Genetic engineering of *Saccharomyces cerevisiae* for efficient polysaccharide utilisation

by

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DECLARATION

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

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SUMMARY

Biomass is the sole foreseeable sustainable source of organic fuels, chemicals and materials. It is a rich and renewable energy source, which is abundant and readily available. Primary factors motivating the use of renewable enrgy sources include the growing concern over global climate change and the drastic depletion of non-renewable resources. Among various forms of biomass, cellulosic feedstocks have the greatest potential for energy production from.

The biggest technological obstacle to large-scale utilisation of cellulosic feedstocks for the production of bioethanol as a cost-effective alternative to fossil fuels is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass and fermentation of resulting sugars to ethanol in a single process step via a single microorganism or consortium. Such “consolidated bioprocessing” (CBP) offers very large cost reductions if microorganisms, such as the yeast *Saccharomyces cerevisiae*, can be developed that possess the required combination of efficient cellulose utilisation and high ethanol yields.

Cellulose degradation in nature occurs in concert with a large group of bacteria and fungi. Cellulolytic microorganisms produce a battery of enzyme systems called cellulases. Most cellulases have a conserved tripartite structure with a large catalytic core domain linked by an *O*-glycosylated peptide to a cellulose-binding domain (CBD) that is required for the interaction with crystalline cellulose. The CBD plays a fundamental role in cellulose hydrolysis by mediating the binding of the cellulases to the substrate. This reduces the dilution effect of the enzyme at the substrate surface, possibly by helping to loosen individual cellulose chains from the cellulose surface prior to hydrolysis. Most information on the role of CBDs has been obtained from their removal, domain exchange, site-directed mutagenesis or the artificial addition of the CBD. It thus seems that the CBDs are interchangeable to a certain degree, but much more data are needed on different catalytic domain-CBD combinations to elucidate the exact functional role of the CBDs. In addition, the shortening, lengthening or deletion of the linker region between the CBD and the catalytic domain also affects the enzymatic activity of different cellulases.

Enzymes such as the *S. cerevisiae* exoglucanases, namely EXG1 and SSG1, and the *Saccharomycopsis fibuligera* β-glucosidase (BGL1) do not exhibit the same architectural domain organisation as shown by most of the other fungal or bacterial cellulases. EXG1 and SSG1 display β-1,3-exoglucanase activities as their major activity and exhibit a significant β-1,4-exoglucanase side activity on disaccharide substrates such as cellobiose, releasing a

free glucose moiety. The BGL1 enzyme, on the other hand, displays β-1,4-exoglucanase activity on disaccharides.

In this study, the domain engineering of EXG1, SSG1 and BGL1 was performed to link these enzymes to the CBD2 domain of the *Trichoderma reesei* CBHII cellobiohydrolase to investigate whether the CBD would be able to modulate these non-cellulolytic domains to function in cellulose hydrolysis. The engineered enzymes were constructed to display different modular organisations with the CBD, either at the N terminus or the C terminus, in single or double copy, with or without the synthetic linker peptide, to mimic the multi-domain organisation displayed by cellulases from other microorganisms. The organisation of the CBD in these recombinant enzymes resulted in enhanced substrate affinity, molecular flexibility and synergistic activity thereby improving their ability to act and hydrolyse cellulosic substrates, as characterised by adsorption, kinetics, thermostability and scanning electron microscopic (SEM) analysis.

The chimeric enzyme of CBD2-BGL1 was also used as a reporter system for the development and efficient screening of mutagenised *S. cerevisiae* strains that overexpress CBD-associated enzymes such as *T. reesei* cellobiohydrolase (CBH2). A mutant strain WM91 was isolated showing up to 3-fold more cellobiohydrolase activity than that of the parent strain. The increase in the enzyme activity in the mutant strain was found to be associated with the increase in the mRNA expression levels. The CBH2 enzyme purified from the mutant strain did not show a significant difference in its characteristic properties in comparison to that of the parent strain.

In summary, this research has paved the way for the improvement of the efficiency of the endogenous glucanases of *S. cerevisiae*, and the expression of heterologous cellulases in a hypersecreting mutant of *S. cerevisiae*. However, this work does not claim to advance the field closer to the goal of one-step cellulose processing in the sense of technological enablement; rather, its significance hinges on the fact that this study has resulted in progress towards laying the foundation for the possible development of efficient cellulolytic *S. cerevisiae* strains that could eventually be optimised for the one-step bioconversion of cellulosic materials to bioethanol.

OPSOMMING

Biomassa is die enigste voorsienbare volhoubare bron van organiese brandstof, chemikalieë en materiale. Dit is 'n ryk en hernubare energiebron wat volop en geredelik bekombaar is. Die primêre faktore wat die gebruik van hernubare energiebronne motiveer, sluit in die toenemende kommer oor die globale klimaatsveranderinge en die drastiese uitputting van nie-hernubare bronne. Die vorm van biomassa met die grootste potensiaal vir energieproduksie is substrate ryk in sellulose.

Die grootste tegnologiese hindernis vir die grootskaalse gebruik van sellulose-ryke substrate vir die produksie van bioetanol as 'n koste-effektiewe alternatief vir olie- en steenkool-gebaseerde brandstof is die algemene afwesigheid van laekoste-tegnologie om die weerspannigheid van sellulose-ryke biomassa te oorkom. 'n Belowende strategie om hierdie hindernis te oorkom behels die produksie van sellulotiese ensieme, die hidrolise van biomassa en die gisting van die gevolglike suikers na etanol in 'n enkel-stap proses via 'n enkele mikro-organisme of konsortium. So 'n "gekonsolideerde bioprosessering" ("consolidated bioprocessing (CBP)") bied baie groot verminderings in koste indien mikro-organismes, soos die gis *Saccharomyces cerevisiae*, ontwikkel kan word wat die vereiste kombinasie van doeltreffende sellulose-verbruik en hoë etanolopbrengste bevat.

Die afbreking van sellulose in die natuur vind in samewerking met 'n groot groep bakterieë en swamme plaas. Sellulotiese mikro-organismes produseer 'n groot aantal ensiemsisteme wat as sellulases bekend staan. Die meerderheid sellulases besit 'n gekonserveerde driedelige struktuur met 'n groot katalitiese kerndomein wat deur 'n *O*-glikosileerde peptied gekoppel word aan 'n sellulosebindingsdomein (SBD) wat vir die interaksie met kristallyne sellulose benodig word. Die SBD speel 'n fundamentele rol in die hidrolise van sellulose deur die binding van die sellulases aan die substraat te bemiddel. Dít verminder die verdunningseffek van die ensiem by die substraatoppervlak, moontlik deur behulpsaam te wees met die losmaking van individuele sellulose-kettings van die sellulose-oppervlak vóór hidrolise plaasvind. Die meeste inligting oor die rol van SBD's is verkry deur die verwydering, domein-uitreiling, ligginggerigte mutagenese of die kunsmatige byvoeging van die SBD. Dit blyk dus dat die SBD's in 'n mate uitruilbaar is, hoewel baie meer data oor verskillende katalitiese domein-SBD kombinasies benodig word om die spesifieke funksionele rol van die SBD's te verklaar. Boonop beïnvloed die verkorting, verlenging of verwydering van die koppelgebied tussen die SBD en die katalitiese domein ook die ensiematiese aktiwiteit van verskillende sellulases.

Ensieme soos die *S. cerevisiae*-eksoglukanases, naamlik EXG1 en SSG1, en die *Saccharomycopsis fibuligera* β-glukosidase (BGL1) vertoon nie dieselfde argitektoniese domeinorganisasie as wat deur die meerderheid ander fungus- of bakteriële sellulases

vertoon word nie. EXG1 en SSG1 vertoon β-1,3-eksoglukanase-aktiwiteit as hulle vernaamste aktiwiteit en vertoon 'n noemenswaardige β-1,4-eksoglukanase newe-effek op disakkariedsubstrate soos sellobiose, met die vrystelling van 'n vry glukose gedeelte. In teenstelling vertoon die BGL1-ensiem β-1,4-eksoglukanase-aktiwiteit op disakkariede suikers.

In hierdie studie is domeinmanipulasie op EXG1, SSG1 en BGL1 uitgevoer om hierdie ensieme aan die CBD2-domein van die *Trichoderma reesei* CBHII-sellobiohidrolase te koppel in 'n poging om vas te stel of die SBD hierdie nie-sellulolitiese domeine sou kon moduleer om in sellulose-hidrolise te funksioneer. Die gemanipuleerde ensieme is gekonstrueer om verskillende modulêre rangskikkings met die SBD te vertoon – óf by die N-terminus óf die C-terminus, in dubbel- of enkelkopie, en met of sonder die sintetiese koppelpeptied, om sodoende die multidomein-organisering wat deur sellulases afkomstig van ander mikro-organismes vertoon word, na te boots. Die organisasie van die SBD in hierdie rekombinante ensieme het verhoogde substraataffiniteit, molekulêre buigsaamheid en sinergistiese aktiwiteit tot gevolg gehad en het daardeur hulle vermoë om op sellulose-ryke substrate te werk en hulle te hidroliseer, verbeter, soos gekarakteriseer deur adsorpsie-, kinetiese, termostabliteits- en skanderingselektronmikroskopie (SEM) analises.

Die chimeriese ensiem van CBD2-BGL1 is ook gebruik as 'n verklikkersisteem vir die ontwikkeling en doeltreffende sifting van mutageniseerde *S. cerevisiae*-rasse wat SBD-verwante ensieme soos *T. reesei* sellobiohidrolase (CBH2) ooruitdruk. 'n Mutante ras, WM91, is geïsoleer wat tot drievoud meer sellobiohidrolase-aktiwiteit as die ouer-ras vertoon het. Die verhoging in die ensiemaktiwiteit in die mutantras is gewys om met die verhoging in die mRNA-uitdrukkingsvlakke geassosieer te wees. Die CBH2-ensiem wat uit die mutantras gesuiwer is, het nie 'n noemenswaardige verskil in kenmerkende eienskappe in vergelyking met die ouer-ras getoon nie.

Opsommend kan gesê word dat hierdie navorsing die weg baan vir die verbetering van die doeltreffendheid van die endogene glukanases van *S. cerevisiae*, en die uitdrukking van heteroloë sellulases in mutantrasse van *S. cerevisiae* met verhoogde uitskeidingsvermoëns. Hierdie werk maak egter geen aanspraak daarop dat dit die veld nader bring aan die doel van een-stap selluloseverwerking in die sin van tegnologiese in staat stelling nie; die betekenisvolheid daarvan hou eerder verband met die feit dat hierdie studie gelei het tot vooruitgang in die verskaffing van 'n fondament vir die moontlike ontwikkeling van doeltreffende sellulotiese *S. cerevisiae*-rasse wat uiteindelik geoptimaliseer kan word vir die een-stap bio-omskakeling van sellulose-ryke materiale na bioetanol.

"Maatru Devo Bhava, Pitru Devo Bhava, Acharya Devo Bhava"

"Mother Is Divine, Father Is Divine, Teacher Is Divine”

ज्ञानं ज्ञेयं परिज्ञाता त्रिविधः कर्मचोदना ।
करणं कर्म कर्तेति त्रिविधः कर्मसंग्रहः ॥१८॥

“jnanam jneyam parijnata tri-vidha: karma-codana
karanam karma karteti tri-vidhah: karma-sangrahah”

“Knowledge, the object of knowledge and the knower
are the three factors which motivate action;
the senses, the work and the doer
comprise the threefold basis of action."

- *Bhagavad Gita*

BIOGRAPHICAL SKETCH

Sarath Babu Gundllapalli was born in Kerala, India on 30 May 1977. He matriculated at the ICF Higher Secondary School, Chennai, India in May 1994. Sarath obtained his Bachelor's degree in Microbiology from Bharathidasan University, Tiruchirapalli, India in May 1997 and his Master's degree in Biotechnology from the same university in May 1999. On completion of his Master's degree, he joined the Institute for Wine Biotechnology at Stellenbosch University in 2001 to continue his studies towards a PhD.

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PREFACE

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal to which it was submitted for publication. Additional information can be found in the appendix.

Chapter 1 **INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**
Microbial cellulose utilisation &
Cellulose-binding domains: functions and applications

RESEARCH RESULTS

Chapter 3 Domain engineering of *Saccharomyces cerevisiae* exoglucanases

Chapter 4 Effect of the cellulose-binding domain on the catalytic activity of a β-glucosidase from *Saccharomycopsis fibuligera*

Chapter 5 Isolation of a *Saccharomyces cerevisiae* mutant over expressing *Trichoderma reesei* cellobiohydrolase II

Chapter 6 **GENERAL DISCUSSION AND CONCLUSION**

Appendix Different genetic backgrounds influence the secretory expression of the *LKA1*-encoded *Lipomyces kononenkoae* α-amylase in industrial strains of *Saccharomyces cerevisiae*

CONTENTS

CHAPTER 1. INTRODUCTION AND PROJECT AIMS

CHAPTER 2. MICROBIAL CELLULOSE UTILISATION & CELLULOSE-BINDING DOMAINS: FUNCTIONS AND APPLICATIONS

CHAPTER 3. DOMAIN ENGINEERING OF *SACCHAROMYCES CEREVISIAE* EXOGLUCANASES

CHAPTER 4. EFFECT OF THE CELLULOSE-BINDING DOMAIN ON THE CATALYTIC ACTIVITY OF THE β-GLUCOSIDASE FROM *SACCHAROMYCOPSIS FIBULIGERA*

CHAPTER 5. ISOLATION OF A *SACCHAROMYCES CEREVISIAE* MUTANT OVER EXPRESSING *TRICHODERMA REESEI* CELLOBIOHYDROLASE II

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

INTRODUCTION

Life on earth depends on photosynthesis, which results in the production of plant biomass. Biomass contains a variety of polysaccharides as structural or storage compounds. The amount and ease of availability make biomass a rich, renewable energy source (Sheehan and Himmel, 1999). About half of the carbonaceous compounds in terrestrial biomass are cellulose, which is the most prominent single organic compound on earth. Enzymes secreted by microorganisms mineralise almost all of the biomass produced. The carbon cycle is closed primarily as a result of the action of cellulose-degrading microorganisms present in the soil and in the guts of animals. Microbial cellulose utilisation is responsible for one of the largest material flows in the biosphere and is of interest in relation to the analysis of carbon flux on both local and global scales. It is also an integral component of widely-used processes, such as ensiling, anaerobic digestion and composting. Thus, polysaccharide hydrolysis is one of the most important enzymatic processes on earth.

Although cellulose is chemically homogeneous, no single enzyme is able to hydrolyse it, whereas soluble cellulose derivatives are easily degraded by a single endo-β-1,4-glucanase. The extensive level surface of the insoluble crystalline microfibrils is an unusually resilient substrate for hydrolytic enzymes. The crystalline material is only hydrolysed by a cluster of simultaneously present, interacting enzymes, or, alternatively, by a multi-enzyme complex. Only by cooperation with non-catalytic, specific binding modules are the enzymes able to disrupt the crystal surface at the solid-liquid interphase to make single cellulose fibres accessible for hydrolysis. However, insoluble cellulose is not a homogeneous crystal. Rather, it is a polymorphous, insoluble material, adding to the difficulty of binding to it. The investigation of the hydrolysis mechanisms of cellulolytic enzymes opens up a new way of looking at enzymatic activity: the duality between the mechanical and structural “preparation” of the insoluble (crystalline) substrate, followed by hydrolytic activity on the released molecule. The research on cellulolytic enzymes is aimed at the enzymatic mechanisms at the surface of the insoluble substrate. It also tries to solve the problems relating to the direct conversion of biomass into valuable products using isolated enzymes or cellulolytic microorganisms (Sheehan and Himmel, 1999). An artificial formulation of combined cellulolytic enzymes that works effectively at low concentrations is a goal not yet accomplished. To be cost effective in a commercial process, it would particularly have to speed up the step-by-step process into a consolidated processing step, namely breaking up the crystal surface and releasing single cellulose molecules to the subsequent free glucose molecules (Lynd *et al*., 2002).

Cellulolytic enzymes are important and unique enzymes that are capable of degrading crystalline cellulose to glucose. Various fungi and bacteria produce a variety of cellulolytic enzymes. These enzymes have an interesting conserved tripartite structure with a

large catalytic core domain, linked by a relatively long *O*-glycosylated linker peptide to a small cellulose-binding domain (CBD), which is required for the interaction with crystalline cellulose. The CBD is present either at the N terminus or at the C terminus of these enzymes. Although the exact function of this CBD is not known, it has been confidently suggested that it mediates the binding and stabilisation of the cellulolytic enzymes, thus playing a fundamental role in the hydrolysis of the complex cellulolytic substrate. The addition and deletion of the CBD from cellulolytic enzymes have previously been illustrated to significantly increase and decrease the activity on recalcitrant substrates.

Although more than 600 cellulase genes are known, a detailed biochemical characterisation has been undertaken for only a very limited number of endo- and exoglucanases. Recent nomenclature allows the unequivocal description of enzymes composed of various modules and simplifies comparisons between them (Henrissat *et al*., 1998).

The last decade has seen marked advances in the depth and breadth of the scientific understanding of the structure, function and genetics associated with the components of cellulase systems. Such advances include solving the 3-D structures of over two dozen cellulolytic enzymes, leading to a much better understanding of the reaction mechanisms; the availability of many new protein sequences (300 in 1990, over 5 000 in 2001), meaningful new classification schemes based on structural features; and a better understanding of the regulation of cellulase genes, especially in fungal systems. Significant progress has also been made since 1990 with respect to understanding interactions among cellulase components. This includes a better understanding of synergistic interactions for an increasing number of non-complexed cellulase systems, as well as a better and broader understanding of the structure and composition of cellulosomes. Understanding cellulose hydrolysis as a microbial phenomenon builds on the foundation of knowledge pertaining to cellulose hydrolysis at a sub-cellular and cellular level. In many cases, important tools for understanding microbial cellulose utilisation have become available only recently or have not yet been developed. Such tools include systems that allow foreign genes to be expressed in cellulolytic microorganisms, which are established for the aerobic *T. reesei* (Penttila, 1998), but not for most cellulolytic anaerobes. Methods to independently quantify cells and cellulase can be expected to result in a second set of new insights, particularly in the areas of bioenergetics, metabolic control and kinetics.

Studies in which heterologous cellulase expression confers the ability to grow on non-native substrates have begun to appear only in the last few years and represent an exciting frontier with the potential to become an important tool for fundamentally-oriented investigations, while also being relevant to applied goals. The substantial potential of quantitative analysis to contribute to our understanding of cellulose hydrolysis at both the enzymatic and cellular levels has been realised only to a very limited extent to date. These

measures can be expected to shed light on several fundamental issues of considerable interest about which there is currently substantial uncertainty. The molecular diversity of cellulose enzyme systems is logically sought in an understanding of the niches and adaptive strategies of the microorganisms in which these systems evolved. Conversely, the results of molecular studies substantially enhance the depth and clarity with which the adaptive strategies of cellulolytic microorganisms can be understood.

Several factors motivate the development of microorganisms possessing the properties required for the cost-effective implementation of consolidated bioprocessing (CBP) in an industrial setting. The primary savings anticipated for CBP in comparison to other described process configurations featuring enzymatic hydrolysis result from the elimination of costs associated with a process step dedicated to cellulose production. Other benefits that might be realized include higher product yields, higher rates, and the improved stability of cultures and strains. The often underestimated diversity of yeast species encompasses organisms with a broad range of properties that differ from *S. cerevisiae* and could be useful for CBP. However, *S. cerevisiae* has received the most attention with respect to heterologous cellulase expression as well as the production of ethanol and other commodity products. Although various attempts have so far been made to develop *S. cerevisiae* as a potential candidate organism for the consolidated bioprocessing of cellulosic substrates, the attempts have been seriously mulled by the failure to express the cellulolytic enzymes at an economical scale. This is a result of the poor secretion, hyperglycosylation and non-functionality of these cellulolytic enzymes in the yeast system.

AIMS AND APPROACHES

In the light of all the developments achieved to date, it is reasonable to pursue the expression of cellulase in *S. cerevisiae* at levels sufficient to enable growth on crystalline cellulose, as required for CBP. At the same time, achieving elevated secretion levels in this organism has to date often been a hit-or-miss proposition without a strong mechanistic basis. The integrated advancement of the fundamentals of the cellulolytic enzymes, along with the investigation of strategies to increase secretion levels, is likely to be a particularly fruitful direction for future research in the context of organism development for CBP. Thus, the fundamental objective of this project was to develop *S. cerevisiae* as a potential candidate organism for CBP. CBP requires a microbial culture that combines properties related to both substrate utilisation and product formation.

The following aims were formulated towards achieving this goal

1. The development and characterisation of recombinant enzymes with the ability to hydrolyse cellulosic substrates.

2. The development of a *S. cerevisiae*-over-expressing mutant for the efficient production of naturally available cellulolytic enzymes.

The project involved three approaches, which are set out in different chapters.

I. Engineering of yeast exoglucanase enzymes to mimic the organisational structure of cellulolytic enzymes in nature

As fungi, *S. cerevisiae* exoglucanases, such as EXG1, EXG2 and SSG1, do not possess the organisational structure shown by other fungal or bacterial cellulolytic enzymes. Instead, these enzymes display β-1,3-exoglucanase activities and they also have been shown to possess a residual activity of β-1,4-exoglucanase on disaccharide substrates such as cellobiose to release a free glucose moieties. Mentioning this, the aim is to modify these *S. cerevisiae* exoglucanases to mimic the organisational structure displayed by cellulolytic enzymes in nature. Domain engineering was performed to construct a library of chimeric molecules linking the catalytic domains of the *S. cerevisiae* exoglucanases, EXG1 and SSG1, to the cellulose-binding domain (CBD2) of *Trichoderma reesei* cellobiohydrolase CBH2, with and without the synthetic linker molecule. It was hypothesised that the modification might change the original activity of these enzymes similar to β-1,4-exoglucanases or that it might show an improved or extended residual activity of β-1,4-exoglucanases on higher polysaccharides.

II. Study the effect of the cellulose-binding domain on the enzymatic activity of the β-glucosidase enzyme

Enzyme engineering was performed to link the non-cellulolytic β-glucosidase domain from the *Saccharomycopsis fibuligera* BGL1 enzyme to the cellulose-binding domain (CBD2) of *T. reesei* cellobiohydrolase (CBH2) in an attempt to investigate whether the CBDs would be able to impart a wider range of specificity to this non-cellulolytic domain to function in cellulose hydrolysis.

III. Development of a mutant of *S. cerevisiae* over-expressing cellulolytic enzymes.

Numerous attempts have been made in the past to successfully express cellulolytic enzymes like endoglucanses and cellobiohydrolases in *S. cerevisiae*. At the same time, achieving elevated expression and secretion levels in this organism have thus far often been a hit-or-miss proposition without a strong mechanistic basis. The integrated advancement of the fundamental understanding of the cellulolytic enzymes, along with an investigation of strategies to increase expression and secretion levels, is likely to be a particularly fruitful direction for future research in the context of organism development for CBP. As a most ideal situation, in our present study we used the chimeric β-glucosidase enzyme fused to the

cellulose-binding domain (CBD2-BGL1) as an efficient and simple reporter system for the development of mutant yeast strains over-expressing CBD-associated enzymes such as *T. reesei* cellobiohydrolase (CBH2).

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CHAPTER 2

LITERATURE REVIEW

Microbial Cellulose Utilisation & Cellulose Binding Domains: Functions and Applications

The section, Cellulose-binding Domains: Functions and Applications, will shortly be submitted for possible publication in *Biotechnology Advances*

1. Microbial cellulose utilisation

1.1 Introduction

Plant biomass is the only foreseeable sustainable source of organic fuels, chemicals and materials, as well as food, available to humanity, and is also a non-exclusive sustainable source of electrical power and hydrogen. Primary factors motivating the use of bio-energy include that it is a sustainable resource supply (both responding to depletion of non-renewable resources as well as avoiding emissions of greenhouse gases), it enhances security, and there are macroeconomic benefits for rural communities and society at large. The use of biomass for energy production and utilisation has the greatest potential to positively address these factors, with displacement of oil a particularly critical need and opportunity in this context. Among the various forms of biomass, cellulosic feedstocks have the greatest potential relative to energy production and oil displacement.

A structural analysis of the paths by which sustainable resources can be converted to human needs reveals that biomass is the only foreseeable sustainable source of energy. Furthermore, a dimensional analysis suggests that the land required for biomass production expressed as a ratio of the land required to meet human needs in relation to the land that is available is in fact a much more dynamic quantity than is usually assumed. Indeed, this ratio can plausibly vary by several hundred folds when considering future scenarios. Agricultural productivity (for both biomass feedstocks and food), the population, and end-use efficiency (especially vehicular efficiency) are particularly important variables impacting on biomass resource availability. Taking these factors into consideration, it is quite possible to be optimistic that, with an aggressive and reasonably successful research effort together with societal choices that allow the realisation of some, although not all, of the opportunities to increase land availability, humankind can reasonably expect to meet all of its food, organic material and transportation energy needs from biomass, while co-producing substantial quantities of power and still leaving abundant land for nature (Review by Lynd et al., 2002).

The growing interest in biomass energy is the result of a combination of underlying factors, including:

i. rapid changes in the energy market worldwide, driven by privatisation, deregulation and decentralisation;
ii. greater recognition of the current role and future potential contribution of biomass as a modern energy carrier, combined with a general interest in other renewables and its availability, versatility and sustainability;
iii. better understanding of its global and local environmental benefits and perceived potential role in climate stabilisation;
iv. existing and potential development and entrepreneurial opportunities; and

v. technological advances and knowledge, which have recently evolved in many aspects of biomass energy and other sources of renewable energy (RE).

In addition, there are other, more specific factors that favour the development of biomass energy:

i. growing concern about global climate change, which may eventually drive a global policy on pollution abatement;
ii. growing recognition among established conventional institutions of the importance of biomass energy; and
iii. expected increases in energy demand, combined with current rapid growth of RE. The Global Environmental Facility (GEF) predicts that developing countries alone will need as much as five million MW of new electrical generation capacity in the next 40 years, most of which could be supplied by RE. Environmental pressures will increase the price of fossil fuels as the cheaper sources are depleted. Also, as the external costs are progressively incorporated into the final costs of energy, RE will be put onto a more equal footing with fossil fuels.

1.2 Cellulosic biomass

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g. tunicates) and a few bacteria. Despite great differences in composition and in the anatomical structure of cell walls across plant taxa, a high cellulose content – typically in the range of approximately 35 to 50% of plant dry weight – is a unifying feature (Lynd et al., 1999, Van Soest, 1994). In a few cases (notably cotton bolls), cellulose is present in a nearly pure state. In most cases, however, the cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30% of plant dry weight (Van Soest, 1994). Although these matrix interactions vary with plant cell type and with maturity (Wilson, 1993), they are a dominant structural feature limiting the rate and extent of utilisation of whole, untreated biomass materials.

Cellulose in native plant material shares many characteristics across plant taxa, including its potential for complete hydrolysis and utilisation under the proper microbial and environmental conditions. An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure. Cellulose is synthesised in nature as individual molecules (linear chains of glucosyl residues), which undergo self-assembly at the site of biosynthesis (Brown and Saxena, 2000). An important feature of the crystalline array is that the component molecules of individual microfibrils are packed sufficiently tightly to prevent penetration, not only by enzymes, but also by small molecules such as water. Although cellulose forms a distinct crystalline structure, cellulose fibres in nature are not

purely crystalline. The degree of departure from crystallinity is variable and has led to the notion of a “lateral order distribution” of crystallinity, which portrays a population of cellulose fibres in statistical terms as a continuum from purely crystalline to purely amorphous, with all degrees of order in between (Marchessault and Howsmon, 1957).

Notwithstanding its central position in ecological, agricultural and technological contexts, fundamental understanding of microbial cellulose utilisation is in many respects rudimentary. This is a result of the inherent complexity of microbial cellulose utilisation as well as the methodological challenges associated with its study. The understanding of cellulose hydrolysis can be approached at several levels of aggregation: components of cellulase enzyme systems, unfractionated cellulase complexes, pure cultures of cellulolytic microorganisms and mixtures of cellulolytic microorganisms. In general, our understanding is progressively less complete at more highly aggregated levels of study. Therefore, although much remains to be elucidated at the level of enzyme components and the underlying genetics of such components, our understanding of cellulose hydrolysis by unfractionated cellulase complexes is even less complete, our understanding of hydrolysis by pure cultures is even more limited, and hydrolysis in mixed cultures is understood least of all. There is a natural tendency for science to proceed over time toward finer levels of aggregation – e.g. from pathways to enzymes to genes – and this “reductionist” approach has yielded tremendous insights with respect to the life sciences generally and to cellulose hydrolysis in particular. An alternative, “integrative” approach, involving the development of an understanding of aggregated systems based on an understanding of their less aggregated components, is also a valid and important focus for scientific endeavour. With respect to cellulose hydrolysis, such integration is essential in order for research advances to be translated into advances in technological, ecological and agricultural domains (Review by Lynd et al., 2002).

1.3 Consolidated bioprocessing (CBP)

The dominant impact of the cost of substrate in the economics of biomass conversion emphasises high yields of products per unit substrate. From a metabolic perspective, this means that almost all of the reducing equivalents initially present in the substrate need to end up in the product of interest, rather than being transferred to oxygen or some other electron acceptor.

Four biologically-mediated events occur in the course of converting cellulosic biomass into fuels and chemicals in processes featuring enzymatic hydrolysis: 1) cellulase production, 2) hydrolysis of cellulose and other insoluble polysaccharides, 3) fermentation of soluble cellulose hydrolysis products, and 4) fermentation of soluble hemicellulose hydrolysis products. Alternative processing configurations can be categorised based on the degree to which these events are consolidated. Separate hydrolysis and fermentation (SHF) involves

four discrete process steps and as many as four different biocatalysts. Simultaneous saccharification and fermentation (SSF) consolidates hydrolysis and the fermentation of cellulose hydrolysis products into one process step, with cellulase production and fermentation of hemicellulose hydrolysis byproducts occurring in two additional, discrete process steps. Simultaneous saccharification and co-fermentation (SSCF) involves two process steps: cellulase production and a second step in which cellulose hydrolysis and the fermentation of both cellulose and hemicellulose hydrolysis products occurs. In consolidated bioprocessing (CBP), cellulase production, hydrolysis and the fermentation of the products of both cellulose and hemicellulose hydrolysis are accomplished in a single-step process (Lynd et al., 2002).

Biomass processing technology has exhibited a trend toward increasing consolidation over time. This trend in most evident in the case of studies on ethanol production, which has received the most attention among biomass-derived fermentation products, but is likely to be applicable to other products as well. Consolidation of all the processes involved in cellulose hydrolysis, as in CBP, involves the largest potential cost reduction, especially for dedicated cellulase production relative to current technology such as SSF. A detailed process design study also projected savings of this magnitude, with CBP reducing overall processing costs by ~two-fold and the cost of biologically-mediated process steps by ~eight-fold compared to a current SSF-based case. The CBP processing concept is a potential breakthrough in the low-cost processing of cellulosic biomass that is, in principle, applicable to the production of any fermentation-derived product. However, this potential cannot be realised by organisms available today, and will require the development of new and improved CBP-enabling organisms (extract from the review of Lynd et al., 2002)

1.4 Cellulase enzyme systems

Natural cellulosic substrates (primarily plant cell materials) are composed of heterogeneous, intertwined polysaccharide chains with varying degrees of crystallinity, hemicelluloses and pectins embedded in lignin. Microorganisms produce multiple enzymes to degrade plant cell materials, known as enzyme systems (Warren et al., 1996). It should also be realised that such systems are also active on hemicellulose and are commonly co-produced by cellulolytic microorganisms.

For microorganisms to hydrolyse and metabolise insoluble cellulose, extracellular cellulolytic enzymes must be produced that are either free (non-complex) or cell associated (complex). The biochemical analysis of cellulase systems from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during the past two decades (Bayer et al., 1994, 1998c; Beguin, 1990; Beguin et al., 1992; Bhat, 2000; Davies, 1998; Doi et al., 1998; Knowles et al., 1987; Felix and Ljungdahl, 1993; Leschine, 1995; Schulein, 2000; Shoham et al., 1999; Teeri, 1997; Tomme et al., 1995b; Warren, 1996; Wood and Garcia-

Campayo, 1994). The components of cellulase systems were first classified on the basis of their mode of catalytic action and have more recently been classified on the basis of structural properties (Henrissat et al., 1998).

Three major types of enzymatic activity are found: (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-β-D-glucan 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-glucoside glucohydrolases (EC 3.2.1.21). Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or non-reducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri, 1997). β-Glucosidases hydrolyse soluble cellodextrins and cellobiose to glucose (Fig. 1).

The following minimum set of components are required for complex cellulolytic enzymes: a scaffolding protein with a cellulose-binding domain, a cell wall-anchoring protein, at least two cohesions and a domain that binds to a cell wall-anchoring protein, at least one exoglucanase and an endoglucanase containing dockerin that is capable of binding to the scaffolding protein. In addition, either a β-glucosidase or cellodextrin and cellobiose phosphorylases together with the appropriate permeases would be required. Cellulolytic enzymes are distinguished from other glycoside hydrolases by their ability to hydrolyse β-1,4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1,4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and a nucleophile or base. The hydrolysis products can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end (Withers, 2001).

The insoluble, recalcitrant nature of cellulose represents a challenge for cellulase systems. A general feature of most cellulolytic enzymes is a modular structure, often including both catalytic and carbohydrate-binding modules (CBMs) linked together by a linker peptide. The CBM effects binding to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate, insoluble cellulose. The presence of CBMs is particularly important for the initiation and processivity of exoglucanases (Teeri et al., 1998). Revisiting the original model of cellulose degradation proposed by Reese et al. (1950), a possible additional non-catalytic role for CBMs in cellulose hydrolysis was proposed: the “sloughing off” of cellulose fragments from cellulosic surfaces of cotton fibres, for example, thereby enhancing cellulose hydrolysis (Din et al.,

1994c). Cellulase systems exhibit higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism.

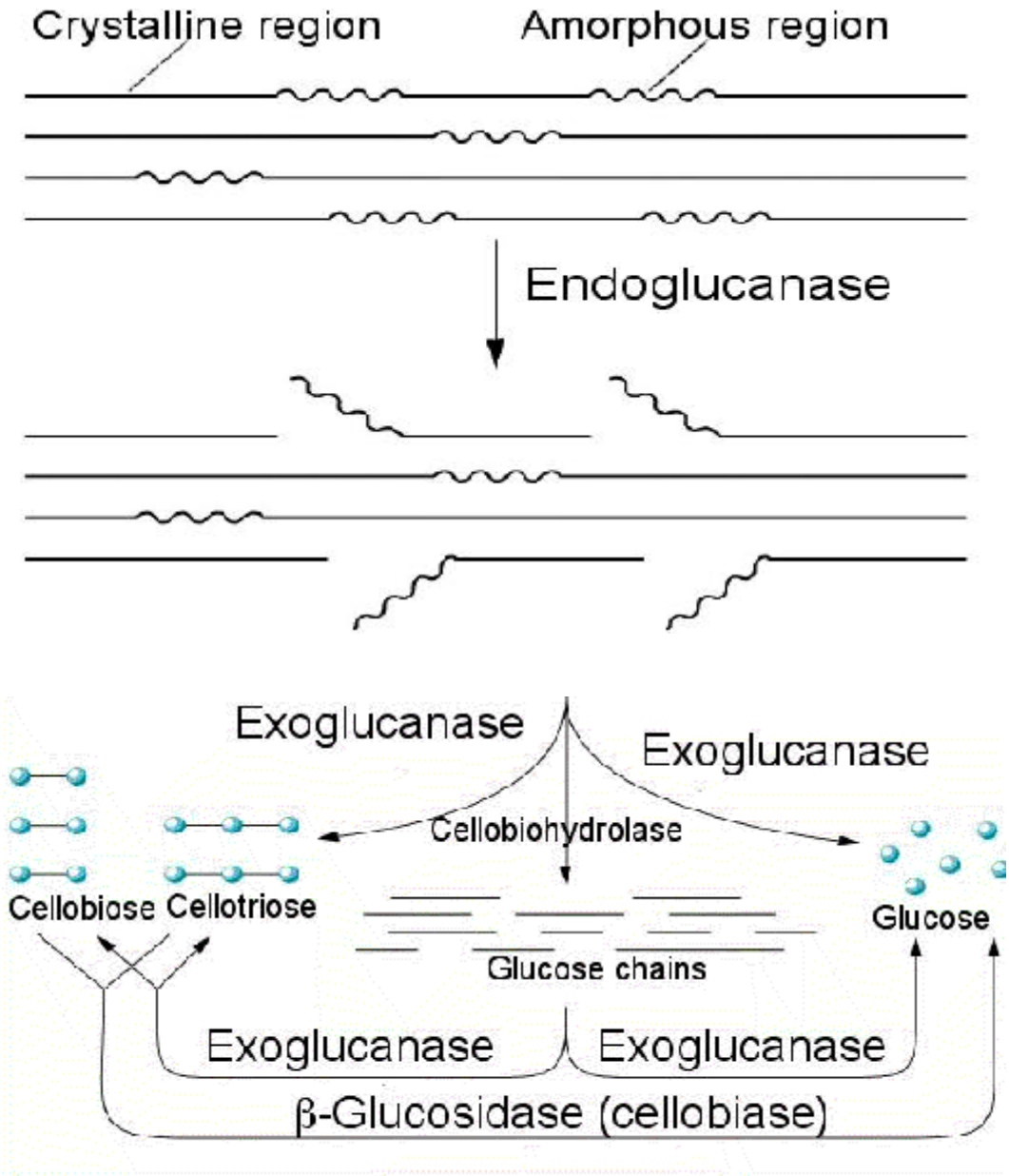

Fig. 1. Systemic degradation of cellulose by cellulolytic enzymes.

Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs (Teeri, 1997).

Cellulase systems are not merely an agglomeration of enzymes representing the three enzyme groups (endoglucanases, exoglucanases and β-glucosidases, with or without CBMs), but rather act in a coordinated manner to efficiently hydrolyse cellulose.

Microorganisms have adapted different approaches to effectively hydrolyse cellulose that occurs naturally in insoluble particles or embedded within hemicellulose and lignin polymers (Tomme et al., 1995b). Cellulolytic filamentous fungi (and actinomycete bacteria) have the ability to penetrate cellulosic substrates through hyphal extensions, thus often presenting their cellulase systems in confined cavities within cellulosic particles (Eriksson et al., 1990). The production of "free" cellulolytic enzymes, with or without CBMs, may therefore suffice for the efficient hydrolysis of cellulose under these conditions. The enzymes in these cellulase systems do not form stable high-molecular weight complexes and therefore are called "non-complexed" systems (Fig. 2). By contrast, anaerobic bacteria lack the ability to effectively penetrate cellulosic material and perhaps have to find alternative mechanisms for degrading cellulose and gaining access to the products of cellulose hydrolysis in the presence of competition from other microorganisms and with limited ATP available for cellulase synthesis. This could have led to the development of "complexed" cellulase systems (called "cellulosomes"), which position cellulase-producing cells at the site of hydrolysis, as observed for clostridia and ruminal bacteria (Fig. 3).

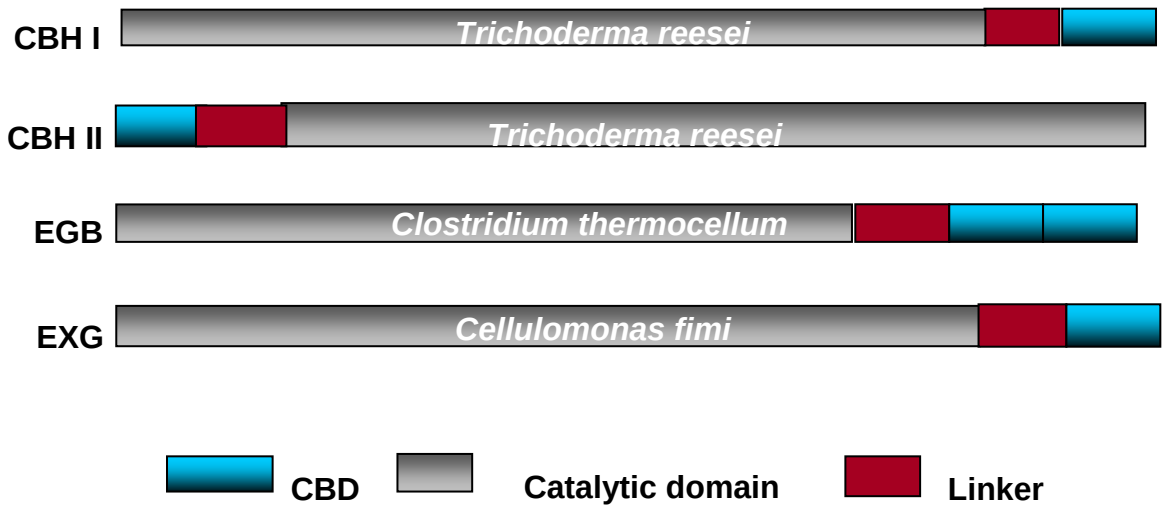

Fig. 2 Schematic representation of multi domain organisation shown by non-complex cellulolytic enzymes.

1.5 Organism development via the recombinant cellulolytic strategy

The recombinant cellulolytic strategy for organism development prior to cellulose conversion via CBP begins with non-cellulolytic microorganisms having excellent product formation properties and involves heterologous expression of a functional cellulase system. Such heterologous expression has been undertaken for a variety of purposes using a variety of microorganisms, such as *S. cerevisiae, Escherichia coli* and *Zymomonas mobiles* (Van Rensburg et al., 1998; Lejeune et al., 1988; Zhou et al., 1999). Our focus on the body of work involves *S. cerevisiae* as the model system, since it encompasses the most advanced

embodiment of the recombinant organism in general and the recombinant cellulolytic organism in particular.

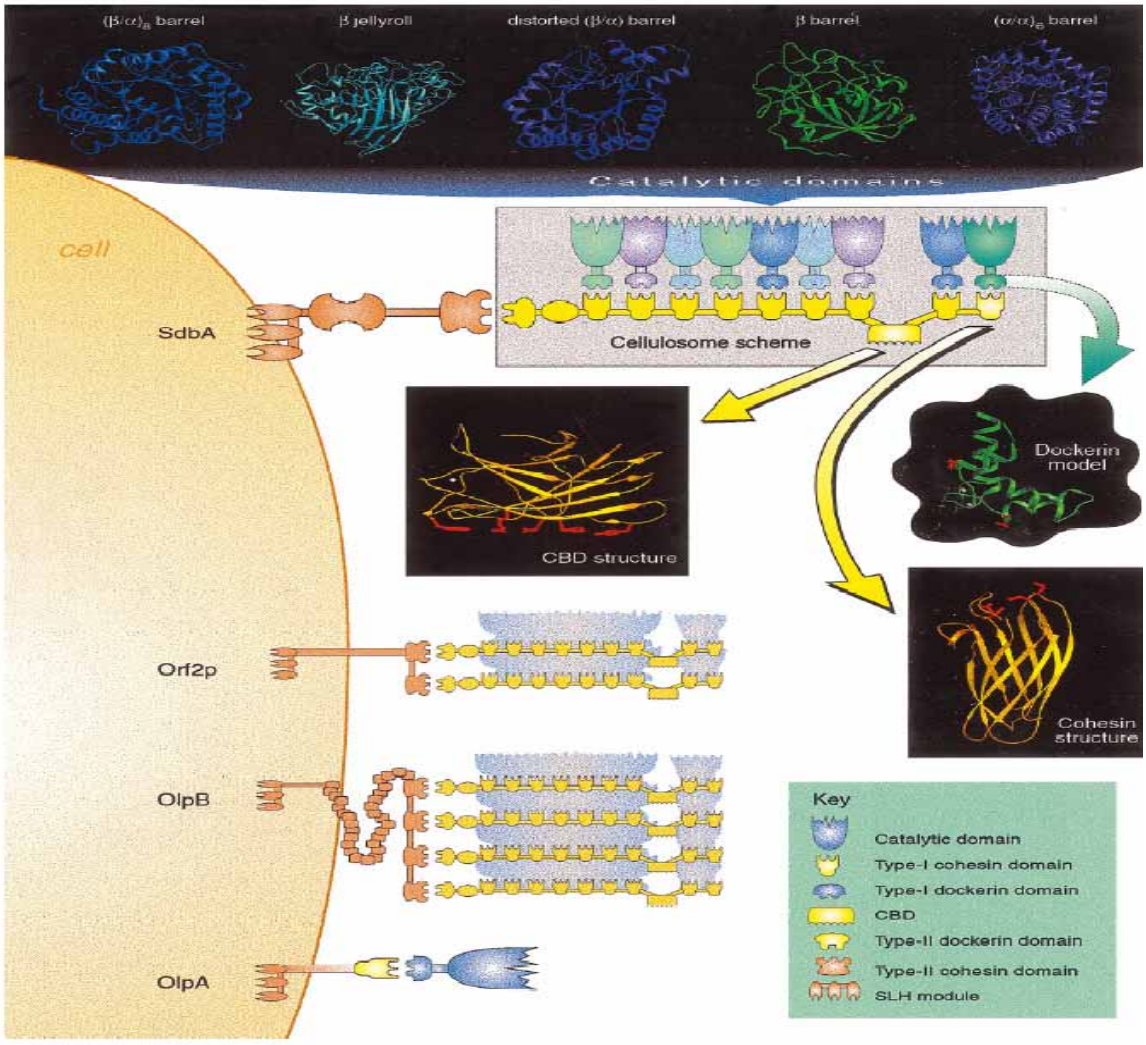

Fig. 3 Schematic representation of complex cellulolytic enzymes – "cellulosomes" (adapted from Bayer et al., 1998b)

Genes encoding cellulolytic enzymes have been cloned from various bacteria, filamentous fungi and plants and expressed in *S. cerevisiae*. Among the bacterial genes the endo-β-1,3-1,4-glucanase gene from *Bacillus subtilis* was expressed in *S. cerevisiae* under its own promoter and signal sequences (Hinchliffe and Box, 1984; Skipper et al., 1985). The synthesis of high levels of β-glucanase in brewing yeast strains was achieved by placing this gene under the control of the *S. cerevisiae ADH1* promoter on a high-copy-number 2μm-based plasmid vector (Cantwell et al., 1986). The fact that no extracellular endo-β-1,3-1,4-glucanase activity could be detected in the cultures of *S. cerevisiae* was attributed to the inability of yeast to process the protein to promote secretion. However, even when the β-glucanase gene was fused to the $ADH1_P$-$MF\alpha1_S$ expression secretion cassette, no

extracellular enzyme activity could be detected in culture fluids of the yeast transformants (Van Rensburg et al., 1997).

Two endo-β-glucanase genes from thermophilic bacteria were cloned and expressed in *S. cerevisiae* without replacing the bacterial gene promoters and secretion signal sequences. The specific activity of the *Clostridium thermocellum* enzyme synthesised in yeast was about 28% of that found in *E. coli* (Sacco et al., 1984). The cellobiohydrolase gene from an unidentified anaerobic bacterium was also expressed in *S. cerevisiae* (Saito et al., 1990). When this gene was placed under the control of the *SUC2* promoter and invertase secretion signal, no cellobiohydrolase was secreted by the yeast transformant, suggesting that the translocation of the hybrid protein was influenced by the protein structure (Uozumi et al., 1993). When the *SUC2* sequences were replaced by the *STA1* promoter and glucoamylase secretion signal, the recombinant yeast secreted approximately 40% of the cellobiohydrolase synthesised into the culture medium (Uozumi et al., 1993). A *S. cerevisiae* strain expressing both the *Cellulomonas fimi cenA*-encoded endo-β-1,4-glucanase (Eng) and the *cex*-encoded exo-β-glucanase (Exg) was able to saccharify filter paper and pre-treated aspen wood chips in reaction mixtures that were supplemented with β-glucosidase (Wong et al., 1988). In a similar study, the endoglucanase gene (Parvez et al., 1994) and the β-glucosidase genes (Rajoka et al., 1998) from *Cellulomonas biazotea* were also cloned and successfully expressed in *S. cerevisiae*.

Several cellulase genes from various fungi have also been expressed in *S. cerevisiae*. Five endo-β-1,4-glucanases (encoded by *egl1, egl2, egl3, egl4* and *egl5*) and two cellobiohydrolases (CBHI and CBHII) from *T. reesei* were efficiently secreted into the culture medium by *S. cerevisiae* transformants (Aho and Paloheimo, 1990; Bailey et al., 1993; Penttila et al., 1987, 1988; Saloheimo et al., 1994, 1997). Similar results were observed when the endo-β-1,4-glucanase gene from *Trichoderma longibrachiatum* was expressed in a wine yeast strain (Pérez González et al., 1993, 1996). These enzymes were not secreted efficiently until the late exponential or stationary growth phase, rendering the yeast cells larger and more irregular in shape (Penttila et al., 1989). Both the endo-β-1,4-glucanases and the cellobiohydrolases carrying the *T. reesei* leader peptides efficiently entered the secretory pathway of *S. cerevisiae*, but they were produced in highly glycosylated forms and were heterogeneous in size. It would seem that these proteins had undergone extensive elongation of the outer mannose chains in the Golgi apparatus. Despite overglycosylation of the cellulolytic enzymes produced by *S. cerevisiae*, the specific activity of the yeast-made endoglucanase was not significantly altered, whereas a slight decrease in the specific activity of the yeast-made CBHII was observed in comparison to the native enzymes (Bailey et al., 1993). The binding of the recombinant CBHII at a concentration of 30 μg/ml to crystalline cellulose also decreased, since only 50 to 70% of the yeast-made enzyme was bound to cellulose under conditions where 100% of the *T. reesei* enzyme was bound (Penttila et al.,

1988). This reduced ability to bind to the substrate was attributed to hyperglycosylation. However, the yeast-produced hyperglycosylated enzyme formed aggregates that bind less efficiently (Penttila et al., 1989). Another fungal cellobiohydrolase gene (*cbh1-4)* that was cloned and expressed in *S. cerevisiae* originated from *Phanerochaete chrysosporium*. The *CBH1*-encoded activity in the recombinant *S. cerevisiae* was, however, rather low (Van Rensburg et al., 1996).

In other work directed toward developing cellulolytic yeasts, a cDNA fragment encoding the FI-carboxymethylcellulase (FI-CMCase) of *Aspergillus aculeatus* was linked to the *GAP* (glyceraldehyde-3-phosphate dehydrogenase) promoter and transformed into *S. cerevisiae* (Ooi et al., 1994). Production of FI-CMCase by *S. cerevisiae* was shown to be growth associated, but 92% of the total enzyme activity was not secreted into the medium. The cloning and expression of cellobiohydrolase (*cbh1*) and β-glucosidase (*bgl1*) genes of *A. aculeatus* in *S. cerevisiae* have been reported in a series of four papers (Murai et al., 1997, 1998; Takada et al., 1998; Ueda and Tanaka, 2000). It was found that by co-expressing the *A. aculeatus* cellobiohydrolase (*cbh1*) and β-glucosidase (*bgl1*) genes in *S. cerevisiae*, the yeast transformants were able to hydrolyse up to 59% of added avicel (Takada et al., 1998). Murai et al. (1997, 1998) succeeded in anchoring these enzymes on the cell surface of *S. cerevisiae*. The cellobiohydrolase and β-glucosidase activities were detected in the cell pellet fraction, not in the culture supernatant. The yeast cells displaying these cell surface-anchored enzymes could grow on cellobiose or water-soluble cellooligosaccharides as the sole carbon source. This report by Murai et al. (1998) demonstrates that the cell surface-engineered yeast with these “immobilised” cellobiohydrolase and β-glucosidase proteins could be endowed with the ability to assimilate cellooligosaccharides.

S. cerevisiae does not contain any β-glucosidase activity and seems to lack an uptake system for cellobiose. With the aim of constructing cellobiose-fermenting strains of *S. cerevisiae*, β-glucosidase genes were isolated from *A. niger, Candida pelliculosa* var. *acetaetherius,* and *Kluyveromyces lactis* (Penttila et al., 1984; Raynal and Guérineau, 1984; Kohchi and Toh-e, 1986; Raynal et al., 1987) and cloned into *S. cerevisiae*. The β-glucosidase gene of *Candida molischiana* has also been cloned and successfully expressed in *S. cerevisiae* (Sanchez-Torres et al., 1998). In another instance, two β-glucosidase genes (*BGL1* and *BGL2*) derived from *Saccharomycopsis fibuligera* were expressed in *S. cerevisiae* (Machida et al., 1988) and it was observed that the *S. cerevisiae* transformant carrying the *BGL1* fermented cellobiose to ethanol, but the transformant carrying *BGL2* did not. Van Rensburg et al. (1998) have introduced into *S. cerevisiae* the following genes chosen with the intention of expressing a rudimentary cellulase system: the *B. fibrisolvens* endo-β-1,4-glucanase (*END1*), the *P. chrysosporium* cellobiohydrolase (*CBH1*), the *Ruminococcus flavefaciens* cellodextrinase (*CEL1*), and the *S. fibuligera* cellobiase (*BGL1*) gene constructs. *S. cerevisiae* transformants secreting biologically-active endo-β-1,4-

glucanase, cellobiohydrolase, cellodextrinase and cellobiase were able to hydrolyse various substrates, including CMC, hydroxyethyl cellulose, laminarin, barley glucan, cellobiose, polypectate, birchwood xylan and methyl-β-D-glucopyranoside.

In a later study, the gene (*cel*) encoding the bifunctional endo/exoglucanase of a *Bacillus* sp. (Han et al., 1995) was co-expressed with the β-glucosidase gene (*bgl*) of *B. circulans* (Cho et al., 1999b). When this recombinant *S. cerevisiae* strain was used in simultaneous saccharification and fermentation, an ethanol concentration of 2 wt% was obtained after 12 h from 50 g of microcrystalline cellulose per litre (Cho et al., 1999a). Petersen et al. (1998) took the concept of constructing a cellulose-degrading yeast one step further by engineering an *S. cerevisiae* strain for the degradation of four polysaccharides, viz. starch, pectin, cellulose and xylan (the main component of hemicellulose). This engineered *S. cerevisiae* strain contained the *Lipomyces kononenkoae* α-amylase gene (*LKA1*), the *Erwinia chrysanthemi* pectate lyase gene (*PEL5*), the *Erwinia carotovora* polygalacturonase gene (*PEH1*), the *B. fibrisolvens* endo-β-1,4-D-glucanase gene (*END1*), the *P. chrysosporium* cellobiohydrolase gene (*CBH1*), the *S. cerevisiae* exo-β-1,3-D-glucanase gene (*EXG1*), the *S. fibuligera* cellobiase gene (*BGL1*) and the *A. niger* endo-β-D-xylanase gene (*XYN4*). This strain was able to grow on starch, pectate and cellobiose, but the degradation of cellulose (Solka Floc and lichenan) and xylan was insignificant.

The expression of cellobiohydrolases (CBHs) in *S. cerevisiae* has been a particular focus of researchers in the field because of the vital role such enzymes play in degrading crystalline cellulose. Takada et al. (1998) expressed the *cbhI* gene of *A. aculeutus* and used the resulting protein in conjunction with additional cellulolytic enzymes produced by *S. cerevisiae* to achieve up to 59% hydrolysis of avicel. Cho and Yoo (1999) reported measurable filter paper activity associated with the production of an endo/exoglucanase originating from *B. subtilis*. Notwithstanding these notable studies, there are few reports on the hydrolysis of high-crystallinity cellulose with enzyme preparations, including CBHs produced by recombinant *S. cerevisiae*, and this has proved more challenging than has the functional production of other classes of cellulase enzymes. At present, functional CBH expression represents a bottleneck to CBP organism development and to growth enablement on crystalline cellulose in particular. Further progress in this area, including an understanding of the basis for both the successes and difficulties encountered in the work to date, is an important goal for future research.

Another objective for current and future research on the development of CBP *S. cerevisiae* strains is the improvement of the secretory expression of the abovementioned saccharolytic enzymes (Lynd et al., 2002). The high-level secretion of heterologous proteins, or of native proteins for that matter, is not as readily achieved in *S. cerevisiae* as in some bacteria, fungi, or other yeast species (e.g. *Pichia pastoris, Hansenula polymorpha, K. lactis* and *Yarrowia lipolytica*). Notwithstanding obstacles such as hyperglycosylation and hindered

secretion due to the cell wall, there are an increasing number of examples of the effective secretion of heterologous proteins by *S. cerevisiae*. Native secretion sequences have been found sufficient to effect proper posttranslational processing and secretion of functional proteins in the case of genes originating from fungal sources, including EgI, EgII and Xyn2 of *T. reesei* (La Grange et al., 2001, 1996; Penttila et al., 1988), XynC, XlnA and Man1 of *Aspergillus* (Crous et al., 1995; Luttig et al., 1997; Setati et al., 2001) and a glucoamylase gene. In-frame fusions to the yeast *MFα1S* secretion sequence have been used to express saccharolytic genes from bacteria in *S. cerevisiae*, including the *end1* gene of *B. fibrosolvens*, the *cel1* gene of *R. flavefaciens*, the *beg1* gene of *B. subtilis*, and the *xlnD* gene of *A. niger* (La Grange et al., 2001, 1996; Van Rensburg et al., 1994, 1995, 1996). Although these proteins were often extensively glycosylated, they were still efficiently secreted through the yeast cell wall into the medium (La Grange et al., 1996). Secretion of a mannanase (Man1) of *A. aculeatus* was recently reported at levels corresponding to about 5% of cellular protein (Setati et al., 2001).

Looking beyond saccharolytic enzymes, researchers have isolated several mutant strains with a "supersecreting" phenotype showing substantially increased secretion of particular proteins. For example, an *ssc1* (*pmr1*) *ssc2* double mutant secreted prochyomosin, bovine growth hormone and scuPA at levels that were five- to 50-fold higher than that secreted by non-mutated controls (Kjeldsen, 2000). Specific manipulations involving both the leader sequence and structural gene resulted in a substantial (up to 4.8-fold) impact on the levels of secretion of single-chain pro-insulin-like molecules into the culture supernatant (Kjeldsen, 2000).

1.6 Protein engineering of cellulolytic enzymes

At present, the protein engineering of cellulolytic enzymes is in its early stages. Protein engineering studies to date have been confined to endoglucanase, exoglucanase and the cellulose-binding domain of exoglucanase of *Trichoderma, Cellulomonas* and *Bacillus* (Pons et al., 1995; Damude et al., 1995). So far, cellulolytic enzymes have been engineered mainly at three levels. Firstly, the catalytically important amino acids have been identified and/or mutated by site-directed mutagenesis in order to understand the active site architecture and the role of active site amino acids in catalysis (Damude et al., 1995). Secondly, cellulolytic enzymes have been engineered to help in the understanding of the contribution of disulphide bridges in the stability of cellulolytic enzymes (Pons et al., 1995). Thirdly, the domains of cellulolytic enzymes are either being engineered or exchanged in order to purify a specific enzyme and also to produce hybrid enzymes. For example, CBD has been used as an affinity tag for the purification of heterologous polypeptides and for the immobilisation of enzymes (Assouline et al., 1993; Levy et al., 2002a). Recently, the CBD gene of either endoglucanase (CenA) or exoglucanase (Cex) from *C. fimi* has been fused to either the

alkaline phosphatase gene of *E. coli* or the β-glucosidase gene of *Agrobacterium* spp. and used for easing the purification of the recombinant proteins (Gilkes et al., 1988). Similarly, the CBD gene of exoglucanase from *C. fimi* has been fused to the Factor-X gene and the resultant fusion protein was purified using cellulose affinity chromatography (Assouline et al., 1993). Binding of CelC, a non-cellulosomal endoglucanase, to CelT, which has identical catalytic properties to CelC, confirms that foreign proteins can be incorporated into the cellulosome of *C. thermocellum* to modify its catalytic properties (Tokattidis et al., 1993). Thus, the CBD appears to be ideal for the production of a variety of affinity tags and the immobilisation of proteins and enzymes. Most of all, CBDs can be used as desired proteins and enzymes into protein complexes for the construction of efficient enzyme cocktails for the collective and synergistic degradation of cellulose. All these possibilities are expected to have tremendous impacts in biotechnology and industry. Other protein engineering studies include the conformational modelling of substrate binding to endocellulase from *Thermonospora fusca* (Taylor et al., 1995) and the molecular dynamics simulation of fungal CBD (Hoffrin et al., 1995). Future protein engineering on cellulolytic enzymes aims to improve their biochemical and catalytic properties through domain engineering, so that cellulolytic enzymes with desired properties and potential applications can be produced in large quantities.

1.7 Conclusion

In many cases, important tools for understanding microbial cellulose utilisation have become available only recently or have not yet been developed. Such tools include systems that allow foreign genes to be expressed in cellulolytic microorganisms, which are established for the aerobic *T. reesei* but not for most cellulolytic anaerobes. The recent development of an electrotransformation system for *C. cellulolyticum* makes possible new studies of microbial cellulose utilisation using homologous recombination-mediated gene knockout technology. Such studies can be expected to yield exciting comparative results as similar systems become available for more cellulolytic microorganisms that are not currently transformable. Studies in which heterologous cellulase expression confers the ability to grow on non-native substrates have begun to appear only in the last few years and represent an exciting frontier with potential to become an important tool for fundamentally-oriented investigation, while also being relevant to applied goals. However, in recent years, a vast majority of studies investigating cellulose hydrolysis and cellulose enzyme systems have proceeded within the context of the enzymatic paradigm. In terms of fundamentals, this paradigm focuses on cellulose hydrolysis primarily as an enzymatic rather than as a microbial phenomenon. In the light of this, several studies to date involved various cellulolytic enzymes from both fungal and bacterial origin. These studies involved various approaches, such as site-directed mutagenesis, random mutagenesis, domain engineering and DNA shuffling which are aimed

at improving a number of different properties, such as enzyme activity, affinity, thermostability and improved expression.

Research on cellulose utilisation at various levels of the microbial and enzymatic paradigm has progressed very rapidly during the past five decades. Remarkable progress has been made in understanding the production of cellulase, the biochemistry of cellulose degradation, the cloning and expression of cellulase genes, as well as the determination of the 3-D structure of a few cellulolytic enzymes. In addition, potential industrial applications of cellulolytic enzymes have been identified. Among the major impediments to exploiting the commercial potential of cellulolytic enzymes are the yield, stability, specificity and cost of cellulase production. Future research in this direction should focus on producing high yields of thermostable, alkaline and acidic cellulolytic enzymes with broad substrate specificity and improved catalytic efficiency, using low cost media and fermentation techniques. Research should also aim at exploiting the commercial potential of existing and new cellulolytic enzymes.

2. Cellulose-binding domains: functions and applications

2.1 Introduction

Cellulose is produced naturally by algae and higher plants, as well as by certain bacteria, marine invertebrates, fungi, slime moulds and amoeba (Richmond, 1991). Different organisms produce cellulose microfibres of characteristically different dimensions and functions. Although simple chemically, the diverse origins of cellulose result in a variety of complex physical forms. This is due to the propensity of cellulose chain to pack together and to form long crystals stabilised by intermolecular forces. In most cases, cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicellulose and lignin, which comprise 20-35 and 5-30 percent of the plant dry weight (Lynd et al., 1999; Van Soest, 1994). Although cellulose forms a distinct crystalline structure, cellulosic fibres in nature are not purely crystalline. The degree of departure from crystallinity is variable, and portrays a population of cellulose fibres in statistical terms as a continuum from purely crystalline to purely amorphous, with all degrees of order in between (Marchessault and Howsmon, 1957).

Microorganisms produce multiple enzymes, known as enzyme systems, to degrade cellulosic materials (Warren et al., 1996). For microorganisms to hydrolyse and metabolise insoluble cellulose, extracellular cellulolytic enzymes must be produced that are either free or cell associated. The biochemical analysis of cellulase systems from aerobic and anaerobic bacteria and fungi have been comprehensively reviewed during the past two decades (Knowles et al., 1987; Bayer et al., 1994, 1998a; Beguin and Lemaire, 1996; Beguin and Alzari, 1998; Belaich et al., 1997; Bhat, 2000; Davies, 1998; Doi et al., 1998; Mosier et al., 1999; Shoham et al., 1999; Schulein, 2000; Schwartz, 2001; Teeri, 1997; Tomme et al., 1995c, 1995b; Warren, 1996; Wood and Garcia-Campayo, 1990; Wood, 1992). Efficient wood-decaying organisms, such as filamentous fungi, typically use batteries of secreted and synergistically acting cellulolytic enzymes, while anaerobic bacteria utilise large multi-enzyme complexes (cellulosomes), which operate at the cell substrate interface (Tomme et al., 1995c; Bayer et al., 1996). Anaerobic fungi also seem to have large cellulosome-like enzyme complexes (Fanutti et al., 1995; Denman et al., 1996; Dijkerman et al., 1996). These cellulolytic systems have been classified on the basis of their enzyme activity on a large range of cellulosic and synthetic substrates. Three major types of enzymatic activities are found. Firstly, endoglucanases (1,4-β-D-glucan glucanohydrolase, EC3.2.1.4), such as *Clostridium thermocellum* endoglucanase D (CelD), act more randomly along the cellulose chain, generating oligosaccharides of varying lengths and consequently new chain ends (Davies and Henrissat, 1995; Harjunpaa et al., 1996; Warren, 1996; Teeri, 1997). Secondly, exoglucanases, or cellobiohydrolases (1, 4-β-D-glucan cellobiohydrolase, EC3.2.1.91), release cellobiose units mainly from the chain ends and degrade preferentially crystalline

cellulose in a processive manner. Exoglucanases can also act on crystalline cellulose, presumable peeling cellulose chains from the microcrystalline structure (Teeri, 1997). Finally, β-glucosidases hydrolyse soluble cellodextrins and cellobiose to glucose. These groups of enzymes cleave β-1,4-glycosidic bonds and their endo/exo specificity seems to be determined by the topology of their active sites. While the endoglucanases have open active site clefts, the exoglucanase active sites are located in tunnels formed by long loops in the protein structures (Divne et al., 1994; Meinke et al., 1995; Rouvinen et al., 1990; Spezio et al., 1993; Teeri, 1997). All potent cellulolytic bacteria and fungi produce a battery of all the abovementioned cellulolytic enzymes, which act synergistically to solubilise crystalline cellulose (Beguin and Aubert, 1994; Henrissat, 1994, 1998; Tomme et al., 1995b 1995c)

Cellulolytic enzymes are composed of independently folding, structurally and functionally discrete units, referred to as either domains or modules (Henrissat et al., 1998). Cellulolytic enzymes generally consist of a catalytic domain, which is responsible for the hydrolysis reaction, and a cellulose-binding domain, which mediates the binding of the enzymes to the substrate. The CBD facilitates cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate (Bolam et al., 1998). The two domains are joined by a linker peptide, which must be sufficiently long and flexible to allow efficient orientation and operation of both domains (Ferreira et al., 1990; Shen et al., 1991; Sridodsuk et al., 1993). These linker peptides are often heavily glycosylated. Sequence and structure comparison studies have revealed remarkable diversity in the structures and respective combinations of the cellulase-catalytic and CBD (Henrissat, 1994; Tomme et al., 1995d).

Cellulolytic enzymes are classified into families according to the amino acid sequence similarities of their catalytic domains. Such a classification system is based on the concept that similarities in sequence reflect the conservation of both the structural fold and the catalytic mechanism (Henrissat and Davies, 1997). Cellulose hydrolysis is accompanied by either the inversion or retention of the configuration of the anomeric carbon and, in both cases, is catalysed primarily by two carboxyl groups located in the active site of the enzyme (Henrissat and Davies, 1997; White and Rose, 1997). Inverting enzymes employ a single-step concerted mechanism, whereas retaining enzymes function via a double-displacement mechanism during which a transient covalent enzyme-substrate intermediate is formed. The understanding of this process has been assisted by the design of non-hydrolysable oligosaccharide derivatives and transition-state analogs, which can be trapped in the active site of the enzyme, and the complex can subsequently be analysed by structural means (Klarskov et al., 1997; Mackenzie et al., 1997; Notenboom et al., 1998; Sulzenbacher et al., 1997). Indeed, the recent advances in crystallographic methods and the new potential to determine the structures of cellulolytic enzymes with relative rapidity, both with and without substrate analogs, have changed our outlook regarding this group of enzymes. The architecture of the cellulolytic enzymes in and around the immediate active-site residues,

along with processive action of the entire cellulose-binding tunnel and/or groove, is critical to our understanding of cellulase action.

The cellulolytic activity of the catalytic domain is modified by accessory modules that can supplement the overall properties of the enzyme. The most common and functionally significant of these modules is the cellulose-binding domain (CBD), for which more than a dozen distinct families have been described on the basis of sequence homology (Tomme et al., 1995d).

Most CBDs occur as structurally well-defined domains linked to either the N or the C terminus of the catalytic domains. In some cellulolytic enzymes, multiple CBD copies or internal CBDs have also been identified (Meinke et al., 1991a, b; Henrissat, 1994). The catalytic domains of cellulosomal enzymes are bound non-covalently to the cellulosome-integrating protein (scaffolding), which carries the CBD, although some of the catalytic domains also have additional CBDs of their own (Lamed et al., 1983; Goldstein et al., 1993; Doi et al., 1994; Morag et al., 1995; Bayer et al., 1994, 1996, 1998c). Over the past few years, the contributions of CBDs to cellulase action on cellulosic substrates have been subjected to intensified investigation. Like their neighbouring catalytic domains, representative structures for each CBD family are now being elucidated either by crystallography or by NMR spectroscopy. The major function of the CBD is simply to deliver its resident catalytic domain to the crystalline cellulose substrate. The binding appears to be extremely stable, although the enzyme may undergo lateral diffusion on the substrate surface. Nevertheless, some CBDs also appear to catalyse the disruption of the non-covalent interactions between the chains of the crystalline substrate, whereas others bind preferentially to non-crystalline substrates (Linder and Teeri, 1997).

It is now well established that living organisms contain complex cellulolytic systems for the management of cellulose-containing materials. One of the pivotal players in these systems is the core catalytic component, such as endoglucanases and cellobiohydrolases, which hydrolyses polysaccharides. Another component that appears to be strongly present and devoted to cellulose degradation is the cellulose-binding domain. This important naturally occurring protein entity is contained within many cellulolytic systems, as well as within other carbohydrate-degrading enzymes: starch-binding domains (Jespersen et al., 1991; Coutinho and Reilly, 1994), chitin-binding domains (Watanabe et al., 1992; Iseli et al., 1993; Blaak and Schremf, 1995). It is thus apparent that CBDs play an essential role in the mechanism of cellulose degradation and have the potential to modify cellulose-containing materials. This article reviews in detail the nature and functions of these CBDs in the modification of polysaccharides and polysaccharide-containing materials and also describes the potential applications of CBDs in diverse fields of biotechnology.

2.2 Cellulose-binding domains

More than 50 years ago, the first stages in the enzymatic degradation of crystalline cellulose were proposed to involve the action of an unknown non-hydrolytic component termed C_1. This component was thought to be responsible for the destabilisation (non-hydrolytic disruption) of the cellulose structure, making the substrate accessible to the enzyme, the C_x component (Reese et al., 1950). Forty years later, the first C_1 component was cloned from *Clostridium cellulovorans* and *Cellulomonas fimi* (Din et al., 1991; Goldstein et al., 1993; Shoseyov and Doi, 1990; Shoseyov et al., 1990). The cloning and expression of individual binding domains offered researchers an opportunity to study this hypothesis.

Cellulose-binding domains are found in nature as discrete domains in cellulolytic enzymes and xylanases, in proteins that are involved in the degradation of plant biomass (Gilkes et al., 1991; Tomme et al., 1995b, 1995b), and in proteins without hydrolytic activity, such as the *Clostridial* cellulose-integrating or -binding proteins Cip and Cbp (Shoseyov et al., 1990; Shoseyov and Doi, 1990; Tomme et al., 1995b; Goldstein et al., 1993). CBDs are involved in targeting the enzymes to their polysaccharide substrates or particular substrate regions, thereby increasing the effective enzyme concentration at the surface of the insoluble cellulose. In Cbp or Cip, the CBD is part of a scaffolding subunit that organises the catalytic subunits in a cohesive multi-enzyme complex called the cellulosome, which then adheres strongly to cellulose (Bayer et al., 1998a, b; Beguin and Aubert, 1994; Beguin and Lemaire, 1996). Structural characterisation of a wide variety of different cellulose-degrading enzymes showed that they shared a modular structure composed of a catalytic domain linked to a distinct cellulose-binding domain: CBD (Tomme et al., 1988, 1995c, b). Interestingly, strikingly similar domain structures have been found in several enzymes active on other insoluble carbohydrates such as raw starch and chitin. Glucoamylases, the enzymes active on insoluble raw starch, have distinct starch-binding domains (Jespersen et al., 1991; Coutinho and Reilly, 1994) and many different chitinases contain a chitin-binding domain (Watanabe et al., 1992; Iseli et al., 1993; Beitema, 1994; Blaak and Schrempf, 1995). Similar to the case in cellulolytic enzymes, the removal of the carbohydrate-binding domains of these enzymes significantly reduced their activities on insoluble substrates, but not on soluble substrates (Iseli et al., 1993; Blaak and Schrempf, 1995), making it apparent that such a modular domain structure offers significant advantages in the degradation of these insoluble substrates.

Cellulose-binding domains have also been found in several other polysaccharide-degrading enzymes. In *T. reesei,* CBD has been identified in hemicellulase, endo-mannase and acetryl-xylanesterase (Stalbrand et al., 1995; Margolles et al., 1996). CBDs have been recognised in xylanase originating from *C. thermocellum* (Kulkarni et al., 1999; Kim et al., 2000), esterase from *P. funiculosum* (Kroon et al., 2000), and pectate lyase in *Pseudomonas cellulose* (Brown et al., 2001). In addition, there is the intriguing

presence of such a domain in β-glucosidase located in *P. chrysosporium* (Lymar et al., 1995). The presence of putative CBDs in plant endoglucanses has also been reported (Catala and Bennett, 1998; Trainotti et al., 1999). Trainotti et al. (1999) described four putative CBDs, two from *Arabidopsis*, one from cotton and one from strawberry. Expansins, which are believed to play a role in non-hydrolytic cell wall expansion, are homologous to CBDs and possess cellulose-binding capabilities *in vitro* (Cosgrove, 2000a, b, c).

CBD sequences are classified into 14 different families comprising more than 200 related members and are classified based on amino acid sequence, binding specificity and structure (Gilkes et al., 1991; Tomme et al., 1995d, a, 1998; Bayer et al., 1998a). Some families contain only one member (families V and VIII), whereas others families are large (families I, II and III), with 40 or more members (Tomme et al., 1995d, 1998). The CBDs contain approximately 30-180 amino acids and exist as a single, double or triple domain in one protein. Their location within the parental protein can be either C- or N-terminal or, occasionally, centrally positioned in the polypeptide chain. CBD members form a part of the larger family of carbohydrate-binding modules that is divided into 39 families. Cellulose-binding members are found among 16 of these families (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). These CBDs appear to be divided into two categories, one consisting of CBDs with affinity for crystalline cellulose, and the other consisting of CBDs with affinity for amorphous cellulose but not for crystalline cellulose. CBDs of families 1, 2 and 3 with affinities for crystalline cellulose have common structural characteristics, although their tertiary structures differ (Tomme et al., 1996). Multidimensional NMR was used to elucidate the three-dimensional structure of the family I CBD from *T. reesei* cellobiohydrolase I (Kraulis et al., 1989). Since then, the 3-D structures of representative members of CBD families IIa, III, IV, V, VI, IX and XV have also been resolved by crystallography and NMR (Fig. 4) (Xu et al., 1995; Tormo et al., 1996; Johnson et al., 1996a, 1999; Brun et al., 1997; Sakon et al., 1997; Mattinen et al., 1997, 1998; Notenboom et al., 2001a; Szabo et al., 2001; Czjzek et al., 2001). CBDs of family I, II and III have a planar cellulose-binding domain surface, including a set of aromatic residues and a group of polar residues. Thermodynamic studies have not been reported for these CBDs with cellooligosaccharides, although the interaction between cellohexaose and family I CBDs from *T. reesei* cellulolytic enzymes was studied by NMR (Mattinen et al., 1998). It was reported that the binding of the family II CBD of *C. fimi* Cex to insoluble cellulose was entropically driven (Creagh et al., 1996).

On the other hand, CBDs with preference for amorphous cellulose and/or soluble saccharides have a carbohydrate-binding cleft where aromatic and polar amino acid residues are conserved (Johnson et al., 1996b; Notenboom et al., 2001a, b). These modules often show affinities for oligosaccharides and even monosaccharides; e.g. the family IV CBD of *C. fimi* CenC has an affinity for cellotetriose with a K_a of 180 M^{-1} (Johnson et al., 1996b) and the CBD of *Clostridium cellulovorans* has an affinity for cellotetraose with a K_a of 1.2 X 10^3 M^{-1}

(Notenboom et al., 2001a). It was found that the interactions of these CBDs with oligosaacharides are driven by enthalpic forces. A recent study by Arai et al. (2003) revealed

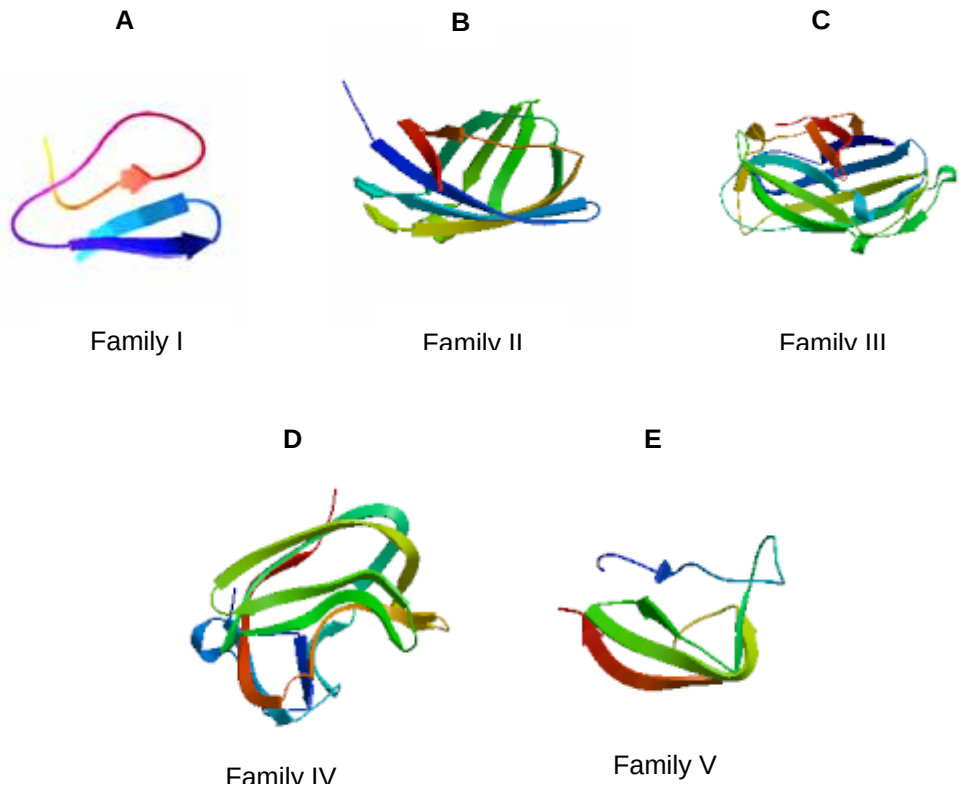

Fig. 4 Cellulose-binding domains from different families: (A) Family I CBD from cellobiohydrolase I of *Trichoderma reesei*; (B) Family II CBD from exoglucanase of *Cellulomonas fimi*; (C) Family IIIa CBD from the scaffolding subunit protein of *Clostridium thermocellum*; (D) Family IV CBD from endoglucanase of *Cellulomonase fimi*; (E) Family V CBD from endoglucanase of *Erwinia chrysanthemi*. (adapted from the weblink **http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html)**

that the thermodynamics of the CBD of *C. thermocellum* CelJ was also found to be consistent with CBDs showing affinity for soluble polymers and oligosaccharides and also showed wide ligand specificity. Recently, a three-dimensional model of the cellulose-binding domain of the rye grass pollen allergen LoI pI built by homology modelling was proposed as a structural scaffold for expansins and other expansin-related proteins (Barre and Rouge, 2002). Cellulose-binding domains have also been classified as a class of small proteins called “Knottins-cystine knot”, along with members of toxins, lectins or small inhibitor molecules. Furthermore, the 3D structure of the cellulose-binding domain of fungal cellobiohydrolase has been shown to share close structural homology to the family of proteins that modify the activity of voltage-gated Na^+, K^+ and Ca^{2+} channels, funnel-web

spider toxin (J-ACTX-Hv1c) and gurmarin, a sweet-taste-supressing peptide from plants (Wang et al., 2000; King et al., 2002; Tedford et al., 2004).

All of the CBDs characterised thus far have structures based on different β-sheet topologies. The CBHI CBD from *T. reesei* folds into a small wedge-shaped structure formed by a small irregular triple-stranded β-sheet and is stabilised by two disulphide bridges. One face of the wedge contains a row of three tyrosines together with a few potential hydrogen bond-forming residues. The spacing of the tyrosines equals the distance of every second glucose moiety in an extended cellulose chain, and site-directed mutagenesis experiments have established their importance in the interaction of the CBD with crystalline cellulose (Linder et al., 1995a, b; Reinikainen et al., 1992, 1995). In several homologous CBDs, some of the tyrosines are replaced by tryptophans or phenylalanines, with a tryptophan instead of a tyrosine contributing to the increased affinity of the EGI CBD (Linder et al., 1995a).

The structural data of these different families reveal the structural similarity between the CBDs and that their cellulose-binding capacity can be attributed, at least in part, to several aromatic amino acids that compose their hydrophobic surface. The CBDs of both the *C. fimi* Cex, CenC and the scaffolding (Cip) of *C. thermocellum* have similar architectures, consisting of two β-sheets packed face-to-face to form a β-sandwich. The structure of CBD_{Cex} contains a single disulphide bridge connecting the N and C termini and the CBD_{Cip} has a high affinity-binding site for Ca^{2+} (Tormo et al., 1996). The CBDs exhibit a planar surface exposing two or three aromatic residues and some potential hydrogen bond-forming residues, and several lines of evidence indicate that these residues mediate their binding interactions with crystalline cellulose (Fig. 5) (Poole et al., 1993; Din et al., 1994b; Xu et al., 1995; Bray et al., 1996; Tormo et al., 1996). The positions and angles between these aromatic residues differ between various CBD members. One good example is between the CBD_{Cex} from family IIa and the CBD_{N1} from family V, in which the former contains a β-barrel-type backbone that displays aromatic amino acids on a relatively flat surface (Din et al., 1994b; Tormo et al., 1996; Nagy et al., 1998) and the latter displays its aromatic residues in a narrow groove (Johnson et al., 1996b; Tomme et al., 1996). CBDs from the same organism can differ in their binding specificity (Carrard et al., 1999) and, occasionally, two CBDs located on the same enzyme can also exhibit this distinction (Brun et al., 2000). Structural comparison shows that, in spite of their different sizes and topologies, the CBDs binding to crystalline cellulose have apparently come up with a very similar solution for the binding interaction (Tormo et al., 1996). The main interaction seems to be the stacking of the aromatic rings with the glucose rings along a single glucan chain assisted by the hydrogen-bonding interactions of other CBD residue to the neighbouring glucan chains (Tormo et al., 1996). Aromatic amino acids are also involved in the carbohydrate interactions of the raw starch-binding domains (Sogaard et al., 1993) and are commonly observed in several other carbohydrate-protein interactions (Vyas, 1991; Quiocho, 1993).

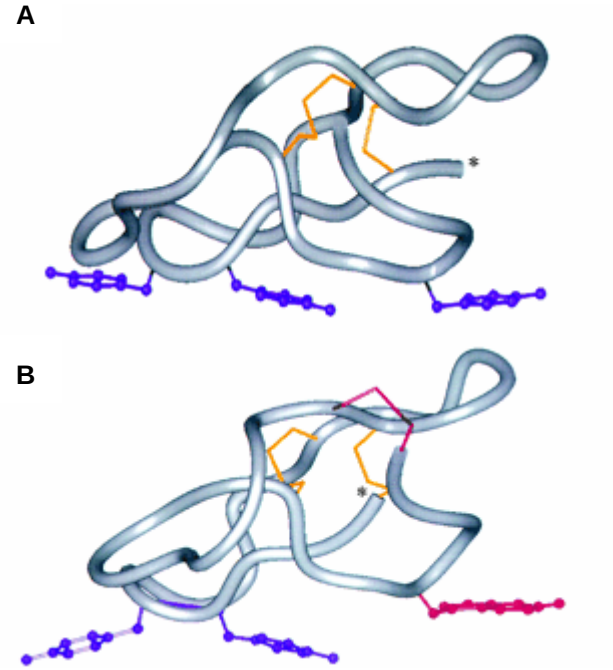

Fig. 5 (A) α-Carbon trace of CBD_{CBHI} determined by NMR. The three tyrosyl side chains present on the binding surface are shown with the stick and ball representation and the two disulphide bridges are in yellow. (B) Predicted model of the α-carbon trace of CBD_{CBHII}. The Tyr33, Tyr34 and Trp7 side chains present on the binding surface are shown in stick and ball representation (adapted from Carrard and Linder, 1999).

2.3 Functions of CBD

The depolymerisation of cellulose into its monomeric glucose units by cellulase enzymes is a complex phenomenon mediated by inter- and intramolecular synergism among the main components of the cellulase system endoglucanases, cellobiohydrolases and β-glucosidases (Riedel and Bronnenmeier, 1998a). Both the enzyme mixture and the solid substrate have attributes that can strongly influence the dynamics of the reaction (Mansfield et al., 1999; Yoshihiko and Takahisa, 2002). In addition, the reaction heterogeneity arising from the interaction between a solid substrate and the enzyme solution further adds to the complexity of an enzymatic hydrolysis system. Adsorption of the enzyme onto the solid surface, penetration into the crystalline structure, occurrence of the chemical reaction, desorption/readsorption or movement of proteins on the cellulose structure and, finally, the diffusion of the hydrolysis products (glucose, cellobiose or short oligomer chains) out of the porous structure seems to be the logical order of events during hydrolysis (Esteghlalian et al., 2001). The simultaneous or consecutive occurrence of these events on various reaction sites on the substrate creates an intense nonlinear dynamic within the first few hours of the reaction. At any moment, different fractions of the enzyme are engaged in different actions. It

has been shown that almost all the enzyme activities, except β-glucosidase, are adsorbed onto the substrate very rapidly (Esteghlalian et al., 2001).

During the first few hours of cellulose hydrolysis, the rate of reaction is linear with time. The intensity of the reaction, however, subsides after a few hours and the reaction gradually slows down, hence exhibiting the typical biphasic pattern observed in any cellulose hydrolysis profile (Esteghlalian et al., 2001). The cause of this gradual drop in the reaction rate was postulated to be both enzyme and substrate driven. Examples of substrate-related factors include the degree of crystallinity and polymerisation of cellulose chains, the accessible surface area, substrate porosity and the presence of extraneous materials such as lignin and hemicellulose. Enzyme-related factors include the thermal or mechanically-induced deactivation of enzymes, sieving of enzyme components by the substrate and the loss of synergism following this separation, as well as feedback inhibition of the hydrolytic products in the reaction medium. Conflicting observations have prevented scientists from identifying a single factor as the sole cause of the gradual loss of efficiency during the hydrolysis of cellulose (Esteghlalian et al., 2001).

The first attempt at establishing a mechanistic model for the enzymatic hydrolysis of cellulose was made by Elwyn Reese in 1950 (Reese et al., 1950). The theory, known as the C_1-C_x model, furnished indirect evidence for the functional role of the cellulose-binding domain, suggesting that a mechanical action by the C_1 factor precedes the actual hydrolytic action by the C_x factor. This theory, although groundbreaking at the time, failed to describe the nature of these factors. Building on this model, numerous attempts were made to attribute the C_1 and C_x activities to different components of an enzyme mixture. For example, Wood and McCrae equated the C_1 and C_x factors with exo- and endo-acting enzymes respectively, suggesting that it was the C_x (endoglucanases) that initiated the attack on the cellulose chains, as they are more random in their action and can create susceptible chain ends suitable for C_1 (exoglucanases) to act on (Wood and McCrae, 1972). This was a clear deviation from the original assumption of the C_1-C_x model, namely that C_1 acts first, followed by C_x. In 1994, Din et al. re-demonstrated the C_1-C_x mechanistic model of enzymatic hydrolysis in the enzyme endoglucanase A from the bacterium *C. fimi* (Din et al., 1994a).

In 1985, Coughlan suggested a direct role for the cellulose-binding domain in the hydrolytic reaction by introducing the concept of amorphogenesis as a prerequisite for the hydrolytic action of the enzyme. His theory was that amorphogenesis (i.e. the swelling, segmentation or destratification of the substrate) renders the crystalline cellulose more accessible to the enzymes for hydrolytic dissolution (Coughlan et al., 1985). Thus, amorphogenesis was the equivalent of the C_1-induced mechanical dispersion proposed by Reese (Reese et al., 1950). Amorphogenesis has been attributed to the H_2O_2 produced by some enzymes (Koenigs, 1975), some iron-containing proteins in fungal filtrates (Griffin et al., 1984), and even to CBHI produced by *T. reesei* (Chanzy et al., 1983). Various studies have tried to elucidate the

nature of a non-hydrolytic factor responsible for the mechanical dispersion of a substrate prior to catalytic reaction. Several groups of researchers have been able to isolate a component or a portion of a component from *T. reesei* filtrate that had the ability to cause dispersion in the substrate. The 5 kDa microfibril-generating factor (MGF) isolated by Krull et al. (1988) increased the microfibril content of filter paper and also released soluble carbohydrates from the filter paper, but did not improve the digestibility of filter paper. In 1998, Banka et al. isolated a larger non-hydrolytic component (11-12 kDa), referred to as fibril-forming protein (FFP), from *T. reesei* that was able to disrupt filter paper and produce free fibrils (Banka et al., 1998). Thus the MGF and FFP played the role of the C_1 factor in Reese's model and were identified as cellulose-binding domains. Observations by Woodward et al. (1992) showed that the catalytic domain of CBHII from *T. reesei* had no catalytic activity toward avicel and cotton linters; nevertheless, the cotton fibres were dispersed upon treatment with this protein. This implied that the hydrolytic activity of this particular catalytic domain is contingent on the presence of its related binding domain. It was also observed that the catalytic domain of *C. fimi* endoglucanase could hydrolyse cotton to a limited extent, even in the absence of its binding domain (Din et al., 1991). This functional difference in the cellulose-binding domains (C_1 factor) could be owing to the differences in the origin of the two enzymes and also to the process of isolation of their domains.

An interesting study by Klyosov et al. (1986) attempted to define a role for the CBD by shifting the concept of the C_1 factor from a component to a property. They suggested that the C_1 factor was not an individual substance or enzyme with a particular specificity, but rather a property of already known enzymes, namely the capacity of cellulolytic enzymes for binding onto the surface of insoluble cellulose. It was proposed that the cellulose-binding component of the enzyme initiated the attack at the disturbed regions of the crystalline cellulose and dispersed the structure by means of a mechanochemical action, creating more accessible areas of attack for the weakly bound, more mobile enzymes that carry out the catalytic reaction. Klyosov also reported that not only the quantity of cellulolytic enzymes, but also their ability to be adsorbed tightly onto crystalline cellulose, was essential for effective hydrolysis. In addition, it was proposed that the multiple enzyme-associated cellulose-binding domains with various adsorption capacities directly mediated the synergistic effect exhibited by the enzyme mixture in cellulose hydrolysis (Carrard et al., 2000; Pages et al., 1997; Fierobe et al., 2002).

The pioneering study by Din et al. (1991) on the cellulose-binding domain of endoglucanase A (CenA) from the bacterium *C. fimi* established that the binding activity resides not in a system distinct from the catalytic domain, but in a discrete domain of each enzyme. It was shown that the isolated CBD_{CenA} causes a roughening of the surface of cellulosic fibres and releases small particles into the solution that can be detected spectrophotometrically. It was also proposed that CBD functions by penetrating into the

cellulose structure and creating free chain ends; however, it could not be verified whether the CBD is indeed capable of destabilising the hydrogen bonds within the structure. This non-hydrolytic effect was observed only with the binding domain and not with the isolated catalytic core of the same enzyme. Unlike with cellulosic fibres from cotton, it was observed that CBD_{CenA} did not exhibit disruptive effects in the treatment of highly crystalline cellulose from *Valonia ventricosa* (Jervis et al., 1997). However, it was noticed that the majority (70%) of bound CBDs were mobile on the crystalline surface, hence undermining the binding site exclusion theory (McGhee and Von Hippel, 1974). The study revealed that the binding of a CBD molecule does not permanently exclude the corresponding binding sites from the pool of substrate-binding sites. Instead, the mobility of the CBD makes those sites available for new binding upon its displacement. Later, this phenomenon was also demonstrated for other CBDs relating to other families (Beguin, 1990; Gao et al., 2001; Xiao et al., 2001; Boraston et al., 2004). Another piece of supporting evidence showed that CBD_{CenA} could prevent the flocculation of microcrystalline cellulose (Gilkes et al., 1993). On the basis of CBD structure-function studies and the model of cellulose structure, it has been proposed that the CBDs interact with the flat surface of the cellulose crystal (Reinikainen et al., 1995; Tormo et al., 1996). In 1992, Gilkes et al. proposed the theory of overlapping binding sites for the CBD (Gilkes et al., 1992). Furthermore, it was also reported by Carrard et al. (2000) that different CBDs increase the range of available sites to various extents. The sites targeted for hydrolysis by different CBDs can differ, implying that each CBD possesses its own specificity for the heterogeneous sites present on the surface of the substrate. Consequently, mixtures of complexes containing the same catalytic domain but different CBDs were shown to access a more extended range of cleavable sites than each complex by itself. A recent report by Lehtio et al. (2003) provides unequivocal evidence that the fungal family I CBDs, as well as the family III CBD from *C. thermocellum* CipA, have defined binding sites on two opposite corners of *Valonia* cellulose crystals.

Recently, Levy et al. (2002b) described the role of cellulose-binding domains in the modification of polysaccharides and the plant cell wall. The cellulose-disruption effect was earlier demonstrated for expansins in growing plant cell walls, as well as in isolated cell wall fractions (McQueen and Cosgrove, 1994; Cosgrove, 2000a; 2000b). Expansins were shown to possess significant sequence identity with microbial cellulolytic enzymes (Cosgrove, 2000a; Wilson and Irwin, 1999). Interestingly, expansins, which are thought to lack endoglucanase activity, bind to cellulose and exhibit significant homology with microbial CBDs and, on some occasions, exhibit a weakening effect on pure cellulose material (Whitney et al., 2000). Swanson et al. (1999) reported the cloning and identification of a fungal protein with alleged novel activity. This plant expansin-like 50 kDa protein, comprising an N-terminal CBD, called swollenin, was reported in *T. reesei* and resulted in an opening and swelling of fibre structure without hydrolysis when incubated with cotton fibres

(Saloheimo et al., 2002). It was therefore suggested that the effect of both expansin and swollenin on cellulose is simply the non-hydrolytic disruption activities proposed over 50 years ago by Reese et al. (1950) and later demonstrated by Din et al. (1991).

Sphigel et al. (1998b) demonstrated that, like other organic cellulose-binding substances, CBDs (family III CBD_{Clos} from *C. cellulovorans*) could modulate cellulose biosynthesis. The CBD increases the rate of cellulose synthesis activity in *Acetobacter xylinum* by up to five-fold compared with controls. The mechanism by which the CBD affects cell wall metabolism remains unknown, although it was postulated that a physico-mechanical mechanism would have allowed the CBD to slide between the cellulose fibres, thus separating them in a wedge-like action. This postulate is supported by in vitro experiments in which the application of recombinant CBD significantly reduced the wet-tensile strength of cellulose paper. The observed results resembled the in vivo effects of expansin. Furthermore, petunia cell suspension treated with an increasing concentration of CBD displayed abnormal shedding of cell wall layers, indicating that the CBD can cause non-hydrolytic cell wall disruption activity. Low concentrations of CBD enhance the elongation of pollen tubes and roots, whereas high concentrations inhibit root elongation in a dose-dependent manner. It has also been shown that the CBD and CBD-like expansins can modulate the growth of transgenic plants (Shoseyov et al., 2001; Cho and Cosgrove, 2000, Sphigel et al., 1998b).

2.3.1 The CBD and cellulose hydrolysis

Although the primary role of the cellulose-binding domain has been defined as targeting the enzyme and/or increasing the local concentration of enzyme at the site of hydrolysis (Stahlberg et al., 1991; Pages et al., 1997; Levy et al., 2002b; Kataeva et al., 2002; Fierobe et al., 2002; Carrard et al., 2000), or the disruption of single glucan chains from the cellulose surface (Knowles et al., 1987; Teeri et al., 1987; Reinikainen et al., 1995; Tormo et al., 1996; Pages et al., 1997; Xiao et al., 2001), it has been postulated, and in some cases shown, that the binding domains of cellulolytic enzymes perform functions other than physical or chemical binding (Din et al., 1991; Teeri et al., 1992) (Fig. 7). These additional functions also seem important for initiating and maintaining efficient hydrolysis. For example, it has been proposed that the CBDs of exoglucanases can act as a plough, delaminating cellulose layers and releasing free chain ends. The enzyme's catalytic domains will then act on these free ends and depolymerise the cellulose structure (Teeri et al., 1992). It is now well established that the removal of the CBD has little influence on the activity of cellulolytic enzymes towards soluble substrates, while their binding and activity towards insoluble cellulose is clearly decreased (Gilkes et al., 1988; Tomme et al., 1988; Irwin et al., 1994; Reinikainen et al., 1992, 1995; Stahlberg et al., 1993; Srisodsuk et al., 1997). A comparative study between an endoglucanase IV (Cel5) of *Ruminococcus albus* and its fused protein complex, Cel5-CBD6,

confirmed previous findings and provided further evidence suggesting that Cel5-CBD6 exhibits enhanced activity with insoluble cellulose compared to native Cel5 (Bae et al., 2003). The report further suggests that CBD6 increases the affinity of Cel5 for insoluble substrates, and this increased binding capacity seems to result in increased catalytic activity. While a targeting function can be proposed for the CBDs of various non-cellulolytic enzymes, assigning a single primary role for the CBDs of the cellulolytic enzymes has been made difficult due to the diverse mode of action exhibited by these enzymes in the hydrolytic process. It has also been suggested that the properties of different CBDs must have been optimised according to the demands of their catalytic domains (Coutinho et al., 1992; Irwin et al., 1994; Linder et al., 1995a,1995b; Tomme et al., 1995a, 1995c).

Most of the information on the role of the CBD has been obtained by removal (Van Tilbeurgh et al., 1986; Tomme et al., 1988; Gilkes et al., 1988; Stahlberg et al., 1991; Irwin et al., 1994; Srisodsuk et al., 1993), domain exchange (Fig. 6) (Tomme et al., 1995a; Srisodsuk et al., 1997; Gal et al., 1997; Carrard et al., 2000; Bae et al., 2003; Mangala et al., 2003) or site-directed mutagenesis of the CBDs (Reinikainen et al., 1992, 1995). In the case of the cellobiohydrolase (CBHI) enzyme from *T. reesei*, it has been found that the removal of the CBD has drastically affected the activity of the catalytic domain on insoluble substrates.

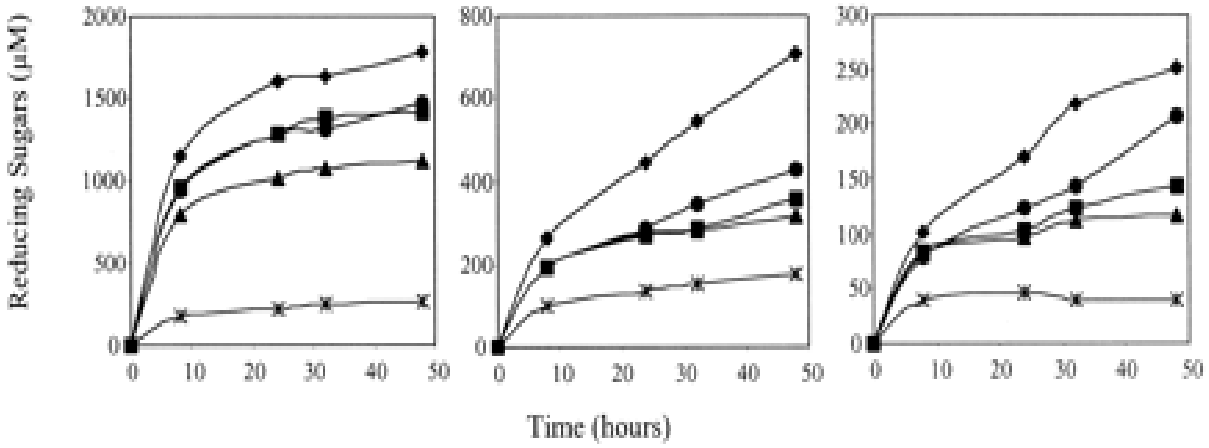

Fig. 6. Stimulation of CelD activity by the different CBDs on insoluble substrate at pH 6, 45°C. Production of reducing sugars was followed on (*Left*) amorphous cellulose, (*Center*) AVICEL, or (*Right*) BMCC. The protein complexes used in this study were: CelD-CBD_{CBH1} (▲), CelD-CBD_{CBH2} (■), CelD-CBD_{Cex} (●), and CelD-CBD_{CipA} (♦). CelD (*) was used as a control (adapted from Carrard et al., 2000)

At saturation, the catalytic domain bonded to an estimated 30% of the surface in comparison to the intact enzyme, thus suggesting different binding sites on the cellulose surface (Stahlberg et al., 1991; Srisodsuk et al., 1993). Recently, Hamada et al. (2001) reinstated this role of the cellulose-binding domain in the exocellulase I from white rot basidiomycete *Irpex lacteus*. Mutational analyses of the CBD of *T. reesei* CBHI have been

shown to produce mutants with gradually decreasing affinities and subsequently decreased affinities on crystalline cellulose (Reinikainen et al., 1992, 1995).

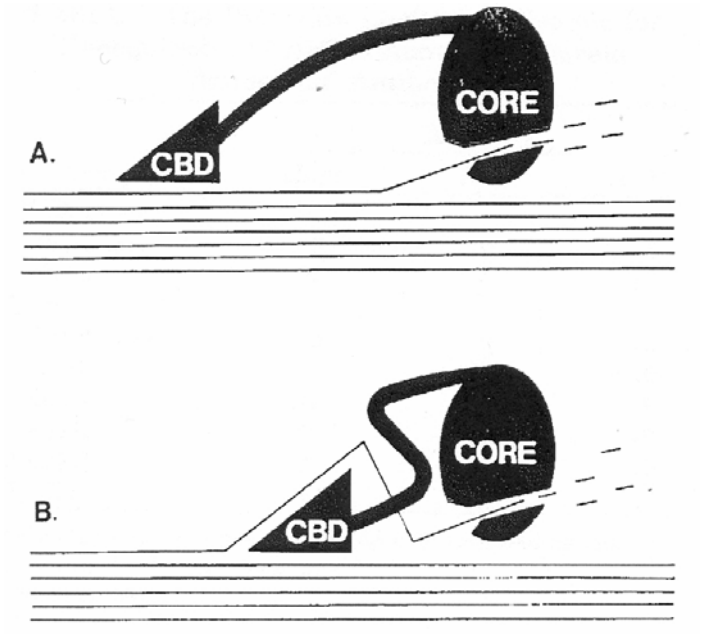

Fig. 7 Hypothesis of the function of the cellulose-binding domains. (A) Crystal binding site model, (B) Crystal disrupting model (Reinikainen et al., 1992).

It has been shown that the addition of a CBD to the catalytic domain of a cellulosomal exoglucanase from *C. thermocellum* and other enzymes such as endoglucanases and chitinases improves its ability to degrade crystalline cellulose (Kruus et al., 1995; Lemos et al., 2003; Limon et al., 2001). The artificial addition of a heterologous CBD (*Thermonospora fusca* E2 CBD) to a catalytic domain of *Pruvotella ruminicola* carboxymethyl cellulase (CMCase) increased its specific activities against insoluble cellulose allomorphs (Maglione et al., 1992). Furthermore, mixing of *Orpinomyces* endoglucanase CelE with the family III cellulose-binding domain (CBDIII) fused with a fibronectin-like repeat (Fn3) increased the efficiency of hydrolysis of acid-swollen cellulose and filter paper (Kataeva et al., 2002). In an attempt to investigate the function of CBDs under various cellulolytic enzyme backgrounds, various domain exchange experiments were performed. Tomme et al. (1995a) reported that the replacement of the family II CBD in the bacterial mixed-function xylanase-exoglucanase, Cex, with family I CBD resulted in slightly decreased binding and activity of the protein on bacterial microcrystalline cellulose, but a clearly enhanced activity on soluble cellulose and xylan. Likewise, swapping of the family I CBD of *T. reesei* CBHI with higher affinity *T. reesei* EGI CBD significantly increased the binding ability of the fusion protein, but did not have any dramatic effect on its activity or synergistic investigation (Srisodsuk et al., 1997). The effect of different cellulose-binding domains on the enzymatic activity of *C. thermocellum* endoglucanase (CelD) towards various cellulosic substrates suggested that the sites made

available for cellulose hydrolysis by each of the CelD-CBD complexes are different or at least partially non-overlapping (Carrard et al., 2000). These reports suggest that the CBDs are interchangeable to a certain degree and that the structural relationship with the associated catalytic domains acts as a prime factor in determining their maximum activity. It is important to mention that another mechanism has been proposed for the CBD, in which the CBD may guide a cellulose chain into the active site of the catalytic module, thereby increasing the catalytic activity (Sakon et al., 1997; Irwin et al., 1998). Further, it was also found that the CBD was able to bestow an extended or modified cellulolytic activity on CBD-fused *S. cerevisiae* exoglucanases and *Saccharomycopsis fibuligera* β-glucosidase to act upon avicel cellulose particles (Gundllapalli et al., 2005).

2.3.2 Effect of the linker domain on the function of the CBD

The enzymatic activity of many different cellulolytic enzymes is also affected by the deletion, shortening or lengthening of the linker region between the CBD and the catalytic domain (Shen et al., 1991; Gilkes et al., 1992; Srisodsuk et al., 1993; Wilson et al., 1995; Rixon et al., 1996; Black et al., 1997; Irwin et al., 1998; Quentin et al., 2002; Kataeva et al., 2002; Receveur et al., 2002). Such data suggest that the two domains act in concert on the cellulose surface during catalysis, and that relatively long linker regions with some flexibility are needed to express full cellulolytic activity. Recently, small-angle X-ray scattering was used to demonstrate this phenomenon of conformational flexibility between the linker domain and the fungal cellulose domain in *Humicola insolens* Cel45 (Receveur et al., 2002). Linker domain or fibronectin type 3 homology domains (Fn3), as found in the cellobiohydrolase of *C. thermocellum*, are common among bacterial extracellular glycohydrolases. The fibronectin-like linker domains have been demonstrated to significantly influence the binding ability and subsequent hydrolysis of acid-swollen cellulose and filter paper by the *Orpimyces* endoglucanase CelE enzyme (Kataeva et al., 2002). The interdomain linker region of many cellulolytic enzymes is sensitive to proteolytic digestion and, consequently, enzyme forms both with and without CBDs have been found in the culture supernatants of several cellulolytic organisms (Saloheimo et al., 1988; Stahlberg et al., 1988; Langsford et al., 1987; Brun et al., 1995; Blaak and Schrempf, 1995; McGinnis and Wilson, 1993). Some cellulolytic organisms have been shown to produce proteases, which specifically remove CBDs (Gilkes et al., 1988; Langsford et al., 1987), and it has been suggested that the presence or the absence of a CBD could also be controlled by differential splicing (Birch et al., 1995). It seems possible that cellulolytic enzymes with CBDs are required in the early stages of cellulose degradation, when most of the substrate is still insoluble (Saloheimo et al., 1988). Recent advances in techniques like atomic force microscopy (AFM) have permitted the experimental study of surface and inter-particle forces in the CBD-substrate interaction (Nigmatullin et al., 2004). AFM imaging revealed an agglomeration of the CBD adsorbed on

the cellulose surface. Despite an increase in surface charge owing to the CBD binding to the cellulose surface, the force profiles were less repulsive for interactions involving, at least, one modified surface. The technique significantly improved the insight into interfacial phenomena in traditional cellulose applications.

2.3.3 Adsorption ability of the CBD

CBDs potentiate the action of cellulolytic enzymes on insoluble substrates. Adsorption to cellulose is a major characteristic feature of the cellulose-binding domain that mediates this action. Numerous studies have established that the aromatic residues on the CBD surface contribute to binding affinity and the question whether this adsorption is reversible or irreversible has often been raised. It is known that, once intact cellulolytic enzymes have adsorbed to cellulose, quite harsh conditions can be needed for their dissociation (Reese, 1982; Chanzy et al., 1984; Beldman et al., 1987). Since the isolated catalytic domains have been easier to elute, it has been concluded that it is the CBD that causes the irreversible binding (Henrissat, 1994; Din et al., 1995). In some studies, the binding of the isolated CBD has indeed appeared irreversible (Ong et al., 1989; Nidetzky et al., 1994a; Millward-Sadler et al., 1994; Palonen et al., 1999), and it has been proposed that the binding of the CBDs occurs through denaturation, leading to irreversibility (Henrissat, 1994). Many models of enzymatic cellulose hydrolysis are based on the adsorption behaviour of the cellulolytic enzymes (Beldman et al., 1987; Beltrame et al., 1982; Kurakake et al., 1995; Nidetzky et al., 1993; Ooshima et al., 1991; Stahlberg et al., 1991). In contrast to the previous assumption of irreversibility, most of the models are derived using the assumptions that cellulase adsorption is a reversible process and that the rates of adsorption and desorption are in equilibrium. These assumptions have been tested experimentally, with conflicting results (Beldman et al., 1987; Beltrame et al., 1982; Stahlberg et al., 1991; Kyriacou et al., 1989; Nidetzky et al., 1994a, b). The reversibility of cellulase binding has been analysed by several investigators, with Kyriacou et al. (1989) using fractionated cellulolytic enzymes from *T. reesei*, and Beltrame et al. (1982) using *T. reesei* crude cellulose, finding adsorption to be an irreversible process. In contrast, Beldman et al. (1987) found cellulase adsorption to be partially reversible. They reported a reversibility range of 27-58% for four endoglucanases from *T. viride* and 75% reversibility for *T. viride* Exo III. Wald et al. (1983) also reported that the adsorption process was reversible. Nidetzky et al. (1994a) conducted reversible adsorption studies with *T. reesei* CBHI, concluding that the partial reversibility of cellulolytic enzymes was due to its bifunctional organisation, a core protein consisting mainly of a catalytic domain linked to a cellulose-binding domain. In fact, the enzyme should undergo a dynamic process of binding and desorption of both domains, allowing processive hydrolysis and/or relocation to new enzymatically accessible sites on the solid substrate surface. The rapid decrease in the degree of polymerisation that is clearly observed in the substrate upon endoglucanase

activity must depend on the enzyme's ability to move (Kleman-Leyer et al., 1994, 1996; Medve et al., 1998; Kotiranta et al., 1999). In the case of the cellobiohydrolases, an irreversibly bound CBD would not allow a processive mode of action, which has been proposed to be a key determinant of their efficient action on the recalcitrant substrates (Divne et al., 1994; Davies and Henrissat, 1995; Harjunpaa et al., 1996; Teeri, 1997; Gilkes et al., 1997; Medve et al., 1998; Kotiranta et al., 1999; Palonen et al., 1999; Tuohy et al., 2002).

It was also proposed recently that the CBDs might behave differently depending upon the catalytic domain they are associated with, thus changing the micro-environment of the enzyme (Carrard et al., 2000). The molecular exchange of the cellulase at the adsorbent surface is another important assumption used in deriving cellulase adsorption models. Surface exchange refers to the dynamic interchange of adsorbed and free cellulolytic enzymes without changing the equilibrium. Kyriacou et al. (1989), using labelled *T. reesei* cellulase fractions, demonstrated the interchange between bound and free cellulase. Moreover, clear cooperativity in the binding interactions has been demonstrated using a double CBD serving as a simplistic model of an intact cellulase (Linder et al., 1996).

In has been suggested (Linder and Teeri 1997) that the diversity of the substrates and the enzymes, as well as the complexities of designing an appropriate experimental system, might have at least contributed in part to the observation of reversible and irreversible binding among the CBD's. Firstly, the variation in the substrates used for the experiment would have offered different binding surfaces for the cellulolytic enzymes used in the binding studies (Beldman et al., 1987: Nidetzky et al., 1993, 1994a; Din et al., 1994a; Linder et al., 1996; Linder and Teeri, 1996; Bothwell et al., 1997). Secondly, similar or differential binding abilities exhibited by both the catalytic and the CBD's in the intact cellulolytic enzymes tend to display an apparent reversibility or irreversibility over the cellulose surface (Stahlberg et al., 1991; Srisodsuk et al., 1993; Bothwell et al., 1997; Carrard et al., 2000).

2.3.4 The CBD as thermostabilising domain

It has been reported previously that many proteins consist of multiple domains or motifs and it has been suggested that the domains interact and affect properties of each other (Wenk et al., 1998; Jaenicke, 1999; Kataeva et al., 2001) and that the domains are not randomly combined (Hayashi et al., 1997). The stability of a protein has always been endowed as an intrinsic property of the whole molecule (Ljungdahl and Sherod, 1976; Ljungdahl 1982). However, domain analyses of various proteins, particularly cellulolytic enzymes, have shown that these domains play a vital role in the thermostability of these enzymes and they were subsequently termed the "thermostabilising domain" (Riedel et al., 1998b; Kataeva et al., 2001). Indeed, recent findings indicate that at least two such domains, one from *Caldibacillus cellulovorans* XynA and an internal domain, X6b, from *C. thermocellum* XynY, are classified

as carbohydrate-binding domains (Charnock et al., 2000). In addition, the characterisation of the *xynX* gene encoding a multidomain xylanase from *C. thermocellum* revealed the presence of a thermostabilising domain (Kim et al., 2000). The characterisation of a cellulose-binding β-glucosidase from cellulose-degrading cultures of *P. chrysosporium* assigned a thermostabilising role for the CBD (Lymar et al., 1995). Our recent work also demonstrated the thermostablising role of the *T. reesei* cellulose-binding domain in the fusion enzymes of *S. cerevisiae* exoglucanases and also in the *Saccharomycopsis fibuligera* β-glucosidase (Gundllapalli et al., 2005).

2.4 Applications of the CBD in biotechnology

Cellulose is the most abundant natural polymer and it is used extensively as a renewable raw material for many products in paper, wood and textile manufacturing. A distinctive structural feature of cellulose materials is heterogeneity, owing to its fibre structure. Cellulose materials form three-dimensional networks with the inclusion of other molecular and particulate additives. The insolubility of cellulose means that technological processes are also based on colloid systems, for example fibre suspension in papermaking. Therefore, interfacial interactions involving cellulose are of particular interest for cellulose material production and utilisation. These interactions not only govern processes of manufacturing, recycling, coating, etc., but also determine the physical properties of cellulose-based products. Recent advances in technology, along with growing interest in new cellulose applications often involving biomolecules or even cells and tissues (drug delivery devices (Urquhart, 2000), affinity separation (Gemeiner et al., 1998) and tissue engineering (Pajulo et al., 1996)), have led to the need for an advanced understanding of the interfacial phenomenon involving complex multicomponent systems based on cellulose polymers.

Non-covalent-specific binding plays an important role in biological processes and is increasingly applied in biotechnology. Specific interactions involving a cellulose matrix can be mediated by CBDs, the auxiliary domains that have been found in cellulose- and hemicellulose-degrading enzymes and provide enzyme binding to the cellulose. The high affinity and stability of specific CBD/cellulose binding have stimulated endeavours to exploit this interaction for cellulose surface modification and the biotechnological application of cellulose. Biotechnological applications also benefit from the fact that isolated CBDs preserve their affinity for cellulose. In addition, CBDs can be fused with enzymes, proteins or polypeptides, producing hybrid biomolecules, which are capable of selective binding to cellulose matrix (Linder et al., 1998).

2.4.1 CBDs as affinity tags

The purification and recovery of biologically active molecules are important aspects of

biotechnology and are a major consideration in the design of fermentation processes. The continued growth and maturation of the pharmaceutical and biotechnology industries has created an increasing need for practical and cost-efficient large-scale processing techniques. The challenges of producing ever-increasing amounts of recombinant biomolecules of extremely high purity and bioactivity from complex backgrounds of contaminants have inspired the development of new or improved separation techniques. Biospecific affinity purification holds an important place in the repertoire of protein and peptide purification techniques and is becoming increasingly popular with the discovery of more specific and efficient affinity tags (Uhlen and Moks, 1990; Uhlen et al., 1992; La Vallie and McCoy, 1995). Besides allowing the affinity purification of the target polypeptides, these affinity tags can also increase the *in vivo* proteolytic stability, modulate the solubility or control the cellular localisation of the target polypeptides in the expression host. In addition, these tags can be used for immobilization of biologically active molecules in continuous bioprocessing applications. To date, numerous affinity tags have been developed (Ford et al., 1991; Nilsson et al., 1992; Terpe, 2003). These vary from whole proteins and protein domains to poly- or single amino acid residues. Some of the more commonly used tags include *Staphylococcus aureus* protein A (Moks et al., 1986; Nilsson et al., 1987), *Schistosoma japonicum* glutathione S-transferase (Smith and Johnson, 1988), maltose-binding protein MalE from *Escherichia coli* (Bedouelle and Duplay, 1988), S-peptide from ribonuclease A (Kim and Raines, 1993), biotin (Cronan, 1990; Wilchek and Bayer, 1988), streptavidin (Sano and Cantor, 1991) and polyamino acid affinity tags (Porath et al., 1975; Hochuli et al., 1988). Although each affinity system offers particular advantages, many share the common drawback of the high cost of the affinity matrix and support components, which limits the use for large-scale applications.

Cellulose, on the other hand, is an attractive and ideal matrix for enzyme immobilisation and for large-scale affinity purposes, mainly because of factors such as its cost effectiveness, excellent physical properties, inertness and low, non-specific affinity for most proteins (Wilchek and Chaiken, 2000; Lowe, 2001). Cellulose is also commercially available with various degree of polymerisation. CBDs therefore constitute an excellent immobilisation tool for linking proteins to cellulose (Bayer et al., 1994; Shoseyov and Warren, 1997; Tomme et al., 1998; Linder et al., 1998; Saleemudin, 1999). Tomme et al. (1998) described the characterisation and use of cellulose-binding domains as alternative and highly versatile tags for affinity applications based on their high and specific affinity for cellulose and the related polysaccharide, chitin.

Many applications using CBD-fused proteins have been developed, including CBD-protein A for IgG purification (Ramirez et al., 1993), CBD-streptavidin for biotinylated molecule applications (Le et al., 1994), and CBD-alkaline phosphatase (Greenwood et al., 1989). These studies have shown that the CBD is a useful affinity tag for immobilising

proteins to cellulose. Cellulose-binding domains have also been used to create a β-glucosidase bioreactor (Ong et al., 1991). The feasibility of employing the CBD as an affinity tag has been reported on a number of occasions: lipase B (Ahn et al., 2004; Rotticci-Mulder et al., 2001), glucoamylase (Jiang and Radford, 2000), organophosphorus hydrolase (Wang et al., 2002; Richins et al., 2000), α-amylase (Bjornvad et al., 1998) and heparinase I (Shpigel et al., 1999) have been expressed as CBD-fused enzymes while retaining their high specific activity. Furthermore, phytochelatins EC20 (Xu et al., 2002), protein A (Maurice et al., 2003; Shpigel et al., 2000), human T cell connective tissue activator peptide III (CTAP-III) (Rechter et al., 1999), human hsp60 epitope (Shpigel et al., 1998a), stem cell factor (SCF) (Fig. 8) (Boraston et al., 2001; Doheny et al., 1999) have also been functionally expressed as CBD-fused proteins.

The purification and immobilisation of biologically active proteins are of great importance to industry and research. CBD fusions facilitate the low cost purification of target proteins by allowing binding to an inert support such as cellulose and chitin. In addition, the CBD fusions bound to cellulose particles may not require the addition of adjuvant to elicit antibody responses. On the basis of the binding and elution characteristics of CBDs and fusion proteins, different expression vectors have been designed for the production of CBD-fusion proteins. Graham et al. (1995) and Hasenwinkle et al. (1997) constructed an expression vector for C- and N-terminal CBD-fused proteins (pTugA and PtugK) based on CBD_{Cex} from *C. fimi*. Novagen has utilised this technology to introduce a new series of expression vectors, such as pET and pBAC, which incorporate CBDs as their fusion tags (Fig. 9) (Novy et al., 1997). A CBD-tagged rabbit polyclonal antibody incorporated with CBD derived from *Clostridium cellulovorans* and suitable for the detection of proteins expressed in pET and pBAC have also been developed (Novagen). These studies established the use of CBDs in fusion proteins as tags for affinity purification or immobilisation, thus illustrating their technical applications.

2.4.2 The CBD as an immobilisation tool

Cell surface engineering of microbes has attracted a lot of attention in recent years, with applications in a wide range of processes and technology, from immunology and vaccinology to biotechnology. Currently, environmental microbiology in particular attracts a lot of attention, since genetically-engineered immobilised cells might be usable as bio-adsorbents for the sequestration of toxic metals and compounds. Although easy to perform, cell immobilisation by simple adsorption has serious drawbacks. Changes in pH, temperature or ionic strength can easily release the cells from the matrix. CBDs provide a new class of immobilisation tags with appealing attributes. The specific adhesion of whole cells to cellulosic materials with high affinity has been demonstrated by anchoring the CBD from *C. fimi* on the surface of *E. coli* (Fig. 10) (Francisco et al., 1993; Wang et al., 2001). Recently, a

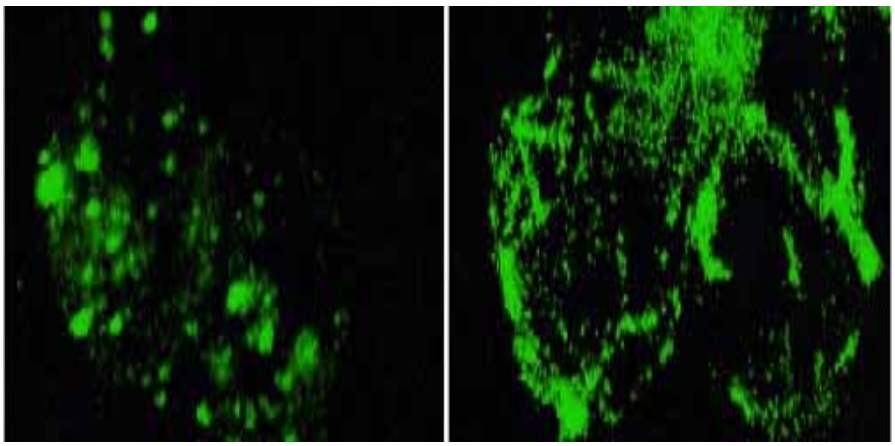

Fig. 8 The cellulose-binding domain used in the affinity targeting of cytokines (SCF) into the cells. Immunofluorescent laser scanning confocal microscopy of cells stimulated with BMCC-bound SCF-CBD_{Cex} (Doheny et al., 1999).

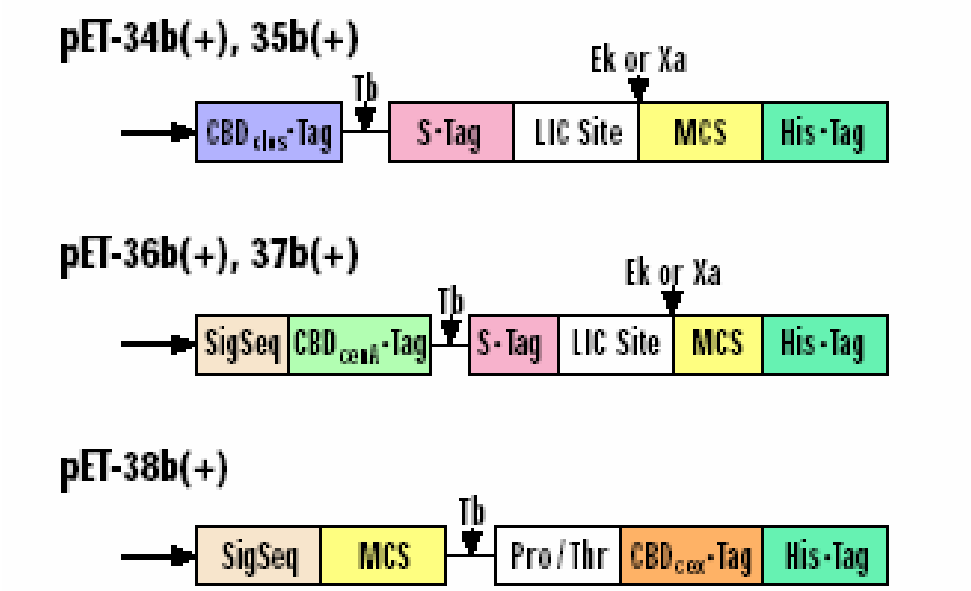

Fig. 9 pET CBD vectors containing three CBD sequences (Novy et al., 1997, *in**Nova**tions 7, Novagen, Inc.*).

fungal CBD derived from the cellobiohydrolase Cel7A of *T. reesei* was surface-displayed to generate nickel-binding *Staphylococci* strains (Wernerus et al., 2001). Similarly, the CBD from cellobiohydrolase I and cellobiohydrolase II of the filamentous fungus *T. reesei* was expressed on the cell surface of the yeast *S. cerevisiae*, which showed an improved affinity towards cellulosic substrates (Jin-Min Nam et al., 2002). These cell surface-engineered strains of *S. cerevisiae* have been shown to display a significant improvement in the fermentative bioprocessing of cellulosic substrates. Using the CBD, *E. coli* and *Staphylococci*

strains with increased ability to bind various other toxic metal ions, such as cadmium, have also been generated (Xu et al., 2002). In addition, *E. coli* cells expressing both organophosphorus hydrolase (OPH) and a CBD on the cell surface enabled the efficient hydrolysis and detoxification of environmentally toxic organophosphorous compounds (Wang et al., 2002).

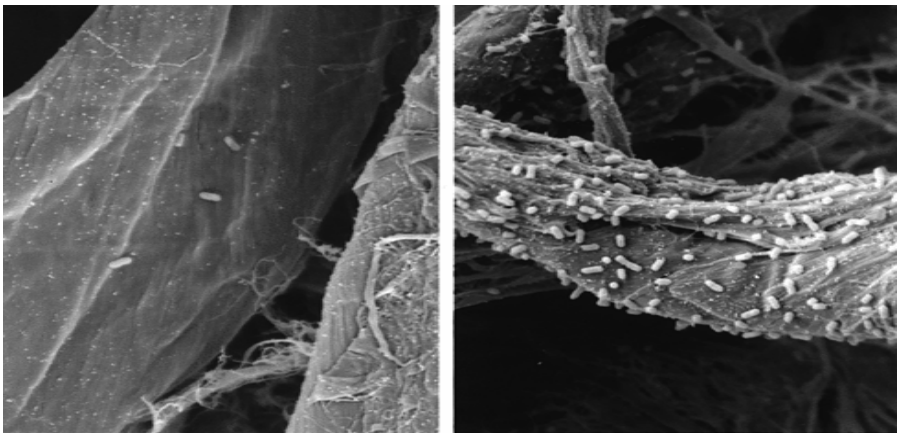

Fig. 10 Scanning electron micrograph demonstrating whole-cell immobilisation using cell surface-exposed cellulose-binding domain. Cellulosic fabric or Whatman filter paper was immobilised with *E. coli*. (Wang et al., 2001).

In a different study, *Staphylococcus carnosus* was chosen to display CBD_{cel6A} from *T. reesei* on its cell surface and the addition of the CBD predisposed the anchoring of bacterial cells to cotton fibres (Lehtio et al., 2001). A new strategy for cell immobilisation with immense application in biomedical research was demonstrated by fusing the cell attachment peptide, RGD (argninine-glycine-aspartic acid), to the CBD for improved cell adhesion (Hsu et al., 2004, Wierzba et al., 1995). Stem cell factor immobilised onto cellulose via the CBD_{Cex} from *C. fimi* is more potent in stimulating the proliferation of factor-dependent cell lines when compared to the soluble unbound growth factor (Doheny et al., 1999). Furthermore, a recombinant form of the outer-membrane protein A associated with atypical *Aeromonas salmonicida* fused to a cellulose-binding domain and immobilised on cellulose particles served as an adjuvant for the parenteral vaccination of fish (Maurice et al., 2003).

Phage display technology has proven to be a powerful tool for the isolation and in vitro evolution of biologically important molecules. However, the general usefulness of this technology is still limited and one of the most debilitating obstacles to the widespread application of the technology is the accumulation of insert loss clones in the libraries. A novel phage-display technology developed by Berdichevsky et al. (1999) employed the fusion of the CBD from *Clostridium thermocellum* to a single chain antibody Fv (scFv) and expressed this as an in-frame fusion protein. The CBD domain served as an immobilising tag, allowing

rapid phage capture and concentration of the recombinant antibodies, thus increasing the efficiently of the screening process. Tailor-made, metal-binding *staphylococcus was* created using a phage display combinatorial library based on a fungal CBD derived from *T. reesei* (Wernerus et al., 2001). The phage display technique was also used to screen for peptides that enable the indirect immobilisation of proteins to a solid surface via the CBD. This technique enabled the production of active protein molecules that are commonly, partially or totally denatured on direct passive coating to surfaces (Levy and Shoseyov, 2002b).

2.4.3 The CBD as a diagnostic tool

Most pathogenic protozoan parasites form a cyst wall that is resistant to environmental stress. Chitin and cellulose are the carbohydrate polymers conveying the required structural toughness to the cyst wall. A recombinant cellulose-binding protein consisting of two CBDs from *T. reesei* cellulolytic enzymes linked together in combination with anticellulase antibody and anti-mouse immunoglobulin fluorescein conjugate was used as a selective immunocytochemical marker for cellulose in protozoa. This demonstrates the potential of CBD as a diagnostic tool for the detection of pathogenic protozoan contaminants (Fig. 11) (Linder et al., 2002). Furthermore, Siegel and Shoseyov (2001) developed a system based on the CBD that enables the rapid detection of pathogenic microbes in food samples. In this method, the CBD is conjugated to a bacteria-binding epitope-specific monoclonal antibody and is loaded onto a cellulose matrix that acts as a cell concentrator. The bound bacteria can later be eluted and identified. The CBD was also used in the development of a novel bifunctional starch-cellulose cross-bridge protein (CSCP) that was comprised of a CBD from *C. cellulovorans* (CBD_{Clos}) and a starch-binding domain from *A. niger* B1 (SBD_{Asp}). This new class of chimeric polysaccharide-binding domains demonstrates cross-bridging ability in different model systems and has tremendous potential for use in a wide range of biomaterials, with applications in biomedicine (e.g. tissue engineering) and drug delivery systems (Levy et al., 2004).

2.4.4 The CBD in affinity targeting

Cellulose is a major constituent of many commercial products, making the targeting to it of functional molecules of great economic significance. TheCBD, with its high affinity for cellulosic substrates, has been shown to have great potential in this context. In the 1980s, cellulolytic enzymes were employed as an alternative to the original abrasive stones for manufacturing stone-washed denim. The use of enzyme mixtures was problematic, since some of the enzymes present in the mixtures contained cellulolytic activity towards insoluble cellulose and this occasionally caused a decrease in fibre strength (Cavaco-Paulo, 1998a; 1998b). The presence of the CBD overcame this drawback by allowing the localised targeting of enzymes such as pectate lyases onto the garment, resulting in a stone-washed appearance (Andersen et al., 2001). The strong affinity that exists between cellulose and the

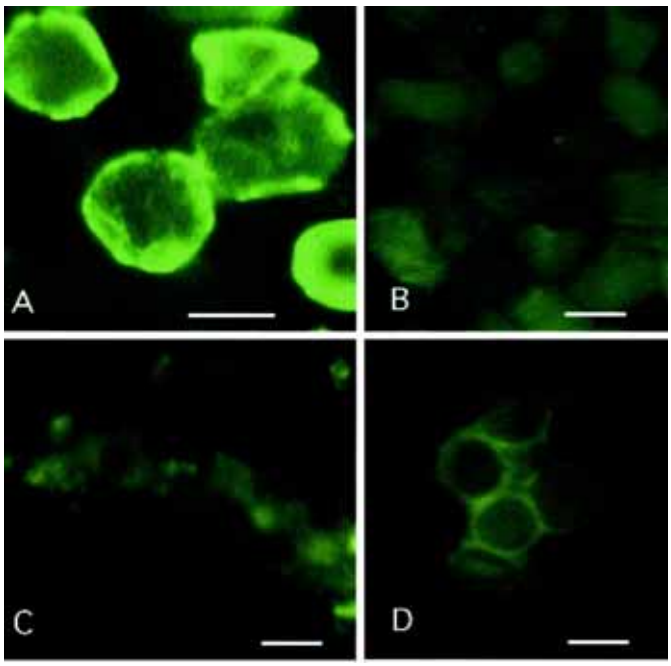

Fig. 11 Staining for cyst wall cellulose in frozen sections of different protozoan cysts using the D-CBD procedure (Linder et al., 2002).

CBD is used in many applications associated with the textile industry. The performance of the recombinant enzymes and other fragrance-bearing particles used in numerous laundry powders can be improved by fusion to CBDs, thereby increasing their affinity for the textile substrate (Von der Osten et al., 2000b; Berry et al., 2001). In the weaving industry, the enzymatic desizing process whereby fabrics are softened prior to the dyeing process is significantly improved by the fusion of the CBD to the target enzymes employed (Von der Osten et al., 2000a; Cavaco-Paulo et al., 1999). Emerson et al. (1998) proposed that antimicrobial agents, such as aldehydes or alcohols, could be effectively targeted to polysaccharide materials with the assistance of the CBD and may be useful for directly impregnating surfaces such as paper or wood. The application of CBD in oral care products was established after the finding that CBD removed and prevented plaque formation by dispersing the oral polysaccharides involved in dental plaque, such as fructan and glucan. In addition, it was also established that the CBD could be employed safely in improved plaque removal by fusing it to enzymes that are capable of dental plaque degradation (Fuglsang and Tsuchiya, 2001). A new direction in the function of CBD as an inducer of biocontrol enzymes has been proposed, in which the addition of CBD from *T. reesei* to the catalytic domain of chitinases from *Trichoderma harzianum* significantly increased the antifungal capabilities of these enzymes (Limon et al., 2004). This introduces an innovative course in the application of CBD as a biocontrol agent.

2.4.5 The CBD in protein expression

The controversial beneficial effect of the CBD on the expression of proteins has been demonstrated in several studies. A new application for the CBD as an enhancer of the secretion of different heterologous proteins was demonstrated in the yeast *S. cerevisiae*. It was found that the secretion of CBD-fused industrial enzymes, such as *Bacillus stearothermophilus* lipase 1, lipase B and cyclodextrin glucosyltransferase, was enhanced significantly when compared with that of the mature proteins without CBD (Ahn et al., 2004). Other studies have also shown that, for the most part, expressing foreign proteins fused to CBD result in high expression levels (Rotticci-Mulder et al., 2001; Levy et al., 2001; Boraston et al., 2001; Sphigel et al., 2002; Richins et al., 2000; Kauffmann et al., 2000). Replacing the CBD of endo-1,4-β-glucanase from *Bacillus subtilis* with the CBD of exoglucanse I from *T. viridae* resulted in high expression levels in *E. coli* (Kim et al., 1998). Similar results were reported by Otomo et al. (1999).

2.4.6 The CBD in plant biotechnology

Several studies were performed to analyse the effect of the CBD on the living plant cell. It was found that family III CBD from *C. cellulovorans*, at low concentrations, enhanced the elongation of *Prunus persica* pollen tubes and *Arabidopsis thaliana* root seedlings, whereas, at high concentrations, the CBD inhibited root elongation in a dose-dependent manner (Atalla et al., 1993; Hayashi and Ohsumi, 1994; Hackney et al., 1994; Whitney et al., 1995). It was also shown that family III CBD could modulate the growth of transgenic plants and also significantly increased the biomass production and wood characteristics in selected clones when compared with wild-type control plants (Shoseyov et al., 2001). The transgenic clones showed a significant increase in the fibre cell length and also in the average degree of polymerisation of cellulose. Paper manufactured from these fibres resulted in properties such as an increase in burst, tear and tensile indices (Shoseyov et al., 2001; Levy et al., 2002a). It was also demonstrated that the direct application of individual CBD in the manufacturing process could modify paper properties. Fusing two CBDs (family III CBD_{Clos} derived from *C. cellulovorans*) and applying them to paper resulted in improved mechanical properties, such as tensile strength and brittleness. Applying a single CBD molecule to the paper also improved its mechanical properties, but to a lesser extent. In addition, paper treated with the double CBD molecule became more hydrophobic and demonstrated water-repellent properties (Levy and Shoseyov, 2002a).

2.5 Conclusion

Cellulose is an abundant biopolymer on earth. Its excellent chemical and physical properties have made it a very useful component in countless applications. Cellulose-based polymers have a range of properties that make them suitable for use in a wide array of biomedical

applications, ranging from tissue engineering to drug delivery systems. In all these applications, a better understanding, fine-tuning and control of the CBD functions could lead to improved technical breakthroughs required for economical feasibility. Furthermore, the large body of biochemical, functional and structural data available on selected CBDs and the vast number of unexplored and uncharacterised families of CBDs in nature with immense potential properties and functions suggest that CBDs can be manipulated and customised for a wider range of applications in industry and medicine. Exploiting the immense potential of the CBD in its entirety would realise the vision as quoted recently in 2004 by the company CBD technologies in the website Globes online that "CBD could dictate to the cellulose: expand the tree trunk, add starch to potato, grow exceptionally juicy tomato, increase the oily component in corn; and even intervene in the laundry process, accelerate diagnostic processes, separate antibodies, and speed up and reduce the price of pharmaceutical processing through chemical conjugation of biological processes".

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CHAPTER 3

RESEARCH RESULTS I

Domain engineering of *Saccharomyces cerevisiae* exoglucanases

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Domain engineering of *Saccharomyces cerevisiae* exoglucanases

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1. Abstract

To illustrate the effect of a cellulose-binding domain (CBD) on the enzymatic characteristics of non-cellulolytic exoglucanases, 10 different recombinant enzymes combining the *Saccharomyces cerevisiae* exoglucanases, EXG1 and SSG1, with the CBD2 from the *Trichoderma reesei* cellobiohydrolase, CBH2, and a linker peptide were constructed. The enzymatic activity of the recombinant enzymes increased with CBD copy number. The recombinant enzymes CBD2-CBD2-L-EXG1 and CBD2-CBD2-SSG1 exhibited the highest cellobiohydrolase activity of 17.5 and 16.3 U mg^{-1} respectively on avicel cellulose, which is approximately 1.5- to 2- fold higher than the native enzymes. The molecular organisation of cellulose binding domain in these recombinant enzymes enhanced substrate affinity, molecular flexibility and synergistic activity, contributing to their elevated action on the recalcitrant substrates as characterised by adsorption, kinetics, thermostability and SEM analysis.

2. Introduction

Cellulolytic microorganisms produce at least three major types of enzymes: endoglucanases, exoglucanases and β-glucosidases. Most cellulolytic enzymes have a conserved tripartite structure with a large catalytic core domain linked by a *O*-glycosylated peptide to a cellulose-binding domain that is required for the interaction with crystalline cellulose. Cellulose binding domain (CBD) mediates the binding of the cellulolytic enzymes to the substrate, thereby playing a fundamental role in cellulose hydrolysis (Tomme *et al*. 1995). It has been suggested that the CBD enhances the enzymatic activity of cellulolytic enzymes merely by reducing the dilution effect of the enzyme at the substrate surface, by promoting the solubilisation of single glucan chains from the cellulose surface or possibly by helping to loosen individual cellulose chains from the cellulose surface prior to the actual hydrolysis of

the polymer. Most information on the role of CBDs has been obtained by removal, domain exchange, site-directed mutagenesis or by artificial addition of the CBD (review by Linder & Teeri 1997). It thus seems that the CBDs are interchangeable to a certain degree, but much more data are needed on different catalytic domain-CBD combinations to elucidate the exact functional role of the CBDs. In addition, the shortening, lengthening or deletion of the linker region between the CBD and the catalytic domain also affects the enzymatic activity of different cellulolytic enzymes (Srisodsuk *et al.* 1993).

Saccharomyces cerevisiae possesses four different glucanases, namely EXG1, BGL2, SSG1 and EXG2, which do not exhibit the same architectural domain organisation showed by most of the other fungal or bacterial cellulolytic enzymes. These enzymes display β-1, 3-exoglucanase activities as their major activity and exhibit a significant β-1,4-exoglucanase side-activity on disaccharide substrates such as cellobiose, releasing free glucose moieties (Larriba *et al.* 1995).

In this study, the domain engineering of two *S. cerevisiae* exoglucanases, EXG1 and SSG1, was performed to link these two enzymes to the CBD2 domain of the *Trichoderma reesei* CBHII cellobiohydrolase in an attempt to investigate whether the CBD would be able to modulate these non-cellulolytic domains to function in cellulose hydrolysis. The engineered enzymes were constructed to display different modular organisations with the CBD, either at the N-terminus or the C-terminus, in single or double copy, and with and without the synthetic linker peptide, in order to mimic the multi-domain organisation displayed by cellulolytic enzymes from other microorganisms. The characterisation of the physicochemical and catalytic properties of these multidomain recombinant enzymes was performed using various cellulose and non-cellulose substrates.

3. Materials and methods

3.1 Strains and plasmids

Escherichia coli HB101 [*F-hsdS20ara-1 recA13 proA12 lacY1 galK2 rspL20 mtl-1xyl-5*] was used as an host for the cloning and expression of the recombinant enzyme gene constructs, using pGEM@-T Easy plasmid (Promega) (Lonn *et al.* 2002), and as the host for the derivative cloning vectors. A modified version of vector YEp352 (Van Rensburg *et al*. 1996) was used for the sub cloning of the different fusion derivatives of the *S. cerevisiae EXG1* and *SSG1* genes. The region of the *CBH2* gene of *T. reesei* encoding the cellulose binding domain (*CBD2*) was obtained from the plasmid pAZ21. *Saccharomyces cerevisiae* VIN7 strain (a commercial wine yeast strain) was used as the source of genomic DNA for the amplification of both the *EXG1* and *SSG1* genes.

3.2 Cloning and construction of the recombinant *EXG1* and *SSG1* genes

The various genetic constructs, which led to the production of native and recombinant enzymes of EXG1 and SSG1, are summarised in Table 2. The lists of primers used are detailed in Table 1. The region of the *CBH2* gene of *T. reesei* between nucleotides 73 and 186, which encodes the cellulose binding-domain (CBD2), was amplified by the polymerase chain reaction (PCR) method with primers CBD-F and CBD-R, to facilitate insertion at the N-terminus. In a similar way the CBD2 domain encoding region was amplified with the primers CBD'-F and CBD'-R, to facilitate the in-frame fusion of the CBD at the C-terminal position. The fragment of the *EXG1* gene (nucleotides 121 to 1347), which encodes the mature EXG1, was amplified using the primers EXG1-F and EXG1-R. In addition, a PCR product using the primers EXG1-F and EXG1'-R was generated to facilitate in-frame fusion to the CBD at the C-terminus. The region between nucleotides 4 and 1347 encoding the mature EXG1 together with the signal peptide (used in this study as inter-domain linker) was amplified using primers L-EXG1-F; EXG1-R and primers L-EXG1-F; EXG1'-R to facilitate the fusion of CBD2 at N- and C- terminus respectively. The region of the *SSG1* gene between nucleotides 4 and 1335, encoding the mature SSG1, was amplified by PCR with the primers SSG1-F and SSG1-R. The PCR reaction contained 1 x PCR buffer, 1.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 2 ng μL^{-1} template and 3.5 U DNA polymerase (Roche) in a total volume of 100 μL. PCR programme is as follows: denaturation, 2 min at 94°C; primer annealing, 30 s at 50°C; and primer extension, 2 min at 68°C. Standard methods of DNA manipulation techniques were followed. Both the coding and non-coding strands were sequenced to ensure the reliable identification of all constructs.

3.3 Enzyme expression, preparation and purifications

Growth conditions, expression and enzyme preparation was performed as described in Lönn *et al.* (2002). Purification of the native and chimeric enzymes was performed as described in Suzuki *et al.* (2001) with slight modification. The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard.

3.4 Preparation of substrates

Avicel and amorphous cellulose were purchased from FMC Co., USA. Carboxy methyl cellulose, laminarin and barley β-glucan were obtained from Sigma. BMCC (bacterial micro crystal cellulose) was obtained from Cellulon (Weyerhaeuser, WA). Avicel, amorphous cellulose and BMCC were weighed out, washed with distilled water and filtered as described by the supplier.

3.5 Enzyme and adsorption assay

The purified native and recombinant enzymes were analysed for their exo-1, 3-β-glucanase activity as described in van Rensburg *et al.* (1997), with little modification. One unit of β-glucanase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar equivalents (expressed as glucose) per minute at pH 5.0 and 55 °C, using β-D-glucan (Sigma).

The cellobiohydrolase activity was assayed using the two-step standard assay with little modification (Boisset *et al.* 2001). Reactions were carried on in 2mL O-ringed screw cap microcentrifuge tubes containing enzyme preparations in synergism with the commercial enzymes, endoglucanase EGII (*Trichoderma longibrachiatum*) and β-glucosidase (*Aspergillus niger*) (Megazyme), in a reaction volume of 750 µL with 1.0 g L^{-1} of substrate (avicel, amorphous, BMCC or CMC) in 50 mM sodium citrate buffer (pH 4.8). The reaction mixes were incubated at 50°C with end-over-end rotation at 40 rpm for 16 h. The samples were filtered through 0.45 µm cellulose acetate filters and analysed for free glucose produced by glucose-oxidase-based method (GAGO kit, Sigma). Each reaction mix was done in triplicate. The unit cellobiohydrolase activity of the native and recombinant EXG1 and SSG1 enzymes were calculated from the standards, performed using purified cellobiohydrolase enzyme from *Trichoderma longibrachiatum* (Megazyme) using three different substrates as avicel, amorphous, BMCC and CMC cellulose separately, under similar assay conditions and expressed as cellobiohydrolase units.

Adsorption assays were performed in a similar manner as described previously by Gal *et al.* (1997). Enzyme preparations of native and recombinant enzymes were incubated with 1 g·L^{-1} of Avicel in 50 mM sodium citrate buffer (pH 4.8), in a final reaction volume of 750 µL, in an end-over-end rotation at 40 rpm. The incubation temperature was maintained at 4°C for 16 h and the preparations were then centrifuged at 5000 × *g* for 15 min. In the binding assays, the free protein fraction was estimated from the residual enzymatic activity in the supernatant. This was subtracted from the initial enzyme activity to estimate the substrate-associated fraction.

3.6 Kinetic parameters

The Michaelis-Menten parameters, V_{max} and K_m were determined for all the recombinant enzymes from Michaelis-Menten plots of specific activities at various substrate concentrations. Rates were measured in triplicate for six to ten concentrations of Avicel, and these generally ranged from 0.2 to 5 times the value of K_m. The values of V_{max} and K_m were determined by nonlinear regression analysis, using the Graph Pad Prism program.

3.7 pH and Temperature profile

The effect of pH on the activity of the native and recombinant enzymes was investigated in the pH ranges 3 - 7, using 1 g L^{-1} Avicel in 50 mM sodium citrate buffer (pH 4.8) at 50°C. The temperature profiles for the native and recombinant enzymes were measured at temperatures between 30 and 60°C, under similar assay conditions.

3.8 Thermostability

The native and recombinant proteins were incubated in 50 mM of sodium citrate buffer (pH 4.8) at 50°C. At regular time intervals, aliquots were taken and centrifuged and the supernatant solution was assayed for residual activity.

3.9 Specimen preparation for scanning electron microscopy (SEM)

The electron micrographs were taken after 16 h of digestion at 50°C at a pH of 6 with the native or the recombinant enzymes added in synergism with commercial endocellulase (*T. longibrachiatum*) and β-glucosidase (*A. niger*). Preparation of the cellulose (Avicel) samples treated with the recombinant enzymes was performed as previously described (Boisset *et al.* 2000). The specimens were examined using a fully analytical S440 Leo Scanning Electron Microscope operated at an accelerating voltage of 10 kV. All the images were visualised at a magnification of 1.00 K x.

4. Results

4.1 Purification and screening of the recombinant enzymes

The native and recombinant enzymes were purified and analysed by SDS-PAGE gels. The protein products ranged between the theoretical values of approximately 45 and 55 kDa (data not shown). Preliminary screening of recombinant enzymes was performed based on its ability to hydrolyze avicel cellulose (Table 2). The hydrolytic assays were performed as mentioned in the material and methods section. Based on the performance of hydrolysis 6 recombinant enzymes (highlighted in Table. 2) were chosen along with the native EXG1 and SSG1 as controls, for further study.

4.2 Hydrolytic activity on different substrates

The hydrolytic activity of the recombinant enzymes towards different cellulosic and non-cellulosic substrates was tested and tabulated (Table 3). It was observed in general that all the recombinant enzymes of EXG1 and SSG1 were able to act on cellulosic substrates and the pattern in the degree of hydrolysis was similar on all the substrates tested. However, the activity increased with the increasing copies of CBD in the recombinant enzymes. CBD2-CBD2-L-EXG1 and CBD2-CBD2-SSG1 display the highest hydrolytic activity among the

group of EXG1 and SSG1 enzymes respectively, followed by the single CBD fusions of these enzymes. In addition, the recombinant enzymes also showed a similar pattern of improved hydrolysis towards β-1,3-exoglucanase specific laminarin and β-glucan.

Table 1. Primers designed for PCR.

Primer	Oligonucleotide sequence[a]	Restriction site
CBD-F	5'-**aagctt**agcgtctggggccaa-3'	*Hin*dIII
CBD-R	5'-**aagctt**aagacactgggagtaata-3'	*Hin*dIII
CBD'-F	5'-a**ctcgag**agcgtctggggccaa-3'	*Xho*I
CBD'-R	5'-a**gtcgac**tcaaagacactgggagtaata-3'	*Sal*I
EXG1-F	5'-cc**atcgat**tactacgattatgaccacgg-3'	*Cla*I
L-EXG1-F	5'-a**atcgat**ctttcgcttaaaacgttactg-3'	*Cla*I
EXG1-R	5'-a**ctcgag**ttagttagaaattgtgccacat-3'	*Xho*I
EXG1'-R	5'-a**ctcgag**gttagaaattgtgccacatt-3'	*Xho*I
SSG1-F	5'-a**ggcgcc**gtttcgttcagagggc-3'	*Nar*I
SSG1-R	5'-a**ctcgag**atgacattggttaggat-3'	*Xho*I

[a] The restriction sites are indicated in bold.

Table 2. Construction and screening of recombinant enzymes.

Plasmids	Chimeric enzyme[a]	Avicelase activity (U mg $^{-1}$)[b]
pCEL71	**EXG1**	9.6 ± 0.7
pCEL72	CBD2-EXG1	9.5 ± 0.5
pCEL72	CBD2-CBD2-EXG1	4.3 ± 0.6
pCEL72	EXG1-CBD2	7.6 ± 0.4
pCEL72	**L-EXG1**	7.6 ± 0.4
pCEL72	**CBD2-L-EXG1**	14.1 ± 0.6
pCEL72	**CBD2-CBD2-L-EXG1**	17.5 ± 0.6
pCEL72	**L-EXG1-CBD2**	15.9 ± 0.8
pCEL72	**SSG1**	9.9 ± 0.5
pCEL72	CBD2-SSG1	6.4 ± 0.3
pCEL72	**CBD2-CBD2-SSG1**	16.3 ± 0.5
pCEL72	**SSG1-CBD2**	14.4 ± 0.6

[a] The highlighted enzymes are used for further characterisation.

[b] The values shown here are expressed as cellobiohydrolase units per mg of protein and are means of three independents assays ± accumulated standard errors.

Table 3. Hydrolytic activity of the recombinant enzymes on different cellulose and non-cellulose substrates.

Enzymes	Hydrolytic activity (U mg $^{-1}$)					
	Avicel[a]	Amorphous[a]	BMCC[a]	CMC[a]	Laminarin[b]	β-Glucan[b]
EXG1	9.1 ± 0.5	16.4 ± 1.0	7.4 ± 0.5	2.8 ± 0.1	32.2 ± 1.5	20.2 ± 1.0
L-EXG1	7.9 ± 0.6	14.8 ± 0.6	6.2 ± 0.5	2.4 ± 0.1	25.2 ± 2.0	14.5 ± 1.1
CBD2-L-EXG1	13.4 ± 0.6	30.2 ± 1.1	9.3 ± 0.5	4.7 ± 0.4	47.9 ± 1.9	26.7 ± 0.9
CBD2-CBD2-L-EXG1	17.0 ± 0.3	38.9 ± 1.8	12.2 ± 0.4	5.2 ± 0.3	59.2 ± 2.7	32.4 ± 1.3
L-EXG1-CBD2	15.7 ± 0.8	28.0 + 0.6	11.0 ± 0.4	4.8 ± 0.3	48.5 ± 2.9	26.2 ± 1.1
SSG1	9.4 ± 0.8	18.3 ± 0.4	6.4 ± 0.3	3.2 ± 0.2	34.9 ± 1.9	18.6 ± 1.3
CBD2-CBD2-SSG1	16.5 ± 1.0	38.1 ± 0.9	11.3 ± 1.0	5.6 ± 0.4	59.6 ± 1.8	32.7 ± 1.3
SSG1-CBD2	14.2 ± 0.7	30.8 ± 1.0	10.4 ± 0.6	4.8 ± 0.3	52.4 ± 2.6	27.4 ± 0.5

[a] The values shown here are given in cellobiohydrolase units per mg of protein.

[b] The values shown here are given in glucanase units per mg of protein.

The values are means of three independents assays ± accumulated standard errors.

4.3 Adsorption as a function of hydrolysis

The adsorption data of recombinant EXG1 and SSG1 enzymes are shown in Table 4. The percentage of adsorption of enzymes CBD2-CBD2-L-EXG1, L-EXG1-CBD2 and CBD2-CBD2-SSG1 towards Avicel increased at a magnitude of two- to three-fold compared with the wild types. However, only a slight increase in binding capacity was observed for CBD2-L-EXG1 and SSG1-CBD2.

Table 4. Adsorption and kinetic parameters of the recombinant enzymes on avicel.

Enzymes	Adsorption (%)	Kinetic parameter	
		V_{max}[a]	K_m[b]
EXG1	12.8	2424 ± 183.0	4.66 ± 0.70
L-EXG1	21.6	1555 ± 41.5	3.18 ± 0.19
CBD2-L-EXG1	35.6	3733 ± 173.3	5.66 ± 0.50
CBD2-CBD2-L-EXG1	42.0	3793 ± 121.9	4.29 ± 0.28
L-EXG1-CBD2	45.4	2578 ± 137.4	2.66 ± 0.33
SSG1	27.1	2249 ± 162.1	4.80 ± 0.69
CBD2-CBD2-SSG1	42.8	3838 ± 174.8	4.64 ± 0.42
SSG1-CBD2	33.7	3627 ± 172.1	5.20 ± 0.48

[a] $\mu mol \cdot L^{-1} \cdot h^{-1}$

[b] $g \cdot L^{-1}$

Values are mean of three determinations ± standard deviation.

4.4 Temperature and pH profile

To investigate the effect of CBD on the temperature and pH optimum of the recombinant enzymes, the profiles were investigated at temperatures 30 to 60°C and pH 3 to 7 respectively (Fig. 1A, 1B). The hydrolytic activity of all the recombinant enzymes was found to be optimum at a temperature of 50°C and pH 6 respectively, similar to the native enzymes. The figures also illustrates that the temperature and pH profiles of the multidomain recombinant enzymes did not show significant deviation when compared with their wild-type counterpart.

4.5 Thermal stability

The thermodenaturation curves of the recombinant enzymes were illustrated in figure 1C. Enzymes CBD2-CBD2-L-EXG1, CBD2-CBD2-SSG1 and CBD2-L-EXG1 exhibited the highest thermal stability, retaining full activity until 30 h of incubation at 50°C while the native enzymes EXG1 and SSG1 showed an immediate loss of activity after 10 h of incubation. Enzyme L-EXG1 showed the least thermal stability among the recombinants.

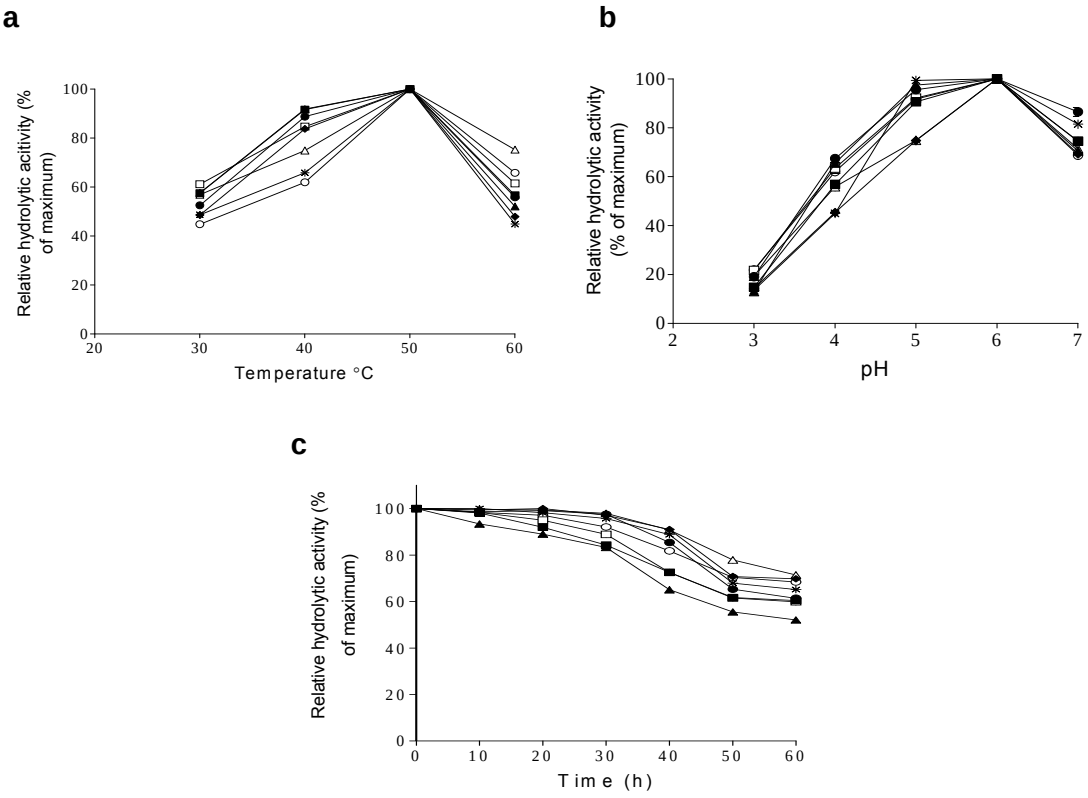

Fig. 1. a) Temperature optimum; b) pH optimum; c) Thermostability: ■, EXG1; ▲, L-EXG1; ●, CBD2-L-EXG1; ◆, CBD2-CBD2-L-EXG1; ∗, L-EXG1-CBD2; □, SSG1; △, CBD2-CBD2-SSG1; ○, SSG1-CBD2. The values shown here are means based on duplicated assays±standard deviation

4.6 Kinetic properties of the recombinant enzymes

The kinetics of the engineered enzymes were displayed in Table 4. Recombinant enzyme L-EXG1 and L-EXG1-CBD2 showed the highest affinity for the cellulosic substrate with the rest of the enzymes showing a slightly improved affinity than the native forms. However, the V_{max} values for all the recombinant enzymes were significantly increased up to 2-fold, except for the enzyme L-EXG1.

4.7 Scanning electron microscopy

The SEM analysis of avicel particles treated with recombinant enzymes clearly shows the hydrolytic function of these enzymes on cellulose (Fig. 2). Recombinant enzymes CBD2-L-EXG1, CBD2-CBD2-L-EXG1 and CBD2-CBD2-SSG1 showed primarily an exo- processive mode of hydrolysis on avicel cellulose as illustrated by the meshy, loosened and brittle appearance of the cellulose particles (Fig 2B, 2C, 2E). Further, it can also be seen that the particles treated with recombinant enzymes are more heterogeneous and that the solubilisation occurred more at the ends than on the surface, unlike the control samples.

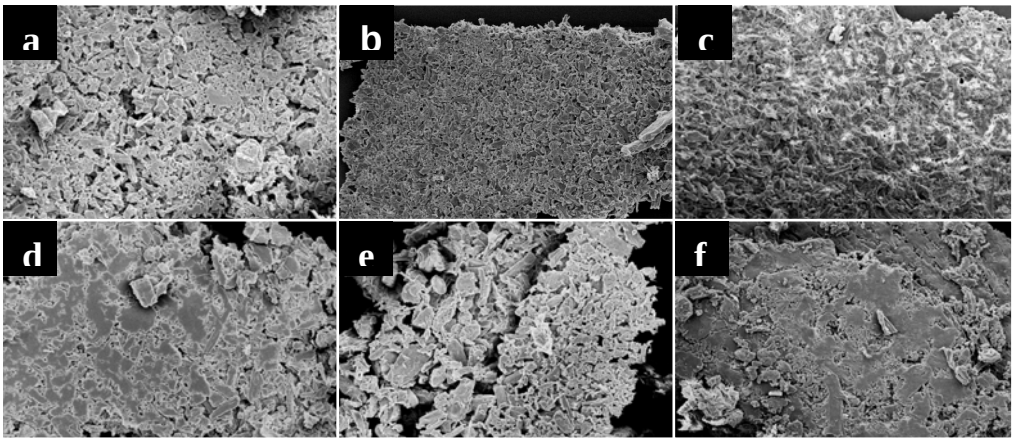

Fig. 2 Scanning electron microscope (SEM) of Avicel particles treated with different recombinant enzyme. All the images were visualised at a magnification of 1.000 K x. A, EXG1; B, CBD2-L-EXG1; C, CBD2-CBD2-L-EXG1; D, SSG1; E, CBD2-CBD2-SSG1; F, control enzyme treatment (endocellulase and β-glucosidase only)

5. Discussion

The results reported in this study show that both the CBD and the linker sequences coupled to a non-cellulolytic catalytic domain of EXG1 and SSG1 imparted the ability to act and hydrolyse β-1, 4-exoglucanase specific cellulosic substrates. The data clearly suggest that the degree of hydrolysis and mode of action of these recombinant enzymes differed with regard to the position and number of copies of the CBD. Recombinant enzymes with two CBD copies might have acquired increased adsorption ability thus promoting better stability

of the molecules on the surface of the substrates, leading to improved hydrolysis (Linder *et al*. 1996). The lower activities observed for the single-CBD enzymes probably reflect the fact that they have a less rigid stability and subsequent low hydrolysis on recalcitrant substrates. These results agree with previous reports, which state that the efficiency of cellulolytic enzymes is directly related to their affinity for the substrate (Black *et al*. 1997).

There is a close relationship between molecular flexibility and function. The distance between the catalytic domain and the CBD, caused by the linker domain would have enhanced the cellulolytic activity of the engineered enzymes by increasing the flexibility between them and also the proximity to the substrate, while facilitating the dynamic adsorption process led by the CBD (Srisodsuk *et al.* 1993). On the other hand, the limited flexibility of the molecules without a linker sequence would have forced the enzyme to bind less efficiently to the surface of substrate. Kinetic analysis also demonstrated that the increase in the relative hydrolytic activity of the recombinant enzymes was caused not only by increased affinity but also by improved catalytic efficiency on the substrate. These factors also illustrate the significant increase in the hydrolytic activity towards β-1, 3-exoglucanase specific substrates like glucan and laminarin by the engineered enzymes. This also led us to assume that neither the folding pattern of the enzymes nor the 3-D conformations were affected by the fusion of both the CBD and the inter-domain linker.

Recombinant enzymes like thermophilic enzymes are also rigid and gained sufficient molecular flexibility for improved thermostability. We suggest that the cellulose-binding domain endowed a thermostable property to the recombinant enzymes, and this reiterates the designation of CBDs as the “thermostabilising domains” (Kataeva *et al.* 2001).

The substantial reduction in the size and morphology of the hydrolysed avicel particles as shown in the SEM indicates well-coordinated and synergistic activity by the enzymes present in the mix (Boisset *et al.* 2000). The finding illustrates that recombinant enzymes primarily engaged in an exo- processing mode of action as shown by the pattern on hydrolysis. The engineered enzymes should have undergone a dynamic process of binding and desorption, allowing successive hydrolysis and/or relocation to new, enzymatically accessible sites on the avicel cellulose surface, as has been suggested for the cellulolytic enzymes boarding different CBD modules (Lehtio *et al.* 2003). The molecular structure of these enzymes might thus be balanced between the requirement for stability and dynamics.

In conclusion, the present study suggests that the engineered multidomain *S. cerevisiae* β-1,3-exoglucanases would have gained sufficient molecular stability, flexibility and intramolecular interaction between the domains to act synergistically in optimised enzymatic mixtures for the biodegradation of crystalline cellulose. It was also clear that the CBD sequence played the role of a thermo-stabilising domain in the engineered enzymes. A more detailed understanding of the role and function of CBD in these enzymes would further

enable the development of efficient engineered enzymes adapted to bioprocess-efficient microbial system like *S. cerevisiae*.

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CHAPTER 4

RESEARCH RESULTS II

Effect of the cellulose binding domain on the catalytic activity of a β-glucosidase from *Saccharomycopsis fibuligera*

This manuscript will shortly be submitted for possible publication in
APPLIED MICROBIOLOGY AND BIOTECHNOLOGY

Effect of the cellulose binding domain on the catalytic activity of a β-glucosidase from *Saccharomycopsis fibuligera*

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1. Abstract

Enzyme engineering was performed to link the β-glucosidase enzyme (BGL1) from *Saccharomycopsis fibuligera* to the cellulose-binding domain (CBD2) of *Trichoderma reesei* cellobiohydrolase (CBHII) to investigate the effect of a fungal CBD on the enzymatic characteristics of this non-cellulolytic yeast enzyme. Recombinant enzymes were constructed with single and double copies of CBD2 fused at the N terminus of BGL1 to mimic the two-domain organisation displayed by cellulolytic enzymes in nature. The engineered S. *fibuligera* β-glucosidases were expressed in *Saccharomyces cerevisiae* and characterised using a range of cellulosic and non-cellulosic substrates to illustrate the effect of the CBD on their enzymatic activity. The organisation of the CBD in these recombinant enzymes resulted in enhanced substrate affinity, molecular flexibility and synergistic activity, thereby improving the ability of the enzymes to act on and hydrolyse cellulosic substrates, as characterised by adsorption, kinetics, thermal stability and scanning electron microscopic (SEM) analysis.

2. Introduction

Cellulolytic enzymes belong to the family of enzymes that are capable of degrading cellulose. Cellulolytic microorganisms produce at least three major types of enzymes: endoglucanases, exoglucanases and β-glucosidases. Endo- and exoglucanases have a conserved tripartite structure with a large catalytic core domain linked by O-glycosylated peptide to a cellulose-binding domain (CBD), which is required for interaction with crystalline cellulose. The CBD mediates the binding of the cellulolytic enzymes to the substrate, thereby playing a fundamental role in its hydrolysis (Tomme et al. 1995). It has been suggested that

the CBD enhances the enzymatic activity of cellulolytic enzymes simply by reducing the dilution effect of the enzyme at the substrate surface (Stahlberg et al. 1991), by promoting the solubilisation of single glucan chains off the cellulose surface (Tormo et al. 1996), or by loosening individual cellulose chains from the cellulose surface prior to its actual hydrolysis (Teeri et al. 1992).

Most information on the role of CBDs has been obtained from the removal (Arai et al. 2003), domain exchange (Mangala et al. 2003) and site-directed mutagenesis of the CBDs (Reinikainen et al. 1992), or by artificial addition of the CBD (Lemos et al. 2003). It thus seems that the CBDs are interchangeable to a certain degree, but much more data are needed on different catalytic domain-CBD combinations to elucidate the exact functional role of the cellulose-binding domains. Since there are possibly many factors at work in the interaction between the CBD and the catalytic domain, it would also be interesting to study the role of CBDs in the context of a β-glucosidase functional domain. In addition, the presence of a N-terminal CBD has been found in nature in a β-glucosidase enzyme from the wood-degrading fungi, *Phanerochaete chrysosporium* (Lymar et al. 1995).

In this study, domain engineering was performed to link the non-cellulolytic β-glucosidase domain from the *Saccharomycopsis fibuligera* BGL1 enzyme (Machida et al. 1988) to the cellulose-binding domain (CBD2) of *Trichoderma reesei* cellobiohydrolase (CBHII) to investigate whether the CBDs would be able to modulate these non-cellulolytic domains to function in cellulose hydrolysis. Recombinant enzymes were constructed with single and double copies of CBD2 fused at the N terminus of BGL1 displaying the two-domain organisation, which is shown by many cellulolytic enzymes. The recombinant enzymes were expressed in *Saccharomyces cerevisiae* and characterised for their catalytic and physicochemical properties using various cellulose and non-cellulose substrates.

3. Materials and Methods

3.1 Strains and plasmids

Escherichia coli strain DH5α (F- α80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rK-, mK_+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1) was used as an intermediate host for the cloning of the recombinant β-glucosidase gene constructs and for the propagation of all the plasmids. The *S. cerevisiae* strain CENPK42 (*MATα, leu2-3,112 ura3-52 trp1-289 his3-Δ1*) was used as a host for recombinant enzyme expression. The 2μ multi-copy expression vector pCEL15 (Gundllapalli et al. 2005), a derivative of YEp352 (Van Rensburg et al. 1996) containing the sequences required for replication in *E. coli* and yeast, the ampicillin resistance gene, the yeast *URA3* gene, the *MFα1* secretion signal, and the *PGK1* promoter and terminator was used for expression. Plasmid pEFB19 (Van Rensburg et al. 1998) was

used as the source for the gene coding the β-glucosidase (*BGL1*) from *S. fibuligera*. The DNA fragment coding for the cellulose-binding domain (*CBD2*) of *T. reesei* cellobiohydrolase II was kindly provided by Dr P van Rensburg, Institute for Wine Biotechnology. Sequencing of the recombinant constructs was performed using pGEM®-T Easy plasmid (Promega).

3.2 Cloning, construction and transformation of the recombinant forms of BGL1

The various genetic constructs that led to the production of native and recombinant forms of *BGL1*-encoded enzyme are described. The region of the *CBH2* gene of *T. reesei* between nucleotides 73 and 186, which encodes the cellulose binding-domain (CBD2), was amplified by PCR with primers containing *Hin*dIII at the 5' termini of both CBD-F (5'-AAGCTTAGCGTCTGGGGCCAA-3') and CBD-R (5'-AAGCTTAAGACACTGGGAGTAATA-3') to facilitate multi-copy insertion into pCEL15. The amplification reaction contained standard PCR buffer (provided by the supplier), 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM of each primer, 2 ng·μL^{-1} of template, and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. PCR was carried out in a PCR Express thermal cycler for 15 cycles: denaturation, 2 min at 94°C; primer annealing, 30 s at 48°C; and primer extension, 1 min at 68°C. The plasmids with a single copy and a double copy of CBD2 with the same orientation were chosen and named pCEL16 and pCEL41 respectively. The fragment of the *BGL1* gene (nucleotides 159 to 2535) that encodes the mature BGL1 was amplified using BGL1-F (5'-CCATCGATGTCCCAATTCAAAACTATAC-3'), containing a *Cla*I site, and BGL1-R (5'-CCGCTCGAGTCAAATAGTAAACAGGACAG-3') containing an *Xho*I site. This PCR product was cloned into the *Cla*I and *Xho*I sites of pCEL15, pCEL16 and pCEL41, thereby generating the plasmids pCEL29, pCEL30 and pCEL31 respectively. The PCR mix and programme was the same as mentioned before, except that the annealing temperature was changed to 55°C. Standard methods were used for the isolation, restriction, purification and ligation of DNA, the plasmid transformation into *E. coli* and agarose gel electrophoresis. Both the coding and non-coding strands were sequenced to ensure the reliable identification of all constructs (PE/Applied Biosystems). The transformation of the *S. cerevisiae* CENPK42 strain was carried out by the lithium acetate method (Gietz et al. 2002) and selected on SCD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, amino acids supplemented as required), supplemented with tryptophan, leucine and histidine.

3.3 Purification of native and recombinant β-glucosidases

Purification of the native and recombinant enzymes was performed as described in Suzuki et al. (2001) with modification. *S. cerevisiae* transformants carrying either native or recombinant BGL1 were grown in SCD medium at 30°C for 24 h in a rotary shaker (200 rpm). The pre-culture was used to inoculate 500 ml of YPD (1% yeast extract, 2% peptone, 2% glucose) at an optical density (OD measured at a wavelength of 600 nm) of 0.1. After

incubation for 60 h, the enzymes were purified from the supernatant. The culture fluid was obtained by centrifugation at 5 000 rpm for 10 min and was brought to 80% saturation by the addition of pre-chilled saturated ammonium sulphate solution and left overnight at 4°C. Precipitates were collected by centrifugation and dissolved in 50 mM citrate buffer at pH 6.0. The solution was subsequently diafiltered on a 50 kDa cut-off Amicon membrane (Amicon), with 50 mM citrate buffer at pH 6.0. The proteins were then separated on a Bio-Rad Automated Econo system by anion exchange chromatography equipped with a DEAE sepharose column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl and the monitored fractions showing activity were pooled. The purified enzyme was dialysed against 50 mM citrate buffer at pH 4.8 and concentrated by ultra-filtration (100 kDa cut-off membrane, Amicon). The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard.

3.4 Antibody preparation

Antibodies to almond β-glucosidase (Sigma) were obtained, essentially as described by Bellstedt et al. (1987). Purified β-glucosidase was used for the primary immunisation of the rabbit (1 ml of 1 $mg \cdot ml^{-1}$). Antigen boosts were performed at three weeks and five weeks after primary immunisation, and the antiserum was collected on the 28^{th} and 42^{nd} day.

3.5 Immunochemical identification and quantification of proteins

The partially purified samples of the native and recombinant β-glucosidase were concentrated using Amicon Micron-10 microconcentrators. Samples were subjected for electrophoresis in Tris-Glycine-SDS gels and transferred to polyvinylidene fluoride (PVDF) microporous membrane (ImmobilonTM-P Transfer Membrane, Millipore) by means of the standard procedure (Towbin et al. 1979). Western blot analysis was performed using the ECL Western blotting analysis system (Amersham Pharmacia biotech). Blots were sequentially treated with rabbit anti-β-glucosidase (1:15000) and donkey anti-rabbit IgG conjugated with horseradish peroxidase (1:20000). The HRP/hydrogen peroxide-catalysed oxidation of luminol in alkaline conditions emits enhanced chemiluminescence that was detected by short exposure to blue light-sensitive autoradiography film (Hyperfilm ECL, Amersham Biosciences). Microtiter plates were coated with enzyme samples overnight at 4°C, after which the unbound material was removed and the wells were washed twice with PBS solution. The plates were blocked with 4.5% skim milk powder and incubated at room temperature for 1 h. The residual blocking buffer was removed and the wells were washed four times with PBS solution. The wells were subsequently loaded with PAb in blocking buffer at a dilution of 1:30000 and incubated at room temperature for 1 h. Following incubation, the wells were washed four times with PBS. An anti-rabbit IgG whole molecule

alkaline phosphatase conjugate was used to amplify the signal generated by the bound antibodies of PAbs at an optimal working dilution of 1:15000 in the blocking buffer. The plates were incubated at room temperature for 1 h, followed by the washing steps as mentioned previously. p-Nitrophenyl phosphate substrate (Sigma) suspended in 1 M diethanolamine buffer at a pH of 9.8 and containing 0.5 mM $MgCl_2$ was added to each of the wells. Absorbance at 405 nm was determined with a microtiter plate reader (Bio-Tek Instruments). Pre-immune serum was probed with horseradish peroxidase-conjugated anti-rabbit IgG as the negative control. Standards were prepared using linear concentrations of purified β-glucosidase from almonds (Sigma).

3.6 Enzyme assays

β-Glucosidase activity was determined by monitoring the release of p-nitrophenol from p-nitrophenol-β-glucoside (PNPG) (Lymar et al. 1995). One unit of β-glucosidase activity is defined as that amount of enzyme that will hydrolyse 1 μmol of PNPG per minute. The β-glucosidase activity towards the cellobiose substrate was determined by assaying the amount of reducing sugar (free glucose) released. The reaction mixture contained 50 μl of 1% cellobiose (Sigma) stock solution in 50 mM citrate buffer at pH 4.8 and the enzyme preparation. After 1 h of incubation at 37°C, the free glucose released was measured using the Trinder reducing sugar assay kit (Sigma). The cellobiohydrolase activity was assayed using the two-step standard assay with little modification (Boisset et al. 2001). Reactions were carried on in 2 mL O-ringed screw cap micro-centrifuge tubes containing the enzyme preparations, in synergism with the commercial enzyme endoglucanase EGII (*T. longibrachiatum*) in a reaction volume of 750 μL with 1.0 $g \cdot L^{-1}$ of substrate [avicel, amorphous, bacterial micro-crystal cellulose (BMCC) or carboxy methyl cellulose (CMC)] in 50 mM sodium citrate buffer (pH 4.8). The reaction mixes were incubated at 50°C with end-over-end rotation at 40 rpm for 16 h. The samples were filtered through 0.45 μm cellulose acetate filters and analysed for free glucose with the GAGO kit (Sigma). Each reaction mix was done in triplicate. The unit cellobiohydrolase activity of the native and recombinant enzymes was calculated from the standards that were prepared, using purified cellobiohydrolase enzyme from *Trichoderma longibrachiatum* (Megazyme) on avicel, amorphous, BMCC and CMC cellulose separately under similar assay conditions, and expressed as cellobiohydrolase units. The hydrolytic assays on cellooligosaccharide and cellopentose were performed under similar conditions as mentioned above, except that the hydrolytic mix contained either the native or the recombinant enzymes.

3.7 Preparation of substrates

Avicel and amorphous cellulose were purchased from FMC Co., (USA). CMC, laminarin and barley β-glucan were obtained from Sigma. BMCC was obtained from Cellulon (USA). Avicel, amorphous cellulose and BMCC were weighed out, washed with distilled water and filtered as described by the supplier. Cellooligosaccharides were obtained from Sigma.

3.8 HPLC analysis of hydrolysis products

The HPLC analysis of the products of hydrolysis was performed using a refractive index detector (Agilent technologies). The column used for separation was an Aminex HPX-87H column (Bio-Rad). The HPLC was operated at 30°C using 6 mM of sulphuric acid at a flow rate of 0.8 ml·min^{-1} as the mobile phase.

3.9 Adsorption assay

Adsorption assays were performed in a similar manner as described by Gal et al. (1997). Enzyme preparations of native and recombinant enzymes were incubated with 1 g·L^{-1} of Avicel in 50 mM sodium citrate buffer (pH 4.8), in a final reaction volume of 750 μL, in an end-over-end rotation at 40 rpm. The incubation temperature was maintained at 4°C for 16 h and the preparations were then centrifuged at 5000 × *g* for 15 min. In the binding assays, the free protein fraction was estimated from the residual enzymatic activity in the supernatant. This was subtracted from the initial enzyme activity to estimate the substrate-associated fraction.

3.10 Kinetic parameters

The Michaelis-Menten parameters, V_{max} (μmol·min^{-1}) and K_m (mg·mL^{-1}), were determined for all the recombinant enzymes from Michaelis-Menten plots of specific activities for six to 10 concentrations of avicel, and the rates were measured in triplicate, ranging from 0.2 to five times the value of K_m. The values of V_{max} and K_m were determined by nonlinear regression analysis using the graph pad prism programme.

3.11 pH and temperature profile

The effect of pH on the activity of the native and recombinant enzymes was investigated in the pH range 2-7, using 1 g·L^{-1} cellobiose in 50 mM of sodium citrate buffer at 50°C. The temperature profiles for the native and recombinant enzymes were measured at temperatures between 30 and 60°C at pH 4.8, under similar assay conditions.

3.12 Thermostability

The native and recombinant enzymes were incubated in 50 mM of sodium citrate buffer (pH 4.8) at temperatures between 30 and 70°C for 30 min and snap cooled immediately in ice. The remaining activity was measured at 50°C using cellobiose as substrate.

3.13 Sample preparation for scanning electron microscopy (SEM)

The electron micrographs of the avicel particles were taken after 16 h of digestion at 50°C at a pH of 6, with the native or recombinant enzymes added in synergism with the commercial preparation of the *T. longibrachiatum* endocellulase. The preparation of the avicel samples treated with the recombinant enzyme preparations was performed as described previously (Boisset et al. 2000). The specimens were examined using a fully analytical S440 Leo Scanning Electron Microscope (SEM) operated at an accelerating voltage of 10 kV. All the images were visualised at a magnification of 1.00 K x.

4. Results

4.1 Construction and expression of recombinant enzymes

All the recombinant molecules that were constructed were cloned separately into the yeast expression plasmid and transformed into the *S. cerevisiae* CENPK42 strain (see Materials and Methods). The native and recombinant enzymes of BGL1 were successfully expressed in *S. cerevisiae* and purified from the culture supernatant. The purified enzymes were analysed and confirmed by SDS-PAGE gel and immunoblotting. The molecular weight was determined to be 220 kDa for the native enzyme (Machida et al. 1988) and approximately 225 and 230 kDa (theoretical values) for the recombinant enzymes (Fig. 1).

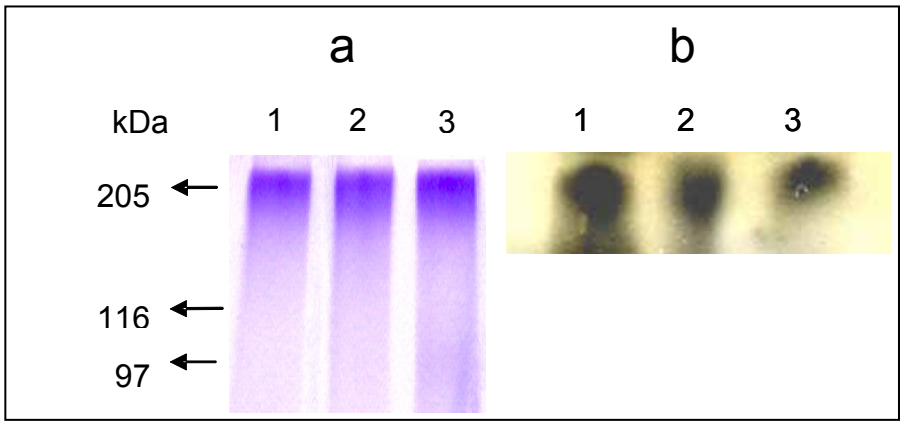


Fig. 1 Analysis of purified native and recombinant β - glucosidase enzymes by (a) SDS – PAGE and (b) Western blot. lane 1, native BGL1; lane 2, CBD2-BGL1; lane 3, CBD2-CBD2-BGL1

4.2 Hydrolytic activity of the recombinant enzymes

The recombinant BGL enzymes were assayed for their ability to hydrolyse β-1,4 linkage-specific cellulosic substrates (Table 1). It was observed that the recombinant CBD2-BGL1 enzyme exhibited the highest rate of hydrolysis on all the celluloses – approximately four-fold higher than the mix containing the native enzyme. The recombinant CBD2-CBD2-BGL1 enzyme showed an activity of approximately two-fold higher than that of the native enzymes. Interestingly, on native cellobiose substrate, the BGL1 enzyme and the recombinant CBD2-BGL1 exhibited comparable hydrolytic activity, but the activity of CBD2-CBD2-BGL1 was decreased more than two-fold. No significant background activity was measured for the CBD2 and CBD2-CBD2 constructs.

Table 1 Hydrolytic activity of the recombinant enzymes on different substrates

Enzyme	Reducing sugars released (μmol glucose min^{-1})[a]				
	Cellobiose	Avicel	Amorphous	BMCC	CMC
BGL1	0.70	0.015	0.055	ND	ND
CBD2-BGL1	0.70	0.068	0.230	0.051	0.044
CBD2-CBD2-BGL1	0.26	0.032	0.170	0.019	0.027
CBD2	ND	ND	0.021	ND	ND
CBD2-CBD2	ND	ND	0.019	ND	ND

[a] Average hydrolytic activities from at least three independent experiments are presented. Standard deviation was less than 10%
ND, Not detected

The native and recombinant enzymes of BGL1 displayed different patterns of hydrolysis on the cellooligosaccharide mix (Table 2). The percentage of soluble product released summarises the improved performance of hydrolysis by the recombinant enzymes CBD2-BGL1 and CBD2-CBD2-BGL1 to act on longer chain β-1,4-specific substrates, which showed three-fold and two-fold higher activity respectively. Although the native BGL1 enzyme released glucose as its major hydrolytic product, most of it was derived from the hydrolysis of cellobiose in the mix. However, the wild-type BGL1 showed less significant activity on the cellotriose substrate, which corresponds with the data published by Machida et al. (1988). The pattern of hydrolysis shown by the native and recombinant enzymes on cellopentose was comparable to the profile shown in cellooligosaccharides (Table 3). Glucose is the major hydrolysis product of both CBD2-BGL1 and CBD2-CBD2-BGL1, followed by cellobiose and cellotriose. The profiles of the recombinant enzymes also reveal a sequence in which cellopentose is hydrolysed into cellobiose and cellotriose, and then further hydrolysed into glucose and cellobiose respectively. The sequential hydrolysis of

cellooligosaccharides leading to the release of glucose as the major hydrolytic product illustrates the bi-functional activity of β-glucosidase and β-1,4-exoglucanase by the recombinant enzymes.

4.3 Adsorption as a function of hydrolysis

Table 4 summarises the measured binding capability of the different peptides. As can be observed from the data, the percentage of adsorption of the recombinant enzymes, CBD2-BGL1 and CBD2-CBD2-BGL1, towards avicel increased 10- and 16-fold respectively, compared to the wild-type BGL1. In addition, the net adsorption of the recombinant enzyme, CBD2-BGL1, was noticed to increase until 16 h, after which a total desorption was observed. However, the chimera CBD2-CBD2-BGL1 does not show any desorption at that time point (data not shown).

Table 2 HPLC analysis of the hydrolysis products using cellooligosaccharide mix as substrate.

Enzyme	Hydrolysis products (g L^{-1})				Soluble products (%)[a]
	Glucose	Cellobiose	Cellotriose	Cellopentose	
BGL1	0.086	0.009	0.031	0.091	21
CBD2-BGL1	0.290	0.095	0.006	0.220	61
CBD2-CBD2-BGL1	0.140	0.150	0.008	0.120	42
Substrate control	0.000	0.016	0.064	0.100	18

[a] soluble sugar is measured only in relation to the total soluble sugar (18%) analyzed above, from the mix of cellooligosaccharides of 1g/L
Standard deviation was less than 5%

Table 3 HPLC analysis of the hydrolysis products using cellopentose as substrate.

Enzyme	Hydrolysis products (g L^{-1})			
	Glucose	Cellobiose	Cellotriose	Cellopentose
BGL1	0.11	0.015	ND	0.88
CBD2-BGL1	0.32	0.17	0.02	0.52
CBD2-CBD2-BGL1	0.14	0.24	0.1	0.59
Substrate control	ND	ND	ND	1.00

Standard deviation was less than 5%

4.4 Kinetic properties of the recombinant enzymes

The recombinant enzymes CBD2-BGL1 and CBD2-CBD2-BGL1 actively catalysed the hydrolysis of avicel (Table 4). CBD2-BGL1 yielded approximately two-fold higher V_{max} values for the avicel substrate than the recombinant enzyme CBD-CBD2-BGL1, although it displayed a similar K_m. As for the wild type BGL1, the values for both K_m and V_{max} were significantly lower than those for the recombinant enzymes.

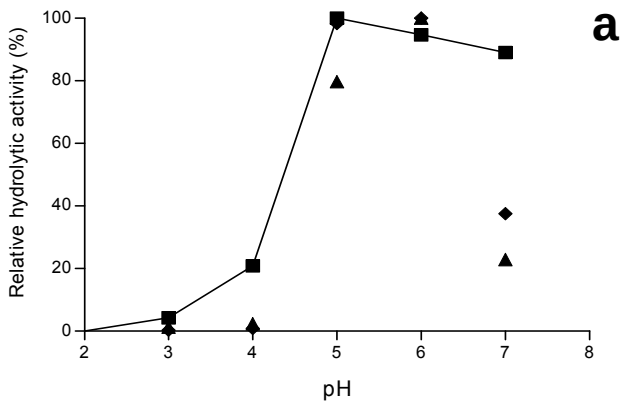
4.5 pH and temperature profile

To investigate the pH- and temperature-dependent activity profile of the recombinant enzymes, the profiles were investigated at pH 2-7 and temperatures of 30 to 60°C respectively (Fig. 2a and 2b).The data illustrate a shift in the pH optimum of both the recombinant enzymes from 5 to 6, in comparison to the native enzyme, which exhibits an optimum of 5. However, the CBD2-BGL1 enzyme showed a broad optimum range, from pH 5-6. The temperature profile obtained shows that the recombinant enzymes exhibit a similar activity profile at all the temperatures, with the optimal activity measured at 50ºC.

Table 4 Adsorption and kinetic parameters of the recombinant enzymes anaslysed using avicel as substrate.

Enzyme	Adsorption (%)	Kinetic parameters	
		V_{max} (μmol min^{-1})	K_m (g L^{-1})
BGL1	3	0.09	1.11
CBD2-BGL1	29	0.30	0.22
CBD2-CBD2-BGL1	51	0.17	0.25

[a] Experiments were performed in triplicates and the standard deviation was less than 10%



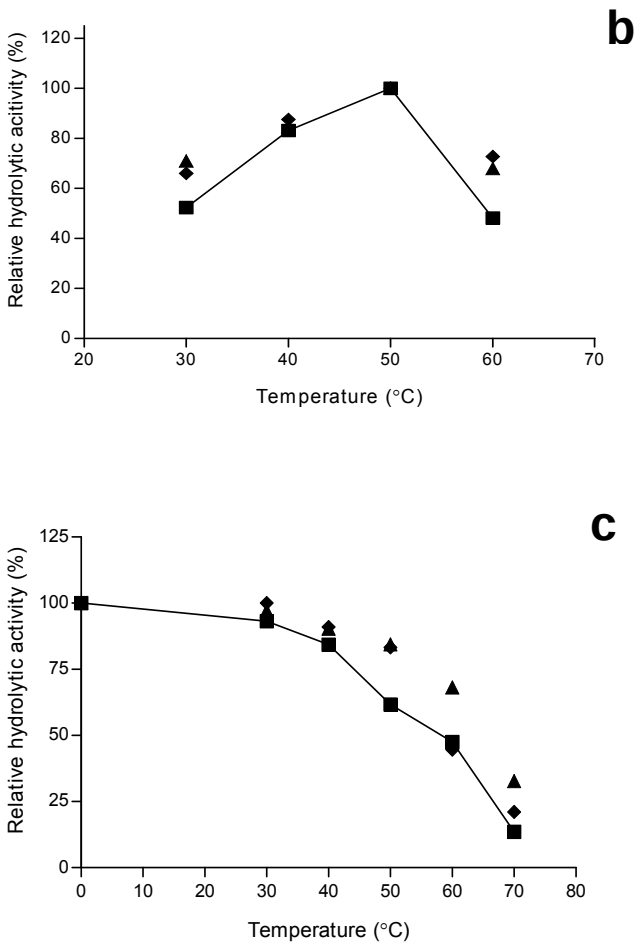


Fig. 2 (a) pH optimum (b) temperature optimum (c) Thermostability: (■) BGL1; (◆) CBD2-BGL1; (▲) CBD2-CBD2-BGL1. Average values from at least three independent experiments are presented and the deviation was less than 10%.

4.6 Thermostability

An investigation of the thermal denaturation kinetics of the enzymes showed that, while the native BGL1 enzyme showed an immediate loss of activity after incubation at 40°C, the recombinant enzymes, CBD2-BGL1 and CBD2-CBD2-BGL1, were stable until 50°C (Fig. 2c). It was also noted that the stability of CBD2-BGL1, dropped significantly to approximately 50% of its maximum activity from 50°C to 60°C, whereas the stability of the CBD2-CBD2-BGL1 decreased gradually.

4.7 Scanning Electron Microscopy

The morphological modification imparted to avicel cellulose particles by the recombinant enzymes in comparison with that of its native forms were illustrated in the series of electron micrographs displayed in Fig. 3. Figure 3B is the result of digestion with the native BGL1 enzyme and EGII mixture, where the surface of the cellulose particle is clearly distorted in

comparison with that of the untreated sample. In Fig. 3c and 3d corresponding to the hydrolytic data of the CBD2-BGL1 and CBD2-CBD2-BGL1 enzymes, a dramatic increase in digestion is clearly noticed on the surface, reflected by meshy, loosened and tiny micron-long particles in the case of CBD2-BGL1 and by more brittle, powdery particles in the case of CBD2-CBD2-BGL1. A significant morphological difference is also noticed between the samples, with the particles in Fig. 3d solubilized more at the ends than on the surface unlike the samples treated with CBD2-BGL1 where it was completely digested.

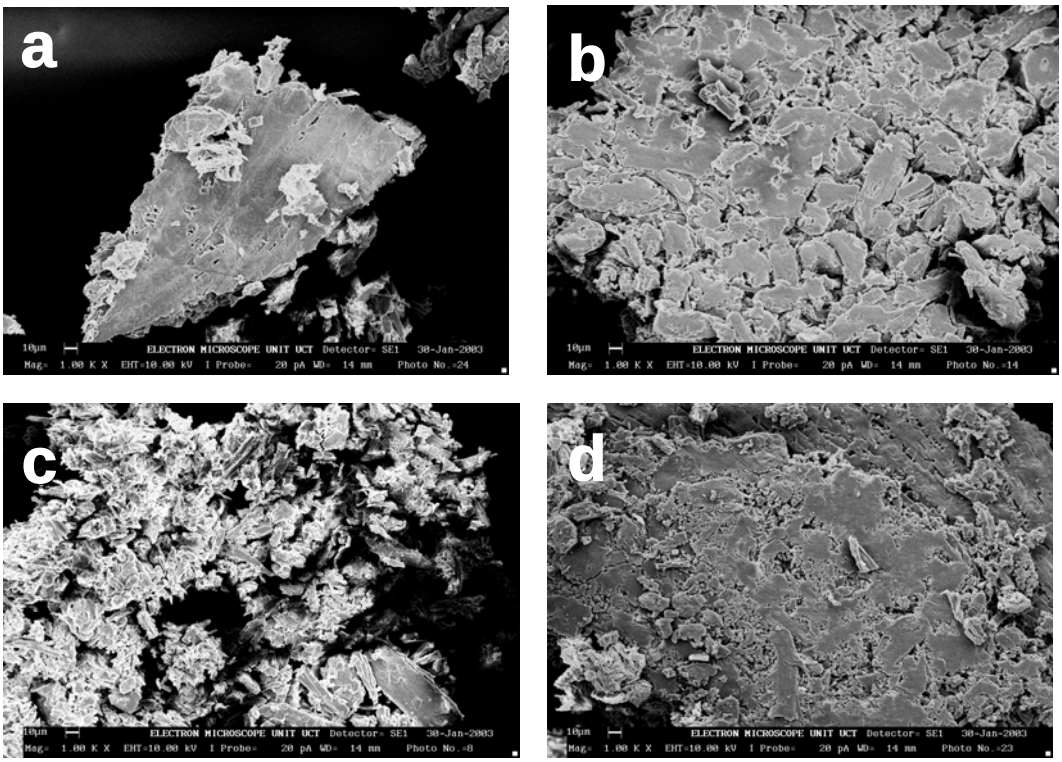


Fig. 3 SEM of the avicel cellulose particles after 16 h digestion by the recombinant enzymes of BGL1: (a) Untreated (b) BGL1 (c) CBD2-BGL1 (d) CBD2-CBD2-BGL1

5. Discussion

The results reported in this article suggest that the CBD coupled to a non-cellulolytic β-glucosidase enzyme modulates its ability to act and hydrolyse cellulosic substrates. Consequently, the data clearly suggest that the degree of hydrolysis and mode of action of these recombinant enzymes differ with regard to the number of copies of the CBD. The ability of these enzymes to sequentially hydrolyse the cellooligosaccharides to free glucose demonstrates their bi-functional activity.

The presence of the CBD in the recombinant enzymes facilitated their binding to the surface of the substrates, thereby promoting better stability and hydrolysis (Linder et al.

1996). The increased affinity of the recombinant enzymes towards the cellulosic substrate clearly demonstrates this aspect. The CBD, as has been reported previously, would also have enhanced the hydrolysis simply by reducing the dilution effect of the enzyme at the substrate surface, thus promoting the solubilisation of single glucan chains from the cellulose surface (Stahlberg et al. 1991; Tormo et al. 1996). The lower rate of hydrolysis observed for the double copy CBD enzyme probably reflects the fact that, although they exhibited the greatest adsorption ability, the fusion of multiple CBDs would have translated themselves into misfolding of the recombinant molecule and consequent distortion of the 3D conformation. In direct support of this, the kinetic data show that the maximum hydrolytic activity of this recombinant enzyme is significantly reduced in comparison to that of its counterpart with single copy CBD. This would also explain the reduced cellobiase activity of the double CBD enzyme. Since the isolated CBDs did not contribute towards the hydrolysis of cellulose, it could be suggested that the ability of the recombinant molecules to act on cellulose was rather a cooperative interaction between the catalytic domain and the CBD. Such interactions between the domains have previously been demonstrated in the cellulolytic enzymes from *T. reesei* (Nidetsky et al. 1994). Further the reduced activity of the double CBD recombinant enzyme could also be attributed to the lack of processivity of this enzyme in *S. cerevisiae* in comparison to the single CBD enzyme.

The release of glucose as the major hydrolytic product of cellooligosaccharides by the recombinant enzymes suggests that, while the hydrolysis product of the modulated exo-cellulase activity was cellobiose, the native activity of β-glucosidase subsequently converted the cellobiose to glucose. The presence of trace amounts of cellotriose as a transient by-product in the hydrolysis of both cellooligosaccharide and cellopentose suggests that the presence of the CBD increased the affinity of the recombinant enzymes for this substrate. The increase in the concentration of cellopentose in the case of cellooligosaccharide hydrolysis also places it alongside cellotriose as a transient product, although the affinity towards this substrate is lower.

The recombinant enzymes, such as the thermophilic enzymes, gained sufficient molecular flexibility for improved thermostability (Lonn et al. 2003). Our data suggest that the CBD endowed the recombinant enzymes with this thermostable property, and this reiterates the designation of CBDs as “thermostabilising domains” (Kataeva et al. 2001). Indeed, the characterisation of a novel cellulose-binding β-glucosidase from cellulose-degrading cultures of *P. chrysosporium* assigned a thermostabilising role to the CBD (Lymar et al. 1998).

The substantial reduction in the size and morphology of the hydrolysed avicel particles, as shown by SEM, indicates synergistic activity between the recombinant enzyme and EGII enzyme present in the hydrolytic mix. The contribution of the CBD to synergistic activity has already been reported for free cellulolytic enzymes from *T. reesei* and *Humicola insulans* (Nidetzky et al. 1994; Boisset et al. 2000). This finding also illustrates that

recombinant enzymes primarily engage in a dominant exo-processing mode of action, as shown by the pattern in hydrolysis. As demonstrated, the engineered enzymes should have undergone a dynamic process of binding and desorption, allowing successive hydrolysis and/or relocation to new, enzymatically accessible sites on the avicel cellulose surface, as has been suggested for the cellulolytic enzymes boarding different CBD modules (Lehtio et al. 2003). The structure of these enzymes might thus be balanced between the requirement for stability and that for dynamics. The fragmentation observed on the surface of the particles can be clearly attributed to the endocellulase action of the EGII enzyme in the mix.

In conclusion, the present study suggests that the engineered β-glucosidase enzymes would have gained sufficient stability and affinity for cellulosic substrates, thus achieving significant hydrolytic efficiency in optimised enzymatic mixtures. It was also clear that the CBD sequence played the role of a thermostabilising domain in the engineered enzymes. A more detailed understanding of these recombinant enzymes like the elucidation of the 3-D structure would enable to illustrate the specific role and function of the CBD in these enzymes. Further this would envisage the development of efficient engineered enzymes adapted to bioprocess-efficient microbial systems.

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CHAPTER 5

RESEARCH RESULTS III

Isolation of a *Saccharomyces cerevisiae* mutant over expressing *Trichoderma reesei* cellobiohydrolase II

This manuscript will shortly be submitted for possible publication in

FEMS YEAST RESEARCH

Isolation of a *Saccharomyces cerevisiae* mutant over expressing *Trichoderma reesei* cellobiohydrolase II

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1. Abstract

In a previous study, we showed that the fusion of the cellulose-binding domain (CBD2) from *Trichoderma reesei* cellobiohydrolase II to a β-glucosidase (BGL1) enzyme from *Saccharomycopsis fibuligera* significantly hindered its expression and secretion in *Saccharomyces cerevisiae*. This suggests that the possible low secretion of heterologous cellulolytic enzymes in *S. cerevisiae* could be attributed to the presence of a CBD in these enzymes. As a most ideal situation, in our present study, we used the chimeric enzyme of CBD2 and BGL1 (designated CBGL1) as a reporter enzyme for screening mutagenised *S. cerevisiae* strains with increased ability to secrete CBD-associated enzymes such as cellulolytic enzymes. A mutant strain, WM91-CBGL1, was isolated showing up to 200 U L^{-1} of total activity, approximately three-fold more than that of the parental host strain. 75% of the activity was secreted into the extracellular medium. The mutant strain transformed with the *T. reesei CBH2* gene produced cellobiohydrolase enzyme up to three-fold more than the parental strain, but with 50% of the total activity retained intracellularly. The cellobiohydrolase enzyme from the parental and mutant strain was partially purified and the characteristic properties were analysed.

2. Introduction

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g. tunicates) and a few bacteria. Its abundance and ease of availability make cellulose a rich, renewable energy source [1]. Almost all of the biomass produced is mineralised by microbial enzymes. Cellulolytic microorganisms produce multiple enzymes, known as enzyme systems, to degrade plant cell materials [2]. Three major types of enzymatic activity are found: (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases or

1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91) and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21). Endoglucanases cut randomly at internal amorphous sites in the cellulose chain, generating oligosaccharides of various lengths, while exoglucanases act in a processive manner on these oligosaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as the major products. β-Glucosidases hydrolyse soluble cellodextrins and cellobiose to glucose.

Cellulosic biomass would likely be the preferred feedstock for fuel production today were it not for the recalcitrance of cellulosic biomass and the difficulty of converting cellulose into reactive intermediates [3]. Approaches and research directed toward overcoming the recalcitrance of cellulosic biomass include gasification, acid hydrolysis, enzyme-mediated hydrolysis and the development of cellulolytic *Saccharomyces cerevisiae* yeast strains for use in an integrated process, known as direct microbial conversion (DMC) or consolidated bioprocessing (CBP). Such an integrated process offers a very large cost reduction and differs from the earlier SHF (separate hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation in which enzymes from external sources are used) strategies in that the production of cellulolytic enzymes, the hydrolysis of cellulosic biomass and the fermentation of the resulting sugars to ethanol take place in a single process via a cellulose-fermenting yeast strain [3].

The expression of cellobiohydrolases (CBHs) in *S. cerevisiae* has been a particular focus of research because of the vital role such enzymes play in degrading crystalline cellulose. Penttilä *et al*. [4] reported the functional expression of the cellobiohydrolase enzymes, CBHI and CBH2, from *T. reesei* in *S. cerevisiae*. Takada *et al*. [5] expressed the *cbhI* gene of *Aspergillus aculeutus* and used the resulting protein in conjunction with additional cellulolytic enzymes produced by *S. cerevisiae* to achieve up to 59% hydrolysis of avicel. However, it has been observed that the extracellular secretion of these enzymes in *S. cerevisiae* is very low. Notwithstanding these notable studies, the hydrolysis of high-crystalline cellulose with enzyme preparations, including CBHs produced by recombinant *S. cerevisiae*, has not been widely reported, and this has proved more challenging than the functional production of other classes of cellulase enzymes. At present, functional CBH expression and secretion represent a bottleneck to organism development and growth enablement, particularly on crystalline cellulose. Looking beyond saccharolytic enzymes, several mutant strains with “over-production” and “supersecreting” phenotypes showing substantially increased expression and secretion of various proteins have been isolated [6, 7, 8, 9].

In order to screen for desired mutant strains, it was necessary to establish an efficient but simple and direct procedure to estimate the level of protein production. Developing an *S. cerevisiae* mutant strain over-expressing cellobiohydrolase has always been a drawback, owing to the lack of an efficient screening procedure. In our previous

study (manuscript submitted), we found that the fusion (designated CBGL1) of a cellulose-binding domain (CBD2) from *T. reesei* cellobiohydrolase II to a β-glucosidase (BGL1) enzyme from *S. fibuligera* significantly hindered its expression and secretion in *S. cerevisiae*, suggesting that the possible low secretion of cellulolytic enzymes in *S. cerevisiae* could be attributed to the presence of a CBD in these enzymes. As a most ideal situation, in our present study, we used this chimeric enzyme (CBGL1) as a reporter system for the development and efficient screening of mutagenised yeast strains over-secreting CBD-associated enzymes such as *T. reesei* cellobiohydrolase (CBH2). Apart from characterising the expression profile of the *S. cerevisiae* mutant, we also describe the enzymatic characteristics of the purified cellobiohydrolase enzyme (CBH2) secreted by this mutant strain.

3. Materials and Methods

3.1 Strains and plasmids

Escherichia coli strain DH5α (F-α80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rK-, mK_+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1) was used as an intermediate host for the cloning of the recombinant β-glucosidase and cellobiohydrolase gene constructs and for the propagation of the plasmids. The *S. cerevisiae* parent strain, W303-1A (*MATa, leu2-3,112 ura3-52 trp1-289 his3-Δ1 ade2-1*), and the selected mutant strain, WM-91 (developed for this study), were used as hosts for the enzyme expression analysis. *S. cerevisiae* strain BY4742 (*MATα, leu2-3, 112 lys2 ura3-52 his3-Δ1*) was used for developing diploid strains. The 5.9 kb yeast integration vector, a derivative of YIp5 containing the sequences required for replication in *E. coli* and yeast, the ampicillin resistance gene, the yeast uracil (*URA3)* gene, the yeast mating factor's secretion signal (*MFα1s)* and the yeast phosphoglycerate kinase I gene (*PGK1*) promoter ($PGK1_P$) and terminator ($PGK1_T$), was used for heterologous expression. The multiple cloning site placed between *MFα1s* and *PGK1t* contained the following restriction sites: *Hin*dIII, *Cla*I, *Xho*I and *Bgl*II. The plasmid pCEL15-CBD2-BGL1 (refer Chapter 4) was used as the source for the gene encoding the CBGL1 recombinant enzyme. The gene encoding the cellobiohydrolase enzyme of *T. reesei* cellobiohydrolase II was obtained from the plasmid pAZ21. Sequencing of the genes was performed using pGEM®-T Easy plasmid (Promega).

3.2 Culture media

The yeast strains were grown in the following media (all percentages are w/v): YPD (1% yeast extract, 2% peptone, 2% glucose); YPC (1% yeast extract, 2% peptone, 2% cellobiose); synthetic glucose medium (0.67% yeast nitrogen base without amino acids, 2% glucose, amino acids supplemented as required); synthetic cellobiose medium (0.67% yeast

nitrogen base without amino acids, 2% cellobiose, amino acids supplemented as required) (2% agar was supplemented for all the above solid media); YP-Avicel (1% yeast extract, 2% peptone, 2% avicel cellulose); and YP-amorphous (1% yeast extract, 2% peptone, 2% amorphous cellulose). For ascospore formation, diploid cells harvested from YPD were suspended in 1% potassium acetate and sporulated for 5-7 days at 23°C.

3.3 Cloning and construction of recombinant plasmids

The recombinant *CBGL1* gene construct was amplified by the polymerase chain reaction (PCR) method from the plasmid pCEL15-CBD2-BGL1 with primers CBD-F (5'-AAGCTTAGCGTCTGGGGCCAA-3') and BGL1-R (5'-CCGCTCGAGTCAAATAGTAAACAGGACAG-3') containing *Hin*dIII and *Xho*I sites (underlined) respectively, and cloned at the respective sites in the expression vector to construct the plasmid pCBGL1. Similarly, the *CBH2* gene was amplified from the plasmid pAZ21 using the primers CBH2-F (5'-AATCGATCAAGCCTGCTCAAGCGTC-3') and CBH2-R (5'-AAGATCTTTACAGGAACGATGGGTTTG-3') containing the restriction sites *Cla*I and *Bgl*II (underlined) respectively, and cloned at the respective sites in the expression vector to construct the plasmid pCBH2. The amplification reactions contained 1 x PCR buffer, 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM of each primer, 2 ng μL^{-1} template DNA and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. PCR was carried out in a PCR Express thermal cycler for 15 cycles: denaturation, 2 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 1 min at 68°C for both the reactions. Standard protocols for DNA manipulation techniques were followed. Both the coding and non-coding strands were sequenced to ensure the reliable identification of all cloned constructs (PE/Applied Biosystems).

3.4 Construction of recombinant S. cerevisiae strains

The recombinant yeast integration plasmids, pCBGL1 and pCBH2, were linearised at the *Nco*1 and *Apa*I restriction sites of the *URA3* marker respectively and transformed into *S. cerevisiae* strains W303-1A (parent) and WM91 (mutant), using the lithium acetate method described in Gietz *et al*. [10]. Transformants were selected on synthetic glucose medium complemented with tryptophan, leucine, histidine and adenine.

3.5 Mutagenesis and screening of the S. cerevisiae strain

The *S. cerevisiae* strain transformed with the plasmid containing the *CBGL1* gene was subjected to EMS mutagenesis as described in Aho *et al*. [6]. Mutated strains producing higher amounts of extracellular β-glucosidase enzyme were preliminarily screened by plating the mutagenised yeast cells on the synthetic cellobiose medium containing 2% cellobiose as the sole carbon source. Ten putative mutants were isolated on the basis of the time of

colony-forming efficiency in comparison to that of the parent strains. The selected mutant strains, grown overnight on synthetic glucose media, were transferred to the liquid YPC medium at a density of 1×10^5 cells/ml and were cultured in a rotary shaker for 60 h at 30°C. The growth was measured as cell density at various time points, and samples collected at these time points were quantitatively analysed for both extracellular and intracellular β-glucosidase activity to isolate the mutant strain with the highest enzymatic activity.

3.6 Enzymatic assays

β-Glucosidase activity of intracellular and extracellular enzymes was determined by monitoring the release of p-nitrophenol from p-nitrophenol-*β*-D-glucopyranoside (PNPG) [11]. Intracellular enzyme samples were prepared as described in Ausubel *et al*. [13]. One unit of β-glucosidase activity is defined as that amount of enzyme that will hydrolyse 1 μmol of PNPG per minute. The Cellobiohydrolase activity of both intracellular and extracellular enzyme fraction was determined by monitoring the release of p-nitrophenol from p-nitrophenol-*β*-D-cellobioside (PNP-Cellobioside), as described in Kataeva *et al*. [12]. The reaction was carried out at 37°C for 60 min. One unit of cellobiohydrolase activity is defined as that amount of enzyme that will hydrolyse 1 μmol of PNP-Cellobioside per minute. The hydrolytic assay on various cellulosic substrates (avicel, amorphous cellulose, carboxymethyl cellulose and hydroxyethyl cellulose) using extracellular cellobiohydrolase enzyme was performed as follows: the reactions were carried out in 2 mL O-ringed screw cap microcentrifuge tubes containing enzyme preparations in a reaction volume of 750 μL with 1.0 g L^{-1} of substrate in 50 mM sodium citrate buffer (pH 5.0). The reaction mixes were incubated at 37°C with end-over-end rotation at 40 rpm. The samples were filtered through 0.45 μm cellulose acetate filters and analysed for reducing sugar (GAGO kit, Sigma). Each reaction mix was done in triplicate. One unit of hydrolytic activity was expressed as 1 μmol of reducing sugar released per minute.

3.7 Plasmid loss and marker recycling technique

5-fluoro orotic acid technique was followed for replacing the hybrid *CBGL* containing cassette with the *BGL* cassette and also for *URA3* marker recycling. The *S. cerevisiae* strain was grown on YPD medium containing 5-fluoro-orotic acid (FOA) for two to three days [13].

3.8 Southern hybridisation

Genomic DNA isolated from each of the parent and mutant strains integrated with pCBGL1 and pCBH2 was digested with restriction enzymes *Pst*I and *Eco*RV respectively, and subjected to agarose gel electrophoresis. The standard procedure for Southern hybridisation was followed. The blots were probed with 2.7 kb DIG-labelled *CBGL1* and 1.5

kb DIG-labelled *CBH2* gene respectively to confirm the integration of the cassette at the *URA3* locus.

3.9 Northern hybridisation

Total RNA was extracted from the yeast cells by disrupting the cells with glass beads in the presence of phenol, chloroform and isoamylalcohol, as described by Ausubel *et al.* [13]. RNA was denatured by incubation with formaldehyde and then electrophoresised on 1.2% agarose containing formaldehyde. The RNA was transferred to a nylon membrane (Bio-Rad) by the capillary blotting procedure using Tris-acetate-EDTA blotting buffer. Blocking and hybridisation with DIG-labelled *CBGL1* and *CBH2* probe DNA along with the actin encoding *ACT1* were performed essentially as described in the DIG application manual (Roche Molecular Biochemicals). The autoradiography film was densitometrically analysed using alpha imager gel documentation and image analysis system (Alpha Innotech Corporation, USA). mRNA levels in different samples were normalized by comparing them to the levels of *ACT1* (actin) mRNA determined from the same blots.

3.10 Thin-layer chromatography

The hydrolysis profile of cellobiose and the avicel substrate was monitored by thin-layer chromatography using a silica gel 60 F_{254} thin-layer chromatography plate (Merck) and a solvent system of n-propanol-ethanol-water (7:1:2). After eight hours of separation, the plate was developed with 5% sulphuric acid in ethanol spray, followed by incubation at 110°C for about 5 min.

3.11 Partial purification of enzymes

The parent and mutant *S. cerevisiae* transformants carrying either the CBGL1 or CBH2 enzymes were cultured in minimal media with selection conditions and with 2% (w/v) glucose at 30°C on a rotary shaker (200 rpm) for 24 h. The pre-culture was used to inoculate 500 ml of YPD at an optical density (OD measured at a wavelength of 600 nm) of 0.1. After 48 h of incubation, the enzyme was purified from the supernatant. The culture fluid was obtained by centrifugation at 5 000 rpm for 10 min and was brought to 80% saturation by the addition of pre-chilled saturated ammonium sulphate solution and left overnight at 4°C. Precipitates were collected by centrifugation and dissolved in 50 mM citrate buffer at pH 6.0. The solution was subsequently dialysed against 50 mM citrate buffer at pH 4.8 and purified by filtering it through ultrafiltration columns with 50 kDa and 100 kDa cut-off respectively. The enzymes were eluted using 50 mM citrate buffer at pH 4.8. The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard. The partially purified enzymes were analysed by SDS-PAGE.

3.12 SDS-PAGE

SDS-PAGE analysis of the extracellular β-glucosidase and cellobiohydrolase enzymes was performed using 5% and 10% gel respectively, as described by Laemmli [14]. The wide-range molecular marker (Sigma) was used for mass determination. The gel was stained using Coomassie brilliant blue R250.

3.13 Antibody preparation

Antibodies to almond β-glucosidase (Sigma) were obtained essentially as described by Bellstedt *et al.* [15]. Purified β-glucosidase was used for the primary immunisation of the rabbit (1 ml of 1 mg ml^{-1}). Antigen boosts were performed at three weeks and five weeks after primary immunisation and antisera were collected on the 28^{th} and the 42^{nd} day.

3.14 Immunochemical identification of proteins

The extracellularly secreted native and recombinant β-glucosidase enzymes were collected at different time intervals and concentrated using Amicon Micron-10 microconcentrators. The samples were electrophoresised in Tris-Glycine-SDS gels and transferred to polyvinylidene fluoride (PVDF) microporous membrane (ImmobilonTM-P Transfer Membrane, Millipore) by means of the standard procedure [16]. Western blot analysis of the proteins was performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech). The blots were sequentially treated with rabbit anti-β-glucosidase (1:15000) and donkey anti-rabbit IgG conjugated with horseradish peroxidase (1:20000). The HRP/hydrogen peroxide-catalysed oxidation of luminol in alkaline conditions emitted enhanced chemiluminescence that was detected by short exposure to blue light-sensitive autoradiography film (Hyperfilm ECL, Amersham Biosciences).

3.15 Optimum pH and temperature determination

The optimum pH for the CBH2 enzyme from the parent and mutant strains was investigated at pH ranges of 2-8, using 1 g L^{-1} avicel in 50 mM sodium citrate buffer at 30°C. The pH stability was examined by pre-incubating the enzyme in the absence of a substrate at 30°C for 30 min in 50 mM sodium citrate buffer (pH 2-8) before determining the CBH2 activity. The temperature profiles of the CBH2 enzyme from the parent and mutant strains were measured at temperatures between 20 and 70°C under similar assay conditions. The temperature stability was determined by pre-incubating the enzyme in the absence of a substrate at temperatures ranging from 20 to 70°C for 30 min in 50 mM sodium citrate buffer (pH 5.0) before determining the CBH2 activity. The stability of the enzyme was also measured at different time points under the incubation temperature of 50°C.

4. Results and discussion

4.1 Isolation and genetic characterisation

A chimeric gene construct (*CBGL1*), encoding the cellulose-binding domain (CBD2) from the *T. reesei* cellobiohydrolase II fused to the *S. fibuligera* β-glucosidase (BGL1), was isolated from plasmid pCEL15-CBD2-BGL1 using sequence-specific primers. The 2.7 kb PCR product was inserted between the $MF\alpha1_S$ secretion signal and the $PGK1_P$-$PGK1_T$ expression cassette, thereby generating a yeast integrating plasmid containing the $MF\alpha1_S$-$PGK1_P$-CBD2-BGL1-$PGK1_T$ gene construct. The resulting plasmid, pCBGL1, was integrated into the *URA3* locus of the *S. cerevisiae* W303-1A strain and the integration was confirmed by Southern blotting (data not shown). The positive transformants (W303-CBGL1) exhibited cellobiase activity on paranitrophenol glucopyranoside substrate, confirming the functional expression and secretion of the chimeric β-glucosidase enzyme (data not shown).

Yeast cells of W303-CBGL1 were treated with ethyl methane sulphonate until the survival rate dropped below 20% and the mutagenesis frequency reached 10^{-3}-10^{-4}. The cells were plated onto YPC medium and 10 putative mutant colonies were selected on the basis of their ability to grow on cellobiose. Mutant strain WM91-CBGL1, which exhibited a dramatic increase in its ability to grow in YPC medium, was chosen for further study.

Characterisation of the WM91-CBGL1 mutant strain revealed that it was able to produce more than three-fold more β-glucosidase than the parent transformant, W303-CBGL1, after 48 h of growth in YPD medium (Table 1). It was also noticed that 75% of the enzyme produced was secreted and that the intracellular fraction constituted approximately 25% of the total activity in both the parent and the mutant transformant.

TABLE 1. β-Glucosidase activity in the parent (W303) and mutant (WM91) strain of *S. cerevisiae* transformed with the chimeric *CBGL1* and native *BGL1* gene. The strains were grown at 30°C in YPD media for 48 h and the activity was measured. The data shown are mean values of triplicate measurements and the standard deviation was less than 10%.

Strain	β-Glucosidase activity (U L^{-1})		
	Intracellular	Extracellular	Total activity
W303-1A	7.1	23.9	31.0
W303-CBGL1	15.6	49.9	65.5
WM91-CBGL1	48.9	151.5	200.4
W303-BGL1	47.7	191.1	238.8
WM91-BGL1	51.5	199.7	251.2

In order to study whether the mutation in WM91-CBGL1 was specific for the chimeric enzyme, the *CBGL1* cassette was replaced with the native *BGL1* gene using the 5-fluoro-orotic acid (FOA) technique. It was interesting to note that the total enzyme activity did not differ significantly between the parent (W303-BGL1) and the mutant (WM91-BGL1) strain and that the extracellular/intracellular ratio also remained the same, except for a small increase in the mutant strain (Table 1). The data clearly illustrates that the mutation in the WM91 strain was not specific for the CBD-fused protein. The little activity exhibited by the parent strain could be attributed to the residual β-glucosidase activity of the native EXG1 enzyme that is secreted into the medium.

To confirm that the increase in enzyme production in WM91-CBGL1 is due to the chromosomal mutations, the selected mutant was grown in 5-fluoro orotic acid medium to delete the pre-existing plasmid pCBGL1 along with the *URA3* marker. The colonies exhibiting a loss of β-glucosidase activity and lacking the ability to grow in the absence of uracil-supplemented medium were selected. Some of these ura$^-$ strains were retransformed with pCBGL1 plasmid that showed the mutant phenotype, confirming that the mutation(s) had not happened in the expression plasmid. Strain WM91-BGL1 was crossed with strain BY4742 and the diploids were grown in the minimal medium with the necessary supplementation of amino acids. All the diploids showed β-glucosidase activity similar to that of the parental strain, suggesting that the mutation(s) are recessive. It was also noticed that the diploid strain lost the ability to form ascospores.

4.2 Characterisation of growth and enzyme production

The enzyme activity profile of the parent and mutant strains was studied to analyse the accumulation of the CBGL1 enzyme with reference to the extracellular and intracellular ratios at various time periods of growth in YPD medium (Fig. 1A).

Although the rate of growth was similar in YPD for both strains (data not shown), the parent strain did not show a major increase in enzyme activity after the 12 h, while the mutant strain showed an increase in activity of almost three-fold from 12 to 60 h of growth. It was also noted that the secreted fraction of the enzyme increased considerably (more than three-fold) in the mutant from 12 to 60 h of growth, while the parent strain showed a modest increase only after 36 h. The enzyme activity of the parent and mutant strains correlated well with their variation shown during growth in YPC medium (Fig. 1A).

The mutant strain reached twice the final cell density obtained by the parental strain, suggesting that the increased expression and secretion of the enzyme by the mutant strain enabled the higher rate of hydrolysis of cellobiose to glucose, thus making it available for growth. The improved growth by the mutant strain in YPC has also been illustrated by a significant reduction of residual cellobiose in the media analysed by TLC (data not shown). After 24 h of growth, the cellobiose concentration in the medium was significantly reduced in

comparison to that of the parent strain, which showed a steady presence of cellobiose in the medium.

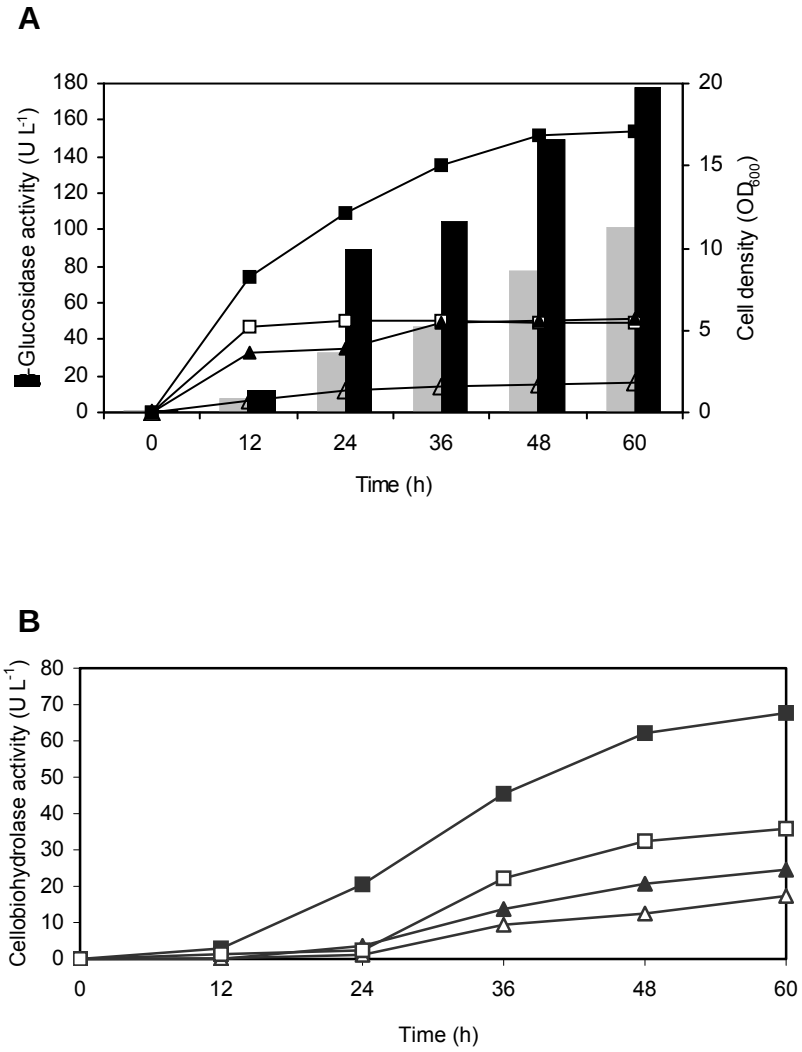

Fig. 1. (A) β-glucosidase activity profile of the parent and mutant strain during growth in YPD medium. Extracellular - W303-CBGL1 (—▲—); WM91-CBGL1 (—■—), intracellular - W303-CBGL1 (—△—); WM91-CBGL1 (—□—). Growth of parent and mutant strains expressing chimeric β-glucosidase enzyme on cellobiose (YP-cellobiose); W303-CBGL1, grey bars; WM91-CBGL1, black bars. (B) Cellobiohydrolase activity profile of the parent and mutant strain during growth in YPD medium. Extracellular - W303-CBH2 (—▲—); WM91-CBH2 (—■—), intracellular- W303-CBH2 (—△—); WM91-CBH2 (—□—).Values are the mean of triplicate measurements and the standard deviation was less than 10%.

The difference in the extracellular enzyme production between the parent and mutant strain was also evident from the Western blot staining (Fig. 3). While the extracellular β-glucosidase enzyme was detected in the mutant strain after 12 h of its growth in YPD medium, the enzyme was detected only after 24 h in the parent strain.

The following six mechanisms were considered as possible explanations for increased enzyme production in the mutant [17], (i) the mutant might have acquired a higher ability in specific chimeric β-glucosidase (CBGL1) mRNA production due to either gene amplification (similar to a previously described copper-resistant mutant *CUP1* [18]) or more efficient transcription; (ii) more efficient translation of CBGL1 mRNA; (iii) a general increase in protein production due to more efficient energy utilisation for protein synthesis at the expense of cell growth, like α-factor-treated *MATa* cells which continue translation and transcription without DNA synthesis and cell division; (iv) the production of large cell mass, resulting in an increased production of proteins; (v) improved secretion due to the reduction of specific protein degradation; and (vi) secretion due to a reduction in the improper transport of heterologous proteins (e.g., to the vacuole). In the investigation of the first possibility, RNA was extracted from both the parent and mutant strains after 12, 24 and 48 h of growth.

Northern blotting analysis revealed a considerable quantitative difference in the transcription profile of *CBGL1* between the two strains (Fig. 2A). In fact, the CBGL1 mRNA transcription in the mutant strain increased markedly, to an average of two- to three-fold in comparison to the parental strain, suggesting the reason for the increase in production of the enzyme. The differential rate in transcription leading to the increased production of the heterologous enzyme has been reported previously using the endoglucanase enzyme from *T. reesei* [6]. In addition, the data also suggest that transcription is most likely the rate-limiting factor in the production of the chimeric BGL1 enzyme in the parent strain.

The hyperglycosylation of heterologous proteins in *S. cerevisiae* has been known to hinder secretion to a significant extent [19]. SDS-PAGE analysis of the partially purified CBGL1 from the supernatant of both the parent and mutant strain did not show any variation in their molecular weights (ca. 220 kDa) [20], suggesting that posttranslational glycosylation is similar in both strains, eliminating the hypothesis that the improved secretion in the mutant was caused by a reduction in protein glycosylation (Fig. 4A).

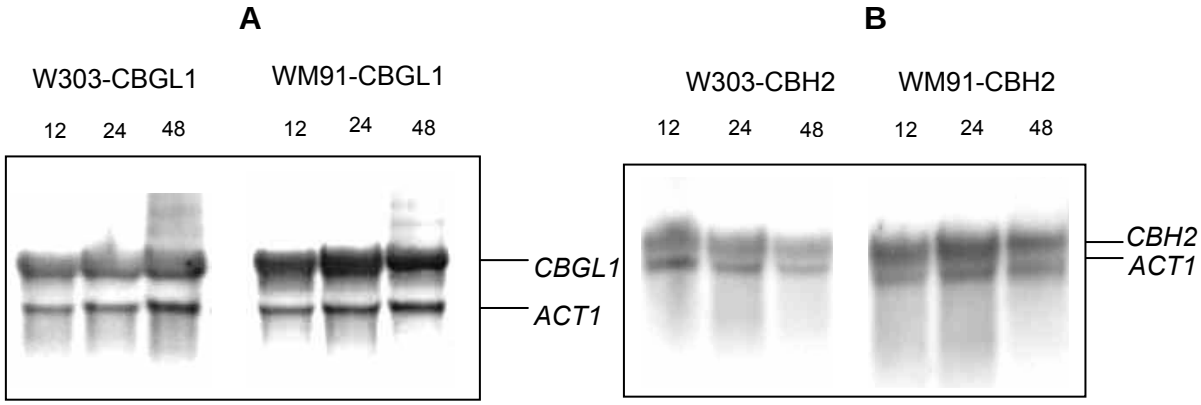

Fig. 2. mRNA expression profile of (A) β-glucosidase gene (*CBGL1*) and (B) cellobiohydrolase gene (*CBH2*) analysed by northern blotting in the parent and mutant strain

of *S. cerevisiae*. RNA was extracted from cells grown in YPD medium at 30°C for the indicated time points in hours. 10 μg of total RNA was probed with *CBGL1* and *CBH2* respectively. *ACT1* was probed as the positive control.

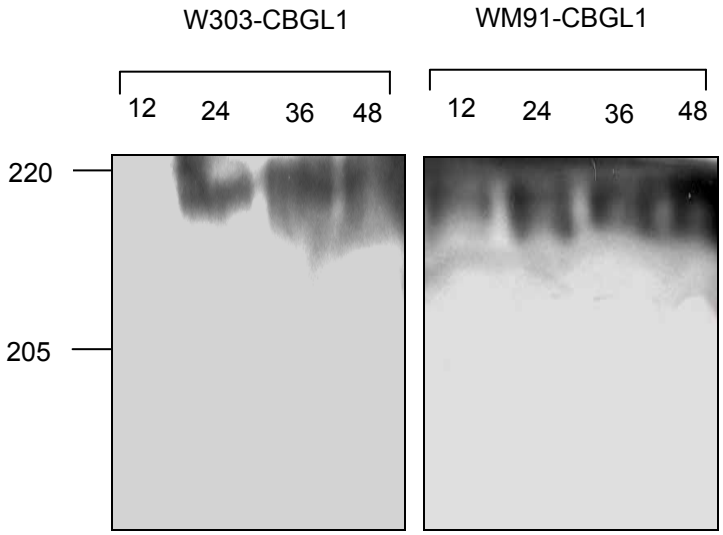

Fig. 3. Western blot analysis of the chimeric β-glucosidase enzyme secreted by the parental (W303-CBGL1) and mutant (WM91-CBGL1) strain at different time periods of growth. The sizes of the molecular markers (kDa) are shown on the left.

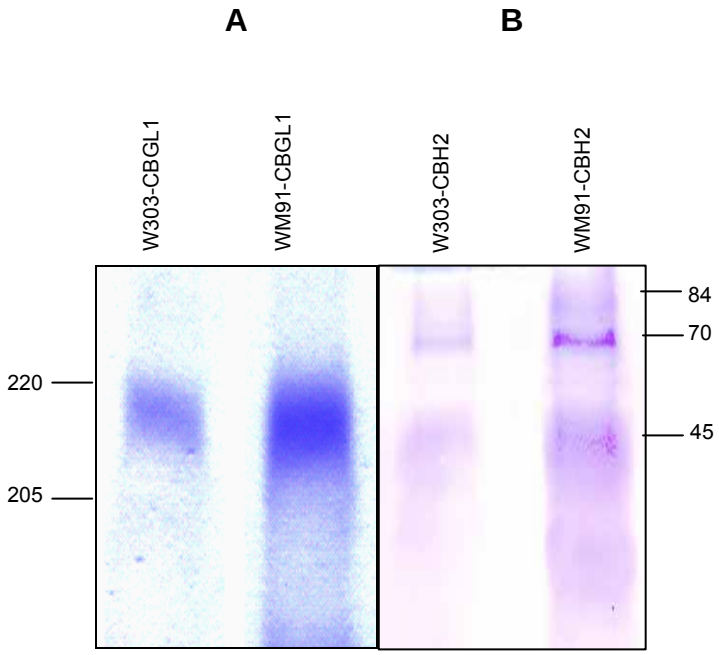

Fig. 4. SDS-PAGE analysis of the partially purified (A) β-glucosidase and (B) cellobiohydrolase enzyme from the parent and mutant strain of *S. cerevisiae*. The sizes of the molecular markers (kDa) are shown.

4.3 Expression and characterisation of the CBH2 enzyme in the mutant

The ability of the parent strain, W303-1A, and the mutant strain, WM91, to produce *T. reesei* cellobiohydrolase enzyme (CBH2) was investigated. The enzyme was also purified from the culture supernatant and characterised for its hydrolytic and physical properties. The 1.5 kb *CBH2* gene was isolated from plasmid pAZ21 using sequence-specific primers and cloned into a yeast integrating vector to construct the plasmid pCBH2. The plasmid was subsequently integrated into the parent and the mutant strain to obtain the transformants W303-CBH2 and WM91-CBH2 respectively. The integrations were confirmed by Southern hybridisation (data not shown).

The cellobiohydrolase activity of the parent and the mutant *S. cerevisiae* transformant was detected using paranitrophenol cellobioside as substrate. It was observed that the mutant strain showed an increase of approximately three- to five-fold in the total productivity of the enzyme when compared to the parent strain at all the time periods of growth (Fig. 1B). It was also noticed that, as in the case of the chimeric β-glucosidase enzyme, the majority of the CBH2 enzyme produced was secreted extracellularly. While an average of 65% of the total activity was extracellular in the WM91-CBH2 strain, the W303-CBH2 strain exhibited about three-fold less activity in the extracellular fraction. The expression pattern of the mutant strain also showed a quick accumulation in extracellular activity from 24 to 48 h, in comparison to the parent strain, which followed a steady pattern. The ratio of intracellular accumulation of the CBH2 enzymes was also found to be reduced considerably in the mutant when compared to the parent strain.

As demonstrated in the WM91-CBGL1 strain, the increased production of the CBH2 enzyme in the mutant strain, WM91-CBH2, was also found to be a result of increased CBH2 mRNA transcription (Fig. 2B). Northern analysis showed that the ratio of the transcribed CBH2 mRNA in the mutant and the parent varied at an average of two- to three-fold in relation to the *ACT1* concentration.

SDS-PAGE analysis of the partially purified enzyme from the supernatant of both transformants is approximately 70 kDa, which is consistent with previous reports [4, 7], excluding that the enhancement of secretion by the WM91-CBH2 strain is a result of differential glycosylation of CBH2 (Fig. 4B).

The ability of the mutant *S. cerevisiae* strain, WM91-CBH2, to grow on cellulosic substrates was analysed using amorphous, avicel and CMC cellulose (data not shown). Although avicel and amorphous cellulose differed vastly in their degree of crystallinity, the rate of growth of both the mutant and the parent strain in these substrates remained similar. However, the mutant strain showed only a small improvement in growth in both substrates. The low growth rate of the mutant strain, in spite of the increased production and secretion of the CBH2 enzyme, could be attributed to a limitation in the availability of accessible sites

in the substrate. Both the mutant and the parent strain did not show any growth on CMC substrate.

4.4 Characteristic properties of the CBH2 enzyme from the mutant S. cerevisiae

The purified CBH2 enzyme was examined for its ability to hydrolyse various cellulosic substrates under standard assay conditions (Table 2). Although the CBH2 enzyme from the mutant strain did not show a significant variation in hydrolytic activity on amorphous, CMC and HEC cellulose, a small difference was noted in the hydrolysis of avicel cellulose.

The enzyme was further studied for the effect of pH and temperature on the avicelase activity (Fig. 5A & B). The optimum pH was found to be between 5.0 and 6.0 for the enzymes isolated from both the W303-CBH2 and the WM91-CBH2 strain. The enzymes were stable between pH 4.0 and 6.0 during incubation at 50°C for 30 min. Nevertheless, the CBH2 enzyme from WM91-CBH2 was found to be less stable at lower pH values than the CBH2 from WM303-CBH2.

The avicelase activity of the CBH2 enzyme was also measured at various temperatures at pH 5.0. The enzymes from both the W303-CBH2 and the WM91-CBH2 strain exhibited an optimum temperature range between 30 and 40°C. Interestingly, the temperature stability assay showed that the CBH2 enzyme from the mutant strain was relatively stable until 40°C, after which the activity dropped rapidly by 80%. The CBH2 enzyme from the parental strain was relatively stable up to 60°C, with more than 90% of the activity being retained (Fig. 5B).

TABLE 2. Hydrolytic activity of the cellobiohydrolase enzyme from the parent (W303) and mutant (WM91) strain of *S. cerevisiae* on various cellulosic substrates. The experiments were done in triplicates and the standard deviation was less than 10%.

Substrate	Cellobiohydrolase activity (1U=μmol min^{-1})	
	W303-CBH2	WM91-CBH2
Amorphous	25.08	25.08
Avicel	18.07	25.7
CMC	22.41	23.84
HEC	24.92	24.61

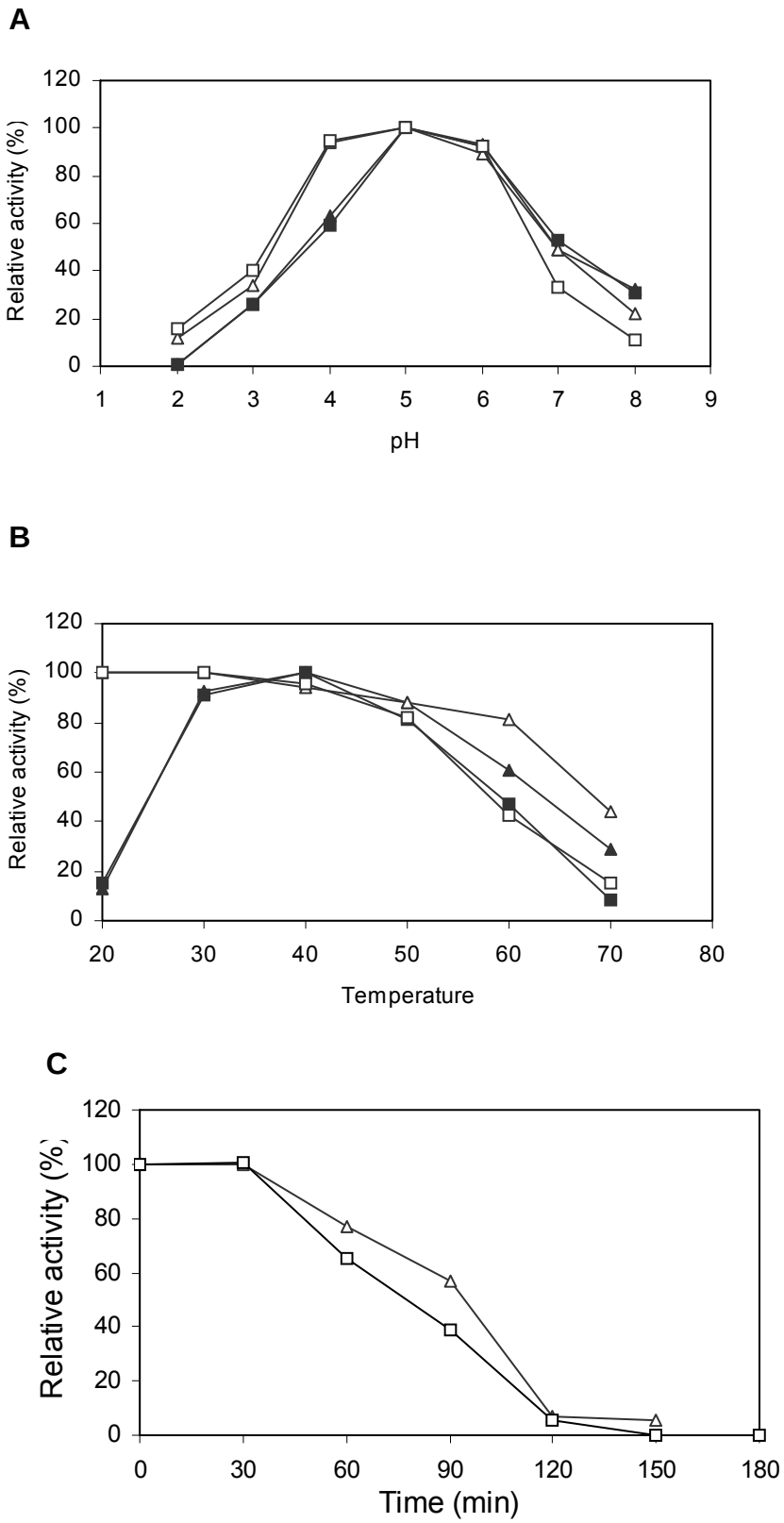

Fig. 5. (A) pH optimum and stability (B) temperature optimum and stability (C) thermostability profile at 50°C, of the CBH2 enzyme from the parent and mutant strain. pH and temperature optimum: W303-CBH2 (—▲—), WM91-CBH2 (—■—). pH and temperature stability: W303-CBH2 (—△—), WM91-CBH2 (—□—).

This suggests a possible structural fragility in the CBH2 molecule produced by the mutant strain, which was further confirmed by its thermal instability at 50°C (Fig. 5C). The pH profile of the CBH2 enzyme from the WM91-CBH2 strain widely resembled that of the CBH2 enzyme secreted by *Schizosaccharomyces pombe*, whereas the optimum temperature and stability of the molecule varied extensively [7].

In conclusion, the chimeric β-glucosidase enzyme (CBGL1) was successfully used as a screening system for the isolation of mutant yeast strains with improved secretion for CBD-associated enzymes such as *T. reesei* cellobiohydrolase (CBH2). Our data suggest that the increased expression of both BGL1 and CBH2 that enhancing the secretion ratio of them in the mutated genetic background was associated with the higher mRNA expression levels. The optimisation of expression of other cellulolytic enzymes such as endoglucanases and xylanases in the *S. cerevisiae* strain(s) could enable the consolidated bioprocessing of cellulosic substrates.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

1. GENERAL DISCUSSION AND CONCLUSION

The vast majority of studies investigating cellulose hydrolysis and cellulase enzyme systems have proceeded within the context of an enzymatically-oriented intellectual paradigm. In terms of fundamentals, this paradigm focuses on cellulose hydrolysis as being primarily an enzymatic rather than a microbial phenomenon. In terms of applications, the enzymatic paradigm anticipates processes featuring the production of cellulase in a step separate from that in which the cellulosic feedstock is hydrolysed for the purpose of conversion to a desired product. This paradigm is clearly manifested in statements accompanying the early work of pioneers in the field. For example, Sternberg wrote in a 1976 statement attributed to Mandels and Weber (1969): “Thousands of microorganisms have the ability to grow on cellulose. Although many of these grow quite rapidly only a few produce extracellular cellulolytic enzymes capable of converting crystalline cellulose to glucose in vitro.” Reese and Mandels wrote in 1971: “The dream of cellulase investigators is to develop a commercially feasible process for converting waste cellulose to glucose.”

The biggest technological obstacle to large-scale utilisation of cellulosic feedstocks for the production of bioethanol as a cost-effective alternative to fossil fuels is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass and fermentation of resulting sugars to ethanol in a single process step via a single microorganism or consortium. Such “consolidated bioprocessing” (CBP) offers very large cost reductions if microorganisms, such as the yeast *Saccharomyces cerevisiae*, can be developed that possess the required combination of efficient cellulose utilisation and high ethanol yields.

The greatest protein engineering successes on cellulolytic enzymes have clearly been in the construction of variant recombinants for structural and mechanistic analyses. Kinetic and structural data have elucidated the catalytic mechanism for all cellulase families to a large extent. The results reported in this study show that both the cellulose binding domain (CBD) and the linker sequences coupled to a non-cellulolytic catalytic domain, such as *S. cerevisiae* EXG1 and SSG1 and *S. fibuligera* BGL1, impart the ability to act and hydrolyse β-1,4-exoglucanase-specific cellulosic substrates. This suggests that the engineered multidomain enzymes would have gained sufficient molecular stability, flexibility and intramolecular interaction between the domains to act synergistically in optimised enzymatic mixtures for the biodegradation of crystalline cellulose. It is also clear that the CBD sequence plays the role of a thermostabilising domain in these engineered enzymes. A more detailed understanding of the role and function of the CBD in these enzymes would

further enable the development of efficient engineered enzymes that are adapted to bioprocess-efficient microbial systems, such as *S. cerevisiae*.

The chimeric enzyme of CBD2-BGL1 was also used as a reporter system for the development and efficient screening of mutagenised *S. cerevisiae* strains that overexpress CBD-associated enzymes such as *Trichoderma reesei* cellobiohydrolase (CBH2). A mutant strain WM91 was isolated showing up to 3-fold more cellobiohydrolase activity than that of the parent strain. The increase in the enzyme activity in the mutant strain was found to be associated with the increase in the mRNA expression levels. The CBH2 enzyme purified from the mutant strain did not show a significant difference in their characteristic properties in comparison to that of the parent strain.

S. cerevisiae has received the most attention as a potential candidate for CBP, with its rapport as an efficient fermentation organism and also as an efficient host for heterologous expression. However, owing to the lack of an endogenous cellulolytic system their employment in CBP has been mulled. With the development of recombinant enzymes of EXG1, SSG1 and BGL1 *S. cerevisiae* can be armed with its own cellulolytic enzymes capable of hydrolysing recalcitrant substrates like cellulose. In addition, expression of these recombinant enzymes in the mutant *S. cerevisiae* strain developed, would also overcome the drawbacks encountered during heterologous cellulase expression like poor expression and secretion.

In summary, this research has paved the way for the improvement of the efficiency of the endogenous glucanases of *S. cerevisiae*, and the expression of heterologous cellulases in a hypersecreting mutant of *S. cerevisiae*. The results does not claim to advance the field closer to the goal of one-step cellulose processing in the sense of technological enablement; rather, its significance hinges on the fact that this study has resulted in progress towards laying the foundation for the possible development of efficient cellulolytic *S. cerevisiae* strains that could eventually be optimised for consolidated bioprocessing.

As a future direction, the recombinant enzymes of EXG1, SSG1 and BGL1 designed in this study can be co-expressed along with other crucial cellulolytic enzymes like endocellulases to build a complete cellulolytic machinery in *S. cerevisiae* that would enable the systemic and synergistic degradation of cellulose chains into soluble sugars. Further this cellulolytic *S. cerevisiae* strain can be engineered to anchor the cellulose binding domain at the cell surface thereby providing a covalent anchorage for the whole cell to the substrate. This would significantly reduce the dilution effect of the expressed enzymes thus increasing the rate of hydrolysis.

Today, biotechnology is a central component of progress toward applied objectives within the context of both the microbial and enzymatic cellulose hydrolysis paradigms. For the microbial paradigm, achieving the organism development milestones that we foresee as being pursuant to both the native and recombinant cellulolytic strategies will be based, to a

very large extent, on the successful application of biotechnological tools. For the enzymatic paradigm, cellulolytic enzymes with higher specific activity than the current commercial preparations are highly desirable and are likely to be required. Developing such cellulolytic enzymes can be approached either by using new heterologous expression systems for cellulolytic enzymes that naturally have high specific activity, or by using protein engineering to create new, improved enzyme systems. Notwithstanding the substantial differences between the microbial and enzymatic paradigms outlined in the preceding paragraphs, it is possible that the results from work undertaken within both these paradigms may be incorporated into advanced technology in a convergent manner. For example, high specific-activity cellulolytic enzymes could, in principle, be incorporated into CBP-enabling microorganisms. In the decades since commercially feasible cellulose conversion processes featuring enzymatic hydrolysis were first envisioned by Reese, Mandels and other pioneers in the field, sustainable resource supply, energy security and global climate change have emerged as dominant issues affecting the wellbeing of humankind. The motivation for realising this vision has thus increased substantially, especially as the magnitude of the challenges involved has become more apparent. In spite of the great effort that has been devoted to the field, there are biotechnological approaches to developing practical processes for the conversion of cellulose to fuels and commodity chemicals in existence today that are both promising and relatively unexplored. An important subset of such approaches involves microbial cellulose utilisation. This is not only important from a fundamental point of view, but it opens up the possibility for complete biomass conversion, which is one of the great environmental goals of the 21st century.

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APPENDIX

Different genetic backgrounds influence the secretory expression of the *LKA1*-encoded *Lipomyces kononenkoae* α-amylase in industrial strains of *Saccharomyces cerevisiae*

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Different genetic backgrounds influence the secretory expression of the *LKA1*-encoded *Lipomyces kononenkoae* α-amylase in industrial strains of *Saccharomyces cerevisiae*

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1. Abstract

A haploid laboratory strain and four industrial (baking, brewing, wine, ATCC) strains of *Saccharomyces cerevisiae* were transformed with the *Lipomyces kononenkoae* α-amylase-encoding gene (*LKA1*). These transformants displayed significant differences in terms of the level of secretory expression of *LKA1* under control of the *PGK1* promoter and terminator, as well as their ability to produce and secrete the *LKA1*-encoded raw-starch-degrading α-amylase and to ferment starch. These results demonstrate the importance of the selection of appropriate host strains for yeast development pursuant to starch conversion into commercially important commodities via consolidated bioprocessing (CBP).

2. Introduction

The synthesis of a functional foreign protein in *Saccharomyces cerevisiae* involves a cascade of complex cellular mechanisms. The insertion of a foreign gene into an expression vector does not guarantee high levels of expression of foreign protein. Since gene expression and protein secretion are complex, multi-step processes, barriers could be encountered at numerous stages, from transcription through to the stability of secretion proteins (Romanos *et al*. 1992). Therefore, the choice of vector, promoter and secretion signal is crucial for optimal gene expression and protein secretion. However, the genetic background of various strains may also contribute to the differential levels of foreign protein synthesis and secretion. To date, two strategies have been used to secrete foreign proteins from yeast: the use of heterologous (non-yeast) and homologous (yeast) signal sequences.

S. cerevisiae lacks the ability to produce extracellular depolymerising enzymes that can efficiently liberate fermentable sugars from abundant, polysaccharide-rich substrates. This limits *S. cerevisiae* to a narrow range of carbohydrates. Exploitation of recombinant yeast strains with the ability to utilise such complex polysaccharides can, in turn, aid in the cost-effective production of various heterologous proteins of biological interest. Several yeasts can degrade starch. The lipid-forming yeast, *Lipomyces kononenkoae* IGC 4052B, is one of the most efficient raw-starch-degrading yeasts known (Spencer-Martins & Van Uden,1977; Horn *et al*.,1988). It secretes a highly active raw starch-degrading α-amylase that liberates reducing groups from glucose polymers containing both α-1,4 and α-1,6 bonds. However, owing to its low ethanol tolerance, slow growth rate, poorly characterised genetics and lack of GRAS (Generally Regarded As Safe) status, the potential of *L. kononenkoae* to be used in one-step bioconversion processes of starch-rich biomass to commercially important commodities is limited.

The α-amylase gene (*LKA1*) from *L. kononenkoae* was previously cloned, characterised and expressed in *S. cerevisiae* (Steyn & Pretorius,1995; Steyn *et al*.,1995, 1996). In the present report we describe the expression of the *L. kononenkoae LKA1* gene and the production of its encoded extracellular α-amylase by four genetically distant recombinant industrial strains of *S. cerevisiae* to show that the genetic background also plays a significant role in heterologous protein production.

3. Materials and methods

3.1 Strains and plasmids

Saccaharomyces cerevisiae strains used in the study were derived from two different backgrounds viz., industrial strains VIN13 (wine), BAK13 (baking), BREW13 (brewing), and ATCC 4126 (a fast-fermenting, ethanol-tolerant strain) as well as a haploid strain (Y294). Chromosome banding patterns confirmed that all the strains varied in their genetic backgrounds (data not shown). *Escherichia coli* DH5α [*supE*44 Δ*lac*U169 (ø80*lacZ*ΔM15) *hsd*R17 *recA1 end*A1 *gyr*A96 *thi*-1 *rel*A1] was used as the host strain for the standard manipulation of the α-amylase *(LKA1)* gene. The integration plasmid pDLG31 was constructed by using the following plasmids: pWX509 [*bla SMR1-410*] (Casey *et al*., 1988) and pAJC2 [*bla URA3 PGK1*$_P$*-LKA1-PGK1*$_T$] (Steyn *et al*., 1995)

3.2 Media and culture conditions

E. coli was grown at 37°C with shaking in Luria-Bertani medium [1% peptone, 0.5% yeast extract, 0.5% NaCl (pH 7.2)] supplemented with 100 µg ml^{-1} of ampicillin. The recombinant yeast cells were grown at 30°C in synthetic media containing 0.67% yeast nitrogen base

without amino acids (Difco). The medium was supplemented with the required amino acids and 2% glucose for SCD. SCDSM plates contained 0.5% glucose and 60-80 µg ml^{-1} (depending on the yeast strain) of sulfometuron methyl (Dupont) dissolved in N-, N-, dimethylformamide. A rich medium (YPD) contained 1% yeast extract, 2% peptone and 2% glucose. The starch hydrolysing activity of enzymes was studied by plating the transformed yeast onto a synthetic complete medium (SC) containing 0.67% yeast nitrogen base, 2% Lintner starch and 2% agar. The plates were incubated for 2 to 6 days at 30°C and were then placed at 4°C for the remaining undigested starch to precipitate. A clear zone around the colonies indicated starch-degrading activity. Likewise, the production of α-amylase was assessed by plating the colonies onto SC medium containing 0.67% yeast nitrogen base, 900 mg phadebas starch per litre (Pharmacia & Upjohn) and 2% agar. The plates were incubated for 2 to 6 days at 30°C and colonies showing activity were identified by a clear zone around them. Growth curve experiments were performed in YPD, with 2% Lintner starch as the sole carbon source.

Starch fermentation trials were done by inoculating the transformed and non-transformed yeast strains in 10 ml YPD in a 250-ml flask and incubating it for 24 h at 30°C and then re-inoculating in 600 ml YPD in a 2-litre flask. After 48 h of growth, the cells were harvested and inoculated into the fermentation medium to give 20 g cells l^{-1}. The fermentation medium contained 6.7 g l^{-1} yeast nitrogen base, 0.42 g l^{-1} Tween 80, 0.01 g l^{-1} ergosterol and 20 g l^{-1} of soluble potato starch.

3.3 DNA manipulations

Standard methods of DNA manipulation, plasmid DNA isolation and transformation of *E. coli* DH5α were used (Sambrook *et al*., 1989). DNA amplification by the polymerase chain reaction (PCR) technique, RNA isolation from yeast cells, and Southern and Northern blot hybridisations were performed as described by Laing and Pretorius (1992) and Sambrook *et al*. (1989).

3.4 Construction of recombinant plasmids

Previously, the *LKA1* gene from *L. kononenkoae* was cloned and expressed in *S. cerevisiae* under control of the phosphoglycerate kinase (*PGK1*) promoter and terminator (Steyn *et al*., 1995). Plasmid pWX509* was constructed by digesting plasmid pWX509 (Casey *et al*., 1988) with *Cla*I and *Sma*I, the overhanging end filled in with DNA polymerase I (Klenow fragment) and then re-ligated. Plasmid pDLG31 was constructed by ligating a 3850-bp *Kpn*I-*Sna*BI fragment from pAJC2 (Steyn *et al*., 1995) into the *Kpn*I-*Nru*I sites of pWX509*. The restriction maps of the recombinant plasmids pWX509* and pDLG31 are shown in Figure 1.

3.5 Yeast transformation

Industrial yeast strains were transformed by electroporation. Yeast cells were inoculated in 10 ml YPD and incubated at 30°C until the stationary phase. Pre-warmed 100 ml YPD was then inoculated with 10 ml of the pre-culture and incubated until mid-growth-phase was reached [absorbance at 600 nm (A_{600}) of 1]. The cells were then harvested, washed with 50 ml sterile water and resuspended in 50 ml of 0.025 M 1,4-dithiothreitol solution and incubated at room temperature for 10 min. Thereafter the cells were harvested again and washed in 50 ml TE-buffer pH 7.5 (0.01 M Tris.HCl, 0.01 M EDTA). Finally, the cells were resuspended in 10 ml TE buffer. Linear DNA (10-15 µg) at a maximum volume of 20 µl was added to 400 µl cell suspension in a microcentrifuge tube and incubated on ice for 10 min. Thereafter 400 µl of a 70% polyethylene glycol-solution was added and mixed thoroughly, but carefully. The mixture was transferred to electroporation cuvettes and incubated on ice for 5 to 10 min. The EasyjecT +450V Twin pulse (EquiBio) apparatus was used for electroporation. The pulse program was as follows: voltage 1300 V with 25 µF capacity, a shunt of 329 ohm and a pulse of 8.2 msec. The yeast cells were then immediately plated on SCDSM and incubated for at least seven days.

3.6 Enzyme assays

Amylase activity was assayed using the PAT assay kit (Pharmacia Diagnostics, Uppsala, Sweden). Samples were collected at different time intervals after inoculation in YPD. The culture supernatants, containing the secreted α-amylase, were removed as 200 µl aliquots (in triplicate) and pipetted into test tubes to which 4 ml of 0.1 M sodium acetate buffer (pH5) was added. The test tubes were pre-incubated for 5 min at 37°C. Phadebas starch (one tablet of 45 mg) was added to each tube, immediately mixed for 10 s and incubated at 37°C for exactly 15 min. Adding 1 ml 0.5 M NaOH terminated the reaction. The reaction solution was mixed and centrifuged at 8000 rpm for 5 min. The absorbance of the supernatant was measured at 620 nm (A_{620}) against the buffer (4.2 ml of buffer). The amylase activity (nkat/ml) was read from the standard curve. Assays were repeated six times and the mean value recorded. The standard deviations were between 5 and 10%.

3.7 Starch utilisation and hydrolysis

Fermentation samples were collected at different time intervals in 2 ml microcentrifuge tubes and centrifuged at 6000 rpm for 2 min. An aliquot (200 µl) of the supernatant was collected in a dry test tube; 1 ml of 2 M HCl was added to each tube and the contents were boiled for 30 min at 100°C to break down the complex starch molecules into simple glucose moieties. One ml of 2 M NaOH was added to stop the reaction. The reducing sugars released by the starch breakdown were determined using the Glucose Trinder Kit.

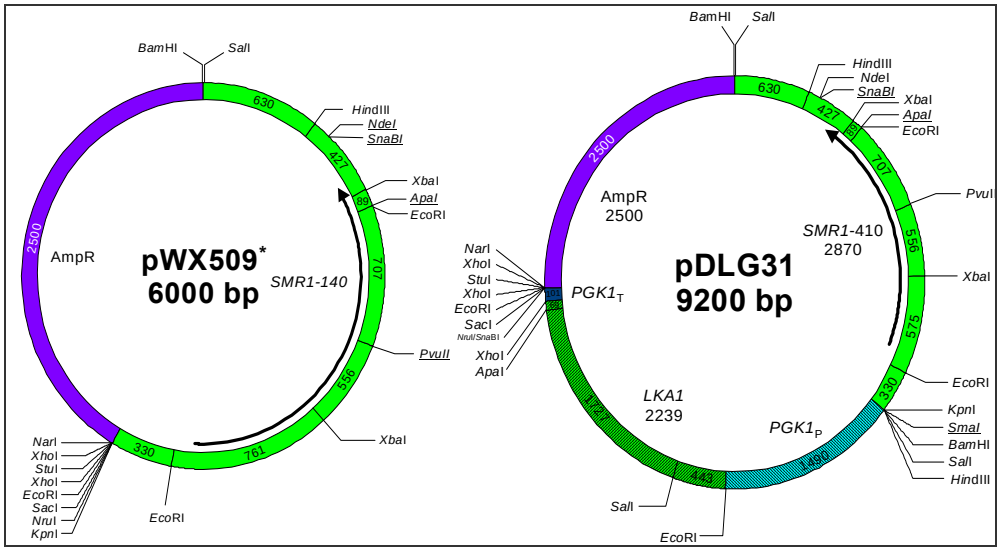

Fig. 1. Restriction map of plasmids pWX509* and pDLG31.

4. Results and Discussion

4.1 Transformation of industrial yeast

An integration plasmid was designed for single copy integration of the *L. kononenkoae LKA1* gene into all four industrial strains of *S. cerevisiae*. The plasmid, designated pDLG31, was devised to achieve single integration by homologous recombination at the *ILV2* locus on chromosome XIII. Figure 1 shows the plasmid pDLG31 containing an appropriate dominant selectable marker *SMR1-410* for selection in industrial yeasts. It consists of a 3850-bp *Kpn*I-*Nru*I fragment containing the *LKA1* gene with the *PGK1* promoter and terminator cassette. Integration was achieved by linearising the plasmid at the *SMR1-410* gene. Southern blot analysis was performed to confirm integration (data not shown).

4.2 Synthesis and secretion of α-amylase in yeast

Plasmid pDLG31 was transformed into four industrial strains and a haploid strain of *S. cerevisiae*. As mentioned previously, the chromosome banding patterns confirmed that these strains consisted of different genetic backgrounds. The mitotic stability of the transformed DNA in the recombinant strains (grown in non-selective medium) was calculated to be 100%. The results indicated that the industrial strains of *S. cerevisiae* transformed by the integration plasmid were able to secrete extracellular α-amylase. The expression of the *LKA1* gene in all of these yeast strains was directed by the phosphoglycerate kinase 1 (*PGK1*) promoter. The native leader peptide was responsible for secretion of the Lka1p. The introduction of the *LKA1* gene did not adversely affect the growth of the transformed strains when grown in YPD, as can be seen in Figure 3.

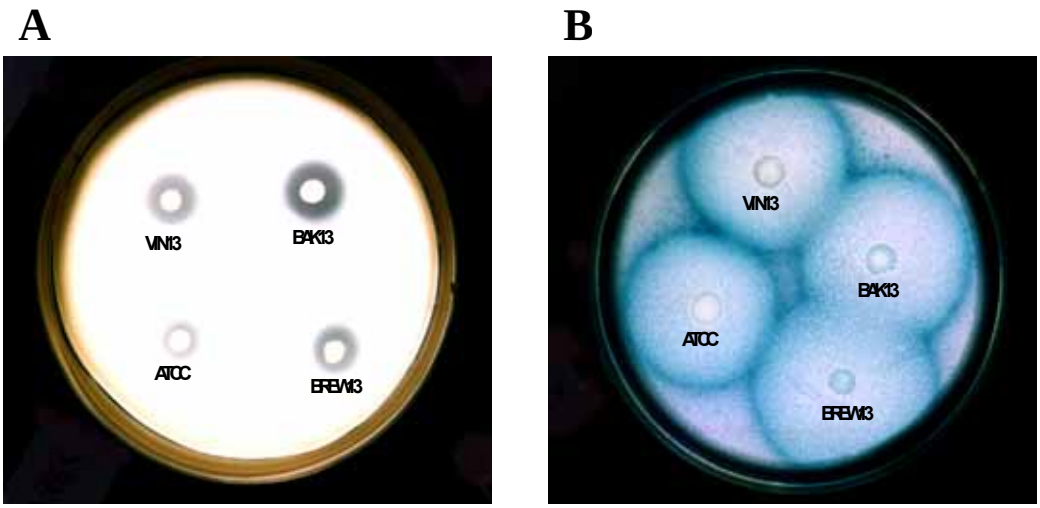

Fig. 2. Functional expression of the α-amylase-encoding gene (LKA1) in S. cerevisiae strains. Plate A contains Lintner starch and plate B Phadebas starch.

Interestingly, although the same cassette was used in all the yeast strains and the growth rate of the four industrial strains was identical, the amylase activity curves differ considerably between the strains (Figure 3). The difference is even more pronounced when the recombinant yeast strains on the agar plates containing the Lintner starch are compared (Figure 2A). The maximum extracellular α-amylase activities were produced after 34 h for all four industrial strains, but only after 72 h for the haploid strain. In the industrial strains, there is nearly a two-fold increase between the lowest (ATCC, 445 nkat ml^{-1}) and the highest producer (VIN13, 1050 nkat ml^{-1}). If the maximum enzymatic activity of the industrial strains is compared to that of the haploid strain [Y294 (pDLG31)], there is a 3.8-fold (ATCC) to 1.6-fold (VIN13) reduced level of activity, respectively. This substantiates the fact that the thick cell walls of the industrial strains tend to hamper the secretion of extracellular enzymes.

4.3 Starch utilisation and hydrolysis

The utilisation of starch by the different yeast transformants was compared (figure 4). The control strains, not secreting Lka1p, consumed almost no starch (data not shown). In all four industrial strains, the fastest utilisation of starch occurred within the first 48 h. Thereafter the starch utilisation was much slower. The BREW13 (57%) and VIN13 (54%) yeast strains were much more effective in utilising the starch compared to the BAK13 (36%) and ATCC (35%) strains. Since the secreted amylolytic enzymes are present continuously during the growth of the yeast cells, the sugar uptake and carbohydrate content of the culture broth at a given time will reflect the activities of the amylolytic enzymes present. It also reflects the ability of the cells to assimilate various products of starch hydrolysis products.

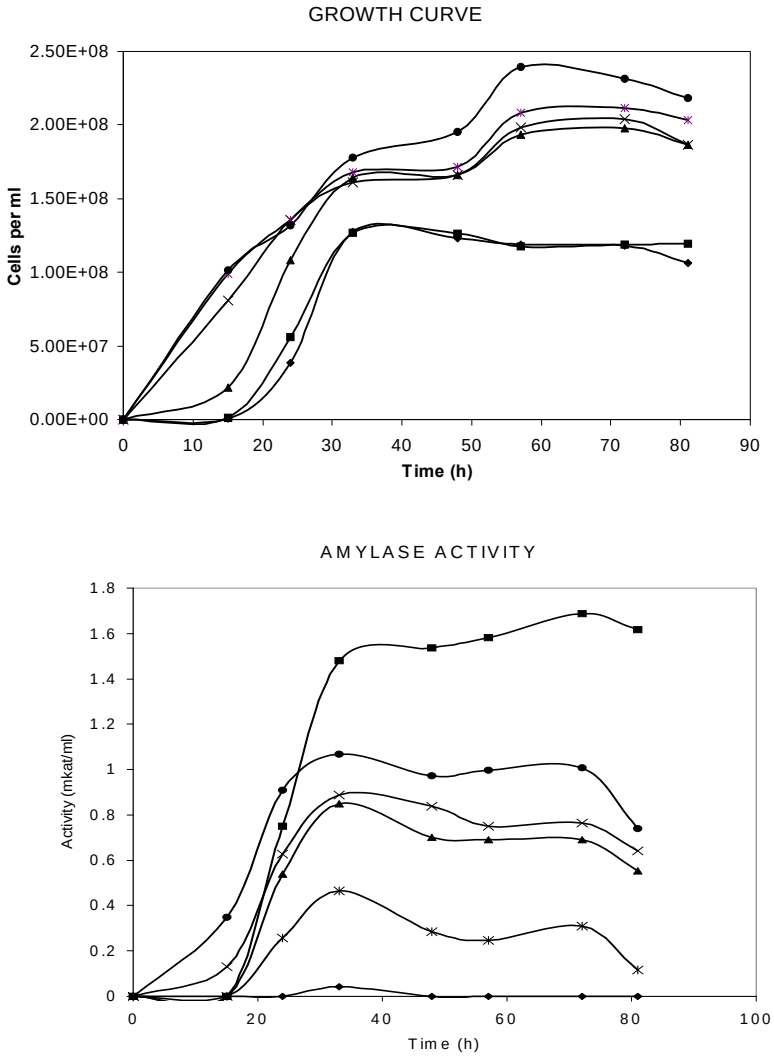

Fig. 3. Schematic representation of the growth curves (A) and the amylase activity curves (B) of the transformants and laboratory yeast strains. ◆, Y294 (control); ■, Y294 (pDLG31); ▲, BREW13 (pDLG31); ×, BAK13 (pDLG31); ✶ ATCC (pDLG31); ●, VIN13 (pDLG31).

This, on the whole, probably explains the inefficiency of VIN13 and BAK13 yeast strains in utilising starch in comparison to brew13 yeast, even though they exhibit the maximum enzymatic activity.

From these data, it is clear that, for the construction of an industrial recombinant yeast strain, the specific genetic background of the strain should be evaluated, as it can play an enormous role in the end result. Although the recombinant industrial strains were able to hydrolyse starch, the preliminary growth curve studies (on Lintner starch) indicated that the breakdown of starch was insufficient to sustain continuous growth of the recombinant industrial strains (data not shown). This can also be attributed to the fact that the native

secretion signal of *L. kononenkoae* may not be optimal for *S. cerevisiae* and the results might be different if an *S. cerevisiae* secretion signal is used.

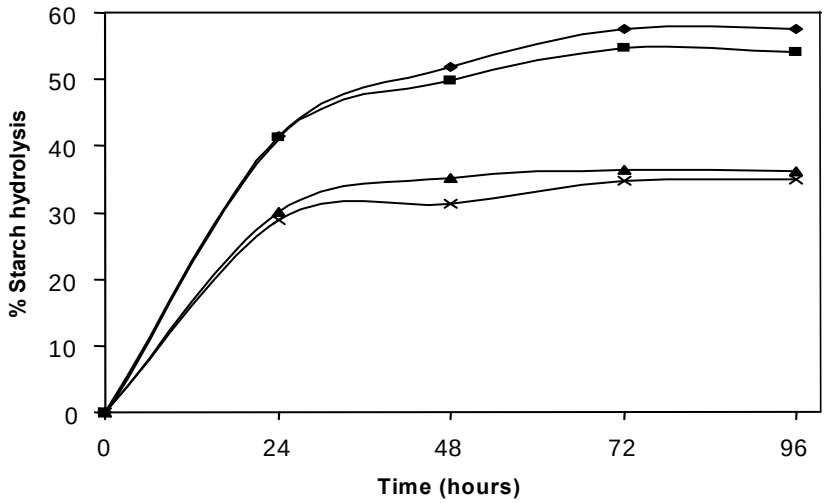

Fig. 4. The utilisation of starch by the different yeast transformants. ♦ Brewing (pDLG31); ■ Wine (pDLG31); ▲ Baking (pDLG31); × ATCC (pDLG31).

In conclusion, the development of amylolytic yeast strains for cost-effective consolidated bioprocessing (CBP) of renewable resources (*e.g.*, polysaccharide-rich biomass) is an important strategy to make the world less dependent on fossil fuels. In this regard, this study contributes towards improving the substrate range of potential CBP *S. cerevisiae* strains. The results presented here demonstrate the importance of the selection of appropriate host strains for yeast development pursuant to starch conversion into fuel ethanol via CBP. However, high-level *secretory* expression of amylolytic enzymes in *S. cerevisiae* represents at present a bottleneck to CBP organism development and to growth enablement on starch. Further progress in this area, including understanding the basis for both the successes and difficulties encountered in work to date, is an important goal for future research. Integrated advancement of fundamental understanding along with investigation of strategies to increase secretion levels is likely to be a particularly fruitful direction for future research in the context of organism development for CBP.

5. Acknowledgements

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