



HAL
open science

**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:	Maischberger, Thomas; Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences; Research Centre Applied Biocatalysis Leitner, Elisabeth; Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences Nitisinprasert, Sunee; Department of Biotechnology, Kasetsart University Juajun, Onladda; Food Biotechnology Laboratory, BOKU University of Natural Resources and Applied Sciences; School of Biotechnology, Suranaree University of Technology Yamabhai, Montarop; Suranaree University of Technology, School of Biotechnology Nguyen, Thu-Ha; Vienna University of Natural Resources and Applied Life Sciences, Department of Food Sciences and Technology, Food Biotechnology Lab Haltrich, Dietmar; Universität für Bodenkultur, Food Sciences and Technology
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

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

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

1
2
3 gastrointestinal microbiota that confers benefits upon host well-being and health”
4
5
6 45 [15]. In practice, the beneficial bacteria that serve as targets for prebiotics and GOS
7
8 are almost exclusively bifidobacteria and lactobacilli. An advantage of the concept of
9
10 prebiotics to modify gut function is that the target bacteria are already commensal to
11
12 the large intestine, whereas with probiotics allochthonous micro-organisms are
13
14 introduced and have to compete against established colonic communities [16]. GOS
15
16
17 50 have been classified as one of the few proven prebiotics fulfilling the three criteria (i)
18
19 resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal
20
21 absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of
22
23 the growth and/or activity of intestinal bacteria associated with health and well-being
24
25 [15]. GOS have been widely investigated for their prebiotic properties and
26
27
28
29 55 physiological effects using *in vitro*, animal, human, and infant studies, and functional
30
31 effects of GOS on human health are summarised in [16], [20] and [21].
32
33
34 GOS are produced from lactose using β -galactosidases (E.C. 3.2.1.23), which in
35
36 addition to their hydrolytic activity catalyse glycosyl transfer reactions. In this reaction
37
38 galactosyl residues are transferred onto suitable acceptors such as another sugar
39
40
41 60 (lactose, the primary reaction products glucose and galactose, or other
42
43 oligosaccharides formed), thus building up complex series of higher oligosaccharides
44
45 [17-19]. The spectrum of the oligosaccharides making up these mixtures strongly
46
47 depends on the source of the β -galactosidase used for the biocatalytic reaction, and
48
49 on the conversion conditions used in their production. Since these differences in
50
51
52
53 65 GOS spectrum and yields are a result of structural and/or mechanistic differences
54
55 between β -galactosidases from different sources, a detailed knowledge on novel, yet-
56
57 unexplored β -galactosidases from various strains can be of significant interest [18].
58
59 Rabiou *et al.* [22] and Tzortzis *et al.* [23] synthesised a range of GOS mixtures from
60
lactose using β -galactosidases from different bifidobacteria. Subsequently it was

1
2
3 70 shown that these different mixtures typically resulted in better growth of that strain
4
5 that had served as the source of the enzyme for GOS production. This concept may
6
7 serve as the basis for a new generation of functionally enhanced, targeted
8
9 oligosaccharides, and has increased the interest in β -galactosidases from beneficial
10
11 probiotic organisms.
12
13

14
15 75 The objective of this work was to study the β -galactosidase from *L. pentosus* in detail
16
17 and to compare it to other lactobacillal β -galactosidases isolated from organisms of
18
19 typical animal origin. Recent data are indicating that *L. pentosus* might be of interest
20
21 as a beneficial LAB making it attractive as a probiotic strain, e.g. based on
22
23 immunomodulating properties [24] or the reduction of pathogens in animals [25].
24
25
26

27 80

29 **2 Material and Methods**

31 **2.1 Strain and culture conditions**

32
33
34
35
36 *Lactobacillus pentosus* KUB-ST10-1 was from the culture collection of Kasetsart
37
38 University (Bangkok, Thailand). It was isolated from soil of a dairy farm in Thailand
39
40 and identified by using both the API 50 CH carbohydrate fermentation stripes test
41 85
42 (bioMérieux, Inc., Marcy l'Etoile, France) and sequence analysis of the amplified
43
44 chromosomal 16S rDNA. It was stored at -70°C in MRS broth medium (Merck,
45
46 Darmstadt, Germany) containing 15% glycerol. To prepare fresh inocula, it was
47
48 transferred twice after an 18-h cultivation period to fresh MRS broth medium
49
50 supplemented with lactose (final concentration of 2%).
51
52
53 90

54
55 *L. pentosus* KUB-ST10-1 was grown anaerobically in 10 L of MRS broth medium
56
57 containing 2% lactose at 37°C with slight agitation and without pH control. A
58
59 preculture, grown overnight in MRS medium (2% lactose (w/v)), was used to
60
inoculate the fresh medium to a final concentration of 1% (v/v) inoculum. Cells were

1
2
3 95 harvested at an optical density (OD_{600}) of 13 by centrifugation at $8800 \times g$ for 15 min
4
5 at 4°C .
6
7

8 9 **2.2 Enzyme purification**

10
11
12 β -Galactosidase was purified from the crude extract using a previously described
13 protocol [9]. Wet biomass (100 g) was resuspended in 200 mL of sodium phosphate
14
15
16
17 100 buffer (50 mM, pH 6.5) and homogenized (APV-2000; Silkeborg, Denmark) to disrupt
18 the cells. Cell debris was removed by ultracentrifugation ($36000 \times g$, 30 min, 4°C) to
19
20 obtain the crude enzyme extract.
21
22

23
24 *Ammonium sulphate precipitation (ASP)*. Ammonium sulphate was added in small
25 portions to the crude extract to a final concentration of 50% saturation under
26
27
28
29 105 continuous stirring at 4°C . The precipitated protein was collected by centrifugation
30 (6200 $\times g$, 15 min, 4°C), and the pellet was re-dissolved in 50 mM sodium phosphate
31
32 buffer (pH 6.5) containing 1 M ammonium sulphate (buffer A).
33
34

35
36 *Hydrophobic Interaction Chromatography (HIC)*. The dissolved pellet was loaded
37 onto a 20-mL phenyl-sepharose fast flow column (50 mm \times 200 mm; Amersham,
38
39
40
41 110 Uppsala, Sweden) that had been pre-equilibrated with buffer A. Proteins were eluted
42 by a linear gradient of 0% to 100% buffer B (50 mM sodium phosphate buffer, pH
43
44
45
46
47
48
49
50
51 high β -galactosidase activity were pooled and concentrated by ultrafiltration (30-kDa
52 cut-off; Amicon, Beverly, MA).
53

54
55
56
57
58
59
60 115 *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

1
2
3 120 10 CV. Active fractions were pooled, desalted and concentrated by ultrafiltration. The
4
5 purified enzyme was stored in 50 mM sodium phosphate buffer (pH 6.5) at 4 °C.
6
7

8 9 **2.3 Enzyme assays**

10
11
12 β -Galactosidase activity was determined using both the artificial chromogenic
13 substrate *o*-nitrophenyl- β -D-galactopyranoside (*o*NPG) and the natural substrate
14
15 lactose as described previously [9]. When *o*NPG was the substrate, the reaction was
16
17 125 performed in 50 mM sodium phosphate buffer (pH 6.5) and using a 10-min incubation
18
19 at 30 °C, after which the reaction was stopped. One unit of *o*NPG activity (U_{oNPG}) is
20
21 defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the
22
23 assay conditions described above. When lactose was used as the substrate, the
24
25 reaction was done in 50 mM sodium phosphate buffer (pH 6.5) for 10 min at 30 °C,
26
27 130 after which the reaction was stopped. The release of D-glucose was determined
28
29 colourimetrically using the glucose oxidase/peroxidase (GOD/POD) assay [26]. One
30
31 unit of lactase activity (U_{Lac}) is defined as the amount of enzyme releasing 1 μmol of
32
33 D-glucose per minute under the conditions listed above.
34
35
36
37
38
39
40

41 42 135 **2.4 Protein analysis and gel electrophoresis**

43
44
45 Protein concentrations were determined by the method of Bradford [27] using bovine
46
47 serum albumin as the standard. The PhastSystem unit (Amersham) and precast
48
49 polyacrylamide gels (Phastgel 8–25, Amersham) were used for performing native
50
51 and SDS-PAGE. Samples were treated as described by Laemmli [28] with slight
52
53 140 modifications. The enzyme was diluted to 1–2 mg of protein per mL and incubated
54
55 with 2x Laemmli buffer at 60 °C for 5 min. Protein bands were stained using either
56
57 Coomassie Blue staining or 4-methylumbelliferyl- β -D-galactopyranoside (MUG) as
58
59 substrate for active staining. For SDS-PAGE and native PAGE molecular masses
60

1
2
3 were estimated using the Precision Plus Protein Dual Color Kit (Biorad) and the High
4
5 145 Molecular Weight calibration Kit (Amersham), respectively.
6
7

8 9 **2.5 Steady-state kinetic measurement**

10
11
12 Catalytic constants were determined at 30 °C and pH 6.5 for the two substrates oNPG
13
14 and lactose with their concentrations in 50 mM phosphate buffer varying from 0.1–22
15
16 mM and 0.1–600 mM, respectively. The kinetic parameters were calculated by non-
17
18
19 150 linear regression, and the obtained data were fit to the Henri-Michaelis-Menten
20
21 equation (using SigmaPlot, SPSS Inc., Chicago, IL, USA).
22
23

24 25 **2.6 Effects of temperature and pH on enzyme activity and stability**

26
27
28 The temperature dependence of β -galactosidase activity (both oNPG and lactose)
29
30 was determined by assaying the enzyme samples over the range of 20 to 70 °C for 10
31
32
33 155 min. To study thermal stability, enzyme samples were incubated in 50 mM sodium
34
35 phosphate buffer, pH 6.5, at 4, 23, 30, 37, and 42 °C. At certain time intervals
36
37 samples were taken and the residual oNPG activity was measured. For measuring
38
39 the pH dependence of β -galactosidase activity three buffer systems (sodium citrate,
40
41 50 mM, pH 4.0–5.5; sodium phosphate, 50 mM, pH 5.5–7.5; glycine, 50 mM, pH 7.5–
42
43 9.0) were used. For the determination of the pH stability enzyme samples were
44
45 160 incubated at various pH values and 37 °C for up to 72 h, and the remaining enzyme
46
47 activity was measured at certain time intervals using oNPG as substrate under
48
49 standard assay conditions.
50
51
52
53

54 55 **2.7 Substrate specificity**

56
57
58 165 To determine the substrate specificity of the *L. pentosus* β -galactosidase for various
59
60 structurally related substrates *p*-nitrophenyl- β -D-mannopyranoside, *p*-nitrophenyl- β -D-

1
2
3 xylopyranoside, *o*-nitrophenyl- β -D-glucopyranoside and *p*-nitrophenyl- β -D-
4
5 cellobioside were used. Activities were determined in accordance to standard *o*NPG
6
7 assay conditions using the respective substrate in concentrations of 22 mM.
8
9

10 11 170 **2.8 Transgalactosylation activity**

12
13
14 Purified β -galactosidase from *L. pentosus* (5 U_{*o*NPG} per mL reaction mixture) was
15
16 used for the discontinuous formation of galacto-oligosaccharides (GOS) at 30 °C
17
18 using 205 g/L of lactose in 50 mM sodium phosphate buffer (pH 6.5, 2 mM MgCl₂) as
19
20 substrate. Samples were taken at various intervals and the composition of the GOS
21
22 mixture was analyzed by capillary electrophoresis (CE) and high-performance anion
23
24 175 exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as
25
26 described previously [13].
27
28
29
30
31
32

33 **3 Results**

34 35 36 37 180 **3.1 Formation and purification of β -galactosidase from *L. pentosus***

38
39
40 *L. pentosus* KUB-ST10-1 was selected for detailed studies on its β -galactosidase
41
42 since this strain grew very well on lactose, it showed high β -gal activity in a
43
44 screening, in which several lactic acid bacteria were compared pertaining to their
45
46 ability to form this enzyme activity (data not shown), and since its β -galactosidase
47
48 had not been studied in any detail previously. Batch cultivations of *L. pentosus* were
49
50 185 carried out in a 10-L laboratory fermenter at 37 °C without pH control using lactose-
51
52 based MRS medium. The biomass was harvested after 18 h of growth when β -gal
53
54 activity reached a maximum of 644 units (U_{*o*NPG}) per litre fermentation broth. β -
55
56 Galactosidase was purified to apparent homogeneity from the crude cell extract using
57
58
59
60
190 a two-step purification protocol based on hydrophobic interaction and affinity

1
2
3 chromatography. The enzyme was purified 16-fold to a specific activity of 97 U/mg of
4
5 protein, using standard assay conditions with *o*NPG as the substrate.
6
7

8 9 **3.2 Properties of β -galactosidase from *L. pentosus***

10
11
12 *L. pentosus* β -galactosidase is a heterodimer (M_r ~107 kDa) consisting of a small
13
14 (~35 kDa) and large (~72 kDa) subunit as judged by SDS-PAGE (**Figure 1A**) and
15 195 (~35 kDa) and large (~72 kDa) subunit as judged by SDS-PAGE (**Figure 1A**) and
16 native PAGE (**Figure 1B**). The additional protein band (~80 kDa) in native PAGE
17 when staining with Coomassie Blue presumably results from degradation of the intact
18
19 ~107-kDa enzyme, as was previously shown for the related β -gal from *L. reuteri* by
20
21 using mass spectrometry [9]. Active staining on native PAGE with MUG yielded only
22
23 one band with β -galactosidase activity, corresponding to the larger band in Figure
24
25
26 200 one band with β -galactosidase activity, corresponding to the larger band in Figure
27
28 1B. Active staining on the SDS-PAGE gel after treating the enzyme in SDS buffer at
29
30 60 °C for 5 min, thus separating the two subunits without unfolding, showed that one
31
32 band corresponding to the large subunit exhibited activity with MUG (not shown). In
33
34 contrast, the small subunit by itself did not show any activity. When the enzyme was
35
36 treated in SDS buffer at ~100 °C for 5 min, no activity could be detected after active
37
38 205 treated in SDS buffer at ~100 °C for 5 min, no activity could be detected after active
39
40 staining on the gel.
41
42
43

44 45 **3.3 Substrate specificity**

46
47
48 To study the substrate specificity of *L. pentosus* β -galactosidase various structurally
49
50 related artificial substrates were tested under standard assay conditions and
51
52 210 compared to *o*NPG. No appreciable activity was detected when using *p*-nitrophenyl-
53
54 β -D-xylopyranoside, *o*-nitrophenyl- β -D-glucopyranoside, *p*-nitrophenyl- β -D-
55
56 mannopyranoside and *p*-nitrophenyl- β -D-cellobioside as substrates for the activity
57
58 assay.
59
60

3.4 Enzyme kinetics

1
2
3 215 Steady-state kinetic studies were performed with the preferred substrates lactose
4 and oNPG and the kinetic constants K_m , v_{max} , and k_{cat} were determined. The k_{cat}
5 values were calculated on the basis of theoretical v_{max} values obtained by nonlinear
6 regression using SigmaPlot. Data are summarized in **Table 1**. The catalytic
7 efficiencies (k_{cat}/K_m) for the two substrates, oNPG and lactose, indicate that oNPG is
8 the preferred substrate in accordance with other microbial β -galactosidases.
9
10
11
12
13
14
15 220
16
17

18 **3.5 Effects of pH and temperature**

19
20
21
22 The pH optimum of *L. pentosus* β -galactosidase activity is pH 8.0 and pH 7.5 in
23 glycine buffer when using lactose and oNPG as substrate, respectively (**Figure 2**). In
24 general, the enzyme is more active in the alkaline range (pH 7.0–8.5), and it showed
25 a pronounced effect of the buffer system used on activity. The effect of different
26 buffers and pH values on stability was investigated subsequently in more detail.
27 Catalytic or kinetic stability, *i.e.*, the length of time an enzyme remains active before
28 undergoing irreversible inactivation, was measured for various pH values at a
29 constant temperature of 37°C, and the inactivation constants (k_{in}) and the half-life
30 times of denaturation ($\tau_{1/2}$) were determined (**Table 2**). β -Galactosidase showed first-
31 order inactivation kinetics at all pH values studied when analysed in the ln(residual
32 activity) versus time plot (not shown). Highest $\tau_{1/2}$ values were calculated in glycine
33 buffer (pH 8.0 and 8.5) where the stability was increased up to 3.2-fold compared to
34 when using sodium phosphate buffer at pH 6.5. Highest inactivation constants and
35 hence least stabilities were obtained in sodium acetate buffer (pH 4.0 and 5.0) and
36 glycine buffer (pH 9.0), with $\tau_{1/2}$ values of less than 1.5 h.
37
38
39
40
41 230
42
43
44
45
46
47
48
49
50
51
52
53 235
54
55
56
57

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

1
2
3 240 investigated at the pH value of milk, pH 6.5, both in the absence and presence of 2
4 mM MgCl₂. Inactivation constants and half-life times were calculated as described
5
6
7
8 above. The addition of 2 mM MgCl₂ to the buffer improved stability significantly,
9
10 increasing the half life times $\tau_{1/2}$ of the enzyme at 30, 37 and 42 °C 3.2-, 4.9- and 5.6-
11
12 fold, respectively (**Table 3; Figure 4**).

16 245 **3.6 Transgalactosylation activity**

18
19 In order to assess the potential of the novel β -galactosidase from *L. pentoses* for the
20 production of GOS, a discontinuous lactose conversion process was studied at 30 °C,
21
22 using an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (+
23
24 2 mM MgCl₂, pH 6.5) and 5 U_{ONPG}/mL of β -galactosidase activity to produce GOS.
25
26
27
28
29 250 The maximum GOS yield (31%) was obtained at a lactose conversion of 78%
30
31 (**Figure 5**). The newly formed sugar mixture contained 31.5% glucose, 15.9%
32
33 galactose, 21.9% lactose, 11.4% non-lactose disaccharides, 18.7% trisaccharides
34
35 and 0.6% tetrasaccharides as analysed by capillary electrophoresis and high-
36
37 performance anion exchange chromatography with pulsed amperometric detection,
38
39
40
41 255 and using authentic sugars and the standard addition technique for comparison. The
42
43 main GOS products identified were non-lactose disaccharides mainly of the β -(1→3)
44
45 and β -(1→6) linkage type as well as the two trisaccharides β -D-Galp-(1→3)-lactose,
46
47 and β -D-Galp-(1→6)-lactose.
48
49
50

53 260 **4 Discussion**

54
55 The genus *Lactobacillus* is the largest among the *Lactobacteriaceae* and includes
56
57 more than a hundred described species [29]. Lactobacilli are nutritionally fastidious,
58
59 metabolize a varying range of different carbohydrates, and are associated with a
60
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

1
2
3 source other than those traditionally used in the dairy industry may have unique
4 properties or characteristics. In a recent phylogenetic analysis based on 16S rRNA
5 genes the genus *Lactobacillus* was grouped into five major divisions [36]. In this
6
7
8
9
10
11
12
13 295 *pentosus* is a member of group C [36], hence might differ in its metabolic activities
14 from the sources of β -galactosidases recently characterised in our group.

15
16
17 The structural features of β -galactosidases from lactobacilli are varying to a great
18 extent. While heterodimeric structures are in general less frequently encountered
19 among microbial β -galactosidases, these seem to prevail among *Lactobacillus* β -
20
21
22
23
24 300 galactosidases. Lactobacillal heterodimeric β -galactosidases are encoded by two
25 genes, *lacL* and *lacM*, and have been described in e.g., *L. acidophilus* [34, 37], *L.*
26
27
28
29
30
31
32
33
34
35
36 305 *salivarius* was reported to be a monomer of ca. 30 kDa [43], the enzyme from *L. delbrueckii*
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60 315 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,

1
2
3 indicating oNPG as the better substrate. The K_m value of 37.8 mM determined for
4
5 lactose compares positively with the Michaelis constants reported for commercially
6
7 employed β -galactosidases, e.g., from *Aspergillus niger* (54–100 mM), *A. oryzae*
8
9 (36–180 mM), *Kluyveromyces fragilis* (15–52 mM) [47] and *Kluyveromyces lactis* (20
10 320 (36–180 mM), *Kluyveromyces fragilis* (15–52 mM) [47] and *Kluyveromyces lactis* (20
11
12 mM) [48]. This value is, however, somewhat higher than the values reported for other
13
14 *Lactobacillus* β -galactosidases: 13 mM for β -gal from *L. reuteri* L103 [9], 1.27 mM for
15
16 β -gal from *L. kefiranofaciens* [49], and 4.04 mM for β -gal from *L. acidophilus* [34].
17
18 The higher $K_{m, \text{lactose}}$ value for *L. pentosus* β -gal as compared to other lactobacillal
19
20 enzymes, especially those of milk origin, could indicate a lesser adaptation of this
21
22 325 soil-isolated strain to lactose utilization. *L. pentosus* β -gal is rather specific for the β -
23
24 (1→4) galactosyl linkage. When using a range of structurally related nitrophenyl
25
26 substrates, essentially no activity was measured for the β -(1→4) linked D-glucoside,
27
28 D-mannoside, and D-xyloside substrates.
29
30
31
32
33
34 330 *L. pentosus* β -galactosidase is optimally active at pH 7.5 (glycine buffer) and 55°C
35
36 when using oNPG as substrate, and pH 8.0 (glycine buffer) and 60–65°C when
37
38 lactose was used as substrate under otherwise standard activity assay conditions
39
40 (10-min assay). This pH optimum is slightly more alkaline than reported for most
41
42 other lactobacillal β -galactosidases, which show an optimum of 6.5–7.0 [9, 34],
43
44
45
46 335 however, we observed a pronounced effect of the buffer system used on the optimal
47
48 pH, and that certainly affects the actual value determined. The temperature optimum
49
50 of *L. pentosus* β -gal of 60–65°C is considerably higher than reported for most other
51
52 β -galactosidases from lactobacilli, which typically are optimally active in the range of
53
54 45–50°C [9, 34, 43, 50]. *L. pentosus* KUB-ST10-1 can grow at temperatures of up to
55
56 45°C. This is higher than typical growth temperatures observed for many
57
58 340 *Lactobacillus* spp. The increased temperature optimum and stability of the *L.*
59
60 *pentosus* β -galactosidase might reflect an adaptation to these higher growth

1
2
3 temperatures. Because of this increased temperature optimum we investigated the
4
5 thermostability of *L. pentosus* β -gal in more detail, determining the inactivation
6
7 kinetics at different conditions. With respect to pH, highest half-life times $\tau_{1/2}$ were
8 345 obtained in glycine buffer at pH 8.0–8.5 and at 37°C (43–47 h). Again, the buffer
9
10 system had a pronounced effect on stability, since the inactivation constants k_{in}
11
12 determined were approximately twice as high at pH 7.5 when using sodium
13
14 phosphate instead of glycine buffer; however the use of glycine buffer might be
15
16 impractical for technical applications. When using the former buffer, highest stability
17
18 was observed at pH 6.5–7.0 with $\tau_{1/2}$ of 14.6 h. Addition of 2 mM Mg^{2+} increased
19
20 350 stability and hence $\tau_{1/2}$ of β -gal activity significantly by a factor of up to 5.6-fold. For
21
22 example, $\tau_{1/2}$ values of e.g. ~75 h were obtained at pH 6.5, 37°C and when using
23
24 phosphate buffer containing 2 mM of Mg^{2+} . This increase in stability and activity of *L.*
25
26
27
28
29
30
31
32 355 *pentosus* β -gal could be a considerable advantage when using technical substrates
33
34 such as cheese whey or whey permeates for the production of GOS, since cheese
35
36 whey contains approximately 1.5 mM of Mg^{2+} . Thus, use of these technical
37
38 substrates could improve the performance of *L. pentosus* β -gal without the need of
39
40 adding exogenous Mg^{2+} .
41
42

43
44 360 β -Galactosidases have recently attained significant interest in biocatalytic processes
45
46 towards the synthesis of oligosaccharides or various galactosylated compounds; for
47
48 this purpose various galactosides can serve as galactosyl donor. Especially galacto-
49
50 oligosaccharides (GOS), which are formed from lactose as a result of
51
52 transgalactosylation, have attracted renewed attention in the field of functional food
53
54 because of their proven benefits on health [16, 17, 18, 20]. As GOS are only
55
56 365 transiently formed and are in turn substrates for the β -galactosidase as well, a
57
58 maximum GOS yield of 31% (w/v) was reached at 78% lactose conversion when
59
60 using the β -gal of *L. pentosus*. The main GOS spectrum formed from lactose by this

1
2
3 enzyme is very similar to those found for β -gal from *L. reuteri* [13] and *L. acidophilus*
4
5
6 370 [34]. *L. pentosus* β -gal shows a high specificity for the formation of β -(1 \rightarrow 6) and β -
7
8 (1 \rightarrow 3) linkages, and hence the main transgalactosylation products detected were the
9
10 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,
11
12 β -D-Galp-(1 \rightarrow 3)-D-Gal, and the trisaccharides β -D-Galp-(1 \rightarrow 3)-D-Lac, β -D-Galp-
13
14 (1 \rightarrow 6)-D-Lac. In a recent study a strong prebiotic effect was attributed to some of
15
16
17 375 these compounds [51], and hence it is likely that the GOS mixture formed by *L.*
18
19 *pentosus* β -gal has a considerable potential as prebiotic.
20
21

22
23 In conclusion, this work presented the characterisation and transgalactosylation
24
25 capacity of a novel β -galactosidase from *L. pentosus*. Although the β -galactosidase
26
27 described here is not derived from a proven probiotic *Lactobacillus* strain, the
28
29 380 physical and biochemical characteristics are quite similar to those derived from
30
31 probiotic strains [9, 34] with the exception that it is considerably more stable at higher
32
33 temperatures. Also the GOS spectrum and yields are comparable with the former
34
35 studies, making this novel enzyme that is produced by a well-known food-grade
36
37 organism interesting for potential applications in food industry.
38
39
40

41 385
42

43
44 **Acknowledgements** This research work was supported financially by the Research
45
46 Centre Applied Biocatalysis (Graz, Austria) and the National Research Council of
47
48 Thailand (to MY). OJ thanks the Duo-Thailand 2008 program for a mobility grant.
49

50
51 The authors have declared no conflict of interest.
52
53
54
55
56
57
58
59
60

390 **5 References**

- 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
- [1] Zanoni, 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
395 *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.
- 400 [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.,
405 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.
- 410 [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 -

- 1
2
3 415 galactosidase gene cloned in *Escherichia coli*. *J. Bacteriol.* 1989, 171, 625-
4 635.
5
6
7
8 [9] Nguyen, T.-H., Splechna, B., Steinböck, M., Kneifel, W., *et al.*, Purification
9 and characterization of two novel β -galactosidases from *Lactobacillus reuteri*.
10 *J. Agric. Food Chem.* 2006, 54, 4989-4998.
11
12
13
14
15 420 [10] David, S., Stevens, H., van Riel, M., Simons, G., de Vos, W. M., *Leuconostoc*
16 *lactis* β -galactosidase is encoded by two overlapping genes. *J. Bacteriol.*
17 1992, 174, 4475-4481.
18
19
20
21
22 [11] Toba, T., Tomita, Y., Itoh, T., Adachi, S., β -Galactosidase of lactic acid
23 bacteria: Characterization by oligosaccharides formed during hydrolysis of
24 lactose. *J. Dairy Sci.* 1981, 64, 185-192.
25
26 425
27
28 [12] Garman, J., Coolbear, T., Smart, J., The effect of cations on the hydrolysis of
29 lactose and the transferase reactions catalysed by β -galactosidase from six
30 strains of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 1996, 46, 22-27.
31
32
33
34
35
36 [13] Splechna, B., Nguyen, T.-H., Steinböck, M., Kulbe, K. D., *et al.*, Production of
37 prebiotic galacto-oligosaccharides from lactose using β -galactosidases from
38 430 *Lactobacillus reuteri*. *J. Agric. Food Chem.* 2006, 54, 4999-5006.
39
40
41
42
43 [14] Rastall, R. A., Maitin, V., Prebiotics and synbiotics: towards the next
44 generation. *Curr. Opin. Biotechnol.* 2002, 13, 490-496.
45
46
47
48 [15] Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., Roberfroid, M. B.,
49 435 Dietary modulation of the human colonic microbiota: updating the concept of
50 prebiotics. *Nutr. Res. Rev.* 2004, 17, 259-275.
51
52
53
54
55 [16] Macfarlane, G. T., Steed, H., Macfarlane, S., Bacterial metabolism and health-
56 related effects of galacto-oligosaccharides and other prebiotics. *J. Appl.*
57 *Microbiol.* 2008, 104, 305-344.
58
59
60

- 1
2
3 440 [17] Park, A. R., Oh, D. K., Galacto-oligosaccharide production using microbial β -
4 galactosidase: current state and perspectives. *Appl. Microbiol. Biotechnol.*
5 2010, *85*, 1279-1286.
6
7
8
9
10 [18] Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., Gras, S. L., Recent
11 advances refining galactooligosaccharide production from lactose. *Food*
12 *Chem.* 2010, *121*, 307-318.
13 445
14
15 [19] Zarate, S., Lopez-Leiva, M. H., Oligosaccharide formation during enzymatic
16 lactose hydrolysis: A literature review. *J. Food Prot.* 1990, *53*, 262-268.
17
18 [20] Nakayama, T., Amachi, T., β -Galactosidase, enzymology, in: Flickinger, M. C.,
19 Drew, S.W. (Eds.) *Encyclopedia of Bioprocess Technology: Fermentation,*
20 *Biocatalysis, and Bioseparation*, John Wiley and Sons, New York, 1999; Vol.
21 450 3, pp. 1291-1305.
22
23
24
25
26
27 [21] Sako, T., Matsumoto, K., Tanaka, R., Recent progress on research and
28 application of non-digestible galacto-oligosaccharides. *Int. Dairy J.* 1999, *9*,
29 69-80.
30
31
32
33
34
35
36
37
38 455 [22] Rabiou, B. A., Jay, A. J., Gibson, G. R., Rastall, R. A., Synthesis and
39 fermentation properties of novel galacto-oligosaccharides by β -galactosidases
40 from *Bifidobacterium* species. *Appl. Environ. Microbiol.* 2001, *67*, 2526-2530.
41
42
43
44
45 [23] Tzortzis, G., Goulas, A. K., Gee, J. M., Gibson, G. R., A novel galacto-
46 oligosaccharide mixture increases the bifidobacterial population numbers in a
47 continuous in vitro fermentation system and in the proximal colonic contents of
48 pigs in vivo. *J. Nutr.* 2005, *135*, 1726-1731.
49 460
50
51
52
53
54
55 [24] Nonaka, Y., Izumo, T., Izumi, F., Maekawa, T. *et al.*, Antiallergic effects of
56 *Lactobacillus pentosus* strain S-PT84 mediated by modulation of Th1/Th2
57 immunobalance and induction of IL-10 production. *Int. Arch. Allergy Immunol.*
58 2008, *145*, 249-257.
59
60 465

- 1
2
3 [25] Casey, P. G., Gardiner, G. E., Casey, G., Bradshaw, B. *et al.*, A five-strain
4 probiotic combination reduces pathogen shedding and alleviates disease
5 signs in pigs challenged with *Salmonella enterica* Serovar Typhimurium. *Appl.*
6 *Environ. Microbiol.* 2007, 73, 1858-1863.
7
8
9
10
11
12 470 [26] Kunst, A., Draeger, B., Ziegenhorn, J., Colorimetric methods with glucose
13 oxidase and peroxidase, in: Bergmeyer, H. U., Bergmeyer, J., Graßl, M.
14 (Eds.), *Methods of Enzymatic Analysis*, VCH Publishers, Weinheim 1988, Vol.
15 3, pp. 178-185.
16
17
18
19
20
21
22 [27] Bradford, M. M., A rapid and sensitive method for the quantitation of
23 microgram quantities of protein utilizing the principle of protein dye binding.
24 475 *Anal. Biochem.* 1976, 72, 248-254.
25
26
27
28
29 [28] Laemmli, U.K., Cleavage of structural proteins during the assembly of the
30 head of bacteriophage T4. *Nature* 1970, 227, 680-685.
31
32
33
34 [29] Felis, G. E., Dellaglio, F., Taxonomy of lactobacilli and bifidobacteria. *Curr.*
35 *Issues Intest. Microbiol.* 2007, 8, 44-61.
36 480
37
38 [30] Konings, W. N., Kok, J., Kuipers, O. P., Poolman, B., Lactic acid bacteria: the
39 bugs of the new millennium. *Curr. Opin. Microbiol.* 2000, 3, 276-282.
40
41
42
43 [31] de Vos, W. M., Vaughan, E. E., Genetics of lactose utilization in lactic acid
44 bacteria. *FEMS Microbiol. Rev.* 1994, 15, 217-237.
45
46
47
48 485 [32] Honda, H., Kataoka, F., Nagaoka, S., Kawai, Y. *et al.*, β -Galactosidase,
49 phospho- β -galactosidase and phospho- β -glucosidase activities in lactobacilli
50 strains isolated from human faeces. *Lett. Appl. Microbiol.* 2007, 45, 461-466.
51
52
53
54 [33] Vasiljevic, T., Jelen, T., Oligosaccharide production and proteolysis during
55 lactose hydrolysis using crude cellular extracts from lactic acid bacteria. *Le*
56 *Lait* 2003, 83, 453-467.
57
58
59
60 490

- 1
2
3 [34] Nguyen, T.-H., Splechtna, B., Krasteva, S., Kneifel, W. *et al.*, Characterization
4 and molecular cloning of a heterodimeric β -galactosidase from the probiotic
5 strain *Lactobacillus acidophilus* R22. *FEMS Microbiol. Lett.* 2007, 269, 136-
6 144.
7
8
9
10
11
12 495 [35] Splechtna, B., Nguyen, T.-H., Zehetner, R., Lettner, H. P. *et al.*, Process
13 development for the production of prebiotic galacto-oligosaccharides from
14 lactose using β -galactosidase from *Lactobacillus* sp. *Biotechnol. J.* 2007, 2,
15 480-485.
16
17
18
19
20
21
22 [36] Canchaya, C., Claesson, M. J., Fitzgerald, G.F., van Sinderen, D., O'Toole, P.
23 W., Diversity of the genus *Lactobacillus* revealed by comparative genomics of
24 500 five species. *Microbiology* 2006, 152, 3185-3196.
25
26
27
28
29 [37] Halbmayr, E., Mathiesen, G., Nguyen, T.-H., Maischberger, T. *et al.* High-level
30 expression of recombinant β -galactosidases in *Lactobacillus plantarum* and
31 *Lactobacillus sakei* using a Sakacin P-based expression system. *J. Agric.*
32 505 *Food Chem.* 2008, 56, 4710-4719.
33
34
35
36
37
38 [38] Corral, J. M., Banuelos, O., Adrio, J. L., Velasco, J., Cloning and
39 characterization of a β -galactosidase encoding region in *Lactobacillus*
40 *coryniformis* CECT 5711. *Appl. Microbiol. Biotechnol.* 2006, 73, 640-646.
41
42
43
44 [39] Fortina, M. G., Ricci, G., Mora, D., Guglielmetti, S., Manichini, P. L., Unusual
45 organization for lactose and galactose gene clusters in *Lactobacillus*
46 510 *helveticus*. *Appl. Environ. Microbiol.* 2003, 69, 3238-3243.
47
48
49
50
51
52 [40] Pridmore, R. D., Berger, B., Desiere, F., Vilanova, D. *et al.*, The genome
53 sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC
54 533. *Proc. Natl. Acad. Sci. U. S. A.* 2004, 101, 2512-2517.
55
56
57
58
59
60

- 1
2
3 515 [41] Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D. *et al.*,
4 Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl.*
5 *Acad. Sci. U. S. A.* 2003, *100*, 1990-1995.
6
7
8
9
10 [42] Obst, M., Meding, E. R., Vogel, R. F., Hammes, W. P., Two genes encoding
11 the β -galactosidase of *Lactobacillus sake*. *Microbiology* 1995, *141*, 3059-3066.
12
13
14
15 520 [43] Bae, H. C., Choi, J. W., Nam, M. S., Purification and characterisation of β -
16 galactosidase from *Lactobacillus salivarius* subsp. *salivarius* Nam27. *Korean*
17 *J. Food Sci. Ani. Resour.* 2007, *27*, 110-116.
18
19
20
21
22 [44] Adams, R. M., Yoast, S., Mainzer, S. E., Moon, K. *et al.*, Characterization of
23 two cold-sensitive mutants of the β -galactosidase from *Lactobacillus delbruckii*
24 subsp. *bulgaricus*. *J. Biol. Chem.* 1994, *269*, 5666-5672.
25
26
27 525
28
29 [45] de Macias, M. E., Manca de Nadra, M. C., Strasser de Saad, A. M., Pesce de
30 Ruiz Holgado, A. A., Oliver, G., Isolation and properties of β -galactosidase of
31 a strain of *Lactobacillus helveticus* isolated from natural whey starter. *J. Appl.*
32 *Biochem.* 1983, *5*, 275-281.
33
34
35
36
37
38
39 530 [46] Acebron, I., Curiel, J. A., de Las Rivas, B., Munoz, R., Mancheno, J. M.,
40 Cloning, production, purification and preliminary crystallographic analysis of a
41 glycosidase from the food lactic acid bacterium *Lactobacillus plantarum* CECT
42 748(T). *Protein Expr. Purif.* 2009, *68*, 177-182.
43
44
45
46
47
48 [47] de Roos, A., Industrial enzymes: enzymes in dairy applications, in: Aehle, W.
49 (Ed.), *Enzymes in Industry*, Wiley-VCH, Weinheim 2004, Vol. 2, p. 144.
50
51 535
52
53 [48] Kim, C. S., Ji, E. S., Oh, D. K. Expression and characterization of
54 *Kluyveromyces lactis* β -galactosidase in *Escherichia coli*. *Biotechnol. Lett.*
55 2003, *25*, 1769-1774.
56
57
58
59
60

- 1
2
3
4
5
6 540
7
8
9
10
11
12
13
14
15
16
17 545
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
- [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

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).

Temperature (°C)	Sodium phosphate buffer (pH 6.5)		Sodium phosphate buffer (pH 6.5, + Mg^{2+})	
	Inactivation constant k_{in} (h^{-1})	Half-life $\tau_{1/2}$ (h)	Inactivation constant k_{in} (h^{-1})	Half-life $\tau_{1/2}$ (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

1
2
3
4
5
6
7
8 565 **Figure 1.** SDS-PAGE (A) and native PAGE (B) of purified β -galactosidase from *L.*
9 *pentosus*. A: lane 1, molecular weight marker (Precision Plus Protein Dual Color,
10 Biorad), CBB staining; lane 2, enzyme sample after treatment in SDS buffer at 100 °C
11 for 5 min, CBB staining. B: lane 1, molecular weight marker (High Molecular Weight
12 Calibration Kit for electrophoresis, Amersham); lane 2, CBB staining.
13
14
15
16
17
18

19
20 570
21
22 **Figure 2.** pH optimum of β -galactosidase from *L. pentosus* using lactose (A) and
23 *o*NPG (B) as the substrate. Symbols: ●, sodium acetate buffer; ○, sodium
24 phosphate buffer; ▼, glycine buffer. Activities are given relative to standard assay
25 conditions (sodium phosphate buffer, pH 6.5).
26
27
28
29
30

31 575
32
33
34 **Figure 3.** Temperature optimum of β -galactosidase from *L. pentosus* when using
35 lactose (●) and *o*NPG (○) as the substrate. Activities are given relative to standard
36 assay conditions at 30 °C.
37
38
39
40
41
42

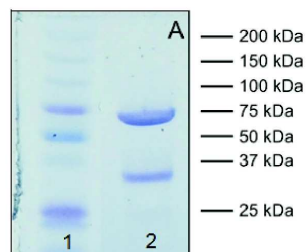
43 580 **Figure 4.** Inactivation kinetics of β -galactosidase from *L. pentosus* in sodium
44 phosphate buffer (A; 50 mM, pH 6.5) and sodium phosphate buffer supplemented
45 with 1 mM MgSO₄ (B; 50 mM, pH 6.5) at different temperatures.
46
47
48 Symbols: (A): ●, 4 °C; ○, 23 °C; ▼, 30 °C; ▽, 37 °C; ■, 42 °C; (B): ●, 30 °C; ○, 37 °C;
49 ▼, 42 °C.
50
51
52
53

54
55 585
56
57 **Figure 5.** Formation of galacto-oligosaccharides (GOS) by β -galactosidase from *L.*
58 *pentosus* during the discontinuous conversion reaction of 205 g/L lactose in 50 mM
59 sodium phosphate buffer at 30 °C. (A) Formation of disaccharides (●), trisaccharides
60

1
2
3 (O), tetrasaccharides (▼) and total GOS (▽) during lactose conversion. (B) Time
4
5 590 course of reaction for lactose conversion; symbols: lactose (▼), glucose (●),
6
7 galactose (O).
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

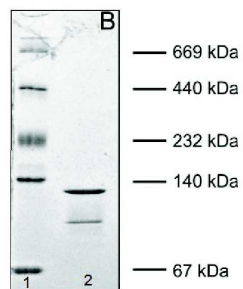
For Peer Review

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



210x297mm (600 x 600 DPI)

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



210x297mm (600 x 600 DPI)