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Galactooligosaccharides formation during enzymatic hydrolysis of lactose: towards a prebiotic-enriched milk

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Abstract

The formation of galacto-oligosaccharides (GOS) in skim milk during the treatment with several commercial β-galactosidases (*Bacillus circulans*, *Kluyveromyces lactis* and *Aspergillus oryzae*) was analyzed in detail, at 4°C and 40°C. The maximum GOS concentration was obtained at a lactose conversion of approximately 40-50% with *B. circulans* and *A. oryzae* β-galactosidases, and at 95% lactose depletion for *K. lactis* β-galactosidase. Using an enzyme dosage of 0.1% (v/v), the maximum GOS concentration with *K. lactis* β-galactosidase was achieved in 1 h and 5 h at 40°C and 4°C, respectively. With this enzyme, it was possible to obtain a treated milk with 7.0 g/L GOS—the human milk oligosaccharides (HMOs) concentration is between 5 and 15 g/L—, and with a low content of residual lactose (2.1 g/L, compared with 44-46 g/L in the initial milk sample). The major GOS synthesized by this enzyme were 6-galactobiose [Gal-β(1→6)-Gal], allolactose [Gal-β(1→6)-Glc] and 6′-O-β-galactosyl-lactose [Gal-β(1→6)-Gal-β(1→4)-Glc].

Keywords: Galacto-oligosaccharides, Prebiotics, Transglycosylation, Beta-galactosidase, Lactose-free milk, Human milk oligosaccharides.
1. Introduction

Prebiotics are non-digestible food ingredients that are selectively fermented by the human gastrointestinal microbiota allowing specific changes, both in its composition and/or activity, conferring benefits upon host well-being and health (Roberfroid, 2007). In particular, galactooligosaccharides (GOS) promote the *Bifidobacteria* and *Lactobacilli* growth (Rodriguez-Colinas, Kolida, Baran, Ballesteros, Rastall, & Plou, 2013) resulting in health benefits such as improvement of mineral absorption, inhibition of pathogens and modulation of immune system (Gosling, Stevens, Barber, Kentish, & Gras, 2010; Rastall et al., 2005).

Commercial GOS contain a mixture of oligosaccharides formed by one or various galactosyl moieties linked to a terminal glucose, or by exclusively galactose units (galactobioses, galactotrioses, etc.) (Park & Oh, 2010; Torres, Goncalves, Teixeira, & Rodrigues, 2010).

Human milk oligosaccharides (HMOs) constitute a family of more than a hundred structurally diverse carbohydrates that exert numerous benefits to breast-fed infants (Bode, 2012). The concentration of HMOs in human milk varies between 5-15 g/L. All HMOs contain lactose at their reducing end, which is normally elongated with N-acetyl-lactosamine or lacto-N-biose and further fucosylated or sialylated (Bode, 2012). To mimic the multiple benefits of HMOs, other related carbohydrates, in particular GOS and fructooligosaccharides (FOS), are currently added to infant formulas (Shadid et al., 2007; Angus, Smart, & Shortt, 2007). Despite their structural differences compared to HMOs, the incorporation of GOS and FOS into baby foods favours the microbiota composition in the infant’s feces and reduce allergenic manifestations (e.g. atopic dermatitis) and infections during the first years of life (Boehm, Stahl, Jelinek, Knol, Miniello, & Moro, 2005).

Apart from lactose hydrolysis, β-galactosidases (EC 3.2.1.23) are able to catalyze a transgalactosylation reaction in which lactose or other carbohydrates in the mixture serve as galactosyl acceptors, yielding GOS with different polymerization degree and type of
glycosidic bonds (Hsu, Lee, & Chou, 2007; Park et al., 2010; Torres et al., 2010). The enzyme source and the reaction operating conditions (lactose concentration, water activity, temperature, pH, etc.) notably influence the yield and composition of the synthesized GOS (Iqbal, Nguyen, Nguyen, Maischberger, & Haltrich, 2010; Urrutia et al., 2013). In general, the GOS yield increases with increasing lactose concentration (Vera, Guerrero, Conejeros, & Illanes, 2012). Under the optimal conditions, GOS yields are between 30-40% (w/w) (Gosling et al., 2010).

The use of generally recognized as safe (GRAS) β-galactosidases to deplete lactose from milk is very extended in the dairy industry as a response to the commonly occurring lactose intolerance (Adam, Rubio-Texeira, & Polaina, 2005). Indeed the pH of milk (approx. 6.7) is appropriate for the activity of many β-galactosidases. Nonetheless the formation of GOS during the treatment of milk with β-galactosidases has been scarcely reported (Kim, Lim, & Kim, 1997; Mlichova & Rosenberg, 2006; Puri, Gupta, Pahuja, Kaur, Kanwar, & Kennedy, 2010; Ruiz-Matute, Corzo-Martínez, Montilla, Olano, Copovi, & Corzo, 2012), probably due to the fact that the lactose content in bovine milk is around 5% (w/v), a value significantly lower compared with typical reported lactose buffered solutions [15-50% (w/v) lactose] employed to promote the transglycosylation reaction (Ganzle, Haase, & Jelen, 2008; Prenosil, Stuker, & Bourne, 1987). The use of milk whey permeates to synthesize GOS is more extended (Lopez-Leiva & Guzman, 1993; Lorenzen, Breiter, Clawin-Rädecker, & Dau, 2013). In this context, Chen, Hsu, & Chiang (2002) developed a multi-step process to increase GOS production by first ultrafiltrating the milk to separate lactose from proteins, followed by a concentration of the permeate and a further transgalactosylation reaction with β-galactosidases.

In this work, we performed a detailed study of GOS formation during lactose hydrolysis in milk catalyzed by several β-galactosidases with different specificity (Bacillus
circulans, Kluyveromyces lactis and Aspergillus oryzae). Previous studies on this subject have not performed a comparative kinetic analysis between different $\beta$-galactosidases or have not employed the required chromatographic methodology (e.g. separation of lactose and allolactose) to determine the complete profile of the GOS synthesized. Our objective was to develop a strategy for obtaining dairy products with a significant presence of GOS and, at the same time, a low content of lactose. Such kind of products with double functionality could be of interest in the dairy market.
2. Materials and methods

2.1. Materials

Biolactase NTL-CONC is a β-galactosidase preparation from *Bacillus circulans* supplied by Biocon (Spain). The β-galactosidase from *Kluyveromyces lactis* Lactozym pure 6500L was kindly supplied by Novozymes A/S (Denmark). Lactase F, a solid β-galactosidase preparation from *Aspergillus oryzae*, was obtained from Amano (Japan). Glucose, galactose and bovine serum albumin were from Sigma-Aldrich. The standards 6-galactobiose, 4-galactobiose, 6-O-β-galactosyl-glucose (allolactose) and 4’-O-β-galactosyl-lactose were from Carbosynth (Berkshire, UK). Skim milk “Hacendado” was purchased from a local Mercadona supermarket (Spain). All other reagents and solvents were of the highest available purity and used as purchased.

2.2. Determination of enzyme activity

The activity of the three enzyme preparations was measured at 25°C with lactose as substrate at the pH of milk (6.7). Lactose (0.1 % w/v, 1 g/L) was dissolved in 1 mL of 10 mM potassium phosphate buffer (pH 6.7). Then, 10 µL of the enzyme (conveniently diluted) was added and the mixture was incubated at 25°C. At different times (5, 20, 35 and 50 min), aliquots were taken and analyzed by HPLC to determine residual lactose concentration using a ternary pump (Varian) coupled to a 4.6 x 250 mm Luna-NH2 column (5 µm, 100 Å) from Phenomenex. Detection was performed using an evaporative light scattering detector ELSD 2000ES (Alltech) equilibrated at 82°C with a nitrogen flow of 2.1 L /min. Acetonitrile/water 75:25 (v/v) was used as mobile phase at 1 mL/min. The column temperature was kept constant at 30°C. A calibration curve of lactose (0-1 g/L) was carried out under the above conditions. Accordingly, one unit of activity was defined as that catalyzing the hydrolysis of 1 µmol lactose per minute.
2.3. Formation of galacto-oligosaccharides from skim milk

Biolactase (20 µL), Lactozym pure (20 µL) or Lactase F (20 mg) were added to 20 mL of skim milk. The lactose concentration in skim milk was between 44-46 g/L as measured by HPAEC-PAD. The mixture was incubated at 4°C or 40°C in an orbital shaker (Vortemp 1550) at 200 rpm. At different reaction times –selected to obtain the complete reaction profile–, 200 µL aliquots were sampled from the reaction vessel. The enzyme present in the aliquots was inactivated by incubating 5 min in a Thermomixer (Eppendorf) at 95°C and 350 rpm. In the case of Biolactase, the reaction was stopped by adding 800 µL of 0.4 M Na₂CO₃ because some residual activity after heating process was observed, probably due to the presence of a thermostable β-galactosidase isoform in B. circulans preparation. Samples were filtered using 0.45 µm cellulose filters coupled to eppendorf tubes (National Scientific) during 5 min at 6000 rpm and then diluted 1:400 with water before HPAEC-PAD analysis.

2.4. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Product analysis was performed by HPAEC-PAD on a ICS3000 Dionex system (Dionex Corp., Sunnyvale, CA) consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 x 250 mm Carbo-Pack PA-1 column (Dionex) connected to a CarboPac PA-1 guard column was used at 30°C. For eluent preparation, MilliQ water and 50% (w/v) NaOH (Sigma-Aldrich) were used. The flow rate was 1.0 mL/min during the analysis. The initial mobile phase was 15 mM NaOH for 12 min. A mobile phase linear gradient from 15 mM to 200 mM NaOH was performed at 1.0 mL/min in 15 min, and the latter eluent was kept constant for 25 min. Analyses were performed in duplicate, and the peaks were analyzed using Chromeleon.
software. Standard deviations were lower than 3% in all cases. Identification of the different carbohydrates was done based on commercial standards and purified GOS as described elsewhere (Rodriguez-Colinas et al., 2011; Rodriguez-Colinas, Poveda, Jimenez-Barbero, Ballesteros, & Plou, 2012; Urrutia et al., 2013).

2.5. Determination of protein concentration

The protein concentration in the enzyme preparations was estimated following the Bradford method (Bradford, 1976) adapted to 96-well plates. Bovine serum albumin (BSA) was used as standard. All samples were prepared in triplicate.
3. Results and discussion

3.1. Kinetics of GOS formation in skim milk with β-galactosidases from different sources

Table 1 summarizes the main properties of the β-galactosidases employed in this study: Biolactase (*B. circulans*), Lactozym pure (*K. lactis*) and Lactase F (*A. oryzae*). We measured the enzymatic activity of the three preparations by an HPLC assay using lactose as substrate. Although the optimum pH for the three β-galactosidases is different (5.5 for *B. circulans*, 6.8 for *K. lactis* and 4.5 for *A. oryzae*), the activity assay was carried out at the pH of our milk sample (6.7). These activity values were more representative for the present work than those typically measured with *o*-nitrophenyl-β-D-galactopyranoside (ONPG). As shown in Table 1, the volumetric activity was 2.5-fold higher for Lactozym compared with Biolactase, probably due to the proximity of the optimum pH of *K. lactis* β-galactosidase to the pH of milk. Lactase F is a solid preparation, and its activity towards milk could be considered low as its optimum pH is almost two units below the pH of milk.

We incubated skim milk with each of the three β-galactosidases at 40°C using an enzyme dosage of 0.1% v/v for the liquid preparations (Biolactase and Lactozym pure) and 0.1% w/v for Lactase F. Considering that the lactose concentration in milk was between 44 and 46 g/L as measured by HPAEC-PAD, the experiments were carried out with 12.3, 29.3 and 2.0 enzyme units (U) per g lactose for Biolactase, Lactozym pure and Lactase F, respectively.

We analyzed the formation of galactooligosaccharides (GOS) in skim milk during lactose hydrolysis catalyzed by the different β-galactosidases. Fig. 1 illustrates the kinetics of lactose removal and total GOS synthesis at 40°C. The three enzymes showed the typical pattern with a point of maximum GOS concentration followed by a progressive decrease in
the amount of GOS due to the competition between hydrolysis and transglycosylation reactions (Mozaffar, Nakanishi, & Matsuno, 1985). The maximum yield of GOS depends basically on the concentration of lactose and the intrinsic enzyme properties, that is, its ability to bind the sugar acceptor (to which a galactosyl moiety is transferred) and to exclude H$_2$O. The time required to get the maximum GOS yield depends inversely on the amount of enzyme; however, the GOS concentration at this maximum is not affected by the dosage of biocatalyst (Ballesteros et al., 2006).

For a fixed enzyme dosage, Lactozym pure was the most active preparation, as lactose was almost completely depleted in 1.5 h, whereas Biolactase and Lactase F required 4.5 and 24 h respectively. These results were in accordance with the activity values shown in Table 1. Fig. 1A shows that the maximum GOS concentration with *B. circulans* preparation was approx. 7.6 g/L—which represents 16% (w/w) of total carbohydrates in the sample—and this maximum was achieved when around 50% of the initial lactose had disappeared. Mozaffar et al. (1985), using a purified β-galactosidase from *B. circulans*, reported a maximum amount of GOS close to 5.5% of total sugars, which was obtained at 39% conversion of lactose.

In contrast, maximum GOS concentration with *K. lactis* β-galactosidase (7.0 g/L, 15% of total sugars, Fig. 1B) was obtained at a significantly higher lactose conversion (95%). The lowest yield of GOS (4.5 g/L) was obtained with the enzyme from *A. oryzae*, and it was achieved at 43% of lactose conversion (Fig. 1C).

### 3.2. GOS specificity of β-galactosidasces

Fig. 2 shows the HPAEC-PAD chromatograms of the reaction mixtures with skim milk at the point of maximum GOS concentration for each of the enzymes. Peaks 1, 2 and 5 correspond to galactose, glucose and lactose, respectively. As illustrated in the chromatogram of Fig. 2A, the three main GOS present in the Biolactase reaction mixture were identified as
the disaccharide 4-galactobiose [Gal-β(1→4)-Gal], the trisaccharide 4’-O-β-galactosyl-lactose [Gal-β(1→4)-Gal-β(1→4)-Glc] and the tetrasaccharide Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc, confirming the specificity of this enzyme for the formation of β(1→4) linkages (Rodriguez-Colinas et al., 2012; Yanahira et al., 1995). Using a buffered 400 g/L lactose solution, other minor GOS containing β(1→3) bonds were detected with this enzyme (Rodriguez-Colinas et al., 2012). An advantage of the B. circulans enzyme in the dairy industries is that it is not inhibited by calcium ions present in milk compared with other β-galactosidases (Mozaffar et al., 1985).

Fig. 2B illustrates that the major GOS synthesized by β-galactosidase from K. lactis were the disaccharides 6-galactobiose [Gal-β(1→6)-Gal] and allolactose [Gal-β(1→6)-Glc] and the trisaccharide 6’-O-β-galactosyl-lactose [Gal-β(1→6)-Gal-β(1→4)-Glc]. These results confirm that this enzyme exhibits a clear tendency to form β(1→6) linkages (Martinez-Villaluenga, Cardelle-Cobas, Corzo, Olano, & Villamiel, 2008; Rodriguez-Colinas et al., 2011).

Regarding the β-galactosidase from A. oryzae (Fig. 2C), a notable specificity towards the formation of β(1→6) bonds was also observed. In a recent paper, using a concentrated lactose solution (400 g/L), we observed that A. oryzae β-galactosidase showed a preference to form glycosidic linkages in the order β(1→6) > β(1→3) > β(1→4) (Urrutia et al., 2013).

Table 2 summarizes the carbohydrate composition of the β-galactosidase-treated milks at their respective points of maximum GOS concentration. As stated before, one of the main differences between Lactozym pure (K. lactis) and Biolactase (B. circulans) refers to the concentration of residual lactose at the point of maximum GOS yield: 2.1 g/L and 28.1 g/L, respectively. This could be related with the fact that the β(1→6) bonds are more resistant to enzymatic hydrolysis than β(1→4) linkages, and is in agreement with the discovery that GOS with β(1→6) bonds were found as residual components in several lactose-free UHT (Ultra-
heat treatment) milks and dairy drinks (Ruiz-Matute et al., 2012). In the case of Lactase F, although $\beta(1\rightarrow6)$ bonds are preferably formed, the enzyme displays a less favourable transglycosylation to hydrolysis ratio.

In this context, Ruiz-Matute et al. (2012) analyzed the formation of GOS in milk at 30°C with Lactozym pure. They reported that a residual lactose content lower than 1000 ppm (1 g/L) can be achieved with a GOS content of nearly 7.8 g/L. In our study, a similar GOS concentration (7.0 g/L) was obtained at 40°C with *K. lactis* $\beta$-galactosidase, but the remaining lactose was 2100 ppm. Further reduction of the lactose content to 360 ppm lowered the GOS concentration to 4.9 g/L (Fig. 1B).

### 3.3. Effect of temperature on GOS formation

From the industrial point of view, some dairy processes are preferably performed at 4°C, e.g. ice creams’ processing in which $\beta$-galactosidase treatment gives a sweeter product that does not crystallize when condensed or frozen and also improves the creaminess of the product. We analyzed the GOS formation at this temperature (Fig. 3), and data was compared with that obtained at 40°C. Fig. 3 illustrates that as reaction temperature decreased, the time required to reach the maximum production of GOS increased.

The maximum GOS yield at 4°C was obtained with *B. circulans* $\beta$-galactosidase (8.1 g/L, 18 % of total carbohydrates) and it was reached again at 50% of lactose conversion. Gosling, Stevens, Barber, Kentish, & Gras (2009) assayed the *B. circulans* $\beta$-galactosidase preparation Biolacta in milk in the temperature range 4-60°C. They observed that GOS yield increased with temperature, as has been described in other transglycosylation studies (Linde, Rodriguez-Colinas, Estevez, Poveda, Plou, & Lobato, 2012; Ning, Wang, Chen, Yang, Jin, & Xu, 2010). However, in our experiments with this enzyme the GOS yield was similar at both temperatures.
In the case of \textit{K. lactis}, the maximum GOS concentration was lower at 4°C compared to that obtained at 40°C (4.8 and 7.0 g/L, respectively). In addition, the reaction time required to reach the maximum GOS yield was 5-fold higher (5 h at 4°C vs. 1 h at 40°C). The kinetics of GOS production by \textit{A. oryzae} β-galactosidase (Fig. 3C) varied significantly between both temperatures, indicating a bad performance of this enzyme at 4°C. GOS formation at 40°C followed the common pattern with a maximum concentration at 1.5 h and total depletion of lactose after 24 h. However, the reaction was much slower at 4°C: after 80 h, the residual lactose was still 25 g/L. The GOS yield was quite similar at both temperatures (4.2-4.5 g/L, respectively).

\textbf{3.4. Effect of lactose conversion on GOS synthesis}

Fig. 4 represents the profile of GOS concentration vs. lactose conversion determined for each enzyme at 4°C and 40°C. These profiles correlate well with those already published with buffered lactose solutions (Rodriguez-Colinas et al., 2011, 2012). With \textit{B. circulans} and \textit{A. oryzae} β-galactosidases, the maximum amount of GOS was produced at approx. 40-50% of lactose conversion. In contrast, when \textit{K. lactis} β-galactosidase was used, the maximum GOS yield was achieved at approximately 95% of lactose conversion. The behaviour was similar at 4°C and 40°C, except for \textit{A. oryzae} enzyme whose activity at 4°C was extremely low.

These results indicate that with Lactozym pure it is possible to obtain a treated milk with a GOS concentration in the range of 4.8-7.0 g/L (the concentration of HMOs in human milk is between 5-15 g/L) and at the same time with a low content of lactose (2.1-2.7 g/L). In addition, the maximum GOS yield can be obtained at 4°C in 5 h using an enzyme dosage of only 0.1% (v/v).

Lactose hydrolysis can be performed before or after UHT treatment. Heating prior to enzymatic treatment is usually preferred because the monosaccharides formed in the
hydrolysis are more susceptible to suffer Maillard reactions during UHT, contributing to the loss of essential amino acids such as lysine. However, Ruiz-Matute et al. (2012), analyzing the presence of several markers (furosine, tagatose, etc.) in lactose-free UHT milks, concluded that UHT treatment is normally carried out after the enzymatic treatment with β-galactosidases. From the data obtained in our work, we suggest to carry out enzymatic treatment before pasteurization, because the β-galactosidase can be inactivated and the reaction stopped by the UHT process when a maximum GOS concentration is reached. On the contrary, when the enzymatic elimination of lactose is performed under aseptic conditions after thermal treatment of milk, it is not possible to stop the reaction at will, which causes hydrolysis of most of the GOS synthesized.

4. Conclusions

A commercial preparation of K. lactis β-galactosidase (Lactozym pure) at a low dosage (0.1% v/v) is able to give GOS-enriched milk in which approximately 95% of the initial lactose has been eliminated. The GOS concentration (formed basically by 6-galactobiose, allolactose and 6′-O-β-galactosyl-lactose) is close to the HMOs content of human milk. Pasteurization after controlled enzymatic treatment could result in a product with a low lactose content and with the extra benefit of a significant presence of prebiotic GOS.

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Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., & Gras, S. L. (2009). Facile pretreatment of *Bacillus circulans* β-galactosidase from *Lactobacillus pentosus*
purification characterization and formation of galacto-oligosaccharides increases the yield of galactosyl oligosaccharides in milk and lactose reaction systems. *Journal of Agricultural and Food Chemistry, 57*, 11570-11574.


Rastall, R. A., Gibson, G. R., Gill, H. S., Guarner, F., Klaenhammer, T. R., Pot, B., Reid, G.,
human colon by probiotics, prebiotics and synbiotics to enhance human health: an
overview of enabling science and potential applications. *FEMS Microbiology Ecology*,
52, 145-152.

837S.*

Rodriguez-Colinas, B., de Abreu, M. A., Fernandez-Arrojo, L., de Beer, R., Poveda, A.,
Plou, F. J. (2011). Production of galacto-oligosaccharides by the β-galactosidase from
*Kluyveromyces lactis*: comparative analysis of permeabilized cells versus soluble

(2012). Galacto-oligosaccharide synthesis from lactose solution or skim milk using the
β-galactosidase from *Bacillus circulans*. *Journal of Agricultural and Food Chemistry*,
60, 6391-6398.

(2013). Analysis of fermentation selectivity of purified galacto-oligosaccharides by in
vitro human faecal fermentation. *Applied Microbiology and Biotechnology, 97, 5743-
5752.*

(2012). Presence of mono-, di- and galactooligosaccharides in commercial lactose-free
UHT dairy products. *Journal of Food Composition and Analysis, 28, 164-169.*

Shadid, R., Haarman, M., Knol, J., Theis, W., Beermann, C., Rjosk-Dendorfer, D., Schendel,
D. J., Koletzko, B. V., & Krauss-Etschmann, S. (2007). Effects of


**Figure captions**

**Fig. 1.** Kinetics of GOS formation in skim milk at 40°C and 0.1 % enzyme dosage catalyzed by several β-galactosidases: (A) Biolactase from *B. circulans*; (B) Lactozym pure from *K. lactis*; (C) Lactase F from *A. oryzae*. The symbols indicate: (●) Lactose; (○) Total GOS. The pH of milk was 6.7 and initial lactose concentration was 46 g/L.

**Fig. 2.** HPAEC-PAD analysis of the reactions of skim milk with different β-galactosidases at 40°C, at the point of maximum GOS concentration: (A) Biolactase from *B. circulans*; (B) Lactozym pure from *K. lactis*; (C) Lactase F from *A. oryzae*. The chromatograms correspond to the reaction mixtures (diluted 1:400) after 0.75 h, 1 h and 1.5 h respectively. The peaks correspond to: (1) Galactose; (2) Glucose; (3) 6-Galactobiose; (4) Allolactose; (5) Lactose; (6) 4-Galactobiose; (7) 6'-O-β-galactosyl-lactose; (8) 4'-O-β-galactosyl-lactose; (9) Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc.

**Fig. 3.** Kinetics of GOS formation in skim milk at 4°C and 0.1 % enzyme dosage catalyzed by several β-galactosidases: (A) Biolactase from *B. circulans*; (B) Lactozym pure from *K. lactis*; (C) Lactase F from *A. oryzae*. The symbols indicate: (●) Lactose; (○) Total GOS. The pH of milk was 6.7 and initial lactose concentration was 46 g/L.

**Fig. 4.** GOS formation vs. lactose conversion using skim milk catalyzed by β-galactosidases from different sources at 40°C (left) and 4°C (right). (A) Biolactase from *B. circulans*; (B) Lactozym pure from *K. lactis*; (C) Lactase F from *A. oryzae*. The pH of milk was 6.7 and initial lactose concentration was 46 g/L.