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ORIGINAL PAPER

# Synthesis of oligosaccharides with lactose and *N*-acetylglucosamine as substrates by using *β*-D-galactosidase from *Bacillus circulans*

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**Abstract** In the present study, *β*-D-galactosidase from *Bacillus circulans* was proved to be a suitable biocatalyst for the production of *N*-acetyl-oligosaccharides with lactose and *N*-acetylglucosamine (GlcNAc) as biocatalyst. During the hydrolysis of lactose, apart from no ultraviolet absorption oligosaccharides such as *β*-D-Gal*p*-(1 → 6)-D-Glc*p* (6′-allolactose) and *β*-D-Gal*p*-(1 → 4)-*β*-D-Gal*p*-(1 → 4)-D-Glc*p* (4′-galactosyl-lactose), the formation of four *N*-acetyl-oligosaccharides was followed by high-performance liquid chromatography with a diode-array detector. The four *N*-acetyl-oligosaccharides were isolated from the reaction mixture and identified to be as *β*-D-Gal*p*-(1 → 4)-D-Glc*p*NAc (LacNAc, **I**), *β*-D-Gal*p*-(1 → 6)-D-Glc*p*NAc (allo-LacNAc, **II**), *β*-D-Gal*p*-(1 → 4)-*β*-D-Gal*p*-(1 → 4)-D-Glc*p*NAc (**III**), *β*-D-Gal*p*-(1 → 4)-*β*-D-Gal*p*-(1 → 4)-*β*-D-Gal*p*-(1 → 4)-D-Glc*p*NAc (**IV**) by authentic standards and the spike technique or high-resolution mass spectrometry with an electrospray ionization source and nuclear magnetic resonance spectroscopy. Furthermore, the effects of synthetic conditions including reaction temperature, concentration of substrate, molar ratio of donor/acceptor and enzyme concentration on the formation of *N*-acetyl-oligosaccharides were examined. We found that the optimal synthetic conditions were different for production of oligosaccharides with *β*-(1 → 4) linkages and *β*-(1 → 6) linkage. The optimal reaction conditions for **I**, **III** and **IV** were 40 °C, 0.50 M lactose and 0.50 M GlcNAc and 1.0 U/mL of enzyme. Under such conditions, the *N*-acetyl-oligosaccharides formed were composed of 28.75% of **I**, 2.29% of **II**, 9.47% of **III** and 5.67% of **IV**. On the other hand, suitable reaction conditions found for **II** were 40 °C, 0.50 M lactose and 0.50 M GlcNAc and 2.0 U/mL of enzyme.

**Keywords** *β*-D-Galactosidase · Transgalactosylation · *N*-acetyl-oligosaccharides · *Bacillus circulans*

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## Introduction

There is a consensus that oligosaccharide-rich foods are beneficial to health. Some have functional effects, similar to receptor analogs [1] or structurally related to naturally occurring cell surface glycoconjugates and immunomodulatory components [2]. For example, human milk oligosaccharides have been reported to have pathogenic adherence–inhibition activity [3, 4]. *N*-Acetyllactosamine (*β*-D-Gal*p*-(1 → 4)-D-Glc*p*NAc, LacNAc, **I**), a representative core structure in oligosaccharide components of human milk oligosaccharides [5, 6], has been shown to be a constituent of glycoconjugates and has been identified as a competitively inhibitor for enteropathogenic *Escherichia coli* [7, 8]. Furthermore, several studies on the metabolic fate of the oligosaccharides in human milk have outlined that these substances resist the digestion [3, 5] and reach the colon where they stimulate the development of the bifidobacteria and lactobacilli, thus representing the paradigm of prebiotics [6, 9]. Recently, some synthetic oligosaccharides have also been reported to have adherence–inhibition activity [1, 10, 11], such as α-D-Gal*p*-(1 → 4)-*β*-D-Gal*p*-(1 → 4)-D-Glc*p*NAc and *β*-D-Gal*p*-(1 → 6)-*β*-D-Gal*p*-(1 → 4)-D-Glc*p* etc., which contain saccharide residues similar to those expressed on the binding site of the epithelial surface receptor for enteropathogenic *Escherichia coli*. Studies concerning the contribution that each

constituent saccharide residue makes toward pathogens binding suggest the importance of the galactose and *N*-acetylglucosamine (D-GlcNAc) residue. Therefore, oligosaccharide-based anti-adhesives containing similar saccharide residues should effectively compete for enteropathogenic *E. coli*, indicating their potential applications as inhibitors of pathogenic adherence or infant dietary additives in food industry [12, 13].

As the understanding of these biological functions increases, the need for practical synthetic procedures of oligosaccharides and their analogs in large quantities has become a major subject. From a practical viewpoint, the use of transglycosylation reactions of glycosidases is more interesting. More results have been reported on oligosaccharides containing galactose and D-GlcNAc residue using $\beta$-D-galactosidase with lactose and D-GlcNAc as substrates during the last few years [14, 15]. Thus far, although a number of studies have been reported on the synthesis of oligosaccharides containing GlcNAc using $\beta$-D-galactosidase, most are focused on the formation of **I** and *N*-acetylallolactosamine ($\beta$-D-Gal*p*-(1 → 6)-D-Glc*p*NAc, allo-LacNAc, **II**), and there are few references on the formation of higher degree of polymerization (DP) oligosaccharides containing GlcNAc other than disaccharide [16, 17]. As it is know, some higher DP oligosaccharides containing GlcNAc have ability against invading pathogens as mentioned above; therefore, it is necessary to gain more insight on the formation not only of disaccharide but also on the production of higher DP oligosaccharides fraction during transgalactosylation reaction. Here, we report in detail the synthesis, purification and characterization of a series of *N*-acetyl-oligosaccharides with lactose and GlcNAc as substrates catalyzed by use of $\beta$-D-galactosidase from *Bacillus circulans*. In addition, the effects of synthetic conditions such as temperature, substrate concentration, ratio of donor/acceptor and enzyme concentration on the formation of transfer products were investigated.

## Materials and methods

### Materials

$\beta$-D-Galactosidase from *B. circulans* was obtained from Daiwa Kasei Co., Ltd. (Osaka, Japan). Lactose, glucose, galactose, GlcNAc, *o*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG) and activated charcoal (Darco G-60, 100 mesh particle size) were purchased from Sigma (St. Louis, MO, USA). Standards of **I**, **II** and $\beta$-D-Gal*p*-(1 → 6)-D-Glc*p* (6′-allolactose) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Celite 535 was got from Fluka Co. (Buchs, Swiltzerland). HPLC grade of acetonitrile was purchased from Hanbon Science and Technology Co., Ltd. (Jiangsu, China). Millipore membrane filters (0.45 µm) were got from Millipore Co. (Bedford, MA, USA). $\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-D-Glc*p* (4′-galactosyllactose) was synthesized according to our reported method [18]. All other chemicals were of analytical grade.

### Determination of enzyme activity

The activity of $\beta$-D-galactosidase was analyzed using ONPG as substrate according to the method reported by Zeng et al. [19]. The reaction was carried out in 5.0 mL 50 mM sodium phosphate buffer (pH 6.0) containing 2.0 mM ONPG and an appropriate amount of enzyme at 37 °C for 10 min. The reaction was stopped by adding 2.0 mL of 1.0 M $Na_2CO_3$ solution. The liberated *o*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µM of *o*-nitrophenol per minute under the assayed conditions. As a result, the commercial enzyme preparation expressed an activity of 860 U per gram of enzyme preparation.

### Synthesis of oligosaccharides

The synthesis of oligosaccharides was carried out with lactose and GlcNAc as substrates with $\beta$-D-galactosidase from *B. circulans* as biocatalyst in eppendorf tube. In order to investigate the influences of synthetic conditions on the formation of transfer products, the enzymatic reactions were done at four temperatures (30, 40, 50 and 60 °C), four concentrations of substrate (0.50, 0.75, 1.00 and 1.25 M), four molar ratios of donor/acceptor (1: 2, 1: 1, 2: 1 and 3: 1) and four enzyme concentrations (0.5, 1.0, 1.5 and 2.0 U/mL). In general, to a solution (1.0 mL) containing substrates dissolved in 50 mM sodium phosphate buffer (pH 6.0, unless otherwise specified) was added $\beta$-D-galactosidase, and the reaction mixture was incubated at the required temperature. Aliquots (10 µL) of samples were withdrawn at various intervals for 12 h and immediately immersed in boiled water for 5 min to inactivate the enzyme. After appropriate dilution, samples were analyzed by high-performance liquid chromatography (HPLC). All assays were run in triplicate.

### Determination of carbohydrates

The carbohydrates in the reaction mixtures were determined by HPLC equipped a refraction index detector (RID) and a diode array detector (DAD), on an Agilent 1100 series HPLC system consisting of a G1311A quatpump, a G1315B DAD, a G1362A RID, a G1379A degasser and a

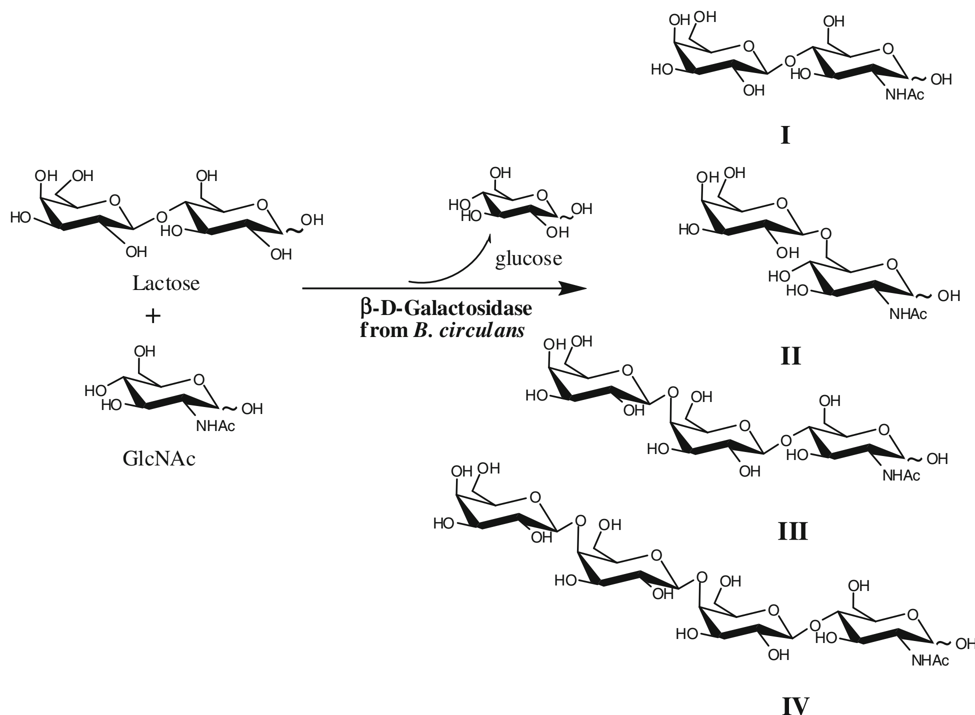
G1316A column oven. Chromatographic separations were performed following the reported method by Li et al. [18] with some modifications. The separation was completed on a Sugar-D column (4.6 × 250 mm, Nacalai Tesque Inc., Japan), using acetonitrile–water (75:25, v/v) as the mobile phase at 40 °C with a flow rate of 1.0 mL/min, or a Shodex Sugar KS-801 column (8.0 × 300 mm, Showa Denko Co., Tokyo, Japan) eluted with ultrapure water at 60 °C with a flow rate of 0.8 mL/min. DAD was monitored at 210 nm (*N*-acetyl group). Injection volume was 20 μL. Sugar compounds in the reaction mixtures were identified by comparing the retention times with those of standard sugars. Quantification of each sugar was performed by an external calibration curve using its corresponding standard solutions.

## Purification and structural characterization of transfer products

Lactose (1.70 g, 0.50 M) and GlcNAc (1.10 g, 0.50 M) were dissolved in 10 mL 50 mM sodium phosphate buffer (pH 6.0), and *β*-D-Galactosidase (10 U) from *B. circulans* was added. The mixture was incubated at 40 °C. After 4-h incubation, the reaction was terminated by heating at 95 °C for 5 min. The reaction mixture was filtrated, and the filtrate was loaded onto an activated charcoal–Celite preparative column (Ø3 × 50 cm, Yamazen Co., Osaka, Japan). The column was first eluted with 1,000 mL of water and then eluted with a linear gradient from 0 to 30% ethanol in water (2,000 mL) at a flow rate of 4 mL/min. After chromatography, the effluent solution was collected and monitored by measuring the absorbances at 210 nm and 485 nm (carbohydrate content, determined by the phenol–sulfuric acid method). Those with sugars were further analyzed by HPLC with a Sugar-D column. As results, three fractions ($F_1$, $F_2$ and $F_3$) containing transfer products were determined. Among the three fractions, $F_3$ was collected, concentrated by a rotary evaporator (Heidolph Laborota 4000 efficient, Schwabach, Germany) and lyophilized to afford the transfer product **IV** (Scheme 1). For $F_1$ and $F_2$, they were collected, concentrated and loaded onto a column of Biogel P2 (1.5 × 90 cm, BioRad, Richmond, USA), respectively. The column was eluted by ultrapure water at a flow rate of 0.4 mL/min, and the eluates were treated as mentioned above. As a result, transfer products **I-III** were obtained.

The structures of transfer products were identified by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance spectroscopy (NMR). $^1$H and $^{13}$C NMR spectra were recorded in $D_2O$ as solvent at 300 K with a Bruker Avance DRX-500 spectrometer (Bruker, Karlsruhe, Germany) using the residual solvent signal as internal standard. The chemical shifts ($\delta$) are given in ppm, and $J$ values are given in Hz. HRMS were obtained by direct injection using the Mariner System 5304 mass spectrometer (Applied Biosystems, Foster City, CA, USA) with electrospray ionization (ESI) source.

Data for **III**: $^1$H NMR $\delta$ 5.07 (d, 1 H, $J$ 3.3, H-1$_\alpha$), 4.52 (d, 1 H, $J$ 7.50, H-1$_\beta$), 4.45 (d, 1 H, $J$ 7.42, H-1″), 4.37 (d, 1 H, $J$ 6.83, H-1′), 1.90 (s, 3 H, $H_3$ of -NH-COCH$_3$); $^{13}$C




**Scheme 1** Synthetic scheme for *N*-acetyl-oligosaccharides by use of *β*-D-galactosidase from *Bacillus circulans* with lactose and GlcNAc as substrates

NMR $\delta$ 175.06 (C=O of -NH-COCH$_3$ due to the $\alpha$ anomer), 174.80 (C=O of -NH-COCH$_3$ due to the $\beta$ anomer), 104.55 (C-1″), 103.25 (C-1′), 95.21 (C-1$_\beta$), 90.86 (C-1$_\alpha$), 79.21 (C-4$_\alpha$), 78.84 (C-4$_\beta$), 77.49 (C-4′), 75.47 (C-5″), 75.18 (C-5$_\beta$), 74.84 (C-5′), 73.28 (C-3″), 73.14 (C-3′), 72.82 (C-3$_\beta$), 71.78 (C-5$_\alpha$), 71.72 (C-2″), 70.60 (C-2′), 69.60 (C-3$_\alpha$), 68.95 (C-4″), 61.33 (C-6″), 61.06 (C-6′), 60.38 (C-6$_\alpha$), 60.26 (C-6$_\beta$), 56.53 (C-2$_\beta$), 54.04 (C-2$_\alpha$), 22.52 (C of -CH$_3$ due to the $\alpha$ anomer) and 22.23 (C of -CH$_3$ due to the $\beta$ anomer); HRMS *m/z* 568.1871 for [M + Na]$^+$ (calcd for $C_{20}H_{35}O_{16}$ N [M + Na]$^+$, 568.1848).

Data for **IV**: $^1$H NMR $\delta$ 5.06 (d, 1 H, *J* 3.3, H-1$_\alpha$), 4.58 (d, 1 H, *J* 6.95, H-1$_\beta$), 4.52 (d, 1 H, *J* 7.50, H-1‴), 4.46 (d, 1 H, *J* 7.42, H-1″), 4.36 (d, 1 H, *J* 6.83, H-1′), 1.90 (s, 3 H, H$_3$ of -NH-COCH$_3$); $^{13}$C NMR $\delta$ 175.07 (C=O of -NH-COCH$_3$ due to the $\alpha$ anomer), 174.79 (C=O of -NH-COCH$_3$ due to the $\beta$ anomer), 104.62 (C-1‴, C-1″), 103.24 (C-1′), 95.18 (C-1$_\beta$), 90.84 (C-1$_\alpha$), 79.21 (C-4$_\alpha$), 78.82 (C-4$_\beta$), 77.86 (C-4″), 77.45 (C-4′), 75.48 (C-5″), 75.116 (C-5$_\beta$), 74.92 (C-5‴) 74.77 (C-5′), 73.59 (C-3‴), 73.35 (C-3″), 73.08 (C-3′), 72.79 (C-3$_\beta$), 72.16 (C-2‴), 71.69 (C-2″, C-5$_\alpha$), 70.57 (C-2′), 69.58 (C-3$_\alpha$), 68.94 (C-4‴), 61.31 (C-6″), 61.21 (C-6‴), 60.85 (C-6′), 60.35 (C-6$_\alpha$), 60.26 (C-6$_\beta$), 56.51 (C-2$_\beta$), 54.04 (C-2$_\alpha$), 22.49 (C of -CH$_3$ due to the $\alpha$ anomer) and 22.20 (C of -CH$_3$ due to the $\beta$ anomer); HRMS *m/z* 730.2426 for [M + Na]$^+$ (calcd for $C_{26}H_{45}O_{21}$N [M + Na]$^+$, 730.2376).

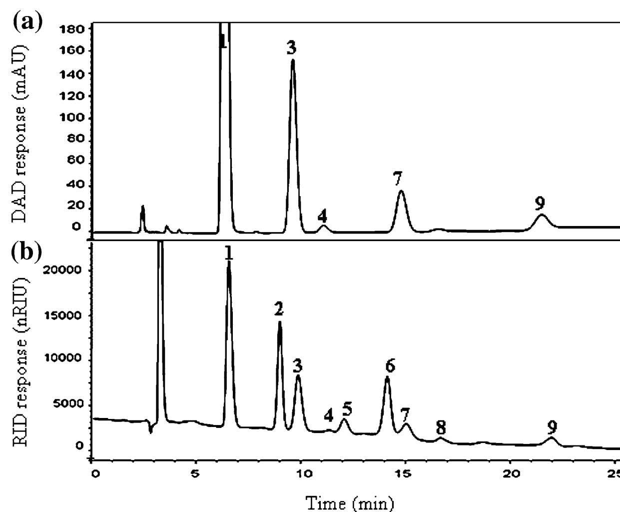



**Fig. 1** HPLC profiles of carbohydrate mixture obtained by the transgalactosylation of $\beta$-D-galactosidase from *B. circulans* after 4 h of reaction. The reaction was performed at 40 °C, pH 6.0, and using 1.00 M of lactose and Glc*p*NAc with the molar ratio of 1: 1 containing 1.0 U/mL enzyme. The reaction mixture was analyzed by a column of Sugar-D with a DAD (**a**) and a RID (**b**). *1* *N*-acetylglucosamine (GlcNAc), *2* glucose and galactose, *3* $\beta$-D-Gal*p*-(1 → 4)-D-Glc*p*NAc (LacNAc, **I**), *4* $\beta$-D-Gal*p*-(1 → 6)-D-Glc*p*NAc (allo-LacNAc, **II**), *5* $\beta$-D-Gal*p*-(1 → 6)-D-Glc*p* (6′-allolactose), *6* lactose, *7* $\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-D-Glc*p*NAc (**III**), *8* $\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-D-Glc*p* (4′-galactosyl-lactose), *9* $\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-D-Glc*p*NAc (**IV**)

## Results and discussion

### Synthesis of oligosaccharides by using $\beta$-D-galactosidase from *B. Circulans*

The reaction was performed by using 1.00 M of lactose and GlcNAc as substrates in a molar ratio of 1:1 and $\beta$-D-galactosidase from *B. circulans* (1.0 U/mL) at 40 °C. Figure 1 shows the typical HPLC chromatograms of reaction mixture. In the present study, two kinds of HPLC columns were used for the separation of the reaction mixture, Sugar-D for the separation of the whole transfer products and Sugar KS-801 for the separation of galactose and glucose (data not shown). After 4 h of reaction, GlcNAc (peak 1), galactose/glucose (peak 2), lactose (peak 6) and oligosaccharides (peak 3–5 and 7–9) as a result of the transgalactosylation were observed. Apart from substrate GlcNAc, peak 3, 4, 7 and 9 had absorbance at 210 nm (Fig. 1a), indicating the presence of GlcNAc. Using authentic standards and with the standard-addition technique, peak 3 and 4 were identified as LacNAc (**I**) and allo-LacNAc (**II**), respectively. The result is similar to those obtained by Hernaiz and Crout [15] and Sakai et al. [20]. However, higher DP transfer products were not detected in their reports. In the present study, we found that transfer products **III** (peak 7) and **IV** (peak 9) showed *m/z* 568.1871 and 730.2426 for [M + Na]$^+$ (Fig. 2), corresponding to *N*-acetyl-trisaccharide and *N*-acetyl-tetrasaccharide, respectively. Furthermore, $^1$H and $^{13}$C NMR analyses were carried out to determine the glycosidic linkage of the two transfer products. Assignment of the NMR data to each sugar including GlcNAc and galactose was performed through a comparison of the chemical shifts with **I** and **II** [14, 20]. The structure of **III** was characterized by the appearance of new proton and carbon signals at $\delta$ 4.45 corresponding to Gal*p* H-1″ and at $\delta$ 104.55, 75.47, 73.28, 71.72, 68.95, 61.33 corresponding to C-1″, C-5″, C-3″, C-2″, C-4″ and C-6″ of the additional terminal Gal unit linked to **I**, respectively. In addition, the characteristic peak at $\delta$ 4.45 ppm (*J* 7.42 Hz) of Gal*p* H-1″ in the $^1$H NMR spectrum represented the presence of the galactosyl residue to **I** through a $\beta$-linkage. The $\beta$-(1 → 4) linkage between the terminal Gal and **I** was confirmed by a downfield shift of the signal for terminal Gal C-4′ from $\delta$ 68.94 to 77.49. Therefore, **III** was identified as a novel oligosaccharide containing **I** unit, $\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-D-Glc*p*NAc. Similarly, **IV** was confirmed to be $\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-$\beta$-D-Gal*p*-(1 → 4)-D-Glc*p*NAc. Apparently, transfer products **III** and **IV** were

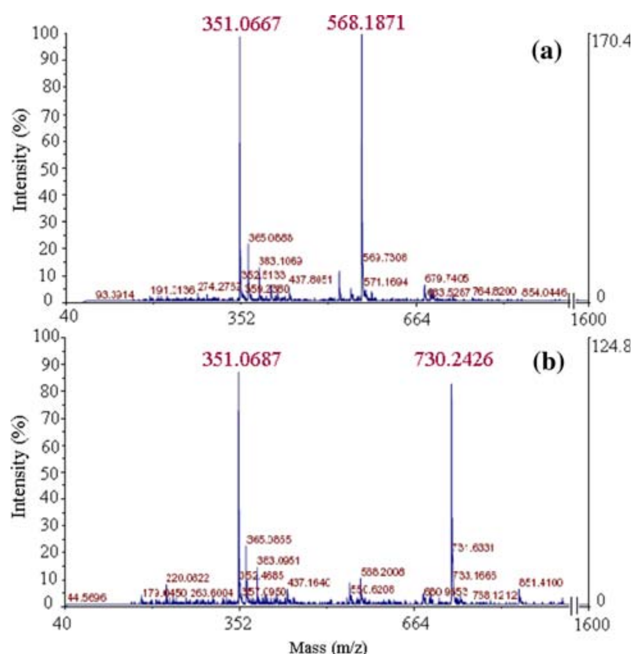



**Fig. 2** Mass spectra of **III** (**a**) and **IV** (**b**) by using ESI-HRMS

produced by further regioselective galactosylation of **I** formed initially. Reuter et al. [16] also reported the presence of higher DP *N*-acetyl-oligosaccharides in the reaction mixtures of transgalactosylation by using $\beta$-D-galactosidase from *Sulfolobus solfataricus*, *Aspergillus oryzae* or *E. coli*, but they did not make any identification.

In the reaction mixture, two oligosaccharides (peak 5 and 8) without ultraviolet absorption were also detected (Fig. 1b). With authentic standards and spike technique, we identified peak 5 as 6′-allolactose and peak 8 as 4′-galactosyl-lactose. They belong to galactooligosaccharides (GOS). The results indicated that not only acceptor GlcNAc but also donor lactose and hydrolytic product (glucose) of the reaction could serve as acceptors of the galactosyl moiety. These oligosaccharides have been reported to be resistant to human digestive enzymes and act as prebiotics. There are a number of papers on GOS production with lactose as substrate [21–24].

Transgalactosylation is thought to involve intermolecular as well as intramolecular reactions [22]. The glycosidic bond of lactose is cleaved and immediately forms again at a different position of the glucose molecule before it diffuses out of the active site by intermolecular transgalactosylation, yielding allolactose (peak 5), whereas by intramolecular transgalactosylation, di- (peak 3 and 4), tri- (peak 7 and 8) and tetra-saccharides (peak 9) are produced. Any sugar molecule in the reaction mixture can be the nucleophile to accept the galactosyl moiety from the galactosyl–enzyme complex. The production of transfer products can be regarded as kinetic intermediates as they are also substrates for hydrolysis. For all these reasons, the transfer products' yield and composition may change dramatically with reaction time and conditions. Although hydrolysis of synthesized oligosaccharides competes with transgalactosylation, the latter can be favored at high substrate concentration, regulating temperature and lower water activity [18, 21]. Hence, the effects of synthetic conditions on the formation of transfer products containing GlcNAc unit were investigated.

## Effects of synthetic conditions on the formation of transfer products I-IV

### *Effect of temperature*

Figure 3a shows the effects of temperature on the formation of compounds **I-IV** catalyzed by $\beta$-D-galactosidase from *B. circulans*. A maximum production for **I** (28.75%), **III** (9.47%) and **IV** (5.67%) was noted in the reaction mixture at 40 °C; however, higher temperature (50 and 60 °C) resulted in a significant reduction in the production, probably due to the hydrolysis of formed transfer products. The optimal temperature for their production is rather close to this enzyme to exhibit its highest hydrolytic activity, which was determined at 40 °C using ONPG as the substrate. This observation was consistent with that of Vetere and Paoletti [25], who demonstrated that a higher yield of **I** was prone to obtain with a lower temperature. As mentioned above, **III** and **IV** were produced by two or more sequential transgalactosylations. Therefore, the yields of higher DP oligosaccharides, to a great extent, depend on the yield of **I**. In contrast, the yield of **II** (1.97%) increased constantly with the increase of temperature, reaching to 6.99% at 60 °C. It indicated that **II** was more stable than **I**. The same tendency has been reported by Usui et al. [14], who investigated $\beta$-D-galactosyl-disaccharide synthesis with lactose as donor and GlcNAc or *N*-acetylgalactosamine (D-GalNAc) as acceptor by use of $\beta$-D-galactosidase from *B. circulans*.

### *Effect of substrate concentration*

One of the most influential factors on yields of transgalactosylation is the concentration of substrate, and an increase in this parameter as high as possible could shift the equilibrium toward the transgalactosylation reaction [16]. Thus, a study on the influence of substrate concentration was performed to select the optimal conditions for the formation of **I-IV**. It was found that the maximum yields of **I**, **III** and **IV** increased significantly with the increase of initial substrate concentration from 0.50 to 1.00 M (Fig. 3b). However, further increase in substrate concentration resulted in the reduction of oligosaccharides

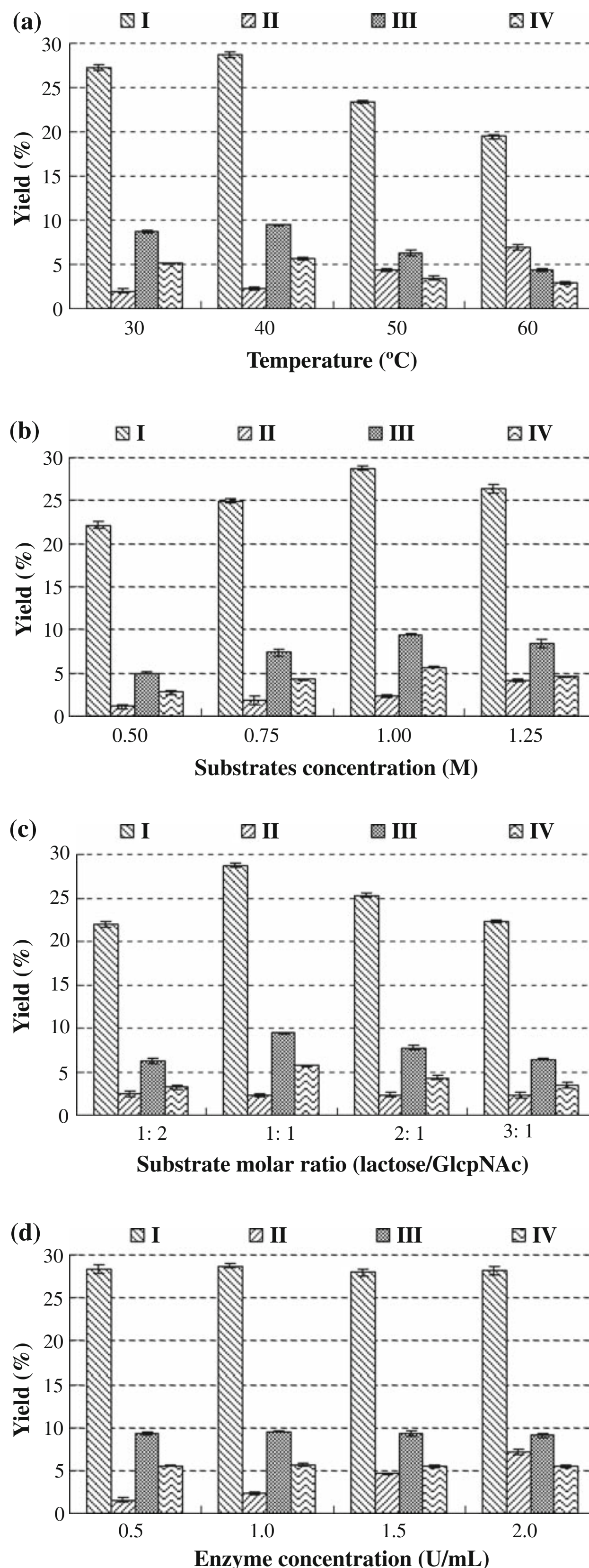



**Fig. 3** **a** Effect of temperature on the *N*-acetyl-oligosaccharides production catalyzed by *β*-D-galactosidase from *Bacillus circulans* (1.0 U/mL). The reactions were performed at pH 6.0 with an initial concentration of 1.00 M of lactose and GlcNAc with the molar ratio of 1: 1 for 12 h. **b** Effect of the initial substrate concentration with lactose and GlcNAc at a molar ratio of 1: 1 on the *N*-acetyl-oligosaccharides production catalyzed by *β*-D-galactosidase from *Bacillus circulans* (1.0 U/mL). The reactions were performed at 40 °C and pH 6.0 for 12 h. **c** Effect of molar ratio of donor/acceptor on the *N*-acetyl-oligosaccharides production catalyzed by *β*-D-galactosidase from *Bacillus circulans* (1.0 U/mL). The reactions were performed at 40 °C using 1.00 M of lactose and GlcNAc and pH 6.0 for 12 h. **d** Effect of enzyme concentration on the *N*-acetyl-oligosaccharides production catalyzed by *β*-D-galactosidase from *Bacillus circulans*. The reactions were performed at 40 °C with an initial concentration of 1.00 M of lactose and GlcNAc with the molar ratio of 1: 1 and pH 6.0 for 12 h. *Bars* indicate standard deviations

production. One possible explanation for this phenomenon is that higher contents of glucose and galactose will be expected with relative abundant lactose. Therefore, the formation of transfer products may decrease under high concentration of substrate, since both glucose and galactose are competitive inhibitors of *β*-D-galactosidase [23, 26]. We also noted that the hydrolytic reaction dominated under low substrate concentration, while oligosaccharide formation dominated in reaction with a higher substrate concentration. Regarding **II**, its production increased with the increase of substrate concentration.

### *Effect of molar ratio of donor/acceptor*

The yields of oligosaccharides are influenced not only by the substrate concentrations but also by the molar ratio of donor/acceptor used. Among the different donor/acceptor ratios, the maximum yields of **I**, **III** and **IV** were obtained with equimolar of lactose and GlcNAc (Fig. 3c). With a ratio of 1:1, yields of **I**, **III** and **IV** were in average 1.3-, 1.5- and 1.8-fold of those with ratios of 2:1, 3:1 and 1:2. These results could be explained by a lower degree of secondary hydrolysis and a higher transglycosylation rate at equimolar concentration. The significant effect of molar ratio of donor/acceptor observed in the present study is different from the report of Zeng et al. [19], who found that the optimal molar ratio of donor/acceptor was 3:1 on *β*-D-galactopyranosyl disaccharides production by *β*-D-galactosidase from porcine liver. However, our observations are consistent with the report of Reuter et al. [16], who found that the optimal molar ratio of donor/acceptor was 1:1 for the formation of *N*-acetyl-oligosaccharides with *β*-D-galactosidase from *S. solfataricus*, *A. oryzae* or *E. coli* as biocatalyst.

### Effect of enzyme concentration

To determine the effect of the enzyme concentration, enzyme concentrations in a range from 0.5 to 2.0 U/mL were tested. It is worth mentioning that except for **II**, maximum yields of **I**, **III** and **IV** show no dependency with enzyme concentration. As shown in Fig. 3d, the maximum production of **I**, **III** and **IV** were 28.32, 9.31 and 5.49%, respectively, whereas the yield of **II** increased gradually from 1.56 to 7.14% with the increase of enzyme concentration. Because of the differences between regioselectivity and transfer specificity of enzyme for different transfer products, the optimum enzyme concentration varied with each other. The amount of enzyme added should be a compromise to reach the maximum trangalactosylation rate in a relatively short time without fast hydrolysis of the transgalactosylation products to facilitate stopping the reaction at the kinetic maximum.

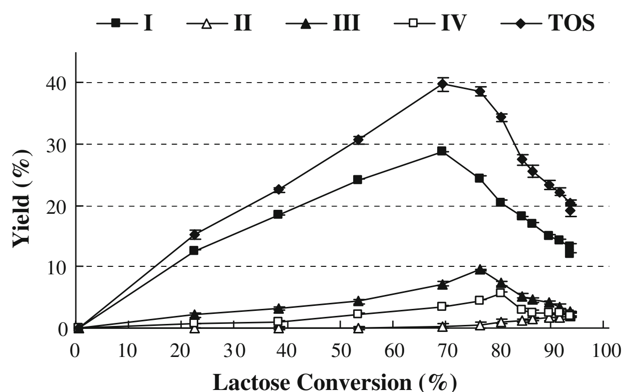



**Fig. 5** Formation and degradation of *N*-acetyl-oligosaccharides during lactose conversion by *β*-D-galactosidase from *Bacillus circulans* (1.0 U/mL). The reactions were performed at 40 °C with an initial concentration of 1.00 M of lactose and GlcNAc with the molar ratio of 1: 1 and pH 6.0. *TOS* total *N*-acetyl-oligosaccharides. *Bars* indicate standard deviations

### Oligosaccharide production by *β*-D-galactosidase from *B. circulans* under optimal conditions

In summary, the optimum conditions for the production of **I-IV** were pH 6.0, 40 °C, 0.50 M lactose and 0.50 M GlcNAc, and 1.0 U/mL *β*-D-galactosidase. Figure 4 shows the hydrolysis of lactose and the formation of transfer products for a typical reaction. Initially, a rapid reduction in lactose concentration was accompanied by a high rate of the formation of transfer products. Up to 69.32% conversion of lactose, the total amount of **I-IV** kept rising. And a maximum total amount of **I-IV** (39.79%) was reached after 4 h of incubation. After that, hydrolysis prevailed over synthesis, which eventually led to the accumulation of galactose and glucose. Meanwhile, the content of glucose in the reaction mixture was found to be much higher than that of galactose, indicating the involvement of galactose in *N*-acetyl-oligosaccharides formation. Furthermore, *N*-acetyl-oligosaccharides formed were no end products. They were only transiently formed as they were also subjected to hydrolysis which became more and more pronounced toward the end of the reaction when the donor lactose became depleted. These observations were similar to those reported for *β*-D-galactosidases from other microorganisms [20, 27].

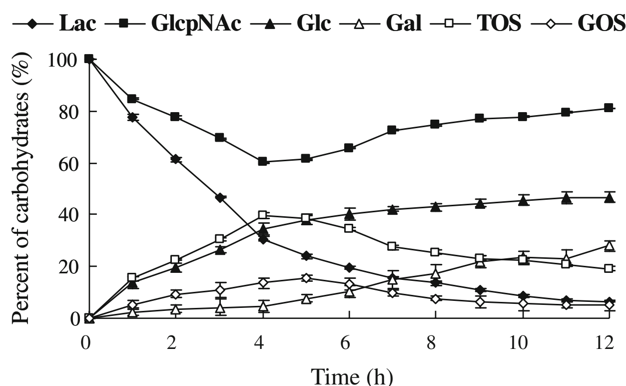



**Fig. 4** Time course of the lactose hydrolysis and the oligosaccharides formation catalyzed by *β*-D-galactosidase from *Bacillus circulans*. The reactions were performed at 40 °C with an initial concentration of 1.00 M of lactose and GlcNAc with the molar ratio of 1: 1 and pH 6.0. *Lac* lactose, *GlcNAc N*-acetylglucosamine, *Glc* glucose, *Gal* galactose, *TOS* total *N*-acetyl-oligosaccharides, *GOS* total galactooligosaccharides. *Bars* indicate standard deviations

When taking a look at the size distribution within the *N*-acetyl-oligosaccharides mixtures, the amounts and composition of *N*-acetyl-oligosaccharides changed dramatically with the degree of lactose conversion which is illustrated in Fig. 5. One can see that the production of **I** dominated at the beginning of the reaction. This is not surprising since GlcNAc is the most abundant sugar in the initial reaction mixture. After 4-h reaction, its yield reached to 28.75% when 69.32% lactose was hydrolyzed (Fig. 4). However, it also acted as galactosyl acceptor by two or three sequential transgalactosylations, resulting in the formation of **III** and **IV** with maximum yields of 9.47 and 5.67%, respectively, through 5-h or 6-h incubation. In contrast, **II** kept increasing during the whole reaction. The reason might be due to its *β*-(1 → 6) linkage. Finally, it should be noted that the yield of GOS reached maximum (15.36%) at 5 h of reaction, corresponded to a consumption of 76.13% lactose (Fig. 4). However, the GOS amount formed was far below average values of previously reports using the same enzyme with lactose as sole substrate [24, 28]. This may relate to the fact that GlcNAc have lower Michaeis constant, which is susceptible to be attacked by the *β*-D-galactosidase than lactose or glucose.

## Conclusion

In the present study, we found that *N*-acetyl-oligosaccharides (**I-IV**) and two oligosaccharides (6′-allolactose and 4′-galactosyl-lactose) without ultraviolet absorption were formed in a reaction mixture with lactose and GlcNAc as substrates using $\beta$-D-galactosidase from *B. circulans* as biocatalyst. We also demonstrated the effects of temperature, substrate concentration, molar ratio of donor/acceptor and enzyme concentration on the *N*-acetyl-oligosaccharides production. The optimal reaction conditions for **I**, **III** and **IV** were 40 °C, 0.50 M lactose and 0.50 M GlcNAc and 1.0 U/mL of enzyme at pH 6.0. Under such conditions, the *N*-acetyl-oligosaccharides formed were composed of 28.75% of **I**, 2.29% of **II**, 9.47% of **III** and 5.67% of **IV**. On the other hand, suitable reaction conditions found for **II** were 40 °C, 0.50 M lactose and 0.50 M GlcNAc and 2.0 U/mL of enzyme at pH 6.0. $\beta$-D-Galactosidase from *B. circulans* presented a high specificity for the formation of oligosaccharides with $\beta$-(1 → 4) linkages, although *N*-acetyl-oligosaccharide with $\beta$-(1 → 6) linkage was also detected in minor amounts. Further works on the potential function of **I-IV** are in progress.

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