DOI:10.1021/jf9018596

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Facile Pretreatment of *Bacillus circulans* β-Galactosidase Increases the Yield of Galactosyl Oligosaccharides in Milk and Lactose Reaction Systems

Aaron Gosling,[†,‡] Johan Alftrén,[§] Geoff W. Stevens,[†] Andrew R. Barber,[∥] Sandra E. Kentish,[†] and Sally L. Gras*[,†,‡]

[†]The Department of Chemical and Biomolecular Engineering and [‡]The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Victoria 3010, Australia, [§]Food Technology, Chemical Centre, Lund University, Post Office Box 124, SE-221 00 Lund, Sweden, and [∥]Innovative Food and Plants Division, South Australian Research and Development Institute, Regency International Centre, Days Road, Regency Park, South Australia 5010, Australia

The commercially available preparation of β-galactosidase from *Bacillus circulans*, known as Biolacta FN5, has been extensively used in the production of prebiotic galactooligosaccharides (GOS). This study focuses on characterizing the production of GOS in two reaction systems: 10% lactose (w/v) in buffer and skim milk. Analysis of the temperature dependence of the GOS yield along with the relative rates of GOS synthesis and degradation leads to the finding that GOS degradation activity was selectively decreased in Biolacta FN5 above 40 °C. Facile heat treatment of Biolacta FN5 solution prior to use allowed for GOS yields to be significantly increased in both reaction systems.

KEYWORDS: Galactooligosaccharides; GOS; prebiotic; galactosyl transfer; β-galactosidase; lactose hydrolysis; *Bacillus circulans*

INTRODUCTION

Galactooligosaccharides (GOS) are nondigestible carbohydrates comprised of one glucose and one to nine galactose molecules linked by glycosidic bonds. GOSs are produced by the activity of the enzyme β-galactosidase (EC 3.2.1.23) on lactose (see ref *1* for a review). GOS are prebiotic, selectively stimulating the proliferation of beneficial colonic microflora, and impart physiological benefits to the consumer (*2*). These established health effects have resulted in a growing commercial interest in GOS as a functional food ingredient, particularly in Japan and Europe (*3*, *4*).

GOS can be produced *in situ* within a dairy product (such as whole or skim milk) by the direct addition of β-galactosidase. This treatment lowers the lactose concentration of the products, which can be beneficial to lactose intolerant consumers (*5*), and can be considered a processing consolidation step that combines both GOS synthesis and lactose reduction. Alternatively, GOS can be synthesized from pure lactose preparations or lactose-enriched solutions (such as whey), although the high costs of lactose purification associated with the former are a considerable disadvantage.

The reaction mechanism of β-galactosidase activity on lactose is well understood (*6*). It involves the galactosyl moiety of lactose covalently binding to the active site of the enzyme, while glucose is released, as shown in **Figure 1**. Many nucleophiles containing hydroxyl functionality can act as an acceptor for this galactosyl moiety. When the acceptor is water, the reaction is hydrolysis. When the acceptor is a carbohydrate, the reaction is galactosyl transfer and a GOS molecule is formed. GOS also serves as a substrate for both hydrolysis and as an acceptor for galactosyl transfer; therefore, complex mixtures result. The yield of a GOS reaction will be a function of both the rate of galactosyl transfer and the rate of hydrolysis.

Previous research on the β-galactosidase-catalyzed transformation of lactose has typically focused on model systems employing lactose buffer solutions at moderate or elevated temperatures, where enzyme activity is often high (*7*−*9*). While these model buffer systems allow insight into the fundamental characteristics of the system, these findings do not necessarily translate to more complex systems, such as the direct treatment of dairy products with β-galactosidase. Low-temperature treatment is also industrially relevant because these conditions minimize milk protein denaturation and vitamin degradation (*10*).

A small number of reports in the literature describe β-galactosidase treatment of milk systems at lower temperature (*11*−*13*), but these reports focus on the hydrolytic activity of β-galactosidase rather than the production of GOS. This may be due to the perception that GOS yield will potentially be reduced in milk at low temperatures relative to in a lactose buffer system. Indeed, three factors can affect the yield under these conditions: first, the GOS yield correlates with the lactose concentration (*1*), which is lower in milk compared to typical reported buffer systems [~5% (w/v) in milk cf. 15% (w/v) in buffer systems; see ref *14* for a tabulation of yields obtained at different lactose concentrations]; milk also contains substances such as calcium, which are

*To whom correspondence should be addressed. Telephone: +61-3-8344-6281. Fax: +61-8344-6281. E-mail: sgras@unimelb.edu.au.

 Published on Web 11/18/2009 © 2009 American Chemical Society

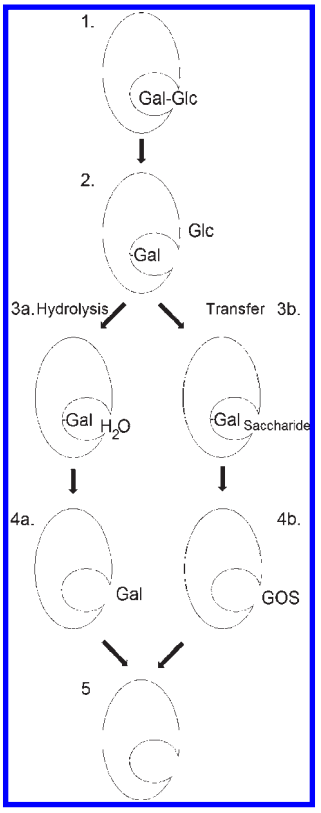

Figure 1. Reaction mechanism of β-galactosidase activity with lactose as the substrate. (1) Lactose enters the active site of β-galactosidase. (2) Galactose becomes covalently bound to β-galactosidase, while glucose diffuses away. (3a) Water enters the active site to act as the galactosyl acceptor as part of the hydrolysis pathway. (4a) Free galactose is released from the enzyme. (3b) A saccharide enters the active site to act as the galactosyl acceptor as part of the transfer pathway. (4b) A GOS molecule is released from the enzyme. (5) β-Galactosidase, cleared of substrate, is ready for another catalytic cycle.

inhibitory for many β-galactosidases (*15*); and finally, the GOS yield is reported to be higher at elevated temperatures (*9*, *16*, *17*).

The choice of the β-galactosidase employed can address some of these caveats, because GOS yield is highly enzyme-dependent (*1*). For example, the β-galactosidase preparation Biolacta FN5, derived from *Bacillus circulans*, gives high GOS yields (*18*). This enzyme preparation is also of interest for the industrial treatment of dairy-based products because it is commercially available at food grade and not inhibited by calcium (*19*). Biolacta FN5 consists of β-galactosidases I, II, and III (*20*) each with different properties: β-galactosidase I produces little GOS; β-galactosidase II produces much GOS (*21*); and β-galactosidase III produces $\beta1-3$ galactosides (*22*). It appears that β-galactosidase II contributes most significantly to GOS synthesis, because the Biolacta FN5 preparation is known to produce predominately $\beta1-4$ galactosides (*23*).

In this study, the feasibility of GOS production in milk at low temperatures is examined. A second aim is to characterize the activity of the Biolacta FN5 enzyme preparation to better understand the increase in GOS yield reported at high temperatures (*16*) and to further optimize the GOS yield for a range of processing conditions.

MATERIALS AND METHODS

Enzymes and Chemicals. Biolacta FN5 was kindly supplied by Vitachem, Sydney, Australia. A commercial pasteurized skim milk product (Skinny Milk, Parmalat Foods, Rowville, Australia) was used as a reaction medium. Supplier data indicated that the milk had the following composition: protein, 3.8% (w/v); fat, 0.1% (w/v); lactose, 5.4% (w/v); sodium, 0.063% (w/v); and calcium, 0.138% (w/v). The pH of the milk was 6.7. Lactose, D-glucose (Chem Supply, Gillman, Australia), and D-galactose (Sigma-Aldrich, Sydney, Australia) standards were analytical-grade, as well as the organic solvents (Merck, Kilsyth, Australia). Milli Q water (resistivity $< 18.2\ \Omega$) was used.

***β*-Galactosidase Reactions.** Reactions were performed in two media: milk and a 10% (w/v) lactose solution in 100 mM sodium acetate buffer at pH 6.6. Rather than use the same lactose concentration as the milk reaction system (5.4%, w/v), 10% (w/v) lactose was selected because this gave GOS yields that more closely matched those from the milk system. Reaction solutions were pre-equilibrated to the required temperature ($\pm$0.1 °C) in a circulating water bath.

A unit of *para*-nitrophenol galactoside (pNPG) activity was defined as previously described (*22*). A total of 0.1 pNPG unit of Biolacta FN5 enzyme preparation was added for every 1 g of lactose to initiate the reaction.

A solvent extraction procedure based on a published method (*24*) was used to prepare reaction samples for analysis. This extraction served the dual purposes of stopping the reaction and removing fat and protein, which could interfere with high-performance liquid chromatography (HPLC) analysis. In this procedure, 150 μL samples withdrawn from reactions at measured time intervals were added to 600 μL of methanol. A total of 150 μL of chloroform was then added to the sample, followed by 450 μL of Milli Q water. After vigorous mixing, the samples were centrifuged at 13000*g* for 3 minutes. The aqueous phase was then removed for HPLC analysis.

The Biolacta FN5 preparation was heat-treated in selected experiments by incubating a 0.45 pNPG unit/mL enzyme solution at 60 ($\pm$1) °C for 20 minutes in a water bath. The enzyme was then cooled in an ice bath before use for kinetic experiments.

Quantification of Saccharides and Data Analysis. HPLC analysis of carbohydrates was performed using a Shimadzu Prominence system equipped with a RID-10A refractive index detector and a 300 $\times$ 7.8 mm Rezex RCM-Monosaccharide Ca^{2+} column (Phenomenex). The mobile phase was Milli Q water, delivered at a flow rate of 0.5 mL/minute. The column and detector cell were maintained at 80 °C and 40 °C, respectively.

Galactose, glucose, and lactose were quantified against external standards, also prepared by the solvent extraction procedure above.

The concentration of total GOS was calculated by mass balance (e.g., the difference between the initial concentration of lactose and the concentration of lactose, galactose, and glucose measured at each reaction time point). The maximum GOS yield was defined as the highest concentration of GOS achieved during the reaction per unit concentration of initial lactose. The initial rates of glucose or galactose formation were also found by the linear regression function of Microsoft Excel using a minimum of three data points from the very start of the reaction.

Statistical significance was calculated using the paired *t*-test function within Microsoft Excel, with a significance of $p < 0.05$.

RESULTS AND DISCUSSION

Activity of Biolacta FN5 Preparation. The Biolacta FN5 preparation was first characterized using lactose as a substrate, as shown in **Figure 2**. The β-galactosidase reaction kinetics show two distinct phases: the initial stage (0$-$3 h for a reaction at 40 °C) and the remainder of the reaction. GOS accumulates in the initial stage, indicating that the rate of galactosyl transfer is higher than the rate of GOS hydrolysis. As expected, the lactose substrate concentration decreases rapidly, generating glucose at an approximately linear rate and a smaller quantity of galactose, consistent with galactose incorporation in GOS. In the second stage of the reaction, the concentration of GOS begins to decline as the rate of GOS hydrolysis becomes greater than the rate of galactosyl transfer.

Increasing the GOS Yield with Increased Temperature. The Biolacta FN5 β-galactosidase preparation was successfully used to produce GOS in milk as well as a buffered lactose solution for a range of temperatures from 4 °C to 60 °C, as shown in **Figure 3**.

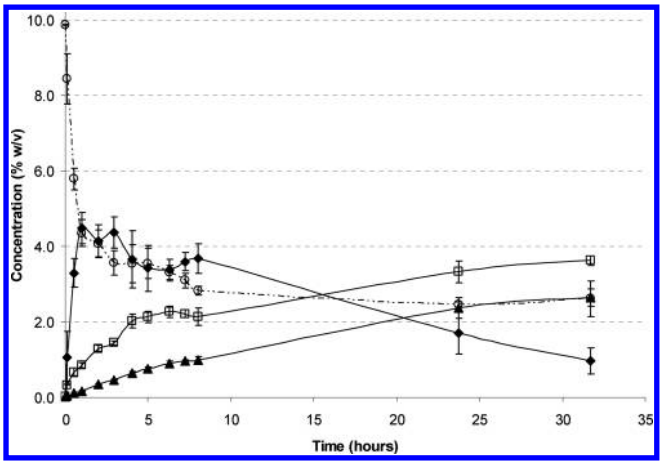

Figure 2. Typical time course of β-galactosidase-catalyzed reaction in a 10% (w/v) lactose solution in buffer at 40 °C. Lactose (○), glucose (□), galactose (▲), and GOS (◆). The error bars indicate an interval of two standard deviations of the mean of triplicate measurements. Lines are for guidance only.

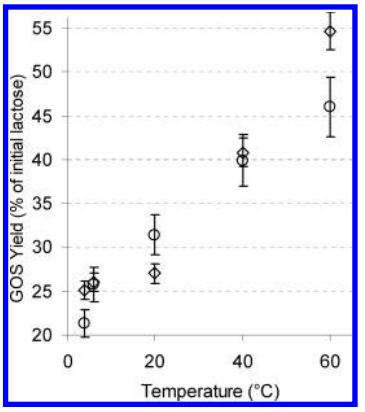

Figure 3. GOS yield as function of the temperature in 10% (w/v) lactose in buffer (◇) and milk (○). The error bars depict 0.4% (w/v) GOS, the typical standard deviation of GOS measurements. The maximum concentrations of GOS were observed at a lactose conversion (%) of 53, 45, 53, 59, and 52 in milk and 44, 47, 50, 66, and 69 in lactose in buffer at 4 °C, 6 °C, 20 °C, 40 °C, and 60 °C, respectively.

A reasonable yield of between 20% and 30% was achieved for milk and a buffered lactose solution at temperatures less than 10 °C. These GOS yields are equivalent or better than those of 25% or 14% obtained from other industrially produced β-galactosidases from *Streptococcus thermophilus* (*25*) and *Kluyveromyces lactis* (*19*) in milk at 37 °C and 25 °C, respectively, and they illustrate the feasibility of β-galactosidase treatment at low temperatures.

The GOS yield obtained from the Biolacta FN5 β-galactosidase increased with temperature. For the temperatures examined, the highest yield was achieved at 60 °C for both the milk and buffered lactose solution. This finding is consistent with the reported temperature dependence of the maximum GOS yield for other β-galactosidases (*9*, *16*, *17*).

Relationship of the GOS Yield to Hydrolytic and Transfer Activity. The initial rate of glucose and galactose formation was examined as a function of the temperature, because these rates and the reaction mechanism (**Figure 1**) can together provide further insight into the enzymatic activity of Biolacta FN5. The change in the galactose concentration over time V^{app}_{Gal} estimates the total rate of hydrolytic activity on all species present, because free galactose is generated by the hydrolysis of a glycosidic bond, regardless of whether the substrate is lactose or GOS. In contrast,

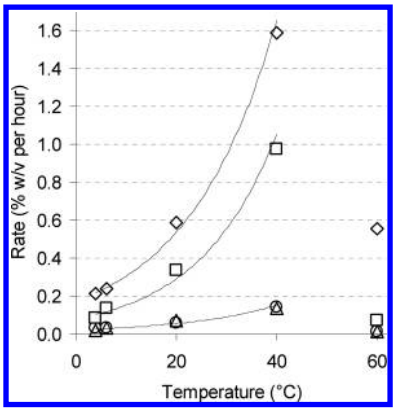

Figure 4. Galactosyl transfer $V^{app}_{Glc-Gal}$ and hydrolysis V^{app}_{Gal} rates as a function of the temperature. $V^{app}_{Glc-Gal}$ in 10% (w/v) lactose in buffer (◇) and milk (□). V^{app}_{Glc} in 10% (w/v) lactose in buffer (○) and milk (△). Lines are for guidance only.

free glucose is formed by β-galactosidase activity on lactose and is the sum of the rates of hydrolysis and transfer activities on lactose. An estimate of the rate of galactosyl transfer $V^{app}_{Glc-Gal}$ can therefore be obtained when the initial rate of galactose formation is subtracted from the initial rate of glucose formation.

As a check, the ratio of the initial galactosyl transfer and hydrolysis rates $V^{app}_{Glc-Gal}/V^{app}_{Gal}$ was calculated at different reaction temperatures. As expected, this ratio increases with temperature in a manner similar to the maximum GOS yield (**Figure 3**). This ratio is also analogous to other transfer and hydrolysis ratios used to characterize β-galactosidase-based reaction systems (*26*−*28*).

At low to moderate temperatures between 4 °C and 40 °C, the estimated galactosyl transfer $V^{app}_{Glc-Gal}$ and hydrolysis V^{app}_{Gal} rates in both milk and buffer reaction systems increased as a function of the temperature, as shown in **Figure 4**. The temperature dependence observed for the buffered lactose and milk system was well-described by the Arrhenius relationship [$R^2 > 0.95$ for an Arrhenius plot of $\ln(k)$ versus $1/T$, where k is the rate constant and T is the absolute temperature]. In both reaction systems, the difference between the galactosyl transfer and hydrolysis rates explains the temperature dependence of the maximum GOS yield between 4 °C and 40 °C (**Figure 3**).

At 60 °C, the hydrolysis V^{app}_{Gal} rate decreased markedly to near zero in both reaction systems (**Figure 4**). The galactosyl transfer $V^{app}_{Glc-Gal}$ rate was also reduced, although to a lesser extent (**Figure 4**). Because the Biolacta FN5 enzyme preparation is derived from the mesophilic *B. circulans*, it seems likely that this thermally induced change corresponds to the inactivation of one or more of the β-galactosidase enzymes.

β-Galactosidase II is the most likely of the three enzymes in Biolacta FN5 to be preserved at 60 °C, leading to the preservation of some galactosyl transfer activity ($V^{app}_{Glc-Gal}$; **Figure 4**) and the increase in the GOS yield observed for both reaction systems at 60 °C (**Figure 3**). This enzyme is known to be the most stable against thermal inactivation (*20*, *21*). It also contributes most significantly to the transfer activity observed in Biolacta FN5, while β-galactosidases I and III display primarily hydrolytic activity.

GOS Yield Is Enhanced by Heat Treatment of Biolacta FN5. Biolacta FN5 solutions were next heated at 60 °C for 20 minutes prior to kinetic analysis to test the hypothesis that elevated temperatures can selectively inactivate β-galactosidases I and III and effectively enhance enzyme-transfer activity.

These experiments confirmed that the hydrolytic activity of Biolacta FN5 was reduced by thermal inactivation. The rate of hydrolysis V^{app}_{Gal} measured at 4 °C was vastly reduced for

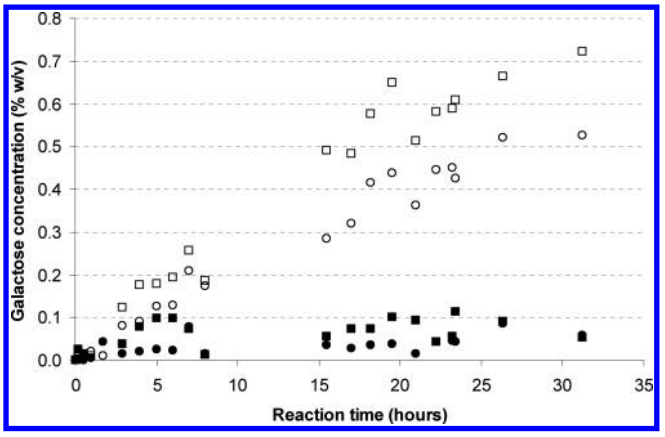

Figure 5. Effect of the heat treatment of the Biolacta FN5 enzyme preparation on galactose formation at 4 °C. Lactose (10%, w/v) in buffer with heat-treated (■) and untreated (□) Biolacta FN5. Milk reaction system with heat-treated (●) and untreated (○) Biolacta FN5.

Table 1. GOS Concentration in Milk and Buffered Lactose Reaction Systems after 30 h of Reaction Time at 4 °C

reaction system	enzyme pretreatment	mean[a] GOS concentration (% w/v)	standard deviation	GOS yield (%)
lactose	heat treated	4.46	0.19	45
	untreated	3.82	0.08	38
milk	heat treated	2.78	0.09	28
	untreated	2.34	0.08	23

[a] Mean of triplicate measurements.

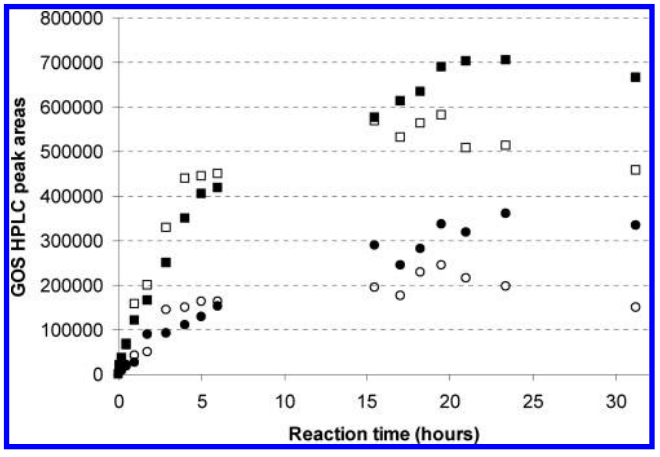

Figure 6. Effect of the heat treatment of the Biolacta FN5 preparation on the formation of GOS at 4 °C. Lactose (10%, w/v) in buffer with heat-treated (■) and untreated (□) Biolacta FN5. Milk reaction system with heat-treated (●) and untreated (○) Biolacta FN5.

heat-treated Biolacta FN5 preparations compared to untreated Biolacta FN5 preparations in both milk and buffered lactose reaction systems, as shown in **Figure 5**.

Heat treatment of the Biolacta FN5 preparation also significantly increased the GOS yield after 30 h at 4 °C, as shown in **Table 1**. An increase of 14% and 18% in the GOS concentration was measured for the heat-treated enzyme added to buffer and milk systems, respectively, translating to a 7% and 5% increase in the GOS yield.

The reaction kinetics for heat-treated Biolacta FN5 at 4 °C in **Figure 6** provide further evidence that GOS degrading hydrolytic activity has been selectively decreased by heat treatment. Only subtle differences are observed between the reaction kinetics for heat-treated and untreated preparations in the first few hours of the reaction, with the largest difference occurring in the second phase of the reaction, where hydrolysis is normally an important factor (**Figure 2**). A significant increase in the GOS peak area is observed by HPLC for heat-treated Biolacta FN5 after 16 h of the reaction compared to the untreated preparation ($p < 0.02$), consistent with the increase in the GOS concentration calculated by the mass balance in **Table 1**. The reduced hydrolytic activity observed for heat-treated preparations in **Figure 6** is also consistent with **Figure 5**. Moreover, the reaction kinetics in **Figure 6** are similar to those reported for purified β-galactosidase II (*20*).

Our experiments show that a simple, cost-effective step of pretreating the mixed Biolacta FN5 enzyme preparation by heating at 60 °C for 20 minutes prior to use is an effective way to increase the GOS yield. This treatment is easier, although possibly as effective as the multiple purification steps previously used (*20*) to purify β-galactosidase II.

In conclusion, we have found that facile heat treatment of the Biolacta FN5 preparation prior to use increases the GOS yield. Heat treatment reduces the net hydrolytic activity of the preparation while preserving the galactosyl transfer activity of β-galactosidase II. Further, GOS production is effective in milk and viable at low temperatures, suggesting that a variety of processing conditions can be used to generate GOS *in situ* within dairy products or other lactose solutions.

ABBREVIATIONS USED

GOS, galactooligosaccharide; HPLC, high-performance liquid chromatography; V^{app}_{Gal}, apparent rate of galactose formation; $V^{app}_{Glc-Gal}$, apparent rate of glucose formation minus the apparent rate of galactose formation.

ACKNOWLEDGMENT

We acknowledge Daniel Otieno for preliminary work leading to this study and Chiranjib Bhattacharjee from Jadavpur University, Kolkata, India, and P. K. Bhattacharya from the Indian Institute of Technology, Kanpur, India, for useful discussions. We also acknowledge the Particulate Fluids Processing Centre, Special Research Centre of the Australian Research Council (ARC), for their support.

LITERATURE CITED

(1) Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. *Food Chem.* **1998**, *63*, 147–154.
(2) Macfarlane, G. T.; Steed, H.; Macfarlane, S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* **2008**, *104*, 305–344.
(3) Siro, I.; Kapolna, E.; Kapolna, B.; Lugasi, A. Functional food. Product development, marketing and consumer acceptance—A review. *Appetite* **2008**, *51*, 456–467.
(4) Crittenden, R. G.; Playne, M. J. Production, properties and applications of food-grade oligosaccharides. *Trends Food Sci. Technol.* **1996**, *7*, 353–361.
(5) Lomer, M. C. E.; Parkes, G. C.; Sanderson, J. D. Review article: Lactose intolerance in clinical practice—Myths and realities. *Aliment. Pharmacol. Ther.* **2008**, *27*, 93–103.
(6) Vasella, A.; Davies, G. J.; Böhm, M. Glycosidase mechanisms. *Curr. Opin. Chem. Biol.* **2002**, *6*, 619–629.
(7) Petzelbauer, I.; Reiter, A.; Splechtna, B.; Kosma, P.; Nidetzky, B. Transgalactosylation by thermostable β-glycosidases from *Pyrococcus furiosus* and *Sulfolobus solfataricus*. *Eur. J. Biochem.* **2000**, *267*, 5055–5066.
(8) Boon, M. A.; Janssen, A. E. M.; van der Padt, A. Modelling and parameter estimation of the enzymatic synthesis of oligosaccharides by β-galactosidase from *Bacillus circulans*. *Biotechnol. Bioeng.* **1999**, *64*, 558–567.

(9) Hsu, C. A.; Lee, S. L.; Chou, C. C. Enzymatic production of galactooligosaccharides by β-galactosidase from *Bifidobacterium longum* BCRC 15708. *J. Agric. Food Chem.* **2007**, *55*, 2225–2230.
(10) Kilshaw, P. J.; Heppell, L. M.; Ford, J. E. Effects of heat treatment of cow's milk and whey on the nutritional quality and antigenic properties. *Arch. Dis. Child.* **1982**, *57*, 842–847.
(11) Kim, S. H.; Lim, K. P.; Kim, H. S. Differences in the hydrolysis of lactose and other substrates by β-D-galactosidase from *Kluyveromyces lactis*. *J. Dairy Sci.* **1997**, *80*, 2264–2269.
(12) Hu, J. M.; Li, H.; Cao, L. X.; Wu, P. C.; Zhang, C. T.; Sang, S. L.; Zhang, X. Y.; Chen, M. J.; Lu, J. Q.; Liu, Y. H. Molecular cloning and characterization of the gene encoding cold-active β-galactosidase from a psychrotrophic and halotolerant *Planococcus* sp L4. *J. Agric. Food Chem.* **2007**, *55*, 2217–2224.
(13) Cieslinski, H.; Kur, J.; Bialkowska, A.; Baran, I.; Makowski, K.; Turkiewicz, M. Cloning, expression, and purification of a recombinant cold-adapted β-galactosidase from antarctic bacterium *Pseudoalteromonas* sp 22b. *Protein Expression Purif.* **2005**, *39*, 27–34.
(14) Prenosil, J. E.; Stuker, E.; Bourne, J. R. Formation of oligosaccharides during enzymatic lactose: Part I: State of art. *Biotechnol. Bioeng.* **1987**, *30*, 1019–1025.
(15) Jakubowski, J.; Giacin, J. R.; Kley, D. H.; Gilbert, S. G.; Leeder, J. G. Effect of calcium, magnesium and whey proteins on the activity of β-galactosidase (*A. niger*) immobilised on collagen. *J. Food Sci.* **1975**, *40*, 467–469.
(16) Boon, M. A.; Janssen, A. E. M.; van't Riet, K. Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. *Enzyme Microb. Technol.* **2000**, *26*, 271–281.
(17) Park, H. Y.; Kim, H. J.; Lee, J. K.; Kim, D.; Oh, D. K. Galactooligosaccharide production by a thermostable β-galactosidase from *Sulfolobus solfataricus*. *World J. Microbiol. Biotechnol.* **2008**, *24*, 1553–1558.
(18) Zarate, S.; Lopez-Leiva, M. H. Oligosaccharide formation during enzymatic lactose hydrolysis: A literature review. *J. Food Prot.* **1990**, *53*, 262–268.
(19) Mozaffar, Z.; Nakanishi, K.; Matsuno, R. Formation of oligosaccharides during hydrolysis of lactose in milk using β-galactosidase from *Bacillus circulans*. *J. Food Sci.* **1985**, *50*, 1602–1606.
(20) Vetere, A.; Paoletti, S. Separation and characterization of three β-galactosidases from *Bacillus circulans*. *Biochim. Biophys. Acta* **1998**, *1380*, 223–231.
(21) Mozaffar, Z.; Nakanishi, K.; Matsuno, R.; Kamikubo, T. Purification and properties of β-galactosidases from *Bacillus circulans*. *Agric. Biol. Chem.* **1984**, *48*, 3053–3061.
(22) Fujimoto, H.; Miyasato, M.; Ito, Y.; Sasaki, T.; Ajisaka, K. Purification and properties of recombinant β-galactosidase from *Bacillus circulans*. *Glycoconjugate J.* **1998**, *15*, 155–160.
(23) Usui, T.; Kubota, S.; Ohi, H. A convenient synthesis of β-galactosyl disaccharide derivatives using the β-galactosidase from *Bacillus circulans*. *Carbohydr. Res.* **1993**, *244*, 315–323.
(24) Wessel, D.; Flugge, U. I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **1984**, *138*, 141–143.
(25) Greenberg, N. A.; Mahoney, R. R. Formation of oligosaccharides by β-galactosidase from *Streptococcus thermophilus*. *Food Chem.* **1983**, *10*, 195–204.
(26) Richard, J. P.; Westerfeld, J. G.; Lin, S.; Beard, J. Structure−reactivity relationships for β-galactosidase (*Escherichia coli*, lac Z). 2. Reactions of the galactosyl−enzyme intermediate with alcohols and azide ion. *Biochemistry* **1995**, *34*, 11713–11724.
(27) Splechtna, B.; Nguyen, T.-H.; Steinbock, M.; Kulbe, K. D.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54*, 4999–5006.
(28) Petzelbauer, I.; Splechtna, B.; Nidetzky, B. Galactosyl transfer catalyzed by thermostable β-glycosidases from *Sulfolobus solfataricus* and *Pyrococcus furiosus*: Kinetic studies of the reactions of galactosylated enzyme intermediates with a range of nucleophiles. *J. Biochem.* **2001**, *130*, 341–349.

Received for review June 1, 2009. Revised manuscript received August 6, 2009. Accepted October 14, 2009. This research was funded by an Australia−India Strategic Research Fund Program BF01-0024 from the Department of Innovation, Industry, and Science Research.