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Domain engineering of *Saccharomyces cerevisiae* exoglucanases

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Abstract

To illustrate the effect of a cellulose-binding domain (CBD) on the enzymatic characteristics of non-cellulolytic exoglucanases, 10 different recombinant enzymes were constructed combining the *Saccharomyces cerevisiae* exoglucanases, EXG1 and SSG1, with the CBD2 from the *Trichoderma reesei* cellobiohydrolase, CBH2, and a linker peptide. The enzymatic activity of the recombinant enzymes increased with the CBD copy number. The recombinant enzymes, CBD2-CBD2-L-EXG1 and CBD2-CBD2-SSG1, exhibited the highest cellobiohydrolase activity (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 CBD 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 scanning electron microscopic analysis.

Introduction

Cellulolytic microorganisms produce at least three major types of enzymes: endoglucanases, exoglucanases and β-glucosidases. 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 mediates the binding of cellulases to the substrate, thereby playing a pivotal role in cellulose hydrolysis (Tomme *et al.* 1995). CBD enhances the enzymatic activity of cellulases by decreasing 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 domain removal, domain exchange, site-directed mutagenesis or by artificial addition of a 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 cellulases (Srisodsuk *et al.* 1993).

Saccharomyces cerevisiae possesses four different glucanases, namely EXG1, BGL2, SSG1 and EXG2 (Lynd *et al.* 2002). These yeast glucanases do not exhibit the same architectural domain organisation showed by most of the other fungal or bacterial cellulases. These enzymes display β-1,3-exoglucanase activity as their major activity and exhibit a significant β-1,4-exoglucanase side-activity, releasing free glucose moieties from disaccharide substrates such as cellobiose (Larriba *et al.* 1995).

The purpose of this study was to investigate the functional role of CBDs and their effect on the hydrolytic activity of two *S. cerevisiae* exoglucanases, namely the main extracellular β-1,3-exoglucanase, EXG1, and the sporulation-specific β-1,3-exoglucanase, SSG1. This was done by domain engineering, which entailed the linking of these two exoglucanases to the CBD of the *Trichoderma reesei* cellobiohydrolase (CBHII). To mimic the multi-domain organisation displayed by cellulases from other microorganisms, the modular organisation of these engineered yeast exoglucanases differed in terms of the location (either at the *N*-terminus or the *C*-terminus) and copy number of the heterologous CBD, as well as the absence or presence of a synthetic linker peptide. The characterisation of the physicochemical and catalytic properties of these multi-domain recombinant enzymes was performed using various cellulose and non-cellulose substrates.

Materials and methods

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 *EXG1* and *SSG1* gene constructs, using pGEM-T Easy plasmid (Promega) (Lönn *et al.* 2002), and as the host for the derivative cloning vectors. A modified version of vector YEp352 (Malherbe *et al.* 2003) was used for the subcloning of the different fusion derivatives of the *S. cerevisiae EXG1* and *SSG1* genes. The gene fragment encoding the cellulose binding domain (CBD2) of *T. reesei* cellobiohydrolase II was obtained from plasmid pAZ21. A commercial wine yeast strain, *S. cerevisiae* VIN7, was used as the source of genomic DNA for the amplification of both the *EXG1* and *SSG1* genes.

Cloning and construction of the recombinant EXG1 and SSG1 gene cassettes

The primers that were used to construct the *EXG1* and *SSG1* gene cassettes are listed in Table 1, while the various recombinant EXG1 and SSG1 glucanases are tabulated in Table 2. 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 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 121–1347), 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 an inter-domain linker) was amplified using primers L-EXG1-F; EXG1-R and primers L-EXG1-F; EXG1′-R to facilitate the fusion of

Table 1. PCR primers designed for DNA amplification.

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′-ag**tcgact**caaagacactgggagtaata-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	Enzymes[a]	Avicelase activity (U mg^{-1})[b]
pCEL71	**EXG1**	9.6 ± 0.7
pCEL72	CBD2-EXG1	9.5 ± 0.5
pCEL73	CBD2-CBD2-EXG1	4.3 ± 0.6
pCEL74	EXG1-CBD2	7.6 ± 0.4
pCEL75	**L-EXG1**	7.6 ± 0.4
pCEL76	**CBD2-L-EXG1**	14.1 ± 0.6
pCEL77	**CBD2-CBD2-L-EXG1**	17.5 ± 0.6
pCEL78	**L-EXG1-CBD2**	15.9 ± 0.8
pCEL79	**SSG1**	9.9 ± 0.5
pCEL80	CBD2-SSG1	6.4 ± 0.3
pCEL81	**CBD2-CBD2-SSG1**	16.3 ± 0.5
pCEL82	**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 independent assays ± accumulated standard errors.

CBD2 at the *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 standard PCR buffer, 1.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 2 ng template μl^{-1} and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. The PCR programme was 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.

Enzyme expression, preparation and purification

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). The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard.

Preparation of substrates

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

Enzyme and adsorption assay

The purified native and recombinant enzymes were analysed for their exo-1,3-β-glucanase activity as described by Van Rensburg *et al.* (1997). One unit of β-glucanase activity was defined as the amount of enzyme required to release 1 μmol reducing sugar equivalents (expressed as glucose) per min at pH 5 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 out in 2 ml *O*-ringed screw cap micro-centrifuge tubes containing the purified enzyme preparations together with commercial preparations of the *Trichoderma longibrachiatum* endoglucanase EGII and the *Aspergillus niger* β-glucosidase (Megazyme), in a reaction volume of 750 μl with the substrate (Avicel, amorphous cellulose, BMCC or CMC) at 1 g l^{-1} 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 soluble reducing sugars. Each reaction mix was done in

triplicate. The calculation of the units for cellobiohydrolase activity of the native and recombinant EXG1 and SSG1 enzymes was based on standard curves obtained with purified *T. longibrachiatum* cellobiohydrolase (Megazyme). One unit of cellobiohydrolase activity was defined as the amount of enzyme required to release 1.11 μmol glucose per min under the assay conditions mentioned. Adsorption assays were performed in a similar manner as described previously by Gal *et al.* (1997).

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 6–10 concentrations of Avicel, and these generally ranged from 0.2 to 5 times the K_m value. The V_{max} and K_m values were determined by nonlinear regression analysis, using the Graph Pad Prism program.

pH and temperature optimum

The effect of pH on the activity of the native and recombinant enzymes was investigated in the pH ranges 3–7, using 1 g Avicel l^{-1} 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.

Thermostability

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

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 (*Aspergillus niger*). A 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 an analytical S440 Leo Scanning Electron Microscope operated at an accelerating voltage of 10 kV. All the images were visualised at a magnification of 1000.

Results

Purification and screening of thenative and recombinant yeast exoglucanases

The native and recombinant *S. cerevisiae* EXG1 and SSG1 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 the recombinant enzymes was performed based on their ability to hydrolyse Avicel cellulose (Table 2). The hydrolytic assays were performed as mentioned in the Material and methods section. Based on the performance of hydrolysis, six recombinant enzymes were chosen along with the native EXG1 and SSG1 as controls, for further study.

Hydrolytic activity on different substrates

The hydrolytic activity of the recombinant enzymes towards different cellulosic and non-cellulosic substrates was tested and is tabulated in Table 3. All the recombinant EXG1 and SSG1 enzymes acted on the cellulosic substrates and the pattern in the degree of hydrolysis was similar on all the substrates tested. However, the increase in activity correlated with an increase in copy number of CBD present in the engineered enzymes. As compared to the activities of their respective wild-type and single CBD enzymes, CBD2-CBD2-L-EXG1 and CBD2-CBD2-SSG1 displayed the highest hydrolytic activity. In addition, the recombinant enzymes also showed a similar pattern of improved hydrolysis towards β-1,3-exoglucanase specific laminarin and β-glucan.

Adsorption as a function of hydrolysis

The adsorption data of recombinant EXG1 and SSG1 enzymes are shown in Table 4. The

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	7.4 ± 0.5	2.8 ± 0.1	32.2 ± 1.5	20.2 ± 1
L-EXG1	7.9 ± 0.6	14.8 ± 0.6	6.2 ± 0.5	2.4 ± 0.1	25.2 ± 2	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 ± 0.3	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 ± 0.6	11 ± 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	38.1 ± 0.9	11.3 ± 1	5.6 ± 0.4	59.6 ± 1.8	32.7 ± 1.3
SSG1-CBD2	14.2 ± 0.7	30.8 ± 1	10.4 ± 0.6	4.8 ± 0.3	52.4 ± 2.6	27.4 ± 0.5

The values are means of three independent assays ± accumulated standard errors.
[a] The values shown here are expressed as cellobiohydrolase units per mg of protein.
[b] The values shown here are given in glucanase units per mg of protein.

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

Enzymes	Adsorption (%)	Kinetic parameter	
		V_{max}[a]	K_m[b]
EXG1	12.8	2424 ± 183	4.66 ± 0.70
L1-EXG1	21.6	1555 ± 41.5	3.18 ± 0.19
CBD2-L1-EXG1	35.6	3733 ± 173.3	5.66 ± 0.50
CBD2-CBD2-L1-EXG1	42	3793 ± 121.9	4.29 ± 0.28
L1-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

The values are mean of three determinations ± standard deviation.
[a] $\mu mol\ l^{-1}\ h^{-1}$.
[b] gl^{-1}.

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.

Temperature and pH profile

To investigate the effect of the CBD on the temperature and pH optimum of the recombinant EXG1 and SSG1 enzymes, the profiles were investigated at temperatures 30–60 °C and pH 3–7 respectively (Figure 1a, b). As in the case of the native enzymes, the hydrolytic activity of all the recombinant enzymes was found to be optimal at a temperature of 50 °C and pH 6. The figure illustrates that the temperature and pH profiles of the multi-domain recombinant enzymes did not show significant deviation when compared with their wild-type counterpart.

Thermal stability

The thermodenaturation curves of the recombinant enzymes are 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

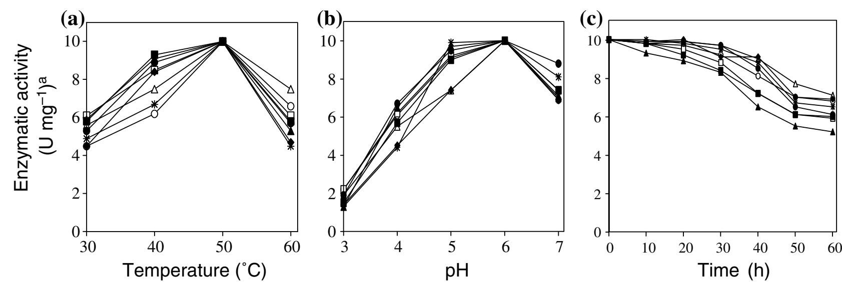

Fig. 1. (a) Temperature optimum; (b) pH optimum; (c) thermostability: ■, EXG1; ▲, L-EXG1; ●, CBD2-L-EXG1; ◆, CBD2-CBD2-L-EXG1; *, L-EXG1-CBD2; □, SSG1; △, CBD2-CBD2-SSG1; ○, SSG1-CBD2. The values are expressed as cellobiohydrolase units and are means of three independent assays ± accumulated standard errors.

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.

Kinetic properties of the recombinant glucanase

The kinetics of the engineered yeast glucanases is given 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 increased up to two-fold, except for the enzyme L-EXG1.

Scanning electron microscopy

The SEM analysis of Avicel particles treated with recombinant enzymes clearly shows the hydrolytic function of these enzymes on cellulose (Figure 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 (Figure 2b, c, e). Unlike the control samples (Figure 2a, d, f), the particles treated with the recombinant enzymes are also more heterogeneous and it appears that the solubilisation occurred more at the ends than on the surface reflecting a better synergy between the endo- and exo-activity.

Discussion

The results reported in this article show that both the CBD and the linker sequences coupled to a non-cellulolytic catalytic domain of EXG1 and SSG1 imparted the abililty 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.

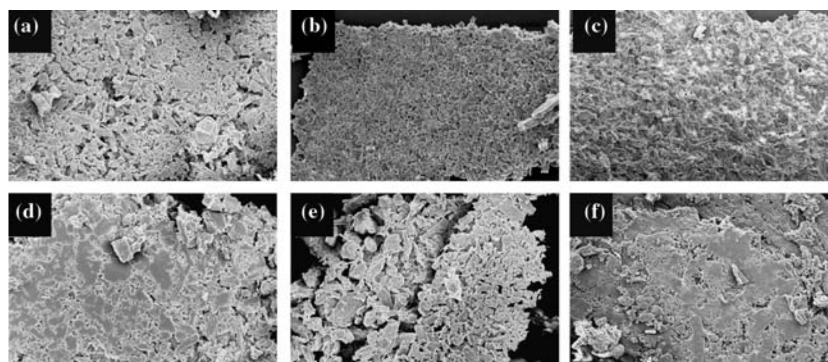

Fig. 2. Scanning electron microscopic (SEM) analysis of Avicel particles treated with different recombinant yeast exoglucanases. All the images were visualised at a magnification of 1000. (a) EXG1, (b) CBD2-L-EXG1, (c) CBD2-CBD2-L-EXG1, (d) SSG1, (e) CBD2-CBD2-SSG1, (f) control enzyme treatment (endocellulase and β-glucosidase only).

The recombinant yeast glucanases with two CBD copies might have acquired increased adsorption ability thereby promoting better stability of the molecules on the surface of the substrates, leading to improved hydrolysis (Linder *et al*. 1996). The lower activities on recalcitrant substrates observed for the single-CBD enzymes probably reflect the fact that the enzyme–substrate complexes are less stable. 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 EXG1 and SSG1 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 CBD endowed the 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). This 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 multi-domain *S. cerevisiae* β-1,3-exoglucanases would have gained sufficient molecular stability, flexibility and intra-molecular 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 thermostabilising 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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