 (Cwp2) peptides. The maximum specific growth rates (μ_{max}) of the recombinant *S. cerevisiae* strains were determined in batch cultivation. *S. cerevisiae* secreting the recombinant *S. fibuligera* BGL1 enzyme sustained growth aerobically and anaerobically, in minimal medium containing 5 g L^{-1} cellobiose at 0.23 h^{-1} (compared to 0.29 h^{-1} on glucose) and 0.18 h^{-1} (compared to 0.25 h^{-1} on glucose), respectively. Substrate consumption and product formation were determined to evaluate product yields in glucose and cellobiose.
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Keywords: *Saccharomyces cerevisiae*; Heterologous protein production; β-Glucosidase cellobiose-fermenting yeast

1. Introduction

The production of fuel ethanol from cheap and renewable biomass, such as plant material or lignocellulose, has become a major challenge for the biotechnology industry (Zaldivar et al., 2001; Wheals et al., 1999). The two-step conversion of biomass to ethanol involves the enzymatic hydrolysis of cellulosic biomass to produce reducing sugars, and the conversion of the resulting sugars to ethanol. However, this is a very costly process due to the recalcitrance of cellulose, and therefore the low yield and high cost of the enzy-

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matic hydrolysis process (Lynd et al., 2002). Significant research efforts have been made to improve the hydrolysis of lignocellulosic materials. Pretreatment of lignocellulosic materials to remove lignin and hemicellulose can considerably enhance the hydrolysis of cellulose. Optimization of the cellulase enzymes and the enzyme loading can also improve the hydrolysis (Zacchi et al., 2003; Sun and Cheng, 2002). The process of simultaneous saccharification and fermentation (SSF) combines enzymatic hydrolysis of pretreated lignocellulose by cellulases and hemicellulases with conversion of the resulting hexoses and pentoses to ethanol. SSF gives higher reported ethanol yields and requires lower amounts of enzyme, since the sugars released during lignocellulose hydrolysis are continuously converted to ethanol, and therefore prevent feedback-inhibition of the hydrolytic enzymes (Hari et al., 2001). Another promising strategy to develop an inexpensive process involves the production of cellulolytic enzymes, hydrolysis of biomass and conversion of resulting sugars to desired products in a single process step via a cellulolytic microorganism or consortium. Such consolidated bioprocessing (CBP) may offer cost reductions if microorganisms can be engineered to possess the necessary combination of substrate utilization and product formation properties (Lynd et al., 2002).

The development of a yeast strain capable of producing ethanol by fermenting cellulosic substrates has received a great deal of interest over recent years. The advantages of yeasts include (i) their high ethanol productivity and tolerance, (ii) larger cells size, which simplify their separation from the culture broth and (ii) resistance to viral infection (Hahn-Hägerdal et al., 2001). Although *S. cerevisiae* is a good choice, it has a few shortcomings, such as inability to degrade polysaccharides. Since cellobiose (and longer chain cello-oligosaccharides) is the major soluble by-products of cellulose hydrolysis, its efficient utilization is of primary importance to CBP development. Enzymatic hydrolysis of cellobiose requires the action of β-glucosidases. This heterogeneous group of enzymes displays broad substrate specificity towards cellobiose, cello-oligosaccharides and different aryl- and alkyl-β-D-glucosides. β-Glucosidases occur widely in animals, plants, fungi and bacteria (Bhatia et al., 2002). In this work, the main interest in β-glucosidases was its involvement in the biological saccharification of cellulosic material. β-Glucosidases work synergistically with endoglucanases (EC 3.2.1.4) and exoglucanases (EC 3.2.1.91) on the degradation of cellulose. They not only catalyze the final step in the degradation of cellulose, but also stimulate the extent of cellulose hydrolysis by relieving the cellobiose-mediated inhibition of exoglucanase and endoglucanase (Sternberg et al., 1977; Yan et al., 1998).

β-Glucosidases genes from various cellulolytic organisms were amplified by PCR for expression in *S. cerevisiae*. A multi-copy yeast expression vector containing the *Trichoderma reesei xyn2* secretion signal together with *PGK1* promoter and terminator was used to construct a cellobiose-fermenting strain that produce the recombinant β-glucosidases. Previous work from our laboratory indicated that the 33-amino-acid leader peptide of the *xyn2* secretion signal was properly recognized and cleaved at the Kex2-like Lys-Arg residues, enabling the efficient secretion and glycosylation of a heterologous β-xylanase in *S. cerevisiae* (La Grange et al., 1996). Hahn-Hägerdal (1984) demonstrated the advantages of co-immobilizing β-glucosidase together with *S. cerevisiae* in alginate beads compared to separately immobilizing biocatalysts for the efficient conversion of cellobiose to ethanol. The co-immobilized system was also desirable for recovering both β-glucosidase and *S. cerevisiae* during continuous cultivation of cellobiose to ethanol. In this paper, the same principle was applied using recombinant DNA technology, by fusing the *xyn2* secretion signal to the β-glucosidase enzymes, and thus targeting it to *S. cerevisiae* periplasmic space. Finally, the activities of the recombinant β-glucosidases produced by *S. cerevisiae* were compared with regards to catalytic activity and the ability to sustain growth of the yeast on cellobiose as a sole carbon source.

2. Materials and methods

2.1. Strains and media

The genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. *S. cerevisiae* Y294 was cultivated in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or selective synthetic complete (SC) medium (2% glucose, 0.67% yeast nitrogen base [Difco] containing amino

Table 1
Microbial strains and plasmids used in the present study

Strain or plasmid	Relevant genotype	Source of reference
Strains		
S. cerevisiae Y294	α *leu2-3, 112 ura3-52 his3 trp1-289*	ATCC 201160
Candida wickerhamii	Wild type	UOFS Y0652
Trichoderma reesei QM6a	Wild type	ATCC 13631
E. coli XL1-Blue	MRF′ *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lac*^{q} Z*ΔM15* T*n10* (*tet*)]	Strategene
Plasmids		
pGEM-T-easy®	*bla*	Promega
YEp352	*bla URA3*	Hill et al. (1986)
yAZ4	*bla URA3* $PGK1_{PT}$	This investigation
pBS-XYNSEC	*bla* $xyn2_S$	This investigation
pYbglA21	*bglA*	IWBT[a]
pBGL1	*BGL1*	Van Rensburg et al. (1998)
pDLG82	*bla URA3* $PGK1_P$-*xyn2*-$ADH2_T$	This investigation
pDLG1-$ENO1_{PT}$	*bla URA3* $ENO1_{PT}$	This investigation
pDLG5	*bla URA3* $ADH2_P$-*xyn2*-$ADH2_T$	La Grange et al. (1996)

[a] Institute of Wine Biotechnology, Stellenbosch University, South Africa.

acid supplements). Recombinant plasmids were constructed and amplified in *Escherichia coli* XL1-Blue cultivated at 37 °C in Luria-Bertani liquid medium or on Luria-Bertani agar (Sambrook et al., 1989). Ampicillin for selecting and proliferating resistant bacteria was added to a final concentration of 100 μg mL^{-1}.

2.2. DNA manipulations and vector construction

Standard protocols were followed for DNA manipulations (Sambrook et al., 1989). The enzymes for DNA cleavage and ligation were purchased from Roche and used as recommended by the supplier. Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Benson (1984).

A multi-copy, yeast expression plasmid, designated yXYNSEC (Fig. 1) was constructed as follows: the alcohol dehydrogenase (*ADH2*) terminator in pDLG82 was replaced with a *Bgl*II/*Hin*dIII-fragment containing the *PGK1* terminator from pJC1. The 132-bp *xyn2* secretion signal was isolated from pBS-XYNSEC and inserted into the *Eco*RI-*Bgl*II site.

2.3. PCR amplification

The sequence-specific primers used to isolate the genes, encoding the β-glucosidase enzymes of interest, are presented in Table 2. The PCR reaction mixture

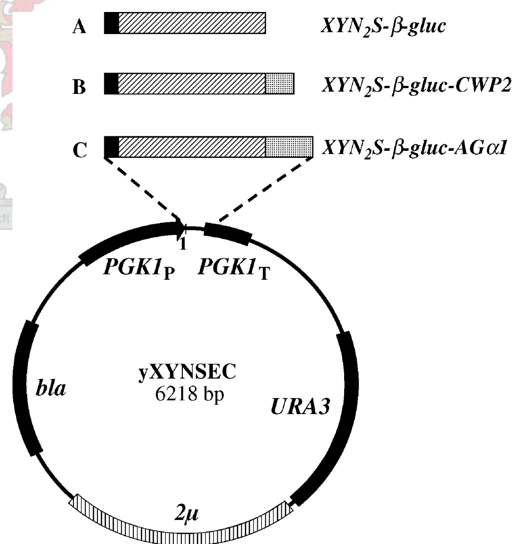

Fig. 1. Schematic summary of the construction of the different β-glucosidase expression cassettes used in this study. (A) The expression cassette used for the production and secretion of the β-glucosidase enzyme. The open-reading-frame (ORF) sequence of the respective β-glucosidase gene (hatched box) was fused to the secretion signal of the *T. reesei xyn2* gene (black box). (B and C) The expression cassettes constructed for anchoring the β-glucosidase genes on the cell wall. The mature peptide sequence was fused at the C-terminus to either the cell wall protein 2 (Cwp2) or the α-agglutinin (AGα1) anchoring moieties.

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Table 2
β-Glucosidase genes, their origin and the PCR primers used for their amplification

Gene (size)	Enzyme	Organism	Overlap primer	Primer sequence
bglA (2526-bp)	BglA	*Aspergillus kawachii*		5′-GTCA**TACGTA**GATGAATTGGCCTACTCCC-3′
				5′-TGGA**AGATCTT**AGTGAACAGTAGGCAGAGAC-3′
			XYNSEC-L	5′-CT**GAATTC**AGGCCTCAACATGGTCTCCTTCACC-3′
			AKAWBGL-AG1	5′-AAAGAGCTTTTGGCGCTGTGAACAGTAGGCAGAGACG-3′
			AG1-R	5′-**TACGTA**GATCTGTTTTAGAATAGCAGGTACGAC-3′
			AKAWBGLCWP2	5′-CCGTCAGTGATTTGAGAAATGTGAACAGTAGGCAGAGACG-3
			CWP2-R	5′-**TACGTA**GATCTTTCTTATAACAACATAGCAGCAG-3′
bglB (1767-bp)	BglB	*Candida wickerhamii*		5′-GTC**TCGCGA**GCTGATGACGCTTCTAAAC-3′
				5′-CCGC**TCGAGA**TCTTCAAGCTCTATTATCATTAATAAAAC-3′
BGL1 (2580-bp)	BGL1	*Saccharomycopsis fibuligera*		5′-GAC**TCGCGA**GTCCCAATTCAAAACTATACC-3′
				5′-CCG**CTCGAG**CGGTCAAATAGTAAACAGGACAGATG-3′
			XYNSEC-L	5′-CT**GAATTC**AGGCCTCAACATGGTCTCCTTCACC-3′
			SFIBBGLAG1	5′-AAAGAGCTTTTGGCGCTAATAGTAAACAGGACAGATGTCTT-3′
			SCAG1-R	5′-AAGCCT**CTCGAG**TGTACAGTACCCGTTTTAG-3′
			SFIBBGLCWP2	5′-CCGTCAGTGATTTGAGAAATAATAGTAAACAGGACAGATGTC-TT-3′
			CWP2Xho-R	5′-GATAT**CTCGAG**TTCTTATAACAACATAGCAGCA-3′
bgl (2139-bp)	Bgl	*Trichoderma reesei*		5′-GTCA**TACGTA**GTTGTACCTCCTGCAGGG-3′
				5′-CCG**CTCGAG**TTACGCTACCGACAGAGTG-3′

The restriction sites included in the primers are indicated in bold face.

(50 μL) was as follows: 200 ng template, 100 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate, the reaction buffer supplied by the manufacturer and 3.5 U of EXPAND® DNA polymerase (Roche). Plasmids, pYbglA21 and pBGL1, containing the complete *Aspergillus kawachii bglA* and *S. fibuligera BGL1* genes, respectively, were obtained from the Institute of Wine Biotechnology (IWBT) (Stellenbosch University, South Africa) and used as a template for PCR. *Candida wickerhamii* genomic DNA and the *T. reesei* first-strand cDNA (La Grange et al., 1996) were used as template for amplification of the *bglB* and *bgl1* genes, respectively.

2.4. Construction of vectors expressing different β-glucosidase genes

PCR products obtained from each reaction were initially cloned into a commercial vector, pGEM-Teasy®, and subcloned as follows. The 2526-bp fragment amplified from plasmid pYbglA21, was digested with *Sna*BI and *Bgl*II and ligated into plasmid yXYNSEC digested with *Nru*I and *Bgl*II. The recombinant plasmid was designated yAKA. The 2139-bp fragment amplified from *T. reesei* first-strand cDNA, was digested with *Sna*BI and *Xho*I, and inserted into yXYNSEC digested with *Nru*I and *Xho*I. The resulting plasmid was designated yTRE. The 2580-bp fragment amplified from plasmid pBGL1 was digested with *Nru*I and *Xho*I and inserted into yXYNSEC digested with the same enzymes to generate plasmid ySFI. The 1767-bp PCR product amplified from *C. wickerhamii* genomic DNA was digested with *Nru*I and *Xho*I prior to introduction into the N*ru*I/*Xho*I site of yXYNSEC. The final recombinant plasmid was designated yCWI.

The *A. kawachii bglA* gene containing the *xyn2* secretion signal from *T. reesei* in plasmid yAKA was fused in frame with the 960-bp 3′ anchor sequence from the *S. cerevisiae* α-agglutinin gene (AG1) (Van der Vaart et al., 1997). The fusion protein was constructed with overlap-PCR using primers XYNSEC-L, AKAWBGL-AG1, AG1-R (sequences presented in Table 2) and *bglA* from yAKA as template. The 3620-bp fusion gene was digested with *Eco*RI and *Bgl*II and ligated into the corresponding sites of yXYNSEC to give yAKA(A) The *A. kawachii bglA* gene was also fused to the 210-bp 3′ anchor sequence from the *S. cerevisiae* cell wall 2 protein (CWP2). This 2860-bp fusion

was made using PCR primers XYNSEC-L, AKAW-BGLCWP2 and CWP2-R and *bglA* from yAKA as template. The resulting fusion gene was digested with *Eco*RI and ligated to the corresponding sites of pDLG1-$ENO1_{PT}$ to give yAKA(C).

The *S. fibuligera* glucosidase from ySFI was used as template to constructs fusion genes in order to anchor the enzyme to the *S. cerevisiae* cell wall. The AG-α anchor was added using PCR primers XYNSEC-L, SFIBBGLAG1 and SCAG1-R. The resulting 2915-bp fusion gene was digested with *Eco*RI-*Xho*I ligated into the corresponding sites of yXYNSEC to yield ySFI(A). The CWP2-SFI fusion was made using PCR primers XYNSEC-L, SFIBBGLCWP2 and CWP2Xho-R. The 3700-bp fusion gene was digested with *Eco*RI-*Xho*I ligated into the corresponding sites of yXYNSEC to give ySFI(C).

2.5. DNA sequencing

The nucleotide sequence of the individual constructs were determined by amplifying DNA fragments with the Big Dye Terminator cycle-sequencing reader reaction with AmpliTaq DNA polymerase F5 (Applied Biosystems kit) using fluorescently labeled nucleotides, and the reaction mixtures were subjected to electrophoresis on an Applied Biosystems automatic DNA sequencer (model ABI Prism 377). Sequence data were analyzed by using the PC/GENE software package (IntelliGenetics Inc., Mountain View, California).

2.6. Yeast transformation

S. cerevisiae Y294 was transformed with the individual recombinant plasmids by the dimethyl sulfoxide-lithium acetate method described by Hill et al. (1991) and the transformants were confirmed with PCR. Disruption of the uracil phosphoribosyltransferase (*FUR1*) gene in the plasmid-containing *S. cerevisiae* transformants was performed to ensure autoselection of the *URA3*-bearing expression plasmids in non-selective medium (Kern et al., 1990). *fur1*::*LEU2* autoselective transformants were screened for on selective plates deficient in uracil and leucine.

2.7. Enzyme assay

β-Glucosidase activity was measured by incubating appropriately diluted cells or cell extracts with 5 mM of *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (Berghem and Pettersson, 1974) in 50 mM citrate buffer at optimal pH and temperature for each of the enzymes for 2 min. The *p*-nitrophenol released from pNPG was detected at 405 nm after adding 1 mL of 2% sodium carbonate to raise the pH and stop the reaction. One unit of enzyme activity was defined as the amount of enzyme required for producing 1 μmol of *p*-nitrophenol/min at optimal pH and temperature. Yeast spheroplasts were generated as described by Smith and Robinson (2002). Spheroplast and supernatant samples were separated by centrifugation at 400 g for 5 min.

2.8. Medium and inoculum

Growth was measured as absorbance at 600 nm. Batch cultivation was conducted in defined medium (Verduyn et al., 1992) containing either 5.26 g L^{-1} glucose or 5 g L^{-1} cellobiose as the sole carbon source. The medium was supplemented with amino acids, both according to the auxotrophic requirements of the yeast strains (histidine [165 mg L^{-1}] and tryptophan [664 mg L^{-1}]) and to enhance heterologous protein production (aspartic acid [257 mg L^{-1}], glutamic acid [64 mg L^{-1}], glycine [33 mg L^{-1}] and serine [108 mg L^{-1}]) (Gorgens et al., 2001).

Approximately 1 mL of a stationary phase culture was used to inoculate the bioreactor to an OD_{600} of 0.05. The cells were aerobically pre-cultured in the bioreactor on 1 g L^{-1} glucose. After depletion of the glucose, filter-sterilized cellobiose (or glucose) solution was injected into the bioreactor.

2.9. Cultivations

Cultivations were conducted in a computer-controlled glass bioreactor (Belach Bioteknik AB, Stockholm, Sweden) with a total volume of 500 mL and a working volume of 100 mL. Temperature and pH were controlled at 30 °C and pH 5.0–5.2 (by the addition of 3 M potassium hydroxide), respectively. The culture broth was magnetically agitated at 250 rpm and aerated with either 0.5 L min^{-1} airflow or 0.2 L min^{-1} nitrogen gas for aerobic and anaerobic conditions, respectively. Dow-Corning antifoam (BDH) was added to control foaming. All cultivations were done in duplicate.

2.10. Analytical methods

Samples for determination of cell density, substrate consumption and product formation were taken from the bioreactor at 2–3 h intervals. Samples were treated as previously described (Gorgens et al., 2001). During late exponential and stationary phase, the dry cell weight of each culture was determined (Meinander et al., 1996).

2.11. Substrate consumption and product formation

Cellobiose, glucose, glycerol, acetate and ethanol concentrations were determined by column liquid chromatography (CLC), with a Gilson CLC system (Gilson, Villiers-le-Bel, France). The compounds were separated on an Aminex HPX-87H column (Bio-Rad, Richmond, CA), and subsequently detected with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The column temperature was 45 °C and 5 mM H_2SO_4 was used as a mobile phase at a flow rate of 0.6 mL min^{-1}.

2.12. Calculations

Specific growth rates were calculated at separate points on the growth curve (ln OD_{600} versus time) by using four adjacent points (two on each side) on the curve to determine the slope at the particular point. The maximum of these specific growth rates for the individual cultivations was selected, and an average calculated for each strain. The yields of biomass and fermentation products on glucose and cellobiose were estimated from the slopes of straight-line sections in the [product] versus [substrate] curves. The rates of glucose and cellobiose consumption and ethanol production were calculated by fitting a limited population growth model (Hirsch and Smale, 1974) to the cultivation data.

3. Results

3.1. Cloning of the β-glucosidase genes

The primers used for isolating the β-glucosidase genes, were based on the sequence data available in Genbank (http://www.ncbi.nlm.nih.gov/entrez/) and designed to exclude the native secretion signals from the amplified products. The PCR products obtained from each reaction were consistent with the predicted sizes of the respective genes: *bglA* of *A kawachii* (2526-bp), *bglB* of *C. wickerhamii* (1767-bp), *BGL1* of *S. fibuligera* (2580-bp) and *bgl1* of *T. reesei* (2139-bp). The β-glucosidase genes were inserted in frame with the *T. reesei xyn2* secretion signal in yXYNSEC for constitutive expression under the transcriptional control of the *S. cerevisiae PGK1* promotor and terminator. The resulting constructs were designated yAKA, yCWI, ySFI and yTRE. In addition, the *S. fibuligera xyn2s-BGL1* and *A. kawachii xyn2s-bglA* genes were also fused to the cell wall protein 2 and α-agglutinin (AGα1) peptide encoding regions in order to anchor the respective enzymes to the yeast cell wall.

3.2. Construction of recombinant yeast strains producing β-glucosidase

Recombinant plasmids were used to transform *S. cerevisiae* Y294 to uracil prototrophy (Ura^+). The Ura^+ transformants carrying a plasmid capable of producing β-glucosidase activity were selected by their ability to form yellow halos around colonies on SC^{-ura} selection plates containing 5 mM *p*-nitrophenyl-β-D-glucopyranoside (chromogenic analog of cellobiose). Several halo-forming *S. cerevisiae* transformants were obtained and sub-cultured to single colonies. PCR with total DNA from the respective transformants were performed to confirm the presence of the recombinant plasmids. The *FUR1* gene of the recombinant transformants was disrupted to create the autoselective strains summarized in Table 3. The *fur1* deletions were confirmed with PCR on genomic DNA isolated from the respective transformants. Y294[HOST] was used as reference strain (Crous et al., 1995).

3.3. Recombinant β-glucosidase production

The β-glucosidase activity was determined by using *p*-nitrophenyl-β-D-glucopyranoside substrate. The properties of each of the recombinant enzymes are summarized in Table 4. No activity was detected in the reference yeast strain harboring the plasmid YEp352. The culture supernatants did not yield β-

Table 3
Summary of the recombinant *S. cerevisiae* strains constructed in this study

Recombinant *S. cerevisiae* strain	Genotype	Strain designation
Y294[*fur1*::*LEU2* yAKA]	*bla URA3 PGK1*P-*xyn2*s-*bglA-PGK1*T[a]	Y294[AKA]
Y294[*fur1*::*LEU2* yAKA(A)]	*bla URA3 PGK1*P-*xyn2*s-*bglA-AGα1-PGK1*T[b]	Y294[AKA(A)]
Y294[*fur1*::*LEU2* yAKA(C)]	*bla URA3 ENO1*P-*xyn2*s-*bglA-cwp2-ENO1*T[c]	Y294[AKA(C)]
Y294[*fur1*::*LEU2* yCWI]	*bla URA3 PGK1*P-*xyn2*s-*bglB-PGK1*T[d]	Y294[CWI]
Y294[*fur1*::*LEU2* ySFI]	*bla URA3 PGK1*P-*xyn2*s-*BGL1-PGK1*T[e]	Y294[SFI]
Y294[*fur1*::*LEU2* ySFI(A)]	*bla URA3 PGK1*P-*xyn2*s-*BGL1-AGα1-PGK1*T[f]	Y294[SFI(A)]
Y294[*fur1*::*LEU2* ySFI(C)]	*bla URA3 PGK1*P-*xyn2*s-*BGL1-cwp2-PGK1*T[g]	Y294[SFI(C)]
Y294[*fur1*::*LEU2* yTRE]	*bla URA3 PGK1*P-*xyn2*s-*bgl-PGK1*T[h]	Y294[TRE]
Y294[*fur1*::*LEU2* YEp352]	*bla URA3*	Y294[HOST]

[a] Fusion enzyme *xyn2*s-*bglA* was designated AKA.
[b] Fusion enzyme *xyn2*s-*bglA-AGα1* was designated AKA(A).
[c] Fusion enzyme *xyn2*s-*bglA-cwp2* was designated AKA(C).
[d] Fusion enzyme *xyn2*s-*bglB* was designated CWI.
[e] Fusion enzyme *xyn2*s-*BGL1* was designated SFI.
[f] Fusion enzyme *xyn2*s-*BGL1-AGα1* was designated ySFI(A).
[g] Fusion enzyme *xyn2*s-*BGL1-cwp2* was designated SFI(C).
[h] Fusion enzyme *xyn2*s-*bgl* was designated TRE.

glucosidase activity for any of the strains. These results suggest that active β-glucosidase activity was associated with the cells without leakage into the culture medium. To more accurately identify the location of the different enzymes, the cells were treated with zymolyase to produce spheroplasts. For the enzymes that were fused to the *T. reesei xyn2* secretion signal, the β-glucosidase activity was detected in the supernatant harvested from the spheroplasts, but no activity could be detected in the cell debris containing the cell wall fraction. It was therefore concluded that the enzymes were transported through the secretion pathway and located in the periplasmic space. For the AKA(A), AKA(C), SFI(A) and SFI(C) fusion proteins the pNPG-hydrolyzing activity were exclusively assigned to the fraction containing the cell wall.

3.4. Growth kinetics of recombinant β-glucosidase-producing strains grown on cellobiose and glucose

Though all recombinant *S. cerevisiae* strains produced active β-glucosidases, they were not all able to sustain growth on cellobiose (Table 5). Growth of Y294[HOST] on 5.26 g L^{-1} glucose gave an indica-

Table 4
Properties of the various recombinant β-glucosidases produced in *S. cerevisiae*

Strain	Properties of the recombinant enzymes				
	Location of activity	pH optimum	Temperature optimum (°C)	K_m (pNPG) (mM)	V_{max}[a]
Y294[AKA]	Periplasmic space	4.5	60–65	1.03 ± .009	0.15
Y294[AKA(A)]	Cell wall	4.5	60–65	1.91 ± 0.007	1.75
Y294[AKA(C)]	Cell wall	4.5	60–65	1.45 ± 0.006	4.33
Y294[CWI]	Periplasmic space	4.5	40	1.46 ± 0.058	0.969
Y294[SFI]	Periplasmic space	5.4	55	0.378 ± 0.002	8.70
Y294[SFI(A)]	Cell wall	5.4	55	0.215 ± 0.009	3.14
Y294[SFI(C)]	Cell wall	5.4	55	0.223 ± 0.009	28.76
Y294[TRE]	Periplasmic space	4.5	45–50	0.741 ± 0.004	0.132

[a] V_{max} was measured as nmol product min^{-1} mg $cells^{-1}$.

Table 5
Fermentation profiles from batch cultivation of the *S. cerevisiae* recombinant strains on glucose (5.26 g L^{-1}) and cellobiose (5.0 g L^{-1})

Strain	Max. specific growth rate μ_{max} (h^{-1})	Product yields (g/g sugar)				Carbon balance: C_{out}/C_{in}
		Biomass	Ethanol	Glycerol	Acetate	
Aerobic						
Y294[Host][a]	0.29	0.44 ± .05	0.30 ± 0.04	0.04 ± 0.02	0.02 ± 0.02	0.95
Y294[AKA]	No growth	–	–	–	–	–
Y294[AKA(A)]	0.06	n.d.	n.d.	n.d.	n.d.	n.d.
Y294[AKA(C)]	0.07	n.d.	n.d.	n.d.	n.d.	n.d.
Y294[CWI(B)]	No growth	–	–	–	–	–
Y294[SFI]	0.23	0.42 ± 0.05	0.26 ± 0.02	0.04 ± 0.002	0.01 ± 0.02	0.83
Y294[SFI(A)]	0.20	0.60 ± 0.03	0.09 ± 0.04	0.03 ± 0.02	0.08 ± 0.03	0.87
Y294[SFI(C)]	0.20	0.61 ± 0.03	0.12 ± 0.03	0.02 ± 0.02	0.03 ± 0.01	0.88
Y294[TRE)]	No growth	–	–	–	–	–
Anaerobic						
Y294[Host][a]	0.25	0.11 ± 0.02	0.46 ± 0.05	0.08 ± 0.01	0.00 ± 0.01	1.10
Y294[AKA]	No growth	–	–	–	–	–
Y294[AKA(A)]	No growth	–	–	–	–	–
Y294[AKA(C)]	No growth	–	–	–	–	–
Y294[CWI(B)]	No growth	–	–	–	–	–
Y294[SFI]	0.18	0.12 ± 0.03	0.41 ± 0.03	0.05 ± 0.02	0.02 ± 0.01	0.98
Y294[SFI(A)]	0.10	0.13 ± 0.01	0.42 ± 0.02	0.05 ± 0.03	0.01 ± 0.01	0.98
Y294[SFI(C)]	0.11	0.14 ± 0.01	0.39 ± 0.05	0.08 ± 0.02	0.01 ± 0.01	0.99
Y294[TRE)]	No growth	–	–	–	–	–

[a] The reference strain Y294[Host] was cultivated on glucose as carbon source.

tion of the maximum growth rate that can be expected of the host when all the available cellobiose is converted to glucose in a non-rate limiting step. The μ_{max} of the Y294[SFI] strain on cellobiose was significantly higher than the other strains under both aerobic and anaerobic conditions. Y294[SFI] sustained growth aerobically at 0.23 h^{-1} (versus 0.29 h^{-1} on glucose) and anaerobically at 0.18 h^{-1}(versus 0.25 h^{-1} on glucose) on 5.0 g L^{-1} cellobiose as sole carbon source. Fig. 2 illustrates the anaerobic cultivation of the SFI-producing *S. cerevisiae* strains in minimal medium containing cellobiose. Anchoring the SFI(A) and SFI(C) fusion enzymes on the yeast cell wall did not result in any improved growth on cellobiose. In contrast, the Y294[AKA] strain that secreted the AKA enzyme was not able to sustain growth, but the two strains where AKA(A) and AKA(C) fusion enzymes were anchored to the cell wall, i.e. Y294[AKA(A)] and Y294[AKA(C), were able to grow aerobically on cellobiose at 0.06 and 0.07 h^{-1}, respectively (Table 5). Anchoring the AKA enzyme to the cell wall thus had a significant effect on the ability of the strain to utilize cellobiose.

3.5. *Biomass and by-product yields during growth on cellobiose and glucose*

The product yields of the recombinant strains during aerobic and anaerobic growth on glucose (positive

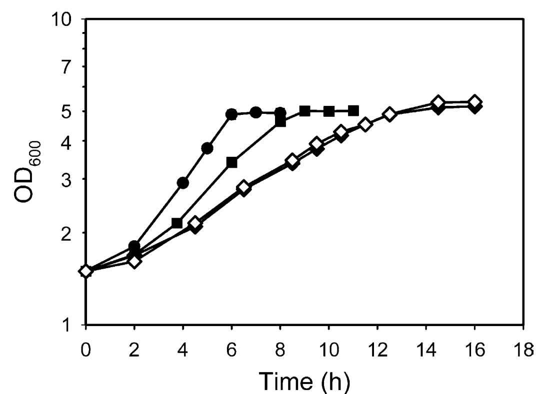

Fig. 2. Anaerobic growth of recombinant *S. cerevisiae* strains on defined medium with cellobiose as sole carbon source. The reference strain Y294[HOST] (●) was grown in 5.26 g L^{-1} glucose. Y294[SFI] (■), Y294[SFI(C)] (◇) and Y294[SFI(A)] (◆) were cultivated in 5 g L^{-1} cellobiose.

control) and cellobiose are presented in Table 5. Closed carbon balances obtained for the anaerobic cultivations indicate that all the carbon from the sugar (glucose or cellobiose) was converted to biomass, ethanol, glycerol and acetate. The less desirable carbon balance values obtained for the aerobic cultivations can be explained by values for carbon dioxide (CO_2) that were not included in the calculations due to the unavailability of an oxygen probe.

During aerobic cultivation, Y294[SFI] yielded 0.42 g biomass per gram cellobiose compared to Y294[HOST] that produced 0.44 g biomass per gram glucose consumed. Anchoring the BGL1 enzyme to the cell wall resulted in an increased biomass yield (~0.6 g biomass per gram cellobiose) and lowered ethanol production. Anaerobic growth of Y294[SFI] yielded 0.41 g of ethanol per gram of cellobiose consumed. This corresponds to 89% of the maximum yield (0.46 g of ethanol) when Y294[HOST] was grown anaerobically on glucose.

4. Discussion

A viable and cost-effective strategy for the production of bio-ethanol is dependent on the production of cellulolytic enzymes, hydrolysis of biomass, and conversion of resulting sugars to desired products in a single process step via a cellulolytic microorganism or consortium. Since cellobiose (and cello-oligosaccharides) is a potent inhibitor of cellulose hydrolysis, it is a key substrate with regards to ethanol production. The enzyme β-glucosidase that converts cellobiose and soluble cellodextrins to glucose has been shown to be one of the major rate-limiting steps in the saccharification of cellulose. β-Glucosidase not only determines the rate, but also the extent of cellulose hydrolysis by relieving end-product inhibition of endoglucanases and cellobiohydrolases (Lynd et al., 2002). We genetically engineered *S. cerevisiae* for anaerobic ethanol production from cellobiose as sole carbon source.

Four β-glucosidase genes of fungal origins were successfully expressed in *S. cerevisiae*. We used an isogenic approach, where all four genes were fused to the *xyn2* secretion signal sequence (La Grange et al., 1996) and expressed constitutively from a multicopy yeast expression vector under transcriptional control of the *S. cerevisiae PGK1* promoter and terminator. Enzyme assays indicated that the recombinant enzymes were trapped in the periplasmic space so that the β-glucosidase activity was cell-associated. Enzymatic removal of the cell wall released the β-glucosidase activity to the supernatant. Similar results were reported with recombinant β-glucosidases from *C. wickerhamii* (Skory et al., 1996), *C. pelluculosa* (Kohchi and Toh-e, 1986) and *A. kawachii* (Iwashita et al., 1999) when produced in *S. cerevisiae*. The recombinant *A. kawachii* AKA, *C. wickerhamii* CWI, *S. fibuligera* SFI and *T. reesei* TRE enzymes were characterized with regards to their pH and temperature optimum as well as their kinetic parameters. According to the K_m values, *S. fibuligera* SFI had the highest affinity for the pNPG substrate (a chromogenic analog of cellobiose). The SFI enzyme had optimal activity at pH 5.4, which corresponds to optimal pH range (5.0–5.5) for growth of *S. cerevisiae*. The K_m and V_{max} values obtained with pNPG substrate are not an accurate assessment of the kinetic parameters on cellobiose. The data presented in Table 6 illustrates a clear difference in the substrate affinity (K_m) of various β-glucosidases for pNPG and cellobiose, respectively. However, the kinetic parameters in this study (Table 4) using pNPG as substrate provided a useful prediction of the performance of the recombinant strains when cultivated on cellobiose as substrate.

The recombinant *S. cerevisiae* strains were cultivated in high-performance bioreactors to determine if the amount of β-glucosidase activity produced by the respective strains was sufficient to enable growth

Table 6
Difference in substrate affinity (K_m) of microbial β-glucosidases for pNPG and cellobiose

Organism	K_m (pNPG) (mM)	K_m (cellobiose) (mM)	Reference
Aspergillus oryzae (HGT-BG)	0.55	7.0	Riou et al. (1998)
Thermoascus aurantiacus	0.114	0.637	Parry et al. (2001)
Kluyveromyces fragilis (bgl1)	0.52	79.0	Leclerc et al. (1987)

on cellobiose as sole carbon source. During aerobic cultivation, Y294[AKA(A)], Y294[AKA(C)] and Y294[SFI] were able to grow in minimal medium containing $5\,g\,L^{-1}$ cellobiose. Y294[SFI] was identified as the best strain and grew at $0.23\,h^{-1}$ on cellobiose (versus $0.29\,h^{-1}$ of the reference strain Y294[HOST] on glucose). More significantly was the ability of this strain to anaerobically utilize cellobiose at $0.18\,h^{-1}$ (versus $0.25\,h^{-1}$ of the reference strain on glucose). The ethanol yield of Y294[SFI] was 0.41 g per gram of cellobiose consumed which corresponds to 89% of the maximum yield (0.46 g of ethanol) when Y294[HOST] was anaerobically grown on glucose. Although micro-aerobic conditions are more suitable when ethanol production is the main objective (Alfenore et al., 2004), it is significant that the recombinant strain was able to effectively ferment cellobiose despite the anaerobic stress. The cellulosome concept of anaerobic cellulolytic *Clostridium* spp. (Bayer et al., 1998) has focused a lot of attention to the construction of a cell surface display system that enables different cellulolytic enzymes to be anchored to the cell wall of *S. cerevisiae* (Fujita et al., 2004, 2002a,b). In a similar approach, we fused the *S. fibuligera* SFI and *A. kawachii* AKA enzymes to the C-terminus sequence of two different cell wall mannoproteins, α-agglutinin (AGα1) and cell wall protein 2. α-Agglutinin is involved in sexual adhesion between cells of the opposite mating type while Cwp2 is a general component of cell wall. The C-terminus of various cell wall proteins in yeast contains the glycosylphosphatidyl-inositol (GPI) anchor that mediates cross-linking of the proteins to the cell wall. The C-terminal regions of these proteins have been used in several studies to display heterologous proteins on the cell surface of *S. cerevisiae* (Fujita et al., 2004, 2002a,b; Shibasaki et al., 2000; Ueda and Tanaka, 2000; Ram et al., 1998). Fusing of the SFI and AKA enzymes to AGα1 and Cwp2 peptides had a prominent effect on the kinetic parameters of the recombinant β-glucosidases. For SFI(A) and SFI(C) the substrate specificity (K_m) for pNPG was slightly increased, but the opposite was true for AKA(A) and AKA(C). The highest maximum specific activity (V_{max}) per milligram cells was obtained with the *S. fibuligera* SFI. The SFI(C) fusion had a three-fold increase on the V_{max} value when compared to the periplasmic-associated SFI. The V_{max} value of the *A. kawachii* AKA was also significantly increased when fused to the AGα1 or Cwp2 proteins.

Anchoring the enzyme to the cell wall enabled the Y294[AKA(A)] and Y294[AKA(C)] strains to grow on cellobiose. According to the V_{max} values, anchoring the AKA(A) and AKA(C) enzymes to the cell wall resulted in an increased amount of β-glucosidase activity per milligram cells. Since the substrate affinity (K_m on pNPG, Table 3) of the enzymes did not increased to the same extent, it is suggested that the amount of functionally active enzyme at the cell surface must have been increased. It is suspected that the GPI anchor sequence, present in the BglA-AGα1 and BglA-Cwp2 fusion proteins, might have aided in more effective secretion (or less miss-folding and degradation) of active β-glucosidase.

The anchoring of the SFI and AKA enzymes could influence the secretion efficiency and/or stability of the respective enzymes, resulting in the diverse effects on their respective activity. Assuming that the GPI anchor will promote more effective secretion, improvements in AKA secretion together with concentrating the active enzyme on the cell wall via the GPI anchor could have resulted in a significant improvement on both the kinetic parameters (Table 4) as well as the host's ability to utilize cellobiose (Table 5).

Heterologous production of SFI resulted in an enzyme with high specific activity and affinity for cellobiose (Table 4). The additional effect of fusing a GPI anchor onto SFI therefore would be less evident, since SFI is efficiently secreted and functional in *S. cerevisiae* (McBride et al., 2005). Concentrating the SFI(A) and SFI(C) fusion enzymes on the cell wall may not improve the host's ability to metabolize glucose (the product of cellobiose hydrolysis), since the enzyme activity is already in excess (McBride et al., 2005). However, over-expression of the fused proteins destined for cell wall anchoring could impose a metabolic burden on the cell, or hinder diffusion of the hydrolysis products at the cell wall.

This work describes the successful construction and characterization of a recombinant *S. cerevisiae* [SFI] strain that is able to effectively utilize cellobiose as a sole carbon source. This is the first report, according to our knowledge, that describes the successful anaerobic cultivation of a recombinant β-glucosidase producing *S. cerevisiae* in defined medium with cellobiose as sole carbon source. This cellobiose-fermenting *S. cerevisiae* strain is of great importance regarding the construction

of a cellulolytic microorganism that will play an important role in consolidated bioprocessing.

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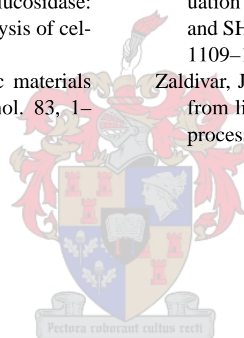

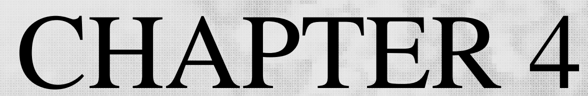

Manuscript 2

Adaptation and characterization of a recombinant *Saccharomyces cerevisiae* strain that transports and utilizes cellobiose intracellularly

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Adaptation and characterization of a recombinant *Saccharomyces cerevisiae* strain that transports and utilizes cellobiose intracellularly

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ABSTRACT

The construction of a *Saccharomyces cerevisiae* β-glucosidase secreting strain that is able to grow on cellobiose as a sole carbon source has been demonstrated before [Van Rooyen et al., 2005]. In a new approach, the secretion signal from the *Saccharomycopsis fibuligera* β-glucosidase (*BGL1*) was removed and expressed constitutively from a multi-copy yeast expression vector under transcriptional control of the *S. cerevisiae PGK1* promotor and terminator. Consequently, the BGL1 enzyme was functionally produced within the intracellular space of the recombinant *S. cerevisiae* strain, designated SIGMA[SSFI]. A strategy employing continuous selection pressure was used to adapt the native *S. cerevisiae* disaccharide transport system(s) for cellobiose uptake and subsequent intracellular utilization. The adapted strain had a significantly improved growth rate of 0.09 h^{-1} in 10 $g.L^{-1}$ cellobiose shake-flask culture. RNA Bio-Dot results revealed the induction of the native α-glucoside (AGT1) and maltose (MAL) transporters in the adapted SIGMA[SSFI] strain, capable of transporting and utilizing cellobiose intracellularly. Aerobic batch cultivation of SIGMA[SSFI] resulted in a maximum specific growth rate (μ_{max}) of 0.17 h^{-1} and 0.30 h^{-1} when grown in cellobiose- and cellobiose/maltose-medium, respectively. The addition of maltose significantly improved the uptake of cellobiose, suggesting that cellobiose transport (presumable via a high/low affinity co-transport system that involves the maltose and α-glucosidase transporters) is the rate-limiting step when SIGMA[SSFI] is grown on cellobiose as sole carbon source. The adapted strain also displayed several interesting phenotypical characteristics, for example, flocculation, pseudohyphal growth and biofilm-formation. These features resemble some of the properties associated with the highly efficient cellulase enzyme systems of cellulosome-producing anaerobes and can be an additional advantage when considering SIGMA[SSFI] as a potential host for future CBP technology.

INTRODUCTION

The potential of plant biomass as a cheap and renewable substrate for the production of fuel and chemicals is gaining considerable interest in recent years. The biological saccharification of cellulose, the main component of plant biomass, is of particular interest to the field of fuel ethanol production. Four biologically mediated process steps are involved in the current cellulose-to-ethanol technology: (i) cellulase enzyme production; (ii) enzymatic saccharification of cellulose; (iii) fermentation of hexose sugars (end-products of cellulose hydrolysis); and (iv) fermentation of pentose sugars (end-products of hemicellulose hydrolysis) to ethanol [Lynd et al., 2002]. The aim would be to combine all four process steps into a one-step conversion of cellulose to fuel ethanol (called consolidated bioprocessing (CBP)), and thereby significantly reduce the processing costs [Lynd et al., 2002].

Saccharomyces cerevisiae has superior ethanol formation properties, but is non-cellulolytic. The expression of cellulases in *S. cerevisiae* is a prerequisite for cellulose conversion via CBP. *S. cerevisiae* has received a great deal of interest regarding heterologous protein expression as well as the production of ethanol and other commodity products [Lynd et al., 2002; Romanos et al., 1992]. Expression of a functional cellulase system in *S. cerevisiae* would require the co-expression of at least three groups of enzymes, namely endoglucanases (EC 3.2.1.4); exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). These enzymes act synergistically to efficiently degrade cellulose [Mansfield and Meder; 2003]. β-Glucosidases catalyse the hydrolysis of soluble cellodextrins and cellobiose to glucose. β-Glucosidases from various origins, e.g. *Aspergillus niger* [Dan et al., 2000], *Aspergillus kawachii* [Van Rooyen et al., 2005; Iwashita et al, 1999] *Candida pelliculosa* var. *acetaetherius* [Kohchi and Toh-e, 1986], *Candida wickerhamii* [Van Rooyen et al., 2005], *Saccharomycopsis fibuligera* and *Trichoderma reesei* [Van Rooyen et al., 2005] have been successfully expressed in *S. cerevisiae*. Raynal and Guérineau (1984) genetically engineered *S. cerevisiae* to produce the *Kluyveromyces lactis* β-glucosidase intracellularly, but the recombinant strain was unable to grow on cellobiose.

Previously we described the construction of cellobiose-fermenting strains by introduction of secreted β-glucosidases from various fungal origins [Van Rooyen et al., 2005]. The accumulation of extracellular cellobiose has two major disadvantages: (i) it causes feedback

inhibition of endoglucanases and cellobiohydrolases and therefore limits the rate and extent of cellulose hydrolysis [Yan et al., 1998]; and (ii) the action of β-glucosidases releases glucose in the external environment that increase the risk of contamination.

In a new approach, the mature *S. fibuligera* β-glucosidase (BGL1) was functionally expressed in the intracellular space of *S. cerevisiae*. The resulting recombinant strain was subjected to continuous selective pressure aimed at adapting its native disaccharide transporter(s) for cellobiose uptake and subsequent hydrolysis by the intracellular β-glucosidase. RNA Bio-dot analysis was used to determine the expression levels of genes encoding native transporter(s) involved in cellobiose uptake by the adapted strain. Finally, the adapted recombinant strain was evaluated for enhanced growth on cellobiose.

MATERIALS AND METHODS

Strains and media. The genotypes of the microbial strains used in the present study are presented in Table 1.

TABLE 1. Microbial strains and plasmids used in the present study

Strain or plasmid	Relevant genotype	Source of reference
Strains		
S. cerevisiae L5366h1 (SIGMA)	α *leu2-3, 112 ura3-52*	Echard Boles, University of Duesseldorf, Germany
E. coli XL1 Blue	MRF' *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB* lac^{q} Z*ΔM15* T*n10* (*tet*)]	Strategene
Plasmids		
pGEM-T-easy®	*bla*	Promega
yAZ4	*bla URA3* $PGK1_{PT}$	Van Rooyen et al., 2005
ySFI	*bla URA3* $PGK1_{P}$-*xyn2*s-*BGL1*-$PGK1_{T}$	Van Rooyen et al., 2005

Recombinant plasmids were constructed and amplified in *Escherichia coli* XL1-Blue cultivated at 37°C in Luria-Bertani liquid medium or on Luria-Bertani agar [Sambrook et al., 1989]. Ampicillin for selecting and proliferating resistant bacteria was added to a final concentration of 100 $\mu g.mL^{-1}$.

S. cerevisiae SIGMA was cultivated in either YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic complete (SC) medium (0.67% yeast nitrogen base [Difco] containing amino acid supplements, 2% glucose). Recombinant *S. cerevisiae* was grown on YP medium (1% yeast

extract, 2% peptone) containing either 10 g.L^{-1} glucose, cellobiose or maltose as sole fermentable sugars.

DNA manipulations and vector construction. Standard protocols were followed for DNA manipulations [Sambrook et al., 1989]. The enzymes for DNA cleavage and ligation were purchased from Roche and used as recommended by the supplier. Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Benson (1984).

PCR amplification. PCR products were amplified from either plasmid DNA (15 ng) or *S. cerevisiae* genomic DNA (200 ng) with the aid of sequence specific primers. The reaction mixture (50 µl) contained the following components: 10 x reaction buffer, 500 µM of each of the nucleotide triphosphates, 0.25 µM of each primer, DNA template and 2.5 U EXPAND polymerase (Roche). DIG-labelled probes were synthesized with the aid of the "PCR DIG Probe Synthesis Kit" (Roche). Table 2 summarizes the different PCR primers used in this study.

TABLE 2. Summary of the PCR primers used.

Primer name	Primer Sequence	Tm (product size)	Gene targeted
SSFI	L: 5'-TCGCGAATTCATGGTCCCAATTCAAAACTATACC-3' R: 5'-CCGCTCGAGCGGTCAAATAGTAAACAGGACAGATG-3'	64 (2583 bp) 65	*BGL1*
ACT	L: 5'-ACTGAAGCTCCAATGAACC-3' R:5'-CATCGACATCACACTTCATG-3'	65 (549 bp) 65	*ACT1*
AGT	L: 5'-ATGATTGCTGTGGGACAA-3' R: 5'-GTCTCGTTCTTCTTCCATTAA-3'	64 (486 bp) 64	*AGT1* (α-Glucoside transporters)
MAL	L: 5'-ATGATTGCTGTGGGACAA-3' R: 5'-AGACAAGTAATTCTCGTTCTTCT-3'	64 (499 bp) 65	*MAL61*, *MAL11* (Maltose transporters)

Construction of vector for intracellular β-glucosidase production. A 2 583-kb DNA fragment, containing the open reading frame that encodes for the mature *Saccharomycospis fibuligera* BGL1 enzyme, was amplified with primers SSFI-L and SSFI-R from plasmid ySFI. The PCR product was digested with *Eco*RI and *Xho*I and ligated into the corresponding sites of plasmid yAZ4 to yield ySSFI.

DNA sequencing. The nucleotide sequence of the individual constructs were determined by amplifying DNA fragments with the Big Dye Terminator cycle-sequencing reader reaction with AmpliTaq DNA polymerase F5 (Applied Biosystems kit) using fluorescently labelled

nucleotides, and the reaction mixtures were subjected to electrophoresis on an Applied Biosystems automatic DNA sequencer (model ABI Prism 377). Sequence data were analyzed by using the PC/GENE software package (IntelliGenetics, Inc., Mountain View, Calif.).

Yeast transformation. *S. cerevisiae* SIGMA was transformed with the recombinant plasmid by the dimethyl sulfoxide-lithium acetate method described by Hill et al. (1991) and the transformants were confirmed with PCR. Disruption of the uracil phosphoribosyltransferase (*FUR1*) gene in the plasmid-containing *S. cerevisiae* transformants was performed to ensure auto-selection of the *URA3*-bearing expression plasmids in non-selective medium [Kern et al., 1991]. Autoselective (*fur1::LEU2*) transformants were screened for on SC plates deficient in uracil and leucine. The resultant *BGL1*-expressing yeast strain was designated SIGMA[SSFI].

Selection for cellobiose utilization. *S. cerevisiae* transformants expressing the *BGL1* intracellularly were grown in rich medium (YPD) to mid-log phase. The cells were appropriately diluted and plated onto YPC-medium (1% yeast extract, 2 % peptone, 1% cellobiose). The plates were incubated at room temperature. After 10 days colony-forming transformants were transferred to fresh YPC-plates. The fastest-growing transformants were selected and subsequently transferred to fresh cellobiose-containing medium every 14 days. This procedure was repeated for 8 months.

Pectora roborant cultus recti

Enzyme assay. β-Glucosidase activity was measured by incubating appropriately diluted cells or cell extracts with 5 mM of p-nitrophenyl-β-D-glucopyranoside (pNPG) in 50 mM citrate buffer at optimal pH and temperature for the specific enzyme according to the method previously described [Van Rooyen et al., 2005].

Total RNA isolation. RNA was isolated from shake flask cultures (YP-medium containing 10 $g.L^{-1}$ of either glucose, maltose or cellobiose) of *S. cerevisiae* SIGMA[SSFI] 48 h after inoculation. RNA isolations were performed as described by Sambrook et al. (1989).

Slot blot analysis. Slot blot hybridizations and autoradiography were performed according to Sambrook et al. (1989), using the SCR072/0 Minifold II slot blotter (Schleicher and Schuell). The DNA probes were labelled using a random primed DIG- labelling kit (Roche) in accordance with the manufacturer's recommendations.

Medium and inoculum. Growth was measured as absorbance at 600 nm. Batch cultivation was conducted in YP-medium containing either 10 $g.L^{-1}$ cellobiose or 7 $g.L^{-1}$ cellobiose + 3 $g.L^{-1}$ maltose. Approximately 2 ml of a stationary phase culture was used to inoculate the bioreactor to an OD_{600} of 0.05.

Fermentation. Batch cultivations were conducted in a 1.3 L computer-controlled glass bioreactor (Bioflow 110 Non-Jacketed Vessels, New Brunswick Scientific Co.) with a total volume of 900 mL. Temperature and pH were controlled at 30°C and pH 5.0 (by the addition of 1 M potassium hydroxide), respectively. The culture broth was mechanically agitated at 500 rpm and aerated with 0.5 $L.min^{-1}$ airflow for aerobic conditions. Dow-Corning antifoam (BDH) was added to control foaming. All cultivations were done in duplicate.

Flow cell experiment. *S. cerevisiae* SIGMA[SSFI] grown in minimal medium (0.67% yeast nitrogen base [Difco] with amino acid supplements; 1% cellobiose) for 48 hours on a rotary shaker at 30°C was used for inoculating the flow cell. With the peristaltic pump (Watson Marlow 205S) switched off, 200 µl of the yeast inoculum was injected into the multiple channel flow cell. The cells were allowed to establish themselves in the flow cell for 12 hours, after which the flow was resumed at 3 $mL.h^{-1}$. The yeast biofilms were maintained in minimal medium containing different carbon sources (1%): (i) glucose, (ii) maltose and (iii) cellobiose for 7 days at room temperature.

Analytical methods. Samples for determination of cell density, dry cell weight and substrate consumption were taken from the fermentor at 3-4 hour intervals. Cells were removed from the samples via filtration through 0.22 µm disposable filters as previously described [Görgens et al., 2001]. The dry cell weight of each culture was determined according to the method described by Meinander et al., 1996.

Substrate consumption and product formation. Cellobiose, maltose, ethanol, glycerol and acetic acid concentrations were determined by high-performance liquid chromatography (HPLC), with a Waters 717 injector (Milford, MA, USA) and Agilent 1100 pump (Palo Alto, CA, USA). The compounds were separated on an Aminex HPX87-H column (Biorad, Richmond, CA) at 45°C, with 5 mM H_2SO_4 at 0.6 ml min^{-1} as mobile phase, and detected with a Waters 410 refractive index detector.

Calculations. Specific growth rates were calculated at individual points on the growth curve (ln OD_{600} vs. time) by using the four surrounding points (two on each side) on the curve to determine the slope at the specific point. The maximum of these specific growth rates for the individual fermentations was selected, and an average calculated for the strain's specific performance on each carbon source. The rates of substrate consumption were calculated from the slope of the (ln [substrate concentration]/[Dry weight] vs. time) graph.

RESULTS

Construction of yeast expression vector for intracellular production of β-glucosidase. Primers, SSFI-L and SSFI-R, were designed to amplify a DNA fragment encoding the mature peptide sequence of the *S. fibuligera* β-glucosidase from plasmid ySFI (constructed during a previous study) [Van Rooyen et al., 2005]. The DNA fragment was successfully isolated and introduced into plasmid yAZ4 for constitutive expression under the transcriptional control of the *S. cerevisiae PGK1* promotor and terminator. The resulting construct was designated ySSFI.

Construction of recombinant yeast strain producing intracellular β-glucosidase. The recombinant ySSFI plasmid was used to transform *S. cerevisiae* SIGMA to uracil prototrophy (Ura^+). Ura^+ transformants were lysed and subsequently assayed for intracellular β-glucosidase activity using *p*-nitrophenyl-β-D-glucopyranoside substrate. No β-glucosidase activity was detected in the cell-free supernatant. The presence of the recombinant plasmid was confirmed by amplifying the *BGL1* gene by PCR from total DNA isolated from recombinant *S. cerevisiae*. The *FUR1* gene of the recombinant yeast strain was disrupted in order to yield an auto-selective strain, SIGMA[ySSFI].

Selection of cellobiose-growing recombinant strain. Recombinant SIGMA[ySSFI] was grown in rich medium and subsequently diluted and plated onto YP-medium containing 10 $g.L^{-1}$ cellobiose as sole carbon source. The plates were incubated at room temperature to select for transformants that can transport and consequently grow on cellobiose-medium. A number of cellobiose-utilizing transformants appeared after 10 days of incubation. The resulting colonies were transferred to fresh cellobiose-plates and incubated at room temperature. This procedure was repeated every 14 days. After 3 months, the cellobiose-utilizing transformants showed a significant improvement with regards to the rate of colony formation as well as colony size. One

of the selected cellobiose-utilizing colonies displayed wrinkled colony morphology on the cellobiose-plates as shown in Figure 1. During shake-flask cultivations, this particular strain showed significantly improved growth on cellobiose compared to the smooth colony-forming strains and the wrinkled colony-forming SIGMA[SSFI] strain was selected for the remainder of the study.

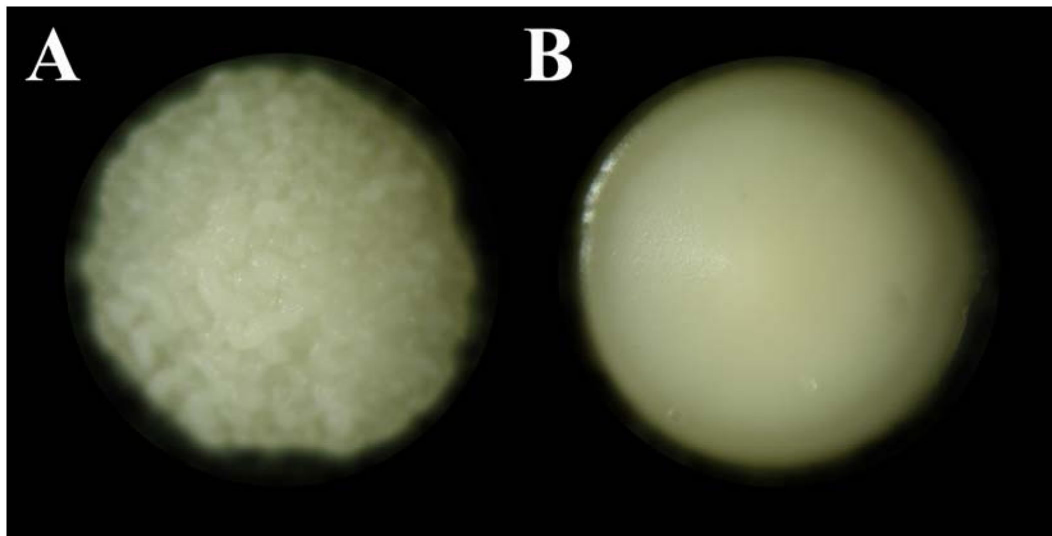

Figure 1. (A) Wrinkled colony morphology of the enhanced cellobiose-utilizing strain after the 3 month selection procedure. (B) The smooth colony morphology of the other is indicated at the right.

Identification of native *S. cerevisiae* transporters possibly involved in cellobiose uptake. Two native disaccharide transporters from *S. cerevisiae*, the maltose permease (encoded by *MALx1*, with "x" designating the MAL locus present) and α-glucoside transporter (encoded by *AGT1*), were identified as the most probable candidates used by the adapted strain to transport cellobiose. The *S. cerevisiae* actin gene (*ACT1*) was selected as an internal gene expression control. The DNA sequence data for the aforementioned transporters and actin gene were obtained from Genbank (http://www.ncbi.nlm.nih.gov/entrez/) and used to design PCR primers for synthesis of DIG-labelled hybridization probes. The corresponding probes, named MAL (maltose permease), AGT (α-glucosidase transporter) and ACT (actin), were amplified from *S. cerevisiae* genomic DNA using DIG-labelled nucleotide triphosphates. RNA Bio-dot analysis was performed on the total RNA isolates from the adapted SIGMA[SSFI] strain cultivated in YP-medium containing 10 $g.L^{-1}$ glucose, maltose and cellobiose, respectively. From the RNA Bio-dot results presented in Figure 2 it is evident that both the MAL and AGT transporters were

induced in the adapted strain. However, neither of the two transporters were induced in the control where SIGMA[SSFI] was grown in glucose-containing medium.

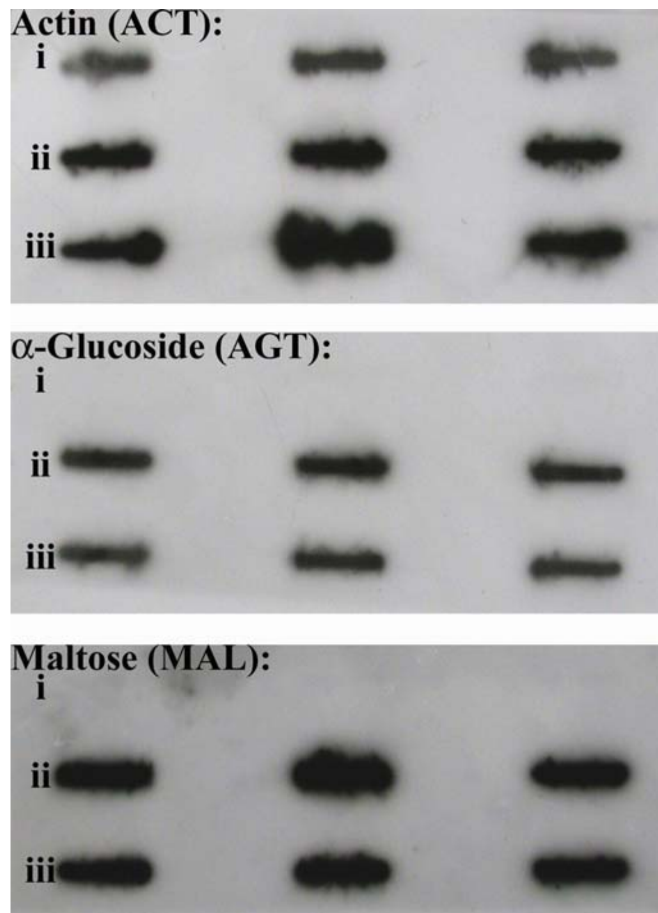

Figure 2. RNA Bio-dot analysis of the SIGMA[SSFI] strain grown in YP-medium containing (i) glucose, (ii) maltose and (iii) cellobiose as sole carbon sources, respectively. 10 µg Total RNA from the individual cultivations were loaded in each lane. The DIG-labelled probes for the different transporters are indicated in the left corner of each blot.

Aerobic batch cultivation of SIGMA[SSFI]. The SIGMA[SSFI] adapted for cellobiose uptake and utilization was grown in high-performance bioreactors to evaluate its growth in minimal medium containing 10 $g.L^{-1}$ cellobiose. Medium containing a mixture of cellobiose and maltose (7 $g.L^{-1}$ cellobiose + 3 $g.L^{-1}$ maltose) was also used to study the effect of maltose addition on cellobiose consumption and utilization in the adapted strain.

Figure 3 shows both the growth and substrate consumption of the SIGMA[SSFI] strain when cultivated in minimal medium containing either cellobiose or a mixture of cellobiose and maltose as sole carbon source. The adapted strain was able to sustain growth aerobically on cellobiose as

sole carbon source at 0.23 h^{-1} (compared to 0.30 h^{-1} on the cellobiose/maltose mixture). SIGMA[SSFI] consumed 0.25 g of cellobiose per grams of dry weight per hour (g.g $DW^{-1}.h^{-1}$) (compared to 0.37 g cellobiose/ maltose.g $DW^{-1}.h^{-1}$).

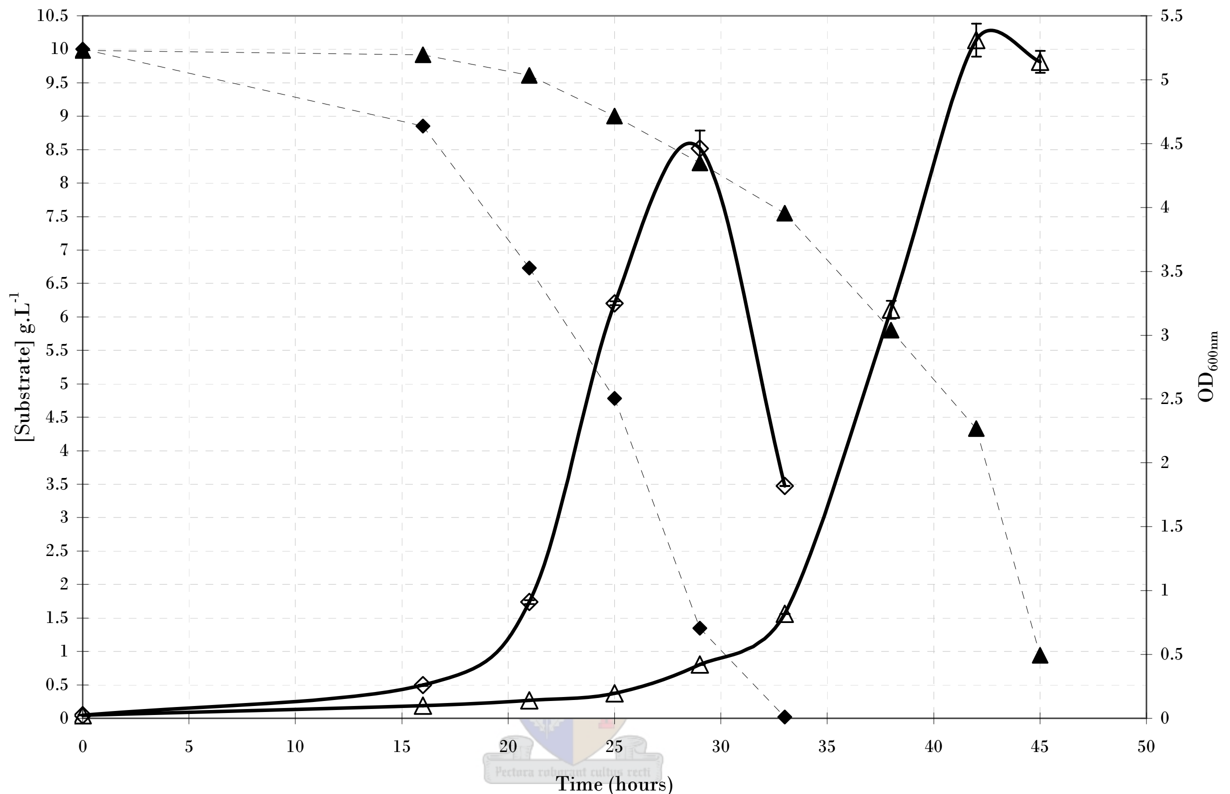

Figure 3. Growth (open symbols) and substrate consumption (filled symbols) of SIGMA[SSFI] during aerobic batch cultivation in minimal medium containing (i) 10 $g.L^{-1}$ cellobiose (△) and (ii) 7 $g.L^{-1}$ cellobiose + 3 $g.L^{-1}$ maltose (◇).

Flocculation, pseudohyphal growth and biofilm formation properties of the SIGMA[SSFI] strain. During aerobic batch cultivation of the adapted strain, flocculation of the yeast cells occurred at cell densities above 1.39 x 10^{8} $cells.ml^{-1}$ and 1.17 x 10^{8} $cells.ml^{-1}$ when grown in cellobiose and cellobiose/maltose-medium, respectively. Yeast cells from both fermentations exhibited the pseudohyphal phenotype when observed under the microscope (Figure 4).

The *S. cerevisiae* SIGMA[SSFI] strain was also inoculated into a multiple channel flow cell. Channels were fed (in duplicate) with minimal medium containing 1% glucose, 1% maltose and 1% cellobiose, respectively. Biofilm development was observed after 2 days of growth on maltose and cellobiose as sole carbon source. No biofilm formation was observed when

SIGMA[SSFI] was grown on glucose. Figure 5 shows the level of biofilm development after 2 days. The biofilms were maintained for 7 days at room temperature.

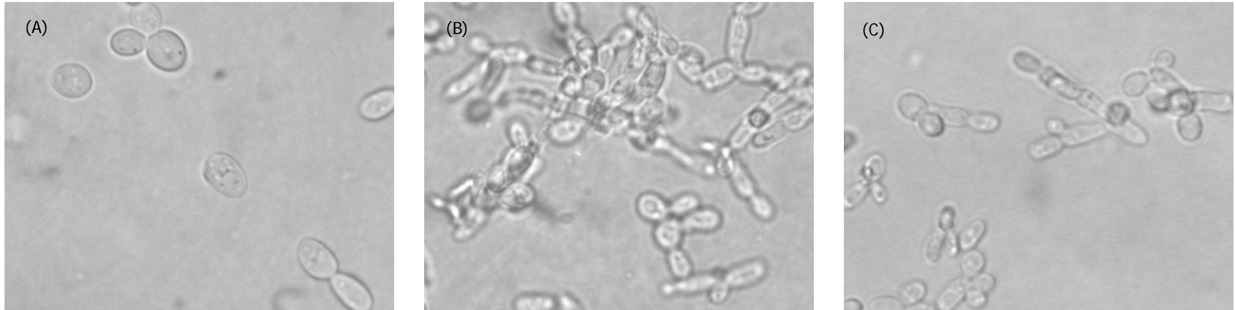

Figure 4. Cell morphology of *S. cerevisiae* SIGMA[SSFI] grown in medium containing (A) glucose, (B) maltose and (C) cellobiose.

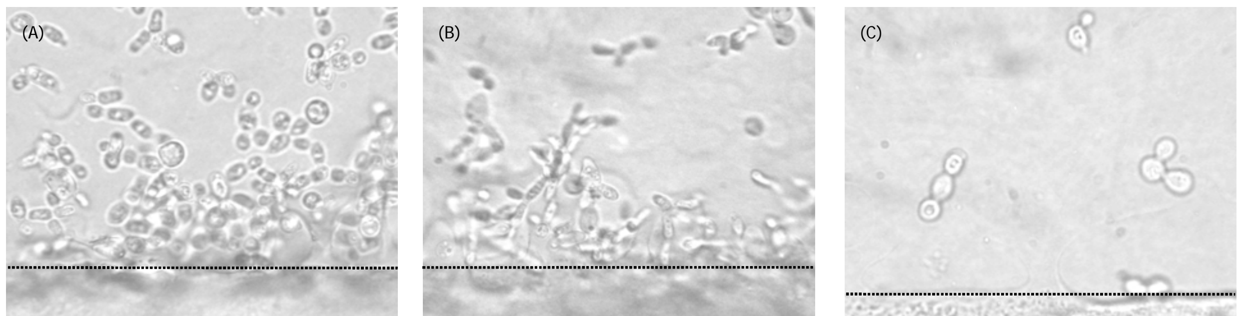

Figure 5. Biofilm formation observed during growth of *S. cerevisiae* SIGMA[SSFI] after 2 days in minimal medium containing (A) maltose and (B) cellobiose. (C) is the control grown in glucose where no biofilm development took place. The surface area of the flow cell channel is highlighted with a black line in each of the photos.

DISCUSSION

Engineering *S. cerevisiae* for efficient cellobiose utilization is an important goal towards the development of a recombinant host that will be able to hydrolyse cellulose. Since it is widely recognized that *S. cerevisiae* do not produce a dedicated cellobiose permease/transporter, the obvious strategy was to produce a secretable β-glucosidase that will catalyze the hydrolysis of cellobiose to glucose extracellularly. However, extracellular cellobiose hydrolysis has disadvantages, such as feedback inhibition on cellulase activity and high risk contamination upon hydrolysis to glucose.

The mature peptide sequence of the *Saccharomycopsis fibuligera* β-glucosidase (BGL1) was produced in the intracellular space of the recombinant *S. cerevisiae* strain, designated SIGMA[SSFI]. The SIGMA[SSFI] strain was selected on cellobiose-containing plates for uptake and subsequent utilization of cellobiose. Continuous selective pressure over a period of 3 months resulted in a strain with a significantly improved growth rate of 0.09 h^{-1} in 10 $g.L^{-1}$ cellobiose shake-flask culture. One of the 10 best cellobiose-utilizing SIGMA[SSFI] colonies displayed an unusual wrinkled colony morphology (Figure 1). Halme et al. (2004) described a similarly wrinkled colony morphology phenotype that resulted from loss-of-function mutations in either *IRA1* or *IRA2*, the genes encoding the yeast Ras GTPase-activating proteins. This particular cell surface alteration causes increased adhesion of *S. cerevisiae* to the agar.

In order to explain the mechanism by which the adapted SIGMA[SSFI] strain is able to transport and utilize cellobiose intracellularly, we investigated the native disaccharide transporters of *S. cerevisiae*. Cellobiose and maltose are both disaccharides of glucose and differ only with regards to their 1,4-linkage (α- and β-linked in maltose and cellobiose, respectively). As a result, cellobiose and maltose display similar stereochemistry as indicated in Figure 6. Subsequent RNA Bio-Dot analysis on the total RNA from the adapted strain when grown on cellobiose and maltose as sole carbon source confirmed the induction of RNA transcripts for the native β-glucoside and maltose transporters (Figure 2). The recombinant strain grown in glucose-medium did not show induction of either of the transporters. These results suggest that the transport of cellobiose (presumably via the maltose permease and α-glucoside transporter) is the rate-limiting step in the utilization of cellobiose by SIGMA[SSFI]. In addition, no β-glucosidase activity could be detected in the supernatant of the cellobiose-growing culture, but only in the

intracellular space and therefore confirmed that the cellobiose has to be transported across the plasma membrane in order to be utilized.

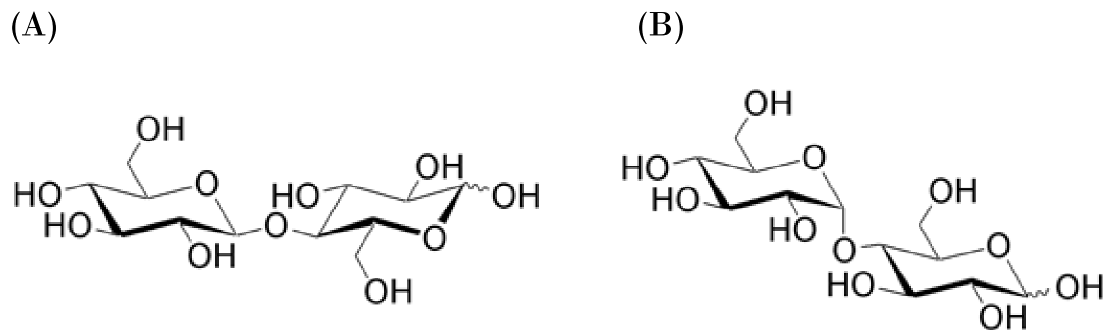

Figure 6. The stereochemistry of the disaccharides, cellobiose (A) and maltose (B).

Previous studies have shown that maltose is taken up via a maltose-H^+ symporter that actively transports maltose together with a proton molecule into the cell [Van Leeuwen et al., 1992; Serrano, 1977]. The cost of maltose transport is therefore 1 ATP per H^+ due to the plasma membrane ATPase required for pumping back the protons to the extracellular space. The genes encoding the different components needed for maltose transport and utilization are located on five unlinked, highly conserved loci, designated *MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6* [Vanoni et al., 1989]. Each locus consists of three genes: (i) a maltose-proton symporter; (ii) a maltase (α-glucosidase); and (iii) a maltose-dependent transcription factor (positive regulatory protein) [Stambuk and de Araujo, 2001]. The presence of at least one of the five loci is required for maltose utilization by *S. cerevisiae*. In *S. cerevisiae* the number of *MAL* loci and their homology is strain dependent [Naumov et al., 1994]. It is worth mentioning that the PCR primers used to amplify the maltose permease (MAL) probe used in the present study targeted a highly conserved region present in these *MAL* loci.

Interestingly, the α-glucoside-H^+ symporter (encoded by the *AGT1* gene) has also been implicated in maltose transport in *S. cerevisiae* [Jespersen et al., 1999; and Han et al., 1995]. To date, all sugar transport activities in *S. cerevisiae* have shown non-linear Eadie-Hofstee kinetic plots, thereby suggesting the existence of different components with different substrate affinities [Stambuk and de Araujo, 2001]. Maltose is no exception and is actively transported by either the high affinity maltose permease or low affinity α-glucoside transporter depending on the substrate concentration. Typically the maltose permease has a K_m of ~5 mM [Stambuk et al., 2001; Van der Rest et al., 1995; and Cheng and Michels, 1989] and the α-glucoside transporter a K_m of

~18 mM for maltose [Stambuk and de Araujo, 2001; and Crumplen et al., 1996]. The RNA Bio-Dot results obtained with the cellobiose-utilizing SIGMA[SSFI] showed that both the maltose permease and α-glucoside transporter were induced in the adapted strain (Figure 2). Presumably, these native transporters have different affinities for cellobiose and therefore co-facilitate sugar transport at different concentrations of cellobiose, suggesting that cellobiose transport in the adapted strain is in agreement with the multi-component model described for sugar transport in *S. cerevisiae*.

Maltose utilization in *S. cerevisiae* is negatively regulated by glucose, both at the transcriptional level and at enzyme activity level [Lucerno et al., 2002; Medintz et al., 2000; Klein et al., 1999; Brondijk et al., 1998; Klein et al., 1996; and Yao et al., 1994]. It was shown that addition of glucose to yeast cells grown in maltose triggers a mono-ubiquitination of the maltose permease [Lucerno et al., 2000] and consequently endocytosis and proteolysis of the transporter in the vacuole [Riballo et al., 1995; and Lucerno et al., 1993]. According to the auto-radiograph shown in Figure 2, neither the maltose or α-glucoside transporter could be detected in SIGMA[SSFI] strain grown in glucose-medium. Since the maltose transporter is rapidly degraded in the presence of glucose, it is of utmost importance that no cellobiose is hydrolyzed to glucose outside the cell in order for this uptake system to function.

The adapted SIGMA[SSFI] strain was cultivated in high-performance bioreactors to determine if the rate of transport and subsequent utilization of cellobiose was sufficient to enable growth on cellobiose as sole carbon source. During aerobic cultivation, SIGMA[SSFI] was able to grow in YP-medium containing 10 $g.L^{-1}$ cellobiose at a maximum specific growth rate (μ_{max}) of 0.17 h^{-1} (Figure 3). Interestingly, when the carbon source was changed to a mixture of cellobiose and maltose (7 $g.L^{-1}$ cellobiose and 3 $g.L^{-1}$ maltose) the μ_{max} increased to 0.30 h^{-1}. The addition of maltose significantly improved the uptake of cellobiose. It is therefore assumed that the transport of cellobiose (via the combined action of the maltose permease and α-glucosidase transporter) is the rate-limiting step in the utilization of cellobiose by SIGMA[SSFI]. The argument is also supported by the significantly increased substrate consumption rate of 0.37 $g.g\,DW^{-1}.h^{-1}$ when SIGMA[SSFI] was grown in the cellobiose/maltose-medium, compared to 0.25 $g.g\,DW^{-1}.h^{-1}$ when grown in cellobiose medium.

The phenotypical characteristics displayed by the cellobiose-utilizing SIGMA[SSFI] strain, namely pseudohyphal growth, flocculation and biofilm formation, are typical adaptations that occur in *S. cerevisiae* in response to nutrient limitation [Gagiano et al., 2002]. A large number of genes, which are mostly involved in the signalling pathways that regulate the dimorphic switch from yeast to hyphal form, have been linked with these adaptations. It has been hypothesized that pseudohyphae grow invasively into the solid agar medium and away from the colony in order to search for nutrient-rich substrates [Bauer and Pretorius, 2001; Pan et al., 2000]. Industrial processes that require easy separation of yeast cells (by sedimentation) and subsequently immobilization of the cells onto biomass support particles derive major benefit from using flocculent yeast strains [Kondo et al., 2002; Liu et al., 1998; and Furuta et al., 1997].

This work describes the successful construction and characterization of a recombinant *S. cerevisiae* SIGMA[SSFI] strain that is able to effectively transport and utilize cellobiose intracellularly. Extracellular hydrolysis of cellobiose has two major disadvantages, namely glucose's inhibitory effect on the activity of cellulase enzymes as well as the increased risk of contamination associated with external glucose release. Therefore, SIGMA[SSFI]'s ability to transport cellobiose is of particular interest when the final goal of industrial-scale ethanol production from cellulosic waste is considered. This is the first report, according to our knowledge, that describes the adaptation of native *S. cerevisiae* transporters to facilitate efficient transport of cellobiose across the yeast cell membrane. This novel cellobiose-utilizing *S. cerevisiae* strain is an important link in the construction of a cellulolytic yeast with properties resembling those associated with the highly efficient cellulase enzyme systems of cellulosome-producing anaerobes. SIGMA[SSFI]'s ability to efficiently remove cellobiose from the extracellular space together with its flocculating, pseudohyphae- and biofilm-forming properties could contribute significantly to the development of *S. cerevisiae* that degrades cellulose.

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CHAPTER 5

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

Biomass conversion has received major research attention due to its abundance and huge potential for conversion into sugars and fuels. Development of a cost-effective process has many challenges as discussed previously. The objective of this study was to construct a cellobiose-fermenting host with superior ethanol formation properties as a starting point for CBP (consolidated bioprocessing) microbial host development. The recombinant cellulolytic strategy, as described by Lynd et al. (2002), focuses on the genetic engineering of non-cellulolytic microorganisms with desirable product formation properties. The non-cellulolytic yeast, *S. cerevisiae*, is the microorganism most commonly used for ethanol fermentation. Genetic engineering of *S. cerevisiae* to ferment cellobiose (the major soluble by-product of cellulose hydrolysis) to ethanol is a critical step in the development of a recombinant host for cellulose conversion via CBP. *S. cerevisiae* lacks the ability to transport cellobiose due to the absence of a dedicated permease for cellobiose. This study described two genetic engineering strategies towards the construction of a cellobiose-fermenting strain. The first involved the introduction of secreted β-glucosidases [Chapter 3], whereas the second was aimed at cellobiose transport and intracellular utilization [Chapter 4].

Isolation and functional expression of secretable β-glucosidases in *S. cerevisiae*

The genes encoding β-glucosidase from *A. kawachii* (*bglA*), *C. wickerhamii* (*bglB*), *S. fibuligera* (*BGL1*) and *T. reesei* (*bgl*) were isolated and heterologously expressed in *S. cerevisiae*. The genes were fused to the secretion signal from the *T. reesei* xylanase (*xyn2*) gene and expressed under control of the *S. cerevisiae PGK1* promoter and terminator sequences. Using this isogenic approach the activities of the recombinant β-glucosidases produced by *S. cerevisiae* were compared on the basis of enzyme kinetics. According to the K_m values, *S. fibuligera* BGL1 had the highest affinity for the pNPG substrate (a chromogenic analog of cellobiose). In addition, the optimum activity of BGL1 was at pH 5.4, which is consistent with the pH range (5.0 to 5.5) for optimal growth of *S. cerevisiae*.

Evaluation of the recombinant cellobiose-fermenting strains

The performances of the recombinant *S. cerevisiae* strains were evaluated in high-performance bioreactors under fermentative conditions during batch cultivation on cellobiose.

It was suggested that the amount of cellobiose-hydrolyzing activity produced by the respective strains would ultimately determine their ability to sustain growth on cellobiose as sole carbon source. During aerobic cultivation, only the strains producing the recombinant *A .kawachii* and *S. fibuligera* enzymes were able to grow in minimal medium containing 5 $g.L^{-1}$ cellobiose. The *BGL1*-expressing strain was identified as the best strain and grew at 0.23 h^{-1} on cellobiose (vs. 0.29 h^{-1} for the reference strain on glucose). More notable was the ability of this strain to anaerobically ferment cellobiose at 0.18 h^{-1} (vs. 0.25 h^{-1} for the reference strain on glucose). The ethanol yield of this strain was 0.41 g per g of cellobiose consumed, which corresponds to 89% of the maximum yield (0.46 g of ethanol) when the reference strain was anaerobically grown on glucose.

Intracellular β-glucosidase production in *S. cerevisiae*

The second strategy was aimed at the efficient removal of cellobiose from the extracellular environment. Extracellular cellobiose has two major disadvantages: (i) it causes feedback inhibition of other cellulases and therefore determines the rate and extent of cellulose hydrolysis [Yan et al., 1998]; and (ii) the action of β-glucosidases releases external glucose that poses an increased contamination risk. In an alternative approach, the mature peptide sequence from the *S. fibuligera* β-glucosidase (*BGL1*) was constitutively expressed in *S. cerevisiae*. The resulting strain produced a β-glucosidase that remained in the intracellular space of the recombinant *S. cerevisiae* strain.

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Cellobiose transport and intracellular metabolism

S. cerevisiae has several native α-glucoside transporters, but does not have a dedicated transporter for cellobiose. The recombinant strain was subjected to a simple selection procedure that aimed at the adaptation of its native transporters for cellobiose uptake and subsequent utilization. Continuous selective pressure over a period of 3 months resulted in a recombinant strain that was able to grow on cellobiose at a rate of 0.09 h^{-1}. β-Glucosidase activity assays confirmed that BGL1 activity was limited to the intracellular space and therefore cellobiose had to be transported across the plasma membrane in order to be utilized. RNA Bio-Dot analysis on the total RNA from the adapted strain when grown on cellobiose and maltose as sole carbon source confirmed the induction of RNA transcripts for the native α-glucoside (AGT1) and maltose (MAL) transporters. Since cellobiose and maltose are both disaccharides of glucose and differ only with regard to their 1,4-linkage, it was not surprising

that the latter permeases could be adapted to facilitate cellobiose transport. The reference strain (before adaptation) grown in glucose-medium did not show induction of either of the transporters.

Evaluation of a novel cellobiose utilizing *S. cerevisiae* strain

The adapted *S. cerevisiae* strain was cultivated in high-performance bioreactors to establish whether the rate of cellobiose transport and subsequent utilization was sufficient to sustain growth on cellobiose as sole carbon source. During aerobic cultivation, the adapted strain was able to grow in YP-medium containing 10 $g.L^{-1}$ cellobiose at a rate of 0.17 h^{-1}. Interestingly, when the carbon source was substituted with a mixture of cellobiose and maltose (7 $g.L^{-1}$ cellobiose and 3 $g.L^{-1}$ maltose) the growth rate increased to 0.30 h^{-1}. The addition of maltose resulted in a considerable improvement in the rate of cellobiose uptake. It was suggested that cellobiose transport (via the combined action of the maltose permease and α-glucosidase transporter) is the rate-limiting step in the utilization of cellobiose by the recombinant strain. The claim is also supported by the significantly increased substrate consumption rate of 0.37 $g.g\ DW^{-1}.h^{-1}$ when the strain was grown in the cellobiose/maltose-medium, compared to 0.25 $g.g\ DW^{-1}.h^{-1}$ when grown in cellobiose medium.

Flocculation, pseudohyphal growth and biofilm formation properties

During aerobic batch cultivation of the adapted strain, flocculation of the yeast cells occurred at cell densities above 1.39×10^8 $cells.ml^{-1}$ and 1.17×10^8 $cells.ml^{-1}$ when grown in cellobiose and cellobiose/maltose-medium, respectively. Yeast cells from both fermentations exhibited the pseudohyphal phenotype when observed under the microscope. These are typical adaptations that occur in *S. cerevisiae* in response to nutrient limitation [Gagiano et al., 2002]. Flocculation can be of particular benefit to industrial processes that require easy separation of yeast cells. Inoculation of the adapted strain into a multiple channel flow cell resulted in biofilm formation when fed with cellobiose or maltose-medium. Biofilm formation has been described for *S. cerevisiae* [Reynolds and Fink, 2001]. The wrinkled colony morphology displayed by the adapted strain has been previously described [Halme et al., 2004] and is the result of a particular cell surface alteration that causes increased adhesion of *S. cerevisiae* to the agar. This alteration of the yeast cell surface might also play a role in biofilm formation by the adapted strain.

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CONCLUSIONS

The objective of this study was the functional expression of β-glucosidase genes in *S. cerevisiae* and subsequent evaluation of the recombinant cellobiose-fermenting strains in order to obtain a suitable candidate for future CBP technology.

From the data presented in this study, the following can be concluded:

⇨ Four β-glucosidase genes of fungal origins were successfully isolated and functionally expressed in the yeast *S. cerevisiae*;

⇨ A comparative analysis of the kinetic properties of the individual secreted enzymes identified the *S. fibuligera* β-glucosidase (BGL1) as the enzyme with the highest activity and substrate affinity for the chromogenic substrate (pNPG);

⇨ The BGL1-producing strain was able to grow on cellobiose at 0.23 h^{-1} (vs. 0.29 h^{-1} of the reference strain on glucose) and 0.18 h^{-1} (vs. 0.25 h^{-1} of the reference strain on glucose) under aerobic and anaerobic conditions, respectively. The ethanol yield of the strain was 89% of the maximum yield.

⇨ The mature peptide sequence of BGL1 was functionally expressed in the intracellular space of *S. cerevisiae*;

⇨ The native disaccharide transport system(s) of the recombinant *S. cerevisiae* strain was successfully adapted for cellobiose uptake and subsequent intracellular utilization;

⇨ RNA Bio-dot analysis revealed the induction of both the maltose permease and α-glucoside transporter in the adapted strain when grown on cellobiose;

⇨ During batch cultivation, the adapted *S. cerevisiae* sustained growth aerobically on cellobiose at 0.17 h^{-1} (vs. 0.30 h^{-1} on cellobiose/maltose-medium);

⇨ The addition of maltose to the cellobiose-containing medium increased the substrate consumption rate of 0.25 $g.g\,DW^{-1}.h^{-1}$ (cellobiose-medium) to 0.37 $g.g\,DW^{-1}.h^{-1}$ (cellobiose/maltose-medium). It seems that cellobiose transport is the rate-limiting step in the utilization of cellobiose by the adapted strain;

⇨ In addition to the recombinant strain's ability to remove cellobiose from the extracellular space, it displays several interesting phenotypical characteristics, namely, flocculation, pseudohyphal growth and biofilm-formation that resembles some of the

properties associated with the highly efficient cellulase enzyme systems of cellulosome-producing anaerobes;

⇨ The novel cellobiose-utilizing *S. cerevisiae* strain constructed during this study is an important step towards the development of a cellulolytic yeast for future CBP technology.

FUTURE RESEARCH

Genetic engineering of a robust industrial strain of *S. cerevisiae* for cellobiose uptake and subsequent utilization would be the next logical step towards recombinant host development. A whole-cell biocatalyst that could effectively convert cellulose to ethanol in a single process step would require the co-expression of endoglucanases and cellobiohydrolases with high synergistic action towards microcrystalline cellulose. The latter will include efficient transformation and high-level expression of the different cellulase enzymes in *S. cerevisiae*. Evolutionary engineering can be applied to the ethanol-producing organism as a whole or to specific proteins, in particular those with regulatory functions to optimize recombinant cellulase production in *S. cerevisiae*.

Ultimately, the aim would be to develop a robust bioconversion process, which will involve identifying and overcoming the major conversion restrictions of metabolic flux and product, temperature and pH tolerances. According to the U.S. Department of Energy's "Biofuels Joint Roadmap" (2006) the following requirements are needed for the development of a future cost-effective biomass-to-ethanol process:

⇨ High ethanol yield (>95% of theoretical yield) with complete hexose and pentose consumption, minimal by-product formation, and minimal loss of carbon into cell biomass;

⇨ Final ethanol titers in the range of 10 to 15 wt %;

⇨ Ethanol productivity of 2-5 $g.L^{-1}.h^{-1}$ under high-solid conditions;

⇨ Tolerance to inhibitors present in hydrolysates;

⇨ The ability to grow in minimal medium or on actual hydrolysates (with only minerals as added nutrients).

In conclusion, to achieve the above targets a cooperative research effort, from both fundamental and applied disciplines, is required. The end result will not only depend on the successful construction a multi-talented robust microorganism with the ability to grow in inhibitory environments of high sugar and ethanol concentrations but also on the insights gained into the molecular basis of these processes that will allow the optimization of flux through the sugar-to-ethanol metabolic pathway. From metabolic engineers to systems biologists, everyone has a vital contribution to make to the future of sustainable energy technology!

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Pectora roborant cultus recti

APPENDICES

Appendix A

Enzyme Properties

pH optima of the recombinant enzymes

Aspergillus kawachii

pH	OD1	OD2	OD3	Average	%Activity	St.Dev	%St.Dev
3.4	0.134	0.133	0.130	0.132	24.34	0.002	0.38
4	0.295	0.298	0.301	0.298	54.81	0.003	0.55
4.4	0.535	0.556	0.540	0.544	100.00	0.011	2.02
5	0.500	0.490	0.488	0.493	90.62	0.006	1.18
5.4	0.471	0.488	0.495	0.485	89.15	0.012	2.27
6	0.372	0.371	0.380	0.374	68.85	0.005	0.91
7	0.120	0.123	0.150	0.131	24.10	0.017	3.04

Candida wickerhamii

pH	OD1	OD2	OD3	Average	%Activity	St.Dev	%St.Dev
3.4	0.145	0.142	0.147	0.145	20.11	0.003	0.35
4	0.292	0.288	0.308	0.296	41.15	0.011	1.47
4.4	0.725	0.717	0.716	0.719	100.00	0.005	0.69
5	0.670	0.684	0.693	0.682	94.86	0.012	1.61
5.4	0.554	0.555	0.560	0.556	77.34	0.003	0.45
6	0.360	0.356	0.364	0.360	50.05	0.004	0.56
7	0.177	0.171	0.176	0.175	24.28	0.003	0.45

Saccharomycopsis fibuligera

pH	OD1	OD2	OD3	Average	%Activity	St.Dev	%St.Dev
3.4	0.084	0.091	0.096	0.090	9.39	0.006	0.63
4	0.168	0.173	0.175	0.172	17.88	0.004	0.37
4.4	0.352	0.360	0.346	0.353	36.66	0.007	0.73
5	0.876	0.880	0.890	0.882	91.68	0.007	0.75
5.4	0.970	0.966	0.950	0.962	100.00	0.011	1.10
6	0.750	0.778	0.796	0.775	80.53	0.023	2.41
7	0.450	0.446	0.444	0.447	46.43	0.003	0.32

Trichoderma reesei

pH	OD1	OD2	OD3	Average	%Activity	St.Dev	%St.Dev
3.4	0.175	0.175	0.173	0.174	37.76	0.001	0.25
4	0.283	0.280	0.290	0.284	61.59	0.005	1.11
4.4	0.461	0.464	0.460	0.462	100.00	0.002	0.45
5	0.441	0.430	0.450	0.440	95.38	0.010	2.17
5.4	0.421	0.425	0.410	0.419	90.69	0.008	1.68
6	0.316	0.322	0.319	0.319	69.10	0.003	0.65
7	0.172	0.166	0.173	0.170	36.90	0.004	0.82

Neg. Control

pH	OD1	OD2	OD3	Average	St.Dev
3.4	0.085	0.091	0.095	0.090	0.005
4	0.196	0.182	0.194	0.191	0.008
4.4	0.330	0.323	0.327	0.327	0.004
5	0.311	0.303	0.316	0.310	0.007
5.4	0.284	0.284	0.270	0.279	0.008
6	0.226	0.222	0.214	0.221	0.006
7	0.088	0.095	0.093	0.092	0.004

Temperature optima of the recombinant enzymes

Aspergillus kawachii

Temp	OD1	OD2	OD3	Average	% Activity	St.dev	% St.dev
30	0.447	0.445	0.449	0.4470	18.67	0.0020	0.08
35	0.692	0.635	0.665	0.6640	27.73	0.0285	1.19
40	1.008	0.991	0.986	0.9950	41.56	0.0115	0.48
45	1.374	1.342	1.373	1.3630	56.93	0.0182	0.76
50	1.704	1.714	1.691	1.7030	71.13	0.0115	0.48
55	2.054	2.095	2.06	2.0697	86.44	0.0221	0.92
60	2.397	2.392	2.394	2.3943	100.00	0.0025	0.11
65	2.39		2.366	2.3780	99.32	0.0170	0.71
70	1.429	1.492	1.442	1.4543	60.74	0.0333	1.39

Candida wickerhamii

Temp	OD1	OD2	OD3	Average	% Activity	St.dev	% St.dev
30	0.625	0.621	0.627	0.6243	63.11	0.0031	0.31
35	0.854	0.86	0.867	0.8603	86.96	0.0065	0.66
40	0.991	0.984	0.993	0.9893	100.00	0.0047	0.48
45	0.92	0.928		0.9240	93.40	0.0057	0.57
50	0.627	0.628	0.647	0.6340	64.09	0.0113	1.14
55	0.44	0.474	0.466	0.4600	46.50	0.0178	1.80

Saccharomycopsis fibuligera

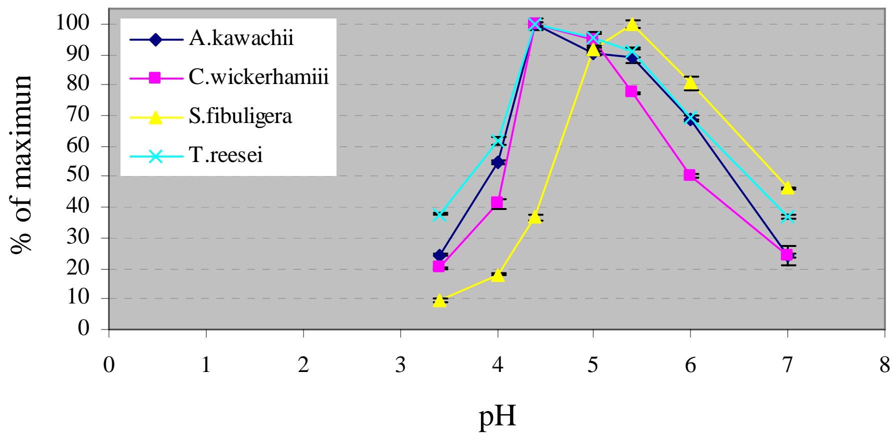
Temp	OD1	OD2	OD3	Average	% Activity	St.dev	% St.dev
30	0.33	0.335	0.331	0.3320	20.41	0.0026	0.16
35	0.423	0.454		0.4385	26.95	0.0219	1.35
40	0.619	0.631	0.661	0.6370	39.15	0.0216	1.33
45	1.094	1.077	1.036	1.0690	65.70	0.0298	1.83
50	1.422	1.4	1.388	1.4033	86.25	0.0172	1.06
55	1.618	1.636		1.6270	100.00	0.0127	0.78
60	0.48	0.454		0.4670	28.70	0.0184	1.13

Trichoderma reesei

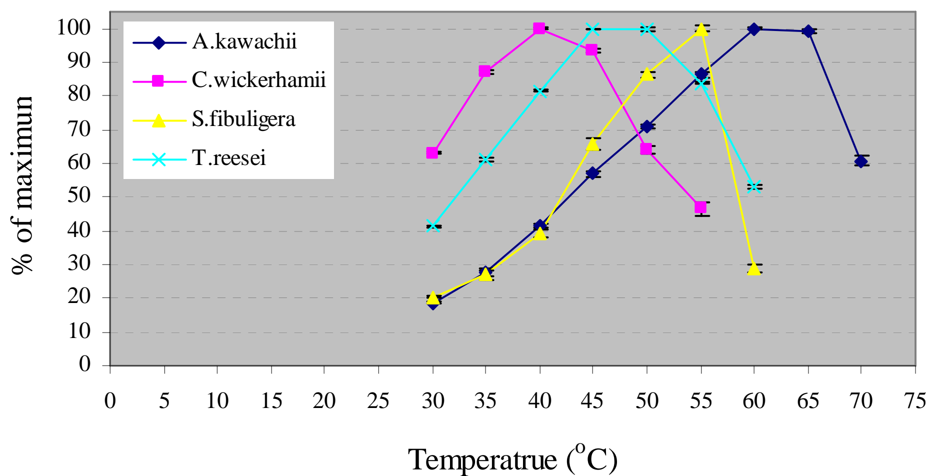
Temp	OD1	OD2	OD3	Average	% Activity	St.dev	% St.dev
30	0.416	0.412	0.42	0.4160	41.37	0.0040	0.25
35	0.628	0.615	0.607	0.6167	61.33	0.0106	0.65
40	0.819	0.822	0.818	0.8197	81.52	0.0021	0.13
45	1.005	1.006		1.0055	100.00	0.0007	0.04
50	1.014	0.999	1	1.0043	99.88	0.0084	0.52
55	0.84	0.844	0.847	0.8437	83.91	0.0035	0.22
60	0.527	0.541	0.53	0.5327	52.98	0.0074	0.45

pH and temperature optima graphs of the recombinant enzymes

pH optima of the recombinant enzymes



Temperature optima of the recombinant enzymes



Kinetic properties of the recombinant enzymes

Aspergillus kawachii

Time	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0864
25	0.364	0.379		0.3715	2.1608	0.0106	
2 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0796
25	0.347	0.338	0.343	0.3427	1.9891	0.0045	
1 mM							
0	0	0	0	0.0000	0.0000	0.0000	#DIV/0!
25				#DIV/0!	#DIV/0!	#DIV/0!	
0,75 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0568
25	0.248	0.246	0.247	0.2470	1.4193	0.0010	
0,5 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0408
25	0.183	0.177	0.18	0.1800	1.0203	0.0030	
0,25 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0235
25	0.106	0.104	0.112	0.1073	0.5875	0.0042	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.0235	42.5566
0.5000	2.0000	0.0408	24.5038
0.7500	1.3333	0.0568	17.6144
1.0000			
2.0000	0.5000	0.0796	12.5686
4.0000	0.2500	0.0864	11.5697

y = 8.4209x + 8.1501 R^2 = 0.9913

1/Vmax	Vmax	St.dev
8.1501	0.1227	0.0087
1/Km	Km	St.dev
0.9678	1.0332	0.0087

Aspergillus kawachii AKA(A)

Time	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.2017
15	0.514	0.517	0.519	0.5167	3.0254	0.0025	
2 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.1670
15	0.432	0.431	0.425	0.4293	2.5053	0.0038	
1 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.1117
15	0.288	0.292		0.2900	1.6754	0.0028	
0,75 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0910
15	0.236	0.238	0.24	0.2380	1.3657	0.0020	
0,5 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0676

15	0.178	0.179	0.18	0.1790	1.0143	0.0010	
0,25 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0369
15	0.1	0.102	0.103	0.1017	0.5537	0.0015	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.0369	27.0904
0.5000	2.0000	0.0676	14.7886
0.7500	1.3333	0.0910	10.9834
1.0000	1.0000	0.1117	8.9531
2.0000	0.5000	0.1670	5.9874
4.0000	0.2500	0.2017	4.9580

y = 5.9553x + 3.1111 R^2 = 0.9993

1/Vmax	Vmax	St.dev
3.1111	0.3214	0.0007
1/Km	Km	St.dev
0.5224	1.9142	0.0007

Aspergillus kawachii AKA(C)

Time	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.4374
11	0.817	0.816		0.8165	4.8112	0.0007	
2 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.3667
11	0.688	0.683	0.687	0.6860	4.0339	0.0026	
1 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.2487
11	0.467	0.469		0.4680	2.7356	0.0014	
0,75 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.2135
11	0.4	0.403	0.406	0.4030	2.3484	0.0030	
0,5 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.1586
11	0.303	0.302	0.3	0.3017	1.7449	0.0015	
0,25 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0906
11	0.174	0.17	0.184	0.1760	0.9964	0.0072	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.0906	11.0395
0.5000	2.0000	0.1586	6.3041
0.7500	1.3333	0.2135	4.6840
1.0000	1.0000	0.2487	4.0211
2.0000	0.5000	0.3667	2.7269
4.0000	0.2500	0.4374	2.2863

y = 2.3496x + 1.6199 R^2 = 0.9996

1/Vmax	Vmax	St.dev
1.6199	0.6173	0.0004
1/Km	Km	St.dev
0.6894	1.4505	0.0004

Candida wickerhamii

Time	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.1008
25	0.43	0.435	0.431	0.4320	2.5211	0.0026	
2 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0863
25	0.367	0.375	0.371	0.3710	2.1578	0.0040	
1 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0649
25	0.284	0.281	0.278	0.2810	1.6218	0.0030	
0,75 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0548
25	0.242	0.234	0.24	0.2387	1.3697	0.0042	
0,5 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0407
25	0.177	0.18	0.181	0.1793	1.0163	0.0021	
0,25 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.0222
25	0.103	0.103	0.1	0.1020	0.5557	0.0017	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.0222	44.9893
0.5000	2.0000	0.0407	24.5995
0.7500	1.3333	0.0548	18.2526
1.0000	1.0000	0.0649	15.4150
2.0000	0.5000	0.0863	11.5857
4.0000	0.2500	0.1008	9.9161

$y = 9.4559x + 6.4779$ $R^2 = 0.996$

1/Vmax	Vmax	St.dev
0.6894	1.4505	0.0040
1/Km	Km	St.dev
1.6199	0.6173	0.0040

Saccharomycopsis fibuligera

Time (min)	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0.0000	0.0000	0.0000	5.6044
2	1.882	1.89	1.9	1.8907	11.2089	0.0090	
2 mM							
0	0	0	0	0.0000	0.0000	0.0000	4.9711
2	1.668	1.682	1.684	1.6780	9.9422	0.0087	
1 mM							
0	0	0	0	0.0000	0.0000	0.0000	4.3825
2	1.438	1.508	1.495	1.4803	8.7649	0.0372	
0,75 mM							
0	0	0	0	0.0000	0.0000	0.0000	3.8335
2	1.267	1.331	1.29	1.2960	7.6671	0.0324	
0,5 mM							
0	0	0	0	0.0000	0.0000	0.0000	3.5285
2.1667	1.294	1.293	1.29	1.2923	7.6452	0.0021	

Saccharomycopsis fibuligera (continued)

Time (min)	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
0,25 mM							
0	0	0	0	0.0000	0.0000	0.0000	2.3822
2.1667	0.851	0.871	0.904	0.8753	5.1616	0.0268	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	2.3822	0.4198
0.5000	2.0000	3.5285	0.2834
0.7500	1.3333	3.8335	0.2609
1.0000	1.0000	4.3825	0.2282
2.0000	0.5000	4.9711	0.2012
4.0000	0.2500	5.6044	0.1784

$y = 0.0629x + 0.1667$ $R^2 = 0.9941$

1/Vmax	Vmax	St.dev
0.1667	5.9988	0.0059
1/Km	Km	St.dev
2.6502	0.3773	0.0059

Saccharomycopsis fibuligera SFI(A)

Time (min)	10 mM			Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0.0000	0.0000	0.0000	#DIV/0!
3				#DIV/0!	#DIV/0!	#DIV/0!	
2 mM							
0	0	0	0	0.0000	0.0000	0.0000	1.4267
3	0.722	0.733	0.727	0.7273	4.2801	0.0055	
1 mM							
0	0	0	0	0.0000	0.0000	0.0000	1.2464
3	0.634	0.639		0.6365	3.7391	0.0035	
0,75 mM							
0	0	0	0	0.0000	0.0000	0.0000	1.2047
3	0.616	0.615		0.6155	3.6141	0.0007	
0,5 mM							
0	0	0	0	0.0000	0.0000	0.0000	1.0945
3	0.56	0.56		0.5600	3.2835	0.0000	
0,25 mM							
0	0	0	0	0.0000	0.0000	0.0000	0.8344
3	0.432	0.426		0.4290	2.5033	0.0042	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.8344	1.1984
0.5000	2.0000	1.0945	0.9137
0.7500	1.3333	1.2047	0.8301
1.0000	1.0000	1.2464	0.8023
2.0000	0.5000	1.4267	0.7009

$y = 0.1384x + 0.6446$ $R^2 = 0.9958$

1/Vmax	Vmax	St.dev
0.6446	1.5513	0.0042
1/Km	Km	St.dev
4.6575	0.2147	0.0042

Saccharomycopsis fibuligera SFI(A)

Time	10 mM			Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0	0	0	1.4081795
3	0.717	0.719		0.718	4.224538416	0.0014142	
2 mM							
0	0	0	0	0	0	0	1.3870028
3	0.705	0.705	0.712	0.7073333	4.161008537	0.0040415	
1 mM							
0	0	0	0	0	0	0	1.2070015
3	0.615	0.61	0.625	0.6166667	3.621004566	0.0076376	
0,75 mM							
0	0	0	0	0	0	0	1.1745748
3	0.601	0.597	0.603	0.6003333	3.523724439	0.0030551	
0,5 mM							
0	0	0	0	0	0	0	1.0521474
3	0.536	0.541	0.539	0.5386667	3.156442327	0.0025166	
0,25 mM							
0	0	0	0	0	0	0	0.7947191
3	0.4	0.416	0.411	0.409	2.384157236	0.0081854	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.7947	1.2583
0.5000	2.0000	1.0521	0.9504
0.7500	1.3333	1.1746	0.8514
1.0000	1.0000	1.2070	0.8285
2.0000	0.5000	1.3870	0.7210
4.0000	0.2500	1.4082	0.7101

$y = 0.148x + 0.6625$ $R^2 = 0.996$

1/Vmax	Vmax	St.dev
0.6625	1.5094	0.0040
1/Km	Km	St.dev
4.4764	0.2234	0.0040

Trichoderma reesei

Time	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
4 mM							
0	0	0	0	0	0	0	0.0916816
30	0.478	0.463		0.4705	2.750446694	0.0106066	
2 mM							
0	0	0	0	0	0	0	0.0651777
30	0.34	0.334		0.337	1.955330554	0.0042426	
1 mM							
0	0	0	0	0	0	0	0.047707
30	0.25	0.248		0.249	1.431209053	0.0014142	
0,75 mM							
0	0	0	0	0	0	0	0.0421481
30	0.224	0.218		0.221	1.264443121	0.0042426	
0,5 mM							
0	0	0	0	0	0	0	0.0366885
30	0.193	0.194		0.1935	1.100655152	0.0007071	

Trichoderma reesei (continued)

Time	OD1	OD2	OD3	Average	Product (mM pNP)	St.dev.	Vo (slope)
0,25 mM							
0	0	0	0	0	0	0	0.0277877
30	0.15	0.15	0.146	0.1486667	0.83363113	0.0023094	

[S]	1/[S]	Vo	1/Vo
0.2500	4.0000	0.0230	43.4783
0.5000	2.0000	0.0367	27.2565
0.7500	1.3333	0.0470	21.2721
1.0000		0.0477	
2.0000	0.5000	0.0652	15.3427
4.0000	0.2500	0.0770	12.9870

y = 8.1092x + 10.957 R^2 = 0.9994

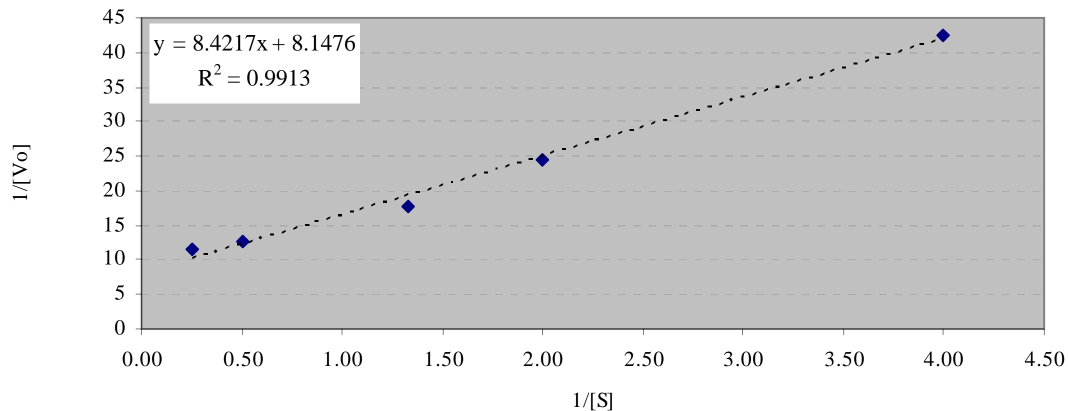
1/Vmax	Vmax	St.dev
10.9570	0.0913	0.0006
1/Km	Km	St.dev
1.3512	0.7401	0.0006

Summary of the kinetic properties of the recombinant enzymes

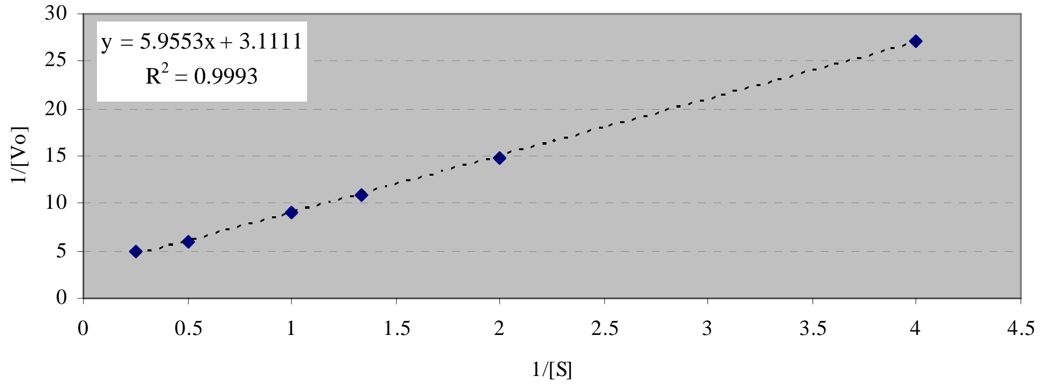
Enzyme	Km (mM)	St.dev	Vmax (graph)	Total OD	[cells] mg/ml	mg/100 µL		nmol/min/mg cells	Vmax
AKA	1.033	0.009	0.123	93.00	16.27	1.627	0.000123	0.076	0.151
AKA(A)	1.914	0.001	0.321	21.00	3.67	0.367	0.000321	0.875	1.749
AKA(C)	1.450	0.000	0.617	16.30	2.85	0.285	0.000617	2.165	4.330
CWI(B)	0.617	0.004	0.154	18.20	3.18	0.318	0.000154	0.484	0.969
SFI	0.377	0.004	0.6	7.90	1.38	0.138	0.0006	4.348	8.696
SFI(A)	0.215	0.004	1.55	5.65	0.99	0.988	0.00155	1.569	3.138
SFI(C)	0.223	0.004	1.51	6.03	1.05	0.105	0.00151	14.381	28.762
TRE	0.740	0.001	0.091	78.75	13.77	1.377	0.000091	0.066	0.132

Km and Vmax graphs of the recombinant enzymes

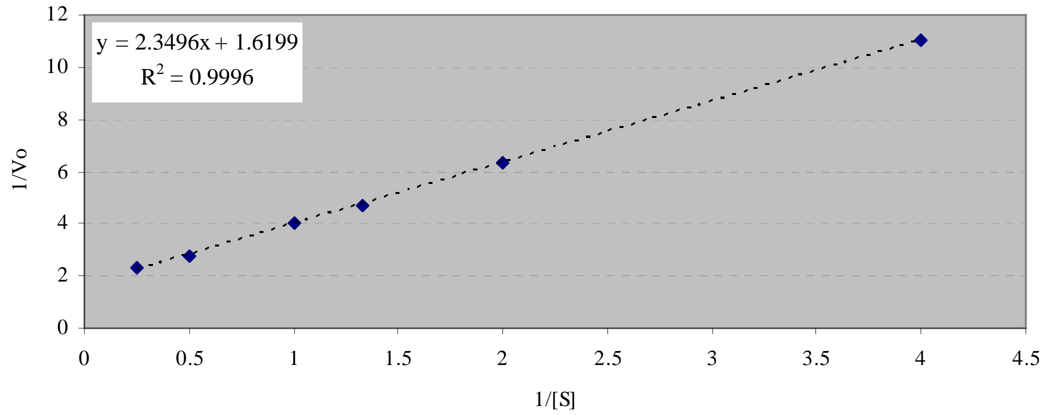
Aspergillus kawachii



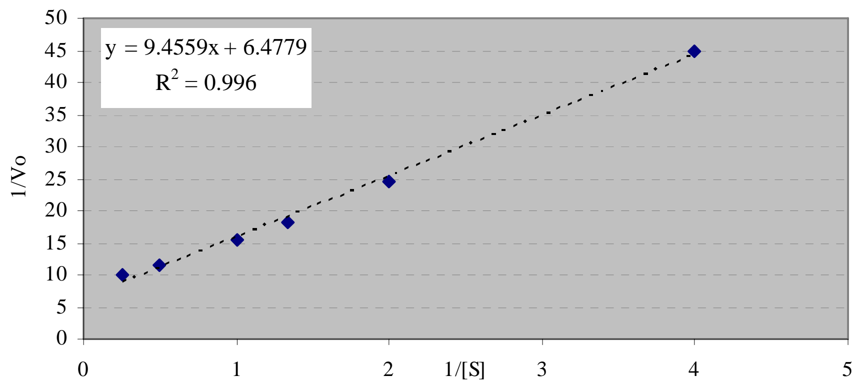
Aspergillus kawachii AKA(A)

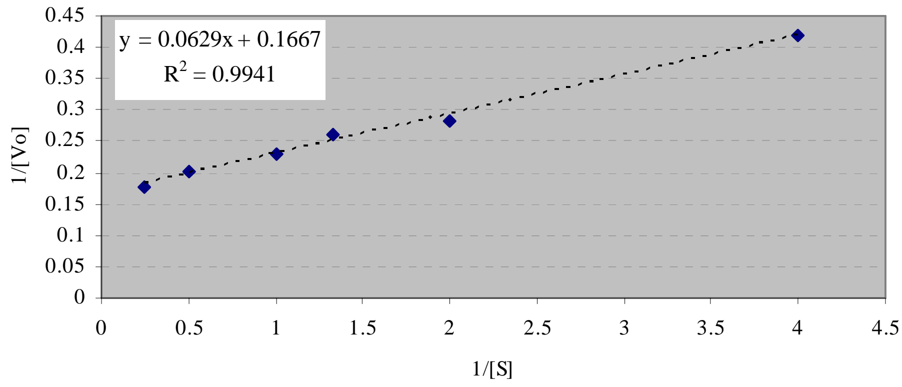


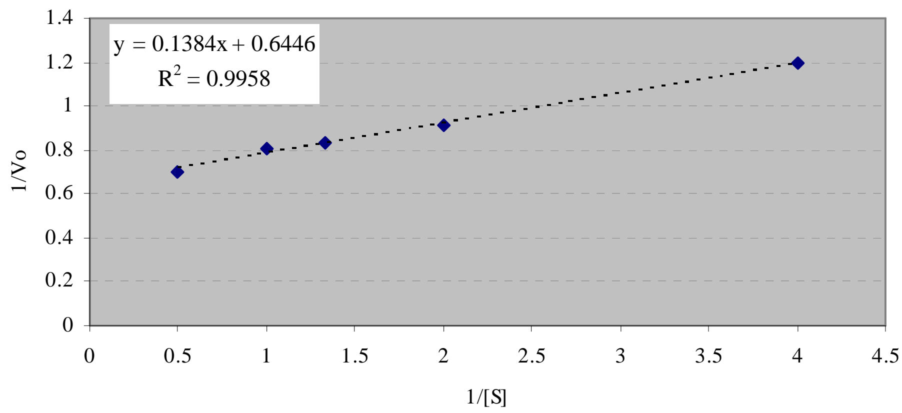
Aspergillus kawachii AKA(C)

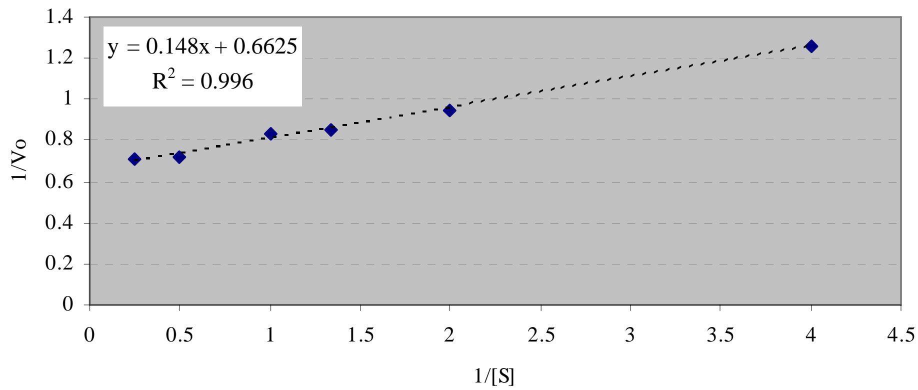


Candida wickerhamii

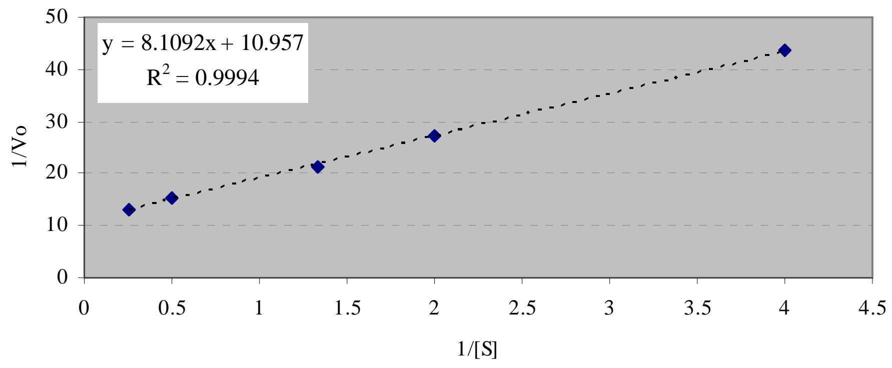


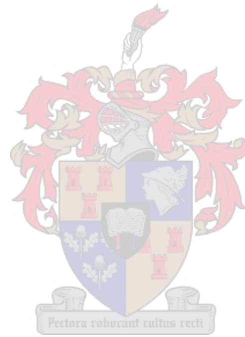

Saccharomycopsis fibuligera
y = 0.0629x + 0.1667
R2 = 0.9941
1/[Vo]
1/[S]
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5


Saccharomycopsis fibuligera SFI(A)
y = 0.1384x + 0.6446
R2 = 0.9958
1/Vo
1/[S]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5


Saccharomycopsis fibuligera SFI(C)
y = 0.148x + 0.6625
R2 = 0.996
1/Vo
1/[S]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Trichoderma reesei





Appendix B

Fermentation Results

Fermentation results: Recombinant Y294 strains secreting β-glucosidases

Aerobic fermentations on defined medium with 5 g.L-[1] carbon source (cellobiose/glucose)
*Each fermentation value represents an average of 3 OD values

Y294[Host]

Time	Ferm1	Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.55	1.54	1.545	1.500	0.007	4.44E+07		0.176
2	2.05	2.06	2.055	2.010	0.007	6.11E+07	0.698	0.303
4	3.74	3.78	3.760	3.715	0.028	1.12E+08	1.312	0.570
6	6.75	6.85	6.800	6.755	0.071	2.03E+08	1.917	0.830
8	11.58	11.4	11.490	11.445	0.127	3.44E+08	2.441	1.059
9	15.2	15.2	15.200	15.155	0.000	4.55E+08	2.721	1.181
11	15.2	15.3	15.250	15.205	0.071	4.57E+08		1.182
13	15.4	15.5	15.450	15.405	0.071	4.63E+08		1.188

Y294[AKA]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.695	1.68	1.688	1.500	0.011	5.01E+07		0.176
2	1.9	1.915	1.908	1.720	0.011	5.67E+07	0.646	0.236
4	1.965	2.02	1.993	1.805	0.039	5.92E+07	0.689	0.256
6	2.03	2.015	2.023	1.835	0.011	6.01E+07	0.704	0.264
9.25	2.155	2.185	2.170	1.983	0.021	6.45E+07	0.775	0.297
12	2.205	2.22	2.213	2.025	0.011	6.58E+07	0.794	0.306
15	2.27	2.315	2.293	2.105	0.032	6.82E+07	0.830	0.323
21	2.325	2.325	2.325	2.138	0.000	6.92E+07		0.330
34	2.45	2.425	2.438	2.250	0.018	7.26E+07		0.352
36	2.345	2.365	2.355	2.168	0.014	7.01E+07		0.336
39	2.355	2.34	2.348	2.160	0.011	6.99E+07		0.334
50	2.32	2.38	2.350	2.163	0.042	6.99E+07		0.335
60	2.29	2.36	2.325	2.138	0.049	6.92E+07		0.330

Y294[AK(A)]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	2.06	1.98	2.020	1.500	0.057	6.00E+07		0.176
19	2.94	2.82	2.880	2.360	0.085	8.58E+07	1.058	0.373
22	3.4	3.4	3.400	2.880	0.000	1.01E+08	1.224	0.459
25	4.075	4.15	4.113	3.593	0.053	1.23E+08	1.414	0.555
29	4.95	4.95	4.950	4.430	0.000	1.48E+08	1.599	0.646
33	6.44	6.32	6.380	5.860	0.085	1.91E+08	1.853	0.768
37	7.84	8.08	7.960	7.440	0.170	2.38E+08	2.074	0.872
42	11.12	10.64	10.880	10.360	0.339	3.26E+08	2.387	1.015
47.5	14.1	13.8	13.950	13.430	0.212	4.18E+08		1.128
54.5	15.9	15.5	15.700	15.180	0.283	4.70E+08		1.181

65.5	17.1	16.3	16.700	16.180	0.566	5.00E+08		1.209

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.835	1.865	1.850	1.500	0.021	5.49E+07		0.176
2	2.07	2.08	2.075	1.725	0.007	6.17E+07		0.237
4	2.165	2.195	2.180	1.830	0.021	6.48E+07		0.262
6	2.24	2.265	2.253	1.903	0.018	6.70E+07		0.279
9.25	2.41	2.415	2.413	2.063	0.004	7.18E+07		0.314
12	2.48	2.505	2.493	2.143	0.018	7.42E+07		0.331
15	2.59	2.63	2.610	2.260	0.028	7.77E+07	0.959	0.354
21	3.215	3.315	3.265	2.915	0.071	9.74E+07	1.183	0.465
27.5	5.42	5.38	5.400	5.050	0.028	1.61E+08	1.686	0.703
34	8.93	8.95	8.940	8.590	0.014	2.68E+08	2.191	0.934
36	9.85	10.35	10.100	9.750	0.354	3.02E+08	2.313	0.989
39	11.98	12.1	12.040	11.690	0.085	3.61E+08	2.488	1.068
41	13.48	13.37	13.425	13.075	0.078	4.02E+08	2.597	1.116
50.75	17.28	18.04	17.660	17.310	0.537	5.29E+08		1.238
64	17.4	18.4	17.900	17.550	0.707	5.36E+08		1.244

Y294[CWI(B)]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.42	1.5	1.460	1.500	0.057	4.32E+07		0.176
15	2.1	1.9	2.000	2.040	0.141	5.94E+07		0.310
20	2.1	1.98	2.040	2.080	0.085	6.06E+07		0.318
24	2	1.9	1.950	1.990	0.071	5.79E+07		0.299
28	2	1.95	1.975	2.015	0.035	5.87E+07		0.304
25	1.9	1.94	1.920	1.960	0.028	5.70E+07		0.292
40	2.1	1.9	2.000	2.040	0.141	5.94E+07		0.310
50	2.05	2	2.025	2.065	0.035	6.02E+07		0.315

Y294[SFI]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.5	1.51	1.505	1.500	0.007	4.46E+07		0.176
2	1.76	1.77	1.765	1.760	0.007	5.24E+07		0.246
4	2.43	2.46	2.445	2.440	0.021	7.28E+07		0.387
6	4.48	4.6	4.540	4.535	0.085	1.36E+08	1.513	0.657
7	5.85	6.1	5.975	5.970	0.177	1.79E+08	1.788	0.776
8	7.55	7.7	7.625	7.620	0.106	2.28E+08	2.031	0.882
9	8.85	9	8.925	8.920	0.106	2.67E+08	2.189	0.950
11	10.15	10.3	10.225	10.220	0.106	3.06E+08		1.009
13	10.2	10.25	10.225	10.220	0.035	3.06E+08		1.009
15	11.05	11	11.025	11.020	0.035	3.30E+08		1.042
17	11.5	11.6	11.550	11.545	0.071	3.46E+08		1.062
19.75	12.1	12	12.050	12.045	0.071	3.61E+08		1.081
22	11.8	12.3	12.050	12.045	0.354	3.61E+08		1.081
28	12.7	12.8	12.750	12.745	0.071	3.82E+08		1.105
33	12.7	12.8	12.750	12.745	0.071	3.82E+08		1.105

Y294[SFI(A)]

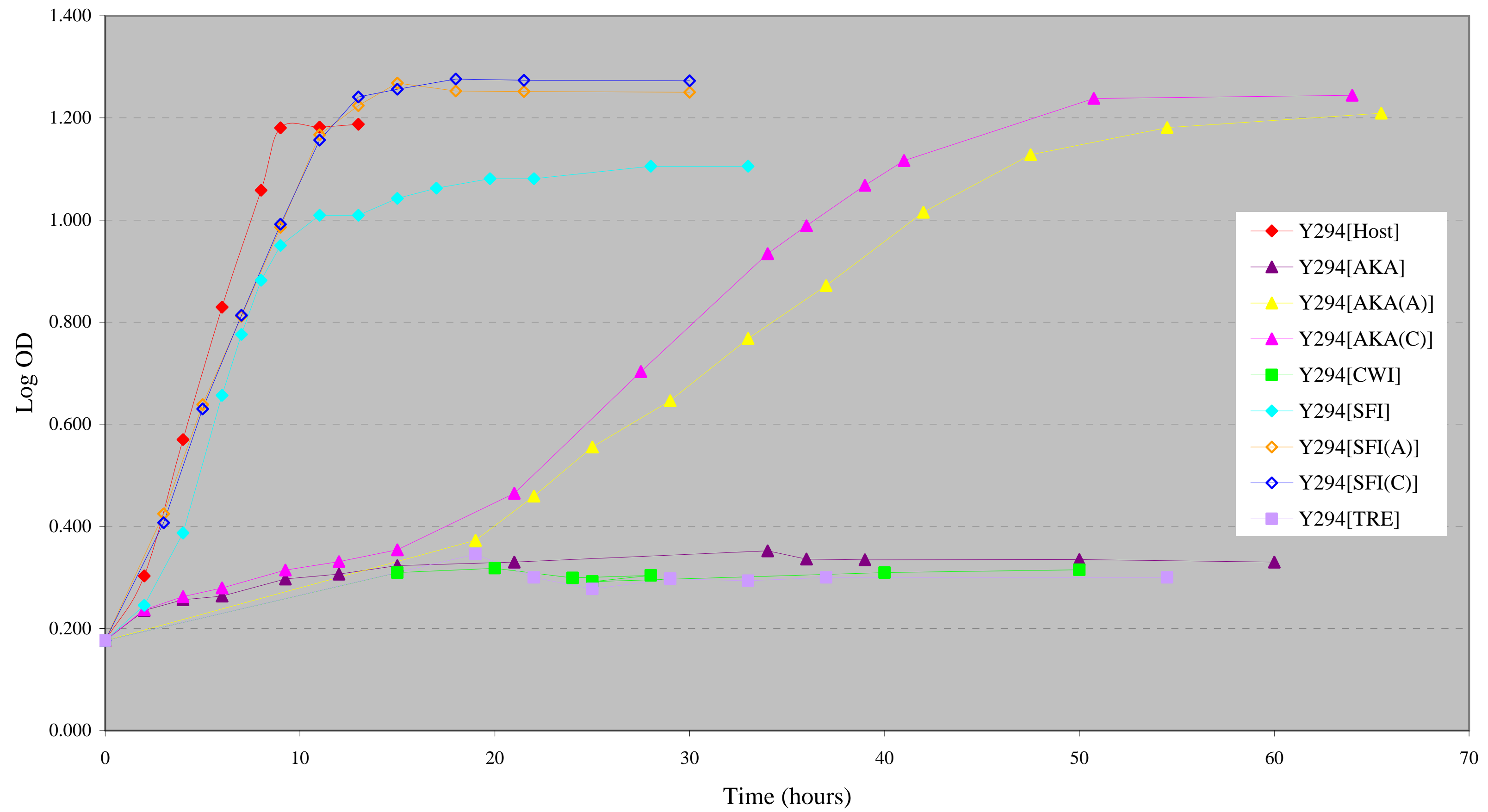
Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.75	1.75	1.750	1.500	0.000	5.19E+07		0.176
3	2.9	2.92	2.910	2.660	0.014	8.67E+07		0.425
5	4.6	4.6	4.600	4.350	0.000	1.37E+08	1.526	0.638
7	6.7	6.75	6.725	6.475	0.035	2.01E+08	1.906	0.811
9	9.9	9.95	9.925	9.675	0.035	2.97E+08	2.295	0.986
11	15	14.9	14.950	14.700	0.071	4.48E+08	2.705	1.167
13	17.1	16.9	17.000	16.750	0.141	5.09E+08		1.224
15	18.9	18.7	18.800	18.550	0.141	5.63E+08		1.268
18	18.2	18.1	18.150	17.900	0.071	5.44E+08		1.253
21.5	18	18.2	18.100	17.850	0.141	5.42E+08		1.252
30	18	18.1	18.050	17.800	0.071	5.41E+08		1.250

Y294[SFI(C)]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.8	1.83	1.815	1.500	0.021	5.39E+07		0.176
3	2.86	2.88	2.870	2.555	0.014	8.55E+07		0.407
5	4.56	4.6	4.580	4.265	0.028	1.37E+08	1.522	0.630
7	6.8	6.85	6.825	6.510	0.035	2.04E+08	1.921	0.814
9	10.1	10.15	10.125	9.810	0.035	3.03E+08	2.315	0.992
11	14.7	14.6	14.650	14.335	0.071	4.39E+08	2.684	1.156
13	17.7	17.8	17.750	17.435	0.071	5.32E+08		1.241
15	18.4	18.3	18.350	18.035	0.071	5.50E+08		1.256
18	19.3	19.1	19.200	18.885	0.141	5.75E+08		1.276
21.5	19.2	19	19.100	18.785	0.141	5.72E+08		1.274
30	19	19.1	19.050	18.735	0.071	5.71E+08		1.273

Y294[TRE]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.48	1.55	1.515	1.500	0.049	4.49E+07		0.176
19	2.34	2.12	2.230	2.215	0.156	6.63E+07		0.345
22	2.04	1.98	2.010	1.995	0.042	5.97E+07		0.300
25	1.92	1.9	1.910	1.895	0.014	5.67E+07		0.278
29	2	2	2.000	1.985	0.000	5.94E+07		0.298
33	1.98	1.98	1.980	1.965	0.000	5.88E+07		0.293
37	2.04	1.98	2.010	1.995	0.042	5.97E+07		0.300
54.5	2	2.02	2.010	1.995	0.014	5.97E+07		0.300


Aerobic growth of the recombinant strains
Log OD
Time (hours)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
Y294[Host]
Y294[AKA]
Y294[AKA(A)]
Y294[AKA(C)]
Y294[CWI]
Y294[SFI]
Y294[SFI(A)]
Y294[SFI(C)]
Y294[TRE]

Anaerobic fermentations on defined medium with 5 g.L-[1] carbon source (cellobiose/glucose)
*Each fermentation value represents an average of 3 OD values

Y294[Host]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.67	1.73	1.700	1.500	0.042	5.04E+07		0.176
2	1.83	1.9	1.865	1.665	0.049	5.54E+07		0.221
3.97	2.8	2.96	2.880	2.680	0.113	8.58E+07	1.058	0.428
5	3.7	3.7	3.700	3.500	0.000	1.10E+08	1.308	0.544

Y294[Host]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
6	4.72	4.74	4.730	4.530	0.014	1.41E+08	1.554	0.656
7	5.45	5.2	5.325	5.125	0.177	1.59E+08		0.710
8	5.25	5.25	5.250	5.050	0.000	1.57E+08		0.703

Y294[AKA(A)]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.62	1.6	1.610	1.500	0.014	4.77E+07		0.176
2	1.68	1.7	1.690	1.580	0.014	5.01E+07		0.199
5	1.65	1.68	1.665	1.555	0.021	4.94E+07		0.192
10	1.7	1.67	1.685	1.575	0.021	5.00E+07		0.197
15	1.6	1.65	1.625	1.515	0.035	4.82E+07		0.180

Y294[AKA(C)]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.55	1.58	1.565	1.500	0.021	4.64E+07		0.176
2	1.6	1.65	1.625	1.560	0.035	4.82E+07		0.193
5	1.62	1.6	1.610	1.545	0.014	4.77E+07		0.189
10	1.7	1.66	1.680	1.615	0.028	4.98E+07		0.208
15	1.65	1.64	1.645	1.580	0.007	4.88E+07		0.199

Y294[SFI]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.5	1.52	1.510	1.500	0.014	4.47E+07		0.176
2	1.7	1.69	1.695	1.685	0.007	5.03E+07		0.227
4	2.04	2.025	2.033	2.023	0.011	6.04E+07	0.709	0.306
6	3.06	3.18	3.120	3.110	0.085	9.30E+07	1.138	0.493
8	4.1	4.15	4.125	4.115	0.035	1.23E+08	1.417	0.614
9	4.75	4.8	4.775	4.765	0.035	1.43E+08	1.563	0.678
11	4.95	5.1	5.025	5.015	0.106	1.50E+08		0.700
13	4.95	5	4.975	4.965	0.035	1.49E+08		0.696
16	5	4.95	4.975	4.965	0.035	1.49E+08		0.696

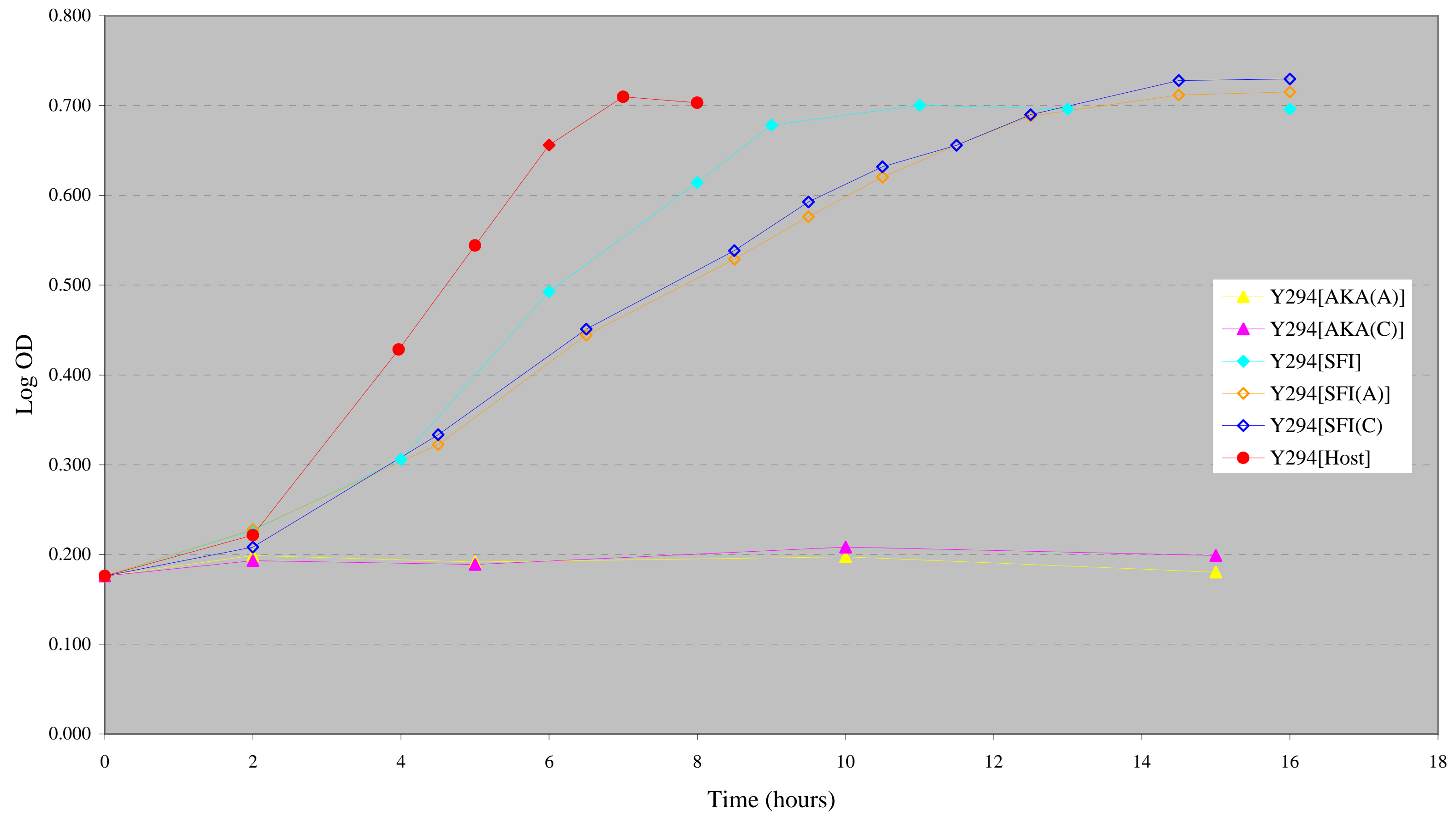
Y294[SFI(A)]

Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.72	1.72	1.720	1.500	0.000	5.10E+07		0.176
2	1.9	1.92	1.910	1.690	0.014	5.67E+07	0.647	0.228
4.5	2.31	2.33	2.320	2.100	0.014	6.90E+07	0.842	0.322
6.5	3	3	3.000	2.780	0.000	8.94E+07	1.099	0.444

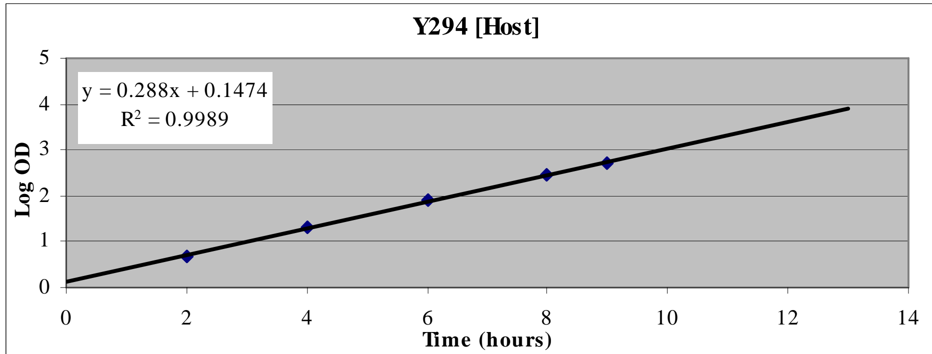
8.5	3.6	3.6	3.600	3.380	0.000	1.07E+08	1.281	0.529
9.5	4	3.98	3.990	3.770	0.014	1.19E+08	1.384	0.576
10.5	4.38	4.4	4.390	4.170	0.014	1.31E+08	1.479	0.620
11.5	4.76	4.74	4.750	4.530	0.014	1.42E+08	1.558	0.656
12.5	5.08	5.12	5.100	4.880	0.028	1.52E+08		0.688
14.5	5.36	5.38	5.370	5.150	0.014	1.61E+08		0.712
16	5.38	5.44	5.410	5.190	0.042	1.62E+08		0.715

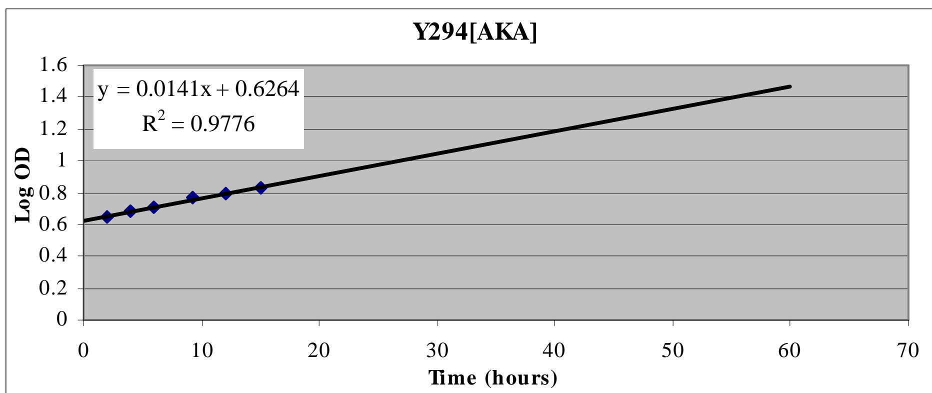
Y294[SFI(C)]

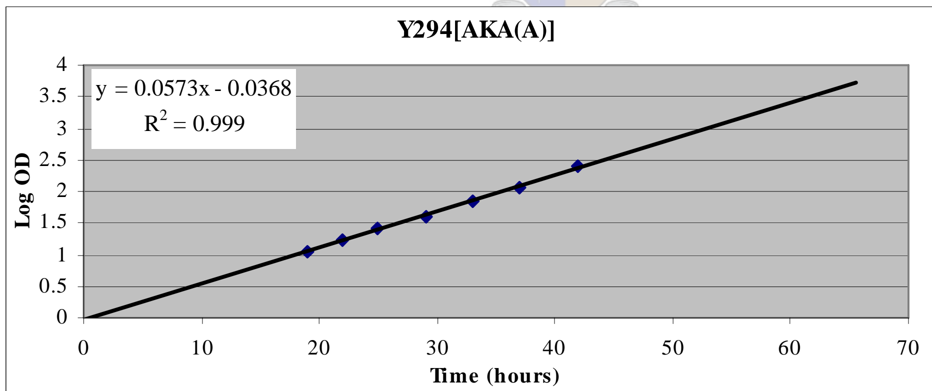
Time	*Ferm1	*Ferm2	Average	Standardised	Std.dev	Cells/ml	Growth Rate	Log OD
0	1.74	1.77	1.755	1.500	0.021	5.21E+07		0.176
2	1.86	1.88	1.870	1.615	0.014	5.55E+07	0.626	0.208
4.5	2.41	2.41	2.410	2.155	0.000	7.17E+07	0.880	0.333
6.5	3.08	3.08	3.080	2.825	0.000	9.18E+07	1.125	0.451
8.5	3.72	3.7	3.710	3.455	0.014	1.11E+08	1.311	0.538
9.5	4.16	4.18	4.170	3.915	0.014	1.25E+08	1.428	0.593
10.5	4.5	4.58	4.540	4.285	0.057	1.36E+08	1.513	0.632
11.5	4.78	4.78	4.780	4.525	0.000	1.43E+08	1.564	0.656
12.5	5.14	5.16	5.150	4.895	0.014	1.54E+08		0.690
14.5	5.6	5.6	5.600	5.345	0.000	1.67E+08		0.728
16	5.62	5.62	5.620	5.365	0.000	1.68E+08		0.730

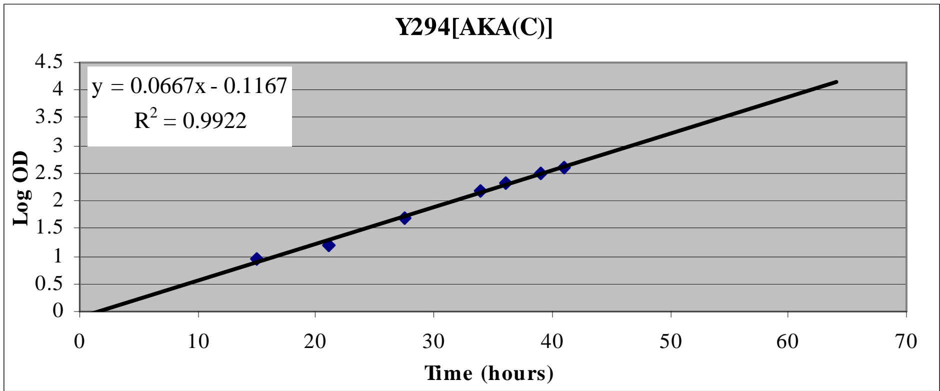

Anerobic growth of the recombinant strains
Log OD
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
2
4
6
8
10
12
14
16
18
Time (hours)
Y294[AKA(A)]
Y294[AKA(C)]
Y294[SFI]
Y294[SFI(A)]
Y294[SFI(C)
Y294[Host]

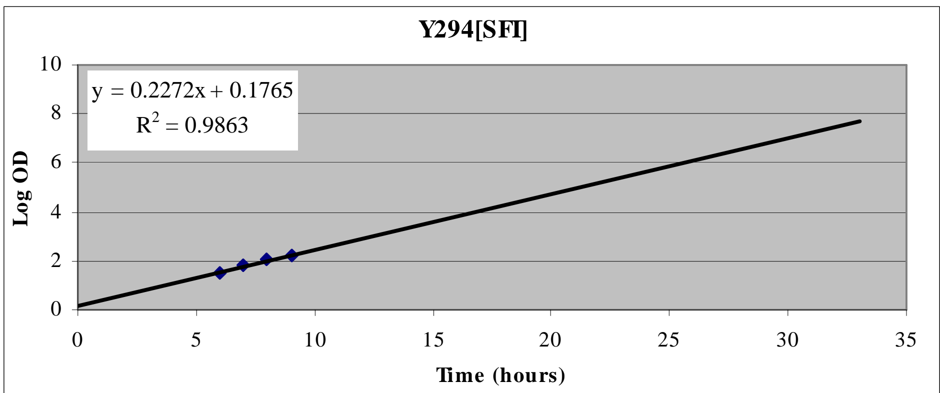
Maximum specific growth rate (μ_{max}) graphs of the recombinant strains during aerobic cultivation

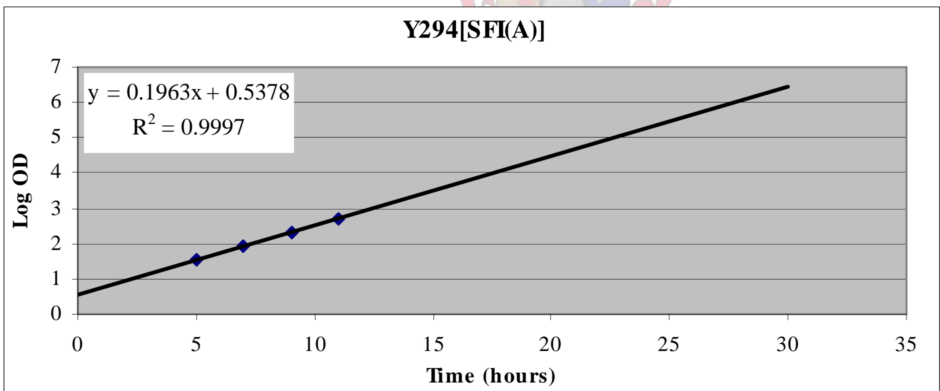


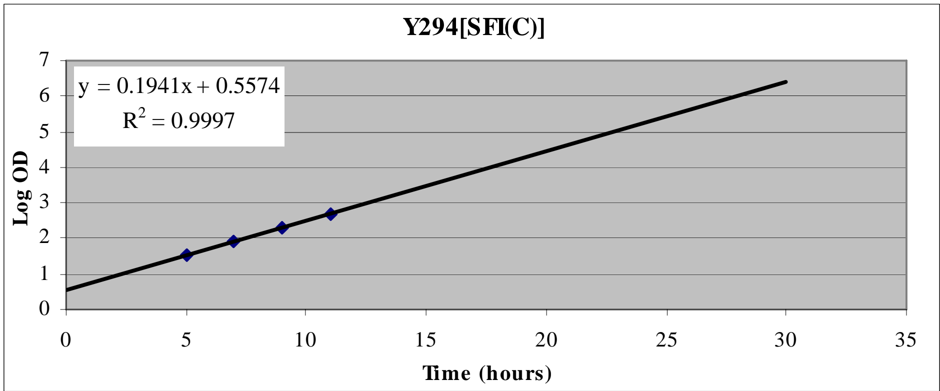




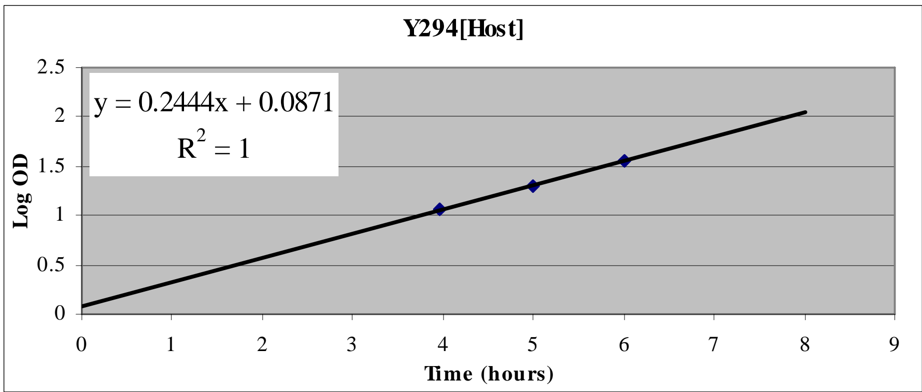

Y294[AKA(C)]
y = 0.0667x - 0.1167
R2 = 0.9922
Log OD
Time (hours)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70

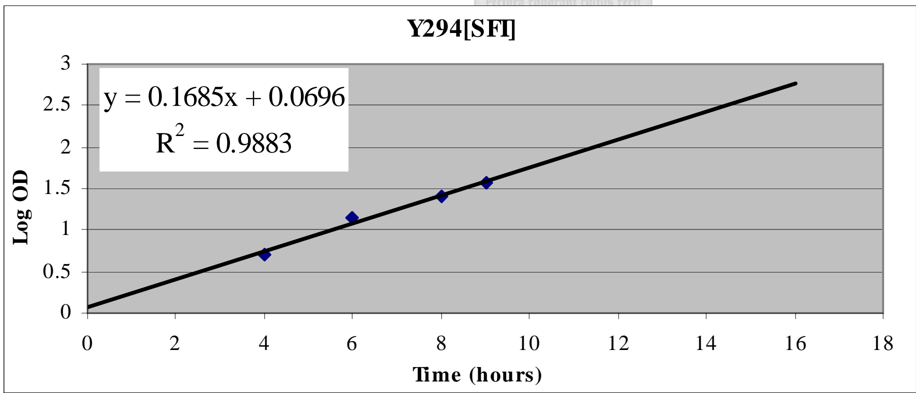

Y294[SFI]
y = 0.2272x + 0.1765
R2 = 0.9863
Log OD
Time (hours)
10
8
6
4
2
0
0
5
10
15
20
25
30
35

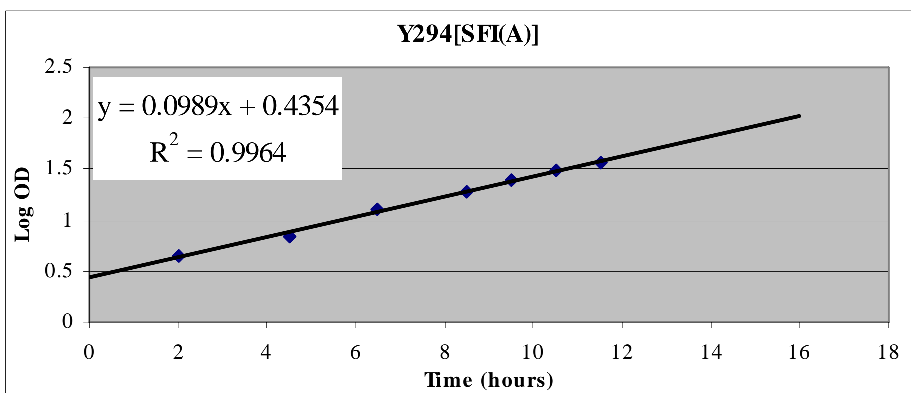

Y294[SFI(A)]
y = 0.1963x + 0.5378
R2 = 0.9997
Log OD
Time (hours)
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35

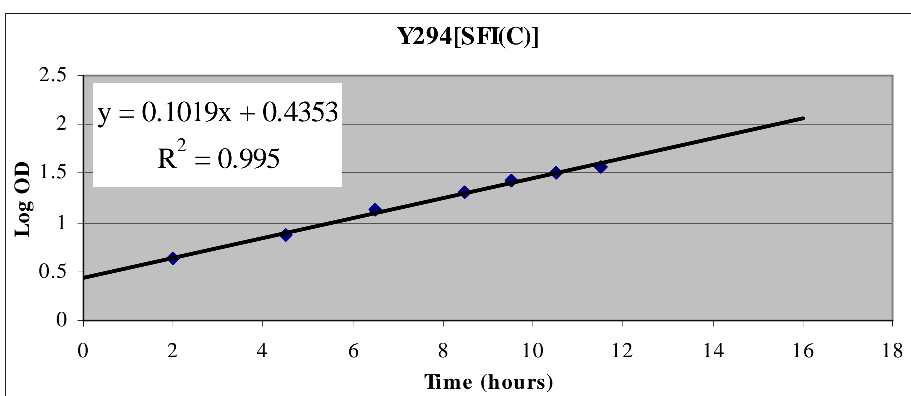


Maximum specific growth rate (μ_{max}) graphs of the recombinant strains during anaerobic cultivation









Pectora roborant cultus recti

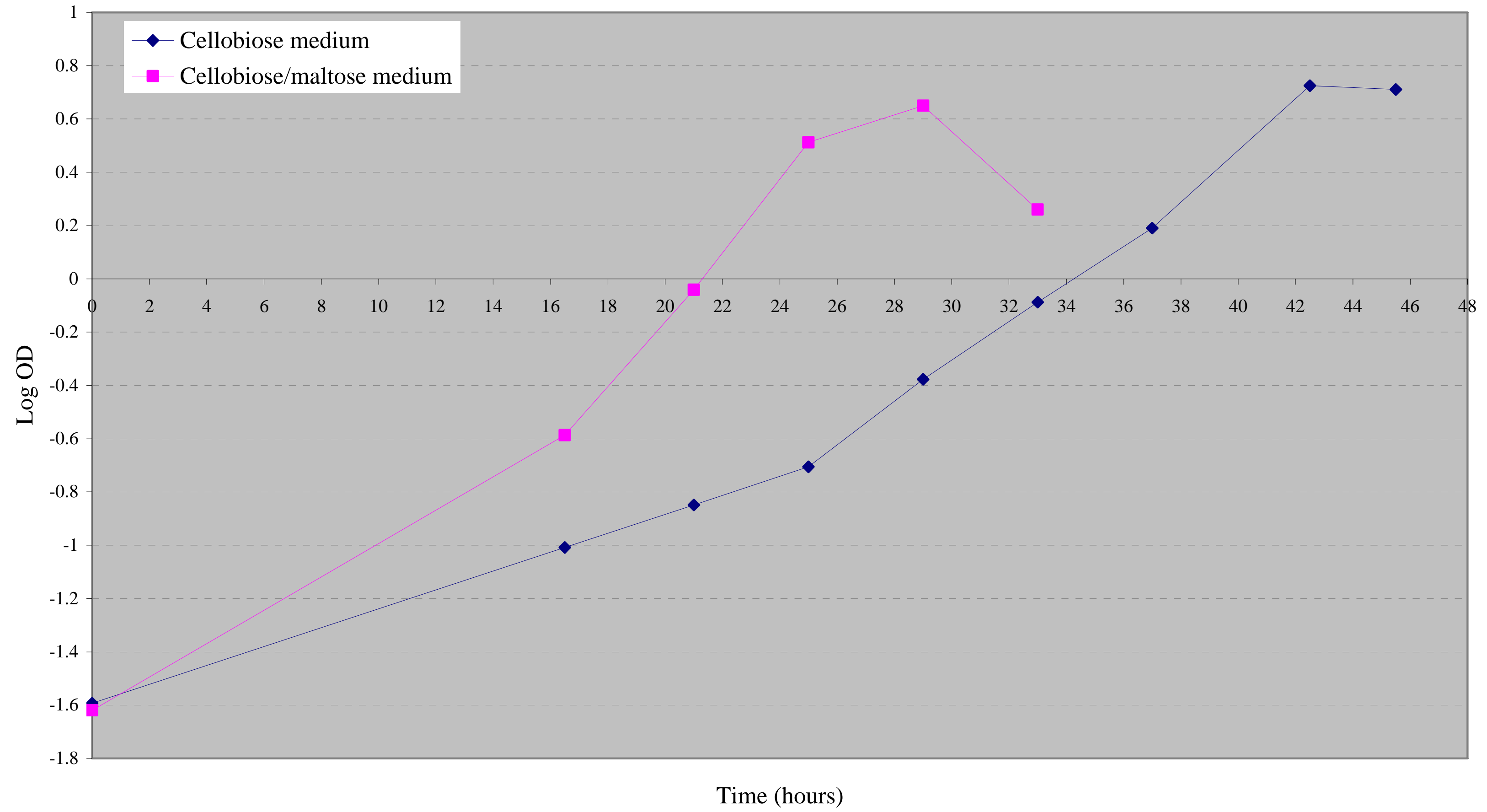
Fermentation results: Recombinant SIGMA strains expressing an intracellular β-glucosidase

SIGMA[SSFI] on 10 g.L-1 cellobiose

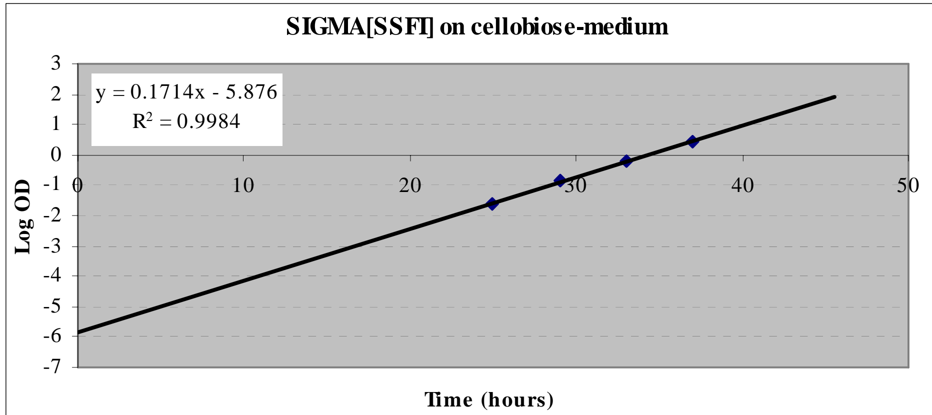
Time	Ferm1	Ferm2	Average	St.dev	Growth Rate	Log OD
0	0.027	0.024	0.026	0.002		-1.593
16.5	0.099	0.097	0.098	0.001		-1.009
21	0.141	0.142	0.142	0.001		-0.849
25	0.2	0.194	0.197	0.004	-1.625	-0.706
29	0.419	0.421	0.420	0.001	-0.868	-0.377
33	0.817	0.818	0.818	0.001	-0.202	-0.088
37	1.79	1.31	1.550	0.339	0.438	0.190
42.5	5.22	5.4	5.310	0.127		0.725
45.5	5.08	5.2	5.140	0.085		0.711

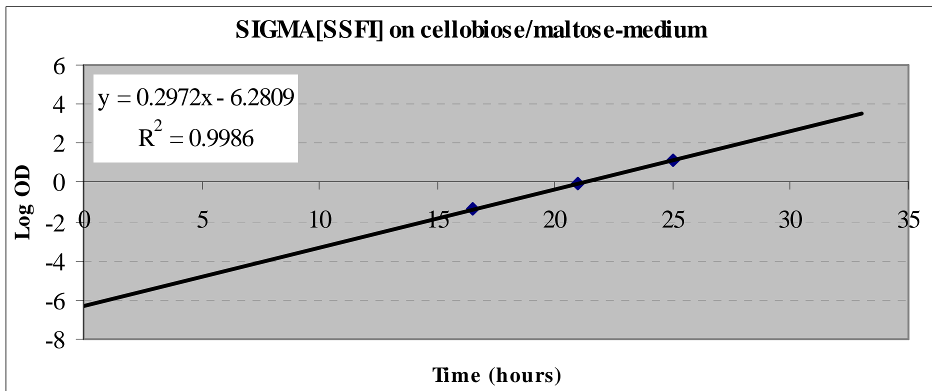
SIGMA[SSFI] on 7 g.L-1 cellobiose + 3 g.L-1 maltose

Time	Ferm1	Ferm2	Average	St.dev	Growth Rate	Log OD
0	0.024	0.024	0.024	0.000		-1.620
16.5	0.262	0.256	0.259	0.004	-1.351	-0.587
21	0.92	0.9	0.910	0.014	-0.094	-0.041
25	3.24	3.26	3.250	0.014	1.179	0.512
29	4.56	4.36	4.460	0.141		0.649
33	1.82	1.82	1.820	0.000		0.260


Aerobic growth of the recombinant SIGMA[SSFI] strain
Cellobiose medium
Cellobiose/maltose medium
Log OD
Time (hours)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.8
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48

Maximum specific growth rate (μ_{max}) graphs of the recombinant SIGMA strain during aerobic cultivation





Appendix C

High-performance Liquid Chromatography (HPLC) Results

HPLC results: Recombinant Y294 strains

Aerobic fermentations

Formulas:	Cellobiose	Glycerol	HAc	Ethanol	Biomass
	180	92	60	46	24.614

Y294[Host] - samples @ 10h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass
Sample 1	5.140	0.230	0.100	1.480	2.24
Sample 2	5.050	0.190	0.120	1.530	2.3
Sample 3	5.250	0.210	0.080	1.550	2.21
Average (g/L)	5.15	0.21	0.10	1.52	2.25
St.dev	0.10	0.02	0.02	0.04	0.05
g/g sugar consumed		0.04	0.02	0.30	0.44
cmole/L	0.17	0.01	0.00	0.07	0.09
Carbon balance	0.95				

Y294[SFI] - samples @ 10h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass
Sample 1	5.000	0.190	0.067	1.270	2.11
Sample 2	4.850	0.210	0.058	1.300	2.02
Sample 3	4.860	0.230	0.031	1.260	2.07
Average (g/L)	4.90	0.21	0.05	1.28	2.07
St.dev	0.08	0.02	0.02	0.02	0.05
g/g sugar consumed		0.04	0.01	0.26	0.42
cmole/L	0.16	0.01	0.00	0.06	0.08
Carbon balance	0.88				

Y294[SFI(A)] - samples @ 11h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass
Sample 1	4.960	0.189	0.340	0.456	2.9
Sample 2	4.880	0.166	0.400	0.418	2.96
Sample 3	4.860	0.142	0.370	0.380	2.91
Average (g/L)	4.90	0.17	0.37	0.42	2.92
St.dev	0.05	0.02	0.03	0.04	0.03
g/g sugar consumed		0.03	0.08	0.09	0.60
cmole/L	0.16	0.01	0.01	0.02	0.11
Carbon balance	0.91				

Y294[SFI(C)] - samples @ 11h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass
Sample 1	5.110	0.148	0.154	0.662	3.119
Sample 2	5.180	0.114	0.139	0.598	3.15
Sample 3	5.320	0.112	0.162	0.622	3.18
Average (g/L)	5.203	0.125	0.152	0.627	3.150
St.dev	0.107	0.020	0.012	0.032	0.031

g/g sugar consumed		0.02	0.03	0.12	0.61	
cmole/L	0.17	0.00	0.01	0.03	0.12	
Carbon balance	0.91					

Anerobic fermentations

Formulas:	Cellobiose	Glycerol	HAc	Ethanol	Biomass
	180	92	60	46	24.614

Y294[Host] - samples @ 10h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass	CO2
Sample 1	5.210	0.487	0.005	2.621	0.625	
Sample 2	5.270	0.483	0.004	2.647	0.634	
Sample 3	5.110	0.461	0.004	2.550	0.588	
Average (g/L)	5.197	0.477	0.004	2.606	0.616	
St.dev	0.081	0.014	0.001	0.050	0.024	
g/g sugar consumed		0.08	0.00	0.46	0.11	
cmole/L	0.17	0.02	0.00	0.11	0.02	0.06
Carbon balance	1.21					

Y294[SFI] - samples @ 10h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass	CO2
Sample 1	4.870	0.260	0.111	1.985	0.569	
Sample 2	4.810	0.251	0.092	1.949	0.601	
Sample 3	4.870	0.215	0.103	2.010	0.636	
Average (g/L)	4.850	0.242	0.102	1.981	0.602	
St.dev	0.035	0.024	0.010	0.031	0.034	
g/g sugar consumed		0.05	0.02	0.41	0.12	
cmole/L	0.16	0.01	0.00	0.09	0.02	0.04
Carbon balance	1.02					

Y294[SFI(A)] - samples @ 11h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass	CO2
Sample 1	5.090	0.291	0.040	2.101	0.65	
Sample 2	4.950	0.268	0.042	2.090	0.64	
Sample 3	4.810	0.227	0.041	2.061	0.665	
Average (g/L)	4.950	0.262	0.041	2.084	0.652	
St.dev	0.140	0.032	0.001	0.021	0.013	
g/g sugar consumed		0.05	0.01	0.42	0.13	
cmole/L	0.17	0.01	0.00	0.09	0.03	0.05
Carbon balance	1.04					

Y294[SFI(C)] - samples @ 11h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass	CO2
Sample 1	5.230	0.423	0.054	2.020	0.728	
Sample 2	5.139	0.419	0.062	2.066	0.709	
Sample 3	5.080	0.420	0.048	1.972	0.722	
Average (g/L)	5.150	0.421	0.055	2.019	0.720	
St.dev	0.076	0.002	0.007	0.047	0.010	

Y294[SFI(C)] - samples @ 11h minus 0h

Time	Cellobiose	Glycerol	HAc	Ethanol	Biomass	CO2
g/g sugar consumed		0.08	0.01	0.39	0.14	
cmole/L	0.17	0.01	0.00	0.09	0.03	0.04
Carbon balance	1.02					

HPLC results: Recombinant SIGMA[SSFI] strain

SIGMA[SSFI] on cellobiose-medium

Time	Cellobiose (g.L^{-1})		Average	St.dev
0	10.05	9.90	9.975	0.106
16.5	9.95	9.89	9.920	0.042
21	9.67	9.55	9.610	0.085
25	9.33	9.23	9.280	0.071
29	9.10	8.90	9.000	0.141
33	8.40	8.26	8.330	0.099
37	7.59	7.50	7.545	0.064
42.5	4.37	4.28	4.325	0.064
45.5	0.98	0.89	0.935	0.064

SIGMA[SSFI] on cellobiose/maltose-medium

Time	Cellobiose/Maltose (g.L^{-1})		Average	St.dev
0	9.98	9.85	9.915	0.092
16.5	8.88	8.81	8.845	0.049
21	6.79	6.67	6.730	0.085
25	4.84	4.71	4.775	0.092
29	1.41	1.29	1.350	0.085
33	0.04	0.00	0.020	0.028
37	0.04	0.00	0.020	0.028