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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MAY 2012

Impact Factor: 2.91 · DOI: 10.1021/jf300852s · Source: PubMed

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Evaluation of Oligosaccharide Synthesis from Lactose and Lactulose Using β-Galactosidases from *Kluyveromyces* Isolated from Artisanal Cheeses

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ABSTRACT: The β-galactosidase activity of 15 *Kluyveromyces* strains isolated from cheese belonging to *Kluyveromyces lactis* and *Kluyveromyces marxianus* species was tested for the production of oligosaccharides derived from lactose (GOS) and lactulose (OsLu). All *Kluyveromyces* crude cell extracts (CEEs) produced GOS, such as 6-galactobiose and 3′-, 4′-, and 6′-galactosyl-lactose. At 4 h of reaction, the main trisaccharide formed was 6′-galactosyl-lactose (20 g/100 g of total carbohydrates). The formation of OsLu was also observed by all CEEs tested, with 6-galactobiose, 6′-galactosyl-lactulose, and 1-galactosyl-lactulose being found in all of the reaction mixtures. The synthesis of trisaccharides predominated over other oligosaccharides. *K. marxianus* strain O3 produced the highest yields of GOS and OsLu after 4 h of reaction, reaching 42 g/100 g of total carbohydrates (corresponding to 80% lactose hydrolysis) and 45 g/100 g of total carbohydrates (corresponding to 87% lactulose hydrolysis), respectively. Therefore, the present study contributes to a better insight into dairy *Kluyveromyces* β-galactosidases and shows the feasibility of these enzymes to transglycosylate lactose and lactulose, producing high yields of prebiotic oligosaccharides.

KEYWORDS: *Kluyveromyces lactis, Kluyveromyces marxianus, transgalactosylation, GOS, OsLu, lactose, lactulose*

INTRODUCTION

β-Galactosidase (EC 3.2.1.23) is a hydrolase that attacks the terminal non-reducing β-D-galactosyl residues of oligosaccharides and transfers the galactosyl moiety to suitable acceptors. These enzymes have several applications in the food fermentation and dairy industries, and mainly because of their ability to hydrolyze lactose, they have attracted the attention of researchers and dairy product manufacturers.[1] Transgalactosylation is favored over hydrolysis in the presence of high substrate concentrations, and in the case of lactose, β-galactosidases produce galacto-oligosaccharides (GOS).[2] GOS are mainly disaccharides (allolactose and galactobiose), trisaccharides (4′- and 6′-galactosyl-lactose), and longer chain oligosaccharides consisting of four or more monosaccharide units.[3]

Although transgalactosylation of lactose has been known for more than 50 years,[4] GOS production is gaining importance because of their recognition as prebiotics.[5] Moreover, the influence of the GOS structure on prebiotic selectivity has been demonstrated.[6] Other health benefits, such as the improvement of mineral absorption, prevention of intestinal infections, and enhancement of immune function, among others, have been described.[7−10]

Recently, the synthetic disaccharide lactulose (4-*O*-β-D-galactopiranosyl-D-fructose) has been proposed as an enzymatic substrate for lactulose-derived oligosaccharide (OsLu) production.[11−13] Although lactulose has been recognized as prebiotic,[14,15] gas production associated with its fermentation in the proximal colon may represent a disadvantage for lactulose ingestion.[16] In this context, synthesis of OsLu may provide a new group of active compounds with health beneficial effects complementary to those provided by GOS[17] and probably without the inconvenience of lactulose consumption.

Nowadays, microbial β-galactosidases represent a feasible alternative to the chemical synthesis of GOS, with the benefits of enzymatic stereospecificity and higher final yields. β-Galactosidases have been frequently characterized in lactic acid bacteria and bifidobacteria related to milk, milk products, and the intestine of neonates.[18,19] The genus *Kluyveromyces* and specifically the species *Kluyveromyces lactis* have received considerable attention as both a genetic model and industrial yeast as a source of different metabolites and enzymes.[20] Similarly, the species *Kluyveromyces marxianus* has been explored because of its potential biotechnological applications, although the accumulated knowledge on *K. marxianus* is much smaller compared to that on *K. lactis*.[21] Both species, present in dairy products, are considered generally recognized as safe (GRAS) microorganisms and present a good growth yield and a higher β-galactosidase activity compared to other yeasts.[22] Thus, both species are relevant industrial sources of β-galactosidase activity, and they have been traditionally used

Received: February 27, 2012
Revised: May 4, 2012
Accepted: May 4, 2012
Published: May 4, 2012

dx.doi.org/10.1021/jf300852s | J. Agric. Food Chem. 2012, 60, 5134−5141

to produce low-lactose products and for the biological treatment of cheese whey waste. With respect to oligosaccharide synthesis, lactose and to a lesser extent lactulose transgalactosylation by *K. lactis* commercial enzymatic preparations has been evaluated,[13,23−25] whereas *K. marxianus* β-galactosidases have only recently been tested for lactose transgalactosylation.[26,27]

Previous studies have demonstrated that the transgalactosylation/hydrolysis ratio varies depending upon the different sources of β-galactosidase and that different enzymes can achieve different degrees of transgalactosylation, leading to variations in the level and composition of synthesized GOS.[28] However, there is little information about the feasibility of food-isolated *Kluyveromyces* strains with potentially different metabolic characteristics to transgalactosylate different substrates.

The aim of the present work was to evaluate the β-galactosidase activity from different strains of *K. lactis* and *K. marxianus* isolated from artisanal cheeses and to screen their potential to produce GOS and OsLu by hydrolysis and transgalactosylation of lactose and lactulose, respectively.

MATERIALS AND METHODS

Chemicals. Lactose was obtained from Scharlau (Barcelona, Spain). Lactulose, D-glucose, raffinose, D-fructose, and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) were purchased from Sigma-Aldrich Co. (Steinheim, Germany). D-Galactose was acquired from Fluka (Steinheim, Germany). D-Glucose and lactose for yeast culture media were obtained from Panreac (Barcelona, Spain). Bacteriological peptone was purchased from Cultimed (Barcelona, Spain). Yeast extract and agar were acquired from Pronadisa (Madrid, Spain).

Yeast Strains. A total of 15 yeast strains belonging to *K. lactis* and *K. marxianus* species were isolated from artisanal ewes' and goats' milk cheeses produced by the cheese company "Los Corrales" from rural Castelló province (Spain). *K. lactis* CECT 1961^T was obtained from the Spanish Type Culture Collection and was included in the study as a control. Isolation sources are shown in Table 1.

Table 1. β-Galactosidase Activity against *o*NPG of Yeast Species CCEs Screened for Oligosaccharide Production

species	strain[a]	isolation source	specific activity (units/mg)
K. lactis	CECT 1961^T	gassy cheese, U.K.	11.8
	BP1	ewes' milk cheese whey	3.5
	BP2	ewes' milk cheese	5.8
	BP3	ewes' milk cheese	7.4
	BP4	ewes' milk cheese	13.1
	BP5	ewes' milk cheese	3.9
	BP6	ewes' milk cheese	4.6
	BP7	ewes' milk cheese	3.3
	BP8	ewes' milk cheese	4.8
	O1	ewes' milk cheese	4.2
	O2	ewes' milk cheese	2.7
	C1	goats' milk cheese	3.4
	C2	goats' milk cheese	3.0
K. marxianus	O3	ewes' milk cheese	1.3
	O4	ewes' milk cheese whey	1.6

[a]All yeast strains, except CECT 1961^T, were isolated from Spanish cheeses.

***Kluyveromyces* Crude Cell Extracts (CCEs).** Yeasts were grown overnight in GPY medium (2% glucose, 0.5% peptone, and 0.5% yeast extract) at 28 °C. Afterward, yeast cells were transferred to LPY medium (2% lactose, 0.5% peptone, and 0.5% yeast extract) and incubated overnight at 28 °C. For preparation of CCEs, cells were resuspended in 50 mM potassium phosphate at pH 6.5 with 1 mM $MgCl_2$ and disrupted with glass beads (0.5 mm) in a bead-beater cell disrupter (model 1107900, Bio Spec Products, Inc., Bartlesville, OK). Disruption was achieved at 4 °C by subjecting the cells to three bursts of 45 s, with resting periods of 5 min. The resulting homogenates were centrifuged at 5000*g* for 20 min at 4 °C, and the supernatants, considered as CCEs, were kept at −20 °C until further analysis.

Determination of β-Galactosidase Activity and Protein Content. β-Galactosidase activity from CCEs was quantified using *o*NPG as the substrate according to Martinez-Villaluenga et al.[23] One enzyme unit is defined as the amount of enzyme releasing 1 μmol of *o*NP per milliliter per minute at 40 °C and pH 6.5. Activity against oNPG was used to adjust the activity of the different CCEs (6 units/mL) for the transgalactosylation reaction (see below). The protein content of CCEs was determined using the Bradford assay, with bovine serum albumin as the standard.[29]

Synthesis of Oligosaccharides Derived from Lactose (GOS) and Lactulose (OsLu). Production of lactose- and lactulose-derived oligosaccharides was carried out using 250 g/L of substrate in 0.1 M phosphate buffer at pH 6.5 and 6 units/mL β-galactosidase activity during incubation at 50 °C up to 24 h, as described by Cardelle-Cobas et al.[13] and Martínez-Villaluenga et al.[23] Lactose and lactulose solutions were heated before the enzyme extract was added and were maintained at the required temperature throughout the experiment. Reactions were performed in individual Eppendorf tubes and incubated in an orbital shaker at 400 rpm. Samples of 200 μL were withdrawn from the reaction mixtures at 0, 2, 4, 6, and 24 h and immediately immersed in boiling water for 5 min to inactivate the enzyme. After appropriate dilution, 20 μL was injected into the chromatograph described below. Control samples were prepared in the same manner, except no CCE was added. All experiments were performed in duplicate.

Chromatographic Determination of Carbohydrates. GOS and OsLu were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC−PAD) in a ICS2500 Dionex system (Dionex Corp., Sunnyvale, CA) consisting of a GP50 gradient pump and an ED50 electrochemical detector with a gold working electrode and a Ag/AgCl reference electrode. Data were acquired and processed with Chromeleon 6.7 software (Dionex Corp.). Separations were performed on a CarboPac PA-1 column (250 × 4 mm) connected to a CarboPac PA-1 guard column (50 × 4 mm) following the method described by Splechtna et al.[30] Detection time and voltage parameters were set as follows: E_1 = 0.1 V (t_1 = 400 ms), E_2 = 2.0 V (t_2 = 10 ms), E_3 = 0.6 V, and E_4 = −0.1 V (t_4 = 60 ms); t_t = 500 ms. Samples and standard solutions were filtered through a nylon Millipore FH membrane (0.22 μm) (Bedford, MA) before injection. Quantification of carbohydrates was performed by external calibration using standard solutions of galactose, fructose, lactose, lactulose, and raffinose. The regression coefficients of the curves for each standard were always greater than 0.99. The amount of lactose or lactulose remaining and the yield of GOS and OsLu were expressed as grams per 100 g of the total carbohydrate content in the reaction mixtures.

Statistical Analysis. Bonferroni test was used for mean comparison at the 95% confidence level (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

RESULTS AND DISCUSSION

β-Galactosidase Activity. As shown in Table 1, all CCEs hydrolyzed *o*NPG. *K. lactis* CCEs showed higher *o*NPG hydrolysis than the two *K. marxianus* CCEs. *K. lactis* BP4 showed the highest β-galactosidase activity (13.1 units/mg), followed by the reference strain CECT 1961^T (11.9 units/mg), whereas *K. marxianus* strains showed values of 1.3 and 1.6 units/mg.

Synthesis of Oligosaccharides Derived from Lactose (GOS). Lactose transgalactosylation by yeast CCEs was

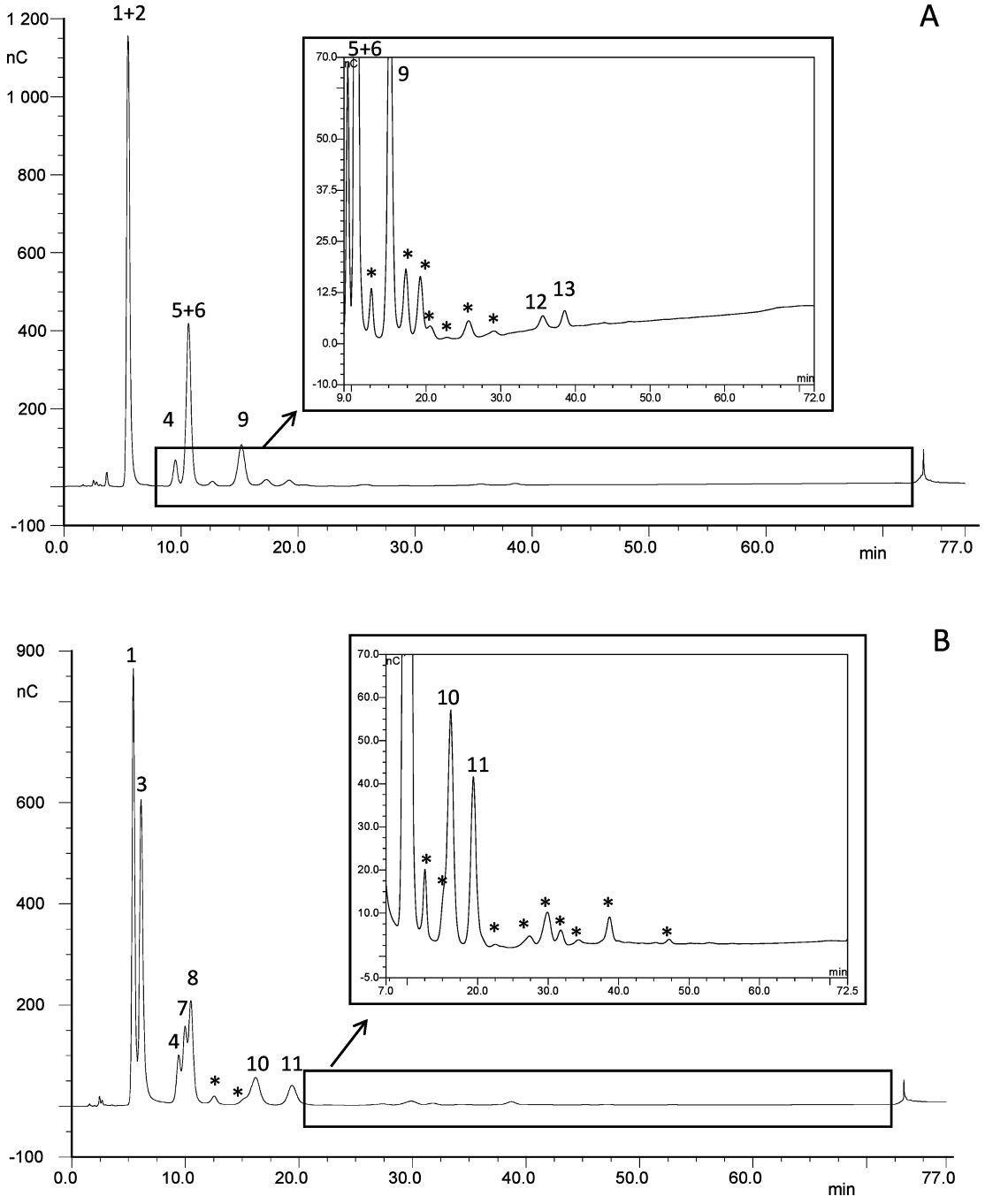

Figure 1. HPAEC–PAD profiles of carbohydrate mixtures obtained by enzymatic hydrolysis of (A) lactose and (B) lactulose by *K. marxianus* O3 β-galactosidase. (A) Compounds: 1, galactose; 2, glucose; 4, 6-galactobiose; 5, allolactose; 6, lactose; 9, 6′-galactosyl-lactose; 12, 4′-galactosyl-lactose; 13, 3′-galactosyl-lactose; and ∗, other oligosaccharides. (B) Compounds: 1, galactose; 3, fructose; 4, 6-galactobiose; 7, allolactulose; 8, lactulose; 10, 6′-galactosyl-lactulose; 11, 1′-galactosyl-lactulose; and ∗, other oligosaccharides.

followed by HPAEC–PAD. Under the conditions tested (pH 6.5, 50 °C, 250 g/L of lactose, and 6 units/mL of β-galactosidase activity), which were previously optimized in the laboratory for a *K. lactis* commercial preparation,[23] GOS production from lactose was compared after 4 h of reaction. All yeast CCEs synthesized galactosyl derivatives of lactose, showing similar chromatographic profiles of GOS production. A representative chromatogram corresponding to lactose transgalactosylation catalyzed by *K. marxianus* O3 CCE after 4 h of reaction is shown in Figure 1A. The peak 1 + 2 corresponded to co-eluting galactose and glucose, whereas peak 5 + 6 was assigned to lactose and allolactose. Peak 4 was identified as 6-galactobiose. Peak 9 corresponded to trisaccharide 6′-galactosyl-lactose. Peaks 12 and 13 corresponded to 4′-galactosyl-lactose and 3′-galactosyl-lactose, respectively. These assignments were made by comparing relative retention times to those found in previous studies.[31] Unidentified di- or trisaccharides as well as high retention time oligosaccharides (peaks marked with an asterisk) were also detected. Table 2 summarizes GOS yields after 4 h of reaction for all yeast β-galactosidases studied. Total GOS yields ranged from approximately 26 to 42 g/100 g of total carbohydrates, in

dx.doi.org/10.1021/jf300852s | J. Agric. Food Chem. 2012, 60, 5134–5141

Table 2. Carbohydrate Composition (g/100 g of Total Carbohydrates) of the Reaction Mixtures during Lactose Hydrolysis (4 h)[a]

strain	monosacharides	lactose + allolactose	6-galactobiose	6′-galactosyl-lactose	other GOS[b]	total GOS[c]
CECT 1961^T	53.69 ± 2.18 bc	13.95 ± 0.45 e	5.63 ± 0.33 c	16.92 ± 0.84 e	8.82 ± 0.45 ab	32.36 ± 1.74 cd
BP1	59.38 ± 1.86 de	10.41 ± 0.55 cd	5.40 ± 0.19 abc	14.37 ± 0.82 bcd	9.44 ± 0.30 abcd	30.21 ± 1.31 bc
BP2	54.57 ± 0.18 bc	13.92 ± 0.20 e	5.29 ± 0.00 abc	16.01 ± 0.04 de	9.21 ± 0.09 abc	31.51 ± 0.02 c
BP3	55.54 ± 1.03 cd	13.08 ± 0.38 e	5.27 ± 0.04 abc	15.64 ± 0.48 de	9.48 ± 0.10 abcd	31.39 ± 0.65 c
BP4	66.27 ± 0.50 f	8.12 ± 0.26 a	4.92 ± 0.07 a	11.82 ± 0.33 a	8.03 ± 0.16 a	25.61 ± 0.24 a
BP5	63.03 ± 0.95 ef	9.12 ± 0.17 abcd	5.09 ± 0.04 abc	13.53 ± 0.23 abc	8.33 ± 0.51 a	27.85 ± 0.78 ab
BP6	62.68 ± 1.42 ef	9.90 ± 0.84 bcd	5.01 ± 0.15 ab	13.40 ± 0.44 abc	8.17 ± 0.11 a	27.41 ± 0.58 ab
BP7	57.33 ± 0.46 cd	10.66 ± 0.13 d	5.57 ± 0.06 bc	15.14 ± 0.17 cde	10.29 ± 0.10 bcd	32.00 ± 0.32 cd
BP8	59.51 ± 0.88 de	10.42 ± 0.18 cd	5.32 ± 0.26 abc	14.55 ± 0.37 cd	9.24 ± 0.05 abc	30.07 ± 0.70 bc
O1	65.21 ± 0.64 f	8.52 ± 0.03 ab	5.32 ± 0.11 ab	12.01 ± 0.14 a	8.42 ± 0.40 a	26.26 ± 0.67 a
O2	57.67 ± 0.05 cd	10.59 ± 0.14 d	5.16 ± 0.09 abc	15.49 ± 0.40 de	10.15 ± 0.25 bcd	31.73 ± 0.19 cd
C1	57.83 ± 0.66 cd	10.28 ± 0.02 cd	5.45 ± 0.07 abc	14.70 ± 0.25 cd	10.73 ± 0.30 cd	31.90 ± 0.64 cd
C2	63.34 ± 0.48 ef	8.81 ± 0.13 abc	5.05 ± 0.03 abc	12.64 ± 0.15 ab	9.26 ± 0.15 abc	27.85 ± 0.35 ab
O3	40.54 ± 0.14 a	17.65 ± 0.82 f	4.90 ± 0.12 a	20.74 ± 0.39 f	15.15 ± 0.95 e	41.81 ± 0.67 e
O4	50.58 ± 0.56 b	14.42 ± 0.09 e	5.48 ± 0.03 abc	16.57 ± 0.05 e	10.96 ± 0.46 d	35.01 ± 0.47 d

[a]Different letters indicate significant differences for the carbohydrate group (Bonferroni test; $p < 0.05$). [b]3′- and 4′-galactosyl-lactose are included. [c]These values include 6-galactobiose, 6′-galactosyl-lactose, and other GOS.

agreement with the range described for other microbial β-galactosidases.[28] Moreover, in a previous work, total GOS yields of approximately 30 g/100 g of total carbohydrates were obtained with the commercial *K. lactis* preparation,[23] which is in agreement with the total GOS yields found with most of the cheese-isolated yeast strains evaluated in the present work. Recently, a maximum GOS yield of 44 g/100 g of total carbohydrates using 400 g/L of lactose and permeabilized *K. lactis* cells has been reported.[24]

K. marxianus O3 CCE stood out as the best GOS producer (42 g/100 g of total carbohydrates from 250 g/L of lactose), but also *K. marxianus* O4 and *K. lactis* CECT 1961^T, BP7, O2, and C1 CCEs were good GOS producers, as indicated by the Bonferroni test. Remarkably, the yield obtained with *K. marxianus* O3 β-galactosidase was more than 2-fold higher than that described using permeabilized cells of *K. marxianus* at an initial lactose concentration of 500 g/L.[26] Some *K. marxianus* strains were also pointed out by Petrova and Kujumdzieva[27] as the most effective strains in GOS production among yeast species isolated from dairy products.

For all reactions, the main GOS product was the trisaccharide 6′-galactosyl-lactose (12−21 g/100 g of total carbohydrates yield), with *K. marxianus* O3 and O4 and *K. lactis* CECT 1961^T CCEs being the best producers. Maximum 6-galactobiose yields corresponded to *K. lactis* CECT 1961^T and BP7 CCEs, whereas the lowest levels were formed by *K. marxianus* O3 CCE. Both oligosaccharides were also the main GOS described for *Aspergillus aculeatus* β-galactosidase[31] and *K. lactis* commercial enzyme.[23] With respect to other GOS (including 4′- and 3′-galactosyl-lactose), yields ranged from 8 to 15 g/100 g of total carbohydrates, with *K. marxianus* O3 CCE being the best producer. Although *K. marxianus* β-galactosidases with enhanced transgalactosylation activity have been recently described,[26,27] individual oligosaccharides formed were not identified.

The time course (up to 24 h) of lactose hydrolysis and GOS production was evaluated for β-galactosidase extracts from the best GOS producers, *K. marxianus* O3 and O4 and *K. lactis* BP7 and CECT 1961^T (Figure 2). Lactose was rapidly hydrolyzed (Figure 2A) to monosaccharides (glucose and galactose) (Figure 2B), in which levels increased along the reaction time. *K. marxianus* O3 β-galactosidase hydrolyzed lactose to a lesser extent (90 g/100 g of total carbohydrates) than the rest of CCEs, which almost completely hydrolyzed lactose (around 3 g/100 g of total carbohydrates remaining). As a general trend, the production of 6′-galactosyl-lactose (Figure 2D) reached a maximum value (15−20 g/100 g of total carbohydrates) after 2 h of reaction, with the level of remaining lactose being 20−28 g/100 g of total carbohydrates. In contrast, the formation of 6-galactobiose (Figure 2C) reached maximum yields after 4 h (with the remaining lactose of 10−18 g/100 g of total carbohydrates), except for *K. marxianus* O3 β-galactosidase, which reached its optimal time production after 24 h (with the remaining lactose around 11 g/100 g of total carbohydrates). The maximum production of other GOS (Figure 2E) was reached after 2 h (*K. lactis* BP7 CCE) or 4 h (*K. lactis* CECT 1961^T and *K. marxianus* O3 and O4 CCEs) of reaction. The same pattern was observed for the maximum formation of total GOS (Figure 2F). Total GOS production decreased slightly from 4 to 24 h of incubation for *K. marxianus* O3 CCE, whereas for the rest of β-galactosidases tested, a higher GOS hydrolysis along the incubation time was observed. After 24 h of incubation, *K. marxianus* O3 CCE stood out as the best GOS producer (40 g/100 g of total carbohydrates).

Synthesis of Oligosaccharides Derived from Lactulose (OsLu). Once the ability of CCEs to produce GOS by lactose transgalactosylation was tested, the time course (up to 24 h) of lactulose transgalactosylation by β-galactosidases from CECT 1961^T, BP7, O3, and O4 was also evaluated. In a previous study of our research group, transgalactosylation of lactulose with β-galactosidases from commercial *K. lactis* and *A. aculeatus* preparations was studied and new structures, such as the trisaccharides 6′galactosyl-lactulose and 1-galactosyl-lactulose, were characterized.[11,12] In the present work under the experimental conditions used (pH 6.5, 50 °C, 250 g/L of lactulose, and 6 units/mL of β-galactosidase activity),[12] the HPAEC−PAD analysis of reaction mixtures showed that all CCEs produced galactosyl derivatives from lactulose. Similar to GOS formation, products obtained from lactulose were the same in all reactions. Figure 1B shows a representative recording of the HPAEC−PAD profiles of the products formed by *K. marxianus* O3 CCE after 24 h. Peaks 1, 3, 7, and 8 were assigned to galactose, fructose, allolactulose, and lactulose, respectively. Peak 4 was identified as the disaccharide 6-galactobiose,

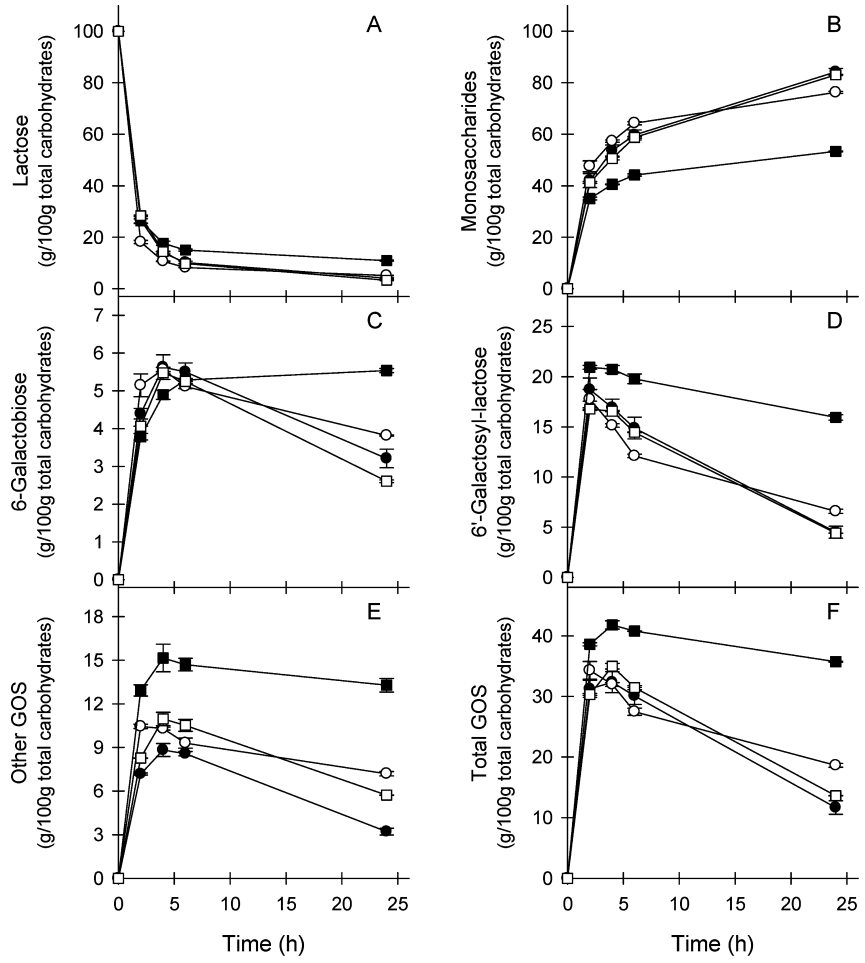

Figure 2. Carbohydrate yields during lactose hydrolysis by selected yeast β- galactosidases: (●) *K. lactis* CECT 1961^T, (○) *K. lactis* BP7, (■) *K. marxianus* O3, and (□) *K. marxianus* O4. Error bars indicate standard deviations.

while peaks 10 and 11 were assigned to the trisaccharides 6′-galactosyl-lactulose and 1-galactosyl-lactulose, respectively.[11] Two unidentified di- or trisaccharides (retention times of 12.5 and 14.8 min) and other high-retention-time oligosaccharides (peaks marked with an asterisk) were also detected.

Figure 3 shows the time course of lactulose conversion and OsLu synthesis for the yeast β-galactosidases. Lactulose (Figure 3A) was hydrolyzed to galactose (Figure 3B) and fructose (Figure 3C). A comparison of Figures 2 and 3 demonstrates that lactulose hydrolysis was slower than that of lactose. *K. lactis* CECT 1961^T β-galactosidase hydrolyzed lactulose to a lesser extent (60% hydrolysis) than the rest of CCEs (80−90% hydrolysis). The amount of galactose present in the reaction mixtures was lower than that of fructose in all analyzed samples. As a general trend, trisaccharide formation (6′-galactosyl-lactulose and 1-galactosyl-lactulose) predominated over the formation of the disaccharide 6-galactobiose. Synthesis of 6′-galactosyl-lactulose (Figure 3E) and 6-galactobiose (Figure 3D) increased with time, reaching a maximum after 24 h of reaction. The production of 1-galactosyl-lactulose (Figure 3F) increased gradually and attained a maximum value after 2 h of reaction in the case of *K. marxianus* O3 CCE (with the remaining value of lactulose being 52 g/100 g of total carbohydrates) and after 6 h of reaction (with the remaining value of lactulose being 47 g/100 g of total carbohydrates) for *K. marxianus* O4 β-galactosidase. In contrast, the formation of this trisaccharide by *K. lactis* CECT 1961^T and BP7 β-galactosidases increased through the reaction time. The formation of other OsLu (including non-identified di- and trisaccharides and high-retention-time oligosaccharides) followed a similar trend to that of the disaccharide, with maximum yields after 24 h of reaction, and dependent upon the β-galactosidase extract used, OsLu mixtures with different compositions can be achieved. As in the case of lactose, the main OsLu were trisaccharides. The maximum production of 6′-galactosyl-lactulose (13 g/100 g of total carbohydrates) at 24 h as well as 1-galactosyl-lactulose (17 g/100 g of total carbohydrates) at 2 h was observed for *K. marxianus* O3 CCE. Maximum disaccharide levels (around 5 g/100 g of total carbohydrates) were produced by *K. marxianus* CCEs. As in the case of GOS, the best OsLu producer was *K. marxianus* O3 β-galactosidase, which yielded 45% total OsLu based on an amount of lactulose consumed of 87.5 g/100 g of total carbohydrates.

Several studies have demonstrated that glycosidic linkages and molecular weights of carbohydrates contribute toward the selectivity of fermentation by beneficial gut bacteria. Our study demonstrates that the main oligosaccharides produced from lactose or lactulose transgalactosylation using enzyme extracts from cheese-isolated yeasts are trisaccharides, which have been reported to show the highest selectivity toward bifidobacteria.[32,33] Moreover, GOS with $\beta 1 \rightarrow 6$ linkages, as those described in this work, can be easily cleaved by β-galactosidases from bifidobacteria[34,35] and, thus, exhibit prebiotic character.

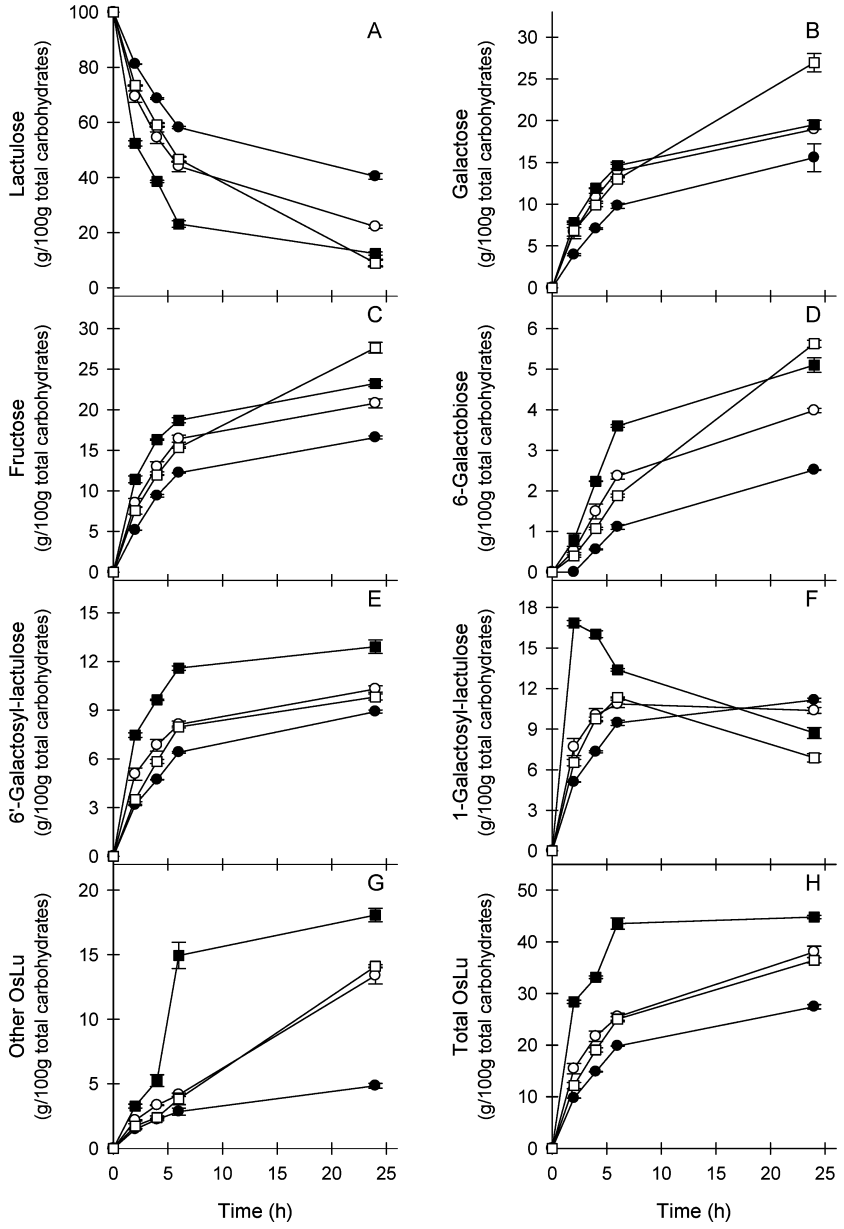

Figure 3. Carbohydrate yields during lactulose hydrolysis by selected yeast β-galactosidases: (●) *K. lactis* CECT 1961^T, (○) *K. lactis* BP7, (■) *K. marxianus* O3, and (□) *K. marxianus* O4. Error bars indicate standard deviations.

Cardelle Cobas et al.[36] reported that the *in vitro* growth of different *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* strains was enhanced by β-galactosyl residues $\beta1 \rightarrow 6$ and $\beta1 \rightarrow 1$ linked over those with $\beta1 \rightarrow 4$ linkages. Likewise, Cardelle-Cobas et al.[37] demonstrated the *in vitro* fermentation of OsLu by mixed fecal microbiota and proposed them as a new generation of prebiotics for improving the composition of gut microbiota.

The present study shows the feasibility of β-galactosidases from *K. lactis* and *K. marxianus* strains isolated from cheese to transgalactosylate lactose and lactulose and produce reaction mixtures with different levels of individual oligosaccharides. To the best of our knowledge, this is the first time that *K. marxianus* β-galactosidases are tested for lactulose transgalactosylation. Furthermore, *K. marxianus* O3 enzyme yielded the highest total oligosaccharide amount when lactose or lactulose was used as an acceptor carbohydrate. Moreover, *K. marxianus* is considered as a thermophilic microorganism, which suggests the possibility of developing transgalactosyl reactions at higher temperatures than 50 °C, with the benefit of using higher substrate concentrations for oligosaccharide yield improvement.

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Funding

This work was supported by Grants Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063, INIA RM2007-00010, and the

R + D program of the Comunidad de Madrid, Project ALIBIRD 2009/AGR-1469. Beatriz Padilla and Ana I. Ruiz-Matute thank their JAE Predoc and JAE Doc contracts from CSIC, respectively.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to the cheese company "Los Corrales" for kindly supplying cheese samples.

■ ABBREVIATIONS USED

CCE, crude cellular extract; CECT, Spanish Type Culture Collection; GOS, oligosaccharides derived from lactose; GPY, glucose, peptone, and yeast extract; GRAS, generally recognized as safe; HPAEC−PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; LPY, lactose, peptone, and yeast extract; OsLu, oligosaccharides derived from lactulose; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl β-D-galactopyranoside

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