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Original article

Oligosaccharide production and proteolysis during lactose hydrolysis using crude cellular extracts from lactic acid bacteria

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Abstract – The extent of the lactose hydrolysis and oligosaccharide formation through transgalactosyl reactions by crude cellular extracts (CCE) containing intracellular β-galactosidase from disrupted *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842, *Lactobacillus delbrueckii* subsp. *lactis* DMF 3078 or *Streptococcus thermophilus* 143 was investigated in lactose-containing buffered solutions or skim milk. Lactose hydrolysis was performed with lactose concentration ranging from 5 to 30% (w/w) at 30, 40, 50 or 60 °C and terminated after 120 min. The proteolytic activities of the CCEs in skim milk systems were also assessed. All CCEs produced di-, tri- and tetrasaccharides in addition to monosaccharides as major products of the lactose hydrolysis in buffered lactose solutions. However, only *Lb. lactis* produced tetrasaccharides at detectable levels in skim milk preparations. The amount and the rate of oligosaccharide formation were significantly ($P < 0.05$) affected by the origin of the enzyme, lactose concentration and temperature. The maximum oligosaccharide production by all CCEs was reached at 50 °C in 30% (w/w) lactose solution. *St. thermophilus* CCE produced significantly higher ($P < 0.05$) amounts of oligosaccharides than the other two CCE preparations at all lactose concentrations studied. However, *Lb. bulgaricus* CCE showed better lactose-hydrolyzing ability and higher proteolytic activity than the other two CCEs. The lactose hydrolysis generally proceeded faster at 50–60 °C than at 30–40 °C as opposed to the proteolytic maxima reached at 40 °C.

Crude cellular extract / β-galactosidase / thermophilic dairy culture / lactose hydrolysis / transferase reaction / proteolytic activity

Résumé – Production d'oligosaccharides et protéolyse lors de l'hydrolyse de lactose par extraits cellulaires bruts de bactéries lactiques. Le degré d'hydrolyse du lactose et la formation d'oligosaccharides par réaction de transgalactosylation sous l'action d'extraits cellulaires bruts (ECB) contenant de la β-galactosidase obtenue par rupture de *Lactobacillus delbruekii* subsp. *bulgaricus* 11842, *Lactobacillus delbruekii* subsp. *lactis* DMF 3078 et *Steptococcus thermophilus* 143 ont été étudiés sur des solutions tamponnées de lactose ou sur le lait écrémé. L'hydrolyse du lactose a été effectuée sur des concentrations de 5 à 30 % (masse) à des températures de 30, 40, 50 et 60 °C pour une durée totale de 120 min. L'activité protéolytique des ECBs sur les systèmes à base de lait écrémé a également été évaluée. Tous les ECBs ont produit des di-, tri- et tetrasaccharides en plus des monosaccharides comme composants majeurs de l'hydrolyse du lactose contenu dans les solutions tamponnées. Toutefois, seul *Lb. lactis* a produit des tetrasaccharides à un niveau

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significatif avec le lait écrémé. La quantité et la vitesse de formation d'oligosaccharides ont été affectées de manière significative ($P < 0.05$) par la provenance de l'enzyme, la concentration en lactose et la température. La production maximale d'oligosaccharides, pour tous les ECBs a été obtenue à 50 °C et une concentration en lactose de 30 % (masse). Les ECBs provenant de *St. thermophilus* ont produit de façon significative ($P < 0.05$) plus d'oligosaccharides que les deux autres préparations, ceci pour toute concentration en lactose. Toutefois, *Lb. bulgaricus* ECB a montré une plus grande capacité d'hydrolyse et une activité protéolytique supérieure aux deux autres ECBs. L'hydrolyse du lactose s'effectue généralement plus rapidement à 50–60 °C qu'à 30–40 °C alors que l'activité protéolytique atteint son maximum à 40 °C.

Extrait cellulaire brut / β-galactosidase / bactérie thermophile / hydrolyse du lactose / reaction de transférase / activité protéolytique

1. INTRODUCTION

The lactose-hydrolyzing ability of crude cellular extracts (CCE) containing β-galactosidase (EC 3.2.1.23, β-gal) from mechanically disrupted *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 (Lb11842) has been explored extensively [14–16, 25, 33]. However, little attention has been paid to date to the catalytic ability of the CCE regarding the transferase reactions, another important property of bacterial β-gal. The proteolytic activity of the Lb11842 CCE preparation has been described in a preliminary way [33]. Such activity, if not controlled properly, could interfere with lactose hydrolysis due to β-gal digestion [7]. Alternatively, for some uses of the CCE, the proteolytic reactions may be desirable and thus these should be well characterized.

The transferase reactions include the internal rearrangement of the lactose molecule with the formation of different disaccharides, as well as transgalactosyl reactions, resulting in creation of tri- and higher oligosaccharides [21]. The transferase products are hydrolyzed very slowly by the human β-galactosidase in the small intestine, which may result in gastrointestinal discomfort and lactose intolerance-like symptoms [18]. More recently, several studies showed the positive effect of oligosaccharide addition on the microbial microflora, influencing the increase in fecal bifidobacteria and modifying the colonic fermentation metabolism in the gut of healthy humans [3], or reducing the colon cancer risk in carcinogen-treated rats [5].

The transferase reactions have been described for highly purified β-gal preparations obtained from a number of microbial sources [6, 11, 20, 27, 29, 31]. Using β-gal in the batch mode was suggested for maximization of oligosaccharide production [22]. No attempt has been made so far to study the oligosaccharide-producing capability of the β-gal-containing CCE, although the work of Kreft et al. [16] offered some indications that oligosaccharides were indeed produced during lactose hydrolysis by Lb11842 CCE in buffered lactose solutions. The oligosaccharides could also be produced by glycosyltransferases, involved in the biosynthesis of exopolysaccharides by numerous LAB strains [2], that would be found in the CCE after cell disruption.

Thermophilic LAB, especially mixed yogurt cultures, possess appreciable proteolytic activity [26]. The cell-enveloped proteases and different intracellular peptidases result in an efficient breakdown of casein, major milk protein, into different amino acids and peptides required for cell growth [17, 28]. Several studies reported pH and temperature dependence of the protease activity of several thermophilic LAB species [1, 4]. The high proteolytic activity of a β-gal preparation could be undesirable during lactose hydrolysis in milk since it may result in bitterness [19] as well as the digestion of the β-gal enzyme. The proteolytic activity in CCE preparations would be expected, since CCE are mixtures of liberated intracellular enzymes as well as cell debris. The minimization or maximization

of the proteolytic activity by controlling the environmental conditions (temperature, time and substrate concentration) during lactose hydrolysis may therefore be required, depending on a targeted use of the β-gal-containing CCE.

The main objectives of our study were to (1) compare the lactose-hydrolyzing ability of the β-gal-containing CCEs prepared using three alternative thermophilic LAB sources in buffered lactose and skim milk systems at different temperatures; (2) characterize the transferase reactions during lactose hydrolysis in buffered lactose and skim milk systems at different temperatures of the three β-gal-containing CCE preparations; and (3) describe the proteolytic activity of the three CCE preparations during lactose hydrolysis in skim milk. The well-characterized *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 was included in these studies for comparison with *Lactobacillus delbrueckii* subsp. *lactis* DMF 3078 and *Streptococcus thermophilus* 143, two other organisms with known high β-gal-producing capability.

2. MATERIALS AND METHODS

2.1. Culture cultivation and cell collection

Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842 (Lb11842) was obtained from the University of Alberta, Department of Agricultural, Food and Nutritional Science. *Lactobacillus delbrueckii* ssp. *lactis* DMF 3078 (Lb3078) and *Streptococcus thermophilus* 143 (St143) were provided by the Department of Milk and Fat Technology, Institute of Chemical Technology, VSCHT, Prague, Czech Republic. The cultures were cultivated in pasteurized commercial skim milk (Lucerne milk processing plant, Edmonton, AB, Canada) as described by Geciova et al. [8]. Fermentations were conducted for 10 h in a 50 L fermentor at 43 °C and pH was maintained by 10 mol·L^{-1} KOH at 5.6 for *Lactobacillus* sp. and 5.8 for *Streptococcus*. After the fermentations, the cell collection and handling were performed as reported previously [34]. Before the production of the CCE, the cell paste samples from each fermentation were randomly selected for the determination of the total solids and the β-gal activity by oven drying and the ONPG (o-nitrophenyl-β-D-galactopyranoside) method, respectively [32]. A unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze 1 μmol of ONPG min^{-1} at 37 °C under conditions described previously [33].

2.2. Preparation of crude cellular extracts and reaction mixtures

The crude β-galactosidase preparations were obtained in a similar way to that described before [34]. Prior to cell disruption for the liberation of the intracellular β-gal, the frozen cell paste (14–16% dry matter) was thawed at 37 °C in a water bath and reconstituted either in pH 6.8 skim milk salt buffer – SMSB [24] or a 10% (w/v) solution of low heat skim milk powder (Dairyworld foods, Vancouver, BC, Canada) resulting in preparations containing approximately 10% cell paste total solids in both cases. The liberation of intracellular β-gal was achieved using a one-pass treatment with two ceramic disrupting chambers of a microfluidizer (Model M-110EH, Microfluidics, Newton, MA, USA) as described by Geciova et al. [8]. These treated preparations, denoted as crude cellular extracts (CCE) in further text, were used without further purification.

2.3. Lactose hydrolysis and quantification of reaction products

The reaction systems for lactose hydrolysis were prepared either by dissolving appropriate amounts of lactose monohydrate (Fisher Scientific Limited, Nepean, ON, Canada) in SMSB to give 5, 12.5, 20 or 30% (w/w) final lactose concentration, or by reconstituting the low temperature

skim milk powder (Dairyworld foods) in deionized water to prepare solutions giving 10, 20 or 30% (w/w) final total skim milk solids content upon recombining with the CCE preparations.

The CCE preparations were combined with the lactose or skim milk solutions, giving reaction mixtures containing approximately 10 $U \cdot mL^{-1}$ β-gal activity, which corresponded to the final cell paste total solids content ranging between 2 and 3% (w/v). The reaction mixtures were held at 30, 40, 50, or 60 °C for 120 min. Samples (1 mL) were taken after 30, 60 and 120 min and the reaction was terminated by alcohol precipitation [33]. The time frame selected was based on the report of Greenberg and Mahoney [10], indicating that maximum oligosaccharide production in milk was achieved within 2 h of the lactose hydrolysis process. This reaction period would probably minimize the likelihood of the oligosaccharide formation by other glycosyltransferases.

The formation of monosaccharides and oligosaccharides was quantified by high performance liquid chromatography (HPLC) equipped with Shimadzu Ezchrom Chromatography processing system (Shimadzu Precision Instruments, Torrance, CA, USA). The mono- and disaccharides were separated by a Supelcosil LC-NH_2-5 μm column (Supelco, Bellefonte, PA, USA; 25 cm length and 4.6 mm diameter) as previously reported [35]. An oligosaccharide column (Jordi Gel, DVB Polyamine column, 250 mm length and 4.6 mm diameter, Bellingham, MA, USA) was used to separate oligosaccharides. Elution was accomplished similarly to the determinations by the Supelcosil column, using a gradient of two mobile phase solvents: deionized water and acetonitrile (HPLC grade). The concentration of acetonitrile was decreased linearly over 25 min from 90 to 60%, increa-sed to 100% at 26 min, and then decreased to 90% at the end of the determination. The total run time was 30 min. The total flow rate was constant at 1 $mL \cdot min^{-1}$. In all cases, a 25 μL sample was injected by a Hewlett Packard Series 1050 autosampler (HP, Mississauga, ON, Canada). The peaks were identified and concentrations determined by using external standard solutions of glucose, galactose, lactose, maltotriose, maltotetraose, maltopentaose and maltohexaose and corresponding calibration curves. The degree of lactose conversion, expressed as a percentage of the initial lactose content of the sample, was calculated as previously reported [33] by dividing the total monosaccharide concentration ($mg \cdot mL^{-1}$) by the initial lactose concentration ($mg \cdot mL^{-1}$).

2.4. Total proteolytic activity

The proteolytic activities of the CCE preparations during lactose hydrolysis in skim milk reaction systems were assessed by HPLC peptide mapping of the hydrolyzed products. The conditions employed during the HPLC determinations were described previously [33]. The samples (1 mL) were taken in a similar way to the samples for the determination of the rate of lactose hydrolysis, diluted ten-fold in deionized water and filtered through a 0.22 μm Millipore filter. The HPLC determinations were performed using a Sephasil reverse phase peptide column (Amersham Biosciences, Piscataway, NJ, USA) and Shimadzu Ezchrom Chromatography processing system. The change of absorbance was observed by an ultraviolet/visible wavelength detector (Shimadzu SPD-10A) operating at 220 nm wavelength. Total peak area was obtained by integration of all the peaks observed. The relative proteolytic activity (RPA, %), expressing the relative increase in the total peptide content, was calculated from the following equation:

$$\text{RPA, \%} = \frac{TPA_t - TPA_0}{TPA_0} \times 100, \quad (1)$$

where TPA_t and TPA_0 are total peak areas at time t (30, 60 or 120 min) and at the beginning of the hydrolysis, respectively.

2.5. Kinetic parameters

In order to compare the efficiency of lactose hydrolysis by the three selected β-gal containing CCE preparations in buffered lactose and skim milk systems, the kinetic parameters, K_m and k_{cat}, were evaluated from the concentrations of the monosaccharides released during the first 30 min of the hydrolysis for all lactose concentrations and temperatures examined. The constants were inferred from the Michaelis-Menten equation using the Lineweaver-Burk method [32, 36].

2.6. Statistical analysis

All experiments were at least replicated and all subsequent analyses were carried out at least in duplicate resulting in n = 4 or more. Statistical analysis using all available data was performed by the General Linear Model of SAS (SAS Institute, 1992) as a full factorial, split-plot in time design. The model included all main effects (strain, lactose concentration, temperature and time) and corresponding interactions. Covariate analysis, using the enzyme activity or the cell paste total solids as a covariate, was employed if necessary. The statistical significance was preset at $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1. Monosaccharide formation

The lactose-hydrolyzing effectiveness of the CCEs from the three different LAB sources was compared using the determination of rate constants for lactose hydrolysis as K_m and k_{cat} values and by measuring the monosaccharide formation during the course of the lactose hydrolysis. Generally, the enzyme origin, temperature and medium in which the lactose hydrolysis was performed had a significant ($P < 0.01$) effect on both kinetic parameters (Tab. I). The covariate analysis showed no significant effect ($P = 0.4478$) of the initial β-gal activity in the CCEs on the kinetic parameters. Shah and Jelen [25] and Garman et al. [6] also reported substantial differences in the β-gal activity in several different LAB species. The K_m and k_{cat} values obtained for the Lb11842 CCE in the present study were broadly similar to those determined by a different methodology for the same CCE preparation during lactose hydrolysis in 10% skim milk [33]. The temperature change from 30 to 60 °C resulted in an increase of approximately 2.5 times in k_{cat} regardless of the strain and medium examined (Tab. I). The K_m values were also positively affected by the temperature increase with an apparent difference among the strains and reaction systems. Notably, the maximum k_{cat} and K_m values were obtained at 50 °C for all tested strains (Tab. I). The initial rate of lactose hydrolysis was generally higher in skim milk than in the buffered lactose solutions. Mozaffar et al. [20] reported opposite findings as they noted a substantial decrease in the initial velocity of the lactose hydrolysis by β-gal obtained from *E. coli* or *K. lactis* in milk in comparison with that in a buffered lactose solution. The product inhibition [12], disregarded in our calculations, as well as different enzymes and/or the proteolytic activity of the CCE preparations [33] may likely have important ramifications on the rate of the lactose hydrolysis. The temperature dependence of the catalytic constant k_{cat} followed the Arrhenius plot, resulting in significant differences ($P < 0.05$) for the three LAB species studied. The energy of activation (E_a) also differed significantly ($P < 0.05$) between the CCE preparations examined (Tab. I). The values obtained in the present study were lower than those determined previously [33], using a different methodology.

The considerable difference between the lactose-hydrolyzing capabilities of the three studied CCEs, described by the different rate constants, K_m and k_{cat}, significantly ($P < 0.01$) affected the extent of the monosaccharide formation in all examined preparations. Tables II and III show the final concentrations of glucose and galactose

Table I. The estimation of Michaelis-Menten-type kinetic parameters, K_m and k_{cat}, and the energy of activation, E_a, by Arrhenius plot for lactose hydrolysis in lactose and skim milk preparations at different temperatures using β-gal-containing CCE produced from three different thermophilic dairy cultures.

CCE*/ Temperature (°C)	Lactose			Skim milk		
	K_m (mmol·L^{-1})	k_{cat} (μmol·U^{-1}·min^{-1})	E_a (kJ·mol^{-1})	K_m (mmol·L^{-1})	k_{cat} (μmol·U^{-1}·min^{-1})	E_a (kJ·mol^{-1})
Lb11842						
30	201.2	257.5		97.4	203.9	
40	244.6	294.4		198.4	293.4	
50	320.9	654.9		127.5	469.5	
60	185.3	614.0	28.68	78.8	443.2	23.71
St143						
30	575.1	272.0		28.6	124.6	
40	861.3	404.1		303.4	237.8	
50	612.4	664.3		122.9	304.0	
60	567.1	659.6	26.67	96.5	328.4	26.75
Lb3078						
30	343.5	225.0		32.0	119.6	
40	407.0	249.3		241.0	202.6	
50	1672.5	550.4		133.9	294.9	
60	431.3	411.8	23.94	94.7	300.1	26.57
SEM**	37.39	15.31	0.78	11.45	8.42	0.74
R^{2***}	0.911	0.921	0.861	0.978	0.963	0.898

* CCE = β-galactosidase-containing crude cellular extracts from Lb11842: *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143: *St. thermophilus* 143, and Lb3078: *Lb. delbrueckii* ssp. *lactis* 3078; ** SEM = adjusted standard error of the mean; *** R^2 = coefficient of the determination; n = 12 or more.

present in lactose and skim milk solutions after the termination of the lactose hydrolysis trials. These data were also used for the calculation of the degree of lactose conversion as described above. The highest degree of lactose conversion into total constitutive monosaccharides (58.7 and 62.5 ± 0.65%, respectively) was achieved by Lb11842 CCE in 5% (w/w) lactose and 10% (w/w) skim milk preparations. Lactose conversion into glucose was similar for the three tested strains at low lactose concentration (50 mg·mL^{-1}) in skim milk, but this conversion substantially increased for Lb11842 CCE in comparison with the other two CCEs with the increase of the lactose concentration (Tab. III). The lactose concentrations and temperatures affected the conversion of lactose to galactose significantly ($P < 0.05$), while the enzyme origin had no apparent effect. The glucose to galactose ratio differed from the theoretical value of 1:1 and generally ranged from 1.44 to 2.32 ±0.10. The ratio obtained during lactose hydrolysis in skim milk was slightly but not significantly ($P > 0.05$) lower than in lactose preparations. Similar observations about the increased glucose/galactose ratio

Table II. The content of monosaccharides as identified by HPLC analysis after termination of lactose hydrolysis in buffered lactose solutions conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures.

	Monosaccharides (mg·mL^{-1})							
Lactose/ CCE*	Glucose				Galactose			
	30**	40	50	60	30	40	50	60
300***								
Lb11842	17.22	19.74	33.26	32.15	8	9.17	19.11	18.48
St143	15.05	15.96	24.58	20.33	7.12	7.54	14.11	10.81
Lb3078	12.59	14.73	22.35	20.08	7.33	6.68	11.25	13.08
200								
Lb11842	15.3	15.25	29.65	29.44	7.34	9.82	17.56	18.32
St143	9.99	14.09	25.61	21.72	4.37	6.48	11.05	13.82
Lb3078	8.78	12.7	17.92	17.86	5.45	5.65	10.94	12.61
125								
Lb11842	13.55	13.02	24.99	19.62	6.44	8.38	15.81	12.83
St143	10.99	13.09	23.33	15.5	6.4	6.18	11.56	10.95
Lb3078	10.13	15.32	14.59	12.8	5.54	2.63	9.77	8.54
50								
Lb11842	10.74	11.1	18.27	18.24	5.26	5.67	9.87	11.33
St143	7.5	8.2	11.99	10.56	3.52	3.98	6.17	7.32
Lb3078	7.05	7.48	8.77	9.13	3.78	4.05	4.26	5.48
SEM+	0.53				0.38			

* CCE = β-galactosidase-containing crude cellular extracts from Lb11842: *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143: *St. thermophilus* 143, and Lb3078: *Lb. delbrueckii* ssp. *lactis* 3078; ** temperature in °C; *** lactose concentration in mg·mL^{-1}; + SEM = adjusted standard error of the mean; n = 4 or more.

during the early stages of lactose hydrolysis observed by others [11, 27] were attributed to the formation of the transferase products containing mainly galactose.

3.2. Transferase reactions by CCE preparations

Lactose hydrolysis by all β-gal-containing CCEs in all reaction mixtures resulted in the formation of other carbohydrates in addition to the major hydrolysis products glucose and galactose. Typical chromatograms from the HPLC analysis of the lactose-hydrolyzed samples obtained using the Lb11842 CCE are shown in Figure 1; the use of the other two CCEs resulted in similar chromatograms (data not shown). The chromatogram "A", acquired using the Jordi oligosaccharide column, shows separation of all carbohydrates into size classes; however, separation within the classes was not achieved. A better separation for mono- and disaccharides was obtained using the Supelcosil column (chromatogram "B"), but the peak for tetrasaccharides did not appear and trisaccharides (peak 3) were partitioned poorly. The formation of tetrasaccharides in skim milk at all concentrations and temperatures was revealed only for Lb3078 CCE (Tab. V). The reason for this observation remained unclear; either oligosaccharides formed by Lb11842 or St143 CCE were below a detectable limit or different kinetic parameters at low lactose concentrations were responsible for a kinetic shift towards the formation of monosaccharides [11]. The peaks 2b and 2c appeared in all

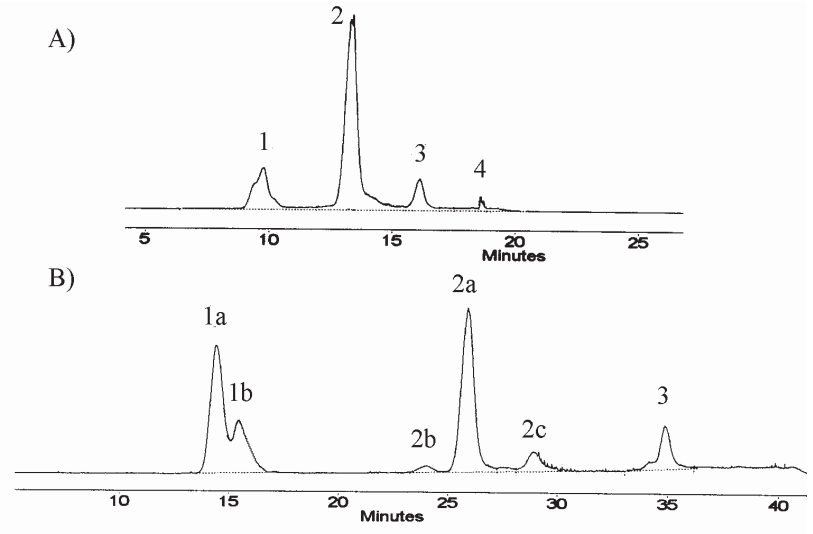

Figure 1. Typical HPLC chromatograms obtained when analyzing products of the lactose hydrolysis by the Lb11842 CCE preparation as acquired by (A) Jordi oligosaccharide or (B) Supelcosil carbohydrate columns (peaks identified as follows: 1 = monosaccharides; 2 = disaccharides; 3 = trisaccharides; 4 = tetrasaccharides; 1a = glucose; 1b = galactose; 2a = lactose; 2b and 2c = unidentified disaccharides).

Table III. The content of monosaccharides as identified by HPLC analysis after termination of lactose hydrolysis in skim milk preparations conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures.

	Monosaccharides (mg·mL^{-1})							
Lactose/ CCE*	Glucose				Galactose			
	30**	40	50	60	30	40	50	60
150***								
Lb11842	13.87	18.6	27.76	29.83	7.24	9.61	17.09	16.09
St143	7.86	12.23	14.27	23.35	3.69	6.29	6.65	11.76
Lb3078	7.41	11.27	13.5	14.54	3.64	5.37	7.21	8.2
100								
Lb11842	12.3	14.01	24.39	22.35	6.89	8.17	14.07	13.19
St143	7.23	10.56	20.17	19.88	3.82	5.72	10.31	10.33
Lb3078	6.97	10.39	18.33	15.78	3.75	5.48	10.89	9.58
50								
Lb11842	14.77	11.47	19.5	18.7	8.98	7.01	11.58	11.3
St143	13.83	8.33	15.83	16.8	7.85	4.8	8.4	9.97
Lb3078	11.85	7.89	13.48	14.97	6.82	4.68	8.62	9.51
SEM+	0.26				0.34			

* CCE = β-galactosidase-containing crude cellular extracts from Lb11842: *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143: *St. thermophilus* 143, and Lb3078: *Lb. delbrueckii* ssp. *lactis* 3078; ** temperature in °C; *** lactose concentration in mg·mL^{-1}; + SEM = adjusted standard error of the mean; n = 4 or more.

Table IV. The content of oligosaccharides formed during lactose hydrolysis in buffered lactose solutions as identified by HPLC analysis after termination of lactose hydrolysis reactions conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures.

	Sugars ($mg \cdot mL^{-1}$)											
Lactose/ CCE*	Total disaccharides**				Trisaccharides				Tetrasaccharides			
	30***	40	50	60	30	40	50	60	30	40	50	60
300+												
Lb11842	13.07	19.42	22.98	14.67	8.14	16.29	26.83	16.94	1.22	1.31	1.52	1.7
St143	13.37	20.05	25.36	20.52	9.21	18.46	32.77	25.45	1.32	1.36	1.95	1.79
Lb3078	9.61	13.15	15.91	13.55	6.04	9.61	20.42	12.34	0.73	0.73	0.91	1.16
200												
Lb11842	8.17	9.66	14.66	13.47	6.92	11.11	16.75	8.7	1.03	1.02	0.91	1.14
St143	9.59	12.70	15.96	14.72	7.59	11.96	15.83	12.44	1.32	1.06	0.96	1.16
Lb3078	7.38	8.59	12.26	12.90	2.65	7.88	7.59	6.66	0.67	0.67	0.79	0.92
125												
Lb11842	6.35	6.91	8.82	8.71	6.91	6.17	10.03	9.74	1.44	1.04	1.15	0.87
St143	7.09	7.16	10.08	9.75	3.79	7.19	9.13	6.28	1.52	1.04	1.26	0.87
Lb3078	4.95	5.03	7.93	7.73	2.3	5.75	5.96	3.48	0.57	0.57	0.61	0.77
50												
Lb11842	3.13	3.29	3.59	3.52	1.7	2.27	3.76	3.41	0.47	1.11	1.02	0.9
St143	3.33	4.21	4.79	4.08	1.47	2.46	3.05	2.7	0.6	1.19	1.27	0.86
Lb3078	2.97	2.72	3.98	3.21	1.59	1.75	2.33	2.29	0.37	0.37	0.54	0.68
SEM++	0.16				0.11				0.13			

* CCE = β-galactosidase-containing crude cellular extracts from Lb11842: *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143: *St. thermophilus* 143, and Lb3078: *Lb. delbrueckii* ssp. *lactis* 3078; ** other than lactose; *** temperature in °C; + lactose concentration in $mg \cdot mL^{-1}$; ++ SEM = adjusted standard error of the mean; n = 4 or more.

processed systems, with combined maxima up to 9.5 ±0.16% of the total sugars, and could likely represent disaccharides other than lactose. Huber et al. [11] and Greenberg and Mahoney [10] reported formation of allolactose (6-*O*-galactosyl-β-D-glucopyranose) and 6-*O*-galactosyl-β-D-galactopyranose as major products of the transferase reactions during lactose hydrolysis. The formation of pentasaccharides or hexasaccharides was not detected in any of the processed systems, although their formation by *St. thermophilus* β-gal was reported [30]. Also, our results were in contrast to the report of Greenberg and Mahoney [10], who detected only disaccharides and no higher oligosaccharides in the *St. thermophilus* β-gal lactose-hydrolyzed skim milk, possibly due to the different detection methodologies used. Figures 2A and 2B show the rate of the oligosaccharide production by the three tested CCEs, indicating that the St143 CCE had a greater oligosaccharide producing ability than the other two CCEs. Consequently, the HPLC-generated-chromatograms resulted in different peak heights, but the HPLC-patterns of the transferase product formation by all three examined CCE preparations were similar, indicating at least the size similarity of the formed oligosaccharides.

The formation of oligosaccharides was significantly ($P < 0.001$) affected by all examined variables (enzyme origin, lactose concentration, temperature and time). The highest oligosaccharide production was achieved at 50 °C in 30% (w/w) lactose solution for all CCEs examined. The St143

Table V. The content of oligosaccharides formed during lactose hydrolysis in skim milk preparations as identified by HPLC analysis after termination of lactose hydrolysis reactions conducted at different temperatures by β-gal-containing CCE preparations from three thermophilic dairy cultures.

	Sugars (mg·mL^{-1})											
Lactose/ CCE*	Total disaccharides**				Trisaccharides				Tetrasaccharides			
	30***	40	50	60	30	40	50	60	30	40	50	60
150+												
Lb11842	6.67	8.80	10.34	10.09	7.26	9.08	8.4	6.6		ND++		
St143	7.15	9.10	12.28	10.40	8	8.98	12.58	9.89		ND		
Lb3078	6.59	8.52	6.25	6.45	3.86	6.6	7.58	8	0.61	0.63	0.73	0.82
100												
Lb11842	3.92	5.59	7.11	5.90	4.51	5.27	5.07	4.82		ND		
St143	4.21	6.73	7.27	6.90	3.74	4.15	5.06	4.81		ND		
Lb3078	3.77	5.37	5.47	2.90	4.29	4.29	4.69	4.81	0.52	0.53	0.68	0.74
50												
Lb11842	2.17	3.61	3.88	3.06	1.14	1.09	1.15	1.38		ND		
St143	3.39	4.01	3.99	3.24	2.2	2.46	2.49	2.38		ND		
Lb3078	2.17	3.41	3.28	2.47	1.93	2.11	2.2	2.38	0.34	0.35	0.5	0.65
SEM+++	0.34				0.21				0.05			

*CCE = β-galactosidase-containing crude cellular extracts from Lb11842: *Lb. delbrueckii* ssp. *bulgaricus* 11842, St143: *St. thermophilus* 143, and Lb3078: *Lb. delbrueckii* ssp. *lactis* 3078; ** other than lactose; *** temperature in °C; + lactose concentration in mg·mL^{-1}; ++ ND = not detected; +++ SEM = adjusted standard error of the mean; n = 4 or more.

CCE was a significantly better ($P < 0.05$) total oligosaccharides producer – including newly formed disaccharides – than the other two CCEs originating from *Lactobacillus* sp. at all lactose concentrations and temperatures studied. The maximum oligosaccharide concentration, approx. 20% of the total carbohydrates (60.1 mg·mL^{-1}) produced by St143 CCE, was reached in 30% (w/w) lactose preparation at 50 °C after 120 min, in comparison with Lb11842 or Lb3078 CCE (51.3 or 37.2 mg·mL^{-1}, respectively), under the same conditions (Tab. IV). Toba et al. [31] and later Garman et al. [6] also reported the high transferase activity of the *St. thermophilus* β-gal.

The effect of the lactose concentration has been recognized previously [11, 13, 23] with the temperature effect appearing to be species-dependent [22]. In the present study, all three CCE preparations followed a similar pattern, achieving the oligosaccharide production maxima at the same temperature (Tabs. IV and V). The maximum oligosaccharide production (approx 20% and 16.5% of the total sugars in lactose and skim milk systems, respectively) achieved by St143 CCE in our study was lower than the values reported previously for *St. thermophilus* β-gal – up to 25% in milk [10] or 40% in a buffered lactose solution [25] – but comparable with those for *E. coli* and *K. lactis* β-gal in lactose solutions and skim milk [19]. Noticeably, oligosaccharide production was lower in skim milk than in lactose preparations even at the same (50 mg·mL^{-1}) lactose concentration (Figs. 2A and 2B; Tabs. IV and V). Although lower in the total amount, the maximum of the oligosaccharide production by the β-gal-containing CCEs was reached sooner (after 60 min) at low (50 mg·mL^{-1}) rather than high (300 mg·mL^{-1}) lactose concentrations and remained fairly constant until termination (Fig. 2A). Indicatively, the maximum of the oligosaccharide production was not reached for Lb11842 and

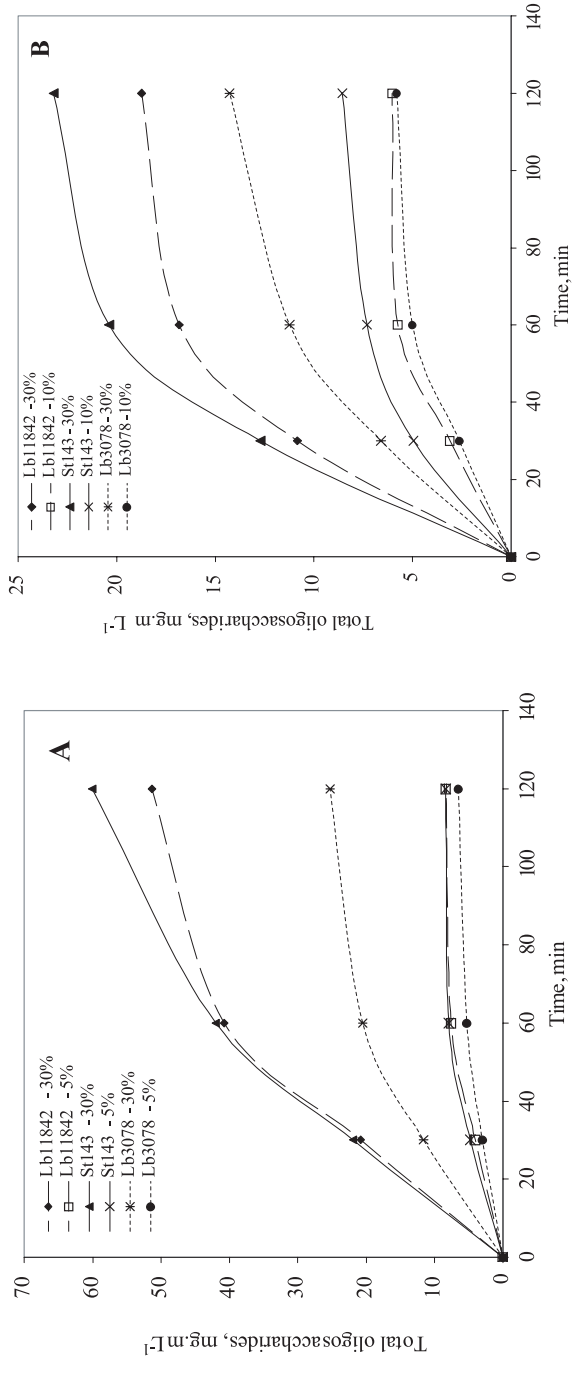

Figure 2. Oligosaccharide formation by the β-galactosidase-containing CEEs from three thermophilic dairy cultures during lactose hydrolysis in (**A**) 5 or 30% (w/w) buffered (pH 6.8) lactose solution and (**B**) 10 or 30% (w/w) skim milk total solids preparation at 50 °C (Lb11842 = *Lb. delbrueckii* ssp. *bulgaricus 11842*; St143 = *St. thermophilus* 143; Lb3078 = *Lb. delbrueckii* ssp. *lactis* 3078; note different scales in **A** and **B**; error bars showing adjusted standard error of the means are the size of the data marker points).

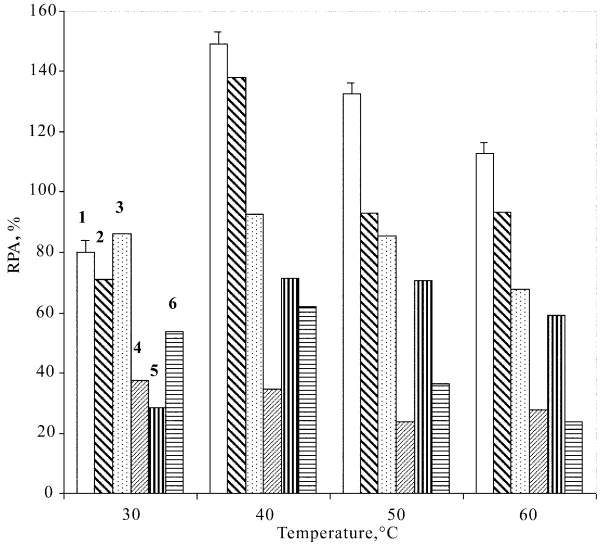

Figure 3. Relative proteolytic activity (RPA, %) of the β-gal-containing CEEs from three thermophilic dairy cultures during lactose hydrolysis in 10 or 30% (w/w) skim milk preparation performed at different temperatures (numbers on top of bars indicate CCE containing reaction systems as follows: 1 = Lb11842: *Lb. delbrueckii* ssp. *bulgaricus* 11842 – 10%; 2 = Lb11842: 30%; 3 = St143: *St. thermophilus* 143 – 10%; 4 = St143 – 30%; 5 = Lb3078: *Lb. delbrueckii* ssp. *lactis* 3078 – 10%; 6 = Lb3078 – 30%; same order used for all temperature sets; bars present adjusted standard error of the means; n = 4 or more).

St143 CCE, likely due to insufficient lactose hydrolysis. Prenosil et al. [22] correlated the oligosaccharide production with a high degree of lactose hydrolysis, achieving maxima when up to 80% of lactose was hydrolyzed. Based on their findings, a higher amount of oligosaccharides might have been produced in our study by prolonging the lactose hydrolysis reaction time.

3.3. Total proteolytic activity of CCE preparations

All three β-gal-containing CCE preparations exhibited appreciable proteolytic activity in skim milk systems. The proteolytic activity of the CCE preparations resulted in a steady increase in the total surface area under the HPLC peptide peaks over the time of the hydrolysis, reaching maxima at the 120 min reaction time endpoint. Figure 3 shows the maxima obtained for the proteolytic activities of the three CCEs in 10 or 30% skim milk reaction systems, after the termination of the hydrolysis. Generally, the values obtained for the 20% skim milk preparations were between the respective data pairs presented in Figure 3 (data not shown). Although covariate adjustment using the cell paste total solids was significant ($P = 0.0329$), the adjusted proteolytic activity still differed significantly ($P < 0.05$) between the CCE sources, with Lb11842 CCE being the most proteolytic. Such a high capability of the CCE preparations to hydrolyze milk proteins is obviously related to the absence of the purification step during the CCE production [32, 33]. On the other hand, the substantial activity differences follow from the origin of the CCE preparations. Shihata and Shah [26], comparing proteolytic activity of several LAB species, showed that *Lb. bulgaricus* and *St. thermophilus* strains were highly proteolytic in comparison with *Lb. acidophilus* and *Bifidobacterium* sp. strains.

The concentration of the skim milk total solids had a significant ($P < 0.05$) effect on the amount of peptides formed through the proteolysis, higher concentrations resulting in lower proteolytic activity (Fig. 3). Such an effect of the increased skim milk total solids might have resulted from the concomitant decrease in water activity, which could have had an inhibitory effect on the proteolytic activity of the studied CCE preparations [9]. The proteolytic activity

of all CCE preparations clearly showed temperature dependence, reaching maxima for all three CCE preparations at 40 °C, in concert with previously reported findings [33]. Similarly, Abraham et al. [1] and Fira et al. [4] reported enhanced proteolytic activity of *Lb. bulgaricus* around 40 °C as opposed to *Lb. acidophilus* strains with maximum at 50 °C [1]. The differences in temperature optima between the proteolysis and transferase reactions for all three CCEs used in our study give a possible tool for manipulating these activities depending on the desired end-use of the CCE.

4. CONCLUSIONS

All the β-gal-containing CCE preparations obtained from three thermophilic dairy cultures studied were able to catalyze the formation of oligosaccharides. The formation rate and the amount of oligosaccharides were origin-specific while the effects of lactose concentration and temperature were substantial in all cases. The highest oligosaccharide production was achieved by St143 at 30% lactose concentration, followed by Lb11842 and Lb3078 CCEs. The maximum oligosaccharide production (approximately 20% of the total sugars) was reached at 50 °C with 30% (w/w) lactose concentration using the St143 CCE. The amount of the oligosaccharides formed by all CCE preparations was substantially lower in skim milk in comparison with corresponding lactose solutions; no tetrasaccharides were detected in skim milk preparations when using Lb11842 or St3078 CCE. The lactose hydrolysis by all CCE preparations resulted in the formation of at least two different disaccharides, likely allolactose and 6-*O*-galactosyl-β-D-galactopyranose. The rate of production of the constitutive monosaccharides achieved by Lb11842 CCE was faster than for the other two enzyme preparations, subsequently resulting in higher k_{cat} values at corresponding temperatures. Similarly, Lb11842 CCE exhibited higher proteolytic activity than the other two studied CCEs. The maximum proteolytic activity was generally reached at 40 °C in 10% skim milk and decreased at higher concentrations of skim milk total solids (20 or 30% w/w). The characterization and simultaneous optimization of the lactose hydrolysis, oligosaccharide formation and proteolytic activity of different CCEs in various lactose-containing preparations may be important for some industrial applications, such as using the CCE preparations as a fermentation enhancer.

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