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# Modeling the minimum enzymatic requirements for optimal cellulose conversion

R den Haan<sup>1</sup>, J M van Zyl<sup>2</sup>, T M Harms<sup>2</sup> and W H van Zyl<sup>1</sup>

<sup>1</sup> Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa

<sup>2</sup> Department of Mechanical and Mechatronic Engineering, University of Stellenbosch, Stellenbosch, South Africa

E-mail: [whvz@sun.ac.za](mailto:whvz@sun.ac.za)

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## Abstract

Hydrolysis of cellulose is achieved by the synergistic action of endoglucanases, exoglucanases and  $\beta$ -glucosidases. Most cellulolytic microorganisms produce a varied array of these enzymes and the relative roles of the components are not easily defined or quantified. In this study we have used partially purified cellulases produced heterologously in the yeast *Saccharomyces cerevisiae* to increase our understanding of the roles of some of these components. CBH1 (Cel7), CBH2 (Cel6) and EG2 (Cel5) were separately produced in recombinant yeast strains, allowing their isolation free of any contaminating cellulolytic activity. Binary and ternary mixtures of the enzymes at loadings ranging between 3 and 100 mg g<sup>-1</sup> Avicel allowed us to illustrate the relative roles of the enzymes and their levels of synergy. A mathematical model was created to simulate the interactions of these enzymes on crystalline cellulose, under both isolated and synergistic conditions. Laboratory results from the various mixtures at a range of loadings of recombinant enzymes allowed refinement of the mathematical model. The model can further be used to predict the optimal synergistic mixes of the enzymes. This information can subsequently be applied to help to determine the minimum protein requirement for complete hydrolysis of cellulose. Such knowledge will be greatly informative for the design of better enzymatic cocktails or processing organisms for the conversion of cellulosic biomass to commodity products.

**Keywords:** cellulose, cellulases, synergy, consolidated bioprocessing, cellobiohydrolase, endoglucanase

## Nomenclature

		$[C]_{CBH2,f}$ (g l <sup>-1</sup> )	Concentration of free cellulose for CBH2
$[C]_{CBH1,f}$ (g l <sup>-1</sup> )	Concentration of free cellulose for CBH1	$[C]_{CBH2}$ (g l <sup>-1</sup> )	Total concentration of available cellulose for CBH2
$[C]_{CBH1}$ (g l <sup>-1</sup> )	Total concentration of available cellulose for CBH1	$[C]_{EG2,f}$ (g l <sup>-1</sup> )	Concentration of free cellulose for EG2
		$[C]_{EG2}$ (g l <sup>-1</sup> )	Total concentration of available cellulose for EG2
		$[E]_{CBH1,f}$ (g l <sup>-1</sup> )	Free concentration of CBH1



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$[E]_{CBH1}$ (g l <sup>-1</sup> )	Total concentration of CBH1
$[E]_{CBH2,f}$ (g l <sup>-1</sup> )	Free concentration of CBH2
$[E]_{CBH2}$ (g l <sup>-1</sup> )	Total concentration of CBH2
$[E]_{EG2,f}$ (g l <sup>-1</sup> )	Free concentration of EG2
$[E]_{EG2}$ (g l <sup>-1</sup> )	Total concentration of EG2
$[EC]_{CBH1}$ (g l <sup>-1</sup> )	Concentration of CBH1-cellulose complex
$[EC]_{CBH2}$ (g l <sup>-1</sup> )	Concentration of CBH2-cellulose complex
$[EC]_{EG2}$ (g l <sup>-1</sup> )	Concentration of EG2-cellulose complex
$k_{CBH1}$ (h <sup>-1</sup> )	Reaction activity for CBH1
$k_{CBH2}$ (h <sup>-1</sup> )	Reaction activity for CBH2
$k_{CCb}$ (g l <sup>-1</sup> )	Inhibition factor for cellobiose
$k_{CEt}$ (g l <sup>-1</sup> )	Inhibition factor for ethanol
$k_{EG2}$ (h <sup>-1</sup> )	Reaction activity for EG2
$r_{[C]_{CBH1}}$ (g l <sup>-1</sup> )	Rate of CBH1 related cellulose conversion
$r_{[C]_{CBH2}}$ (g l <sup>-1</sup> )	Rate of CBH2 related cellulose conversion
$r_{[C]_{EG2}}$ (g l <sup>-1</sup> )	Rate of EG2 related cellulose conversion
$r_{[EC]_{CBH1}}$ (g l <sup>-1</sup> )	Rate of CBH1-cellulose adsorption/desorption rate
$r_{[EC]_{CBH2}}$ (g l <sup>-1</sup> )	Rate of CBH2-cellulose adsorption/desorption rate
$r_{[EC]_{EG2}}$ (g l <sup>-1</sup> )	Rate of EG2-cellulose adsorption/desorption rate
$r_{[C]}$ (g l <sup>-1</sup> )	Rate of Avicel conversion
$\theta_{CBH1}$	Coefficient for cellulose released by CBH1 and CBH2 to CBH1
$\theta_{CBH2}$	Coefficient for cellulose released by CBH1 and CBH2 to CBH2
$\theta_{EG2}$	Coefficient for cellulose released to EG2 by CBH1 and CBH2
$\sigma_{CBH1}$ (g <sub>protein</sub> /g <sub>cellulose</sub> )	Maximum enzyme capacity for CBH1
$\sigma_{CBH2}$ (g <sub>protein</sub> /g <sub>cellulose</sub> )	Maximum enzyme capacity for CBH2
$\sigma_{EG2}$ (g <sub>protein</sub> /g <sub>cellulose</sub> )	Maximum enzyme capacity for EG2
$[C]$ (g l <sup>-1</sup> )	Concentration of Avicel in the solution
$[C]_0$ (g l <sup>-1</sup> )	Initial concentration of Avicel in the solution

$[Cb]$ (g l <sup>-1</sup> )	Concentration of cellobiose
$[Eth]$ (g l <sup>-1</sup> )	Concentration of ethanol
$[G]$ (g l <sup>-1</sup> )	Concentration of glucose
$n$	Exponent adjusting for the reduced reactivity of Avicel
$x$	Ratio of remaining cellulose relative to initial cellulose
$\delta$	Ratio of cellulose released by EG2 during amorphous conversion
$\varphi$	Distribution of cellulose released by EG2 to CBH1 and CBH2

## 1. Introduction

Lignocellulosic biomass holds much promise as an abundant and potentially sustainable resource for the production of liquid fuels and chemicals if technologies to overcome its recalcitrance can be established (Stephanopoulos 2007). Lignocellulose consists mainly of the polymers lignin (10–40%), cellulose (40–55%) and hemicelluloses (25–50%), with the composition varying with plant origin (Sun and Cheng 2002). Improvements in biomass conversion technologies involve combining two or more of the biologically mediated steps that follow biomass pretreatment namely: (i) the production of hydrolyzing enzymes, (ii) enzymatic hydrolysis of the carbohydrate polysaccharides, (iii) fermentation of the hexose sugars, and (iv) fermentation of the pentose sugars (Lynd *et al* 2002, 2005). The hydrolysis and fermentation steps can be combined in the simultaneous saccharification and fermentation (SSF) of hexoses or the simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses if an optimal fermentative organism is available. The ultimate objective is one-step consolidated bioprocessing (CBP) of lignocellulose to bioethanol, in which a single microorganism or microbial consortium converts pretreated biomass to a commodity product, such as ethanol, without the need for added enzymes. This would represent a breakthrough for low-cost biomass processing due to the economic benefits of process integration and by avoiding the high costs of enzymes (Hamelinck *et al* 2005, Van Zyl *et al* 2011a). The yeast *Saccharomyces cerevisiae* has long been used as an industrial ethanologen due to its high rate of ethanol production from glucose, high ethanol tolerance, general robustness and its favorable GRAS (generally regarded as safe) status (Kuyper *et al* 2005, Van Dijken *et al* 2000). Therefore, *S. cerevisiae* is a good candidate to develop for CBP. However, the drawbacks that must be overcome include the engineering of pentose sugar utilization and the production of the necessary enzymes to hydrolyze cellulose and hemicellulose (La Grange *et al* 2010). While there has been considerable success in the development of strains that hydrolyze cellulosic substrates, complete conversion of cellulosic substrates via engineered yeasts strains without added cellulase has remained elusive (Fujita *et al* 2004, Den Haan *et al* 2007a, Olson *et al* 2012).

The hydrolysis of (semi)crystalline cellulose is achieved by the synergistic actions of (i) endoglucanases (EGs EC 3.2.1.4), which act in the amorphous regions of cellulose to release cellodextrins and provide free chain ends; (ii) exoglucanases, including cellodextrinases and cellobiohydrolases (CBHs EC 3.2.1.91), which act on crystalline cellulose in a processive manner starting at the free chain ends and releasing mainly cellobiose; and (iii)  $\beta$ -glucosidases (BGLs EC 3.2.1.21), which hydrolyze cellobiose and small cello-oligosaccharides to glucose (Lynd *et al* 2002, Zhang and Lynd 2004). In free enzyme systems, the process is initiated when free EGs and CBHs in the bulk solution adsorb to the cellulose surface forming an enzyme–substrate complex which allow the entry of water molecules into their active sites. The subsequent hydrolytic reactions then yield free chain ends, oligosaccharides and cellobiose that are the substrates for BGLs. Fungal and bacterial EGs and CBHs usually have a modular organization where an active-site-containing core is connected by a glycosylated peptide linker to the carbohydrate binding moiety (CBM) (Teeri *et al* 1992, Nidetzky *et al* 1994b, Zhang and Lynd 2004). The CBM promotes adsorption of the cellulases to crystalline regions of the cellulosic substrate and may also cause a disturbance in the substrate surface to allow entry of the glucan chain into the tunnel of the catalytic domain (Lehtio *et al* 2003). Some important factors influencing the adsorption of cellulases are the crystallinity of cellulose and other physical parameters such as temperature, ionic strength and pH (Nidetzky *et al* 1994b).

Mathematical modeling of cellulose hydrolysis has been attempted previously (Lynd *et al* 2002, Zhang and Lynd 2004, Nidetzky *et al* 1994b, Van Zyl *et al* 2011b). Recently, modeling the hydrolysis and fermentation of Avicel, an artificial microcrystalline cellulose polymer, in an SSF configuration was attempted for the first time (Van Zyl *et al* 2011b). The authors utilized dynamic adsorption models to describe the adsorption behavior of the cellulase enzymes, while also accounting for enzyme competitive inhibition. The model assumed a constant specific enzyme activity to provide improved approximations to experimentally determined values. This numerical model was unique in that it separated the EG and CBH enzyme kinetics, allowed for competitive inhibition, and was capable of predicting adsorbed enzyme concentrations with reasonable accuracy. Comparison of predicted values to experimental measurements indicated that the numerical model was capable of capturing the significant elements involved with Avicel conversion to ethanol in a SSF configuration. It accounted for the synergistic effects between the EGs and CBHs and it was proposed that the primary limiting factor in cellulose conversion is the availability of enzyme binding sites. Both experimental measurements and numerical predictions indicated a significant decrease in adsorbed exoglucanase after 20 h, attributed to the depletion of free cellulose chain ends early in the reactions.

While heterologous cellulase titers in recombinant *S. cerevisiae* strains have generally been low (Van Zyl *et al* 2007), a recent report showed that production of both CBH1 and CBH2 could be improved to levels where the barrier to

CBH sufficiency in the hydrolysis of cellulose was overcome (Ilmén *et al* 2011). Ilmén *et al* (2011) described the high-level secretion of a CBH1 originating from *Talaromyces emersonii* and a CBH2 originating from *Chrysosporium lucknowense*. The CBH1 was engineered to contain a C-terminal CBM originating from *Trichoderma reesei* CBH1. In an earlier study, we have also described the successful expression of the *T. reesei* EG2 in *S. cerevisiae* (Du Plessis *et al* 2010). The present study focuses on the interaction of these three cellulases that can be produced at relatively high levels in *S. cerevisiae* at temperature and pH levels optimal for yeast growth and is therefore pertinent to the engineering of a CBP yeast. We have tested the interaction of the yeast produced cellulases on Avicel separately and in combination and developed a numerical model related to the one described by Van Zyl *et al* (2011b) to describe the enzyme–substrate interaction and synergy. This information can subsequently be applied to help determine the minimum protein requirement for complete hydrolysis of cellulose. Such knowledge will be greatly informative for the design of better enzymatic cocktails or processing organisms for the conversion of cellulosic biomass to commodity products. To our knowledge, this is the first attempt to produce a mathematical model based on yeast produced cellulases to better understand enzymatic interaction on insoluble microcrystalline particles and to inform the engineering of cellulose degrading yeasts.

## 2. Materials and methods

### 2.1. Media, yeast strains and culture conditions

All chemicals, media components and supplements were of analytical grade. The construction of the three yeast expression plasmids used in this study: pMI529, pMI574 and pRDH147 was previously described (Ilmén *et al* 2011, Brevnova *et al* 2011). *S. cerevisiae* Y294 (genotype: a *leu2-3112 ura3-52 his3 trp1-289*) was transformed using the three plasmids separately, with the lithium acetate dimethylsulfoxide method described by Hill *et al* (1991) and transformants were selected on SC<sup>-URA</sup> plates (1.7 g l<sup>-1</sup> yeast nitrogen base w/o amino acids and ammonium sulfate (Difco laboratories, Detroit, MI, USA), 5 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 g l<sup>-1</sup> glucose, 15 g l<sup>-1</sup> agar, and supplemented with amino acids as required). Autoselective strains were constructed by subsequent transformation with pDF1 (La Grange *et al* 1996), to ensure maintenance of the *URA3*-bearing expression vector under non-selective conditions (Kern *et al* 1990, La Grange *et al* 1996). Yeast transformants were confirmed by PCR as described previously (Ilmén *et al* 2011, Du Plessis *et al* 2010).

### 2.2. Preparation of partially purified enzymes

Yeast strains were cultivated in 2 l Erlenmeyer flasks in 500 ml double strength SC media (3.4 g l<sup>-1</sup> yeast nitrogen base w/o amino acids and ammonium sulfate, 10 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 g l<sup>-1</sup> glucose and supplemented with amino acids as required), buffered to pH 6 with 0.17 M succinate buffer. After four days of cultivation at 30 °C with orbital

shaking at 200 rpm, cultures were centrifuged and filtered through 0.45  $\mu\text{m}$  filters (Millipore) to remove yeast cells. Ultrafiltration initially proceeded using the Minitan (Millipore) system with a 10 kDa cut-off membrane to concentrate samples to  $\sim 200$  ml. Ultrafiltration then proceeded using the Amicon ultrafiltration system (Millipore) using a 30 kDa cut-off membrane to concentrate samples from 200 to 50 ml, dialyzing the samples with 0.05 M Na-acetate buffer (pH 5) in the process. Enzyme samples were diluted as required with 0.05 M Na-acetate buffer pH 5. When higher protein concentrations were required, samples were further concentrated using Vivaspin 20 centrifuge columns (Sartorius Stedim) with a molecular weight cut-off of 10 kDa as directed by the manufacturer.

### 2.3. Protein concentration determinations and enzyme assays

Endoglucanase activity was measured on carboxy-methyl cellulose (CMC) as described previously (Den Haan *et al* 2007b). To determine endoglucanase and exoglucanase activity on a polymeric substrate, Avicel assays were performed as described by Van Wyk *et al* (2010) with Avicel PH-105, in 0.05 M acetate buffer pH 5.0 at 30 °C to mimic yeast SSF conditions. Samples (100  $\mu\text{l}$ ) of the enzyme–substrate mixture were taken at the 0 h, 24 h and other time intervals as required to determine background sugars that were present and the amount of sugars released, respectively, using a modified DNS method (Miller *et al* 1960, Van Wyk *et al* 2010). Absorbance values at 540 nm were read on an X-mark<sup>TM</sup> (Biorad, Hercules, CA, USA) microtiter plate reader. Glucose was used to set a standard curve in the range of 0.125–4 g l<sup>-1</sup>, from which the amount of glucose released during the assay was determined. The amount of activity was expressed as the percentage of Avicel hydrolyzed. Significant glucose accumulation in the assay solution could cause inhibition of the BGL leading to a cellobiose accumulation that would in turn inhibit the other cellulases. However, in all assays Novozyme 188 BGL was added to such an excess that glucose build-up could not become a significant inhibitory factor. BGL assays in samples showed that a large proportion of the enzyme remained active and HPLC analysis showed no significant cellobiose accumulation (not shown).

Protein concentrations in cell-free broths were measured with Bio-Rad protein reagent as directed by the manufacturer, using bovine immunoglobulin as the standard. To determine the enzyme adsorption to Avicel, 450  $\mu\text{l}$  of the enzyme preparations were added to deep-well microtiter plates with each well containing a 450  $\mu\text{l}$  solution with 2% Avicel PH-105 in 0.05 M acetate buffer pH 5.0. The deep-well plate was sealed and incubated on a microtiter plate shaker and shaken at  $\sim 1000$  rpm at 4 °C to prevent hydrolysis of the substrate. Samples (150  $\mu\text{l}$ ) of the enzyme–substrate mixture were taken at 0, 10, 20, 30 and 60 min and thereafter hourly for up to 6 h. The samples were transferred to a 96-well PCR plate using a multi-channel pipette and were centrifuged at 1910g for 2 min. Ten microliter of the supernatant were subsequently

pipetted along with 200  $\mu\text{l}$  Bio-Rad protein reagent solution into a clean 96-well PCR plate and the protein concentrations were determined according to the manufacturer's microtiter plate protocol.

### 2.4. SDS-PAGE and protein deglycosylation

To remove *N*-linked glycans from the enzymes, 1  $\mu\text{l}$  Endoglycosidase H (New England Biolabs) was added into a 9  $\mu\text{l}$  enzyme preparation and incubated for 1 h at 37 °C according to the manufacturer's instructions. In control samples, Endoglycosidase H was replaced by water. Protein samples were subsequently separated by electrophoresis on 10% SDS-PAGE gels according to the Laemmli (1970) method with modifications as described previously (Den Haan *et al* 2007a). Gels were visualized with silver staining. First, the gel was incubated in a fixing solution (30% v/v ethanol, 0.5% v/v acetic acid) for 90 min, replacing the solution twice. Subsequently the gel was incubated in 20% (v/v) ethanol for 10 min and then in water for 10 min. The gel was then incubated in 0.2 g l<sup>-1</sup> sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) for 1 min, followed by two brief washes with water. The gel was subsequently incubated in 1 g l<sup>-1</sup> silver nitrate solution for 30 min. After a brief water wash, developer (20 g l<sup>-1</sup> Na<sub>2</sub>CO<sub>3</sub>, 973  $\mu\text{l}$  formaldehyde) was added and color was allowed to develop to an appropriate level. Color development was fixed by incubation in a final fixer solution (50 g l<sup>-1</sup> tris base, 2.5% (v/v) acetic acid).

### 2.5. Modeling

Modeling was done using C script with the gcc 4.6.3 compiler on the Ubuntu 12.04 operating system. The least squared errors method was used to estimate the parameters for the numerical model based on empirical data.

### 2.6. Adsorption

The adsorption coefficients of CBH1, CBH2 and EG2 to Avicel were determined in a three-step fashion. First, the amount of free enzyme was calculated based on the difference between the total concentration of enzyme present and the amount of enzyme attached to the enzyme–substrate complex by

$$[E]_{\text{CBH1},f} = [E]_{\text{CBH1}} - [EC]_{\text{CBH1}} \frac{\sigma_{\text{CBH1}}}{1 + \sigma_{\text{CBH1}}}, \quad (1)$$

$$[E]_{\text{CBH2},f} = [E]_{\text{CBH2}} - [EC]_{\text{CBH2}} \frac{\sigma_{\text{CBH2}}}{1 + \sigma_{\text{CBH2}}}, \quad (2)$$

$$[E]_{\text{EG2},f} = [E]_{\text{EG2}} - [EC]_{\text{EG2}} \frac{\sigma_{\text{EG2}}}{1 + \sigma_{\text{EG2}}}, \quad (3)$$

where  $\sigma_{\text{CBH1}}$ ,  $\sigma_{\text{CBH2}}$  and  $\sigma_{\text{EG2}}$  are the maximum enzyme capacity for the respective enzymes, which was determined from the synergistic parameter optimizations. Next, free cellulose binding sites were calculated based on the difference between the total binding sites and binding sites occupied by

adsorbed cellulase by

$$[C]_{CBH1,f} = [C]_{CBH1} - \frac{[EC]_{CBH1}}{1 + \sigma_{CBH1}}, \quad (4)$$

$$[C]_{CBH2,f} = [C]_{CBH2} - \frac{[EC]_{CBH2}}{1 + \sigma_{CBH2}}, \quad (5)$$

$$[C]_{EG2,f} = [C]_{EG2} - \frac{[EC]_{EG2}}{1 + \sigma_{EG2}}. \quad (6)$$

Finally, the rate at which enzyme–substrate complexes are formed was calculated using dynamic adsorption equations

$$r_{[EC]_{CBH1}} = k_{f,CBH1}[E]_{CBH1,f}(1.0 + \sigma_{CBH1})[C]_{CBH1,f} - k_{CBH1} \frac{[EC]_{CBH1}}{(1.0 + \sigma_{CBH1})} \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \times \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right], \quad (7)$$

$$r_{[EC]_{CBH2}} = k_{f,CBH2}[E]_{CBH2,f}(1.0 + \sigma_{CBH2})[C]_{CBH2,f} - k_{CBH2} \frac{[EC]_{CBH2}}{(1.0 + \sigma_{CBH2})} \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \times \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right], \quad (8)$$

$$r_{[EC]_{EG2}} = k_{f,EG2}[E]_{EG2,f}(1.0 + \sigma_{EG2})[C]_{EG2,f} - k_{EG2} \frac{[EC]_{EG2}}{(1.0 + \sigma_{EG2})} \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \times \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right]. \quad (9)$$

where  $k_{f,CBH1}$ ,  $k_{f,CBH2}$  and  $k_{f,EG2}$  are the coefficients for the forward adsorption rates.

### 2.7. Synergism

The effects of synergism between the three cellulases can be described using the assumptions that only CBH1 and CBH2 are capable of hydrolyzing the cellulose to cellobiose and that the role of EG2 is to clip amorphous cellulose connections to reveal new reducing and non-reducing ends for the CBH1 and CBH2 respectively. Furthermore as CBH1 and CBH2 proceed to cleave the cellobiose from the cellulose particles, new exposed cellulose is revealed containing both crystalline and amorphous cellulose which can be accessed by the three cellulases.

To capture these effects each cellulase enzyme was modeled with its respective effects as described above. The equations representing these interactions are for CBH1, CBH2 and EG2 respectively given by

$$r_{[C]_{CBH1}} = k_{CBH1} \frac{[EC]_{CBH1}}{(1.0 + \sigma_{CBH1})} - x \left[ \varphi \delta k_{EG2} \frac{[EC]_{EG2}}{(1.0 + \sigma_{EG2})} + \theta_{CBH1} \left( k_{CBH1} \frac{[EC]_{CBH1}}{(1.0 + \sigma_{CBH1})} + k_{CBH2} \frac{[EC]_{CBH2}}{(1.0 + \sigma_{CBH2})} \right) \right] \times \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right], \quad (10)$$

$$r_{[C]_{CBH2}} = \left\{ k_{CBH2} \frac{[EC]_{CBH2}}{(1.0 + \sigma_{CBH2})} - x \left[ (1 - \varphi) \delta k_{EG2} \frac{[EC]_{EG2}}{(1.0 + \sigma_{EG2})} + \theta_{CBH2} \left( k_{CBH1} \frac{[EC]_{CBH1}}{(1.0 + \sigma_{CBH1})} + k_{CBH2} \frac{[EC]_{CBH2}}{(1.0 + \sigma_{CBH2})} \right) \right] \right\} \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \times \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right], \quad (11)$$

$$r_{[C]_{EG2}} = \left\{ k_{EG2} \frac{[EC]_{EG2}}{(1.0 + \sigma_{EG2})} - x \left[ k_{CBH1} \frac{[EC]_{CBH1}}{(1.0 + \sigma_{CBH1})} + k_{CBH2} \frac{[EC]_{CBH2}}{(1.0 + \sigma_{CBH2})} \right] \right\} \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right], \quad (12)$$

where  $k_{CBH1}$ ,  $k_{CBH2}$ ,  $k_{EG2}$  are the specific reaction rates for the three enzymes,  $x$  takes into account the effects of particle shrinkage based on a simplistic spherical model,

$$x = \left( \frac{[C]}{[C]_0} \right)^n, \quad (13)$$

$\varphi$  along with  $\delta$  represents the ratio of available cellulose released to CBH1 and CBH2 by EG2 and  $\theta_{CBH1}$ ,  $\theta_{CBH2}$  and  $\theta_{EG2}$  representing the ratio of cellulose made available by the CBH1 and CBH2 cellulose conversion.

The actual amount of cellulose released during the hydrolysis process was captured using

$$r_{[C]} = \left\{ k_{CBH1} \frac{[EC]_{CBH1}}{(1.0 + \sigma_{CBH1})} + k_{CBH2} \frac{[EC]_{CBH2}}{(1.0 + \sigma_{CBH2})} \right\} \left[ \frac{k_{CCb}}{([Cb] + k_{CCb})} \right] \times \left[ \frac{k_{CEt}}{([Et] + k_{CEt})} \right]. \quad (14)$$

The remaining reactions including the production of cellobiose and glucose was modeled as indicated by Van Zyl *et al* (2011b).

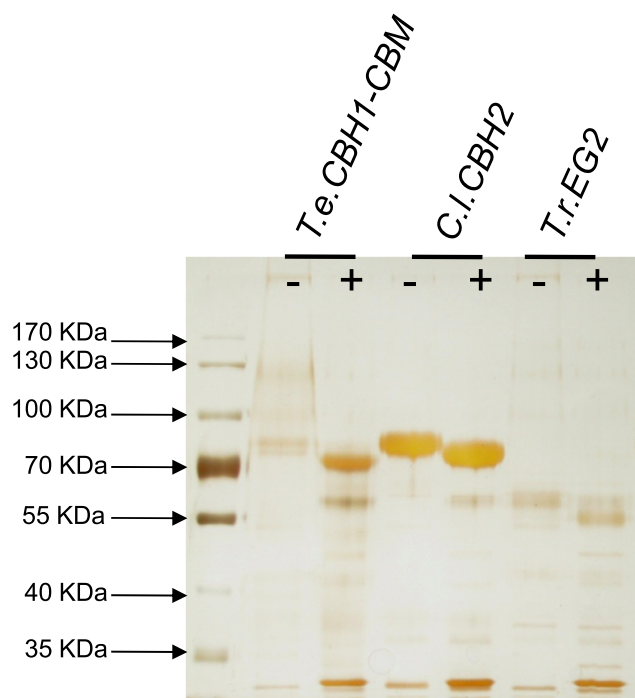
### 2.8. Optimal enzymatic requirements

Optimal enzyme combinations were determined using the model as described above and iteratively evaluating through various enzyme ratios which totaled 35 mg protein/g Avicel. This was done by iteratively evaluating the CBH enzymes combinations first in the absence of and secondly in the presence of the endoglucanase. The results from the model were then compared to the experimental measurements taken of selected combinations.

## 3. Results

### 3.1. Heterologous cellulase production and verification

A modified version of the *T. emersonii* CBH1 (with C-terminally attached CBM from *T. reesei* CBH1), the



**Figure 1.** Reducing 10% SDS-PAGE of enzyme preparations (20  $\mu$ l) visualized by silver staining. Samples were either deglycosylated with endoH (+) or non-treated (-). Sizes of the bands of the molecular weight marker are shown on the left.

*C. lucknowense* CBH2b and the *T. reesei* EG2 enzymes were produced separately by *S. cerevisiae* transformants that were previously constructed (Ilmén *et al* 2011, Brevnova *et al* 2011). These were then tested for activity and interaction on a crystalline cellulose substrate (Avicel) with the addition of a saturating amount of BGL (Novozyme 188). Yeast strains were cultured in minimal media to ensure that very few other protein species would be present in the culture supernatants. Supernatants of the strains were centrifuged and filtered to remove cells and were then concentrated 10 times using ultrafiltration. To assert the relative purity of the enzyme preparations, they were separated by SDS-PAGE in native and *N*-deglycosylated form (figure 1). As can be seen there were very few other protein species present in the samples other than the cellulases of interest. The *T.e.*CBH1–CCBM was shown to be heterogeneous in size due to hyperglycosylation but could be observed as a single band of approximately 70 kDa once *N*-linked glycans were removed. The *C.l.*CBH2 was present as a defined protein band of approximately 75 kDa but a slight band-shift upon deglycosylation revealed that some *N*-glycosylation was present. These observations were in line with what was previously reported for these two heterologously produced enzymes (Ilmén *et al* 2011). The *T.r.*EG2 was also shown to be heterogeneous in size due to hyperglycosylation but could be observed as a single band of approximately 54 kDa once *N*-linked glycans were removed. While all three enzyme preparations were shown to have activity on Avicel in initial assays, only the *T.r.*EG2 preparation had activity on CMC and none of the samples had  $\beta$ -glucosidase activity (not shown). Therefore, due to

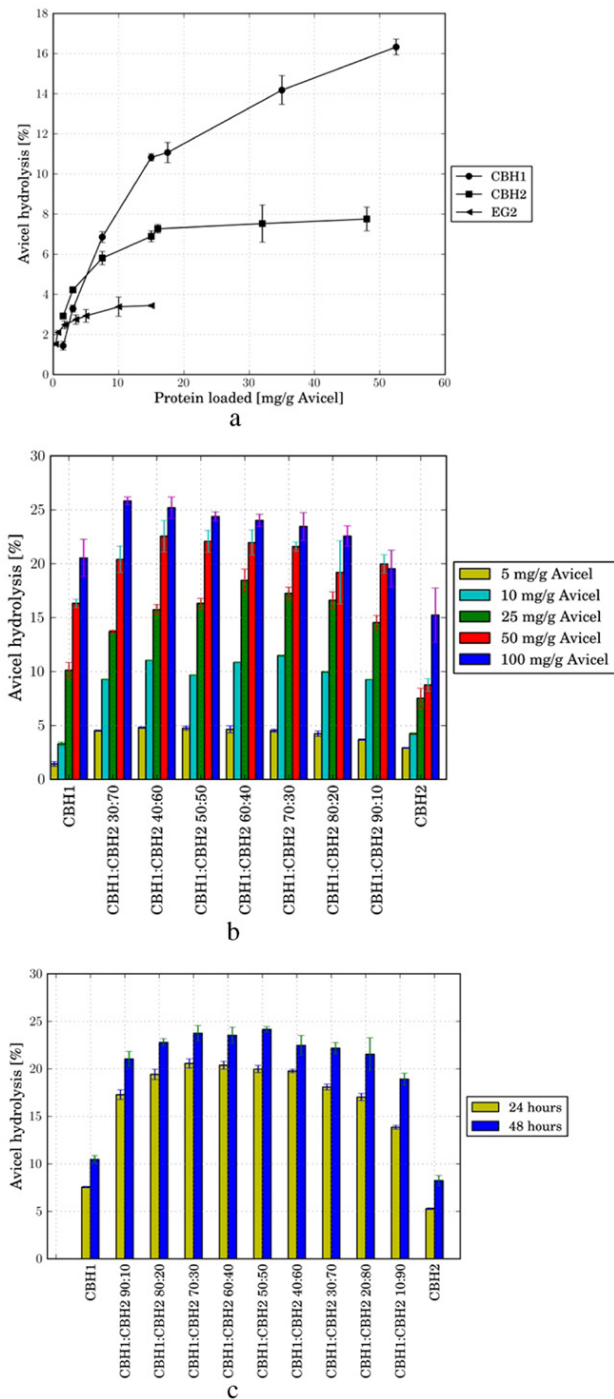
the relative purity of these samples they were used for the subsequent quantitative assays for Avicel hydrolysis. The *T.e.*CBH1–CCBM, *C.l.*CBH2b and *T.r.*EG2 are henceforth referred to simply as CBH1, CBH2 and EG2.

### 3.2. Quantitative activity of CBH1, CBH2 and EG2 on Avicel, separately and in combination

Activities of the three enzymes on Avicel in 24 h were assayed at various protein loadings (figure 2(a)). All three enzymes were active on this substrate but it was clear that increased loading of the cellulases in isolation did not yield comparatively higher levels of Avicel hydrolysis. There was therefore a non-linear relationship between the amount of cellulase loaded and the amount of Avicel hydrolyzed. As expected CBH1 gave the highest levels of Avicel hydrolysis but the levels of hydrolysis also became saturated at higher protein loadings. Subsequently we investigated the synergy of CBH1 with CBH2 that was previously reported (Zhang and Lynd 2004, Ilmén *et al* 2011) and attempted to find an optimal ratio of the CBHs (figure 2(b)). Interestingly, the optimal ratio shifted somewhat with the amount of cellulases loaded. At loadings under 30 mg g<sup>-1</sup>, ratios of CBH1:CBH2 of 60:40–70:30 were optimal. However, any ratio of CBH1:CBH2 of 30:70–70:30 worked equally well at loadings of 50 or 100 mg g<sup>-1</sup> Avicel. The amount of synergy observed was also less apparent at higher protein levels. These effects are likely due to the saturation of enzyme binding sites at higher cellulase loadings. A ratio of CBH1:CBH2 of 70:30 was selected to work with as an ‘optimal ratio’ as it worked well at all protein loadings tested and is also in line with the usual ratios of these enzymes produced by natural cellulose degraders such as *T. reesei* (Nidetzky *et al* 1994b, 1994a).

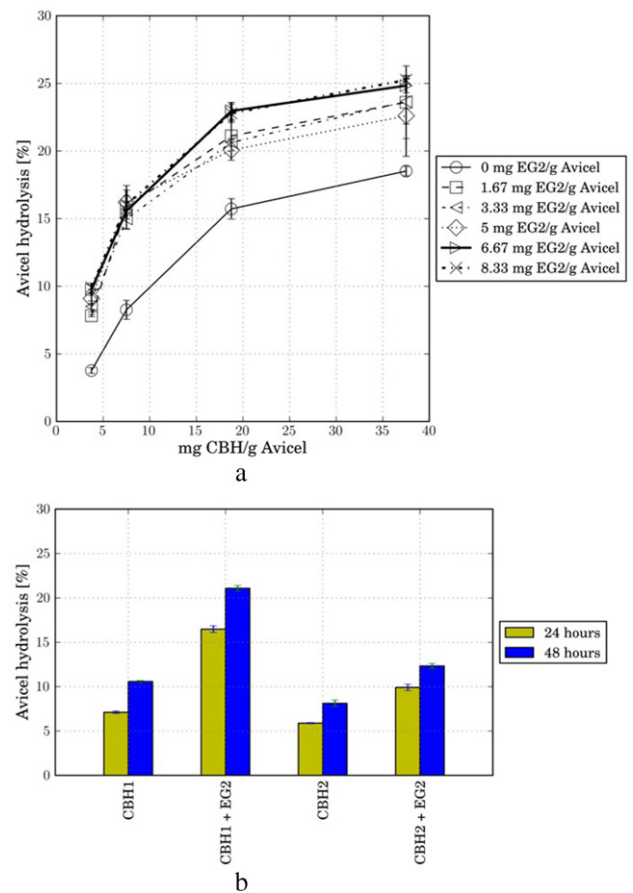
We subsequently tested the addition of EG2 in various loadings to this mixture (figure 3(a)). It was shown that the addition of EG2 had a significant positive effect but the reaction was quickly saturated over 1.67 mg g<sup>-1</sup> Avicel for all loadings tested. In addition, we tested the endo–exo synergy of the CBH1 and CBH2 with EG2 separately (figure 3(b)). It was observed that there was a strikingly positive synergy in both cases. However, the CBH1 + EG2 combination yielded far greater synergy with 16.5% Avicel hydrolysis in 24 h while CBH1 on its own at the same loading yielded only 7.1% hydrolysis. The CBH2 + EG2 combination yielded 9.9% Avicel hydrolysis in 24 h while CBH2 on its own at the same loading yielded 5.9% hydrolysis. The amount of Avicel hydrolyzed by EG2 at this loading was negligible.

Next, we tested the hydrolysis of Avicel by the yeast cellulases over a significantly longer time frame (figure 4). Complete hydrolysis of the substrate was not attained. As all the assays contained an abundance of BGL activity in the form of added Novozyme 188, one might have expected complete hydrolysis of the samples containing CBH1, CBH2 and EG2 in combination. This did not occur and even the highest loading of cellulases tested arrested at ~70% substrate conversion at about 20 days. In these samples, approximately 35% of the Avicel was hydrolyzed in 48 h and with only an additional 35% hydrolyzed over the subsequent 20 days. It



**Figure 2.** Activity of the yeast produced cellulases on Avicel. (a) Activities of the separate cellulases were determined at various protein concentrations. (b) Synergistic activity of combinations of yeast produced CBH1 and CBH2 on Avicel at various combinations and protein concentrations in 24 h. (c) Synergistic activity of combinations of yeast produced CBH1 and CBH2 on Avicel in 24 and 48 h. Activities of the cellulases were determined at total protein loadings of 35 mg g<sup>-1</sup> Avicel. Activities were determined with shaking at 30 °C over 24 h and are indicated as percentage of Avicel hydrolyzed. Each treatment was performed in triplicate and values represent the mean value. Error bars indicate standard deviation.

was also seen that when only 50% or 10% of this amount of cellulases were applied to the substrate there was not a comparatively lower amount of hydrolysis. These two



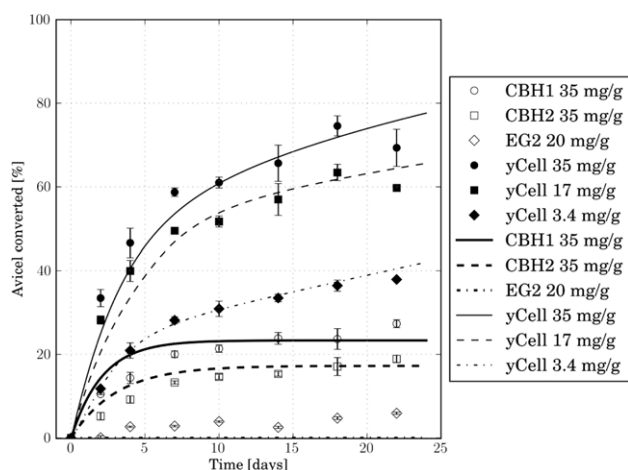
**Figure 3.** Synergism of CBH1 and CBH2 with EG2. (a) Activity of a combination of CBH1 and CBH2 in a 70:30 ratio on Avicel is enhanced by addition of yeast produced EG2. (b) Effects of synergism of EG2 with CBH1 and CBH2 in binary mixtures. CBH loadings were 35 mg g<sup>-1</sup> Avicel in all treatments. EG2 was added as required at 5 mg g<sup>-1</sup> Avicel. Each treatment was performed in triplicate and values represent the mean value. Error bars indicate standard deviation.

treatments lead to levels of hydrolysis of approximately 60% and 38%, respectively over the 22 day period. This again illustrated the non-linear behavior of enzyme hydrolysis on this substrate. This assay also strikingly illustrated the synergy of the CBH1:CBH2:EG2 combination: the lowest loading of the combination only contained ~3.4 mg cellulases/g Avicel, yet it was able to hydrolyze ~38% of the substrate whereas 35 mg CBH1 per gram Avicel alone could only hydrolyze ~27% of the substrate. This once more highlighted the importance of having the optimal blend of cellulases available.

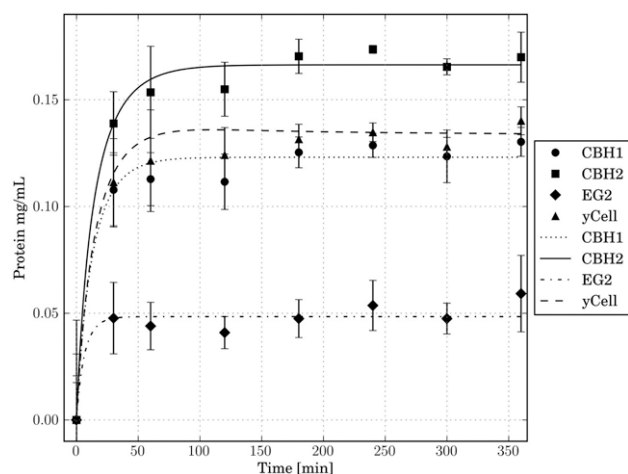
### 3.3. Modeling

Enzyme adsorption coefficients for the maximum enzyme capacity [ $\sigma$ ] and the forward [ $k_f$ ] enzyme adsorption rates are provided in table 1. Either the number of enzymes or the number of available sites for their attachment will always be limited. We assumed that whenever a free enzyme comes into contact with an available site an enzyme–cellulose complex will form, and that there will not be a desorption equilibrium,





**Figure 4.** Activity of single cellulases and a combination of CBH1, CBH2 and EG2 at various loadings on Avicel up to 22 days. yCell refers to mixtures of the yeast produced cellulases in a ratio of CBH1:CBH2:EG2 of 11:5:1. Symbols represent experimentally determined values and simulated results for hydrolysis predicted by the model are superimposed and indicated by lines. Activities were determined with shaking at 30 °C and are indicated as percentage of Avicel hydrolyzed. Each treatment was performed in triplicate and values represent the mean value. Error bars indicate standard deviation.



**Figure 5.** Adsorption of the enzymes to Avicel. Symbols represent experimentally determined values and simulated results for adsorption predicted by the model are superimposed and indicated by lines. yCell refers to mixtures of the yeast produced cellulases in a ratio of CBH1:CBH2:EG2 of 11:5:1. Each treatment was performed in triplicate and values represent the mean value. Error bars indicate standard deviation.

except when the enzyme detaches once the available site has been depleted. EG2 was found to have the lowest enzyme capacity (i.e. amount of enzyme protein which can adsorb to its available substrate on a gram protein per gram substrate basis) even though it presented the highest substrate affinity due to its high  $k_f$  value. CBH1 and CBH2 had similar adsorption capacity and affinity for the crystalline cellulose. Comparison of the model predictions and the experimental measurements are shown in figures 4 and 5. Here the results for the isolated CBH1, CBH2 and EG2 enzymes are shown

**Table 1.** Coefficients for the adsorption models.

Symbol	Value	Source
$\sigma_{CBH1}$	0.0196 g g <sup>-1</sup>	This work
$\sigma_{CBH2}$	0.0697 g g <sup>-1</sup>	This work
$\sigma_{EG2}$	0.019 g g <sup>-1</sup>	This work
$k_{f,CBH1}$	15 g <sup>-1</sup> h <sup>-1</sup>	This work
$k_{f,CBH2}$	13.1 g <sup>-1</sup> h <sup>-1</sup>	This work
$k_{f,EG2}$	51 g <sup>-1</sup> h <sup>-1</sup>	This work
$k_{CCb}$	5.85 g l <sup>-1</sup>	Phillippidis <i>et al</i> (1992)
$k_{CEt}$	50.35 g l <sup>-1</sup>	Phillippidis <i>et al</i> (1992)
$\delta$	2.2	This work
$\varphi$	0.7	This work
$[C]_{CBH1}$	18.5%	This work
$[C]_{CBH2}$	14.5%	This work
$[C]_{EG2}$	9.36%	This work
$\theta_{CBH1}$	0.231	This work
$\theta_{CBH2}$	0.169	This work
$\theta_{EG2}$	0.6	This work
$k_{CBH1}$	-0.6 h <sup>-1</sup>	This work
$k_{CBH2}$	-0.388 h <sup>-1</sup>	This work
$k_{EG2}$	-0.08 h <sup>-1</sup>	This work
$n$	0.666	This work

along with a mixture of the enzymes with a CBH1:CBH2:EG2 ratio of 11:5:1.

The effects of synergism for the selected enzymes increased the efficiency of CBH1. The synergism between the CBH1 and CBH2 significantly increased the binding capacity of CBH1. This indicates that CBH2 contributed to opening up the cellulose structure to allow more of CBH1 to bind and hydrolyze the substrate. It could further be noticed that the largest contributing factor for successful synergism was the EG2 cellulase (figure 3). This enzyme is highly efficient even at very low dosages. Without CBH1 and CBH2 present EG2 was capable of releasing some cellobiose and glucose, likely due to repeated action on the released cello-oligomers. However, the release of glucose by this enzyme was significantly lower than that produced by the CBHs.

#### 4. Discussion and conclusions

The pursuit to enable *S. cerevisiae* to hydrolyze cellulose through the secretion of a heterologous cellulases system has brought about a number of challenges unique to this problem. Though it has long been proposed in literature that hydrolysis of cellulose requires the synergistic action of exoglucanases, endoglucanases and  $\beta$ -glucosidases (Zhang and Lynd 2004), heterologous production of these in *S. cerevisiae* at high levels has been problematic (Van Zyl *et al* 2007). While relatively high-level expression of EGs and BGLs were reported, CBH secretion levels have been poor until recently. Ilmén *et al* (2011) expressed a varied array of CBHs and observed that some candidates were more compatible with yeast expression than others. Furthermore, Yamada *et al* (2010) demonstrated that producing cellulase activities in the correct ratios improved PASC hydrolysis more than simply greater overexpression of the cellulase encoding

genes. Therefore, we can now select candidate cellulases representing the three required types that express well in *S. cerevisiae*, test their apparent synergistic action and apply this knowledge in the construction of cellulose degrading strains of this yeast.

The yeast produced enzymes used in this study were relatively free of other proteins as *S. cerevisiae* generally secretes very low levels of native proteins when cultured in minimal media (figure 1) (Romanos *et al* 1992). The preparations also contained only the hydrolytic activity heterologously expressed in the corresponding strain with no contaminating side activities as is often the case when using enzyme preparations derived from native cellulose utilizing organisms. This allowed determining the activities in isolation and co-operation of these enzymes with no influence from other cellulolytic components and deriving the mathematical model accordingly. Figure 2 showed the clear non-linear relationship between the amount of cellulase loaded in isolation and the amount of Avicel hydrolyzed with the levels of hydrolysis becoming saturated at higher protein loadings. While testing exo–exo synergy of the CBHs we observed that the optimal ratio shifted somewhat with the amount of cellulases loaded, likely due to the limited available binding sites for the enzyme on the substrate that become saturated at higher cellulase loadings. In previous studies it was also shown that optimal degrees of synergism appeared at non-saturating enzyme concentrations (Nidetzky *et al* 1994a). Optimal ratios of the cellulases were determined, among other factors, by the total enzyme concentration reflecting the degree of substrate saturation. Interestingly, the ratio effect was also diminished with longer incubation times. When the CBH1:CBH2 ratio experiment was allowed to react for 48 h instead of 24 h, much smaller differences in activity were seen between 20:80 and 80:20 ratios and all points in between (figure 3(b)). It appears that CBH1 has an initial burst of activity on Avicel that is higher than that of CBH2. This may explain the bias towards higher CBH1:CBH2 ratios in non-saturating enzyme concentrations.

Endo–exo synergy tests of the CBH1 and CBH2 with EG2 separately showed an interesting phenomenon (figure 3(b)). There was noticeably more synergy between the CBH1 + EG2 combination (132% and 100% more hydrolysis than CBH1 alone in 24 and 48 h, respectively) than between the CBH2 + EG2 combination (68% and 52% more hydrolysis than CBH2 alone in 24 and 48 h, respectively). Surprisingly, CBH2 displayed superior binding kinetics with Avicel (figure 5, CBH2 had the highest protein adsorption capacity followed by CBH1 and then EG2). This is likely due to the large degree of hyperglycosylation observed on CBH1 produced in the yeast (figure 1) as was reported previously (Penttilä *et al* 1988, Ilmén *et al* 2011). Hyperglycosylation may cause steric hindrance on the interaction of CBH1 with the substrate leading to limited mobility and restricted access of the enzyme. This is most likely relieved through the action of EG2 opening the substrate structure. The model predicts that further addition of CBH2 also opened up the structure appreciably to allow the efficiency of CBH1 to increase significantly, while the efficiency of the other enzymes remained mostly constant.

While CBH1 and CBH2 act from the reducing and non-reducing ends of the substrate, respectively, these enzymes theoretically compete for binding to the same sites immediately after EG hydrolysis produces new chain ends (Lynd *et al* 2002, Zhang and Lynd 2004). This is because when new chain ends are formed, there are sites available at the same location for both CBH1 and CBH2. As both enzymes are larger than the gap between the two newly formed ends (Zhang and Lynd 2004), only one of the two enzymes can occupy the site initially. This explains why in the yCell enzyme combination, adsorbance capacity was only slightly higher than that of CBH1 only (figure 5). Once the attached CBH1 or CBH2 enzyme start to hydrolyze the cellulose chain the gap between the ends becomes larger to the extent that a CBH can attach to the opposite chain end. Assuming this happens relatively quickly under normal hydrolysis, the effect of one enzyme blocking the other becomes negligible and hence was not included in the reaction model. Furthermore, it could also be shown that while CBH1 and CBH2 binding may negatively affect the binding of EG2, the endoglucanase is extremely efficient and even at very low concentrations it was capable of producing sufficient sites to not influence the overall reaction rates.

Hydrolysis of Avicel by the yeast cellulases over a long period of time showed that complete hydrolysis of the substrate was not attained (figure 4). As all the assays contained an abundance of BGL activity in the form of added Novozyme 188, one might have expected complete hydrolysis of the samples containing combinations of CBH1, CBH2 and EG2 as it has been reported in literature that these are the activities required for crystalline cellulose hydrolysis. This did not occur and hydrolysis arrested at ~70% even in the best case. A part of the explanation may reside with the substrate itself which has been shown to be quite resistant to complete hydrolysis. Van Zyl *et al* (2011b) and Ouyang *et al* (2010) both tested hydrolysis of Avicel using the commercial enzyme preparations Spezyme CP and Celluclast 1.5 l (Novozymes). Though both studies showed high rates of hydrolysis initially, neither reported complete Avicel hydrolysis. However, over 90% hydrolysis of Avicel can be achieved by the enzyme systems of several filamentous fungi in a relatively short time (Arantes and Saddler 2010). It is also known that filamentous fungi secrete a vast collection of enzymes when growing on this substrate (Herpoël-Gimbert *et al* 2008). It is therefore likely that other protein elements, such as swollenin or endoglucanases of different glycosyl hydrolase families may be required for complete hydrolysis of Avicel. It may also be possible that enzymes originating from the same host may interact better on the substrate and yield greater levels of hydrolysis.

In this study a mathematical model was generated to simulate the interactions of yeast produced cellulases on crystalline cellulose, both under isolated and synergistic conditions. Several models for cellulose hydrolysis have been described previously (reviewed by Lynd *et al* 2002, Zhang and Lynd 2004), though these mostly utilize enzyme cocktails at their optimal temperatures (>40 °C). Various mixtures of the enzymes at a range of protein:substrate loadings

allowed refinement of our mathematical model. The model predicted the conversion of Avicel using the isolated CBH1 and CBH2 with reasonable accuracy (figure 4). The model assumed that the available substrate for each enzyme is separate (i.e. that CBH1 cannot convert the substrate used by CBH2 and vice versa) and that combinations of the CBH1, CBH2 and EG2 enzymes interact in a synergistic method to convert more cellulose than each enzyme separately. It also assumed that CBH1 and CBH2 are responsible for the production of cellobiose, which is further converted to glucose by the added BGL and that EG2 functions by generating new available substrate for CBHs at a fixed ratio based on the available amorphous cellulose. The model currently does not predict the optimal enzyme loading requirements prior to the 48 h timeline but does show good agreement with the experimentally determined optima after 48 h. This discrepancy may be attributed to the burst of CBH1 activity found within the first 24 h period of the reactions. Results from Van Zyl *et al* (2011b) indicated this was due to a large amount of substrate sites available in the first 20 h of the reaction, after which the number of available sites decreased considerably. As with most available models, the model is relatively specific to the enzymes and substrate used. However, the model provides an excellent tool to investigate enzyme synergy in the temperature and pH conditions relevant to a CBP process with *S. cerevisiae* and uses cellulases that are produced heterologously by the yeast. The insights gained from the model and experimental data can thus be applied to help determine the minimum protein requirement for complete hydrolysis of cellulose. Such knowledge will be greatly informative for the design of better enzymatic cocktails or cellulose CBP-stains of *S. cerevisiae*.

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