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Production of Galacto-oligosaccharides by the β-Galactosidase from *Kluyveromyces lactis*: Comparative Analysis of Permeabilized Cells versus Soluble Enzyme

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ABSTRACT: The transgalactosylation activity of *Kluyveromyces lactis* cells was studied in detail. Cells were permeabilized with ethanol and further lyophilized to facilitate the transit of substrates and products. The resulting biocatalyst was assayed for the synthesis of galacto-oligosaccharides (GOS) and compared with two soluble β-galactosidases from *K. lactis* (Lactozym 3000 L HP G and Maxilact LGX 5000). Using 400 g/L lactose, the maximum GOS yield, measured by HPAEC-PAD analysis, was 177 g/L (44% w/w of total carbohydrates). The major products synthesized were the disaccharides 6-galactobiose [Gal-β(1→6)-Gal] and allolactose [Gal-β(1→6)-Glc], as well as the trisaccharide 6-galactosyl-lactose [Gal-β(1→6)-Gal-β(1→4)-Glc], which was characterized by MS and 2D NMR. Structural characterization of another synthesized disaccharide, Gal-β(1→3)-Glc, was carried out. GOS yield obtained with soluble β-galactosidases was slightly lower (160 g/L for Lactozym 3000 L HP G and 154 g/L for Maxilact LGX 5000); however, the typical profile with a maximum GOS concentration followed by partial hydrolysis of the newly formed oligosaccharides was not observed with the soluble enzymes. Results were correlated with the higher stability of β-galactosidase when permeabilized whole cells were used.

KEYWORDS: glycosidase, galacto-oligosaccharides, prebiotics, transglycosylation, β-galactosidase, oligosaccharides

INTRODUCTION

β-Galactosidases (β-D-galactoside galactohydrolases, EC 3.2.1.23) catalyze the hydrolysis of the galactosyl moiety from nonreducing termini of oligosaccharides. These enzymes have attracted attention from researchers and dairy product manufacturers primarily due to their ability to remove lactose from milk.[1] In addition, β-galactosidases are employed for oligosaccharide synthesis,[2,3] because the normal hydrolytic reaction of glycosidases can be reversed toward glycosidic bond synthesis under appropriate conditions.[4−6] Thus, β-galactosidase catalyzes transgalactosylation reactions in which lactose (as well as the released glucose and galactose) serve as galactosyl acceptors, yielding a series of di, tri-, and tetrasaccharides (and eventually of higher polymerization degree) called galacto-oligosaccharides (GOS).[7,8] Depending on the source of β-galactosidase, the yield and composition of GOS vary notably.[9−11] GOS constitute the major part of oligosaccharides in human milk.[12] Among other health benefits, GOS are noncariogenic, exhibit prebiotic properties, reduce the level of cholesterol in serum, and prevent colon cancer.[13,14]

The major commercial source of β-galactosidase by far is the mesophile yeast *Kluyveromyces lactis*.[15−17] Due to its intracellular nature, the production of cell-free *K. lactis* β-galactosidase is limited by the high cost associated with enzyme extraction and downstream processing as well as the low stability of the enzyme.[8,18] The hydrolytic activity of *K. lactis* β-galactosidase is very high.[19] The production of GOS using batch and continuous bioreactors of *K. lactis* β-galactosidase has been reported.[15,16] Several studies on the immobilization of this enzyme with different carriers have been also described.[17,20,21]

Biochemical reactions using whole cells have advantages over purified enzymes in many industrial bioconversion processes, allowing the reuse of the biocatalyst and continuous processing. However, the permeability barrier of the cell envelope for substrates and products often causes very low reaction rates, especially in yeasts. To increase the volumetric activity, permeabilization of yeast cells is an economical, simple, and safe process that usually facilitates substrate access to the intracellular enzymes;[22−24] the enzyme yields trisaccharides as the main transglycosylation products.[16,25,26] The permeabilizing agent may decrease the phospholipid content in the membrane, thus allowing the transit of low molecular weight compounds in and out of the cells.[27]

In this context, ethanol-permeabilized *K. lactis* cells have been evaluated for lactose hydrolysis[22,23,28,29] and for the bioconversion

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of lactose and fructose to the disaccharide lactulose.[30] However, to our knowledge, the use of *K. lactis* permeabilized cells for the synthesis of GOS has not been investigated in detail. In this context, permeabilized cells of *Kluyveromyces marxianus* were recently employed for GOS production, although the structure of the synthesized products was not reported.[27]

In this work, we have studied the transgalactosylation activity of ethanol-permeabilized cells of a strain of *K. lactis*. The synthetic activity of the crude preparation was compared with that of two soluble β-galactosidases from *K. lactis* (Lactozym 3000 L HP G and Maxilact LGX 5000). Structural characterization of the synthesized galacto-oligosaccharides was performed in detail.

EXPERIMENTAL PROCEDURES

Materials. The commercial β-galactosidases from *K. lactis* Lactozym 3000 L HP G (batch DKN08677) and Maxilact LGX 5000 (batch 409-240001) were kindly supplied by Novozymes A/S and DSM Food Specialties, respectively. As both preparations contained 50% (v/v) glycerol, they were dialyzed prior to use in 0.1 M potassium phosphate buffer (pH 6.8). Glucose, galactose, lactose monohydrate, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from Sigma-Aldrich. 6-Galactobiose, 4-galactobiose, 3-galactobiose, 6-*O*-β-galactosyl-glucose (allolactose), and 4-*O*-β-galactosyl-lactose were from Carbosynth (Berkshire, U.K.). All other reagents and solvents were of the highest available purity and used as purchased.

Culture Conditions and Preparation of Permeabilized Cells. The *K. lactis* CECT1931 strain was obtained from the Spanish CETC (Colección Española de Cultivos Tipo). Yeasts were grown on YEPL [2% (w/v) lactose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract] at 26 °C. Growth was monitored spectrophotometrically at a wavelength of 660 nm (A_{660}). For enzyme preparations, cells from 200 mL cultures growing logarithmically at an A_{660} of 2.0−2.5 were harvested (3000*g* during 5 min) and washed with distilled water. The cells were permeabilized following the protocol described by Fontes et al.[20] with slight modifications. The cells were resuspended in 50% (v/v) ethanol, stirred for 15 min at 4 °C, washed with distilled water, and lyophilized. A total protein extract was also obtained from the cell mass using Yeast Buster Protein Extraction Reagent (Novagen) following the manufacturer's instructions.

Activity Assay. The enzymatic activity toward ONPG was measured at 40 °C following *o*-nitrophenol release at 405 nm. For the permeabilized cells, the reaction was started by adding 1.5 mg of cell mass to 2 mL of 15 mM ONPG in 0.1 M potassium phosphate buffer (pH 6.8). The mixture was incubated for 20 min with orbital shaking (600 rpm), and the reaction was stopped by immersion in a water bath for 5 min at 95 °C. For the soluble enzymes (Lactozym 3000 L HP G or Maxilact LGX 5000), the reaction was started by adding 10 μL of the dialyzed enzyme (conveniently diluted) to 190 μL of 15 mM ONPG in 0.1 M potassium phosphate buffer (pH 6.8). The increase of absorbance at 405 nm was followed in continuous mode at 40 °C during 5 min. In both cases the absorbance was measured using a microplate reader (Versamax, Molecular Devices). The molar extinction coefficient of *o*-nitrophenol at pH 6.8 was determined (1627 M^{-1} cm^{-1}). One unit (U) of activity was defined as that corresponding to the hydrolysis of 1 μmol of ONPG per minute.

Thermostability of *K. lactis* β-Galactosidase. Soluble enzymes or permeabilized cells were incubated at 40 °C in 100 mM Tris-HCl buffer, pH 7.0. Aliquots were harvested at different times, and the remaining activity toward ONPG was determined. The same experiments were carried out in the presence of 200 g/L glucose or galactose.

Production of Galacto-oligosaccharides. The reaction mixture (20 mL) contained 400 g/L lactose (34.7% w/w) in 0.1 M potassium phosphate buffer (pH 6.8). The biocatalyst was then added (110 mg of permeabilized cell mass, 46 μL of Lactozym 3000 L HP G, or 14 μL of Maxilact LGX 5000) to adjust the β-galactosidase activity in the reaction mixture in the range of 1.2−1.5 U/mL. The mixture was incubated at 40 °C in an orbital shaker (Vortemp 1550) at 200 rpm. At different times, 750 μL aliquots were harvested from the reaction mixture and incubated for 5 min at 95 °C and 350 rpm in a Thermomixer (Eppendorf) to inactivate the enzyme. Samples were filtered using 0.45 μm PVDF filters coupled to Eppendorf tubes (National Scientific) during 5 min at 6000 rpm. For each sample, two dilutions with water (1:400 and 1:4000) were prepared for high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis.

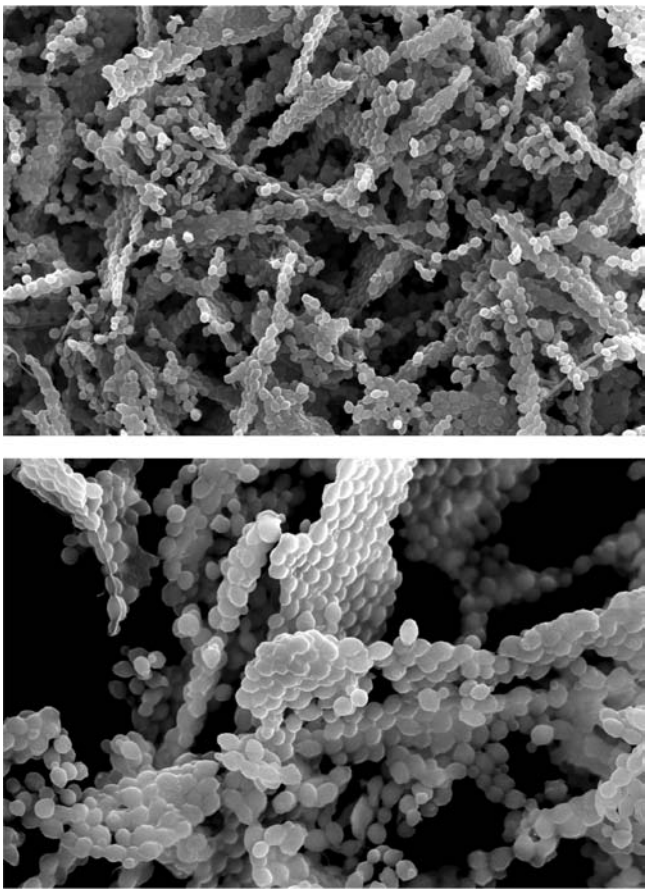

Figure 1. SEM micrographs of permeabilized *K. lactis* cells at 1000× (top) and 2000× (bottom).

HPAEC-PAD. Product analysis was performed by HPAEC-PAD on a ICS3000 Dionex system (Dionex Corp., Sunnyvale, CA) consisting of an 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 × 250 mm Carbo-Pack PA-1 column (Dionex) connected to a CarboPac PA-1 guard column was used at 30 °C. For eluent preparation, Milli-Q water and 50% (w/v) NaOH (Sigma-Aldrich) were used. The initial mobile phase was 15 mM NaOH at 1.0 mL/min for 12 min. A mobile phase gradient from 15 to 200 mM NaOH was performed at 1.0 mL/min in 15 min, and the latter was maintained as mobile phase for 25 min. The separation of lactose and allolactose was achieved with an isocratic run (35 min) with 15 mM NaOH at 1.0 mL/min, followed by a 25 min elution with 200 mM NaOH to remove the rest of the carbohydrates. The peaks were analyzed using Chromeleon software. Identification of the different carbohydrates was done on the basis of glucose,

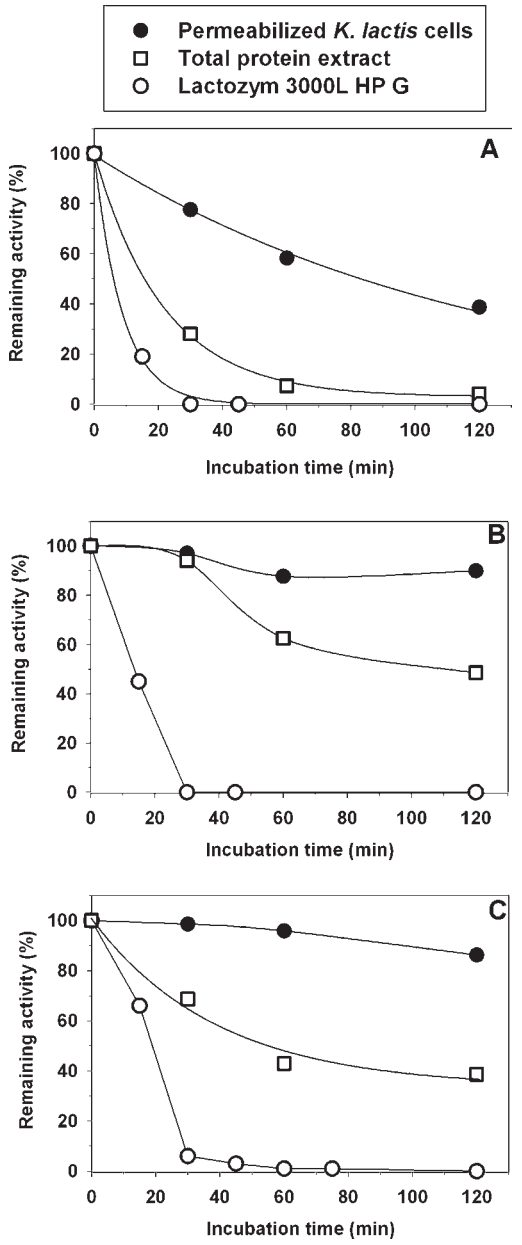

Figure 2. Thermoinactivation of *K. lactis* β-galactosidase at 40 °C in 100 mM Tris-HCl buffer, pH 7.0: (A) in the absence of sugars; (B) in the presence of 200 g/L galactose; (C) in the presence of 200 g/L glucose. Initial activity was 9.2 U/mL for permeabilized cells, 8.9 U/mL for the total protein extract, and 1.3 U/mL for Lactozym 3000 L HP G. Remaining activity was determined at the indicated times using the ONPG assay.

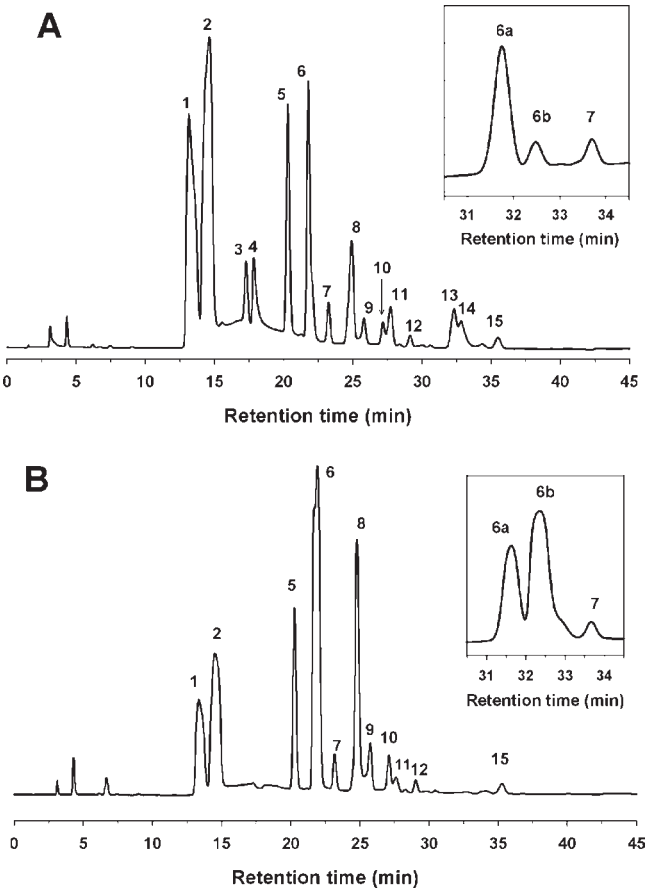

Figure 3. HPAEC-PAD analysis of the reaction of lactose with (A) permeabilized *K. lactis* cells and (B) Lactozym 3000 L HP G. Peaks: (1) galactose; (2) glucose; (3, 4) disaccharides (unknown); (5) 6-galactobiose; (6) lactose and allolactose; (7) 3-galactobiose; (8) 6-galactosyl-lactose; (10) 3-galactosyl-glucose; (14) 4-galactosyl-lactose; (9, 11−13, 15) other GOS (unknown). (Inset) Separation of allolactose (6a) and lactose (6b) under isocratic conditions. The chromatograms correspond to reaction times of 25 and 7 h for *K. lactis* cells and Lactozym 3000 L HP G, respectively.

galactose, lactose, 6-*O*-β-galactosyl-glucose (allolactose), 3-galactobiose, 4-galactobiose, 6-galactobiose, and 4-*O*-β-galactosyl-lactose standards.

Purification of GOS by Hydrophilic Interaction Chromatography. For the isolation of unknown GOS in the mixture (not coeluting with any of the standards), the reaction was scaled up to 5 mL. When the GOS yield reached the maximum value, the biocatalyst was inactivated by boiling the solution for 5 min. The reaction mixture was filtered, and the mixture was purified by semipreparative HPLC. A quaternary pump (Delta 600, Waters) coupled to a Lichrosorb-NH2 column (5 μm, 10 × 250 mm, Merck) was used. The column temperature was kept constant at 25 °C. Acetonitrile/water 85:15 (v/v), conditioned with helium, was used as mobile phase (flow rate = 8.1 mL/min) for 8 min. Then, a gradient to acetonitrile/water 75:25 (v/v) was performed in 2 min, and this eluent was maintained during 6 min. Finally, a gradient from this composition to acetonitrile/water 70:30 (v/v) was performed in 5 min and maintained for 20 min. Peaks were detected using an evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 60 °C. A three-way flow splitter (model Acurate, Dionex) and a fraction collector II (Waters) were employed. The fractions containing the main peaks were pooled, the solvent (acetonitrile/water) was eliminated by rotary evaporation, and the resulting sugars were dissolved in a small amount of methanol and a droplet of water. Ice-cold acetone was added to initiate precipitation. The resulting suspension was filtered, and the crystals were dried overnight in vacuo.

Mass Spectrometry. Samples were analyzed by MALDI-TOF mass spectrometry (Bruker, model Ultraflex III TOFTOF) using 2,5-dihydroxybenzoic acid doped with sodium iodide as matrix, in positive reflectron mode.

Nuclear Magnetic Resonance (NMR). The structure of the oligosaccharides was elucidated using a combination of ^{1}H, ^{13}C, and 2D NMR (COSY, TOCSY, NOESY, HSQC) techniques. The spectra of the samples (ca. 10 mM), dissolved in deuterated water, were recorded on a Bruker AVANCE DRX500 spectrometer equipped with a tunable broadband ^{1}H/X probe with a gradient in the *Z* axis, at a temperature of

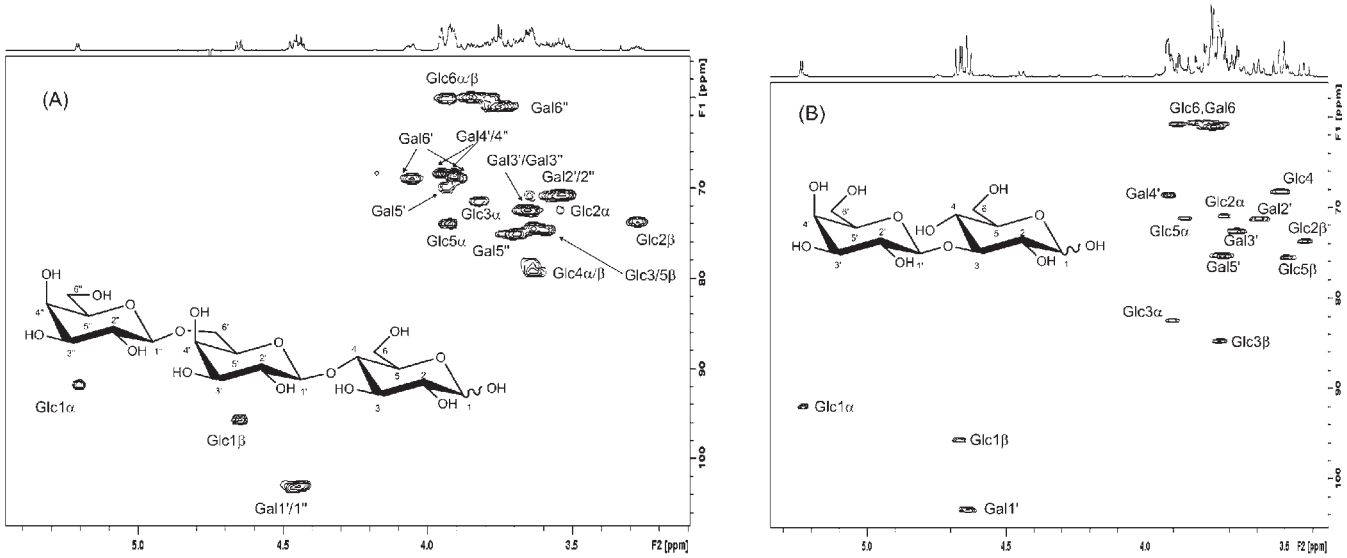

Figure 4. 2D NMR heteronuclear single quantum coherence (HSQC) analysis of two galacto-oligosaccharides obtained in the reaction of lactose with *K. lactis* cells: (A) trisaccharide 6-galactosyl-lactose; (B) disaccharide 3-galactosylglucose.

298 K. Chemical shifts were expressed in parts per million with respect to the 0 ppm point of DSS, used as internal standard. COSY, TOCSY, NOESY, and HSQC sequences were provided by Bruker. COSY, TOCSY (80 ms mixing time), and NOESY (500 ms mixing time) experiments were performed with 16, 8, and 48 scans, respectively, with 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral widths were 9 ppm in both dimensions. The HSQC experiment (16 scans) also used 256 increments in the indirect dimension and 1024 points in the acquisition dimension. The spectral width was 120 ppm in the indirect dimension and 9 ppm in the acquisition one.

Scanning Electron Microscopy (SEM). The permeabilized cells were mounted on aluminum SEM stubs. The mounted samples were sputter-coated with a thin layer of gold at completed Torr vacuum and examined by SEM using an XL3 microscope (Philips) at an acceleration potential of 20 kV.

RESULTS AND DISCUSSION

Activity and Stability of *K. lactis* Cells. Different methods of permeabilizing cells that do not significantly affect enzymatic activity have been described, including concentration, drying, treatment with solvents or surfactants, lyophilization, ultrasonic treatment, mechanical disruption, etc.[29,31] In this work, *K. lactis* cells growing logarithmically on lactose as carbon source at 26 °C were exposed to 50% (v/v) ethanol for 15 min at 4 °C, washed, and lyophilized. Solvent treatment is an inexpensive method that can be applied on a large scale.[32] Depending on the solvent nature and concentration, as well as the incubation time, the permeability of membranes may be increased enough to lead to disruption of cells, with the concomitant release of enzyme molecules into the supernatant.[18,33,34] In our study, the ethanol treatment was not so intense as to produce cell disruption. Although cells treated with short-chain alcohols become nonviable in many cases, intracellular enzymes may resist such treatment and, as a consequence, are not inactivated. In addition, the permeability barriers of cell walls are lowered, so the cells themselves can be utilized as a biocatalyst repeatedly.

Yeast morphology was examined by SEM (Figure 1). The typical oval shape of budding yeast cells was visualized at 1000× (upper picture) and 2000× (bottom picture). A control of yeasts not subjected to ethanol treatment was performed, and similar micrographs (not shown) were obtained.

It is well-known that activity in the permeabilized cell mass is low compared with the soluble enzyme.[23] In this work, the hydrolytic activity of the permeabilized cells, using ONPG as substrate, was 210 U/g of cell mass. The thermal stability of *K. lactis* β-galactosidase in permeabilized cells was studied at 40 °C in 100 mM Tris-HCl buffer (pH 7.0) (Figure 2). This neutral pH has been reported to be optimal for β-galactosidase activity.[19,35] The results were compared with those of the total protein extract obtained by cell lysis and with a soluble β-galactosidase from *K. lactis* (Lactozym 3000 L HP G) (Figure 2). As shown in Figure 2A, the soluble enzyme inactivates very quickly under such conditions (especially Lactozym 3000 L), and most of its activity was lost in <60 min. The permeabilized cells exert a protecting effect on the intracellular β-galactosidase, as the activity remaining after 120 min was approximately 40%.

To assay the enzyme stability under conditions similar to those employed in the biotransformations, studies were also performed in the presence of 200 g/L glucose or galactose, which are the two main monosaccharides present in the reaction mixture when using lactose. It is well-known that sugars stabilize proteins;[36] therefore, the operational conditions are usually favorable for the stability of glycosidases.[37] In the presence of 200 mg/mL galactose (Figure 2B) or glucose (Figure 2C), the stabilizing effect of whole cells was even more pronounced; the activity of the permeabilized cells remained significantly stable during 120 min, whereas the soluble β-galactosidases displayed a significant loss of activity in the same time.

Production of GOS by *K. lactis* Cells. GOS are simultaneously synthesized and degraded by β-galactosidases; as a consequence, the GOS concentration and the composition of the sample change dramatically with reaction time and can hardly be predicted.[8,9] The time at which reaction is harvested has a crucial effect on GOS yield.[14] The maximum GOS yield is basically determined by the intrinsic enzyme properties (transgalactosylation to hydrolysis ratio) as well as lactose concentration.[38,39]

We studied the behavior of *K. lactis* cells in GOS synthesis compared with two soluble β-galactosidases (Lactozym 3000 L

HP G and Maxilact LGX 5000). The experiments were carried out at 1.2−1.5 U/mL, which is a lower enzyme concentration than typically used in similar experiments with *K. lactis* β-galactosidase (e.g., 3−9 U/mL in ref 15, 2.9−8.7 U/mL in ref 16, and 12.5 U/mL in ref 30). At higher enzyme concentrations reactions are so fast (<240 min) that it is difficult to observe any effect of enzyme stability on reaction progress. It has been demonstrated that although the enzyme concentration exerts a marked influence on the reaction time at which the maximum oligosaccharide concentration is achieved, it does not affect the absolute value of GOS yield.[16,40] The rest of the conditions used in our experiments were similar to those previously described for soluble *K. lactis* β-galactosidase (400 mg/mL lactose, pH 7.0, 40 °C).

Figure 3A shows the HPAEC-PAD chromatogram of the reaction mixture with permeabilized cells after 25 h. Peaks 1, 2, and 6 were assigned to galactose, glucose, and lactose, respectively. However, we observed that the shape of peak 6 varied throughout the reaction; in fact, by changing the elution conditions, we were able to split peak 6 into 6a [the lactose isomer allolactose, Gal-β(1→6)-Glc] and 6b (lactose), as shown in the insets of Figure 3. We found that the most abundant GOS synthesized by the permeabilized cells corresponded to peak 5 (the disaccharide 6-galactobiose [Gal-β(1→6)-Gal]), peak 6a (allolactose), and peak 8. Purification of peak 8 was performed by semipreparative HILIC. Its mass spectrum showed that it was a trisaccharide. The 1D and 2D ^{1}H NMR spectra displayed four anomeric signals, two of them corresponding to the two α,β anomers of the reducing glucose moiety and two more corresponding to β-linked galactose residues. From the combination of the signals from COSY, TOCSY, NOESY, and HSQC it could be deduced that the ^{1}H and ^{13}C resonance signals belong to the different residues. The NOESY cross peaks from the two galactose anomeric signals also allowed the connectivities to their contiguous moieties to be distinguished. In fact, one of them was clearly connected with two H-6 signals, the ^{13}C resonance of which was shifted downfield (HSQC, Figure 4A), whereas the other one was connected to the H-4 of the glucose residue, the ^{13}C resonance of which was unambiguously identified (HSQC). Thus, peak 8 showed a galactosyl moiety β-(1→6)-linked to the galactose ring of lactose.

As a consequence, the three major products synthesized by *K. lactis* cells contained a β-(1→6) bond between two galactoses or between one galactose and one glucose. It is worth emphasizing that the chemical structure of the obtained oligosaccharides (composition, number of moieties, and types of linkages between them) may affect their fermentation pattern by probiotic bacteria in the gut.[15] In this context, it was reported that β(1→6) linkages are cleaved very quickly by β-galactosidases from bifidobacteria,[15] a key factor in the prebiotic properties.

With authentic standards, it was also possible to identify peak 7 as the disaccharide 3-galactobiose [Gal-β(1→3)-Gal] and peak 14 as the trisaccharide 4-galactosyl-lactose [Gal-β(1→4)-Gal-β(1→4)-Glc]. Peak 10 was also purified, and mass spectrometry analysis indicated it was a disaccharide. From the combination of the signals from COSY, TOCSY, NOESY, and HSQC (Figure 4B) the structure of peak 10 was attributed to the disaccharide Gal-β-(1→3)-Glc.

The complete identification of the GOS synthesized by a particular enzyme is a difficult task. In fact, considering the formation of Gal-β(1→2)-, β(1→3)-, β(1→4)-, and β(1→6)-bonds, the theoretical number of synthesized GOS accounts for 7 disaccharides, 32 trisaccharides, 128 tetrasaccharides, and so on.

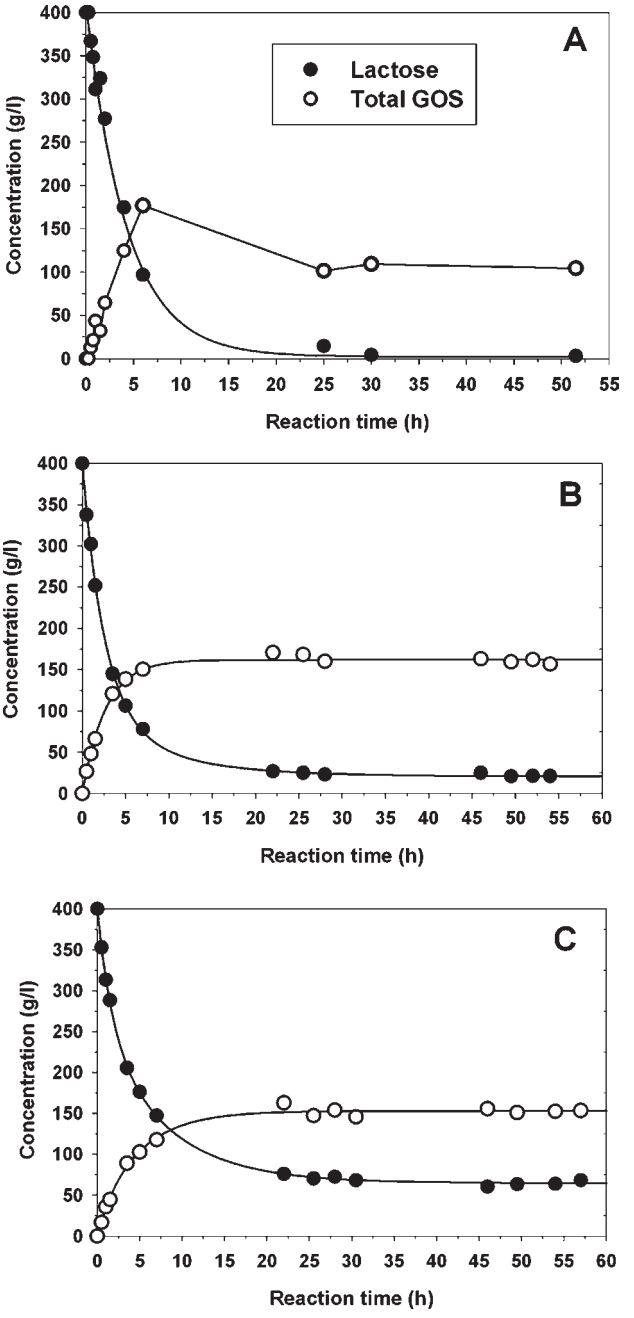

Figure 5. Galacto-oligosaccharides production from lactose by (A) permeabilized *K. lactis* cells, (B) Lactozym 3000 L HP G, and (C) Maxilact LGX 5000. Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.8), 1.2−1.5 U/mL, 40 °C.

Minor peaks 3, 4, 9, 11−13, and 15 could not be identified. As the response factors of GOS isomers in HPAEC-PAD are closely similar, the quantification of the unknown products was carried out using lactose as standard for the disaccharides and 4-galactosyl-lactose for the trisaccharides.

Chockchaisawasdee et al.[16] were the first to perform a preliminary structural analysis of the GOS formed by soluble *K. lactis* β-galactosidase (Maxilact L2000), concluding that the major bonds were β(1→6). Maugard et al.[17] and Cheng et al.[41] detected the formation of disaccharides by *K. lactis*, but no structural identification was done. Martinez-Villaluenga et al.[15]

Table 1. Carbohydrate Composition (Percent w/w) of the Reaction Mixture Using Permeabilized Cells from *K. lactis*[a]

reaction time (h)	galactose	glucose	lactose	6-galactobiose	allolactose	6-galactosyl-lactose	other GOS	total GOS
0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
0.5	2.1	3.0	91.7	0.3	0.0	2.9	0.0	3.2
0.75	3.4	4.2	87.1	0.8	0.0	4.5	0.0	5.3
1	5.6	5.7	77.8	1.2	0.0	7.5	2.2	10.8
2	7.5	7.1	69.3	1.4	0.0	8.6	6.2	16.1
4	10.8	14.4	43.6	3.8	6.1	12.3	8.9	31.1
6	13.9	17.7	24.2	6.4	10.0	15.7	12.1	44.2
25	33.0	38.1	3.6	5.9	6.5	3.6	9.4	25.3
30	33.2	38.5	1.1	10.1	4.3	4.7	8.2	27.3
51.5	35.0	38.1	0.8	10.3	4.3	2.7	8.8	26.1

[a] Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.8), 1.2 U/mL (5.5 mg cell mass/mL), 40 °C.

Table 2. Carbohydrate Composition (Percent w/w) of the Reaction Mixture Using Lactozym 3000 L HP G[s]

reaction time (h)	galactose	glucose	lactose	6-galactobiose	allolactose	6-galactosyl-lactose	other GOS	total GOS
0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
0.5	3.4	5.6	84.4	0.6	0.0	5.4	0.7	6.7
1	6.0	6.5	75.5	2.0	0.0	7.7	2.3	12.0
1.5	6.9	13.6	62.9	1.2	0.0	11.2	4.2	16.5
3.5	11.5	22.0	36.2	3.3	5.6	15.1	6.2	30.2
5	13.3	25.5	26.6	4.1	7.2	15.9	7.3	34.6
7	13.2	29.8	19.5	4.3	7.5	16.5	9.3	37.5
22	16.4	34.4	6.7	6.2	10.6	14.3	11.5	42.6
49.5	18.3	36.7	5.2	5.9	10.2	14.3	9.2	39.7
70	18.5	37.3	5.3	5.9	10.1	13.4	9.5	38.9

[s] Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.8), 1.5 U/mL, 40 °C.

Table 3. Carbohydrate Composition (Percent w/w) of the Reaction Mixture Using Maxilact LGX 5000[a]

reaction time (h)	galactose	glucose	lactose	6-galactobiose	allolactose	6-galactosyl-lactose	other GOS	total GOS
0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
0.5	2.8	4.8	88.2	0.3	0.0	3.6	0.4	4.2
1	4.5	8.2	78.3	1.1	0.0	6.9	0.9	8.9
1.5	5.8	10.9	72.0	0.7	0.0	8.9	1.6	11.2
3.5	8.9	17.5	51.4	2.5	3.2	12.7	3.9	22.3
5	10.1	20.1	44.1	3.8	3.4	13.8	4.6	25.7
7	11.2	22.6	36.8	4.4	4.2	14.7	6.1	29.4
22	13.7	26.5	19.0	4.4	7.0	15.4	14.0	40.7
28	14.8	28.6	18.1	4.8	8.2	16.1	9.4	38.5
49.5	15.6	30.9	15.8	5.1	8.3	15.6	8.7	37.7
70	15.3	28.9	17.3	4.9	8.1	16.3	9.2	38.5

[a] Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.8), 1.5 U/mL, 40 °C.

reported the formation of 6-galactobiose, allolactose, and 6-galactosyl-lactose with *K. lactis* β-galactosidase, in accordance with our findings with permeabilized cells, but the formation of other GOS was not mentioned. To our knowledge, the present study is the most detailed of those published with *K. lactis* β-galactosidase in terms of the chemical structure of the synthesized products.

Figure 5A shows the reaction progress with permeabilized cells. The maximum GOS concentration [177 g/L, which represents 44% (w/w) of the total carbohydrates in the reaction mixture] was observed at 6 h. After that, the amount of GOS diminished by the hydrolytic action of β-galactosidase until reaching an equilibrium GOS concentration of 105 g/L. Interestingly, lactose disappeared almost completely at the end of the process. Table 1 summarizes the carbohydrate composition (in weight) at different reaction times. At the point of maximal GOS concentration (6 h), the mixture was formed by monosaccharides (32%), lactose (24%), 6-galactobiose (6%), allolactose (10%), 6-galactosyl-lactose (16%), and other GOS (12%). Martinez-Villaluenga et al.,[15] using a soluble *K. lactis* β-galactosidase, reported a mixture formed by monosaccharides (49%), residual lactose (21%), the disaccharides 6-galactobiose and allolactose (13%), and 6-galactosyl-lactose (17%).

The upper range of GOS yield is close to 40−45%,[9,42,43] which is lower than that reported for other prebiotics such as fructooligosaccharides (approximately 65%).[44,45] In this work, GOS yield was slightly higher than that reported in previous studies with *K. lactis* β-galactosidase.[15−17] The lower yield reported in such studies could be related with the overestimation of lactose due to its tendency to coelute with allolactose or by not considering the contribution of other minor GOS to the total yield.

Production of GOS by Soluble *K. lactis* β-Galactosidase. The activities of Lactozym 3000 L HP and Maxilact LGX 5000 toward ONPG were 645 and 2145 U/mL, respectively. The reaction profile of both enzymes in the GOS synthesis is shown in Figure 5, panels B and C, respectively. Several differences were found in the behavior of soluble β-galactosidases compared with permeabilized cells. First, panels B and C of Figure 5 do not show the typical pattern in which a maximum GOS yield is followed by the hydrolysis of a part of the synthesized products. Second, lactose is not completely consumed at the equilibrium, with remaining concentrations of 21 and 65 g/L for Lactozym and Maxilact LGX 5000, respectively, whereas only 3 g/L was determined for permeabilized cells. Maximum GOS yield was slightly lower for soluble β-galactosidases (160 g/L for Lactozym and 154 g/L for Maxilact LGX 5000, which represent 42 and 41% w/w of the total carbohydrates present in the mixture) compared with permeabilized cells (177 g/L, 44% w/w).

Figure 3B shows the HPAEC-PAD chromatogram obtained with Lactozym 3000 L HP close to the point of maximum GOS concentration. As illustrated, the profile was similar to that obtained with permeabilized cells. The main transglycosylation products were again 6-galactobiose (peak 5), allolactose (peak 6a), and 6-galactosyl-lactose (peak 8). The main difference of the soluble enzyme compared with the permeabilized cells was the absence of several minor peaks (peaks 3, 4, 13, and 14 in Figure 3A); however, some of them appeared briefly at different reaction times, but they quickly disappeared. A very similar chromatogram was obtained with Maxilact LGX 5000 (data not shown).

Tables 2 and 3 summarize the progress of carbohydrate distribution for both enzymes. The above results seem to indicate that the reaction is stopped before reaching the final equilibrium due to the low stability of soluble β-galactosidases. In contrast, the higher stability of permeabilized cells is manifested by the characteristic reaction profile, with a maximum, when a competition exists between hydrolysis and transglycosylation (kinetic control).[46]

The maximum GOS concentration with permeabilized cells was obtained when lactose conversion was 76%. Lactozym 3000 L HP and Maxilact LGX 5000 reached 95 and 83% of lactose conversion, without displaying a maximum in GOS concentration. This could indicate that the microenvironment of the permeabilized cells also exerts an influence on the transglycosylation to hydrolysis ratio. Thus, in the initial stages of the reaction, permeabilized cells show a higher tendency to form GOS than the soluble enzymes (Figure 5); however, when the GOS accumulate in the system, the whole cells also have a higher tendency to hydrolyze the GOS formed.

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