Development of enzymatically-active bacterial cellulose membranes through stable immobilization of an engineered β -galactosidase

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Abstract

Enzymatically-active bacterial cellulose (BC) was prepared by non-covalent immobilization of a hybrid enzyme composed by a β -galactosidase from *Thermotoga maritima* (TmLac) and a carbohydrate binding module (CBM2) from *Pyrococcus furiosus*. TmLac-CBM2 protein was bound to BC, with higher affinity at pH 6.5 than at pH 8.5 and with high specificity compared to the non-engineered enzyme. Both hydrated (HBC) and freeze-dried (DBC) bacterial cellulose showed equivalent enzyme binding efficiencies. Initial reaction rate of HBC-bound enzyme was higher than DBC-bound and both of them were lower than the free enzyme. However, enzyme performance was similar in all three cases for the hydrolysis of 5 % lactose to a high extent. Reuse of the immobilized enzyme was limited by the stability of the β -galactosidase module, whereas the CBM2 module provided stable attachment of the hybrid enzyme to the BC support, after long incubation periods (3 hours) at 75 °C.

Keywords:

protein immobilization; carbohydrate binding module; bacterial cellulose

1. Introduction

The development of enzymatically-active materials through immobilization technologies expands the range of applications of both the enzyme and the material used as support. Enzyme immobilization provides advantages by reducing operational costs, prolonging enzyme lifetime, increasing enzyme stability and facilitating their recovery and reuse [1]. Specifically, immobilization of β -galactosidases has attracted a great deal of interest, as they are biotechnologically relevant enzymes with important applications in health and food industries [2-5]. β -Galactosidases (EC 3.2.1.23) are glycosidases that hydrolyse terminal non-reducing β -D-galactose residues from di-, oligo- and conjugated-saccharides. They are used in large scale industrial processes for the production of lactose-free milk and milk products, and for the treatment of whey derived from dairy industries, thus reducing pollution load and yielding valuable sugars [6-7]. They are also useful for obtaining functional galacto-oligosaccharides (GOS), which promote the growth of bifidobacteria in vivo [4,8]. For industrial use, thermostable enzymes show distinct advantages since, the use of high temperatures facilitates many processes, increasing product yield by enhancing substrate solubility and reducing the risk of microbial contamination.

Regarding the matrices for enzyme immobilization, cellulose supports are highly attractive, since this carbohydrate, the most abundant biopolymer found in nature, is non-toxic, cheap, inert, stable and biodegradable [9-10]. Bacterial cellulose (BC), which is secreted extracellularly as cellulose fibers by some bacterial species, has a number of advantages for enzyme immobilization, as it has a nanoporous structure and possesses

higher water holding capacity, higher purity and crystallinity and a finer web-like network than plant cellulose [11].

There is a wide range of enzyme immobilization techniques involving physical entrapment or chemical interactions (either covalent or non-covalent) between the enzyme and the support [9,12-16]. These techniques have been applied to many enzymes, including β -galactosidases [5,9,12,17-23]. Among the different methods available for enzyme immobilization onto cellulosic supports, covalent attachment provides the most stable interaction but it requires the addition of chemical coupling agents which may inactivate the enzyme or may not be compatible with food industry requirements. Alternatively, physical adsorption methods are simpler and they usually cause only minor modifications of the enzyme [9]. However, these are often based on weak interactions that make enzyme easily washed out from the support. In all cases, enzyme purification is an unavoidable preliminary step. Another strategy is to use molecular engineering techniques to modify the enzymes to be immobilized by appending a carbohydrate binding module (CBM) which allows attachment to a polysaccharide [24]. CBMs are protein domains with independent fold which are classified in different families, based on sequence similarities, showing diverse substrate specificities [10,25]. Thus, the immobilization of an enzyme containing a carbohydrate binding module (CBM) with cellulose affinity can be a good strategy to develop industrially attractive enzymatically-active materials with potential food and pharmaceutical applications. Moreover, since cellulose adhesion of the CBM-tagged enzyme would be mediated through specific interactions, this strategy may serve as a one-step purification and immobilization procedure. CBM sequences from *Chlostridium cellulovorans* and *Cellulomonas fimi* (CBD-Clos and CBD-Cex,

respectively), currently available in commercial vectors, and other CBMs have been used to generate hybrid enzymes with different catalytic modules (invertase, D-aminoacid oxidase, peroxidase, β -glucosidase and β -galactosidase, among others) that were successfully immobilized on cellulose [26-33]. However, the stability of such attachment at high temperatures, either has not been tested, or has been seen to be very low, with heating resulting in total release of the protein from the cellulose substrate [34], therefore limiting its applicability.

In this work, enzymatically active materials were developed by immobilizing an engineered thermostable hybrid protein, composed by a β -galactosidase fused to a CBM, onto bacterial cellulose. Binding specificity was assessed by comparing the engineered and wild-type enzymes. Different conditions (pH, enzyme concentration and BC hydration state) were tested during the incubation of the engineered enzyme with the bacterial cellulose membranes, and their effects on the binding efficiency and immobilized enzyme activity were studied. Finally, the stability of the immobilization at high temperature and the recyclability of the materials were also analyzed.

2. Material and Methods

2.1 Preparation of TmLac and hybrid TmLac-CBM2 β -galactosidases

The hybrid enzyme used in this work (TmLac-CBM2) was constructed by fusing a β -galactosidase from *Thermotoga maritima* (TmLac) [21] with a carbohydrate binding module (CBM2) from *Pyrococcus furiosus* chitinase [35]. The genetic construct encoding the hybrid protein, as well as the production of both TmLac-CBM2 and the

wild type TmLac by *Escherichia coli*, have been described elsewhere [36]. Because both TmLac and TmLac-CBM2 are thermoresistant proteins, purification was carried out by heat shock treatment of crude extracts at 85 °C during 10 minutes. Endogenous *E. coli* proteins precipitated whereas most of the recombinant protein remained in the soluble fraction. This single step purification procedure was selected because it is much more cost-effective and less time-consuming than chromatographic methods. The soluble fraction was dialyzed against phosphate buffer 50 mM pH 6.5 and concentrated by ultrafiltration using a membrane with a 20 KDa cutoff (Thermo Sci). Enzyme concentration was determined based on β -galactosidase measurements and previous data of specific activity of TmLac ($26 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg enzyme}^{-1}$) and TmLac-CBM2 ($28 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg enzyme}^{-1}$), purified as described in Marín-Navarro et al. [21].

2.2 Preparation of bacterial cellulose nanofiber membranes

The bacterial cellulose pellicles used as immobilizing supports were obtained from the bacterial strain *Gluconacetobacter xylinus* 7351 as described elsewhere [37-38]. The obtained bacterial cellulose pellicles of about 5 cm thick, were cut into small pieces (approx. $2 \times 2 \text{ cm}^2$), and bacterial cells and absorbed culture media were removed from the materials through boiling, first in distilled water and then in a 10 % (v/v) NaOH aqueous solution. Subsequently, the NaOH was removed by 3 rounds of boiling in distilled water. Some of the cellulose fragments were stored in buffered media at pH 7 and 4 °C (HBC), whereas others were freeze-dried and stored in a desiccator (DBC). The crystallinity index of this cellulose (ca. 79%) has been previously determined [38].

2.3 Enzyme immobilization

Binding assays were made with wild type (TmLac) and hybrid (TmLac-CBM2) β -galactosidases. Different volumes (0.5 or 2.5 mL) of enzyme solutions (at concentrations ranging from 0.06 to 1.6 mg/mL) were incubated with approximately 30 mg of DBC or 3 g of HBC (corresponding to approx. 30 mg dry weight) in binding buffer (50 mM phosphate pH 6.5 or 50 mM Tris pH 8.5) under constant agitation (180 rpm) during 4 hours at 37 °C. Afterwards, unbound enzyme was directly recovered by pipetting the remaining solution (around 2 mL) of cellulose incubated with 2.5 mL of enzyme. In the case of cellulose incubated with 0.5 mL virtually all the added solution was embedded within the cellulose matrix after incubation. These observations indicate that under these conditions the absorption capacity of DBC is around 0.5 mL of water / 30 mg of support (semiquantitative estimation). Therefore, unbound enzyme was recovered by washing cellulose with 0.5 mL of the binding buffer (pH 6.5 or pH 8.5) for 15 min at room temperature. Control samples of enzyme without cellulose were incubated in parallel under the same conditions. All the assays were made in triplicate.

2.4 Enzyme Assays

Procedures for the determination of β -galactosidase activity with either the chromogenic compound *p*-nitrophenyl β -D-Galactopyranoside (PNPGal) or lactose (5 % w/v) as the substrate, were carried out in assay buffer (50 mM phosphate pH 6.5, 10 mM NaCl, 1mM MgCl₂), at the optimum pH of the enzyme [39], as described by Marín-Navarro et al. [21].

Assays with PNPGal were performed to quantify the fraction of cellulose bound enzyme, by comparing the activity recovered in unbound and control samples. The ratio of the activity of the unbound sample to that of the control sample represents the fraction (f) of unbound enzyme. Therefore, the percentage of bound enzyme was calculated as $100 \cdot (1 - f)$.

To determine the activity of bound β -galactosidase, the enzyme-loaded cellulose was previously washed with 10 mL of binding buffer at room temperature to remove residual unbound enzyme. Lactose hydrolysing activity assays were carried out with pieces of enzyme-loaded DBC or HBC material corresponding to about 1/5 of the total cellulose weight. Control samples containing similar enzyme amounts of soluble enzyme were analyzed in parallel. When performing serial assays with bound enzyme, cellulose was washed once with assay buffer prior to each step of incubation with lactose.

To quantify the amount of enzyme released from HBC, after incubation with 5% lactose at 75 °C for 3 hours, the soluble fraction was concentrated 100-fold by ultrafiltration through a 20 KDa-cutoff membrane (Pierce Concentrators, 20K MWCO, Thermo Scientific), and diluted back to the initial volume to reduce the concentration of sugars (glucose and galactose) that may inhibit β -galactosidase activity. The sample was finally concentrated again 100-fold (R sample). A control sample of free-enzyme was subjected to the same treatment (C sample). β -Galactosidase activity of the R and C samples was determined using PNPGal as substrate. The percentage of released enzyme (% R) was determined as:

$$\% R = [(AR/AC) \cdot (FE/IE)] \cdot 100,$$

where AR and AC represent the β -galactosidase activity of the R and C samples, respectively, and FE and IE represent the enzyme amount in the control sample (free-enzyme) and in the HBC-bound sample (immobilized enzyme), respectively.

2.5. Fluorescence analysis of immobilized enzymes labelled with FITC

The procedure for protein labelling with FITC (fluorescein isothiocyanate) was adapted from Pinto et al. [40-41]. TmLac and TmLac-CBM2 were dialyzed (Servapor dialysis tubing, MWCO 12 000 – 14 000) against labelling buffer (0.1 M HEPES buffer, pH 9.0). Enzymes (2 mg protein/mL in labelling buffer) were conjugated with FITC by addition of 0.5 mol of FITC per mol of enzyme, in order to keep a protein excess, thus limiting the number of modifications with the fluorophore to one per molecule of enzyme. After incubation at room temperature overnight in the dark, with magnetic stirring, unbound FITC was removed by a second dialysis against 50 mM phosphate buffer, pH 6.5. Both labelled and unlabelled enzymes (250 μ L at 1.3 mg/mL) were incubated with approximately 15 mg of freeze-dried bacterial cellulose in 50 mM phosphate buffer pH 6.5, at 37 $^{\circ}$ C during 4 hours. Treated cellulose was washed twice with the same buffer prior to analysis in a fluorescence microscope (Nikon eclipse 90i).

2.6. Scanning Electron Microscopy (SEM) analysis

Microstructural analysis of the films was carried out through scanning electron microscopy on a Hitachi microscope (Hitachi S-4800). The surface of the samples was

fixed on M4 aluminium specimen mount, gold–palladium coated and observed using an accelerating voltage of 10 kV and a working distance of 10 mm.

2.7. Size determination using dynamic light scattering (DLS)

1 mL aliquots of enzyme solution (either at 0.3 or 1.3 mg/mL) in 20 mM Tris-HCl pH 8.5, 50 mM NaCl were placed in quartz cuvettes and analyzed, using DLS, to estimate the mean hydrodynamic diameter of the enzymes depending on solution concentration. The experiments were performed on a Malvern Zetasizer- Nano ZS (Malvern, UK). Mean hydrodynamic radii were obtained from the raw data using the general-purpose inverse Laplace transform method provided in the instrument software.

2.8. Polyacrylamide gel electrophoresis

Enzyme samples were mixed with protein solvent (125 mM Tris-HCl pH 6.8, 4% SDS, 20 % glycerol, 0.05 % bromophenol blue and 250 mM β -mercaptoetanol) in a 1:1 volume ratio and boiled at 95 °C for 10 minutes. Electrophoresis was carried out in a 10 % polyacrylamide gel, and stained with Coomassie brilliant blue.

2.9. Statistical analysis

IBM SPSS Statistics software (v.23) (IBM Corp., USA) was used to perform the statistical analysis of the data. The significance of the differences observed between samples was assessed through two-sided t-tests at $p < 0.05$.

3. Results and Discussion

Aiming to develop enzymatically active bacterial cellulose membranes, a hybrid enzyme made of the β -galactosidase from *Thermotoga maritima* fused to the carbohydrate binding module CBM2 from *Pyrococcus furiosus* chitinase (TmLac-CBM2) was assayed. Chitin-binding activity of this CBM2 module has been previously shown [36] but other CBMs from the same family also exhibit cellulose-binding function [42]. Previous experiments indicated that thermal stability and intrinsic activity of the chimeric enzyme were similar to those of wild-type β -galactosidase (TmLac) (see Supplementary material). The cellulose-binding affinity of TmLac-CBM2 was measured and compared with that of TmLac. Subsequently, reusability of active membranes was assessed by assaying their activity after consecutive batches.

3.1. Analysis of binding specificity of the hybrid enzyme and pH-dependence of binding efficiency to bacterial cellulose membranes

Binding of hybrid and wild type enzymes to freeze-dried bacterial cellulose (DBC) nanofibrous membranes was measured at two pH values (6.5 and 8.5). These conditions were selected taking as a reference the optimum pH value of TmLac (6.5) [39] and considering that enzyme stability is compromised at acidic (below 5) but not

at basic (up to 9) pH values (results not shown). Bacterial cellulose was selected as enzyme supporting matrix due to the structural features of this material, composed by β -1,4-glucopyranosyl units organized forming a nanofibrous web (with nanofibers of around 40-60 nm width), providing a very high specific area for binding (see Supplementary material). These experiments were performed by incubating 30 mg of the DBC membrane with 2.5 mL of each enzyme solution (0.05 mg enzyme/mL).

Binding efficiency was determined by comparing β -galactosidase activity in the unbound fraction, after incubation with cellulose, with that of a control sample incubated in the same conditions without cellulose. Fig. 1 shows that the affinity of the hybrid enzyme containing the CBM2 module to cellulose was much higher than that of the wild type TmLac, at both pH values. Moreover, it was observed for both enzymes that binding efficiency was significantly higher at pH 6.5 than at pH 8.5. The morphology of the bacterial cellulose membranes was significantly modified after the binding experiments, with a less porous structure observed (see Supplementary Material). These morphological changes seem to correlate with enzyme binding efficiency, since they were more pronounced at pH 6.5.

Physical adsorption of both wild type and hybrid enzymes onto cellulose fibers was also monitored by fluorescence analysis. Wild type β -galactosidase and the hybrid enzyme were stained with fluorescein isothiocyanate (FITC) prior to immobilization. FITC is a fluorescent compound which is reactive towards nucleophiles, including amine and sulfhydryl groups on proteins. Moreover, it has been demonstrated that staining with FITC does not affect the interactions of carbohydrate binding modules with cellulose [40-41,43], as long as FITC labelling is limited to an average of one

modification per molecule. Fig. 2 shows the fluorescence images of cellulose films upon immobilization at pH 6.5 of wild type or hybrid enzymes previously stained with FITC, along with control cellulose membranes incubated with the corresponding unlabeled enzymes. As expected, no fluorescence was observed in the control samples, and on the other hand, the fluorescence intensity detected on the surface of bacterial cellulose nanofibrous membranes bound to FITC-labelled TmLac-CBM2 was higher than that observed for the wild-type enzyme.

The results described show that protein tagging with CBM2 is a useful strategy for immobilization onto cellulose. Moreover, the fact that untagged enzyme shows very low affinity for cellulose, suggests that this approach may be used as a one-step procedure for protein purification and immobilization. Subsequent binding experiments were performed at pH 6.5 with the hybrid TmLac-CBM2 enzyme.

3.2. Binding efficiency of the hybrid enzyme onto bacterial cellulose with different hydration states

Since each DBC fragment (around 30 mg) had an absorption capacity of around 0.5 mL of water we tested whether reducing the incubation volume from 2.5 mL to 0.5 mL of enzyme (0.3 mg/mL) had any effect on the binding capacity of the support. The percentage of bound enzyme to 30 mg of DBC was $42 \pm 2 \%$ and $80 \pm 10 \%$, when 2.5 mL or 0.5 mL were used, respectively. Increased binding with lower volume may be explained by the presence of internal binding sites in the cellulose matrix, which are only accessible to the enzyme present within the sorbed volume. Once the material has been re-hydrated, enzyme diffusion into these internal sites may be hampered by

the highly compact water network connecting cellulose fibers, and only surface sites would bind enzyme effectively. Therefore, as a general rule, volumes below the sorption capacity of DBC seem to improve binding.

Furthermore, the binding capacity of the freeze-dried bacterial cellulose (DBC) was compared to that of hydrated (around 99% water content) bacterial cellulose (HBC). Equivalent amounts (around 30 mg) of the corresponding dry weight were used in all cases. The results (Table 1) show that both DBC and HBC show similar binding efficiencies at different enzyme concentrations (ranging from 0.06 to 1.6 mg/mL). The upper limit was selected as a practical concentration boundary above which enzyme solutions may be not totally soluble. Indeed, the size of the hybrid enzyme in diluted (0.3 mg/mL) and concentrated (1.3 mg/mL) solutions, measured by light scattering (see Supplementary Material), shows the tendency of the enzyme to form aggregates of high molecular mass at higher protein concentration. The amount of enzyme bound to cellulose increased linearly with enzyme concentration, reaching a maximum of 18.5 mg of enzyme bound/g of cellulose (Table 1). This efficiency is in the same range or higher than that reported for different covalent or CBM-mediated immobilization methods. Alkaline phosphatase (AP) was immobilized on nano-fibrillated cellulose (NFC) functionalized with epoxy, amine or carboxylic acid groups with binding efficiencies ranging from 0.003 to 0.1 pmol of enzyme/ μ g of NFC, which is equivalent to 0.3 - 9 mg of AP/ μ g of NFC [44]. Cellulose hydrogel modified with aldehyde groups was able to bind between 8 and 14.6 ng of peroxidase/g of cellulose, depending on the aldehyde content [45]. The binding capacity of Avicel for different chimeric proteins constructed with diverse CBMs fused to the N-terminal end of *Cis*-epoxysuccinic acid hydrolase ranged from 8 to 20 mg of protein/g of Avicel [46].

3.3. Lactase activity of free and immobilized enzyme

The lactase activity of TmLac-CBM2 immobilized in DBC and HBC was assayed in parallel with the enzyme in the free form. Immobilization was carried out with 0.5 mL enzyme (1.6 mg/mL) and cellulose slices of 30 mg (dry weight). After immobilization, lactase assay was carried out with equivalent quantities of enzyme, either in the free or immobilized form (Table 2).

The initial rate of the reaction (after 30 minutes) was significantly lower with HBC or DBC-bound enzymes, showing values of 52 % and 27 % of the activity of the free enzyme, respectively (Table 2). The lower initial activity of the immobilized enzyme may reflect a restriction of the diffusion of substrate and products through the cellulose matrix where the hybrid protein is embedded. Such restriction seems higher for the DBC than for HBC support. Major structural changes seem to occur upon freeze-drying of HBC, since water absorption capacity of DBC is well below water content of HBC. This is probably due to a dense network of hydrogen bonds connecting cellulose fibers, which hinders interaction with water molecules. Moreover, as shown in Supplementary Material, significant morphological changes occurred in the DBC nanofiber membranes after enzyme immobilization, leaving a more compact structure with some fused fibers, which might influence water access to the enzymes bound in the inner parts of the membrane, thus modifying their hydrolytic activity. Furthermore, the different structure of water molecules within the polymeric matrix may also modulate enzyme kinetics, as proposed by Burgos et al. [47]. Enzyme inactivation upon immobilization has been extensively described with diverse techniques and enzymes [3, 5, 48].

Lactose hydrolysis was also evaluated after long-term incubations (Fig. 3). In the case of the free enzyme, the kinetics of glucose release is hyperbolic, whereas for the immobilized enzymes, the initial linear trend is extended in time. As a result, the time required to achieve 70 % of lactose hydrolysis (i.e. around 100 mM glucose released) did not differ significantly among the three forms (Fig. 3), despite the differences in the initial reaction rates (Table 2). The deviation from linear kinetics in the free form may be caused by a combination of thermal inactivation, inhibition by product accumulation and substrate consumption. In the case of immobilized enzymes, either structural changes induced by enzyme attachment to cellulose or the different microenvironment of the enzyme within the polysaccharide matrix may contribute to keep activity constant during a more extended period.

3.4. Analysis of the stability of the immobilized enzyme

Immobilized enzyme could be stored at room temperature during 3 days without any significant loss of activity (not shown), indicating that neither the catalytic action nor the cellulose-attachment of the enzyme were compromised under these conditions. The recyclability of the immobilized enzyme after consecutive incubations with 5 % lactose at 75 °C was tested (Fig. 4). After 2 hours of incubation, around 70 % of the activity was recovered in the second cycle. A slighter but progressive decay of activity was observed during the following cycles, reaching a total loss of 65-70 % after 8 rounds. Such activity decline could be due to inactivation of the β -galactosidase module (TmLac) or enzyme release from the support due to instability of the carbohydrate binding module (CBM2). Therefore, we assayed the strength of the

CBM2-mediated immobilization method. To this end, HBC-bound enzyme was subjected to incubation with 5 % lactose at 75 °C for 3 hours, and the soluble fraction was separated afterwards to analyze whether a significant fraction of TmLac-CBM2 was released from the cellulose matrix. This was initially calculated by determining the β -galactosidase activity of the concentrated sample (R) in relation to the expected activity if 100 % of the enzyme had been released. According to this assay, only 3.6 % of the bound TmLac-CBM2 was detached from the HBC support. To confirm this result, the R sample was also analyzed by SDS-PAGE (Fig. 5). Two dilutions of free TmLac-CBM2 were used as standards, at concentrations of 0.04 mg/mL and 0.14 mg/mL, corresponding to the expected amounts of enzyme in the concentrated supernatant if 3.5 % or 12 % of the enzyme had been released, respectively. The result in Fig. 5 supports again that only a minor fraction (around 3.5 %) of the TmLac-CBM2 hybrid bound to the HBC support is dissociated after 3 hours incubation at 75 °C. This suggests that the activity decay observed for reused immobilized enzyme (Fig. 4) is mainly due to the instability of the β -galactosidase module.

4. Conclusions

This study shows that the fusion of a thermostable CBM2 module to an enzyme (the β -galactosidase TmLac) allows direct immobilization onto a cellulosic substrate with high specificity. Binding efficiency of the hybrid TmLac-CBM2 was similar with hydrated (HBC) or dried (DBC) bacterial cellulose, showing adsorption capacities up to 18.5 mg of enzyme/g of cellulose. Initial activity of the HBC-bound β -galactosidase was higher than that of the DBC-bound enzyme, but still lower than the free protein. However,

the efficiency of lactose hydrolysis to a higher extent was similar for the three forms. Enzyme reuse was restrained by the instability of the β -galactosidase module whereas CBM2 attachment to cellulose was stable even at high temperatures. This study represents a first approach to develop new enzymatically active carbohydrate-based materials with industrial applications not only under mesophilic conditions but also in processes requiring high temperature.

E-supplementary data of this work can be found in online version of the paper

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Table 1. Binding efficiencies of different cellulose types

Cellulose type	Enzyme concentration (mg/mL)	% Binding	mg enzyme/g cellulose
DBC	0.3	80 ± 10	3.6 ± 0.4
	1.6	78 ± 3	18.4 ± 1.1
HBC	0.06	92 ± 3	0.83 ± 0.03
	0.3	81.8 ± 1.6	3.6 ± 0.2
	0.7	82 ± 2	9.2 ± 0.2
	1.6	81 ± 4	18.5 ± 1.3

Table 2. Initial rate of reaction of free and immobilized enzymes with lactose as substrate.

Enzyme	Enzyme amount (mg)	Enzymatic activity ($\mu\text{mol glucose}\cdot\text{min}^{-1}\cdot\text{mg enzyme}^{-1}$) *
Free	$0.08 \pm 0,01$	$63, 2 \pm 1,5^a$ (100 %)
HBC-Bound	$0.11 \pm 0,03$	33 ± 7^b (52 %)
DBC-Bound	$0.15 \pm 0,01$	17 ± 2^c (27 %)

a, b, c represent homogenous groups without significant differences ($\alpha < 0,05$); * relative values compared to the free form in parenthesis

Figure Captions:

Fig. 1. Effect of pH (6.5 and 8.5) on the binding efficiency of wild type (TmLac) or hybrid (TmLac-CBM2) enzymes (0.05 mg enzyme/mL) to lyophilized bacterial cellulose membrane. Error bars represent standard deviation of triplicates.

Fig. 2. Micrographs of the DBC membranes bound to the wild type (A, B) and hybrid (C, D) enzymes, previously labelled with FITC (B, D) or not (A, C). Images were taken under fluorescent (left panels) or bright field (right panels). Scale marks correspond to 200 microns.

Fig. 3. Kinetics of lactose hydrolysis by free and immobilized TmLac-CBM2. Free, DBC-bound or HBC-bound enzymes were incubated with 5 % lactose at 75 °C in a final volume of 10 mL assay buffer using the enzyme amounts indicated in Table 2. Glucose released at different time intervals was quantified. Error bars represent standard deviation of triplicates.

Fig. 4. Analysis of lactase activity of HBC-bound or DBC-bound enzyme after serial incubations with 5% lactose at 75 °C. Glucose released after each 2-hours cycle was quantified, membranes were washed with buffer and subsequently used in the next cycle. Error bars represent standard deviation of triplicates.

Fig. 5. Electrophoretic analysis of enzyme release from HBC membranes. HBC-bound enzyme was incubated at 75 °C for 3 hours with 5 % lactose in assay buffer. Afterwards, the 100-fold concentrated soluble fraction (R) was analyzed in parallel to two TmLac-CBM2 samples at 0.04 mg/mL (S1) and 0.14 mg/mL (S2) and a molecular weight marker (M). The migration of the TmLac-CBM2 hybrid is shown with an arrow. The volume of sample added in each well is indicated on top.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplementary material

Development of enzymatically-active bacterial cellulose membranes through immobilization of an engineered thermostable β -galactosidase

Figure S1. Comparative analysis of thermal stability and intrinsic activity of TmLac and TmLac-CBM2. A) SDS-PAGE analysis was carried out using soluble crude extracts from *E. coli* strains expressing TmLac or TmLac-CBM2, before (C) and after (H) heat treatment at 85 °C for 5 minutes. The electrophoretic mobility of molecular weight standards is indicated on the left. The position of TmLac and TmLac-CBM2 is shown with an asterisk. B) Intrinsic activity with PNPGal as substrate was determined for TmLac and TmLac-CBM2, purified as described in Marin-Navarro et al. 2014.

Figure S2. SEM image of the freeze-dried bacterial cellulose nanofibrous membrane.

A)

B)

Figure S3. SEM images of bacterial cellulose membranes after binding experiment at (A) pH 6.5 and (B) pH 8.5

Figure S4. Size distribution plots of TmLac-CBM2 at two different concentrations: 0.3 mg/mL (solid line) and 1.3 mg/mL (dotted line).