

## Research Article

# $\beta$ -Galactosidase from *Lactobacillus pentosus*: Purification, characterization and formation of galacto-oligosaccharides

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A novel heterodimeric  $\beta$ -galactosidase with a molecular mass of 105 kDa was purified from 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<sub>oNPG</sub>/mg protein. The  $K_m$ ,  $k_{cat}$  and  $k_{cat}/K_m$  values for lactose and *o*-nitrophenyl- $\beta$ -D-galactopyranoside (oNPG) were 38 mM, 20 s<sup>-1</sup>, 530 M<sup>-1</sup>·s<sup>-1</sup> and 1.67 mM, 540 s<sup>-1</sup>, 325 000 M<sup>-1</sup>·s<sup>-1</sup>, respectively. The temperature optimum of  $\beta$ -galactosidase activity was 60–65°C for a 10-min assay, which is considerably higher than the values reported for other lactobacillal  $\beta$ -galactosidases. Mg<sup>2+</sup> ions enhanced both activity and stability significantly. *L. pentosus*  $\beta$ -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  $\beta$ -(1→3) and  $\beta$ -(1→6) linkages, and the main transgalactosylation products identified were the disaccharides  $\beta$ -D-Galp-(1→6)-D-Glc,  $\beta$ -D-Galp-(1→3)-D-Glc,  $\beta$ -D-Galp-(1→6)-D-Gal,  $\beta$ -D-Galp-(1→3)-D-Gal, and the trisaccharides  $\beta$ -D-Galp-(1→3)-D-Lac,  $\beta$ -D-Galp-(1→6)-D-Lac.

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## 1 Introduction

The lactic acid bacterium *Lactobacillus 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 iso-

lates, generally recognised as safe (GRAS), and capable of metabolising different pentoses such as ribose, arabinose and xylose [1]. Yet, some subspecies of *L. pentosus* have been isolated, e.g. from human faeces [2] or from raw milk [3], that have been proposed as potentially probiotic strains. Fred *et al.* [4] previously described very effective growth of *L. pentosus* on lactose, which could imply high  $\beta$ -galactosidase activity, but this property has not been studied to date.

Lactic acid bacteria (LAB) and  $\beta$ -galactosidases have been studied extensively in the past, both from a fundamental and an applied point of view.  $\beta$ -Galactosidases, e.g. from *Bifidobacterium bifidum*, *B. infantis* [5], *Carnobacterium piscicola* [6], *L. acidophilus* [7], *L. bulgaricus* [8], *L. reuteri* [9], and *Leuconostoc lactis* [10], have been cloned and characterised in varying detail. Despite of the importance

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**Abbreviations:**  $\beta$ -Gal,  $\beta$ -galactosidase; GOS, galacto-oligosaccharides; LAB, lactic acid bacteria; MUG, 4-methylumbelliferyl- $\beta$ -D-galactopyranoside; oNPG, *o*-nitrophenyl- $\beta$ -D-galactopyranoside, oNP, *o*-nitrophenol

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  $\beta$ -galactosidases have been characterised in detail pertaining to their biochemical properties or 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 [16–18].

GOS are produced from lactose using  $\beta$ -galactosidases (EC 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 [19–21]. The spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the  $\beta$ -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  $\beta$ -galactosidases from different sources, a detailed knowledge on novel, yet-unexplored  $\beta$ -galactosidases from various strains can be of significant interest [20]. Rabiou *et al.* [22] and Tzortzis *et al.* [23]

synthesised a range of GOS mixtures from lactose using  $\beta$ -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  $\beta$ -galactosidases from beneficial probiotic organisms.

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

## 2 Materials and methods

### 2.1 Strain and culture conditions

*L. pentosus* KUB-ST10-1 was obtained from the culture collection of Kasetsart University (Bangkok, Thailand). It was isolated from soil of a dairy farm in Thailand and identified 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  $-70^{\circ}\text{C}$  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 MRS broth medium containing 2% lactose at  $37^{\circ}\text{C}$  with slight agitation and without pH control. A preculture, grown overnight in MRS medium (2% lactose), was used to inoculate the fresh medium to a final concentration of 1% inoculum. Cells were harvested at an optical density ( $\text{OD}_{600}$ ) of 13 by centrifugation at  $8800 \times g$  for 15 min at  $4^{\circ}\text{C}$ .

### 2.2 Enzyme purification

$\beta$ -Galactosidase was purified from the crude extract using a previously described protocol [9]. Wet biomass (100 g) was resuspended in 200 mL sodium phosphate buffer (50 mM, pH 6.5) and homogenized (APV-2000; Silkeborg, Denmark) to disrupt the cells. Cell debris was removed by ultracentrifuge

gation ( $36\,000 \times g$ , 30 min,  $4^{\circ}\text{C}$ ) to obtain the crude enzyme extract.

Ammonium sulphate was added in small portions to the crude extract to a final concentration of 50% saturation under continuous stirring at  $4^{\circ}\text{C}$ . The precipitated protein was collected by centrifugation ( $6200 \times g$ , 15 min,  $4^{\circ}\text{C}$ ), and the pellet was re-dissolved in 50 mM sodium phosphate buffer (pH 6.5) containing 1 M ammonium sulphate (buffer A).

For hydrophobic interaction chromatography, the dissolved pellet was loaded onto a 20-mL phenyl-Sepharose fast-flow column (50 mm  $\times$  200 mm; Amersham, Uppsala, Sweden) that had been pre-equilibrated with buffer A. Proteins were eluted by a linear gradient of 0–100% buffer B (50 mM sodium phosphate buffer, pH 6.5) over ten column volumes (CV) and at a flow rate of 1.5 mL/min. Fractions with high  $\beta$ -galactosidase activity were pooled and concentrated by ultrafiltration (30-kDa cut-off; Amicon, Beverly, MA).

For affinity chromatography, the concentrated protein solution was applied to a 5-mL column containing cross-linked (4%) beaded agarose immobilized with *p*-aminobenzyl-1-thio- $\beta$ -D-galactopyranoside (Sigma, St. Louis, MO, USA) 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 (0–1 M NaCl in the same buffer) over ten 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  $4^{\circ}\text{C}$ .

### 2.3 Enzyme assays

$\beta$ -Galactosidase activity was determined using both the artificial chromogenic substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside (*o*NPG) and the natural substrate lactose as described previously [9]. When using *o*NPG as the substrate, the reaction was performed in 50 mM sodium phosphate buffer (pH 6.5) and using a 10-min incubation at  $30^{\circ}\text{C}$ , after which the reaction was stopped. One unit of *o*NPG activity ( $U_{\text{oNPG}}$ ) was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  *o*NP/min 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  $30^{\circ}\text{C}$ , 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 ( $U_{\text{Lac}}$ ) was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  D-glucose/min under the conditions listed above.

### 2.4 Protein analysis and gel electrophoresis

Protein concentrations were determined by the method of Bradford [27] using BSA 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 protein/mL and incubated with  $2 \times$  Laemmli buffer at  $60^{\circ}\text{C}$  for 5 min. Protein bands were stained using either Coomassie Blue staining or 4-methylumbelliferyl- $\beta$ -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 (Bio-Rad) and the High Molecular Weight calibration Kit (Amersham), respectively.

### 2.5 Steady-state kinetic measurement

Catalytic constants were determined at  $30^{\circ}\text{C}$  and pH 6.5 for the two substrates *o*NPG 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  $\beta$ -galactosidase activity (both *o*NPG and lactose) was determined by assaying the enzyme samples over 20– $70^{\circ}\text{C}$  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  $42^{\circ}\text{C}$ . At certain time intervals samples were taken and the residual *o*NPG activity was measured. For measuring the pH dependence of  $\beta$ -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 determination of the pH stability, enzyme samples were incubated at various pH values and  $37^{\circ}\text{C}$  for up to 72 h, and the remaining enzyme activity was measured at certain time intervals using *o*NPG as substrate under standard assay conditions.

### 2.7 Substrate specificity

To determine the substrate specificity of the *L. pentosus*  $\beta$ -galactosidase for various structurally related substrates *p*-nitrophenyl- $\beta$ -D-mannopyranosi-

de, *p*-nitrophenyl- $\beta$ -D-xylopyranoside, *o*-nitrophenyl- $\beta$ -D-glucopyranoside and *p*-nitrophenyl- $\beta$ -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  $\beta$ -galactosidase from *L. pentosus* (5 U<sub>*o*NPG</sub>/mL reaction mixture) was used for the discontinuous formation of GOS at 30°C using 205 g/L lactose in 50 mM sodium phosphate buffer (pH 6.5, 2 mM MgCl<sub>2</sub>) as substrate. Samples were taken at various intervals and the composition of the GOS mixture was analyzed by capillary electrophoresis and high-performance anion exchange chromatography with pulsed amperometric detection as described previously [13].

## 3 Results

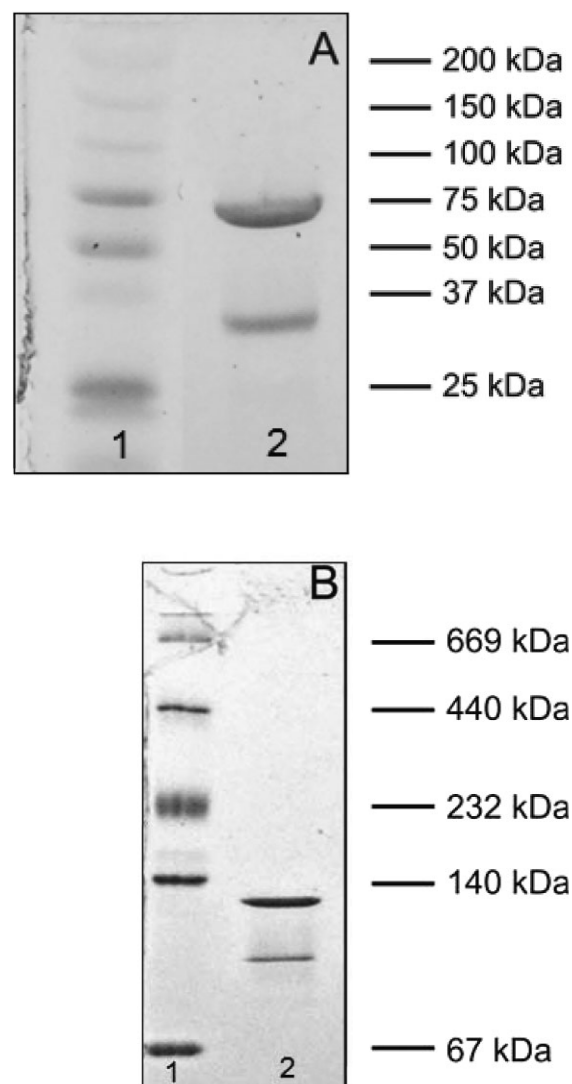
### 3.1 Formation and purification of $\beta$ -galactosidase from *L. pentosus*

*L. pentosus* KUB-ST10-1 was selected for detailed studies on its  $\beta$ -galactosidase since this strain grew very well on lactose, showing high  $\beta$ -gal activity in a screening in which several LAB were compared for their ability to form this enzyme activity (data not shown), and since its  $\beta$ -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  $\beta$ -gal activity reached a maximum of 644 U<sub>*o*NPG</sub>/L fermentation broth.  $\beta$ -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 protein, using standard assay conditions with *o*NPG as the substrate.

### 3.2 Properties of $\beta$ -galactosidase from *L. pentosus*

*L. pentosus*  $\beta$ -galactosidase is a heterodimer ( $M_r$  ~107 kDa) consisting of a small (~35 kDa) and large (~72 kDa) subunit as judged by SDS-PAGE (Fig. 1A) and native PAGE (Fig. 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  $\beta$ -gal from *L. reuteri* us-

ing mass spectrometry [9]. Active staining on native PAGE with MUG yielded only one band with  $\beta$ -galactosidase activity, corresponding to the larger band in Fig 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 was detected after active staining on the gel.



**Figure 1.** SDS-PAGE (A) and native PAGE (B) of purified  $\beta$ -galactosidase from *L. pentosus*. (A) Lane 1, molecular weight marker (Precision Plus Protein Dual Color, Bio-Rad), 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.



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

Substrate	Method for determination of enzyme activity	Kinetic parameter	<i>L. pentosus</i> $\beta$ -galactosidase
Lactose	Release of D-glucose	$v_{\max}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	11.3 $\pm$ 0.75
		$K_m$ (mM)	37.8 $\pm$ 9.41
		$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	20.1
		$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \text{s}^{-1}$ )	0.532
<i>o</i> NPG	Release of <i>o</i> NP	$v_{\max}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	304 $\pm$ 24.6
		$K_m$ (mM)	1.67 $\pm$ 0.64
		$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	543
		$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \text{s}^{-1}$ )	325

### 3.3 Substrate specificity

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

### 3.4 Enzyme kinetics

Steady-state kinetic studies were performed with the preferred substrates lactose and *o*NPG and the kinetic constants  $K_m$ ,  $v_{\max}$ , and  $k_{\text{cat}}$  were determined. The  $k_{\text{cat}}$  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 ( $k_{\text{cat}}/K_m$ ) for the two substrates, *o*NPG and lactose, indicate that *o*NPG is the preferred substrate in accordance with other microbial  $\beta$ -galactosidases.

### 3.5 Effects of pH and temperature

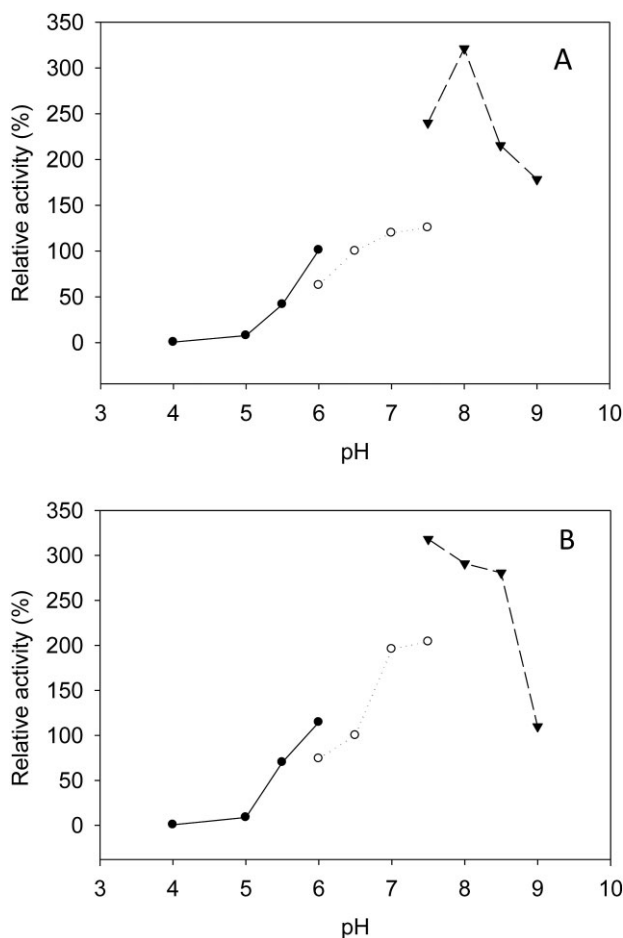
The pH optimum of *L. pentosus*  $\beta$ -galactosidase activity was pH 8.0 and pH 7.5 in glycine buffer when using lactose and *o*NPG as substrate, respectively (Fig. 2). In general, the enzyme was more active in the alkaline range (pH 7.0–8.5), and a pronounced effect of the buffer system used on activity was seen. The effect of different buffers and pH values on stability was subsequently investigated in more detail. Catalytic or kinetic stability, *i.e.* the length of time an enzyme remains active before undergoing irreversible inactivation, was measured at various pH at a constant temperature of 37°C, and the inactivation constants ( $k_{\text{in}}$ ) and the half-life times of denaturation ( $\tau_{1/2}$ ) were determined (Table 2).  $\beta$ -Galactosidase showed first-order inactivation ki-

netics at all pH values studied when analysed in the  $\ln(\text{residual activity})$  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 lowest 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 *o*NPG hydrolysis was 60–65°C and 55°C, respectively (Fig. 3). The effect of temperature on stability of  $\beta$ -galactosidase from *L. pentosus* was investigated at the pH value of milk, pH 6.5, both in the absence and presence of 2 mM  $\text{MgCl}_2$ . Inactivation constants and half-life times were calculated as described above. The addition of 2 mM  $\text{MgCl}_2$  to the buffer improved stability significantly, increasing the half life times  $\tau_{1/2}$  of the enzyme at 30, 37 and 42°C by 3.2-, 4.9- and 5.6-fold, respectively (Table 3; Fig. 4).

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

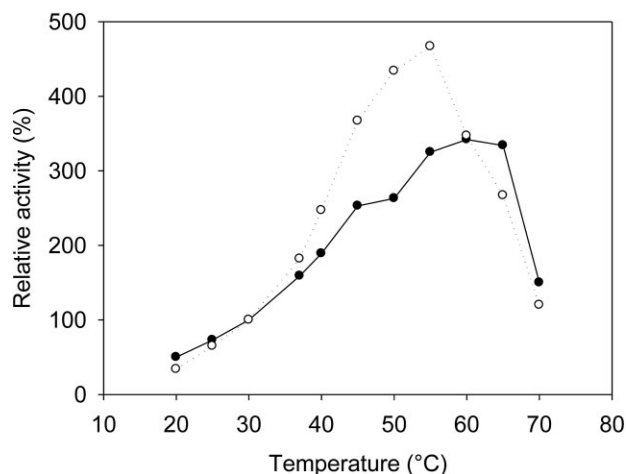
Buffer	pH	Inactivation constant $k_{\text{in}}$ ( $\text{h}^{-1}$ )	Half-life $\tau_{1/2}$ (h)
Sodium acetate	4.0	3770 $\times 10^{-3}$	0.18
Sodium acetate	5.0	504 $\times 10^{-3}$	1.37
Sodium acetate	5.5	131 $\times 10^{-3}$	5.31
Sodium acetate	6.0	91.9 $\times 10^{-3}$	7.54
Sodium phosphate	6.0	63.3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



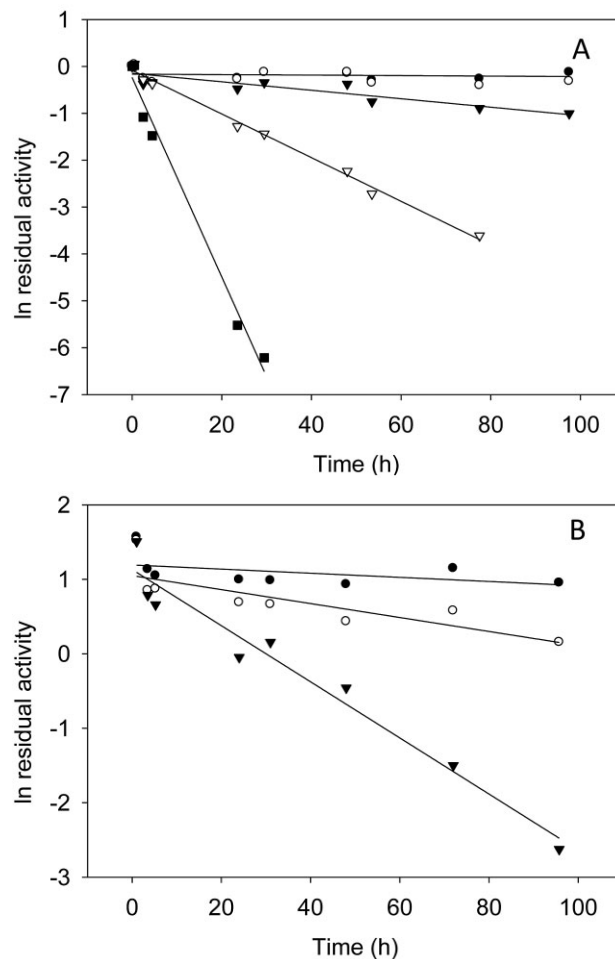
**Figure 2.** pH optimum of  $\beta$ -galactosidase from *L. pentosus* using lactose (A) and oNPG (B) as the substrate. Symbols: (●) sodium acetate buffer; (○) sodium phosphate buffer; (▼) glycine buffer. Activities are given relative to standard assay conditions (sodium phosphate buffer, pH 6.5).

### 3.6 Transgalactosylation activity

To assess the potential of the novel  $\beta$ -galactosidase from *L. pentosus* 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  $U_{oNPG}/mL$   $\beta$ -galactosidase activity to produce GOS. The maximum GOS yield (31%) was obtained at a lactose conversion of 78% (Fig. 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



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

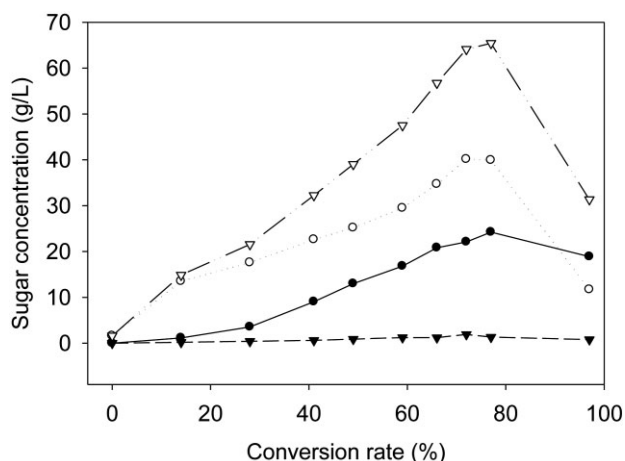


**Figure 4.** Inactivation kinetics of  $\beta$ -galactosidase from *L. pentosus* in sodium phosphate buffer (A; 50 mM, pH 6.5) and sodium phosphate buffer supplemented with 1 mM  $MgSO_4$  (B; 50 mM, pH 6.5) at different temperatures. Symbols: (A): (●) 4°C; (○) 23°C; (▼) 30°C; (▽) 37°C; (■) 42°C; (B): (●) 4°C; (○) 23°C; (▼) 30°C; (▽) 37°C; (■) 42°C.

**Table 3.** Kinetic stability of  $\beta$ -galactosidase from *Lactobacillus pentosus* at various temperatures with and without  $\text{MgCl}_2$  supplementation (2 mM)<sup>a</sup>

Temperature (°C)	Sodium phosphate buffer (pH 6.5)		Sodium phosphate buffer (pH 6.5, + $\text{Mg}^{2+}$ )	
	Inactivation constant $k_{in}$ ( $\text{h}^{-1}$ )	Half-life $\tau_{1/2}$ (h)	Inactivation constant $k_{in}$ ( $\text{h}^{-1}$ )	Half-life $\tau_{1/2}$ (h)
4	$0.5 \times 10^{-3}$	1386	nd	nd
23	$0.8 \times 10^{-3}$	866	nd	nd
30	$9.0 \times 10^{-3}$	77.0	$2.8 \times 10^{-3}$	248
37	$46.6 \times 10^{-3}$	14.9	$9.4 \times 10^{-3}$	73.7
42	$212.7 \times 10^{-3}$	3.26	$37.7 \times 10^{-3}$	18.4

a) nd, not determined.

**Figure 5.** Formation of galacto-oligosaccharides (GOS) by  $\beta$ -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 (○), tetrasaccharides (▼) and total GOS (▽) during lactose conversion. (B) Time course of reaction for lactose conversion; symbols: lactose (▼), glucose (●), galactose (○).

main GOS products identified were non-lactose disaccharides, mainly of the  $\beta$ -(1→3) and  $\beta$ -(1→6) linkage type and the two trisaccharides  $\beta$ -D-Galp-(1→3)-lactose, and  $\beta$ -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 dairy products, meat, fish and plant material such as vegetables, and thus have been investigated intensively for their industrial applications [30]. Lactose utilisation is a primary function of lactobacilli and other LAB used in the fermentation of milk. The mecha-

nism by which lactose is transported into the cell determines largely the subsequent pathway for the hydrolysis of this disaccharide. In several *Lactobacillus* species lactose is transported via phosphotransferase systems, which results in phosphorylation of lactose concomitant with its uptake. The resulting lactose-6'-phosphate is then hydrolysed by phospho- $\beta$ -galactosidase. Alternatively, lactose is taken up by secondary transport systems, and lactose is further metabolised by  $\beta$ -galactosidase within the cell [31, 32]. While the organisation of these lactose genes, which often form operons or operon-like structures with modular organisation, has been studied in much detail, the properties of some of the enzymes encoded by the genes organised in these operons, including  $\beta$ -galactosidases, have received significantly less attention. It is thus surprising that the physical and biochemical properties of  $\beta$ -galactosidases from lactobacilli, which play an eminent role in the metabolism of the principal carbohydrate in milk, have only been characterised in detail in a few instances, and little attention has been paid to the catalytic ability of these enzymes with respect to their transglycosylation reactions [12, 19, 33].

Recently, we reported a comprehensive characterisation of the  $\beta$ -galactosidases from *L. reuteri* and *L. acidophilus*, and pointed out their applicability for the biocatalytic production of GOS [9, 13, 34, 35]. Here, we present the detailed biochemical characterisation and the transgalactosylation activity of a novel  $\beta$ -galactosidase from *L. pentosus*. The strain KUB-ST10-1 used throughout this research was isolated from a soil sample in Thailand, and was selected for further characterisation based on a screening and its high  $\beta$ -galactosidase activity when grown on lactose. It was of interest to determine whether a  $\beta$ -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 it was possible that the latter might differ in its metabolic activities from the  $\beta$ -galactosidases from other sources recently characterised in our group.

The structural features of  $\beta$ -galactosidases from lactobacilli vary extensively. While heterodimeric structures are in general less frequently encountered among microbial  $\beta$ -galactosidases, these seem to prevail among *Lactobacillus*  $\beta$ -galactosidases. Lactobacillal heterodimeric  $\beta$ -galactosidases are encoded by two genes, *lacL* and *lacM*, and have been described in *L. acidophilus* [34, 37], *L. coryniformis* [38], *L. helveticus* [39], *L. johnsonii* [40], *L. plantarum* [41], *L. reuteri* [9] and *L. sakei* [42]. These  $\beta$ -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,  $\beta$ -galactosidase from *L. salivarius* was reported to be a monomer of ca. 30 kDa [43], from *L. delbrueckii* subsp. *bulgaricus* a homodimer of 220 kDa [44], and from *L. helveticus* a homotetramer of 257 kDa [45]; *L. plantarum* forms, in addition to the above-mentioned heterodimeric enzyme, a homohexameric  $\beta$ -galactosidase of ca. 325 kDa [46]. Some of these latter  $\beta$ -galactosidases are members of glycoside family GH42.  $\beta$ -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  $\beta$ -galactosidases reported up to now, and is thus also of the LacLM type.

The steady-state kinetic constants for  $\beta$ -galactosidase from *L. pentosus* were determined for the artificial chromogenic substrate oNPG and the natural substrate lactose. The  $k_{\text{cat}}/K_{\text{m}}$  value for oNPG is significantly higher than that of lactose, indicating that oNPG is the better substrate. The  $K_{\text{m}}$  value of 37.8 mM determined for lactose compares positively with the Michaelis constants reported for commercially employed  $\beta$ -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*  $\beta$ -galactosidases: 13 mM for  $\beta$ -gal from *L. reuteri* L103 [9], 1.27 mM for  $\beta$ -gal from *L. kefirianofaciens* [49], and 4.04 mM for  $\beta$ -gal from *L. acidophilus* [34]. The higher  $K_{\text{m, lactose}}$  value for *L. pentosus*  $\beta$ -gal as compared to other lactobacillal enzymes, especially those of milk origin, could indicate a lesser adaptation of this soil-isolated strain to lactose utilisation. *L. pentosus*  $\beta$ -gal is rather specific for the  $\beta$ -(1 $\rightarrow$ 4) galac-

tosyl linkage. When using a range of structurally related nitrophenyl substrates, essentially no activity was measured for the  $\beta$ -(1 $\rightarrow$ 4)-linked D-glucoside, D-mannoside, and D-xyloside substrates.

*L. pentosus*  $\beta$ -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  $\beta$ -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 affected the actual value determined. The temperature optimum of *L. pentosus*  $\beta$ -gal of 60–65°C is considerably higher than reported for most other  $\beta$ -galactosidases from lactobacilli, which are typically 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*  $\beta$ -galactosidase might reflect an adaptation to these higher growth temperatures. Because of this increased temperature optimum we investigated the thermostability of *L. pentosus*  $\beta$ -gal in more detail, determining the inactivation kinetics at different conditions. With respect to pH, the 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  $k_{\text{in}}$  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, the highest stability was observed at pH 6.5–7.0 with  $\tau_{1/2}$  of 14.6 h. Addition of 2 mM  $\text{Mg}^{2+}$  increased stability and hence  $\tau_{1/2}$  of  $\beta$ -gal activity significantly by a factor of up to 5.6-fold. For example,  $\tau_{1/2}$  values of ~75 h were obtained at pH 6.5, 37°C and when using phosphate buffer containing 2 mM of  $\text{Mg}^{2+}$ . This increase in stability and activity of *L. pentosus*  $\beta$ -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  $\text{Mg}^{2+}$ . Thus, use of these technical substrates could improve the performance of *L. pentosus*  $\beta$ -gal without the need of adding exogenous  $\text{Mg}^{2+}$ .

$\beta$ -Galactosidases have recently attained significant interest for use in biocatalytic processes for synthesising oligosaccharides or various galactosy-



lated compounds; for this purpose various galactosides can serve as the galactosyl donor. In particular, 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 for health [16, 17, 19, 20]. As GOS are only transiently formed and are in turn also substrates for  $\beta$ -galactosidase, a maximum GOS yield of 31% was reached at 78% lactose conversion when using *L. pentosus*  $\beta$ -gal. The main GOS spectrum formed from lactose by this enzyme is very similar to those found for  $\beta$ -gal from *L. reuteri* [13] and *L. acidophilus* [34]. *L. pentosus*  $\beta$ -gal shows a high specificity for the formation of  $\beta$ -(1 $\rightarrow$ 6) and  $\beta$ -(1 $\rightarrow$ 3) linkages, and hence the main transgalactosylation products detected were the disaccharides  $\beta$ -D-Galp-(1 $\rightarrow$ 6)-D-Glc,  $\beta$ -D-Galp-(1 $\rightarrow$ 3)-D-Glc,  $\beta$ -D-Galp-(1 $\rightarrow$ 6)-D-Gal,  $\beta$ -D-Galp-(1 $\rightarrow$ 3)-D-Gal, and the trisaccharides  $\beta$ -D-Galp-(1 $\rightarrow$ 3)-D-Lac,  $\beta$ -D-Galp-(1 $\rightarrow$ 6)-D-Lac. In a recent study [51], a strong prebiotic effect was attributed to some of these compounds, and it is likely that the GOS mixture formed by *L. pentosus*  $\beta$ -gal has a considerable potential as a prebiotic.

In conclusion, this work presented the characterisation and transgalactosylation capacity of a novel  $\beta$ -galactosidase from *L. pentosus*. Although the  $\beta$ -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, which is produced by a well-known food-grade organism, interesting for potential applications in food industry.

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