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Homodimeric β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and Biochemical Characterization

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ABSTRACT: The *lacZ* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081, encoding a β-galactosidase of the glycoside hydrolase family GH2, was cloned into different inducible lactobacillal expression vectors for overexpression in the host strain *Lactobacillus plantarum* WCFS1. High expression levels were obtained in laboratory cultivations with yields of approximately 53000 U of β-galactosidase activity per liter of medium, which corresponds to ∼170 mg of recombinant protein per liter and β-galactosidase levels amounting to 63% of the total intracellular protein of the host organism. The wild-type (nontagged) and histidine-tagged recombinant enzymes were purified to electrophoretic homogeneity and further characterized. β-Galactosidase from *L. bulgaricus* was used for lactose conversion and showed very high transgalactosylation activity. The maximum yield of galacto-oligosaccharides (GalOS) was approximately 50% when using an initial concentration of 600 mM lactose, indicating that the enzyme can be of interest for the production of GalOS.

KEYWORDS: *β-galactosidase, lactase, transgalactosylation, galacto-oligosaccharides, prebiotics*

INTRODUCTION

Lactic acid bacteria (LAB) and especially lactobacilli are important starter and adjunct cultures in the production of foods that require lactic acid fermentation, notably various dairy products, fermented vegetables, fermented meats, and sourdough bread.[1,2] *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), a thermophilic Gram-positive bacterium with an optimal growth temperature of 45 °C, is one of the economically most important representatives of the heterogeneous group of LAB, with a worldwide application in yogurt production and in other fermented milk products.[3] *L. bulgaricus* is a homofermentative LAB, and during growth in milk it rapidly converts lactose into lactic acid for food product preservation. The metabolism of lactose in this organism involves two main enzymes, a lactose antiporter permease (LacS) for the uptake of the sugar and a β-galactosidase (LacZ) for the intracellular cleavage of lactose into glucose and galactose, both of which are part of the *lac* operon.[4] *Lactobacillus* spp. encode β-galactosidases that belong to glycoside hydrolase families GH2 and GH42 according to the CAZy nomenclature (http://www.cazy.org).[5] The predominant GH2 β-galactosidases found in lactobacilli are of the LacLM type, heterodimeric proteins of ∼105 kDa, which are encoded by the two overlapping genes, *lacL* and *lacM*. We recently cloned several lactobacillal β-galactosidase genes of this type, including *lacLM* from *Lactobacillus reuteri*,[6] *Lactobacillus acidophilus*,[7] *Lactobacillus pentosus*,[8] *Lactobacillus plantarum*,[9] and *Lactobacillus sakei*,[10] and characterized the resulting proteins with respect to their biochemical properties. In addition, di- or oligomeric GH2 β-galactosidases of the LacZ type, encoded by the single *lacZ* gene, are sometimes, but not often, found in lactobacilli, whereas they are more frequent in other LAB including *Streptococcus salivarius* and *Streptococcus thermophilus*[11] or bifidobacteria including *Bifidobacterium bifidum*[12] or *Bifidobacterium longum* subsp. *infantis*.[13]

β-Galactosidases catalyze the hydrolysis of the β-1,4-D-glycosidic linkage of lactose and structurally related substrates. β-Galactosidases have two main technological applications in the food industry—the removal of lactose from milk and dairy products[14] and the production of galacto-oligosaccharides (GalOS), exploiting the transglycosylation activity of some of these enzymes.[15,16] GalOS are prebiotic sugars, which are defined as a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-

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Table 1. Strains and Plasmids Used for Cloning and Overexpression of the β-Galactosidase Gene *lacZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus*[a]

strains and plasmids	relevant characteristics and purpose	ref
strains		
L. delbrueckii subsp. *bulgaricus* DSM 20081	original source of *lacZ*	DSMZ
Lactobacillus plantarum WCFS1	host strain, plasmid free	42
E. coli NEB5α	cloning host	New England Biolabs
plasmids		
pJET1.2	for subcloning and PCR fragment synthesis	Fermentas
pSIP403	*spp*-based expression vector, pSIP401 derivative, Em^r, *gusA* controlled by P_{sppA}	33
pSIP409	*spp*-based expression vector, pSIP401 derivative, Em^r, *gusA* controlled by P_{sppQ}	33
pTH101	pSIP403 derivative, *gusA* replaced by *lacZ*	this study
pTH102	pSIP403 derivative, *gusA* replaced by *lacZ* carrying C-terminal His_6-tag	this study
pTH103	pSIP409 derivative, *gusA* replaced by *lacZ*	this study
pTH104	pSIP409 derivative, *gusA* replaced by *lacZ* carrying C-terminal His_6-tag	this study

[a]Em^r, erythromycin resistance; *spp*, sakacin P gene cluster; *gusA*, β-glucuronidase reporter gene; *lacZ*, β-galactosidase gene.

Table 2. Primers Used for Cloning of the β-Galactosidase Gene *lacZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus*[a]

primer	restriction enzyme	sequence (5→3)	ref sequence accession no.
F1	*Bsm*BI	GCTGCGTCTCCCATGAGCAATAAGTTAGTAAAAG	NC_008054, GeneID: 4085367
R1	*Xho*I	CGCGCTCGAGTTATTTTAGTAAAAGGGGCTG	NC_008054, GeneID: 4085367
R2	*Xho*I	CGCGCTCGAG*TTAGTGGTGGTGGTGGTGGTG*TTTTAGTAAAAGGGGC	NC_008054, GeneID: 4085367

[a]Restriction sites are underlined; the His_6-tag sequence is shown in italic.

being and health".[17] GalOS are complex mixtures of different oligosaccharides, and the spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the β-galactosidase used for the biocatalytic reaction as well as on the conversion conditions used in their production.[15,18] Rabiu et al.[19] and Tzortzis et al.[20] produced various GalOS mixtures using lactose as substrate and β-galactosidases from different probiotic bifidobacteria. Subsequently, they showed that these different mixtures typically resulted in better growth of the producer strain of the enzyme for GalOS production. This concept can serve as the basis for a new generation of functionally enhanced, targeted oligosaccharides and has increased interest in β-galactosidases from beneficial probiotic organisms.[21] Because lactobacilli have traditionally been recognized as potentially health-promoting, probiotic bacteria,[22] GalOS produced by their β-galactosidases can be of interest for nutritional purposes. In the present study we report the heterologous expression of the single-gene encoded β-galactosidase (LacZ) from *L. bulgaricus* in *L. plantarum* using pSIP vectors and, thus, the overexpression of this enzyme in a food grade host. In addition, the β-galactosidase was purified, characterized, and compared to the enzymes of the LacLM type, also with respect to the spectrum of GalOS produced by these different β-galactosidases.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals and enzymes were purchased from Sigma (St. Louis, MO) unless otherwise stated and were of the highest quality available. MRS broth powder was obtained from Merck (Darmstadt, Germany). All restriction enzymes, T4 DNA ligase, and shrimp alkaline phosphatase (SAP) were from Fermentas (Vilnius, Lithuania).

Bacterial Strains and Culture Conditions. The type strain *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (synonym *L. bulgaricus*; other collection numbers are ATCC 11842; originally isolated from Bulgarian yogurt in 1919[23]) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). All bacterial strains used in this study are shown in Table 1. *Lactobacillus* strains were cultivated in MRS media at 37 °C, without agitation. *Escherichia coli* NEB5α (New England Biolabs, Ipswich, MA) was grown at 37 °C in Luria−Bertani (LB) medium with shaking at 120 rpm. When needed, erythromycin was supplemented to media in concentrations of 5 μg/mL for *Lactobacillus* or 200 μg/mL for *E. coli*, whereas ampicillin was used at 100 μg/mL for *E. coli*.

DNA Manipulation. Total DNA of *L. bulgaricus* DSMZ 20081 was isolated using chloroform extraction as described by Nguyen et al.[24] with slight modifications. In short, cell pellets from 3 mL overnight cultures were resuspended and incubated at 37 °C for 1 h in 400 μL of 1 mM Tris−EDTA buffer pH 8 (TE buffer) containing 50 μL of lysozyme (100 mg/mL) and 50 μL of mutanolysin (480 U/mL). The mixture was subsequently supplemented with 50 μL of 10% SDS and 10 μL of proteinase K (20 mg/mL) and incubated further at 60 °C for 1 h. After inactivation of proteinase K (at 75 °C for 15 min), 2 μL of RNase (2 mg/mL) was added to the mixture, and incubation was continued at 37 °C for 30 min. Genomic DNA was extracted and purified by using phenol−chloroform and precipitated with 3 M sodium acetate, pH 3.8, and ice-cold isopropanol. The DNA precipitate was washed with cold (−20 °C) 70% ethanol, and the dried DNA pellets were dissolved in 50 μL of TE buffer, pH 7.5, at room temperature with gentle shaking.

The primers used for PCR amplification of *lacZ* from the genomic DNA of *L. bulgaricus* DSM 20081 (NCBI reference sequence no. NC_008054)[23] were supplied by VBC-Biotech Service (Vienna, Austria) and are listed in Table 2. The appropriate endonuclease restriction sites were introduced in the forward and reverse primers as indicated. DNA amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) as recommended by the supplier and using standard procedures.[25] The amplified PCR products were purified by the Wizard SV Gel and PCR Clean-up system kit (Promega, Madison, WI). When needed, the PCR fragments were subcloned into the pJET1.2 plasmid (CloneJET PCR cloning kit, Fermentas), and *E. coli* was used as a host for obtaining the plasmids in sufficient amounts before transformation into *Lactobacillus*. All PCR-generated inserts were confirmed by DNA sequencing performed by a commercial provider.

Plasmid Construction and Transformation. Gene fragments of *lacZ* with or without the His_6-tag were excised from the pJETlacZ plasmid using *BsmB*I and *Xho*I and ligated into the 5.6 kb *Nco*I−*Xho*I

fragments of pSIP403 or pSIP409, resulting in the plasmids pTH101, pTH102, pTH103, and pTH104 (Table 1). The constructed plasmids were transformed into electrocompetent cells of *L. plantarum* WCFS1 according to the protocol of Aukrust and Blom.[26]

β-Galactosidase Assays. β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as the substrates, as described previously.[6] In brief, these 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 575 mM for lactose. Protein concentrations were determined by using the method of Bradford with bovine serum albumin (BSA) as standard.

Expression of Recombinant β-Galactosidase. For the heterologous overexpression of the *lacZ* gene from *L. bulgaricus*, overnight cultures (~16 h) of *L. plantarum* WCFS1 harboring the expression plasmid pTH101, pTH102, pTH103, or pTH104 were added to 15 mL of fresh MRS medium containing erythromycin to an OD_{600} of ~0.1 and incubated at 30 °C without agitation. The cells were induced at an OD_{600} of 0.3 by adding 25 ng/mL of the inducing peptide pheromone IP-673 (supplied by the Molecular Biology Unit, University of Newcastle-upon-Tyne, U.K.). Cells were harvested at an OD_{600} of 1.8−2, washed twice by buffer P (50 mM sodium phosphate buffer, pH 6.5, containing 20% w/v glycerol and 1 mM dithiothreitol),[6] and resuspended in 0.5 mL of the same buffer. Cells were disrupted in a bead beating homogenizer using 1 g of glass bead (Precellys 24; PEQLAB, Germany). Cell-free extracts were obtained after a centrifugation step at 9000*g* for 15 min at 4 °C.

Fermentation and Protein Purification. *L. plantarum* WCFS1 harboring pTH101 or pTH102 was cultivated in 1 L fermentations to obtain sufficient material for purification of LacZ. The cultivation conditions and the induction protocol were identical to those of the small-scale cultivations. Expression of *lacZ* was induced at OD_{600} 0.3, and the cells were harvested at OD_{600} ~6. After centrifugation as above, cells were disrupted by using a French press (Aminco, Silver Spring, MD), and debris was removed by centrifugation (30000*g*, 20 min, 4 °C). The purification of the recombinant enzyme was performed by immobilized metal affinity chromatography using a Ni-Sepharose column (GE Healthcare, Uppsala, Sweden)[8] or substrate affinity chromatography (with the substrate analogue *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized onto cross-linked 4% beaded agarose; Sigma) as previously described.[6] Purified enzymes were stored in 50 mM sodium phosphate buffer, pH 6.5, at 4 °C.

Gel Electrophoresis, Gel Permeation Chromatography, and Activity Staining. Native polyacrylamide gel electrophoresis (PAGE), denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), and activity staining using 4-methylumbelliferyl β-D-galactoside (MUG) as the substrate were carried out as previously described[6] using the Phast System with precast gels (Pharmacia Biotech, Uppsala, Sweden). Gel permeation chromatography was performed on a Superose 12 column (16 × 1000 mm; GE Healthcare) using 20 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl, and with the Sigma Gel Filtration Molecular Markers Kit with standard proteins of 12−200 kDa. In addition, pyranose oxidase with a molecular mass of 250 kDa was used as a standard.[27]

Characterization of Recombinant β-Galactosidase. Steady-state kinetic data for the substrates lactose or *o*NPG were obtained at 30 °C in 50 mM sodium phosphate buffer, pH 6.5, with concentrations ranging from 0 to 600 mM for lactose and from 0 to 25 mM for *o*NPG. Furthermore, the inhibition of the hydrolytic activity of LacZ by D-glucose as well as D-galactose was investigated by adding these sugars into the assay mixture in concentrations ranging from 10 to 300 mM, and the respective inhibition constants were determined. The kinetic parameters and the inhibition constants were calculated using nonlinear regression, fitting the observed data to the Henri−Michaelis−Menten equation using SigmaPlot (SPSS, Chicago, IL).

The pH dependence of β-galactosidase activity was evaluated in the range of pH 3−10 using Britton−Robinson buffer (containing 20 mM each of phosphoric, acetic, and boric acid adjusted to the required pH with NaOH). The temperature dependence of β-galactosidase activity was assessed by measuring activity in the range of 20−90 °C for 10 min. The catalytic stability of β-galactosidase was determined by incubating the enzyme in 50 mM phosphate buffer (pH 6.5) at various temperatures and by subsequent measurements of the remaining enzyme activity (A) at various time points (t) using the standard *o*NPG assay. Residual activities (A_t/A_0, where A_t is the activity measured at time t and A_0 is the initial activity) were plotted versus the incubation time. The inactivation constants k_{in} were obtained by linear regression of ln(activity) versus time. The half-life values of thermal inactivation $\tau_{1/2}$ were calculated using $\tau_{1/2} = \ln 2/k_{in}$.[28]

To study the effect of various cations on β-galactosidase activity, the enzyme samples were assayed at 30 °C for 10 min with 22 mM *o*NPG (10 mM Bis-Tris, pH 6.5, or 50 mM sodium phosphate buffer, pH 6.5) as the substrate in the presence of various cations added in final concentrations of 1−50 mM. The measured activities were compared with the activity blank of the enzyme solution determined under identical conditions but without added cations using the standard *o*NPG assay. Unless otherwise stated, the nontagged enzyme LacZ was used for these characterization experiments.

Lactose Hydrolysis and Transgalactosylation. The synthesis of galacto-oligosaccharides (GalOS) was carried out in discontinuous mode using purified recombinant, nontagged β-galactosidase from *L. bulgaricus* (1.5 lactase U/mL of reaction mixture). Reaction conditions were 600 mM initial lactose concentration in sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$; the incubation temperature was varied from 30 to 50 °C. Continuous agitation was applied at 300 rpm. Samples were withdrawn periodically, and the composition of the GalOS mixture was analyzed by capillary electrophoresis and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), following methods described previously.[29] Individual GOS compounds were identified and quantified by using authentic standards and the standard addition technique.[16,30]

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.

RESULTS AND DISCUSSION

Plasmid Construction and Expression of β-Galactosidase Derived from *L. bulgaricus* in *L. plantarum*. The yields of β-galactosidase activity when using the wild-type strain of *L. bulgaricus* as a producer are rather low; for example, β-galactosidase levels were only ~4000 U of activity/L of medium (MRS containing 2% lactose) after cultivation at 37 °C for 24 h for *L. delbrueckii* subsp. *bulgaricus* DSM 20081. Hence we attempted heterologous overexpression in a food grade organism to obtain higher yields of this biotechnologically attractive enzyme, and we cloned the *L. bulgaricus lacZ* gene into the vectors pSIP403 and pSIP409, which differ only in their promoters.[31−33] The four expression plasmids pTH101, pTH102, pTH103, and pTH104 were constructed by replacing *gusA*, which originally was used as a reporter gene in the pSIP plasmid series, by *lacZ*, both with and without a hexa-histidine tag (Table 1). In these vectors, the transcription of *lacZ* is regulated by the inducible promoters P_{sppA} and P_{sppQ} for the pSIP403 and pSIP409 derivatives, respectively (Figure 1). The expression of *lacZ* with the different vectors was subsequently studied in *L. plantarum* WCFS1 as host, using an inducer concentration of 25 ng/mL of the inducing peptide pheromone IP-673.[25,31] Induced and noninduced cells were harvested in the late stationary phase (OD_{600} of 1.8−2.0), and the intracellular cell-free extracts were analyzed by SDS-PAGE, which showed unique bands of ~100 kDa in induced *L. plantarum* cells (Figure 2) and β-galactosidase activity assays

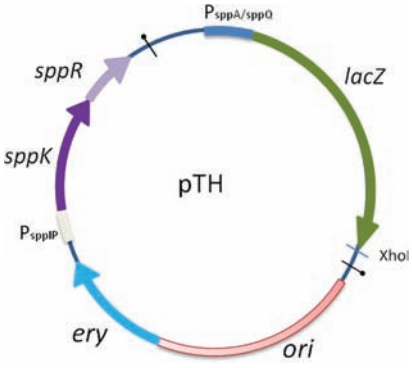

Figure 1. Schematic overview of the pTH plasmids developed in this study. The structural gene *lacZ* (with or without a hexa-histidine tag) is controlled by the inducible promoters P_{sppA} (pSIP403 derivatives) or P_{sppQ} (pSIP409 derivatives). P_{sppIP} controls the structural genes of the two-component regulatory system, *sppK*, a histidine kinase, and *sppR*, a response regulator. *Ery* indicates the erythromycin resistance marker, and transcriptional terminators are marked by lollypop structures.

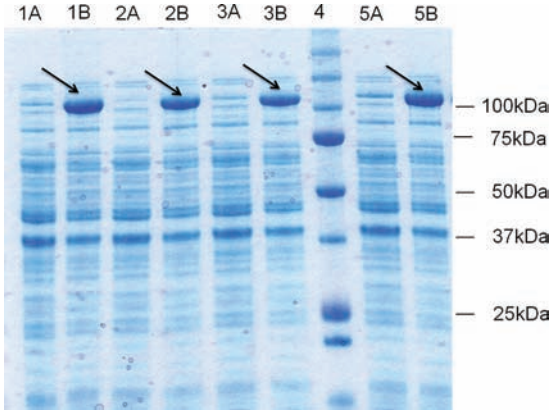

Figure 2. SDS-PAGE analysis of cell-free extracts of noninduced (A) and induced cells (B) of *L. plantarum* WCFS1 harboring pTH101 (lanes 1A, 1B), pTH103 (lanes 2A, 2B), pTH104 (lanes 3A, 3B), and pTH102 (lanes 5A, 5B). Lane 4 shows the Precision Plus Protein standard (Bio-Rad). The gel was stained with Coomassie blue.

(Table 3). Analysis of the crude cell extracts gave volumetric activities in the range of ~15−23 U/mL of cultivation medium and specific activities of ~160−200 U/mg (Table 3). The β-galactosidase activities in *L. plantarum* cells without plasmids were diminishing (0.002 U/mL and 0.07 U/mg), and hence the enzyme activities obtained can be attributed solely to the plasmid-encoded LacZ from *L. bulgaricus*. The choice of the P_{sppA} promoter (pSIP403 derivatives) or P_{sppQ} promoter (pSIP409 derivatives) did not affect the levels of β-galactosidase activity, because expression yields were well comparable and statistically not different for these constructs.

Noninduced cells of *L. plantarum* harboring the various expression vectors were also cultivated and tested for basal expression ("leakage") from the promoters (Table 3). Cells carrying pSIP409-derived vectors containing P_{sppQ} show significantly lower basal activities than cells harboring pSIP403-derived vectors based on P_{sppA}. As a consequence, the highest induction factors, that is, the quotient of specific activity obtained for induced and noninduced cells, of roughly 50 were found for the constructs pTH103 and pTH104 carrying P_{sppQ} as the promoter.

Interestingly, the activities obtained for His-tagged LacZ were always significantly lower by approximately 20−30% despite both protein versions being produced in comparable levels as judged by SDS-PAGE analysis. The reduced activity is most probably caused by the C-terminal His-tag, because specific activities determined for purified, homogeneous nontagged and His-tagged LacZ (306 and 251 U/mg) also differ by ~20%. The exact mechanism of how the His-tag interferes with the activity is, however, not known.

Fermentation and Purification of Recombinant β-Galactosidase LacZ. *L. plantarum* harboring pTH101 or pTH102 was cultivated on a larger scale (1 L cultivation volume), and gene expression was induced in accordance with the previous experiments. Typical yields obtained in 1 L laboratory cultivations were approximately 7.5 ± 0.5 g wet biomass and 53 ± 2 kU of nontagged (pTH101) and 43 ± 2 kU of His-tagged (pTH102) β-galactosidase activity. As judged from the specific activity of the crude cell extract (193 U/mg for nontagged LacZ) and that of the purified enzyme, 63% of the total soluble intracellular protein in *L. plantarum* amounts to the heterologously expressed protein, which was produced at levels of ~170 mg recombinant protein/L of medium.

The recombinant enzymes were purified to apparent homogeneity from cell extracts by single-step purification protocols using either substrate affinity or immobilized metal affinity chromatography. The specific activity of the purified recombinant enzymes was 306 U/mg for wild-type, nontagged LacZ and 251 U/mg for His-tagged LacZ, respectively, when using the standard *o*NPG assay. Both purification procedures yielded homogeneous β-galactosidase as judged by SDS-PAGE (Figure 3A).

Molecular Characterization of the *lacZ* Gene Product, β-Galactosidase LacZ. β-Galactosidase from *L. bulgaricus* is a homodimer, consisting of two identical subunits of ~115 kDa, as judged by denaturing SDS-PAGE (molecular mass of ~115 kDa as judged by comparison with reference proteins; Figure

Table 3. β-Galactosidase Activity in Cell-free Extracts of Induced and Noninduced Cells of *L. plantarum* WCFS1 Carrying Various Expression Plasmids[a]

	volumetric activity (U/mL fermentation broth)		specific activity (U/mg protein)		
plasmid	induced	noninduced	induced	noninduced	induction factor[b]
pTH101	22.5 ± 0.8	1.50 ± 0.04	196 ± 3	10.3 ± 1.1	19
pTH102	15.5 ± 0.6	1.62 ± 0.13	158 ± 3	11.7 ± 0.5	13
pTH103	22.0 ± 1.3	0.63 ± 0.03	193 ± 10	4.11 ± 0.18	47
pTH104	18.0 ± 0.5	0.51 ± 0.04	168 ± 4	3.43 ± 0.13	49

[a]Data are expressed as the average ± standard deviation of three independent cultivations. The specific β-galactosidase activity in cell-free extracts of nontransformed *L. plantarum* was 0.07 U/mg. [b]The induction factors are calculated from the specific β-galactosidase activity obtained under inducing conditions divided by the activity under noninduced conditions in cells harvested at OD_{600} of 1.8−2.0.

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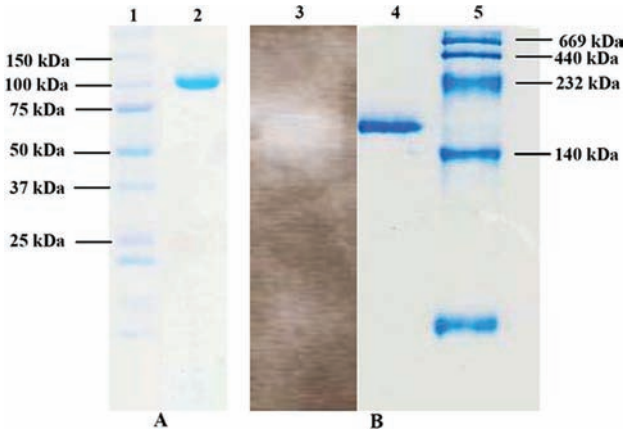

Figure 3. Electrophoretic analysis of purified recombinant *β*-galactosidase from *L. bulgaricus*: (A) SDS-PAGE (lanes: 1, Precision plus Protein standard ladder (Bio-Rad); 2, purified recombinant enzyme); (B) native-PAGE (lanes: 3, activity staining of *β*-galactosidase using 4-methylumbelliferyl *β*-D-galactoside as substrate; 4, purified *β*-galactosidase; 5, high molecular mass protein ladder (GE Healthcare)).

3A) and native PAGE (molecular mass of ~200 kDa; Figure 3B). Gel permeation chromatography and comparison with protein standards of known mass gave a molecular mass of 230 kDa for native LacZ. This is in good agreement with the calculated molecular mass of 114 047 Da deduced for the LacZ subunit from its sequence. Activity staining directly on the native PAGE gel using 4-methylumbelliferyl *β*-galactoside as the substrate indicated furthermore that the protein band of ~200 kDa indeed shows *β*-galactosidase activity (Figure 3B).

Enzyme Kinetics. The steady-state kinetic constants for the hydrolysis of the natural substrate lactose as well as for the artificial substrate *o*-nitrophenol *β*-D-galactopyranoside (*o*NPG) together with the inhibition constants for both end products, D-galactose and D-glucose, for *β*-galactosidase from *L. bulgaricus* are summarized in Table 4. The k_{cat} values were calculated on the basis of the theoretical v_{max} values experimentally determined by nonlinear regression and using a molecular mass of 114 kDa for the catalytically active subunit (115 kDa for His-tagged LacZ). *β*-Galactosidase from *L. bulgaricus* is not inhibited by its substrates lactose in concentrations of up to 600 mM or *o*NPG in concentrations of up to 25 mM as is evident from the Michaelis–Menten plots (not shown). The hydrolysis end products D-galactose and D-glucose competitively inhibit the hydrolytic activity of *β*-galactosidase from *L. bulgaricus*, albeit this inhibition of, for example, D-galactose on cleavage of the natural substrate lactose is only moderate as is evident from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose ($K_{i,Gal}/K_{m,Lac}$ = 3.7). The inhibition by D-glucose is even less pronounced as is obvious from the high inhibition constant measured for the hydrolysis of *o*NPG and the high ratio of K_i to K_m for this reaction ($K_{i,Glc}/K_{m,oNPG}$ = 134).

Effect of Metal Ions on Enzyme Activity. Various mono- and divalent metal ions were tested with respect to a possible stimulating or inhibitory effect on *β*-galactosidase activity. These were added in final concentrations of 1–50 mM to the enzyme in Bis-Tris buffer (Table 5). The monovalent cations

Table 5. Effect of Cations on Activity of *β*-Galactosidase in 10 mM Bis-Tris Buffer, pH 6.5[a]

	relative activity (%)		
cation	1 mM	10 mM	50 mM
blank (none)	100	100	100
Na^+	722	1030	1190
K^+	365	536	507
Mg^{2+}	85	31	nd[b]
Ca^{2+}	77	38	nd
Zn^{2+}	3	0.55	nd

[a]Enzyme activity was determined under standard assay conditions in 10 mM Bis-Tris buffer, pH 6.5, using *o*NPG as the substrate with the respective cation added to give the stated final concentration. Experiments were performed in duplicates, and the standard deviation was always <5%. [b]nd, not determined.

K^+ and especially Na^+ activated *β*-galactosidase activity when using this buffer system considerably; for example, an almost 12-fold increase in activity was found in the presence of 50 mM Na^+ compared to a blank where no metal ion was added to the enzyme sample. When using the standard 50 mM sodium phosphate buffer, pH 6.5, K^+ only resulted in a slight activation of approximately 1.4-fold when added in 10 mM concentrations. The divalent cations Mg^{2+}, Ca^{2+}, and Zn^{2+} showed an inhibitory effect when using Bis-Tris buffer, and especially the latter cation inhibited *β*-galactosidase activity strongly (Table

Table 4. Kinetic Parameters for Recombinant *β*-Galactosidase LacZ from *L. bulgaricus*, both Nontagged and C-Terminally His-Tagged, for the Hydrolysis of Lactose and *o*-Nitrophenyl *β*-D-Galactopyranoside (*o*NPG)

substrate	method for determination of enzyme activity	kinetic parameter[a]	nontagged LacZ	His-tagged LacZ
lactose	release of D-glucose	$v_{max,Glc}$ (μmol min^{-1} mg^{-1})	123 ± 5	111 ± 4
		$K_{m,Lac}$ (mM)	19.2 ± 3.8	19.9 ± 3.8
		k_{cat} (s^{-1})	234 ± 13	211 ± 10
		k_{cat}/K_m (M^{-1} s^{-1})	12300	10600
		$K_{i,Gal}$ (mM)	70.7 ± 16.8	nd
*o*NPG	release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})	317 ± 6	257 ± 5
		$K_{m,oNPG}$ (mM)	0.919 ± 0.088	1.20 ± 0.11
		k_{cat} (s^{-1})	603 ± 15	492 ± 13
		k_{cat}/K_m (M^{-1} s^{-1})	655000	410000
		$K_{i,Glc}$ (mM)	123 ± 9	nd
		$K_{i,Gal}$ (mM)	9.52 ± 1.54	nd

[a]Molecular masses of 114 and 115 kDa were used to calculate k_{cat} from v_{max} for native and His-tagged LacZ, respectively.

5). Interestingly, when using 50 mM sodium phosphate buffer instead of Bis-Tris buffer, Mg^{2+} showed an activating effect (150% relative activity) at concentrations of 1 and 10 mM; this could indicate a synergistic effect with Na^+ present in this buffer.[14]

Effect of Temperature and pH on Enzyme Activity and Stability. The temperature optima of the activity of β-galactosidase from *L. bulgaricus* are 45−50 and 55−60 °C for *o*NPG and lactose hydrolysis, respectively, when using the 10 min assay (Figure 4A). The pH optimum of LacZ activity is pH 7.5 for both substrates lactose and *o*NPG (Figure 4B). Overall, the pH curves show a broad peak with 75% of maximal β-galactosidase activity in the pH range of 6−9 (Figure 4B). Catalytic stability, that is, the length of time the enzyme remains active before undergoing irreversible inactivation, of β-galactosidase from *L. bulgaricus* was measured at a constant pH of 7.0 while the temperature was varied from 37 to 60 °C. In addition, we tested the effect of different buffers and the addition of cations on stability. LacZ activity showed first-order inactivation kinetics when analyzed in the plot of ln(residual activity) versus time (not shown). Data for the inactivation constants k_{in} and half-life times of activity $\tau_{1/2}$ are summarized in Table 6. Regardless of the temperature, stability was comparable in phosphate buffer without added cation and Bis-Tris buffer containing 10 mM Na^+, the metal ion that was found to increase activity significantly. Addition of 10 mM Mg^{2+} to phosphate buffer increased the stability considerably. Under these conditions, *L. bulgaricus* LacZ was well stable at 50 °C with a half-life time of >1 day. When the temperature was increased to 60 °C, activity was, however, lost rapidly (Table 6). This effect of ions such as Mg^{2+} on stability and activity seems common among GH2 β-galactosidases and is also observed for *E. coli* β-galactosidase LacZ[34,35] as well as for some β-galactosidases from *Lactobacillus* spp. of the LacLM type.[6,8] Several metal-binding sites were identified in the structure of *E. coli* LacZ, some of which are located in the direct vicinity of the active site. These ions are thought to take directly part in the catalytic mechanism and also to contribute to subunit interaction and hence stabilization of *E. coli* LacZ.[34,35]

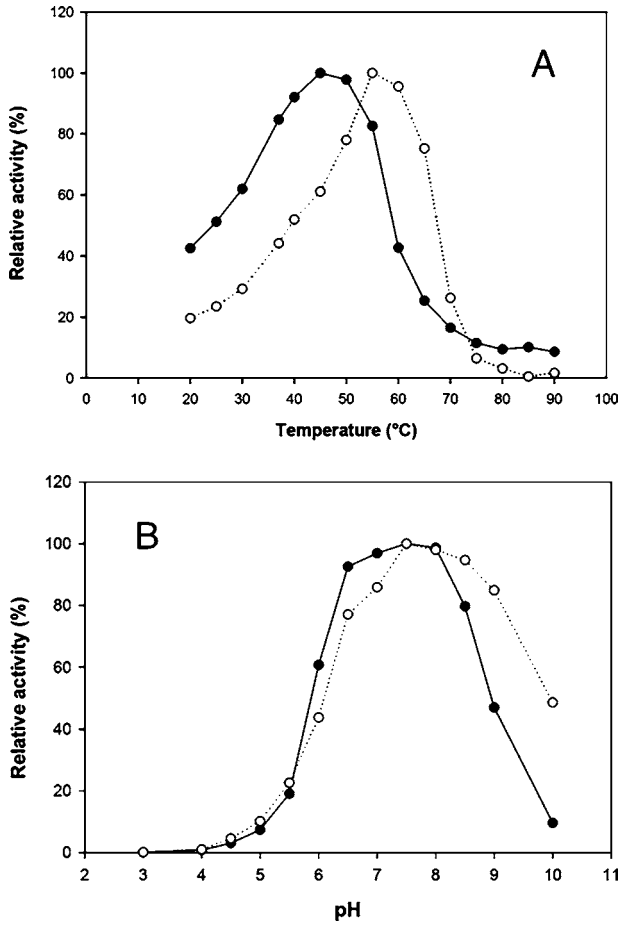

Figure 4. Temperature and pH optima of the activity of recombinant β-galactosidase from *L. bulgaricus*: (○) lactose as substrate; (●) *o*NPG as substrate. Relative activities are given in comparison with the maximum activities measured under optimal conditions (100%), which were 412 and 237 U/mL with *o*NPG and lactose as the substrate, respectively, when determining the temperature optimum (A) and 680 and 106 U/mL with *o*NPG and lactose as the substrate, respectively, for the pH dependence of activity (B).

Lactose Transformation and Synthesis of Galacto-oligosaccharides. The transgalactosylation activity of *L. bulgaricus* LacZ has been described before,[36−38] but has not been studied in much detail; for example, the structures of the main transferase products have not been identified. Lactose conversion and product formation of a typical LacZ-catalyzed reaction, using an initial lactose concentration of 600 mM (205 g/L) in 50 mM sodium phosphate buffer with 10 mM $MgCl_2$, pH 6.5, and 1.5 $U_{lactose}$/mL of β-galactosidase activity at 30 °C, are shown in Figure 5A. During the initial reaction phase, galacto-oligosaccharides (GalOS) are the main reaction products, which are formed together with the primary hydrolysis products D-galactose and D-glucose. The concentration of total GalOS reached a maximum of 102 g/L after 12 h of reaction, when 90% of initial lactose was converted; this corresponds to a yield of almost 50% GalOS. Thereafter, the concentration of GalOS decreased because they are also hydrolyzed by the β-galactosidase. This breakdown of GalOS, however, proceeds only slowly, most probably because of end product inhibition by D-galactose, which at this point of the reaction is present in notable concentrations, and only

Table 6. Catalytic Stability of Recombinant β-Galactosidase from *L. bulgaricus*[a]

	sodium phosphate buffer, pH 7		sodium phosphate buffer, pH 7 + 10 mM Mg^{2+}		Bis-Tris buffer, pH 7 + 10 mM Na^+	
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)
37	0.0053	145	0.0016	345	0.0084	82.5
50	0.925	0.75	0.026	26	1.12	0.62
60	15.3	0.045	1.0	0.32	16.9	0.041

[a]The inactivation constant k_{in} and half-life time of activity $\tau_{1/2}$ were calculated at different temperatures and reaction conditions. Buffer concentrations were 50 mM each. Experiments were performed in duplicates, and the standard deviation was always <5%.

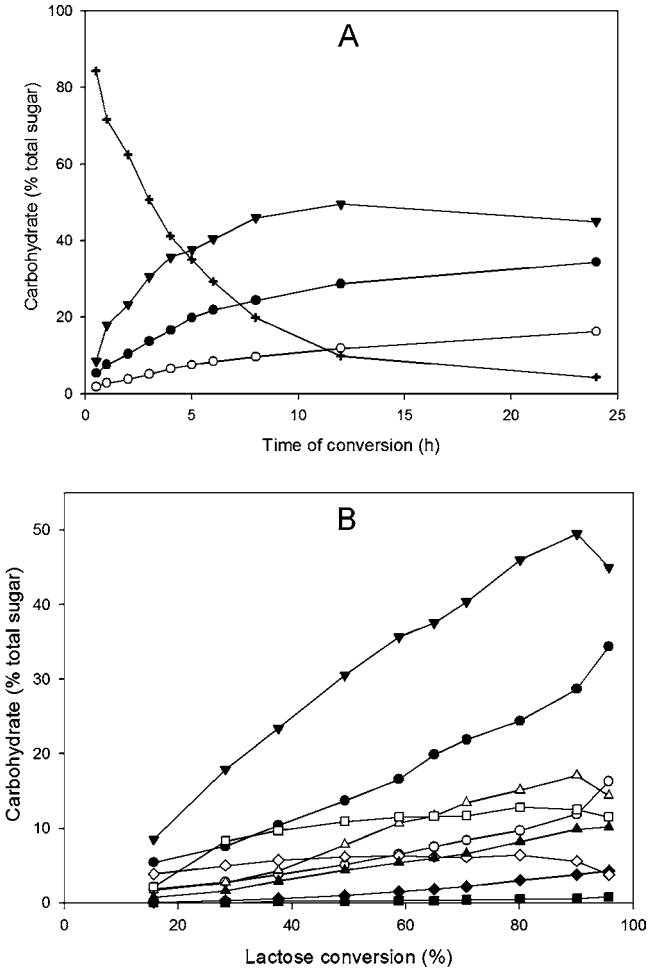

Figure 5. Composition of the sugar mixture during lactose conversion by recombinant β-galactosidase from *L. bulgaricus*. The reaction was carried out at 30 °C with an initial concentration of 600 mM lactose in 50 mM sodium phosphate buffer, pH 6.5, in the presence of 10 mM $MgCl_2$ using ~1.5 $U_{lactose}$/mL of enzyme. (A) Time course of the conversion: (+), lactose; (●), glucose; (○), galactose; (▼) total galacto-oligosaccharides (GalOS). (B) Composition of the sugar mixture and individual GalOS components at different degrees of lactose conversion: (●), glucose; (○), galactose; (▼) total (GalOS); (♦), β-D-Gal*p*-(1→3)-D-Glc; (■), β-D-Gal*p*-(1→3)-D-Gal; (◇), β-D-Gal*p*-(1→3)-Lac; (△), β-D-Gal*p*-(1→6)-D-Glc; (□), β-D-Gal*p*-(1→6)-Lac; (◆), unidentified GalOS. Monosaccharides were measured enzymatically, lactose and GalOS were quantified by HPAEC-PAD and CE. Individual sugars are given as the percentage of total sugars (205 g/L) in the mixture.

approximately 10% of total GalOS are degraded when the reaction proceeds for another 12 h. A detailed analysis of the main transferase products formed is given in Figure 5B. Up to ~90% lactose conversion, the amount of total GalOS, expressed by their relative concentration (percentage of GalOS of total sugars in the reaction mixture) was increasing almost linearly. At the beginning of the reaction, the trisaccharides β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→3)-Lac were formed predominately. With further progress of the reaction, the concentrations of D-galactose and D-glucose increased steadily, and these monosaccharides became important acceptors for the transferase reaction; hence, disaccharides other than lactose are formed as well. Non-lactose disaccharides were prevailing by weight at around 75% lactose conversion and later, with β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Glc as the two main products. In addition to these main GalOS components, β-D-Gal*p*-(1→3)-D-Gal and β-D-Gal*p*-(1→6)-D-Gal were identified in the reaction mixtures; these were, however, minor constituents. GalOS containing new β-(1→4) linkages could not be identified in these mixtures. β-Galactosidase from *L. bulgaricus* formed GalOS structurally similar to those obtained with other β-galactosidases from LAB,[7,9,15,16,39] yet proportions of individual components varied to some extent. The predominant oligosaccharide products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→6)-Lac, together accounting for approximately 60% of the GalOS, indicating that this β-galactosidase has a propensity to synthesize β-(1→6)-linked GalOS.

To examine whether the high thermostability of *L. bulgaricus* LacZ can be exploited for GalOS synthesis, we also ran the lactose conversion experiments at higher temperatures, that is, 40 and 50 °C, using otherwise identical conditions. Table 7 lists these results for a comparable degree of lactose conversion of ~90%. The reaction mixture showed a very similar composition regardless of the reaction temperature. However, the time needed to obtain 90% lactose conversion was reduced, from 12 h of reaction time at 30 °C to 8 h at 40 °C and only 5 h at 50 °C, and therefore the productivity increased from 8.5 to 19.8 g L^{-1} h^{-1} GalOS for the LacZ-catalyzed reaction at the highest temperature tested. It is interesting to note that the reaction temperature hardly affected the maximum GalOS yield or the composition of the GalOS mixture. Several studies have shown that transgalactosylation becomes more pronounced compared to hydrolysis at higher temperatures.[40,41]

Table 7. Oligosaccharide Components (% w/w of Total Sugar) of GalOS Mixtures Obtained with β-Galactosidase of *L. bulgaricus* at Three Different Temperatures[a]

GalOS component	reaction temperature		
	30 °C	40 °C	50 °C
glucose	28.7	31.0	32.5
galactose	11.9	13.5	14.2
total GOS	49.5	48.7	48.2
β-D-Gal*p*-(1→3)-D-Gal	0.6	0.6	0.6
β-D-Gal*p*-(1→3)-D-Glc	3.8	4	3.9
β-D-Gal*p*-(1→3)-Lac	5.6	5.1	4.5
β-D-Gal*p*-(1→6)-D-Gal	1.0	1.3	1.1
β-D-Gal*p*-(1→6)-D-Glc	17.1	15.5	15
β-D-Gal*p*-(1→6)-Lac	12.5	12.5	13.2
unknown OS	8.9	9.7	9.9
lactose conversion	90.1[b]	93.2[c]	94.9[d]

[a]A lactose concentration of 600 mM (205 g/L) and 1.5 U/mL of β-galactosidase activity (determined with lactose as substrate under standard assay conditions) were used in each experiment. Data are given for the maximal yields obtained during the course of the reaction. Experiments were performed in duplicate, and the standard deviation was always <5%. [b]At 12 h. [c]At 8 h. [d]At 5 h.

In conclusion, the properties of β-galactosidase LacZ from *L. bulgaricus* differ in some important aspects from those of lactobacillal β-galactosidases of the LacLM type. Its high activity, modest inhibition by the end product D-galactose, and high transgalactosylation activity together with its thermostability make this enzyme an attractive biocatalyst for various food-related applications.

AUTHOR INFORMATION

Author Contributions

[‖]T.-T.N. and H.A.N contributed equally to this work.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CAZy, Carbohydrate-Active enZYmes Database; CE, capillary electrophoresis; DTT, 1,4-dithiothreitol; GalOS, galacto-oligosaccharides; GOD, glucose oxidase; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; Lac, lactose; MUG, 4-methylumbelliferyl β-D-galactoside; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl-β-D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride.

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