**β -Galactosidase from *Lactobacillus pentosus*:
Purification, characterization and formation of
galacto-oligosaccharides**

Thomas Maischberger, Elisabeth Leitner, Sunee Nitisinprasert, Onladda Juajun, Montarop Yamabhai, Thu-Ha Nguyen, Dietmar Haltrich

► **To cite this version:**

Thomas Maischberger, Elisabeth Leitner, Sunee Nitisinprasert, Onladda Juajun, Montarop Yamabhai, et al.. β -Galactosidase from *Lactobacillus pentosus*: Purification, characterization and formation of galacto-oligosaccharides. *Biotechnology Journal*, 2010, 5 (8), pp.838. 10.1002/biot.201000126 . hal-00560295

HAL Id: hal-00560295

<https://hal.science/hal-00560295>

Submitted on 28 Jan 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

β -Galactosidase from *Lactobacillus pentosus*: Purification, characterization and formation of galacto-oligosaccharides

Journal:	<i>Biotechnology Journal</i>
Manuscript ID:	biot.201000126.R2
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	02-Jul-2010
Complete List of Authors:	<p>Maischberger, Thomas; Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences; Research Centre Applied Biocatalysis</p> <p>Leitner, Elisabeth; Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences</p> <p>Nitisinprasert, Sunee; Department of Biotechnology, Kasetsart University</p> <p>Juajun, Onladda; Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences; School of Biotechnology, Suranaree University of Technology</p> <p>Yamabhai, Montarop; Suranaree University of Technology, School of Biotechnology</p> <p>Nguyen, Thu-Ha; Vienna University of Natural Resources and Applied Life Sciences, Department of Food Sciences and Technology, Food Biotechnology Lab</p> <p>Haltrich, Dietmar; Universität für Bodenkultur, Food Sciences and Technology</p>
Primary Keywords:	Food Biotechnology
Secondary Keywords:	Enzymes
Keywords:	lactase, lactic acid bacteria, transgalactosylation, biocatalysis

Research Article 6532 words

β -Galactosidase from *Lactobacillus pentosus*: Purification, characterization and formation of galacto-oligosaccharides

THOMAS MAISCHBERGER^{1,2}, ELISABETH LEITNER¹, SUNEE NITISINPRASERT³,
ONLADDA JUAJUN^{1,4}, MONTAROP YAMABHAI⁴, THU-HA NGUYEN^{1,2,*} AND DIETMAR
HALTRICH¹

¹ Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences,
Vienna, Austria

² Research Centre Applied Biocatalysis, Graz, Austria

³ Department of Biotechnology, Kasetsart University, Bangkok, Thailand

⁴ School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima, Thailand

Keywords: Lactase · Lactic acid bacteria · Transgalactosylation · Biocatalysis

* **Correspondence:** Dr. Nguyen Thu-Ha (e-mail: thu-ha.nguyen@boku.ac.at).

Address: Lebensmittelbiotechnologie, Department für Lebensmittelwissenschaften und -
technologie, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Vienna, Austria.
Phone: 43-1-47654 6146. Fax: 43-1-47654 6251.

Abbreviations: β -Gal, β -galactosidase; CE, capillary electrophoresis; GOS, galacto-
oligosaccharides; HPAEC-PAD, high-performance anion exchange chromatography with
pulsed amperometric detection; MUG, 4-methylumbelliferyl- β -D-galactopyranoside; oNPG, o-
nitrophenyl- β -D-galactopyranoside, oNP, o-nitrophenol

Abstract

A novel heterodimeric β -galactosidase with a molecular mass of 105 kDa was purified from the crude cell extracts of the soil isolate *Lactobacillus pentosus* KUB-ST10-1 using ammonium sulphate fractionation followed by hydrophobic interaction and affinity chromatography. The electrophoretically homogenous enzyme has a specific activity of 97 U_{oNPG}/mg protein. The K_m , k_{cat} and k_{cat}/K_m values for lactose and oNPG were 38 mM, 20 s⁻¹, 530 M⁻¹·s⁻¹ and 1.67 mM, 540 s⁻¹, 325,000 M⁻¹·s⁻¹, respectively. The temperature optimum of β -galactosidase activity was 60–65 °C for a 10-min assay, which is considerably higher than the values reported for other lactobacillal β -galactosidases. Mg²⁺ ions enhanced both activity and stability significantly. *L. pentosus* β -galactosidase was used for the production of prebiotic galacto-oligosaccharides (GOS) from lactose. A maximum yield of 31% GOS of total sugars was obtained at 78% lactose conversion. The enzyme showed a strong preference for the formation of β -(1→3) and β -(1→6) linkages, and the main transgalactosylation products identified were the disaccharides β -D-Galp-(1→6)-D-Glc, β -D-Galp-(1→3)-D-Glc, β -D-Galp-(1→6)-D-Gal, β -D-Galp-(1→3)-D-Gal, and the trisaccharides β -D-Galp-(1→3)-D-Lac, β -D-Galp-(1→6)-D-Lac.

1 Introduction

The lactic acid bacterium *L. pentosus* is frequently used as starter culture for silage fermentations and various food fermentation processes, including sauerkraut and raw sausage production, or olive and tea leave fermentations, to name a few. Most of *L. pentosus* subspecies are plant isolates, generally recognised as safe (GRAS), and capable of metabolising different pentoses such as ribose, arabinose and xylose [1]. Yet, some subspecies of *L. pentosus* were isolated from e.g. human faeces [2] or from raw milk [3], which were proposed as potentially probiotic strains. Furthermore, Fred *et al.* previously described very effective growth of *L. pentosus* on lactose, which could imply high β -galactosidase activity [4], but this property has not been studied to date.

Lactic acid bacteria (LAB) and β -galactosidases have been studied extensively in the past, both from a fundamental and an applied point of view. β -Galactosidases from e.g. *Bifidobacterium bifidum*, *B. infantis* [5], *Carnobacterium piscicola* [6], *L. acidophilus* [7], *L. bulgaricus* [8], *L. reuteri* [9], and *Leuconostoc lactis* [10] were cloned and characterised to a varying detail. Despite of the importance of LAB and in particular *Lactobacillus* spp. for food technology and dairy applications and despite of numerous studies on the gene clusters involved in lactose utilisation, only few lactobacillal β -galactosidases were characterised in detail pertaining to their biochemical properties or were investigated for their ability to produce galacto-oligosaccharides (GOS) in biocatalytic processes [11-13]. These carbohydrate-based food ingredients are of interest as novel and possibly improved prebiotics, or for the development of synbiotic functional food that could introduce new dimensions of applications [14].

GOS belong to the prebiotics, 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-being and health”

[15]. In practice, the beneficial bacteria that serve as targets for prebiotics and GOS are almost exclusively bifidobacteria and lactobacilli. An advantage of the concept of prebiotics to modify gut function is that the target bacteria are already commensal to the large intestine, whereas with probiotics allochthonous micro-organisms are introduced and have to compete against established colonic communities [16]. GOS have been classified as one of the few proven prebiotics fulfilling the three criteria (i) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being [15]. GOS have been widely investigated for their prebiotic properties and physiological effects using *in vitro*, animal, human, and infant studies, and functional effects of GOS on human health are summarised in [16], [20] and [21].

GOS are produced from lactose using β -galactosidases (E.C. 3.2.1.23), which in addition to their hydrolytic activity catalyse glycosyl transfer reactions. In this reaction galactosyl residues are transferred onto suitable acceptors such as another sugar (lactose, the primary reaction products glucose and galactose, or other oligosaccharides formed), thus building up complex series of higher oligosaccharides [17-19]. The spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the β -galactosidase used for the biocatalytic reaction, and on the conversion conditions used in their production. Since these differences in GOS spectrum and yields are a result of structural and/or mechanistic differences between β -galactosidases from different sources, a detailed knowledge on novel, yet-unexplored β -galactosidases from various strains can be of significant interest [18]. Rabiou *et al.* [22] and Tzortzis *et al.* [23] synthesised a range of GOS mixtures from lactose using β -galactosidases from different bifidobacteria. Subsequently it was

shown that these different mixtures typically resulted in better growth of that strain that had served as the source of the enzyme for GOS production. This concept may serve as the basis for a new generation of functionally enhanced, targeted oligosaccharides, and has increased the interest in β -galactosidases from beneficial probiotic organisms.

The objective of this work was to study the β -galactosidase from *L. pentosus* in detail and to compare it to other lactobacillal β -galactosidases isolated from organisms of typical animal origin. Recent data are indicating that *L. pentosus* might be of interest as a beneficial LAB making it attractive as a probiotic strain, e.g. based on immunomodulating properties [24] or the reduction of pathogens in animals [25].

2 Material and Methods

2.1 Strain and culture conditions

Lactobacillus pentosus KUB-ST10-1 was from the culture collection of Kasetsart University (Bangkok, Thailand). It was isolated from soil of a dairy farm in Thailand and identified by using both the API 50 CH carbohydrate fermentation stripes test (bioMérieux, Inc., Marcy l'Etoile, France) and sequence analysis of the amplified chromosomal 16S rDNA. It was stored at -70°C in MRS broth medium (Merck, Darmstadt, Germany) containing 15% glycerol. To prepare fresh inocula, it was transferred twice after an 18-h cultivation period to fresh MRS broth medium supplemented with lactose (final concentration of 2%).

L. pentosus KUB-ST10-1 was grown anaerobically in 10 L of MRS broth medium containing 2% lactose at 37°C with slight agitation and without pH control. A preculture, grown overnight in MRS medium (2% lactose (w/v)), was used to inoculate the fresh medium to a final concentration of 1% (v/v) inoculum. Cells were

95 harvested at an optical density (OD₆₀₀) of 13 by centrifugation at 8800 x *g* for 15 min
at 4°C.

2.2 Enzyme purification

100 β -Galactosidase was purified from the crude extract using a previously described
protocol [9]. Wet biomass (100 g) was resuspended in 200 mL of sodium phosphate
buffer (50 mM, pH 6.5) and homogenized (APV-2000; Silkeborg, Denmark) to disrupt
the cells. Cell debris was removed by ultracentrifugation (36000 x *g*, 30 min, 4°C) to
obtain the crude enzyme extract.

105 *Ammonium sulphate precipitation (ASP)*. Ammonium sulphate was added in small
portions to the crude extract to a final concentration of 50% saturation under
continuous stirring at 4°C. The precipitated protein was collected by centrifugation
(6200 x *g*, 15 min, 4°C), and the pellet was re-dissolved in 50 mM sodium phosphate
buffer (pH 6.5) containing 1 M ammonium sulphate (buffer A).

110 *Hydrophobic Interaction Chromatography (HIC)*. The dissolved pellet was loaded
onto a 20-mL phenyl-sepharose fast flow column (50 mm x 200 mm; Amersham,
Uppsala, Sweden) that had been pre-equilibrated with buffer A. Proteins were eluted
by a linear gradient of 0% to 100% buffer B (50 mM sodium phosphate buffer, pH
6.5) over 10 column volumes (CV) and at a flow rate of 1.5 mL/min. Fractions with
high β -galactosidase activity were pooled and concentrated by ultrafiltration (30-kDa
cut-off; Amicon, Beverly, MA).

115 *Affinity Chromatography (AC)*. The concentrated protein solution was applied to a 5-
mL column containing crosslinked (4%) beaded agarose immobilized with *p*-
aminobenzyl-1-thio- β -D-galactopyranoside (Sigma, St. Louis, MO) that was pre-
equilibrated with 50 mM phosphate buffer pH 6.5. The enzyme was eluted using a
flow rate of 0.5 mL/min and a linear gradient (0 to 1 M NaCl in the same buffer) over

10 CV. Active fractions were pooled, desalted and concentrated by ultrafiltration. The purified enzyme was stored in 50 mM sodium phosphate buffer (pH 6.5) at 4 °C.

2.3 Enzyme assays

β -Galactosidase activity was determined using both the artificial chromogenic substrate *o*-nitrophenyl- β -D-galactopyranoside (*o*NPG) and the natural substrate lactose as described previously [9]. When *o*NPG was the substrate, the reaction was performed in 50 mM sodium phosphate buffer (pH 6.5) and using a 10-min incubation at 30 °C, after which the reaction was stopped. One unit of *o*NPG activity (U_{oNPG}) is defined as the amount of enzyme releasing 1 μ mol of *o*NP per minute under the assay conditions described above. When lactose was used as the substrate, the reaction was done in 50 mM sodium phosphate buffer (pH 6.5) for 10 min at 30 °C, after which the reaction was stopped. The release of D-glucose was determined colourimetrically using the glucose oxidase/peroxidase (GOD/POD) assay [26]. One unit of lactase activity (U_{Lac}) is defined as the amount of enzyme releasing 1 μ mol of D-glucose per minute under the conditions listed above.

2.4 Protein analysis and gel electrophoresis

Protein concentrations were determined by the method of Bradford [27] using bovine serum albumin as the standard. The PhastSystem unit (Amersham) and precast polyacrylamide gels (Phastgel 8–25, Amersham) were used for performing native and SDS-PAGE. Samples were treated as described by Laemmli [28] with slight modifications. The enzyme was diluted to 1–2 mg of protein per mL and incubated with 2x Laemmli buffer at 60 °C for 5 min. Protein bands were stained using either Coomassie Blue staining or 4-methylumbelliferyl- β -D-galactopyranoside (MUG) as substrate for active staining. For SDS-PAGE and native PAGE molecular masses

were estimated using the Precision Plus Protein Dual Color Kit (Biorad) and the High Molecular Weight calibration Kit (Amersham), respectively.

2.5 Steady-state kinetic measurement

Catalytic constants were determined at 30 °C and pH 6.5 for the two substrates oNPG and lactose with their concentrations in 50 mM phosphate buffer varying from 0.1–22 mM and 0.1–600 mM, respectively. The kinetic parameters were calculated by non-linear regression, and the obtained data were fit to the Henri-Michaelis-Menten equation (using SigmaPlot, SPSS Inc., Chicago, IL, USA).

2.6 Effects of temperature and pH on enzyme activity and stability

The temperature dependence of β -galactosidase activity (both oNPG and lactose) was determined by assaying the enzyme samples over the range of 20 to 70 °C for 10 min. To study thermal stability, enzyme samples were incubated in 50 mM sodium phosphate buffer, pH 6.5, at 4, 23, 30, 37, and 42 °C. At certain time intervals samples were taken and the residual oNPG activity was measured. For measuring the pH dependence of β -galactosidase activity three buffer systems (sodium citrate, 50 mM, pH 4.0–5.5; sodium phosphate, 50 mM, pH 5.5–7.5; glycine, 50 mM, pH 7.5–9.0) were used. For the determination of the pH stability enzyme samples were incubated at various pH values and 37 °C for up to 72 h, and the remaining enzyme activity was measured at certain time intervals using oNPG as substrate under standard assay conditions.

2.7 Substrate specificity

To determine the substrate specificity of the *L. pentosus* β -galactosidase for various structurally related substrates *p*-nitrophenyl- β -D-mannopyranoside, *p*-nitrophenyl- β -D-

xylopyranoside, *o*-nitrophenyl- β -D-glucopyranoside and *p*-nitrophenyl- β -D-cellobioside were used. Activities were determined in accordance to standard *o*NPG assay conditions using the respective substrate in concentrations of 22 mM.

2.8 Transgalactosylation activity

Purified β -galactosidase from *L. pentosus* (5 U_{*o*NPG} per mL reaction mixture) was used for the discontinuous formation of galacto-oligosaccharides (GOS) at 30 °C using 205 g/L of lactose in 50 mM sodium phosphate buffer (pH 6.5, 2 mM MgCl₂) as substrate. Samples were taken at various intervals and the composition of the GOS mixture was analyzed by capillary electrophoresis (CE) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described previously [13].

3 Results

3.1 Formation and purification of β -galactosidase from *L. pentosus*

L. pentosus KUB-ST10-1 was selected for detailed studies on its β -galactosidase since this strain grew very well on lactose, it showed high β -gal activity in a screening, in which several lactic acid bacteria were compared pertaining to their ability to form this enzyme activity (data not shown), and since its β -galactosidase had not been studied in any detail previously. Batch cultivations of *L. pentosus* were carried out in a 10-L laboratory fermenter at 37 °C without pH control using lactose-based MRS medium. The biomass was harvested after 18 h of growth when β -gal activity reached a maximum of 644 units (U_{*o*NPG}) per litre fermentation broth. β -Galactosidase was purified to apparent homogeneity from the crude cell extract using a two-step purification protocol based on hydrophobic interaction and affinity

chromatography. The enzyme was purified 16-fold to a specific activity of 97 U/mg of protein, using standard assay conditions with oNPG as the substrate.

3.2 Properties of β -galactosidase from *L. pentosus*

L. pentosus β -galactosidase is a heterodimer (M_r ~107 kDa) consisting of a small (~35 kDa) and large (~72 kDa) subunit as judged by SDS-PAGE (Figure 1A) and native PAGE (Figure 1B). The additional protein band (~80 kDa) in native PAGE when staining with Coomassie Blue presumably results from degradation of the intact ~107-kDa enzyme, as was previously shown for the related β -gal from *L. reuteri* by using mass spectrometry [9]. Active staining on native PAGE with MUG yielded only one band with β -galactosidase activity, corresponding to the larger band in Figure 1B. Active staining on the SDS-PAGE gel after treating the enzyme in SDS buffer at 60 °C for 5 min, thus separating the two subunits without unfolding, showed that one band corresponding to the large subunit exhibited activity with MUG (not shown). In contrast, the small subunit by itself did not show any activity. When the enzyme was treated in SDS buffer at ~100 °C for 5 min, no activity could be detected after active staining on the gel.

3.3 Substrate specificity

To study the substrate specificity of *L. pentosus* β -galactosidase various structurally related artificial substrates were tested under standard assay conditions and compared to oNPG. No appreciable activity was detected when using *p*-nitrophenyl- β -D-xylopyranoside, *o*-nitrophenyl- β -D-glucopyranoside, *p*-nitrophenyl- β -D-mannopyranoside and *p*-nitrophenyl- β -D-cellobioside as substrates for the activity assay.

3.4 Enzyme kinetics

Steady-state kinetic studies were performed with the preferred substrates lactose and oNPG and the kinetic constants K_m , v_{max} , and k_{cat} were determined. The k_{cat} values were calculated on the basis of theoretical v_{max} values obtained by nonlinear regression using SigmaPlot. Data are summarized in **Table 1**. The catalytic efficiencies (k_{cat}/K_m) for the two substrates, oNPG and lactose, indicate that oNPG is the preferred substrate in accordance with other microbial β -galactosidases.

3.5 Effects of pH and temperature

The pH optimum of *L. pentosus* β -galactosidase activity is pH 8.0 and pH 7.5 in glycine buffer when using lactose and oNPG as substrate, respectively (**Figure 2**). In general, the enzyme is more active in the alkaline range (pH 7.0–8.5), and it showed a pronounced effect of the buffer system used on activity. The effect of different buffers and pH values on stability was investigated subsequently in more detail. Catalytic or kinetic stability, *i.e.*, the length of time an enzyme remains active before undergoing irreversible inactivation, was measured for various pH values at a constant temperature of 37°C, and the inactivation constants (k_{in}) and the half-life times of denaturation ($\tau_{1/2}$) were determined (**Table 2**). β -Galactosidase showed first-order inactivation kinetics at all pH values studied when analysed in the ln(residual activity) versus time plot (not shown). Highest $\tau_{1/2}$ values were calculated in glycine buffer (pH 8.0 and 8.5) where the stability was increased up to 3.2-fold compared to when using sodium phosphate buffer at pH 6.5. Highest inactivation constants and hence least stabilities were obtained in sodium acetate buffer (pH 4.0 and 5.0) and glycine buffer (pH 9.0), with $\tau_{1/2}$ values of less than 1.5 h.

The optimum temperature measured under standard assay conditions (10 min) for lactose and oNPG hydrolysis was 60–65°C and 55°C, respectively (**Figure 3**). The effect of temperature on stability of β -galactosidase from *L. pentosus* was

investigated at the pH value of milk, pH 6.5, both in the absence and presence of 2 mM MgCl_2 . Inactivation constants and half-life times were calculated as described above. The addition of 2 mM MgCl_2 to the buffer improved stability significantly, increasing the half life times $\tau_{1/2}$ of the enzyme at 30, 37 and 42 °C 3.2-, 4.9- and 5.6-fold, respectively (**Table 3; Figure 4**).

3.6 Transgalactosylation activity

In order to assess the potential of the novel β -galactosidase from *L. pentoses* for the production of GOS, a discontinuous lactose conversion process was studied at 30 °C, using an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (+ 2 mM MgCl_2 , pH 6.5) and 5 U_{ONPG} /mL of β -galactosidase activity to produce GOS.

The maximum GOS yield (31%) was obtained at a lactose conversion of 78% (**Figure 5**). The newly formed sugar mixture contained 31.5% glucose, 15.9% galactose, 21.9% lactose, 11.4% non-lactose disaccharides, 18.7% trisaccharides and 0.6% tetrasaccharides as analysed by capillary electrophoresis and high-performance anion exchange chromatography with pulsed amperometric detection, and using authentic sugars and the standard addition technique for comparison. The main GOS products identified were non-lactose disaccharides mainly of the β -(1→3) and β -(1→6) linkage type as well as the two trisaccharides β -D-Galp-(1→3)-lactose, and β -D-Galp-(1→6)-lactose.

4 Discussion

The genus *Lactobacillus* is the largest among the *Lactobacteriaceae* and includes more than a hundred described species [29]. Lactobacilli are nutritionally fastidious, metabolize a varying range of different carbohydrates, and are associated with a large variety of animals and plants. They are extensively used for fermentations of

1
2
3 265 dairy products, meat, fish and plant material such as vegetables, and thus have been
4
5 investigated intensively for their industrial applications [30]. Lactose utilisation is a
6
7 primary function of lactobacilli and other LAB used in the fermentation of milk. The
8
9 mechanism by which lactose is transported into the cell determines largely the
10
11 subsequent pathway for the hydrolysis of this disaccharide. In several *Lactobacillus*
12
13
14
15 270 species lactose is transported via phosphotransferase systems, which results in
16
17 phosphorylation of lactose concomitant with its uptake. The resulting lactose-6'-
18
19 phosphate is then hydrolysed by phospho- β -galactosidase. Alternatively, lactose is
20
21 taken up by secondary transport systems, and lactose is further metabolised by β -
22
23 galactosidase within the cell [31, 32]. While the organisation of these lactose genes,
24
25
26
27 275 which are often forming operons or operon-like structures with modular organisation,
28
29 has been studied in much detail, the properties of some of the enzymes encoded by
30
31 the genes organised in these operons, including β -galactosidases, have received
32
33 significantly less attention. It is thus surprising that β -galactosidases from lactobacilli,
34
35 which play an eminent role in the metabolism of the principal carbohydrate in milk,
36
37
38
39 280 have only been characterised in detail pertaining to their physical and biochemical
40
41 properties in a few instances, and little attention has been paid to the catalytic ability
42
43 of these enzymes with respect to their transglycosylation reactions [12, 17, 33].
44
45
46 Recently, we reported on the comprehensive characterisation of the β -galactosidases
47
48 from *L. reuteri* and *L. acidophilus*, and pointed out their applicability for the
49
50
51 285 biocatalytic production of GOS [9, 13, 34, 35]. In this paper, we present the detailed
52
53 biochemical characterisation and the transgalactosylation activity of a novel β -
54
55 galactosidase from *L. pentosus*. The strain KUB-ST10-1 used throughout this
56
57 research was isolated from a soil sample in Thailand, and was selected for further
58
59 characterisation based on a screening and its high β -galactosidase activity when
60
290 grown on lactose. It was of interest to determine whether a β -galactosidase from a

source other than those traditionally used in the dairy industry may have unique properties or characteristics. In a recent phylogenetic analysis based on 16S rRNA genes the genus *Lactobacillus* was grouped into five major divisions [36]. In this grouping, *L. acidophilus* belongs to group A and *L. reuteri* to group B, while *L. pentosus* is a member of group C [36], hence might differ in its metabolic activities from the sources of β -galactosidases recently characterised in our group.

The structural features of β -galactosidases from lactobacilli are varying to a great extent. While heterodimeric structures are in general less frequently encountered among microbial β -galactosidases, these seem to prevail among *Lactobacillus* β -galactosidases. Lactobacillal heterodimeric β -galactosidases are encoded by two genes, *lacL* and *lacM*, and have been described in e.g., *L. acidophilus* [34, 37], *L. coryniformis* [38], *L. helveticus* [39], *L. johnsonii* [40], *L. plantarum* [41], *L. reuteri* [9] and *L. sakei* [42]. These β -galactosidases of the LacLM type have been classified as members of glycoside family GH2 in the CAZy (Carbohydrate-Active Enzymes) databank (<http://www.cazy.org>). In contrast to this, β -galactosidase from *L. salivarius* was reported to be a monomer of ca. 30 kDa [43], the enzyme from *L. delbrueckii* subsp. *bulgaricus* a homodimer of 220 kDa [44], β -galactosidase from *L. helveticus* a homotetramer of 257 kDa [45], and *L. plantarum* forms – in addition to the above mentioned heterodimeric enzyme – a homohexameric β -galactosidase of ca. 325 kDa [46]. Some of these later β -galactosidases are members of glycoside family GH42. β -Galactosidase from *L. pentosus* KUB-ST10-1 is a heterodimer consisting of two subunits of approximately 35 and 72 kDa, which is in agreement with the majority of lactobacillal β -galactosidases reported hitherto, and is thus also of the LacLM type. The steady-state kinetic constants for β -galactosidase from *L. pentosus* were determined for the artificial chromogenic substrate oNPG and the natural substrate lactose. The k_{cat}/K_m value for oNPG is significantly higher than that of lactose,

indicating oNPG as the better substrate. The K_m value of 37.8 mM determined for lactose compares positively with the Michaelis constants reported for commercially employed β -galactosidases, e.g., from *Aspergillus niger* (54–100 mM), *A. oryzae* (36–180 mM), *Kluyveromyces fragilis* (15–52 mM) [47] and *Kluyveromyces lactis* (20 mM) [48]. This value is, however, somewhat higher than the values reported for other *Lactobacillus* β -galactosidases: 13 mM for β -gal from *L. reuteri* L103 [9], 1.27 mM for β -gal from *L. kefiranofaciens* [49], and 4.04 mM for β -gal from *L. acidophilus* [34]. The higher $K_{m, \text{lactose}}$ value for *L. pentosus* β -gal as compared to other lactobacillal enzymes, especially those of milk origin, could indicate a lesser adaptation of this soil-isolated strain to lactose utilization. *L. pentosus* β -gal is rather specific for the β -(1→4) galactosyl linkage. When using a range of structurally related nitrophenyl substrates, essentially no activity was measured for the β -(1→4) linked D-glucoside, D-mannoside, and D-xyloside substrates.

L. pentosus β -galactosidase is optimally active at pH 7.5 (glycine buffer) and 55°C when using oNPG as substrate, and pH 8.0 (glycine buffer) and 60–65°C when lactose was used as substrate under otherwise standard activity assay conditions (10-min assay). This pH optimum is slightly more alkaline than reported for most other lactobacillal β -galactosidases, which show an optimum of 6.5–7.0 [9, 34], however, we observed a pronounced effect of the buffer system used on the optimal pH, and that certainly affects the actual value determined. The temperature optimum of *L. pentosus* β -gal of 60–65°C is considerably higher than reported for most other β -galactosidases from lactobacilli, which typically are optimally active in the range of 45–50°C [9, 34, 43, 50]. *L. pentosus* KUB-ST10-1 can grow at temperatures of up to 45°C. This is higher than typical growth temperatures observed for many *Lactobacillus* spp. The increased temperature optimum and stability of the *L. pentosus* β -galactosidase might reflect an adaptation to these higher growth

temperatures. Because of this increased temperature optimum we investigated the thermostability of *L. pentosus* β -gal in more detail, determining the inactivation kinetics at different conditions. With respect to pH, highest half-life times $\tau_{1/2}$ were obtained in glycine buffer at pH 8.0–8.5 and at 37°C (43–47 h). Again, the buffer system had a pronounced effect on stability, since the inactivation constants k_{in} determined were approximately twice as high at pH 7.5 when using sodium phosphate instead of glycine buffer; however the use of glycine buffer might be impractical for technical applications. When using the former buffer, highest stability was observed at pH 6.5–7.0 with $\tau_{1/2}$ of 14.6 h. Addition of 2 mM Mg^{2+} increased stability and hence $\tau_{1/2}$ of β -gal activity significantly by a factor of up to 5.6-fold. For example, $\tau_{1/2}$ values of e.g. ~75 h were obtained at pH 6.5, 37°C and when using phosphate buffer containing 2 mM of Mg^{2+} . This increase in stability and activity of *L. pentosus* β -gal could be a considerable advantage when using technical substrates such as cheese whey or whey permeates for the production of GOS, since cheese whey contains approximately 1.5 mM of Mg^{2+} . Thus, use of these technical substrates could improve the performance of *L. pentosus* β -gal without the need of adding exogenous Mg^{2+} .

β -Galactosidases have recently attained significant interest in biocatalytic processes towards the synthesis of oligosaccharides or various galactosylated compounds; for this purpose various galactosides can serve as galactosyl donor. Especially galactooligosaccharides (GOS), which are formed from lactose as a result of transgalactosylation, have attracted renewed attention in the field of functional food because of their proven benefits on health [16, 17, 18, 20]. As GOS are only transiently formed and are in turn substrates for the β -galactosidase as well, a maximum GOS yield of 31% (w/v) was reached at 78% lactose conversion when using the β -gal of *L. pentosus*. The main GOS spectrum formed from lactose by this

enzyme is very similar to those found for β -gal from *L. reuteri* [13] and *L. acidophilus* [34]. *L. pentosus* β -gal shows a high specificity for the formation of β -(1 $\rightarrow$ 6) and β -(1 $\rightarrow$ 3) linkages, and hence the main transgalactosylation products detected were the disaccharides β -D-Galp-(1 $\rightarrow$ 6)-D-Glc, β -D-Galp-(1 $\rightarrow$ 3)-D-Glc, β -D-Galp-(1 $\rightarrow$ 6)-D-Gal, β -D-Galp-(1 $\rightarrow$ 3)-D-Gal, and the trisaccharides β -D-Galp-(1 $\rightarrow$ 3)-D-Lac, β -D-Galp-(1 $\rightarrow$ 6)-D-Lac. In a recent study a strong prebiotic effect was attributed to some of these compounds [51], and hence it is likely that the GOS mixture formed by *L. pentosus* β -gal has a considerable potential as prebiotic.

In conclusion, this work presented the characterisation and transgalactosylation capacity of a novel β -galactosidase from *L. pentosus*. Although the β -galactosidase described here is not derived from a proven probiotic *Lactobacillus* strain, the physical and biochemical characteristics are quite similar to those derived from probiotic strains [9, 34] with the exception that it is considerably more stable at higher temperatures. Also the GOS spectrum and yields are comparable with the former studies, making this novel enzyme that is produced by a well-known food-grade organism interesting for potential applications in food industry.

Acknowledgements This research work was supported financially by the Research Centre Applied Biocatalysis (Graz, Austria) and the National Research Council of Thailand (to MY). OJ thanks the Duo-Thailand 2008 program for a mobility grant. The authors have declared no conflict of interest.

5 References

- [1] Zandoni, P., Farrow, J. A. E., Phillips, B. A., Collins, M. D., *Lactobacillus pentosus* (Fred, Peterson and Anderson) sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* 1987, 37, 339-341.
- [2] Wynne, A. G., Gibson, G. R., Brostoff, J., Composition comprising a *Lactobacillus pentosus* strain and uses thereof. United States Patent 7125708, 10/24/2006, 2006.
- [3] Kim, H.-J., Shin, H.-S., Ha, W.-K., Yang, H.-J., Lee, S.-W., Characterization of lactic bacterial strains isolated from raw milk. *Asian Austral. J. Anim.* 2006, 19, 131-136.
- [4] Fred, E. B., Peterson, W. H., Anderson, A.J., The characteristics of certain pentose-destroying bacteria, especially as concerns their action on arabinose and xylose. *J. Biol. Chem.* 1921, 48, 385-412.
- [5] Møller, P. L., Jørgensen, F., Hansen, O. C., Madsen, S. M., Stougaard, P., Intra- and extracellular β -galactosidases from *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. *Appl. Environ. Microbiol.* 2001, 67, 2276-2283.
- [6] Coombs, J. M., Brenchley, J. E., Biochemical and phylogenetic analyses of a cold-active β -galactosidase from the lactic acid bacterium *Carnobacterium piscicola* BA. *Appl. Environ. Microbiol.* 1999, 65, 5443-5450.
- [7] Nguyen, T.-H., Splechtna, B., Yamabhai, M., Haltrich, D., Peterbauer, C., Cloning and expression of the β -galactosidase genes from *Lactobacillus reuteri* in *Escherichia coli*. *J. Biotechnol.* 2007, 129, 581-591.
- [8] Schmidt, B. F., Adams, R. M., Requadt, C., Power, S., Mainzer, S. E., Expression and nucleotide sequence of the *Lactobacillus bulgaricus* β -

- galactosidase gene cloned in *Escherichia coli*. *J. Bacteriol.* 1989, 171, 625-635.
- [9] Nguyen, T.-H., Splechtna, B., Steinböck, M., Kneifel, W., *et al.*, Purification and characterization of two novel β -galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* 2006, 54, 4989-4998.
- [10] David, S., Stevens, H., van Riel, M., Simons, G., de Vos, W. M., *Leuconostoc lactis* β -galactosidase is encoded by two overlapping genes. *J. Bacteriol.* 1992, 174, 4475-4481.
- [11] Toba, T., Tomita, Y., Itoh, T., Adachi, S., β -Galactosidase of lactic acid bacteria: Characterization by oligosaccharides formed during hydrolysis of lactose. *J. Dairy Sci.* 1981, 64, 185-192.
- [12] Garman, J., Coolbear, T., Smart, J., The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β -galactosidase from six strains of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 1996, 46, 22-27.
- [13] Splechtna, B., Nguyen, T.-H., Steinböck, M., Kulbe, K. D., *et al.*, Production of prebiotic galacto-oligosaccharides from lactose using β -galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* 2006, 54, 4999-5006.
- [14] Rastall, R. A., Maitin, V., Prebiotics and synbiotics: towards the next generation. *Curr. Opin. Biotechnol.* 2002, 13, 490-496.
- [15] Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., Roberfroid, M. B., Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 2004, 17, 259-275.
- [16] Macfarlane, G. T., Steed, H., Macfarlane, S., Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* 2008, 104, 305-344.

- 440 [17] Park, A. R., Oh, D. K., Galacto-oligosaccharide production using microbial β -galactosidase: current state and perspectives. *Appl. Microbiol. Biotechnol.* 2010, 85, 1279-1286.
- [18] Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., Gras, S. L., Recent advances refining galactooligosaccharide production from lactose. *Food Chem.* 2010, 121, 307-318.
- 445 [19] Zarate, S., Lopez-Leiva, M. H., Oligosaccharide formation during enzymatic lactose hydrolysis: A literature review. *J. Food Prot.* 1990, 53, 262-268.
- [20] Nakayama, T., Amachi, T., β -Galactosidase, enzymology, in: Flickinger, M. C., Drew, S.W. (Eds.) *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, John Wiley and Sons, New York, 1999; Vol. 3, pp. 1291-1305.
- 450 [21] Sako, T., Matsumoto, K., Tanaka, R., Recent progress on research and application of non-digestible galacto-oligosaccharides. *Int. Dairy J.* 1999, 9, 69-80.
- 455 [22] Rabi, B. A., Jay, A. J., Gibson, G. R., Rastall, R. A., Synthesis and fermentation properties of novel galacto-oligosaccharides by β -galactosidases from *Bifidobacterium* species. *Appl. Environ. Microbiol.* 2001, 67, 2526-2530.
- [23] Tzortzis, G., Goulas, A. K., Gee, J. M., Gibson, G. R., A novel galacto-oligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. *J. Nutr.* 2005, 135, 1726-1731.
- 460 [24] Nonaka, Y., Izumo, T., Izumi, F., Maekawa, T. *et al.*, Antiallergic effects of *Lactobacillus pentosus* strain S-PT84 mediated by modulation of Th1/Th2 immunobalance and induction of IL-10 production. *Int. Arch. Allergy Immunol.* 2008, 145, 249-257.
- 465

- [25] Casey, P. G., Gardiner, G. E., Casey, G., Bradshaw, B. *et al.*, A five-strain probiotic combination reduces pathogen shedding and alleviates disease signs in pigs challenged with *Salmonella enterica* Serovar Typhimurium. *Appl. Environ. Microbiol.* 2007, 73, 1858-1863.
- 470 [26] Kunst, A., Draeger, B., Ziegenhorn, J., Colorimetric methods with glucose oxidase and peroxidase, in: Bergmeyer, H. U., Bergmeyer, J., Graßl, M. (Eds.), *Methods of Enzymatic Analysis*, VCH Publishers, Weinheim 1988, Vol. 3, pp. 178-185.
- 475 [27] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 1976, 72, 248-254.
- [28] Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680-685.
- 480 [29] Felis, G. E., Dellaglio, F., Taxonomy of lactobacilli and bifidobacteria. *Curr. Issues Intest. Microbiol.* 2007, 8, 44-61.
- [30] Konings, W. N., Kok, J., Kuipers, O. P., Poolman, B., Lactic acid bacteria: the bugs of the new millennium. *Curr. Opin. Microbiol.* 2000, 3, 276-282.
- [31] de Vos, W. M., Vaughan, E. E., Genetics of lactose utilization in lactic acid bacteria. *FEMS Microbiol. Rev.* 1994, 15, 217-237.
- 485 [32] Honda, H., Kataoka, F., Nagaoka, S., Kawai, Y. *et al.*, β -Galactosidase, phospho- β -galactosidase and phospho- β -glucosidase activities in lactobacilli strains isolated from human faeces. *Lett. Appl. Microbiol.* 2007, 45, 461-466.
- [33] Vasiljevic, T., Jelen, T., Oligosaccharide production and proteolysis during lactose hydrolysis using crude cellular extracts from lactic acid bacteria. *Le Lait* 2003, 83, 453-467.
- 490

- [34] Nguyen, T.-H., Splechtna, B., Krasteva, S., Kneifel, W. *et al.*, Characterization and molecular cloning of a heterodimeric β -galactosidase from the probiotic strain *Lactobacillus acidophilus* R22. *FEMS Microbiol. Lett.* 2007, 269, 136-144.
- 495 [35] Splechtna, B., Nguyen, T.-H., Zehetner, R., Lettner, H. P. *et al.*, Process development for the production of prebiotic galacto-oligosaccharides from lactose using β -galactosidase from *Lactobacillus* sp. *Biotechnol. J.* 2007, 2, 480-485.
- 500 [36] Canchaya, C., Claesson, M. J., Fitzgerald, G.F., van Sinderen, D., O'Toole, P. W., Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology* 2006, 152, 3185-3196.
- 505 [37] Halbmayr, E., Mathiesen, G., Nguyen, T.-H., Maischberger, T. *et al.* High-level expression of recombinant β -galactosidases in *Lactobacillus plantarum* and *Lactobacillus sakei* using a Sakacin P-based expression system. *J. Agric. Food Chem.* 2008, 56, 4710-4719.
- [38] Corral, J. M., Banuelos, O., Adrio, J. L., Velasco, J., Cloning and characterization of a β -galactosidase encoding region in *Lactobacillus coryniformis* CECT 5711. *Appl. Microbiol. Biotechnol.* 2006, 73, 640-646.
- 510 [39] Fortina, M. G., Ricci, G., Mora, D., Guglielmetti, S., Manichini, P. L., Unusual organization for lactose and galactose gene clusters in *Lactobacillus helveticus*. *Appl. Environ. Microbiol.* 2003, 69, 3238-3243.
- [40] Pridmore, R. D., Berger, B., Desiere, F., Vilanova, D. *et al.*, The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proc. Natl. Acad. Sci. U. S. A.* 2004, 101, 2512-2517.

- 515 [41] Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D. *et al.*, Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. U. S. A.* 2003, 100, 1990-1995.
- [42] Obst, M., Meding, E. R., Vogel, R. F., Hammes, W. P., Two genes encoding the β -galactosidase of *Lactobacillus sake*. *Microbiology* 1995, 141, 3059-3066.
- 520 [43] Bae, H. C., Choi, J. W., Nam, M. S., Purification and characterisation of β -galactosidase from *Lactobacillus salivarius* subsp. *salivarius* Nam27. *Korean J. Food Sci. Ani. Resour.* 2007, 27, 110-116.
- [44] Adams, R. M., Yoast, S., Mainzer, S. E., Moon, K. *et al.*, Characterization of two cold-sensitive mutants of the β -galactosidase from *Lactobacillus delbruckii* subsp. *bulgaricus*. *J. Biol. Chem.* 1994, 269, 5666-5672.
- 525 [45] de Macias, M. E., Manca de Nadra, M. C., Strasser de Saad, A. M., Pesce de Ruiz Holgado, A. A., Oliver, G., Isolation and properties of β -galactosidase of a strain of *Lactobacillus helveticus* isolated from natural whey starter. *J. Appl. Biochem.* 1983, 5, 275-281.
- 530 [46] Acebron, I., Curiel, J. A., de Las Rivas, B., Munoz, R., Mancheno, J. M., Cloning, production, purification and preliminary crystallographic analysis of a glycosidase from the food lactic acid bacterium *Lactobacillus plantarum* CECT 748(T). *Protein Expr. Purif.* 2009, 68, 177-182.
- [47] de Roos, A., Industrial enzymes: enzymes in dairy applications, in: Aehle, W. (Ed.), *Enzymes in Industry*, Wiley-VCH, Weinheim 2004, Vol. 2, p. 144.
- 535 [48] Kim, C. S., Ji, E. S., Oh, D. K. Expression and characterization of *Kluyveromyces lactis* β -galactosidase in *Escherichia coli*. *Biotechnol. Lett.* 2003, 25, 1769-1774.

- [49] Itho, K., Toba, T., Itho, T., Adachi, S., Properties of β -galactosidase of *Lactobacillus kefiranofaciens* K-1 isolated from kefir grains. *Lett. Appl. Microbiol.* 1992, 15, 232-234.
- [50] Ito, M., Deguchi, Y., Miyamori, A., Matsumoto, K. *et al.*, Effects of administration of galacto-oligosaccharides on the human faecal microflora, stool weight and abdominal sensation. *Microb. Ecol. Health. D* 1990, 3, 285-292.
- [53] Sanz, M. L., Cote, G. L., Gibson, G. R., Rastall, R. A., Influence of glycosidic linkages and molecular weight on the fermentation of maltose-based oligosaccharides by human gut bacteria. *J. Agric. Food Chem.* 2006, 54, 9779-9784

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

550 **Tables**

Table 1. Kinetic properties of β -galactosidase from *Lactobacillus pentosus* for the hydrolysis of lactose and *o*-nitrophenyl- β -D-galactopyranoside (*o*NPG)

substrate	method for determination of enzyme activity	kinetic parameter	<i>L. pentosus</i> β -galactosidase
lactose	release of D-glucose	$v_{\max}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	11.3 ± 0.75
		K_m (mM)	37.8 ± 9.41
		k_{cat} (s^{-1})	20.1
		k_{cat}/K_m ($\text{mM}^{-1} \text{s}^{-1}$)	0.532
<i>o</i> NPG	release of <i>o</i> NP	$v_{\max}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	304 ± 24.6
		K_m (mM)	1.67 ± 0.64
		k_{cat} (s^{-1})	543
		k_{cat}/K_m ($\text{mM}^{-1} \text{s}^{-1}$)	325

555

Table 2. Kinetic stability of β -galactosidase from *Lactobacillus pentosus* at various pH values at 37 °C.

Buffer	pH	Inactivation constant k_{in} (h^{-1})	Half-life $t_{1/2}$ (h)
sodium acetate	4.0	3770×10^{-3}	0.18
sodium acetate	5.0	504×10^{-3}	1.37
sodium acetate	5.5	131×10^{-3}	5.31
sodium acetate	6.0	91.9×10^{-3}	7.54
sodium phosphate	6.0	63.3×10^{-3}	10.9
sodium phosphate	6.5	47.6×10^{-3}	14.6
sodium phosphate	7.0	47.6×10^{-3}	14.6
sodium phosphate	7.5	95.8×10^{-3}	7.24
glycine	7.5	43.6×10^{-3}	15.9
glycine	8.0	14.7×10^{-3}	47.2
glycine	8.5	16.0×10^{-3}	43.3
glycine	9.0	1045×10^{-3}	0.66

Table 3. Kinetic stability of β -galactosidase from *Lactobacillus pentosus* at various temperatures with and without MgCl_2 supplementation (2 mM).

Sodium phosphate buffer (pH 6.5)			Sodium phosphate buffer (pH 6.5, + Mg^{2+})	
Temperature ($^{\circ}\text{C}$)	Inactivation constant k_{in} (h^{-1})	Half-life $t_{1/2}$ (h)	Inactivation constant k_{in} (h^{-1})	Half-life $t_{1/2}$ (h)
4	0.5×10^{-3}	1386	nd	nd
23	0.8×10^{-3}	866	nd	nd
30	9.0×10^{-3}	77.0	2.8×10^{-3}	248
37	46.6×10^{-3}	14.9	9.4×10^{-3}	73.7
42	212.7×10^{-3}	3.26	37.7×10^{-3}	18.4
nd...not determined				

Figure captions

Figure 1. SDS-PAGE (A) and native PAGE (B) of purified β -galactosidase from *L. pentosus*. A: lane 1, molecular weight marker (Precision Plus Protein Dual Color, Biorad), CBB staining; lane 2, enzyme sample after treatment in SDS buffer at 100 °C for 5 min, CBB staining. B: lane 1, molecular weight marker (High Molecular Weight Calibration Kit for electrophoresis, Amersham); lane 2, CBB staining.

Figure 2. pH optimum of β -galactosidase from *L. pentosus* using lactose (A) and oNPG (B) as the substrate. Symbols: ●, sodium acetate buffer; ○, sodium phosphate buffer; ▼, glycine buffer. Activities are given relative to standard assay conditions (sodium phosphate buffer, pH 6.5).

Figure 3. Temperature optimum of β -galactosidase from *L. pentosus* when using lactose (●) and oNPG (○) as the substrate. Activities are given relative to standard assay conditions at 30 °C.

Figure 4. Inactivation kinetics of β -galactosidase from *L. pentosus* in sodium phosphate buffer (A; 50 mM, pH 6.5) and sodium phosphate buffer supplemented with 1 mM MgSO₄ (B; 50 mM, pH 6.5) at different temperatures. Symbols: (A): ●, 4 °C; ○, 23 °C; ▼, 30 °C; ▽, 37 °C; ■, 42 °C; (B): ●, 30 °C; ○, 37 °C; ▼, 42 °C.

Figure 5. Formation of galacto-oligosaccharides (GOS) by β -galactosidase from *L. pentosus* during the discontinuous conversion reaction of 205 g/L lactose in 50 mM sodium phosphate buffer at 30 °C. (A) Formation of disaccharides (●), trisaccharides

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(O), tetrasaccharides (▼) and total GOS (▽) during lactose conversion. (B) Time
course of reaction for lactose conversion; symbols: lactose (▼), glucose (●),
galactose (O).

For Peer Review

210x297mm (600 x 600 DPI)

210x297mm (600 x 600 DPI)