Site-directed mutation of β -galactosidase from *Streptococcus thermophilus* for galactooligosaccharide-enriched yogurt making

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ABSTRACT

β -Galactosidase is one of the most important enzymes used in dairy processing. It converts lactose into glucose and galactose, and also catalyzes galactose to form galactooligosaccharides (GOS), so-called prebiotics. However, most of the β -galactosidases from the starter cultures have low transgalactosylation activities, the process that results in galactose accumulation in yogurt. Here, a site-directed mutation strategy was attempted, to genetically modify β -galactosidase from *Streptococcus thermophilus*. Out of 28 *Strep. thermophilus* strains, a β -galactosidase gene named *bgaQ*, encoded for high β -galactosidase hydrolysis activity (BgaQ), was cloned from the strain *Strep. thermophilus* SDMCC050237. It was 3,081 bp in size, with 1,027 deduced amino acid residuals, which belonged to the GH2 family. After replacing the Tyr⁸⁰¹ and Pro⁸⁰² around the active sites of BgaQ with His⁸⁰¹ and Gly⁸⁰², the GOS synthesis of the generated mutant protein BgaQ-8012 increased from 20.5% to 26.7% at 5% lactose, and no hydrolysis activity altered obviously. Subsequently, the purified BgaQ or BgaQ-8012 was added to sterilized milk inoculated with 2 starters from *Strep. thermophilus* SDMCC050237 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842. The GOS yields with added BgaQ or BgaQ-8012 increased to 5.8 and 8.3 g/L, respectively, compared with a yield of 3.7 g/L without enzymes added. Meanwhile, the addition of the BgaQ or BgaQ-8012 reduced the lactose content by 49.3% and 54.4% in the fermented yogurt and shortened the curd time. Therefore, this study provided a site-directed mutation strategy for improvement of the transgalactosylation activity of β -galactosidase from *Strep. thermophilus* for GOS-enriched yogurt making.

Key words: β -galactosidase, *Streptococcus thermophilus*, transgalactosylation, site-directed mutagenesis

INTRODUCTION

Yogurt, one of the most popular fermented dairy products, is typically fermented using 2 starter cultures, from *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Horiuchi et al., 2009). However, most yogurt starter cultures only metabolize the glucose moiety of lactose and release the galactose in the extracellular medium, resulting in significant amounts of galactose accumulation (Hols et al., 2005; Zhang et al., 2020b). Lactose is not recommended for individuals with symptom of lactose intolerance, which is a common disease in more than half of the world's population (Husain, 2010). In addition, amounts of galactose also reduce the quality of fermented dairy products. For low-sugar yogurt making, several attempts have been made to isolate galactose-positive wild-type *Strep. thermophilus* strains or to co-culture starter cultures with other *Lactobacillus* species (Robitaille et al., 2007; Anbukkarasi et al., 2014; Sørensen et al., 2016; Zhang et al., 2020b). Also, arabinose isomerase has been used during milk fermentation to convert galactose into the low-calorie sweetener tagatose (Rhim et al., 2011).

β -Galactosidase, commonly known as lactase (EC 3.2.1.23), is widely used in the food industry to improve the sweetness, solubility, and flavor of dairy products (Oliveira et al., 2011; Saqib et al., 2017). The commercially used β -galactosidases are mainly from *Aspergillus oryzae* and *Kluyveromyces fragilis* (Horiuchi et al., 2009; Husain, 2010; Saqib et al., 2017; Serey et al., 2021). Many bacterial strains also have the capacity to produce β -galactosidases, including *Bifidobacterium longum*, *Bacillus circulans*, and *Lactobacillus* spp. (Boon et al., 2000; Hsu et al., 2007; Iqbal et al., 2011; Nguyen et al., 2012). In addition to hydrolysis activity, β -galactosidase also has transgalactosylation activity to catalyze galactose to form galactooligosaccharide (GOS), which has benefits for human health (Torres et al., 2010; Xin et al., 2019; Lu et al., 2020). To increase GOS yields, several strategies have been successfully attempted to improve the transgalactosylation activity of β -galactosidase (Gao et al., 2019; Qin et al., 2019).

Received June 22, 2021.

Accepted November 1, 2021.

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Streptococcus thermophilus is one of the most important starters for fermented dairy products with high β -galactosidase hydrolysis activity (Anbukkarasi et al., 2014; Zhang et al., 2020a). However, the transgalactosylation activity of the enzyme in *Strep. thermophilus* is normally low, resulting in galactose accumulation in yogurt. In this study, a β -galactosidase BgaQ was isolated from *Strep. thermophilus* SDMCC050237. After the prediction of structural and active sites via bioinformatics analysis, genetic modification of BgaQ through a site-directed mutagenesis was performed, and the GOS biosynthesis of the generated mutated enzyme in 5% lactose and reconstituted skim milk (RSM) medium were determined. The aim of this study was to improve the transgalactosylation activity of β -galactosidase from *Strep. thermophilus*, to catalyze galactose into GOS for manufacture of GOS-enriched yogurt.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Cultivation Conditions

The strains and plasmids used in this study are listed in Supplemental Table S1 (<https://zenodo.org/record/5720718#.YZzi9sVBw2w>). *Streptococcus thermophilus* strains were cultured in M17 broth (Oxoid) supplemented with 1% lactose at 42°C. The *Lb. bulgaricus* strain ATCC11842 was grown statically in de Man, Rogosa, and Sharpe medium at 37°C. *Escherichia coli* strains were grown aerobically in Luria-Bertani medium at 37°C. If necessary, kanamycin was supplemented to a final concentration of 30 μ g/mL.

Determination of Hydrolysis and Transgalactosylation Activities of β -Galactosidases from *Strep. thermophilus*

Cells of *Strep. thermophilus* strains cultured in lactose-supplemented M17 broth at 42°C for 12 h were harvested by centrifugation at $6,000 \times g$ for 5 min at 4°C, washed twice with PBS (pH 7.4), and suspended in PBS. The cells were disrupted by a cell homogenizer and centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatant was used to assay the hydrolysis and transgalactosylation activities of the β -galactosidases as described previously (Lu et al., 2015; Xin et al., 2019). Briefly, the hydrolysis activity of the β -galactosidase was performed in 200- μ L mixtures containing 50 μ L of the cell supernatant and 150 μ L of lactose solution (200 g/L) dissolved in PBS (pH 7.4), and incubated at 50°C for 10 min. Reactions were stopped by heating at 100°C for 5 min. The amount of glucose released in the

reaction mixture was detected using a glucose assay kit (Robio). One unit of β -galactosidase activity was defined as the amount of enzyme releasing 1 mmol of D-glucose per minute.

The transgalactosylation reaction was performed in 100- μ L mixtures containing 50 μ L of the cell supernatant and 50 μ L of lactose solution (200 g/L) dissolved in PBS (pH 7.4) at 42°C for 4 h, and then stopped by heating at 100°C for 5 min. Five microliters of the reaction solution was spotted onto thin-layer chromatography plates (Merck). The sugars were separated by a developing solvent consisting of n-butanol, isopropyl alcohol, acetic acid, and water (38.9%:27.8%:11.1%:22.2%). Subsequently, the plates were air-dried and sprayed with a chromogenic agent consisting of acetone-aniline-phosphoric acid (50:1:5 by volume) containing 0.018% (wt/vol) diphenylamine. After incubation at 105°C for 5 min, the bands were visualized and then photographed (Lu et al., 2015).

Molecular Manipulation

All molecular manipulations in this study were performed as described previously (Xin et al., 2019). Plasmids from *E. coli* were extracted using a Plasmid Mini Kit (Omega). Taq polymerase, restriction enzymes, and T4 DNA ligase were purchased from TaKaRa and used according to the standard procedures.

For the biochemical property analysis of β -galactosidase, the gene *bgaQ* was PCR amplified from the genomic DNA of *Strep. thermophilus* SDMCC050237 with primers bgaQ F (5'-CATGCCATGGACATGACTGAAAAAATTCAA-3'; underlining indicates the *Nco* I site) and bgaQ R (5'-CCGCTC-GAGATTTAGTGGTTCAATCATGAAGCTT-3'; underlining indicates the *Xho* I site). The PCR product was digested with the restriction enzymes *Nco* I and *Xho* I and then ligated into the corresponding sites of plasmid pET28a, generating the recombinant plasmid pET28a-bgaQ. After transformation into *E. coli* BL21 (DE3), the resulting recombinant *E. coli* BL21 carrying BgaQ was cultured at 37°C until cultures' optical density at 600 nm reached 0.6; then 0.1 mM isopropyl- β -D-thiogalactoside was added to the medium, and culturing continued for 20 h at 16°C for protein expression. Then the cells of *E. coli* BL21 carrying BgaQ were harvested by centrifugation at $6,000 \times g$ for 5 min at 4°C and washed twice with PBS. The cells were disrupted by ultrasonic treatment and centrifuged at $10,000 \times g$ for 20 min at 4°C to remove cell debris. The expression of the protein BgaQ was determined on SDS-PAGE gel after purification by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, according to standard procedures.

Sequence Analysis and Protein Modeling of the β -Galactosidase BgaQ

Sequence identity and similarity searches for the gene *bgaQ* were performed using Blast at NCBI (<http://www.blast.ncbi.nlm.nih.gov>). Multiple-sequence alignment of BgaQ with other related β -glucosidase sequences was performed using ClustalX 2.0 (Larkin et al., 2007). The 3-dimensional structure of BgaQ was generated using the Swiss-Model protein modeling server (<http://swissmodel.expasy.org>). The topology structure of β -galactosidase from *E. coli* (Protein Data Bank identification number 1PX3) was obtained from the RCSB Protein Data Bank (<http://www.rcsb.org>). The modeled structures were analyzed for the active sites of BgaQ by the Pymol molecular graphics system (<http://www.pymol.org>). The amino acid sites associated with transgalactosylation activity were found by autodocking using β -3'-galactosyl-lactose as substrate.

Site-Directed Mutagenesis of the β -Glucosidase BgaQ

To genetic modify the transgalactosylation activity, the Tyr⁸⁰¹ and Pro⁸⁰² around the activity sites in BgaQ sequence were predicted and replaced by His⁸⁰¹ and Gly⁸⁰² using overlap PCR with the mutagenic primer pairs 8012-forward 5'-ACAACGATCGTGCTGCTG-GACATGGATTTCGAAATG-3' and 8012-reverse 5'-TCCAGCCTGCCATTTTCGAATCCATGTCCAG-CACCA-3', with the underlined nucleotides coding the amino acids His and Gly at positions 801 and 802. After digestion with *Nco*I and *Xho*I, the PCR products were ligated into the corresponding sites of the plasmid pET28a, generating the recombinant plasmid pET28-bgaQ-8012, the mutant protein BgaQ-8012 was expressed in *E. coli* BL21 (DE3) and purified as previously described for BgaQ.

Biochemical Properties of BgaQ and Mutant BgaQ-8012

The optimal temperature for hydrolysis activities of the purified β -galactosidase BgaQ and the mutant BgaQ-8012 were determined over the range of 25 to 65°C. For the thermostability assay, the residual activities of the purified enzymes were measured after incubation from 37 to 60°C for 100 min. Three buffer systems, citrate buffer (50 mM, pH 5.5), sodium phosphate buffer (50 mM, pH 6.0–8.0), and Tris-HCl buffer (50 mM, pH 8.5–10.0), were used to measure the optimal pH for hydrolysis activity of the β -galactosidase BgaQ and the mutant BgaQ-8012 at 42°C. The residual activities were measured for pH stability in various

buffers from pH 6.0 to 10.0. For optimization of GOS synthesis conditions, pH from 6.0 to 8.0 and reaction temperatures from 37 to 60°C were adopted.

GOS Biosynthesis with BgaQ and BgaQ-8012

To simulate the fermentation conditions of yogurt processing, GOS production was measured at 5% lactose dissolved in PBS (pH 6.5) at 42°C, containing 1 U of purified BgaQ or BgaQ-8012, for 240 min. Samples were collected at intervals of 40 min. The GOS in the samples were analyzed by HPLC using an Aminex HPX-87H Carbohydrate Column (300 mm $\times$ 7.8 mm; Bio-Rad) at 65°C, with a refractive index detector. The mobile phase was deionized water at a flow rate of 0.5 mL/min. The GOS yield was calculated from the total amount of saccharides eluted at the retention times.

Fermented Milk

BgaQ and BgaQ-8012 were applied to catalyze GOS synthesis in milk. An RSM medium was prepared by reconstituting skim milk powder (10% wt/vol) with distilled water and sterilized at 115°C for 10 min. The 2 starters *Strep. thermophilus* SD MCC050237 and *Lb. bulgaricus* ATCC11842 were cocultured at a ratio of 4:1 in 100 mL of RSM medium to which 1 U of the purified BgaQ or BgaQ-8012 was added; fermentation without purified β -galactosidase was used as control. After incubation at 42°C for 12 h, the curd time was observed visually, indicating the time at which milk changed from a flowing liquid to an immobile semisolid state. The concentrations of lactose, galactose, and GOS in yogurt were determined via HPLC. The pH, titratable acidity, syneresis, and viable cell counts were detected at the end of fermentation, as described by da Costa et al. (2016) and Li et al. (2017).

Statistical Analysis

Each experiment was carried out in triplicate, and data are presented as mean $\pm$ standard deviation. Statistical significance between yogurts fermented by traditional starters with or without β -galactosidase was determined using SPSS version 19.0 (IBM Corp.), with $P < 0.05$ considered as statistically significant.

Amino Acid Sequence Accession Numbers

The β -galactosidase BgaQ and BgaQ-8012 sequences were deposited in GenBank and assigned the following accession numbers: BgaQ, SAMN20952848; BgaQ-8012, SAMN20952849.

Figure 1. Hydrolytic activities of β -galactosidases from various *Streptococcus thermophilus* strains.

RESULTS

Analysis of Hydrolysis and Transgalactosylation Activities of β -Galactosidases from *Strep. thermophilus*

The hydrolysis and transgalactosylation activities of the β -galactosidases from 28 *Strep. thermophilus* strains were determined. Results presented in Figure 1 showed that the hydrolysis activities of β -galactosidases occurred in a strain-specific manner among the *Strep. thermophilus* strains tested, and the strain *Strep. thermophilus* SDMCC050237 exhibited the highest hydrolysis activity, reaching 1.33 U/mL. However, the transgalactosylation activities of the β -galactosidases from the tested *Strep. thermophilus* strains appeared relatively low, as observed by small amounts of GOS production (Figure 2). Therefore, the β -galactosidase (BgaQ) from *Strep. thermophilus* SDMCC050237 was selected for the following analysis.

Cloning and Site-Directed Mutation of the β -Galactosidase BgaQ from *Strep. thermophilus* SDMCC050237

A β -galactosidase gene *bgaQ* was cloned from the *Strep. thermophilus* SDMCC050237 genome. It was

3,081 bp in length and composed of 1,027 amino acid residues. Multiple-sequence alignment showed that the deduced sequence BgaQ shared 99.5% and 47.50% similarities to the β -galactosidase of *Strep. thermophilus* CS9 (accession no. CP030927) and *Lb. bulgaricus* JCM1002 (accession no. CAI98003), respectively (Supplemental Figure S1, <https://zenodo.org/record/5720758#.YZzkXsVBw2w>). Moreover, 6 of the β -galactosidase sequences of *Strep. thermophilus* strains available were retrieved from the GenBank database, and multiple-sequence alignment revealed that those β -galactosidases were highly conserved (Supplemental Figure S1), exhibiting a $(\beta/\alpha)_8$ barrel (TIM barrel) fold consisting of an inner core of 8 parallel β -strands surrounded by 8 α -helices, indicating that the BgaQ from *Strep. thermophilus* SDMCC050237 belonged to the GH2 family.

To improve the transgalactosylation activity of the BgaQ, the catalyzing domain of this enzyme was predicted by comparison with that of the known β -galactosidase 1PX3 from *E. coli*. As shown in Figure 3a, the 2 active sites of glutamate, Glu⁴⁵⁸ and Glu⁵⁴⁶, were identified in BgaQ, and 10 potential transgalactosylation-related sites around them were provided by autodocking using β -3'-galactosyl-lactose as substrate (Figure 3a). Sequence alignment of the BgaQ with the high transgalactosylation activities of the

Figure 2. Transgalactosylation activities of various *Streptococcus thermophilus* strains. GOS = galactooligosaccharide.

β -galactosidases from other bacteria showed that the amino acid residues Tyr⁸⁰¹ and Pro⁸⁰² around the active sites Glu⁴⁵⁸ and Glu⁵⁴⁶ varied significantly in specific strains (Figure 3b), leading us to speculate that the sites Tyr⁸⁰¹ and Pro⁸⁰² were the key sites affecting the transglycosylation activity of the enzyme BgaQ. Thus, the amino acid residues Tyr⁸⁰¹ and Pro⁸⁰² were selected as targets for the site-directed mutagenesis. After replacing the Tyr⁸⁰¹ and Pro⁸⁰² with His⁸⁰¹ and Gly⁸⁰² by overlap PCR, and detecting the transgalactosylation activity, the mutant protein BgaQ-8012 was obtained.

Biochemical Characterization of BgaQ and BgaQ-8012

For biochemical analysis, BgaQ and BgaQ-8012 were expressed in *E. coli* DE3 and purified by Ni-NTA Column (Qiagen), respectively. The 2 purified protein bands appeared the same size, approximately 113 KD, on SDS-PAGE gel, which was in accordance with their theoretical molecular mass (Figure 4). Meanwhile, no statistical difference was detected between the hydrolytic activities of these 2 purified β -galactosidases. The optimum temperature and pH of the purified BgaQ-8012 were also the same as those of the wild-type en-

zyme BgaQ at 50°C and 8.5, respectively (Figure 5). In addition, BgaQ-8012 was shown to be more sensitive to temperature, with only 19% hydrolytic activity at 50°C for 40-min incubation, whereas BgaQ retained 57% activity under the same conditions (Figure 6). For pH stability, both BgaQ and BgaQ-8012 were more stable in acidic pH, showing maximal stability at pH 6.5.

The transgalactosylation conditions of these 2 purified proteins were detected. As shown in Figure 7a, the optimal pH for both BgaQ and BgaQ-8012 was 6.5. However, the optimal temperature showed obvious difference, with the optimal temperature of BgaQ being 50°C and 42°C for BgaQ-8012 (Figure 7b).

GOS Yields at 5% Lactose

To simulate the lactose content of yogurt processing, GOS production was performed at 5% lactose dissolved in PBS (pH 6.5) at 42°C, containing purified BgaQ or BgaQ-8012, for 240 min. Results showed that the GOS yields in these 2 reaction mixtures increased with prolonged time. The GOS yield of BgaQ-8012 was obviously higher than that of BgaQ during the reaction processes: the maximal GOS yield of BgaQ was about 20.5% at 120 min, compared with 26.7% for BgaQ-8012

Figure 3. Autodocking analysis of the transgalactosylation-related sites of BgaQ, the β -galactosidase from *Streptococcus thermophilus* SDMCC050237. (a) Results of autodocking between BgaQ and β -3'-galactosyl-lactose; (b) results of the multiple-sequence alignments of transgalactosylation-related sites of BgaQ.

Figure 4. SDS-PAGE analysis of the purified BgaQ (the β -galactosidase from *Streptococcus thermophilus* SDMCC050237) and BgaQ-8012 (the mutant enzyme of BgaQ). M = protein marker; T = total proteins in the lysate.

at 160 min, indicating that the transgalactosylation activity of the BgaQ increased with the site-directed mutagenesis (Figure 8).

Properties of Yogurt Fermented with BgaQ and BgaQ-8012

Purified BgaQ or BgaQ-8012 were added to the RSM medium in which 2 traditional starters, *Strep. thermophilus* SDMCC050237 and *Lb. bulgaricus* ATCC11842, were inoculated. After incubation at 42°C for 12 h, the contents of lactose and total sugar were significantly reduced by 38.6% and 16.5% (incubated with 2 traditional starters), 49.3% and 26.5%

(incubated with 2 traditional starters + BgaQ), and 54.4% and 40.1% (incubated with 2 traditional starters + BgaQ-8012), respectively (Table 1). Meanwhile, the GOS yields in yogurt fermented with 2 traditional starters and with added BgaQ and BgaQ-8012 were 3.7 g/L, 5.8 g/L, and 8.3 g/L (Table 1). The GOS yields of the mutant enzyme BgaQ-8012 increased compared with the wild enzyme BgaQ (Table 1). However, the galactose contents in all samples increased, particularly in the yogurts with the added β -galactosidase, BgaQ and BgaQ-8012, suggesting that the low pH in yogurt processing or low lactose content limited the GOS synthesis of the β -galactosidases.

Fermented milk performance analysis showed that the addition of the β -galactosidase BgaQ or BgaQ-8012 shortened the curd time significantly compared with the traditional yogurt starters, and there was no significant difference in pH, titratable acidity, and water-holding capacity of yogurt between them (Table 2). In addition, the viable cell counts of *Strep. thermophilus* SDMCC050237 and *Lb. bulgaricus* ATCC 11842 in all the groups reached 8 log cfu/mL (Supplemental Table S2, <https://zenodo.org/record/5720726#.YZzk9sVBw2w>).

DISCUSSION

β -Galactosidase plays a main role in the conversion of lactose into glucose and galactose in dairy products (Sangwan et al., 2015; Lu et al., 2020), as well as in the production of GOS based on its transgalactosylation property (Liu et al., 2017; Rico-Díaz et al., 2017). Most studies have focused on GOS biosynthesis from high concentrations of lactose (Song et al., 2013; Yu and O'Sullivan, 2014; Sangwan et al., 2015; Cao et al., 2019), while little attention has been paid to GOS production in fermented milk, where the low lactose

Figure 5. Optimum temperature (a) and optimum pH (b) for BgaQ (the β -galactosidase from *Streptococcus thermophilus* SDMCC050237) and BgaQ-8012 (the mutant enzyme of BgaQ). Error bars indicate SD.

Figure 6. Temperature stability of BgaQ (the β -galactosidase from *Streptococcus thermophilus* SDMCC050237, a) and BgaQ-8012 (the mutant enzyme of BgaQ, b), and pH stability of BgaQ (c) and BgaQ-8012 (d). Error bars indicate SD.

content (around 5% wt/vol) is not appropriate for optimal transgalactosylation activity (Rodriguez-Colinas et al., 2014). Because the 2 traditional starters of *Strep. thermophilus* and *Lb. bulgaricus* metabolize only the glucose moiety of lactose and release the galactose, resulting in significant amounts of galactose accumu-

lation in yogurt, production of low-sugar yogurt is of increasing interest in the fermented dairy industry. In this study, a novel strategy was adopted to convert residual galactose to GOS through modification of β -galactosidase from *Strep. thermophilus*. As expected, a mutant enzyme BgaQ-8012, modified by site-directed

Figure 7. Effects of various pH (a) and temperatures (b) on the galactooligosaccharide (GOS) yields of the purified proteins. BgaQ = the β -galactosidase from *Streptococcus thermophilus* SDMCC050237; BgaQ-8012 = the mutant enzyme of BgaQ. Error bars indicate SD.

Table 1. Residual sugar content in fermented milks¹

Group ²	Lactose (mmol/kg)	Galactose (mmol/kg)	GOS yield (g/L)	Percentage of lactose reduction (%)	Percentage of total sugar reduction (%)
RSM medium	114.35 ± 3.56	—	—	—	—
Traditional starters	70.09 ± 2.66 ^a	50.53 ± 1.89 ^a	3.7 ± 0.09 ^a	38.5 ^a	16.54 ^a
Traditional starters + BgaQ	56.92 ± 0.47 ^b	63.59 ± 2.56 ^b	5.8 ± 0.11 ^b	49.3 ^b	26.57 ^b
Traditional starters + BgaQ-8012	49.37 ± 1.18 ^c	60.79 ± 2.42 ^c	8.3 ± 0.13 ^c	54.4 ^c	40.14 ^c

^{a-c}Different superscripts indicate significant differences ($P < 0.05$) between yogurt fermented by the 2 traditional starters with BgaQ and BgaQ-8012.

¹Values are presented as mean ± SD of 3 individual experiments ($n = 3$); — indicates data not shown. The percentages of total sugar reduction were calculated by the molar weight of monosaccharides. GOS = galactooligosaccharide.

²RSM = reconstituted skim milk. Traditional starters = *Streptococcus thermophilus* SDMCC050237 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842. BgaQ = the β -galactosidase from *Strep. thermophilus* SDMCC050237; BgaQ-8012 = the mutant enzyme of BgaQ.

mutagenesis from the β -galactosidase BgaQ in *Strep. thermophilus* SDMCC050237, showed high GOS synthesis ability using 5% lactose as substrate, as well as in yogurt processing. Therefore, this study provides an efficient strategy to improve the transgalactosylation activity of β -galactosidase and could be used for GOS-enriched yogurt making.

Site-directed mutagenesis is an invaluable technique to modify genes for the analysis of the structural and functional properties of proteins. Several enzyme variants have been successfully obtained using this strategy, which display enhanced transgalactosylation activity of β -galactosidase or thermal stability compared with the corresponding wild-type enzymes (Yang et al., 2015; Rico-Díaz et al., 2017). But the key of this technique is to identify the targeting mutated sites. Bioinformation

software and internet sites provide the possibility of prediction and analysis of the active sites of enzymes. Using these tools, the potential transgalactosylation-related sites of Tyr⁸⁰¹ and Pro⁸⁰² around the active sites of Glu⁴⁵⁸ and Glu⁵⁴⁶ in BgaQ were identified (Figure 3a). On the basis of results reported previously, indicating that aromatic amino acids around the active sites can reduce the efficiency of enzyme binding to the receptor substrate (Bultema et al., 2014), the aromatic amino acid Tyr⁸⁰¹, which binds to the acceptor substrate, was replaced by His⁸⁰¹. In consideration of the double-displacement reaction of transgalactosylation, including the formation of a glycosylated enzyme reaction intermediate and its deglycosylation either by water molecule (hydrolysis) or by other acceptor (transgalactosylation), it was logical that a reduction in hydrolysis would be beneficial for the transgalactosylation reaction (Qin et al., 2019). Therefore, the amino acid Pro⁸⁰² was substituted by Gly⁸⁰², which could be achieved by reducing the water activity around the active sites. As a result, no obvious difference in hydrolytic activities appeared between BgaQ and the mutant BgaQ-8012. As expected, the transgalactosylation activity of the mutant BgaQ-8012 was enhanced, with GOS yield from 20.5% to 26.7% in 5% lactose as substrate (Figure 8), which was in agreement with previous reports (Bultema et al., 2014; Qin et al., 2019). Meanwhile, the optimal temperature for GOS production decreased from 50°C to 42°C, a promising temperature for yogurt fermentation. These results further demonstrate that site-directed mutagenesis is an effective tool for improvement of the transgalactosylation activity of β -galactosidase in *Strep. thermophilus*.

To investigate the GOS synthesis of the mutant enzyme BgaQ-8012 in yogurt processing, the purified enzymes were added to RSM medium in combination with the 2 traditional starters *Strep. thermophilus* SDMCC050237 and *Lb. bulgaricus* ATCC 11842. As anticipated, the addition of BgaQ or BgaQ-8012 improved the GOS yield, suggesting that the β -galactosidases

Figure 8. Curves of galactooligosaccharide (GOS) biosynthesized by purified BgaQ (the β -galactosidase from *Streptococcus thermophilus* SDMCC050237) and BgaQ-8012 (the mutant enzyme of BgaQ) in 5% lactose as substrate. Error bars indicate SD.

Table 2. Physicochemical characteristics of fermented milks¹

Group ²	Curd time (h)	pH	Titrateable acidity (°T)	Syneresis (%)
Traditional starters	7 ± 0 ^a	3.78 ± 0.02	107.6 ± 2.31	50.9 ± 0.01
Traditional starters + BgaQ	6 ± 0 ^b	3.77 ± 0.02	109.5 ± 1.67	51.9 ± 0.01
Traditional starters + BgaQ-8012	6 ± 0 ^b	3.78 ± 0.02	108.7 ± 2.07	51.4 ± 0.01

^{a,b}Different superscripts indicate significant differences ($P < 0.05$) between yogurt fermented by the 2 traditional starters with BgaQ and BgaQ-8012.

¹Values are presented as mean ± SD of 3 individual experiments ($n = 3$).

²Traditional starters = *Streptococcus thermophilus* SDMCC050237 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842. BgaQ = the β -galactosidase from *Strep. thermophilus* SDMCC050237; BgaQ-8012 = the mutant enzyme of BgaQ.

from *Strep. thermophilus* have abilities to synthesize GOS at low levels of lactose content. In addition, the GOS yield of the mutant enzyme BgaQ-8012 increased from 5.8 g/L to 8.3 g/L compared with the wild-type enzyme BgaQ (Table 1), which indicated that the mutant enzyme BgaQ-8012 was more suitable for GOS-enriched yogurt making. Compared with the addition of artificial GOS to yogurt directly, the addition of BgaQ or BgaQ-8012 also significantly reduced the contents of lactose and total sugar, as has been previously reported (Ruiz-Matute et al., 2012; Rodriguez-Colinas et al., 2014).

Acidification and curdling in a short incubation time are crucial for yogurt making. The fermented milk performance analysis showed that the addition of the BgaQ and BgaQ-8012 shortened the curd time in yogurt processing, due to the β -galactosidases BgaQ and BgaQ-8012 added exogenously to accelerate the hydrolysis of lactose into galactose and glucose, which rapidly promoted the growth of the 2 traditional starters *Strep. thermophilus* SDMCC050237 and *Lb. bulgaricus* ATCC11842 at the beginning of fermentation processing, produced more lactic acid to lower the pH, and subsequently facilitated the acidification processes. It has been reported that cocultures of *Lb. bulgaricus* 2038 and *Strep. thermophilus* 1131 in lactose-hydrolyzed milk resulted in increased cell numbers of *Lb. bulgaricus* 2038 (Yamamoto et al., 2021), but no obvious differences of the cell counts in yogurts with or without addition of the purified enzymes were observed in our study (Supplemental Table S2, <https://zenodo.org/record/5720726#.YZzk9sVBw2w>). Along with the growth of the 2 starters *Strep. thermophilus* SDMCC050237 and *Lb. bulgaricus* ATCC11842, the pH dropped quickly, and the lactose content in milk was reduced, which might inhibit the enzyme activities of the β -galactosylases BgaQ and BgaQ-8012, in turn, with more galactose accumulation in the medium. However, the lactose and total sugar contents were decreased obviously; this phenomenon was also as described previously (Oliveira et al., 2011; Ruiz-Matute

et al., 2012; Rodriguez-Colinas et al., 2014). Therefore, exogenous addition of the β -galactosidases from *Strep. thermophilus*, with high transgalactosylation activity, contributed to GOS-enriched yogurt making.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 31871767; Beijing) and the National Key Research and Development Program of China (2017YFD0400300; Beijing). The authors have not stated any conflicts of interest.

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