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Immobilization of β-Galactosidases from *Lactobacillus* on Chitin Using a Chitin-Binding Domain

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Abstract

Two β-galactosidases from *Lactobacillus*, including a heterodimeric LacLM type enzyme from *Lactobacillus reuteri* L103 and a homodimeric LacZ type β-galactosidase from *Lactobacillus bulgaricus* DSM 20081, were studied for immobilization on chitin using a carbohydrate-binding domain (chitin-binding domain, ChBD) from a chitinolytic enzyme. Three recombinant enzymes, namely, LacLM-ChBD, ChBD-LacLM, and LacZ-ChBD, were constructed and successfully expressed in *Lactobacillus plantarum* WCFS1. Depending on the structure of the enzymes, either homodimeric or heterodimeric, as well as the positioning of the chitin-binding domain in relation to the catalytic domains, that is, upstream or downstream of the main protein, the expression in the host strain and the immobilization on chitin beads were different. Most constructs showed a high specificity for the chitin in immobilization studies; thus, a one-step immobilizing procedure could be performed to achieve up to 100% yield of immobilization without the requirement of prior purification of the enzyme. The immobilized-on-chitin enzymes were shown to be more stable than the corresponding native enzymes; especially the immobilized LacZ from *L. bulgaricus* DSM20081 could retain 50% of its activity when incubated at 37 °C for 48 days. Furthermore, the immobilized enzymes could be recycled for conversion up to eight times with the converting ability maintained at 80%. These results show the high potential for application of these immobilized enzymes in lactose conversion on an industrial scale.

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Notes
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Keywords

β-galactosidase; Lactobacillus; immobilization; chitin-binding domain

Introduction

β-Galactosidases (β-gals) (lactases, EC 3.2.1.23) are known as important enzymes for applications in the dairy industry,1 where they are used for lactose hydrolysis to produce low-lactose or lactose-free products as a response to lactose intolerance of consumers, which affects approximately 70% of the world population.2 Another useful property of β-gals is their transgalactosylation activity, by which health-promoting prebiotic galacto-oligosaccharides (GOS) can be formed from lactose.1,3 Many studies have demonstrated the indirect health benefits of GOS, where GOS promote growth and activity of beneficial intestinal microbes in the host.4–6

It is conceivable that β-gals from probiotic strains produce GOS that are also specific for these probiotics,7 and therefore β-gals from several probiotic *Bifidobacterium* spp.8,9 and prominently *Lactobacillus* spp. such as *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus pentosus*, or *Lactobacillus bulgaricus* have been studied in relation to GOS production during the past decade.10–14 β-Galactosidases from *Lactobacillus* species are reported to be of two main types, consisting of either two different subunits (heterodimeric or LacLM type) or two identical subunits (homodimeric or LacZ type).15,16 Two well-studied examples are the LacLM β-galactosidase from *L. reuteri* L10314 and the LacZ β-galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) DSM 20081,16 both belonging to glycoside hydrolase family GH2.

The recombinant β-gals from *L. reuteri* L103 and *L. bulgaricus* DSM20081 were successfully developed and overexpressed in food grade *Lactobacillus* hosts,16–18 yielding remarkably high levels of activity, for example, ca. 23−53 kU per liter of fermentation broth, respectively.16,17 The lactose conversion toward GOS of both native and recombinant β-gals from *L. reuteri* L103 and *L. bulgaricus* DSM 20081 were studied in detail in both batch and continuous bioreactors.14,16,19–21 Maximum GOS yields achieved were approximately 38% of total sugars (at a lactose conversion rate of 80% and an initial lactose concentration of ~200 g/L) using LacLM from *L. reuteri* L103,20 or 50% GOS for recombinant LacZ from *L. bulgaricus* DSM 20081 (at 90–95% of lactose conversion and an initial lactose concentration of ~200 g/L).16 These GOS mixtures contained mainly (nonlactose) disaccharides, trisaccharides, and tetrasaccharides, in which the transferred galactosyl moiety is attached via β-1,3- and β-1,6-linkages. These structures are important for potential prebiotics.20

To study the application of these enzymes in more detail, we aimed at immobilization of these two β-gals. To date, there have been no efforts on immobilization of β-gal from *L. reuteri* L103, even though β-gals are well-studied enzymes in terms of immobilization.22 Immobilization will enable the reuse of these biocatalysts, and it might also contribute to stabilization of the two β-gals, because their thermostability could be a limiting factor for their application.23

The chitin-binding domain (ChBD) is part of some chitin- or chitosan-degrading enzymes, chitinases or chitosanases, where it is responsible for the tight binding of the enzyme onto the substrate, thus increasing the hydrolytic activity.24,25 The ChBD of *Bacillus circulans* WL-12 chitinase A1 is one of the most well studied carbohydrate-binding domains. It belongs to the carbohydrate-binding module family 12 of the CAZY (carbohydrate-active enzyme) database. The three-dimensional structure of this ChBD has been determined by NMR.26 It consists of 45 amino acids, Ala655–Gln699, including several hydrophobic and aromatic residues with low solvent accessibility, thus forming a rigid and compact structure. 26 Two β-sheets are composed of five strands including Thr660–Tyr662, Gln666–Tyr670, Lys673–Cys677, His681–Ser683, and Trp696–Leu698 residues.26 This ChBD has been reported to bind to only insoluble or crystalline chitin but not to the chitooligosaccharide, soluble derivatives of chitin, or other polysaccharides.27 On the basis of the high affinity to chitin, ChBD from WL-12 has been successfully applied for immobilization of several enzymes such as D-hydantoinase28 or levansucrase29 on chitin material. The procedure for the simultaneous purification and immobilization was a simple mixing of the enzyme solution with the insoluble chitin without the requirement of purified enzyme.28,29 Moreover, this method can overcome the disadvantages of other immobilization methods. For example, the covalent binding method immobilization required the harsh condition to create the bond between the enzyme molecule to the support material.23 The absorption method is based on weak forces such as van der Waals, hydrophobic interaction, or hydrogen interaction; thus, the immobilized enzymes are loosely bound to support materials. 30

This study focuses on the immobilization of heterodimeric *L. reuteri* L103 LacLM and homodimeric *L. bulgaricus* DSM20081 LacZ β-gal on chitin using ChBD of *B. circulans* WL-12 chitinase A1. The recombinant enzymes were fused to the ChBD via DNA-based molecular methods. The biochemical characteristics of these fusion enzymes in comparison to the native enzymes are shown; thus, the effects of the ChBD and the immobilization on the properties of these enzymes are elucidated.

Materials and Methods

Chemicals and Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated and were of the highest quality available. The endonucleases were purchased from New England BioLabs (Ipswich, MA, USA) and used as recommended by the supplier. T4 DNA ligase was from Fermentas (Vilnius, Lithuania).

Bacterial Strains and Media

The bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *L. plantarum* WCFS131 was grown in MRS medium (Oxoid, Basingstoke, UK) at 37 °C without agitation. *Escherichia coli* NEB5 α (New England BioLabs, Ipswich, MA, USA) as cloning host was cultivated in Luria-Bertani (LB) medium at 37 °C with shaking at 200 rpm. Agar media were prepared by adding 1.5% agar to the respective media. Unless otherwise

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stated, the antibiotic concentrations were 5 or 200 μg/mL of erythromycin (Erm) for *Lactobacillus* or *E. coli*, respectively, and 100 μg/mL of ampicillin for *E. coli*.

Oligonucleotide primers for PCR amplification were supplied by VBC-Biotech Service GmbH (Vienna, Austria). The appropriate endonuclease restriction sites were introduced in the forward and reverse primers (Table 2).

Plasmid Construction and Transformation

The DNA amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). Plasmid DNA from *E. coli* was purified using the Gene Elute plasmid Miniprep kit (Sigma, St. Louis, MO, USA). DNA was purified with the Wizard^{R}SV Gel and PCR Clean-up system kit (Promega, Madison, WI, USA). The pJET1.2 plasmid (CloneJET PCR cloning kit, Fermentas) was used for subcloning when necessary.

Three recombinant fusion proteins were constructed. Two are based on LacLM from *L. reuteri* L103, and the chitin-binding domain ChBD was attached upstream of LacL (termed ChBD-LacLM) or downstream of LacM (termed LacLM-ChBD). The third fusion protein was based on LacZ of *L. bulgaricus* DSM20081 with ChBD linked downstream of LacZ (termed LacZ-ChBD).

For construction of the expression plasmids of the fusion protein LacLM-ChBD or LacZ-ChBD, the fragment of *chbd* of *B. circulans* WL-12 chitinase A1 was amplified from the plasmid pTxB1 (New England BioLabs) with the primer pair F1 and R1 (Table 2). After double digestion with *Xho*I and *Acc65*I, this fragment was ligated to the fragment of pSIP403,32 which contained *gusA* as the original reporter gene and was treated by the same endonucleases, resulting in plasmid pSCBD-gusA (Table 1; Figure 1). The *gusA* gene was then excised by double digestion with *Nco*I and *Xho*I to obtain the empty vector pSCBD. This empty vector was ligated to the *Nco*I–*Xho*I fragment of *lacLM* (encoding LacLM from *L. reuteri* L103) from pHA103.1,15 resulting in pSCBDlac1 (Table 1; Figure 1). Similarly, *lacZ* was amplified from pJETlacZ16 by PCR with the two primers F4 and R4 (Table 2) and then digested by *Bsm*BI and *Xho*I prior to ligation into empty pSCBD, resulting in pSCBDlac3 (Table 1; Figure 1).

For construction of the expression plasmid for ChBD-LacLM, *chbd* from pTxB1 was amplified with the two primers F2 and R2, resulting in a fragment of F2-*chbd*-R2. The *lacLM* gene was PCR-amplified from the *lacLM* template in pTH103.1 using the F3 and R3 primers, resulting in the fragment F3-*lacLM*-R3. Because of the 18 nt complementary sequence between F3 and R2 (Table 2), the fragment F2-*chbd*-R2 could be used as a *mega* forward primer in combination with R3 as reverse primer to amplify the F3-*lacLM*-R3 template, resulting in the fragment F2-*chbd*-*lacLM*-R3. The PCR product from this amplification was digested by *Bsa*I and *Xho*I and ligated to *Nco*I–*Xho*I-digested pSIP403, resulting in pSCBDlac2 (Table 1; Figure 1).

This strategy resulted in expression vectors, in which the target gene, *lacLM* or *lacZ* linked to the *chbd* sequence, is controlled by the inducible promoter P_{sppA}, similar to *gusA* in the original pSIP403 vector.32 These plasmids were then electroporated into competent cells of

L. plantarum WCFS1 as previously described.33 The transformants carrying the plasmids were determined and screened by using a colony PCR amplification of the target fusion genes.

Evaluation of the Expression of Recombinant β-Galactosidases

This experiment was performed following a previous method.18

Fermentation

To obtain sufficient amounts of the enzymes for characterization, immobilization, and application, *L. plantarum* WCFS1 harboring different plasmids was cultivated in 1 L of medium to obtain larger amounts of the recombinant enzymes. The bacterial cells were induced at $OD_{600\ nm}$ ~ 0.3 and harvested at $OD_{600\ nm}$ ~ 6. The cell pellets were disrupted by homogenization with a French press (Aminco, Silver Spring, MD, USA). The cell-free extract, clarified by ultracentrifugation (Beckman, USA) at 30000*g* and 4 °C for 20 min, was used either directly for immobilization on chitin beads (New England BioLabs) or for a single-step affinity purification using the *p*-aminobenzyl 1-thio-β-D-galactopyranoside (ABTG) resin (Sigma) as described in a previous study.14

Immobilization on Chitin Beads

Before use, the chitin beads were washed three times with sodium phosphate buffer (50 mM, pH 6.5) and resuspended in the same buffer. Five hundred microliters of chitin bead suspension was mixed with the same volume of diluted cell-free crude extracts of 1000 U/mL of *o*NPG activity. The immobilization experiments were carried out at 4 °C with gentle agitation for 1–18 h. Chitin beads were separated from the supernatant by filtration and rinsed with sodium phosphate buffer. The supernatant and wash solutions were collected and pooled for SDS-PAGE analysis and protein measurement. Chitin beads were resuspended in buffer for further studies.

The immobilization yield (IY) and the activity retention (AR) were calculated according to the methods of Klein et al.34 IY (%) is defined as the ratio of immobilized activity in relation to total applied activity, in which the immobilized activity was determined by subtraction of the residual activity in the supernatant after immobilization from the total applied activity. AR (%) is the percentage of activity measured on chitin beads in relation to the theoretical immobilized activity.

The amount of chitin-bound protein was indirectly estimated by subtraction of the protein concentration in the supernatant after immobilization from that in the crude extract (prior to the immobilization).

Influence of pH and Temperature on the Activity and Stability of Immobilized Enzymes

The pH optimum of β-gal activity was evaluated in Britton–Robinson buffer17 with the pH ranging from 4 and 9. To determine the pH stability, the immobilized enzymes were incubated at 30 or 37 °C in Britton-Robinson buffer of different pH values. At various time intervals, the residual activities were measured with the *o*NPG assay. The inactivation

constants k_{in} were obtained by linear regression of ln(residual activity) versus time. The half-life values $\tau_{1/2}$ were calculated using $\tau_{1/2} = \ln(2)/k_{in}$.35

The optimum temperature for hydrolysis activity of β-gals with both substrates lactose and *o*NPG was determined in the range of 20–80 °C. For estimation of the kinetic thermostability, enzymes were incubated in 50 mM sodium phosphate buffer, pH 6.5, at different temperatures ranging from 20 to 80 °C. At various time intervals, residual activities were measured with *o*NPG as substrate and plotted versus the incubation time. The half-life values of thermal inactivation ($\tau_{1/2}$) were similarly calculated as above.

Steady-State Kinetic Measurements

To estimate the kinetic parameters of recombinant and fusion β-gals for lactose and *o*NPG, substrate concentrations were varied from 1 to 25 mM for *o*NPG and from 10 to 600 mM for lactose.14 The enzyme assays were performed for 10 min at 30 °C in 50 mM sodium phosphate buffer, pH 6.5. The kinetic parameters were calculated by using the Henri–Michaelis–Menten model and nonlinear least-squares regression.

Reusability of the Immobilized Enzymes

To assess the possibility of recycling the immobilized enzyme preparations for conversion, immobilized β-gal LacLM *L. reuteri* L103 was added to sodium phosphate buffer (50 mM, pH 6.5) containing 600 mM lactose and maintained at 30 °C and 600 rpm agitation for the conversion of lactose. Every 24 h, the chitin beads carrying the enzyme were filtered off, rinsed with buffer, and reused in another batch conversion experiment under identical conditions. The filtrates from every conversion cycle were collected to determine the glucose concentration by using a commercial D-glucose assay kit (GOPOD Format, Megazyme, Wicklow, Ireland). The glucose concentration in the filtrate from the first conversion was taken as 100% of converting ability of the immobilized enzyme preparation.

Immobilized LacZ from *L. bulgaricus* DSM 20081 was also tested for its reusability but using the substrate *o*NPG. After 5 min of incubation with *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) at 30 °C, chitin beads carrying LacZ were separated from the liquid phase and reused in the next conversion experiments. The concentration of *o*NPG in the liquid phases after the conversion was measured with a spectrophotometer at 420 nm and used to calculate the residual converting ability of enzyme in each repeating conversion.

Lactose Hydrolysis and Transgalactosylation Experiments

The transformation of lactose was carried out in batch mode. Both chitin-immobilized I-LacLM-ChBD and purified soluble LacLM-ChBD were added at equal concentrations of 1.5 $U_{lactose}$/mL to sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$ and 205 g/L lactose. Lactose conversion experiments were performed at 30 °C for 24 h at 600 rpm of agitation.

The chitin-immobilized β-gal of *L. bulgaricus* was applied in different amounts ranging from 1.7 to 9.7 $U_{lactose}$/mL to sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$ and 50 or 205 g/L lactose. This enzyme preparation was also used for conversion of

lactose in ultrahigh-temperature-treated whole cow's milk. The incubation temperature was varied from 37 to 60 °C.

In all conversion experiments, samples were periodically withdrawn, heated at 99 °C for 5 min, and further analyzed for lactose, galactose, glucose, and GOS present in the samples. Qualitative analysis of sugar was performed by thin-layer chromatography (TLC) as described previously by Nguyen and co-workers.14 Furthermore, samples from conversion experiments were quantitatively analyzed by high-performance liquid chromatography (HPLC) (Dionex, Germany) equipped with a refractive index detector and an Aminex HPX-87K (300 mm × 7.8 mm) carbohydrate analysis column (Bio-Rad, Hercules, CA, USA). The chromatographic separation was performed at 80 °C with ultrapure water used as eluting solvent at a flow rate of 0.5 mL/min. The concentration of saccharides was calculated by interpolation from external standards. Total GOS concentration was calculated by subtraction of the quantified saccharides (lactose, glucose, galactose) from the initial lactose concentration. The GOS yield (%) was defined as the percentage of GOS produced in the samples compared to initial lactose.

β-Galactosidase Assay

β-Galactosidase activity was determined using *o*NPG or lactose as substrate following the method of Nguyen et al.14 In brief, the activity assays were performed in 50 mM sodium phosphate buffer of pH 6.5 at 30 °C, and the final substrate concentrations in the 10 min assays were 22 mM for *o*NPG and 600 mM for lactose. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 μmol of *o*NP per minute, whereas 1 unit of lactase activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per minute under the given conditions.

Protein Measurement

Protein concentration was determined by using the method of Bradford36 with bovine serum albumin (BSA; Sigma) as standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

For visual observation of the expression level of the recombinant β-gals in *L. plantarum* WCFS1 and the effectiveness of the immobilization, cell-free extracts, liquid phases, and chitin beads (after immobilization) were analyzed by SDS-PAGE using 3-(*N*-morpholino)propanesulfonic acid (MOPS) as running buffer.16

Statistical Analysis

All experiments and measurements were performed at least in duplicate, and the data are given as the mean ± standard deviation when appropriate. Student's *t* test was used for the comparison of data with significance value $\alpha = 0.05$.

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Results and Discussion

Plasmid Construction and Expressions of Recombinant β-Galactosidases in *L. plantarum* WCFS1

In this work, the three expression plasmids pSCBDlac1, pSCBDlac2, and pSCBDlac3, containing the sequences of the three recombinant fusion proteins LacLM-ChBD, ChBD-LacLM, and LacZ-ChBD, respectively, were constructed and successfully electroporated into *L. plantarum* WCFS1. The transcription of the encoding sequences *lacLM-chbd*, *chbd-lacLM*, and *lacZ-chbd* is regulated by the inducible promoter P_{sppA} in these plasmids (Figure 1). The expression levels of the different constructs and the recombinant β-galactosidases in *L. plantarum* WCFS1 were investigated.

Induced cells formed intracellular LacLM-ChBD at around 32 U/mL of fermentation broth with a specific activity of ca. 179 U/mg protein (Table 3). ChBD-LacLM was expressed in 3-fold lower yields ($p < 0.05$), giving ca. 11 U of β-galactosidase activities per milliliter of fermentation broth with a specific activity of 54 U/mg. The basal expression from the expression plasmids in noninduced cells was unexpectedly high (7.35 and 4.06 U/mg for LacLM-ChBD and ChBD-LacLM, respectively (Table 3)). The induction ratios (ratio of the β-galactosidase activities obtained under induced conditions divided by the activity under noninduced conditions in cells harvested at similar OD_{600} values of 1.8) were 24 and 14, respectively, which is lower than previously reported for different target genes expressed with the same expression vector pSIP403.16,17 It should be noted that the activity level produced by the wildtype host strain under identical growth conditions is very low (0.07 U/mg),15 and hence the activities measured can be attributed solely to the recombinant enzymes.

The expression of LacZ-ChBD in *L. plantarum* WCFS1 gave a higher volumetric activity (around 65.83 U/mL) but lower specific activity (40.27 U/mg) when compared to the expression of LacLM-ChBD ($p < 0.05$). Again, a rather high basal activity was observed in noninduced cultivation (16.77 U/mL and 9.43 U/mg), even though P_{sppA} is known as a tightly controlled promoter.32,37 As a consequence, a low induction factor of around 4 was obtained with this system (Table 3).

The fusion of ChBD had significant effects on expression levels of the recombinant enzymes. This is obvious from, for example, LacZ, as expression of the *lacZ* gene without fusion to the *chbd* fragment gave much higher expression (i.e., 180-190 U/mg) with the same host, expression system, and induction conditions.16 Moreover, the expression levels of the recombinant proteins are remarkably different depending on the position of ChBD when fused to LacLM. The lower expression levels were observed when *chbd* was fused upstream of *lacL* (ChBD binding N-terminally to the large subunit LacL) (Table 3). It is shown that the nucleotide sequences at the beginning of a gene can change the stability of the secondary structure of the transcribed mRNA and thus affect the translation process.38 The secondary structures of the *chbd-lacLM* mRNA and *lacLM-chbd* mRNA are predicted by the Mfold tool (http://unafold.rna.albany.edu/?q=mfold) (data not shown). With *chbd-lacLM*, a hairpin loop including the ribosome-binding site RBS (AGGAG) is predicted in the region of the 5'UTR and the initial nucleotides of the *chbd*. This hairpin loop might prevent

the binding of ribosome to the mRNA to initiate the translation process. Meanwhile, with *lacLM-chbd*, the RBS is not involved in the hairpin loop; hence, it might be an explanation for the higher expression level of the LacLM-ChBD fusion protein mentioned above.

SDS-PAGE analysis of cell-free extracts of *L. plantarum* WCFS1 harboring the different plasmids clearly indicates strong bands for the recombinant proteins at approximately 72 and 41 kDa (lane 2, Figure 2A) for LacLM-ChBD, 78 and 35 kDa (lane 4, Figure 2A) for ChBD-LacLM, and ~120 kDa (lane 2, Figure 2C) for LacZ-ChBD. These sizes are in agreement with those of LacL (72 kDa) and LacM (35 kDa) from *L. reuteri* L10314,15 and lacZ from *L. bulgaricus* DSM20081 (115 kDa),16 and ChBD (~6 kDa).

The deduced amino acid sequences of the three fusion proteins were used to predict their 3D structures using the RaptorX tool (http://raptorx.uchicago.edu/). The prediction shows that the ChBD domain arranges well separated from the main protein LacM (in the case of LacLM-ChBD) and LacZ (in the case of LacZ-ChBD), but not with LacL (for ChBD-LacLM), where it interacts more tightly with the LacL subunit (see Figure 10). It should be noted that with LacZ-ChBD, the ChBD fold was not predicted accurately, as it was not in agreement with the structure of ChBD alone (see Figure 10D) or in LacM-ChBD (see Figure 10A); nevertheless, it was predicted to be separately positioned from the main protein (Figure 10C). It should be noted that the *chbd* from pTxB1 encodes for ChBD from chitinase A1 of *B. circulans* WL-12 with 45 amino acids, from Ala655 to Gln699, as previously reported26 and 7 extra residues at the N-terminus. Moreover, 5 residues upstream of Thr660 are not involved in the essential structural region of ChBD.26 The sequence of 12 residues might be a flexible linkage between LacM or LacZ to ChBD. Meanwhile, for ChBD-LacLM, the linkage is only the last Gln699 of ChBD, which is not involved in forming the β-sheet.26 These predicted structures indicate that in both LacLM-ChBD and LacZ-ChBD, the interaction of ChBD with chitin beads will affect the main protein to a lesser extent than in ChBD-LacLM. Even though these structures need to be confirmed by crystallization/structure elucidation, they are in agreement with our further results in this study (see below).

Immobilization of β-Galactosidases on Chitin Beads

The three different fusion proteins were used at equal activities of 500 U_{oNPG} to study their immobilization on chitin beads (11 mg of dry weight), and the residual activities in the liquid phase after the immobilization reaction as well as the bound activities were subsequently analyzed for all three constructs (Table 4). An important parameter in immobilization is the immobilization yield (IY) as it is an indication of how much of the applied protein is actually bound to the carrier. Immobilization yields for both LacLM-ChBD and LacZ-ChBD were very high, at >91%, and especially the IY of LacLM- ChBD, which was close to 100% (0.67% of residual activity detected in the supernatant after immobilization), is very promising. In contrast, the IY of ChBD-LacLM was much lower, at ca. 52% (Table 4). These values are further corroborated by SDS-PAGE analysis using samples of the liquid phases and chitin beads after immobilization (Figures 2B and 2C). The bands of ChBD-LacL and lacM or LacZ-ChBD can be seen in the lanes of liquid phase samples (lane 5 in Figure 2B; lane 3, 4, and 5 in Figure 2C). The patterns of chitin bead

samples, with only two bands corresponding to the two subunits of immobilized LacLM (LacL and LacM-ChBD in lane 3 and ChBD-LacL and LacM in lane 6, Figure 2B) or the single strong band of LacZ-ChBD (Figure 2C, lane 6) with only a minor very faint band indicating an impurity, indicate the high specific affinity of ChBD to chitin.25 This observation is in accordance with many other studies using ChBD for enzyme immobilization.28,29,39 LacLM from *L. reuteri* L103 is a heterodimer consisting of two subunits. Fusing the ChBD fragment N-terminally to LacL or C-terminally to LacM clearly had different effects on the immobilization of the recombinant fusion enzymes on chitin beads. A possible explanation for these results could be that in the ChBD-LacLM fusion the ChBD is positioned in such a way that it cannot interact with the chitin beads unperturbed. This is in agreement with the predicted structures of these fusion proteins (see Figure 10). LacZ from *L. bulgaricus* DSM20081 is a homodimer,16 and therefore each of the identical subunits will carry its own ChBD. These two chitin-binding domains per fusion protein might interfere with each other when binding on chitin, which could be the reason for the slightly lower efficiency in immobilization compared to LacLM-ChBD (Table 4).

Even though the immobilization yields were very high for two of the constructs (Table 4), the activity retention (AR) on chitin beads was much lower than expected. The residual activities on chitin beads for ChBD-LacLM and LacLM-ChBD were ~9.19 and 25.89% in comparison with the initially applied activity, respectively. This amounted to AR values of 19–25%. A low AR value of ca. 13.7% was also observed for LacZ-ChBD. However, AR depends on the applied activity of our enzymes, because applying lower activities resulted in higher AR values (data not shown). To date, various methods have been used for immobilization of fungal or bacterial β-gals, yielding different ARs.22,40 For instance, a range of 2.5–9.5% of ARs of β-galactosidase from *E. coli*, which was immobilized on poly(2-hydroxyethyl methacrylate) membrane using entrapment, was reported by Baran and co-workers.41 However, most of these immobilization methods are based on purified enzymes, whereas in this study a single-step procedure was performed for the immobilization.

Partial Characterization of Immobilized β-Galactosidases

The effect of ChBD and of the immobilization on the characteristics of the β-gals was further assessed. Three chitin-immobilized enzymes of LacLM-ChBD, ChBD-LacLM, and LacZ-ChBD are termed I-LacLM-ChBD, I-ChBD-LacLM, and I-LacZ-ChBD, respectively, hereafter. Free LacLM-ChBD was also purified and partially characterized to compare with the immobilized I-LacLM-ChBD.

Steady-State Kinetic Constants

Table 5 presents the kinetic constants of the recombinant fusion β-gals for their two substrates, lactose and *o*NPG. For I-LacLM-ChBD, the v_{max} values for both substrates are higher than those of I-ChBD-LacLM ($p < 0.05$). With 1.74 and 0.72 μmol min^{-1} mg^{-1} these v_{max} values for the natural substrate lactose are much lower than the corresponding value of the free fusion protein (17.8 μmol min^{-1} mg^{-1}), as well as LacLM isolated from its natural source *L. reuteri* L103,14 or expressed recombinantly either in *E. coli*15 or in *L. plantarum* WCFS17 with v_{max} values ranging from 34 to 43 μmol min^{-1} mg^{-1} (Table 5). The Michaelis

constant K_m was also negatively affected by the immobilization, but judging from the K_m values determined, this effect is much less dramatic than for v_{max}. This obviously indicates that the hydrolysis activity of the chitin-bound fusion enzymes is negatively influenced by immobilization. The effect of immobilization on kinetic parameters of I-LacZ-ChBD was also negative, but here the decrease in v_{max} and hence k_{cat} was less drastic (Table 5).

Effect of Temperature and pH on Enzyme Activity

The immobilized fusion enzymes typically showed their highest activity at 50–55 °C with both *o*NPG and lactose as substrate for the 10 min assays (Figure 3); this is also the optimal range of temperature observed with one of the counterparts, the soluble enzyme LacLM-ChBD (data not shown). At higher temperatures (>70 °C) the enzyme activity was rapidly lost. The range of optimal temperature for the activity of the immobilized enzymes is comparable to that of the free enzyme without ChBD from the native strain *L. reuteri* L103 or recombinant LacLM expressed in different hosts.14,15,17 Interestingly, the immobilized preparation I-LacZ-ChBD showed a slightly increased temperature optimum of 65 °C (Figure 3) compared to the free enzyme LacZ without the ChBD with an optimum of 60 °C. 16 We did not observe a similar increase for I-LacLM-ChBD and I-ChBD-LacLM, the reason for which might be that only one subunit is involved in binding to chitin when LacLM is used.

The thermostability of the different immobilized β-galactosidase preparations was tested at various temperatures in 50 mM sodium phosphate buffer, pH 6.5, by determination of the half-life times $\tau_{1/2}$ (Table 6). I-LacZ-ChBD proved to be the most stable preparation at all tested temperatures. For example, $\tau_{1/2}$ of this enzyme was 203 h at 37 °C, whereas it was <170 h for I-LacLM-ChBD and I-ChBD-LacLM at 30 °C. Free β-gal of *L. bulgaricus* was previously shown to be relatively thermostable ($\tau_{1/2}$ for 145 and 345 h without and with, respectively, the presence of 10 mM Mg^{2+} at 37 °C).16 Furthermore, the stability of the immobilized preparation I-LacZ-ChBD was further increased in the presence of 10 mM Mg^{2+} to a half-life time $\tau_{1/2}$ of almost 48 days (1155 h) at 37 °C (Table 7). The soluble fusion protein LacLM-ChBD was considerably less thermostable than its immobilized counterpart I-LacLM-ChBD ($\tau_{1/2}$ of 19 h versus 169 h at 30 °C; Table 6); thus, immobilization onto chitin beads via the chitin-binding domain can improve the kinetic stability of an enzyme significantly.

The immobilized enzyme preparations showed their optimum activity in the pH range of 6–6.5 for both substrates lactose and *o*NPG, with the exception of I-LacZ-ChBD and lactose having an optimal pH of 8 without clear reason (Figure 4). It is likely related to the isoelectric point (pH 9) of ChBD.27 When stored at different pH values, the enzymes were most stable in the pH range that also coincides with the pH optima, that is, 6–6.5 (Table 7). The binding of ChBD from *B. circulans* WL-12 chitinase to chitin was shown to be best at pH 9 (binding capacity of 90%) and less at pH 4 (binding capacity of 80%).27 However, LacLM from *L. reuteri* L103 and LacZ from *L. bulgaricus* were reported to have very low activity at pH 4,14,16 which was also observed with the immobilized enzymes in this study (Figure 4). This suggests that the effect of pH on the fusion enzymes is mainly on the catalytic activity rather than the binding of ChBD to chitin.

Reuse of Immobilized Enzymes

A study on the reusability of the immobilized β-gals, using subsequent hydrolysis steps with *o*NPG or lactose as substrate, was carried out. The residual converting ability of immobilized enzyme preparations was plotted versus the number of cycles of application (Figure 5). In this study I-LacLM-ChBD and I-LacZ-ChBD exhibited better stability, because these immobilized enzymes retained approximately 80% of their initial ability of substrate conversion after four to eight cycles. I-ChBD-LacLM showed a more rapid decrease of relative activity (Figure 5).

These results are comparable with previously reported data. Chiang et al. immobilized levansucrase on chitin using the *B. circulans* WL-12 ChBD, which retained approximately half of its initial activity at the end of the seventh cycle.29

Lactose Transformation

Figures 6 and 7 show a qualitative analysis by TLC of products formed during lactose conversion using different immobilized β-gal preparations under various conditions. In general, the profile of products shown is comparable to the profile of the corresponding free enzymes previously reported14,16 as well as to the profile of Vivinal GOS (Amersfoort, The Netherlands) used as “reference” (Figure 6). This indicates that similar GOS mixtures are formed by the free and immobilized enzymes.

As expected, a higher enzyme activity or higher temperatures led to faster conversion (Figure 6). The β-gal LacZ from *L. bulgaricus* was shown to be a thermostable enzyme,16 and therefore it catalyzed lactose conversion efficiently at the higher temperature of 50 °C. In this study, the chitin-immobilized LacZ again showed good thermostability, thus resulting in a comparable profile of products at 60 °C compared to 37 °C. Regardless of the conditions of the conversion, the maximal GOS yield was around 23–24% (Table 8; Figure 8), which is lower than the yield obtained with the free enzyme LacZ (approximately 50%) as reported by Nguyen and co-workers.16 A possible explanation could be that the immobilization reduces the contact between enzyme and lactose as well as the monosaccharide sugars for transgalactosylation, thus resulting in reduced GOS products. This may be also the reason for the rather high residual lactose concentration at the end of the conversion run (Table 8; Figure 6). This effect of immobilization was also observed by Sheu et al. when using chitosan-immobilized β-gal from *Aspergillus oryzae* for lactose conversion.42 The conversion of lactose in the samples of ~5% w/v, which is the concentration of lactose in milk, using immobilized recombinant β-gal I-LacZ-ChBD with a relatively high activity (9.7 $U_{lactose}$/mL) at a high temperature of 60 °C was fast, as 81% of lactose was converted after only 1 h (Table 8).

When used for batch conversion experiments of lactose, I-LacLM-ChBD and I-ChBD-LacLM also resulted in a profile of GOS products comparable to that of the native β-gal L103 (Figure 7). Lactose conversion was faster with the free enzyme of LacLM-ChBD compared to I-LacLM-ChBD at equal activity loading (Figure 9), which might be explained by limitations in diffusion of the substrate to the active site. A maximum GOS level of ca. 39–40% of total sugars was reached by both enzyme preparations, soluble and immobilized

(Figure 9), yet the time for reaching this maximum was significantly longer for I-LacLM-ChBD at 12 h (Figure 9B) compared to 8 h for LacLM-ChBD (Figure 9A). These maximum yields were obtained at a similar lactose conversion of ca. 85%. This indicates that the conversion of lactose and the yield of GOS obtained by the recombinant enzymes are comparable to the conversion catalyzed by their counterpart, the native enzyme LacLM *L. reuteri* L103.14

In conclusion, this work describes the immobilization of two lactobacillal β-galactosidases, a homodimeric β-galactosidase of the LacZ type and a heterodimeric β-galactosidase of the LacLM type, onto chitin via a chitin-binding domain. This could provide a promising and efficient approach for lactose hydrolysis and production of prebiotic galacto-oligosaccharides because the enzyme can be purified from the crude cell extract and immobilized in one simple step. The immobilized fusion enzyme can be stably reused for several cycles for lactose hydrolysis and transformation. Preliminary results from an ongoing investigation of lactose conversion in continuous mode using these immobilization enzymes also show a high potential for an industrial application of these immobilized enzymes.

Funding

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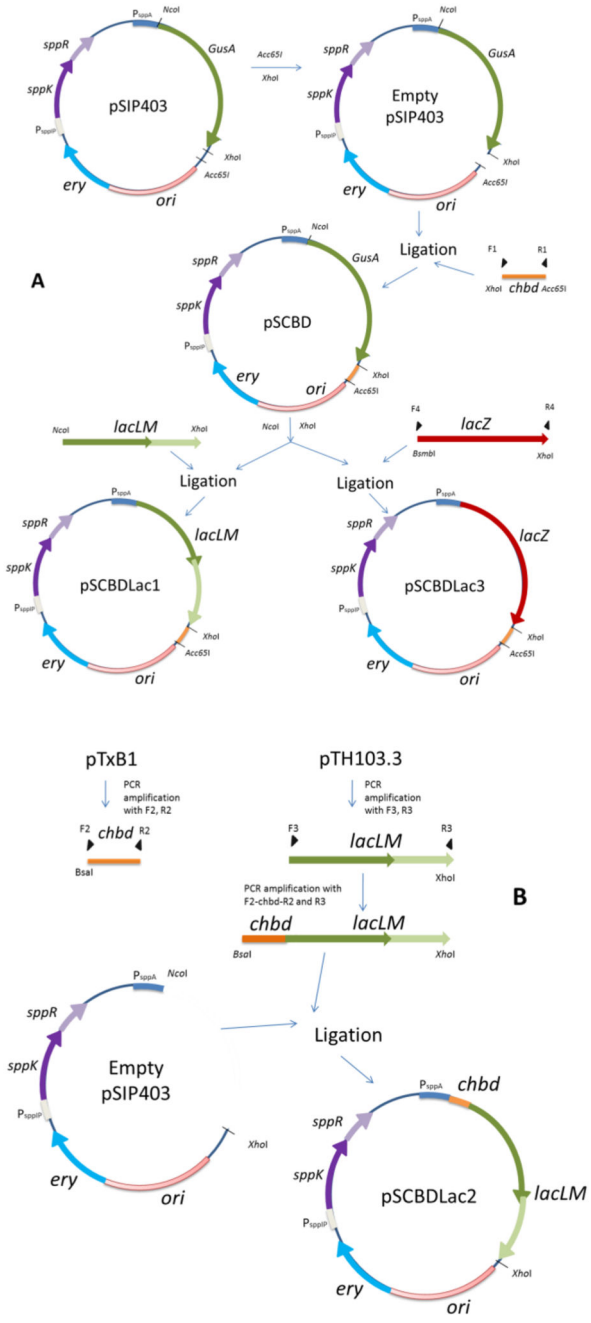

Figure 1.

Construction of expression plasmids: pSCBDlac1 (for LacLM-ChBD) and pSCBDlac3 (for LacZ-ChBD) (A) and pSCBDlac2 (for ChBD-LacLM) (B).

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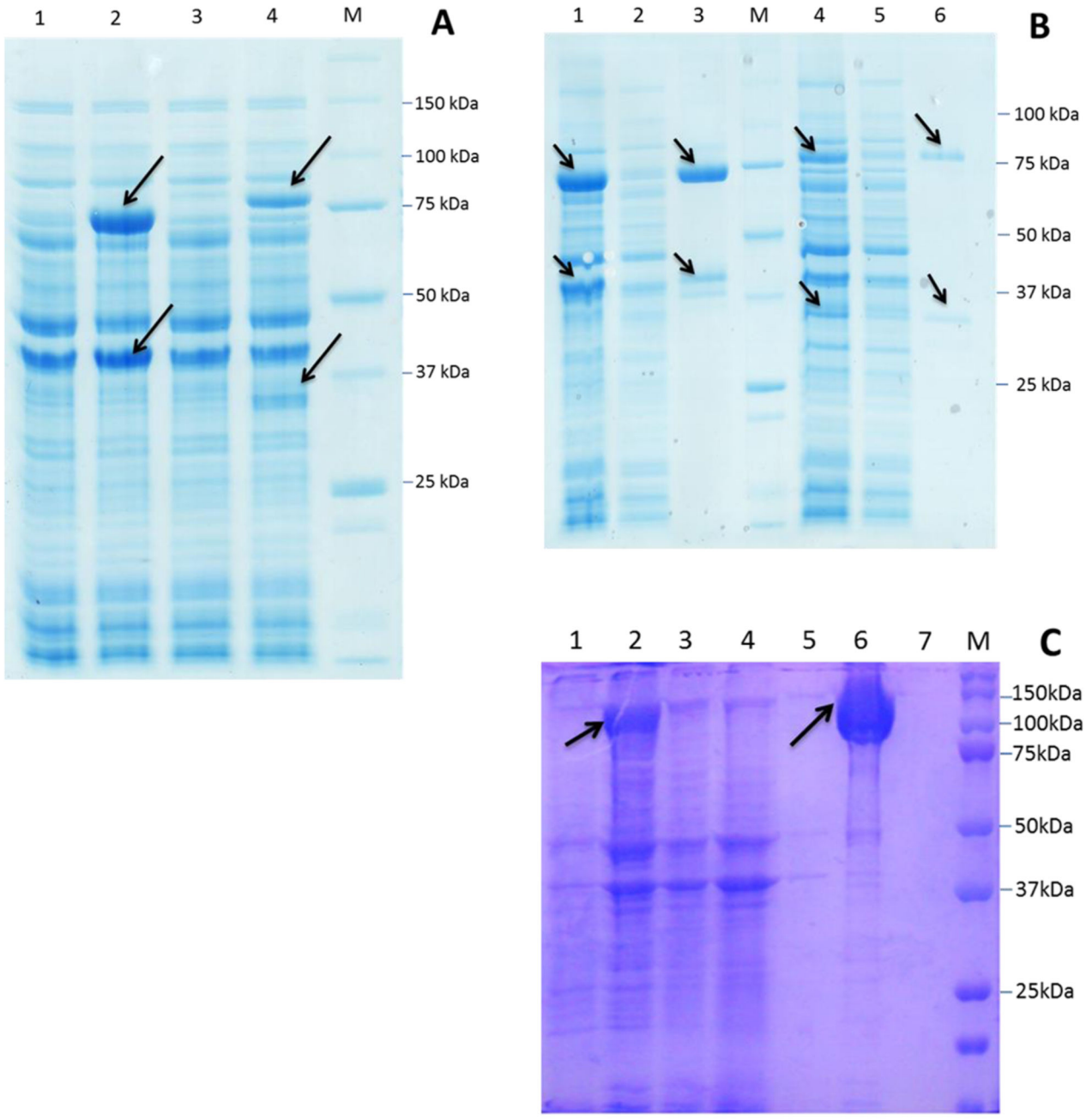

Figure 2.

SDS-PAGE analysis of expression and immobilization of recombinant enzymes. (A) Cell-free extracts of noninduced cells (lanes 1, 3) and induced cells (lanes 2, 4) of *L. plantarum* WCFS1 harboring pSCBlac1 (containing LacLM-ChBD) and pSCBlac2 (containing ChBD-LacLM), respectively. (B) Cell-free crude extracts (lanes 1, 4), flow through during immobilization (lanes 2, 5), and chitin-bound β-gals (lanes 3, 6) of *L. plantarum* harboring plasmids pSCBDlac1 (containing LacLM-ChBD) and pSCBDlac2 (containing ChBD-LacLM), respectively. (C) Cell-free crude extracts of *L. plantarum* WCFS1 harboring

pSCBDlac3 (containing LacZ-ChBD) at OD ~ 0.3 (before induction) (lane 1) and at 16 h of induction (lane 2); flow through during immobilization (lane 3) and rinsing fractions (lanes 4, 5), chitin-bound LacZ (lane 6); and chitin beads (lane 7). Lane M shows the Precision Plus Protein standard (Bio-Rad). The arrows indicate subunits of the recombinant β-galactosidases.

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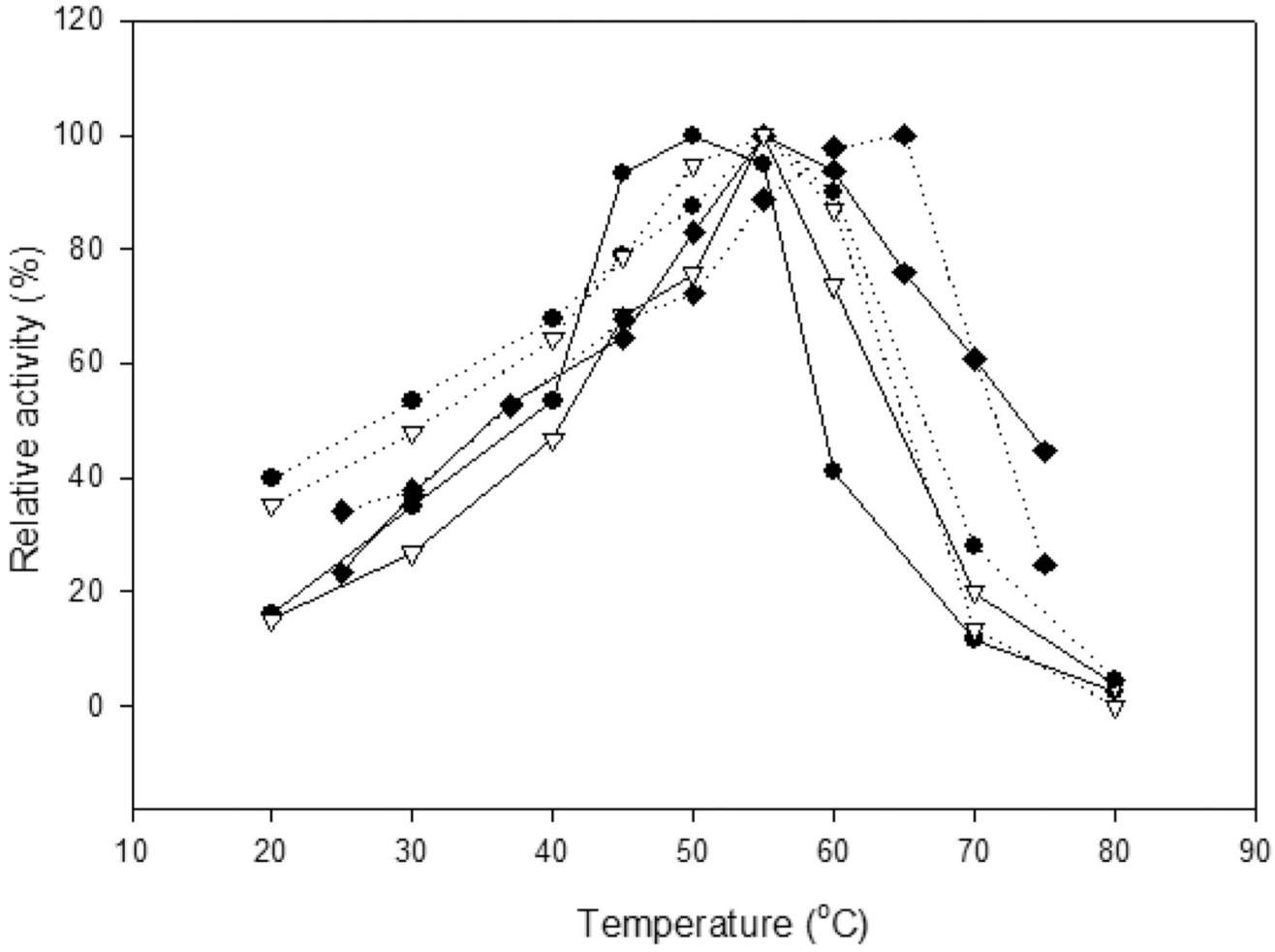

Figure 3.

Temperature optimum of the immobilized enzymes. I-LacLM-ChBD (●) and I-ChBD-LacLM (▽) indicate the recombinant enzymes with the ChBD fused to the C-terminal of LacM and the N-terminal of LacL, respectively. I-LacZ-ChBD (◆) indicates the immobilized LacZ-ChBD. The solid and dotted line indicate the substrate oNPG and lactose, respectively.

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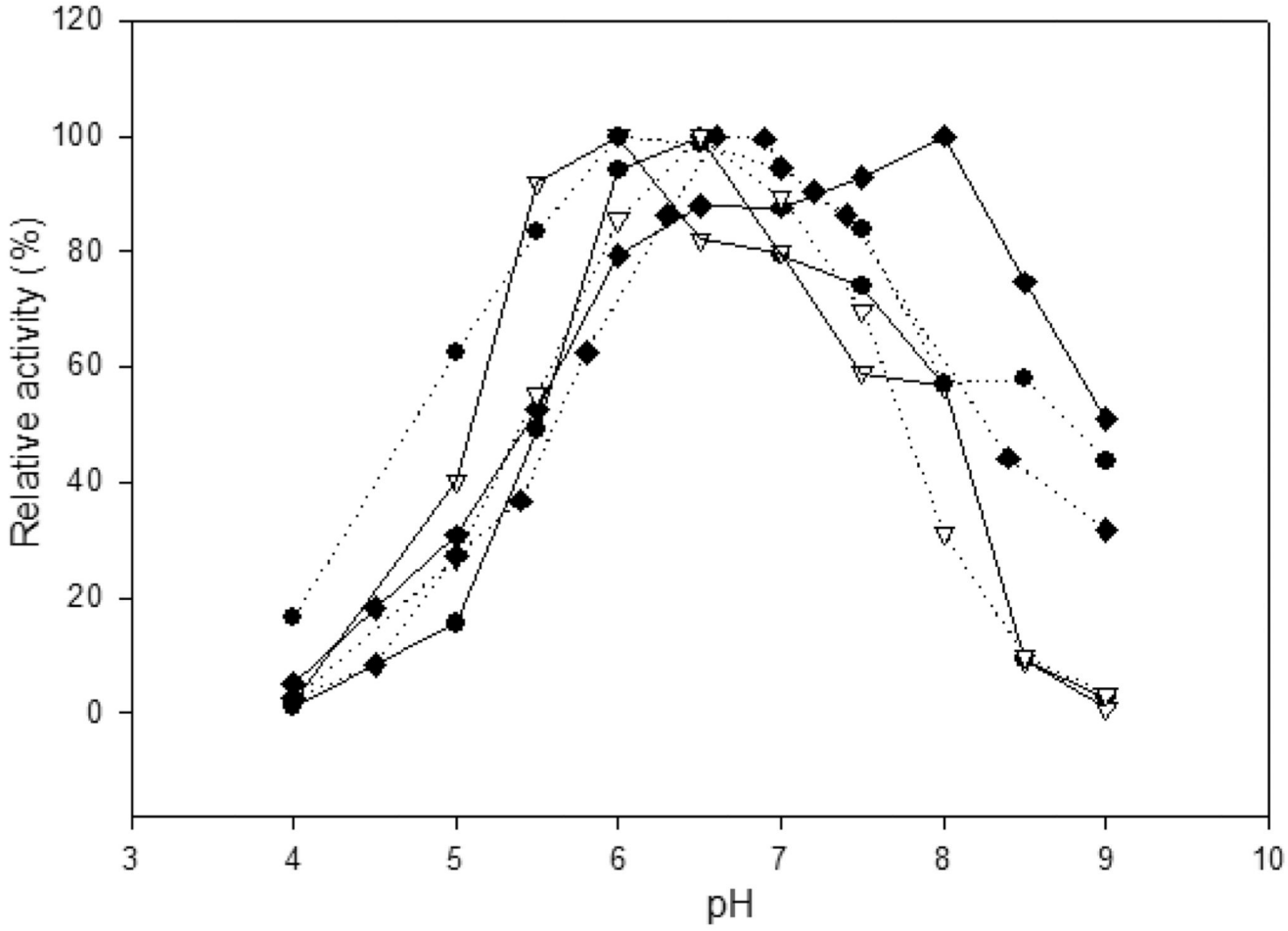

Figure 4.

Optimum pH values of β-galactosidase activity of the immobilized enzymes. I-LacLM-ChBD (●) and I-ChBD-LacLM (▽) indicate the recombinant enzymes with the ChBD fused to C-terminal of the LacM and the N-terminal of the LacL, respectively. I-LacZ-ChBD (◆) indicates the immobilized LacZ-ChBD. The solid and dotted lines indicate substrates *o*NPG and lactose, respectively.

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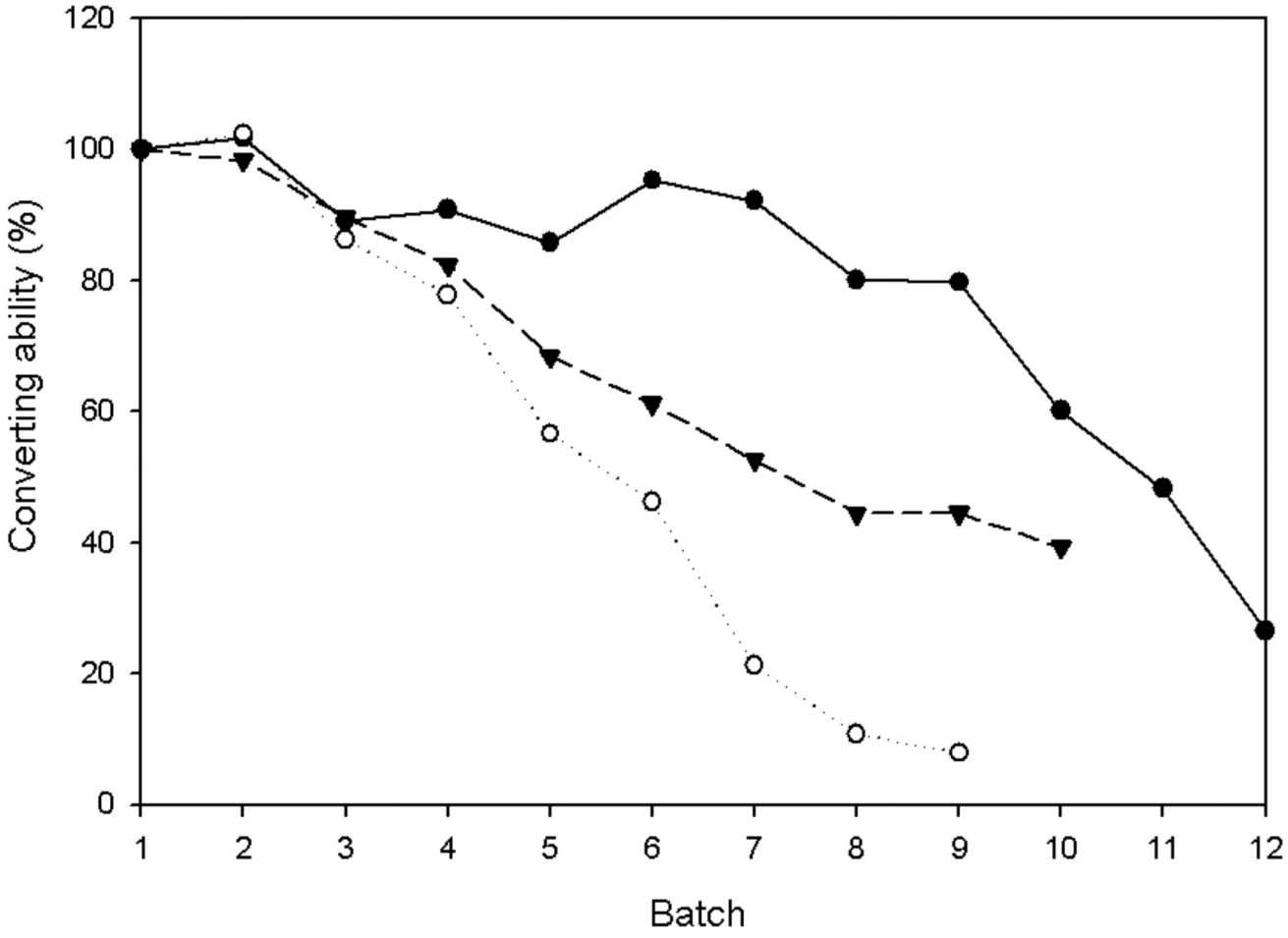

Figure 5.

Reusability of the chitin-immobilized enzymes I-LacLM-ChBD (●), I-ChBD-LacLM (○), and I-LacZ-ChBD (▲). The converting ability (%) was calculated from the ratio of the product released (glucose or *o*NP released from lactose or *o*NPG, respectively) in different reaction cycles to the product obtained at the first batch (assumed as 100%).

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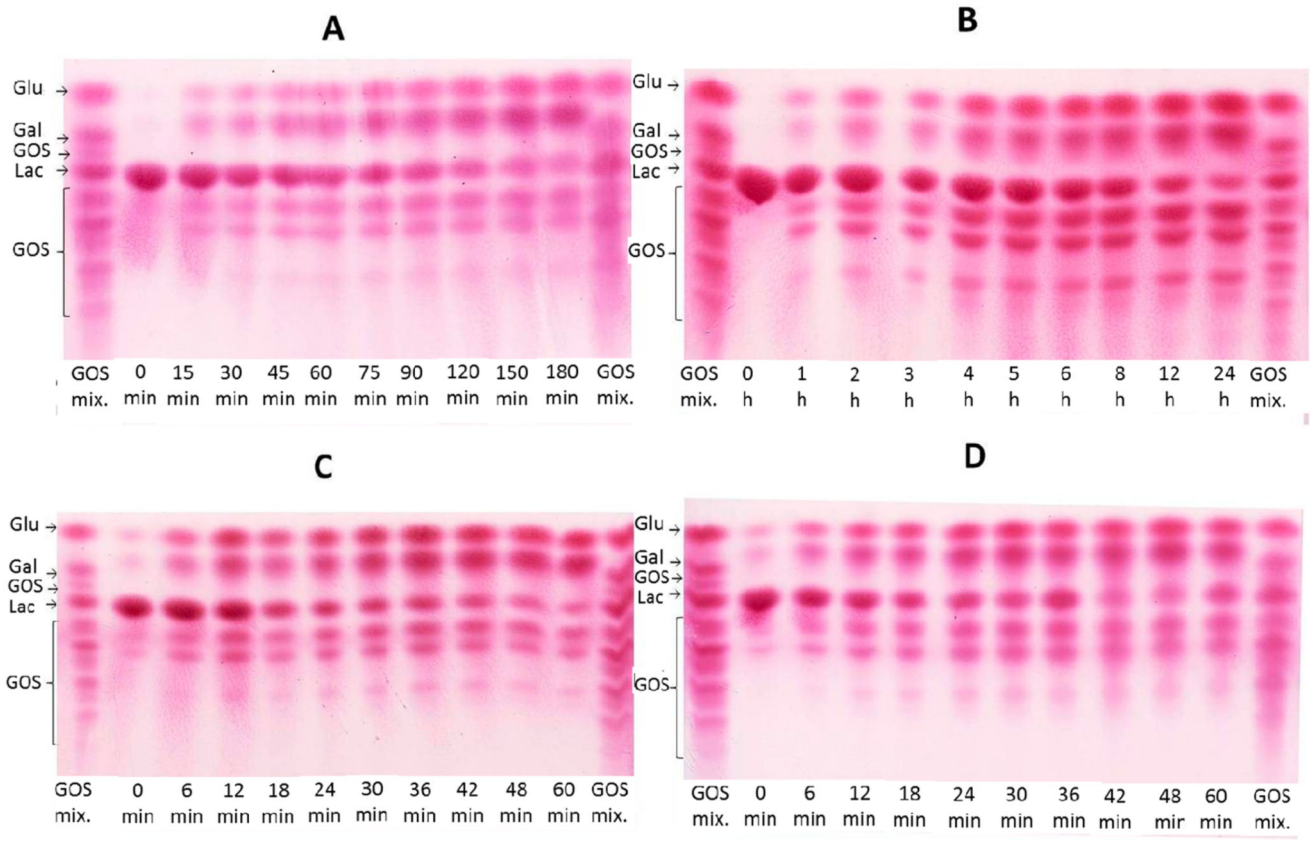

Figure 6.

TLC analysis of different batches of lactose conversion by the immobilized β-galactosidase from *L. bulgaricus*, I-LacZ-ChBD. The batch conversions were carried out in 50 mM sodium phosphate buffer, pH 6.5, 10 mM $MgCl_2$ with various initial lactose concentrations, activities, and temperatures: 50 g/L initial lactose at 55 °C using 3.2 $U_{lactose}$/mL (A); 205 g/L initial lactose concentration at 55 °C using 1.7 $U_{lactose}$/mL (B); 50 g/L initial lactose concentration at 60 °C using 9.7 $U_{lactose}$/mL (C); commercial whole milk, 1 h at 60 °C using 9.7 $U_{lactose}$/mL (D).

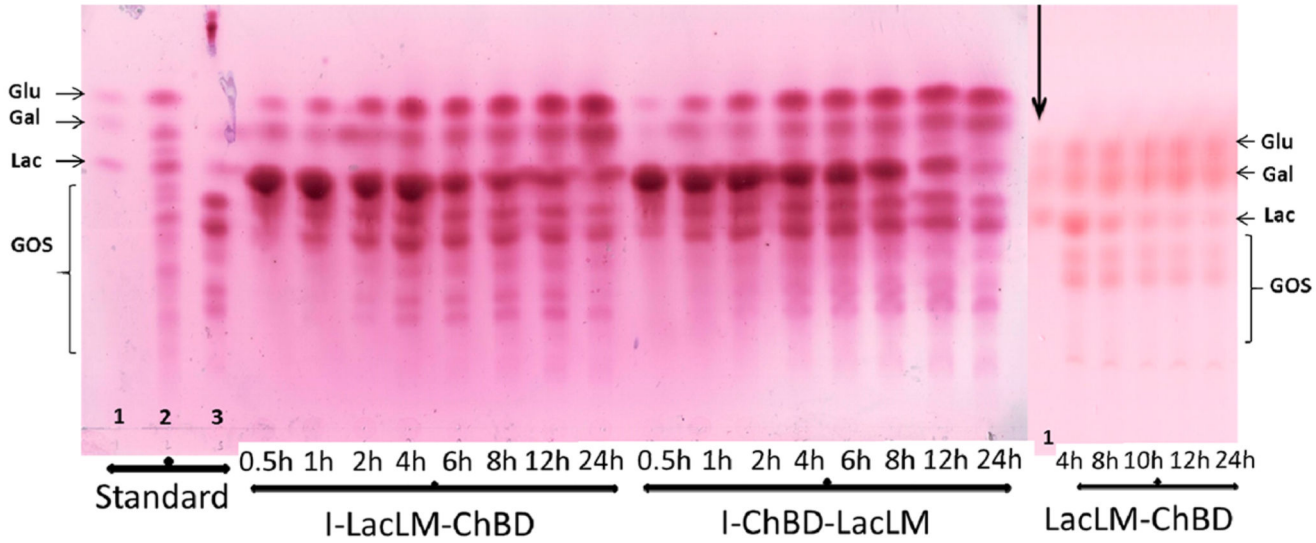

Figure 7.

TLC of products from the batch lactose conversions by the immobilized enzymes I-LacLM-ChBD, I-ChBD-LacLM, and LacLM-ChBD (free soluble enzyme). The conversions were performed in 50 mM sodium phosphate buffer, pH 6.5, 10 mM $MgCl_2$, with an initial concentration of 205 g/L lactose at 30 °C, using 1.5 $U_{lactose}$/ml. Glc, glucose; Gal, galactose; Lac, lactose; GOS, galacto-oligosaccharides. The standards include a mixture of glucose, galactose, and lactose (1); Vivinal GOS (2); and GOS from conversion of free LacLM from *L. reuteri* L103 (3).

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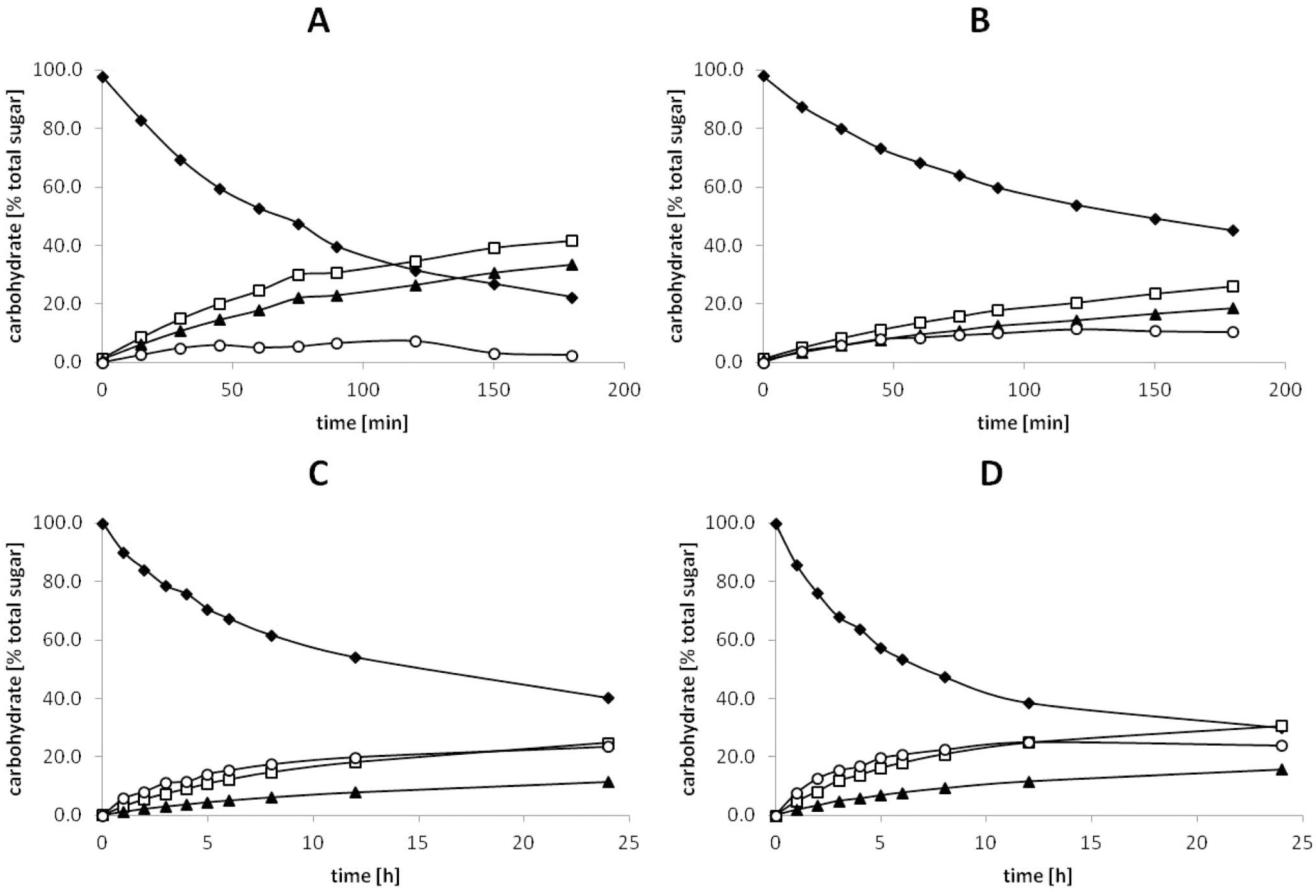

Figure 8.

Carbohydrate composition during lactose transformation by the immobilized *β*-galactosidase from *L. bulgaricus* (I-LacZ-ChBD) determined by HPLC. The batch conversions were carried out in sodium phosphate buffer 50 mM, pH 6.5, in the presence of 10 mM $MgCl_2$ at various temperatures, enzyme activities, and initial lactose concentrations: (A) 50 g/L initial lactose concentration, at 55 °C using 3.2 $U_{lactose}$/mL; (B) 50 g/L initial lactose concentration at 37 °C using 3.2 $U_{lactose}$/mL; (C), 205 g/L initial lactose concentration at 37 °C using 1.7 $U_{lactose}$/mL; (D) 205 g/L initial lactose concentration at 55 °C using 1.7 $U_{lactose}$/mL. The concentrations of lactose (◆), glucose (□), galactose (▲), and total galacto-oligosaccharides (○) were analyzed during the conversion.

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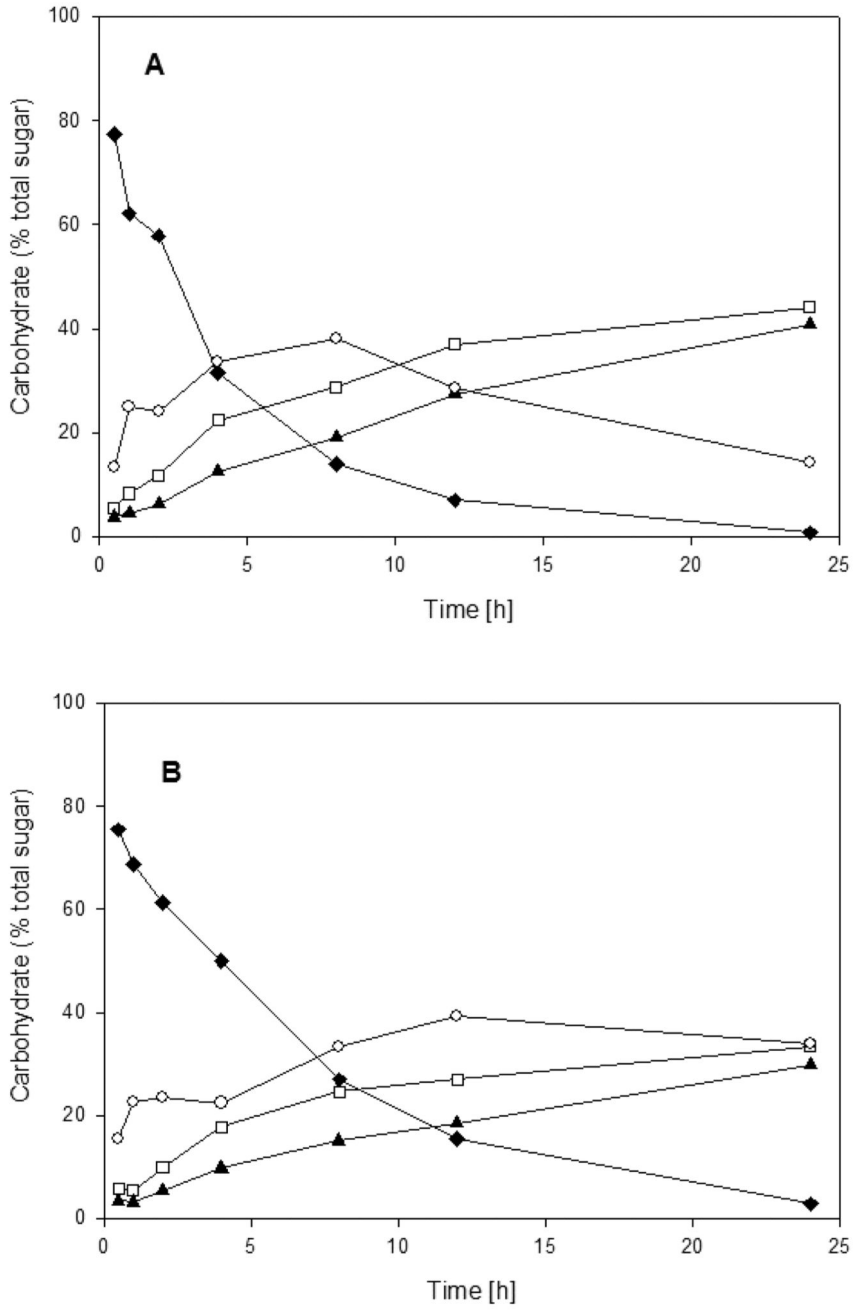

Figure 9.

Carbohydrate composition during lactose transformation by the recombinant β-galactosidase LacLM-ChBD from *L. reuteri* L103 as determined by HPLC. The batch conversions were carried out in 50 mM sodium phosphate buffer, pH 6.5, in the presence of 10 mM $MgCl_2$ at 30 °C; 1.5 $U_{lactose}$/mL, and an initial concentration of lactose of 205 g/L with the free soluble LacLM-ChBD (A) or with the immobilized enzyme I-LacLM-ChBD (B). The concentrations of lactose (◆), glucose (□), galactose (▲), and total galacto-oligosaccharides (○) were analyzed during the conversion.

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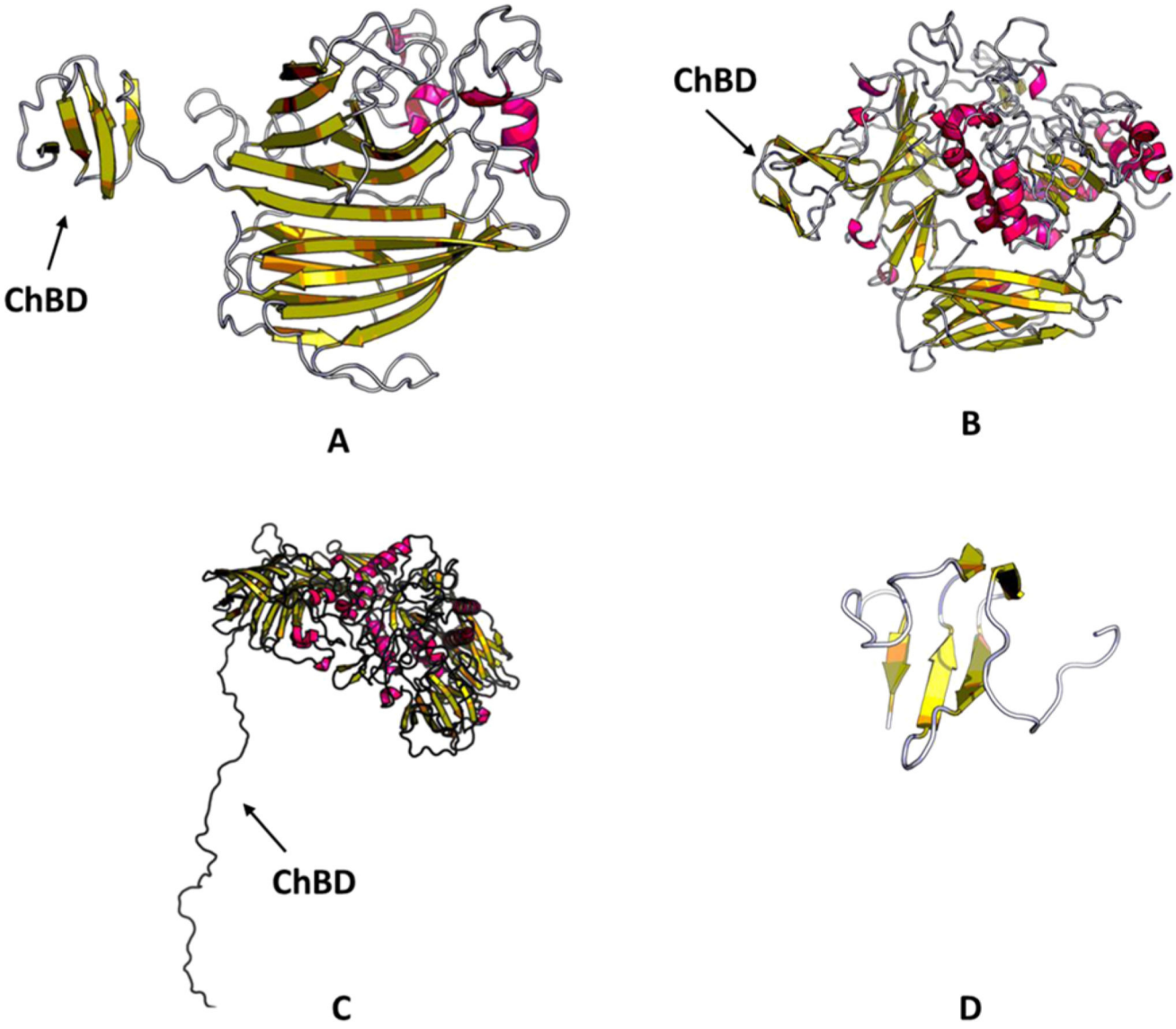

Figure 10.

Predicted 3D structures of LacM-ChBD (A), ChBD-LacL (B), LacZ-ChBD (C), and ChBD (D). The 3D structures were predicted by the RaptorX tool (http://raptorx.uchicago.edu/) on the basis of the deduced amino acid sequences.

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Table 1

Strains and Plasmids Used in This Study

strain or plasmid	relevant characteristic	reference
strains		
L. plantarum WCFS1	wild type	31
E. coli NEB5α	cloning host	NEB
plasmids		
pJETlacZ	source for *lacZ* of *L. bulgaricus* DSM20081	16
pSIP403	Em^r, pSIP401 derivative, *gusA* controlled by P_{sppA}	32
pTH103.3	source for *lacLM* from *L. reuteri*	15
pTxB1	source for *chbd* sequence	NEB
pSCBD	derivative of pSIP403, carrying *chbd*	this study
pSCBDlac1	pSCBD, *gusA* replaced by *lacLM*	this study
pSCBDlac2	pSIP403, *gusA* replaced by fused *chbd_lacLM*	this study
pSCBDlac3	pSCBD, *gusA* replaced by *lacZ* from *L. bulgaricus*	this study

Table 2

Primers Used in This Study

target gene	primer	sequence[a] (5′–3′)
chbd from pTxB1	F1 (*Xho*I)	C TCACTC GAGACGACAAATCCTGGTGTATCCGC
	R1 (*Acc65*I)	C TTCGGTACCTCATTGAAGCTGCCACAAGGC
	F2 (*Bsa*I)	GCAGGTCTC CCATG GCG ACA AAT CCT GGT GTA TCC
	R2	*CCA TTT TAT ATT TGC TTG* TTGAAGCTGCCAC
lacLM of *L. reuteri* L103	F3	G TGG CAG CTT CAA *CAA GCA AAT ATA AAA TGG*
	R3 (*Xho*I)	CG CTCGAG TTA TTT TGC ATT CAA TAC AAA CG
lacZ of *L. bulgaricus* DSM20081	F4 (*Bsmb*I)	GCTGCGTCTCCC ATG AGC AAT AAG TTA GTA AAA G
	R4 (*Xho*I)	GAAGCTCGAG TGA TTT TAG TAA AAG GGG

[a]The *italic sequences* in R2 and F3 are complementary. The restriction enzyme recognizing sites are underlined.

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Table 3

β-Galactosidase Activity in Cell-free Lysate of the Induced and Noninduced Cells of *L. plantarum* WCFS1 Carrying Various Plasmids[a]

	volumetric activity (U/mL fermentation broth)		specific activity (U/mg protein)		
plasmid	**noninduced**	**induced**	**noninduced**	**induced**	**induction factor**
pSCBDlac1	1.45 ± 0.48	32.86 ± 3.55a	7.35 ± 2.44	178.90 ± 10.05e	24
pSCBDlac2	0.77 ± 0.19	11.31 ± 1.20b	4.06 ± 1.09	57.29 ± 6.24f	14
pSCBDlac3	16.77 ± 1.02	65.83 ± 1.53c	9.43 ± 0.14	40.27 ± 2.36g	4

[a]The data are expressed as the mean ± standard deviation from three independent cultivations. The induction factors are calculated by dividing the specific activity (U/mg) of induced and noninduced cells. The letters indicate significant difference ($p < 0.05$).

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Table 4

Immobilization of Recombinant Enzymes on Chitin Beads[a]

enzyme	residual activity in liquid phase (%)	IY[b] (%)	residual activity on bead[c] (%)	AR[d] (%)	residue activity (kU/g chitin bead)
LacLM-ChBD	0.67 ± 0.15e	99.33	25.89 ± 2.49	26.06	11.77
ChBD-lacLM	48.36 ± 4.50f	51.64	9.19 ± 0.50	19.00	4.17
lacZ-ChBD	8.75 ± 0.47g	91.25	12.50 ± 0.61	13.70	6.25

[a]The applied enzyme activity was 500 U, and 11 mg (dry weight) of chitin beads were used. The immobilization was used for 18 h at 4 °C with gentle agitation. Letters e, f, and g indicate significant difference ($p < 0.05$).

[b]IY (%) was calculated by subtraction of the residual enzyme activity (%) in the liquid phase from the total activity applied (100%).

[c]Residual activity (%) on beads is the percentage of total activity on chitin beads to the applied activity. To determine activity on a bead, the bead suspension was used in enzyme assay.

[d]Activity retention, AR (%) is the ratio of residual activity to IY.

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Table 5

Kinetic Parameters of the Immobilized β-D-Galactosidases for the Hydrolysis of Lactose and *o*-Nitrophenyl-β-D-galactopyranoside (*o*NPG) in Comparison to the Respective Free Enzymes without ChBD[a]

substrate	method for determination of enzyme activity	kinetic parameter	LacLM-ChBD[b]	I-LacLM-ChBD	I-ChBD-LacLM	L103[c]	EcoL103[d]	LpL103[e]	I-LacZ-ChBD	LacZ[f]
lactose	release of D-glucose	$v_{max,Glu}$ (μmol min^{-1} mg^{-1})	17.83 ± 0.14	1.74 ± 0.07	0.72 ± 0.01	34	38	43	39.9 ± 0.9	123 ± 5
		$K_{m,Lac}$ (mM)	51.74 ± 1.80	36.82 ± 0.33	12.25 ± 0.39	13	12	12	45.5 ± 4.2	19.2 ± 3.8
		K_{cat} (s^{-1})	9.00 ± 0.07	0.88 ± 0.03	0.36 ± 0.01	18	20	23	79.8 ± 1.8	234 ± 13
		K_{cat}/K_m (s^{-1} M^{-1})	174	24	29	1389	1681	1902	1754	12300
*o*NPG	release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})	103.9 ± 1.82	65.40 ± 1.78	32.09 ± 0.14	nd	nd	nd	87.1 ± 2.8	317 ± 6
		$K_{m,oNPG}$ (mM)	0.91 ± 0.05	2.18 ± 0.45	1.34 ± 0.03	nd	nd	nd	13.7 ± 1.1	0.92 ± 0.09
		K_{cat} (s^{-1})	52.39 ± 0.92	32.98 ± 0.90	16.18 ± 0.07	nd	nd	nd	174.2 ± 5.6	603 ± 15
		K_{cat}/K_m (s^{-1} M^{-1})	54970	15096	12032	nd	nd	nd	12715	655000

[a]The three chitin-immobilized enzymes of LacLM-ChBD, ChBD-LacLM, and LacZ-ChBD are designated I-LacLM-ChBD, I-ChBD-LacLM, and I-LacZ-ChBD, respectively. The bound amount of protein on the chitin beads was calculated by subtraction of the protein content in the cell crude extract (before immobilization) and in the liquid phase (after immobilization). The difference of v_{max} and K_m values among the immobilized enzymes and the free-soluble enzyme is statistically significant ($p < 0.05$). nd, not determined.

[b]Free soluble LacLM-ChBD.

[c]Native enzyme from *L. reuteri* L103.14

[d]Recombinant enzyme expressed and purified from *E. coli*.15

[e]Recombinant enzyme expressed and purified from *L. plantarum* WCFS1.17

[f]Purified recombinant enzyme LacZ from *L. bulgaricus* expressed in *L. plantarum*.16

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Table 6

Thermostability of Recombinant Immobilized β-Galactosidases from *Lactobacillus*[a]

	LacLM-ChBD		I-LacLM-ChBD		I-ChBD-LacLM		I-LacZ-ChBD	
temperature (°C)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	0.036	19.254	0.0041	169.060	0.006	115.524	nd[b]	nd
37	nd	nd	nd	nd	nd	nd	0.0034	203.867
40	0.33	2.100	0.095	7.296	0.188	3.687	nd	nd
50	26.84	0.026	1.92	0.361	3.64	0.190	nd	nd
55	nd	nd	nd	nd	nd	nd	0.9543	0.726
60	115.78	0.006	10.924	0.063	10.82	0.064	nd	nd
65	nd	nd	nd	nd	nd	nd	8.3593	0.083

[a]The immobilized enzymes were incubated in 50 mM sodium phosphate buffer. pH 6.5. at various temperatures.

[b]nd, not determined.

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Table 7

Effect of pH on Enzyme Stability[a]

	I-LacLM-ChBD		I-ChBD-LacLM		I-LacZ-ChBD	
pH	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
4	0.5318	1.303	2.161	0.321	0.0984	7.0
5	0.0373	18.583	0.0685	10.119	0.0035	198.0
6	0.0078	88.865	0.0106	65.391	0.0006	1155.2
6.5	0.0038	182.407	0.0061	113.630	0.0006	1155.2
7	0.077	9.002	0.0432	16.045	0.0008	866.4
8	0.718	0.965	1.0722	0.646	0.0087	79.7

[a]Half-life times of the immobilized β-galactosidases were determined by incubating the enzymes at 30 °C (for I-LacLM-ChBD and I-ChBD-LacLM) or at 37 °C (for I-LacZ-ChBD in the presence of 10 mM $MgCl_2$) at different pH values.

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Table 8

Lactose Conversions Using the Immobilized β-Galactosidase from *L. bulgaricus* under Various Conditions

	initial lactose concentration[a]				
	50 g/L			205 g/L	
temperature (°C)	37	55	60	37	55
$U_{lactose}$/mL	3.2	3.2	9.7	1.7	1.7
run time (h)	3	3	1	24	24
glucose (%)	26.0	41.7	39.3	24.8	30.6
galactose (%)	18.5	33.6	32.8	11.6	15.7
total GOS (%)	10.4	2.5	10.4	23.5	23.9
lactose conversion rate (%)	54.2	77.2	81.2	59.8	70.2

[a] In 50 mM sodium phosphate buffer, pH 6.5, 10 mM $MgCl_2$.