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BbgIV Is an Important *Bifidobacterium* β-Galactosidase for the Synthesis of Prebiotic Galactooligosaccharides at High Temperatures

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ABSTRACT: The individual contributions of four β-galactosidases present in *Bifidobacterium bifidum* NCIMB 41171 toward galactooligosaccharide (GOS) synthesis were investigated. Although the β-galactosidase activity of the whole cells significantly decreased as a function of temperature (40−75 °C), GOS yield was at its maximum at 65 °C. Native-PAGE of the whole cells showed that the contributions of BbgIII and BbgIV to GOS synthesis increased as the temperature increased. Moreover, BbgIII and BbgIV were found to be more temperature stable and to produce a higher GOS yield than BbgI and BbgII, when used in their free form. The GOS yield using BbgIV was 54.8% (percent of total carbohydrates) and 63.9% (percent lactose converted to GOS) at 65 °C from 43% w/w lactose. It was shown that BbgIV is the most important β-galactosidase in *B. bifidum* NCIMB 41171 and can be used for GOS synthesis at elevated temperatures.

KEYWORDS: *transgalactosylation, β-galactosidase, Bifidobacterium, galactooligosaccharides, prebiotic, protein expression, whole-cell biocatalyst*

1. INTRODUCTION

The synthesis of prebiotic galactooligosaccharides (GOS) has recently received better attention because these oligosaccharides have been increasingly used as functional food ingredients.[1] This is due to their emerged health benefits, as GOS have been shown to enhance the growth and metabolic activity of the beneficial bacteria in the gut, particularly bifidobacteria and lactobacilli,[2−4] to increase the bioavailability of minerals, mainly calcium and magnesium,[5−7] to regulate the activity of various bacterial enzymes, such as β-glucuronidase and nitroreductase, which are involved in the production of toxins and carcinogens,[8] to assist in the inhibition of the attachment of pathogenic bacteria to the colonic epithelium,[4,9] to act potentially as therapeutic agents in irritable bowel syndrome (IBS),[10] and to stimulate the immune system.[11]

GOS are mainly produced from lactose through transgalactosylation reactions catalyzed by β-galactosidases (EC 3.2.1.23). Transgalactosylation is the transfer of the galactosyl moiety, after the cleavage of the β-(1→4) glycosidic linkage, found in lactose, to an acceptor molecule containing a hydroxyl group rather than water. Therefore, transgalactosylation is enhanced under conditions of high lactose concentration and low water content.[12−15] Additionally, transgalactosylation is significantly affected by the source of β-galactosidase and the process conditions, such as temperature, reaction time, and pH.[12,16−18]

Among the various microbial β-galactosidases that have been widely used for GOS synthesis, those originating from lactic acid bacteria and bifidobacteria are of valuable interest for the production of GOS. This is because lactic acid bacteria and bifidobacteria are generally recognized as safe (GRAS) microorganisms, which have been long used in food fermentation. Another very interesting reason is that GOS produced using β-galactosidases from lactic acid bacteria and bifidobacteria will most likely show better selectivity for the growth and metabolic activity of these two bacterial genera in the gut, which will lead to improved prebiotic effects.[15,19] Examples of GOS synthesis using β-galactosidases from lactobacilli include, but are not limited to, *Lactobacillus reuteri*,[20,21] *Lactobacillus sakei* LB790,[22] *Lactobacillus fermentum* FUA 3177,[23] and *Lactobacillus ruminis* CHCC8818.[23] Also, various bifidobacterial strains have been used for the synthesis of GOS either as whole-cell biocatalysts, such as *Bifidobacterium bifidum* NCIMB 41171[18,24] and *Bifidobacterium longum* CHCC 8700,[23] or as crude extracts of β-galactosidases, such as *B. longum* BCRC 15708[25] and *Bifidobacterium infantis* DSM-20088.[15] Compared to whole cells, a free enzyme biocatalyst offers various advantages, such as the elimination of possible side reactions and an increase in product purity. On the other hand, whole cells can be much more easily prepared and are generally considered more stable biocatalysts than free enzymes.[26] When the whole-cell biocatalyst carries several β-galactosidases, which take part in GOS synthesis, it is important to understand the role of each of these enzymes, especially if they do not contribute equally to GOS synthesis.

In this context, the cells of *B. bifidum* NCIMB 41171, an industrial microorganism, have been used as the biocatalyst for GOS synthesis.[18,24,27] Moreover, it was previously found that the GOS yield using this strain increased as the synthesis temperature increased from 40 to 60 °C, at any given lactose concentration,[18] making the whole cells a suitable biocatalyst for GOS synthesis at elevated temperatures. It is also known that this bacterial strain contains four β-galactosidases, namely,

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BbgI, BbgII, BbgIII, and BbgIV,[28] which are different in their biochemical characteristics[29] and in their potential location in the bacterial cell.[28] Driven by our previous research, the aim of this work was to investigate the contribution of each of these β-galactosidases to GOS synthesis, particularly at elevated temperatures, to understand the reasons behind the high GOS yield observed at high temperatures using the whole cells, and to evaluate the stability of the four β-galactosidases in GOS synthesis reactions, using both the whole cells and the individual crude enzymes as biocatalysts, with the view of selecting the most suitable enzyme that can be used for GOS synthesis at elevated temperatures, as this will most likely improve control of the production process. The experimental plan involved performing GOS synthesis at various temperatures using whole *Bifidobacterium* cells as well as the four enzymes separately, which were expressed in *Escherichia coli* DH5α and used in a crude form for GOS synthesis. The main parameters measured during syntheses were the GOS yield, the β-galactosidase activities, the operational stability of the biocatalysts, and the rate of lactose conversion.

2. MATERIALS AND METHODS

2.1. Materials. All chemicals were purchased from Sigma-Aldrich (Dorset, U.K.) and were of the highest purity unless otherwise stated. Tryptone, yeast extract, and bacteriological agar were from Oxoid (Basingstoke, U.K.). Sodium acetate and sodium hydroxide, which were used for high-performance anion exchange chromatography coupled with pulsed amperometric detector (HPAEC-PAD) analysis, were from Fischer Scientific (Loughborough, U.K.). The whole-cell biocatalyst, freeze-dried cells of *B. bifidum* NCIMB 41171, was provided by Clasado Inc. (Jersey, U.K.). One gram of the freeze-dried cells had an activity of 1912.72 ± 14.34 units, calculated following the β-galactosidase assay mentioned under section 2.4. *E. coli* DH5α competent cells (genotype F^- φ80lacZΔM15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (r_k-, m_k+) phoA supE44 thi-1 gyrA96 relA1 λ^-) were obtained from Invitrogen (Paisley, U.K.).

2.2. Plasmid Transformation and Preparation of Crude Extracts of BbgI, BbgII, BbgIII, and BbgIV. Plasmids pBL-1-P1, pP2, pS1, and pBL-2-K2, which were constructed previously from pBluescript KS (accession no. X52331) and pBluescript SK (+) (accession no. X52324) plasmids (Stratagene, USA) and which carried the genes encoding for BbgI, BbgII, BbgIII, and BbgIV, respectively,[29] were transformed into chemically competent *E. coli* DH5α cells, according to the manufacturer's instructions. β-Galactosidase positive clones were identified as blue colonies on Luria−Bertani (LB) agar plates containing 50 μL of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and supplemented with 100 μg/mL ampicillin. The transformed *E. coli* cells were grown in shake flasks containing modified LB medium (10 g/L tryptone, 10 g/L yeast extract, 10 g/L glycerol, 5 g/L sodium chloride, 11.36 g/L disodium hydrogen phosphate, 2.39 g/L sodium dihydrogen phosphate), which was supplemented with 100 μg/mL ampicillin (the pH of the medium was 7.0 ± 0.05). The flasks were incubated for 12 h at 37 °C and at 200 rpm. The cells were harvested by centrifugation at 10000*g*, washed twice with phosphate buffer saline (PBS) (pH 7.0), suspended in sodium phosphate buffer (50 mM, pH 6.8), and sonicated at 4 °C (three times for 60 s, each time at 26 amplitude micrometers) using a Soniprep 150 (SANYO Gallenkamp PLS, U.K.). The sonicated solutions were considered as crude extracts of BbgI, BbgII, BbgIII, and BbgIV and were used separately for GOS synthesis, after further concentration with Vivaspin sample concentrators (30000 MWCO) (Sartorius Stedim Biotech, Surrey, U.K.).

2.3. GOS Synthesis. GOS synthesis was conducted using the freeze-dried *Bifidobacterium* cells as well as the crude extracts of BbgI, BbgII, BbgIII, and BbgIV, with 43% w/w of filter-sterilized lactose as the substrate in 0.05 M sodium phosphate buffer, at a pH of 6.8 ± 0.1 and at 150 rpm. The preparation of the lactose solution involved mixing 45.26 g of α-lactose monohydrate with 54.74 g of the buffer at 85 − 90 °C for 10−15 min until lactose was fully dissolved. This was followed by rapid cooling of the lactose solution to the synthesis temperature. When the solution reached the right temperature, the GOS synthesis was immediately initiated by adding 1 g of freeze-dried cells per 100 g of the dissolved lactose solution, which corresponded to about 44 units of β-galactosidase activity per gram of lactose, measured using the β-galactosidase assay described under section 2.4. The same ratio of β-galactosidase activity to lactose was used when the crude extracts of BbgI, BbgII, BbgIII, and BbgIV were separately used as biocatalysts. The synthesis temperature was varied from 40 to 75 °C, at 5 °C intervals. Samples were collected at 1, 2, 4, 8, 12, 16, 20, and 24 h of synthesis. The reactions were run in duplicate. The percentage consumption of lactose (C) and the purity yield of GOS (Y_P) were estimated according to the equations

$$C = \left(\frac{[\mathrm{Lac}]_c}{[\mathrm{Lac}]_i}\right) \times 100 \quad (1)$$

$$Y_P = \left(\frac{\text{GOS produced (g)}}{\text{total carbohydrates (g)}}\right) \times 100 \quad (2)$$

where $[\mathrm{Lac}]_i$ and $[\mathrm{Lac}]_c$ are the molar concentrations of the lactose at time 0 h and the lactose consumed in the reaction, respectively. The GOS yield was also calculated as percent lactose converted to GOS by weight for comparison purposes with other studies. The GOS produced consisted of transgalactosylated disaccharides, trigalactooligosaccharides, tetragalactooligosaccharides, and pentagalactooligosaccharides. The data were statistically treated using Minitab statistical software (release 15, State College, PA). The time required for the conversion of 50% of lactose (TL_{50}) was calculated using nonlinear regression, by plotting the natural logarithm of C versus time, and was used as an indication of the rate of lactose conversion during the reaction.

2.4. β-Galactosidase Activity Assay. Samples were periodically withdrawn throughout the GOS synthesis reactions for determining the β-galactosidase activity. For the syntheses using crude β-galactosidases, the samples were appropriately diluted and used directly for measuring the activity, after the separation of the enzymes from the synthesis solution using Vivaspin concentrators (3000 MWCO) (Sartorius Stedim Biotech, Surrey, U.K.). For the syntheses conducted using whole cells, the enzymatic extract was prepared by sonication as described under section 2.2. The reaction mixture consisted of 250 μL of*o*-nitrophenol-β-galactoside (*o*-NPG) (20 mM), 200 μL of sodium phosphate buffer (0.05 M, pH 6.8), and 10 μL of magnesium chloride (0.05 M). The reaction was initiated by adding 40 μL of the enzymatic extract, which was appropriately diluted, and the mixture was incubated at 40 °C for 10 min. The reaction was terminated by adding 500 μL of sodium carbonate (1 M). The absorbance was immediately measured at 420 nm against a suitable blank. One unit of activity was defined as the amount of enzyme that liberates 1 μmol of *o*-nitrophenol (*o*-NP) per minute, under the above-mentioned conditions. The molar extinction coefficient under these conditions was 4330.2 M^{-1} cm^{-1}. The protein concentration was measured according to the Bradford method[30] using bovine serum albumin (BSA) as a standard. The enzymatic assay measurements were carried out in triplicate.

2.5. Stability of the Whole-Cell Biocatalyst and the β-Galactosidases during GOS Synthesis. The operational stability of the whole cells and the crude BbgI, BbgII, BbgIII, and BbgIV, during the synthesis reactions, was expressed as the operational half-life ($t_{1/2}$) of each biocatalyst. This was calculated by plotting the natural logarithm of the measured β-galactosidase activity for each biocatalyst versus time.

When the *Bifidobacterium* cells were used as the biocatalyst, the individual contributions of each of the four β-galactosidases, found in these bacterial cells, to GOS synthesis were monitored by native polyacrylamide gel electrophoresis (native-PAGE), using 7.5% polyacrylamide gel in an ATTO mini PAGE system (ATTO Corp., Japan). Samples of the whole cells taken from the synthesis reactions at different time points were sonicated as described under section 2.2; the resultant sonicated solutions were loaded and electrophoresed on a nondenaturing PAGE system using 0.05 M Bis-Tris buffer with a pH of 6.8 as the anode running buffer. The cathode running buffer was

0.05 M Bis-Tris containing 0.002% Coomassie G-250 dye, with a pH of 6.8. The individual contribution of each of the four enzymes was evaluated by incubating the gels in 0.05% of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 0.05 M sodium phosphate buffer at a pH of 6.8, for 2 h, and at 25 °C. β-Galactosidase blue bands were visualized and photographed. Protein standards of BbgI, BbgII, BbgIII, and BbgIV were provided by Clasado Inc. (Jersey, U.K.), and were used to assign the β-galactosidase blue bands in the gel to the corresponding enzymes. The gel images were analyzed for the relative area density of the β-galactosidase bands using GelCompare II software (Applied-Maths NV, Belgium). The relative area density was expressed as percent activity of each β-galactosidase, based on the total retained activity of the whole cells, and used as an indication of the contribution of each enzyme to GOS synthesis at different temperatures when using the whole cells as the biocatalyst. All of the analyses were carried out in triplicate.

2.6. Carbohydrate Analysis. Samples were periodically withdrawn from the synthesis solution, heated at 90 °C for 10 min, and filtered through 0.2 μm filter units, and their carbohydrate content was determined by high-performance liquid chromatography (HPLC) using a system consisting of a G1322A degasser (Agilent Technologies, Cheshire, U.K.), a G1310A isocratic pump (Agilent Technologies), and a Shodex RI-71 refractive index detector (Kawasaki, Japan). Separation of the carbohydrates was performed using a Rezex RCM-Monosaccharide Ca^{2+} (8%) column (300 × 7.8 mm) supplied by Phenomenex (Macclesfield, Cheshire, U.K.). The column was maintained at 84 °C; HPLC grade water was used as the mobile phase at a flow rate of 0.5 mL/min. Quantitative determination of each peak was carried out using fructose as the internal standard and maltopentaose, maltotetraose, isomaltotriose (Supelco, Bellefonte, PA), lactose, glucose, and galactose as the external standards.

The carbohydrate composition of the reaction mixture was also determined by HPAEC-PAD to quantify lactose, which was coeluted with the transgalactosylated disaccharides in the same peak using the HPLC method. A Dionex system (Dionex Corp., Surrey, U.K.) consisting of a GS50 gradient pump, an ED50 electrochemical detector with a gold working electrode, an LC25 chromatography oven, and an AS50 autosampler was used. Separation was performed using a pellicular anion-exchange resin based column, CarboPac PA-1 analytical (4 × 250 mm), connected to a CarboPac PA1 Guard (4 × 50 mm) (Dionex Corp.). The column was maintained at 25 °C; elution was performed at a flow rate of 1 mL/min using gradient concentrations of sodium hydroxide and sodium acetate solutions. Under these conditions, lactose was eluted as a separate peak from the other disaccharides, which allowed its quantification using a standard calibration curve. All chromatographic analyses were performed in duplicate.

3. RESULTS

3.1. GOS Synthesis. The maximum GOS yield (Y_P) obtained using the five biocatalysts in GOS synthesis reactions, performed at temperatures ranging from 40 to 75 °C, is depicted in Figure 1. It can be observed that as the temperature increased from 40 to 55 °C, the GOS yield obtained using BbgI and BbgII gradually increased. This trend was similar when using BbgIII, BbgIV, and whole cells, although in these cases Y_P increased to a temperature of 65 °C. At temperatures above 55 °C, in the case of BbgI and BbgII, and above 65 °C, in the case of BbgIII, BbgIV, and whole cells, there was a considerable decline in Y_P, which was also accompanied with a decline in C, meaning that a substantial amount of lactose was left unused under these conditions (Figure 2). In terms of GOS yield, BbgIV was the best enzyme, followed by BbgIII, BbgI, and BbgII (Figure 1). The maximum Y_P values obtained were 41.6, 25.4, 50.2, 54.8, and 52.2% using BbgI, BbgII, BbgIII, BbgIV, and whole cells respectively. This yield was calculated as % GOS based on total carbohydrates and was obtained at about

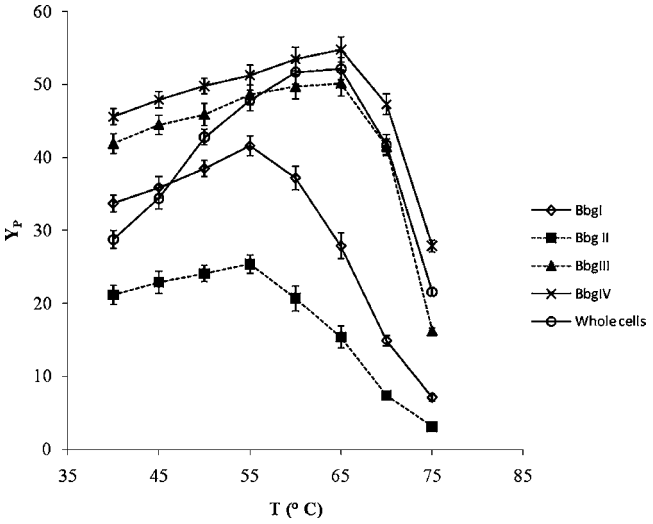

Figure 1. Yields of galactooligosaccharides (Y_P) (expressed as percent of total carbohydrates) as a function of temperature using *Bifidobacterium bifidum* NCIMB 41171 cells and crude BbgI, BbgII, BbgIII, and BbgIV as biocatalysts.

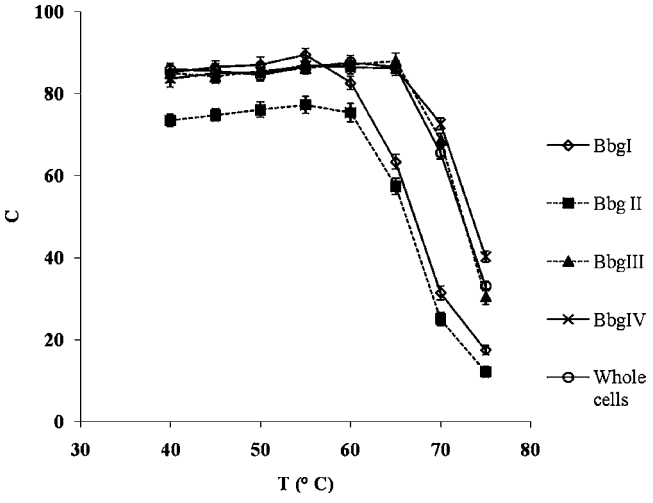

Figure 2. Percentage consumption of lactose (C) as a function of temperature using *Bifidobacterium bifidum* NCIMB 41171 cells and crude BbgI, BbgII, BbgIII, and BbgIV as biocatalysts.

83−89% of lactose conversion for all of the biocatalysts, except for BbgII, for which the maximum Y_P was obtained at about 75−78% of lactose conversion. When the yield was calculated as percent of lactose converted to GOS by weight, for comparison purposes with a few other studies, the maximum yields were 45.7, 29.3, 56.9, 63.9, and 59.4% for BbgI, BbgII, BbgIII, BbgIV, and whole cells, respectively. It should be stated that the maximum GOS yields were obtained at 55 °C using BbgI and BbgII and at 65 °C using BbgIII, BbgIV, and whole cells (Figure 1). Also, the GOS mixture obtained consisted mainly of transgalactosylated disaccharides (DP 2), trisaccharides (DP 3), and, to a lesser extent, higher oligosaccharides (DP ≥4). The different percentages of these oligosaccharides using the five biocatalysts are presented in Table 5.

Compared to the four enzymes, the ranking of the whole *Bifidobacterium* cells in terms of GOS yield was dependent on the synthesis temperature; that is, for 40 and 45 °C, the GOS yield obtained using whole cells was only higher than that obtained using BbgII, whereas for 50 and 55 °C it was higher

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Table 1. Retained Activity of *Bifidobacterium bifidum* NCIMB 41171 Cells, Expressed as Percent β-Galactosidase Activity, Measured at Different Time Points of GOS Synthesis Reactions Taking Place at Various Temperatures[a]

	retained activity of the whole cell biocatalyst (% of initial activity) at							
time (h)	40 °C	45 °C	50 °C	55 °C	60 °C	65 °C	70 °C	75 °C
0	100	100	100	100	100	100	100	100
1	88.3 ± 1.4	84.3 ± 0.8	79.9 ± 0.7	75.2 ± 1.8	64.7 ± 0.7	59.2 ± 0.9	49.9 ± 0.8	41.1 ± 0.4
2	82.9 ± 0.7	78.9 ± 1.1	72.1 ± 0.4	62.7 ± 1.3	51.3 ± 0.6	45.3 ± 1.2	35.4 ± 0.6	27.4 ± 0.7
4	78.6 ± 1.2	70.5 ± 0.6	61.4 ± 1.1	51.1 ± 0.5	38.2 ± 0.5	32.7 ± 0.7	23.6 ± 0.4	13.5 ± 0.3
8	69.1 ± 0.6	63.7 ± 1.2	53.5 ± 1.4	38.7 ± 0.8	26.9 ± 1.1	20.5 ± 0.6	11.1 ± 0.5	4.4 ± 0.1
16	53.3 ± 1.3	49.7 ± 0.5	32.6 ± 1.7	19.6 ± 0.4	12.8 ± 0.5	9.1 ± 0.4	1.8 ± 0.1	ND
24	41.1 ± 1.1	33.3 ± 0.6	19.2 ± 0.5	12.2 ± 0.4	6.4 ± 0.2	2.9 ± 0.2	ND	ND

[a]At 0 h, the whole cell biocatalyst had an activity of 1912.72 ± 14.34 units per g biocatalyst, which corresponds to 100%. ND stands for not detected.

than that obtained using BbgI and BbgII but lower than the yield obtained using BbgIII and BbgIV. At temperatures >60 °C, the yield obtained using whole cells was very high, only lower than that obtained using BbgIV (Figure 1). The hypothesis here is that when using whole cells all four enzymes act at the same time, and therefore the final GOS yield is a result of the different contributions of the four β-galactosidases, found in the whole cells, to GOS synthesis (Figure 1), which is, in turn, partially based on the differences in the temperature optima and operational stabilities of these enzymes (Table 3).

3.2. Operational Stability of Whole Cells during GOS Synthesis. As the temperature increased, the β-galactosidase activity of the whole cells significantly ($P < 0.05$) decreased at all time points (Table 1). The decrease in the activity at temperatures >65 °C was more pronounced than at lower temperatures (Table 1) and was most probably the main reason behind the low GOS yield at these temperatures (Figure 1). The values obtained for the operational half-life ($t_{1/2}$) of the whole cells during GOS synthesis indicated that the cells were less stable as the temperature increased from 40 to 75 °C (Table 3). For example, the $t_{1/2}$ of whole cells decreased by 5.4-, 7.6-, and 12.8-fold when the temperature increased from 40 to 60, 65, and 70 °C, respectively. However, despite the significant decrease in the $t_{1/2}$ values of the whole cells, Y_P still increased as a function of temperature from 40 to 65 °C (Figure 1). This suggested that the retained β-galactosidase activity of the whole cells and hence their stability did not correlate with the high Y_P values. Therefore, the stability and the contribution of the individual β-galactosidases found in the whole cells had to be considered.

3.3. Operational Stability of BbgI, BbgII, BbgIII, and BbgIV during GOS Synthesis. To investigate the high Y_P values obtained using whole cells at high temperatures, samples of whole cells were electrophoresed and specifically stained for the visualization of active β-galactosidases. Three main bands were observed; band 1 was assigned to BbgI, band 2 was assigned to BbgII, and band 3 was assigned to both BbgIII and BbgIV (Figure 3). The relative area density of each band was measured and expressed as percent activity of each β-galactosidase, based on the total retained activity of the whole cells. The results were a good indication of the contribution of each of the β-galactosidases, found in the whole cells, toward GOS synthesis at different temperatures (Table 2).

When using whole cells for GOS synthesis, BbgI and BbgII had, respectively, 21.6 ± 0.4 and 19.5 ± 0.3% of the total β-galactosidase activity of the whole cells at the onset of GOS synthesis, whereas BbgIII and BbgIV had together 58.9 ± 0.8% of the total activity (Table 2). The contribution of BbgI and

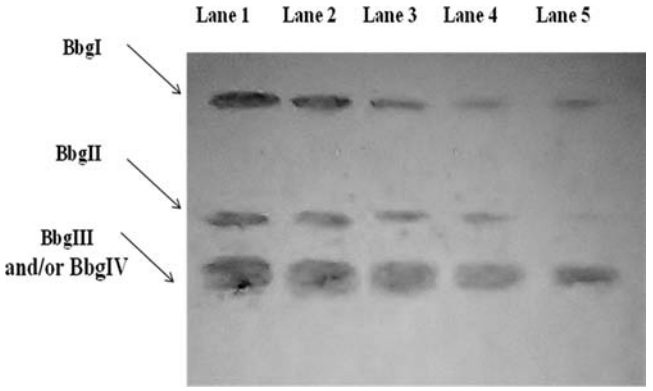

Figure 3. Native gel electrophoresis of the enzymatic extract of *Bifidobacterium bifidum* NCIMB 41171. Lanes 1, 2, 3, 4, and 5 represent the enzymatic extracts of whole cells taken after 2 h from synthesis reactions performed at 50, 55, 60, 65, and 70 °C, respectively, using 43% w/w lactose.

BbgII toward GOS synthesis decreased as the temperature increased for any given time point. In contrast, the contribution of BbgIII and BbgIV toward GOS synthesis increased as the temperature increased (Table 2). BbgIII and BbgIV were almost the only active β-galactosidases at temperatures ≥60 °C. These observations were also supported by the operational half-life ($t_{1/2}$) values of the crude BbgI, BbgII, BbgIII, and BbgIV, when used individually in synthesis reactions (Table 2), after their expression in *E. coli*. Among the four enzymes, BbgII was the least stable one, especially at temperatures >50 °C, and was deactivated at 70 and 75 °C. BbgI was more stable than BbgII, but was also deactivated at 75 °C. On the other hand, BbgIII and BbgIV were the two most stable enzymes. The $t_{1/2}$ of BbgIV in particular decreased only by 2.1-, 2.9-, 3.8-, and 6.9-fold at 55, 60, 65, and 70 °C compared to its $t_{1/2}$ value at 40 °C. The differences in the $t_{1/2}$ values of the four β-galactosidases, for the syntheses using free enzymes, and the differences in the contribution of each enzyme toward GOS synthesis when using the whole cells indicated that GOS was principally synthesized by BbgIII and BbgIV at elevated temperatures of ≥60 °C, as these two β-galactosidases retained considerable activity compared to BbgI and BbgII, which were more susceptible to temperature deactivation.

3.4. Rate of Lactose Conversion in GOS Synthesis. Despite the fact that BbgIII and BbgIV were more temperature stable compared to BbgI and BbgII (Table 3), and thus were principally the main enzymes taking part in GOS synthesis at elevated temperatures (60–70 °C) when using the whole cells and the free β-galactosidases, there was still a significantly

Table 2. Percentage of Activity of BbgI, BbgII, and BbgIII, and BbgIV, Based on the Total Retained Activity of the Whole Cells, Measured at Different Time Points of GOS Synthesis Reactions Taking Place at Various Temperatures[a]

	40 °C			50 °C			60 °C			70 °C		
time (h)	BbgI	BbgII	BbgIII and BbgIV	BbgI	BbgII	BbgIII and BbgIV	BbgI	BbgII	BbgIII and BbgIV	BbgI	BbgII	BbgIII and BbgIV
0	21.6 ± 0.4	19.5 ± 0.3	58.9 ± 0.8	21.6 ± 0.4	19.5 ± 0.3	58.9 ± 0.8	21.6 ± 0.4	19.5 ± 0.3	58.9 ± 0.8	21.6 ± 0.4	19.5 ± 0.3	58.9 ± 0.8
1	18.2 ± 0.2	18.5 ± 0.1	63.3 ± 0.4	17.1 ± 0.2	16.1 ± 0.4	66.8 ± 0.5	16.1 ± 0.2	10.8 ±0.2	73.1 ± 0.6	10.1 ± 0.3	ND	89.9 ± 0.6
2	17.5 ± 0.1	17.9 ± 0.1	64.6 ± 0.5	16.8 ± 0.2	14.5 ± 0.1	68.7 ± 0.9	12.6 ± 0.1	6.9 ± 0.1	80.5 ± 0.4	7.1 ± 0.1	ND	92.9 ± 1.1
4	15.1 ± 0.2	17.4 ± 0.2	67.5 ± 0.4	12.5 ± 0.3	12.8 ± 0.3	74.7 ± 0.6	8.2 ± 0.1	2.1 ± 0.04	89.7 ± 0.8	3.4 ± 0.04	ND	96.6 ± 0.7
8	11.7 ± 0.1	16.2 ± 0.1	72.1 ± 0.2	9.6 ± 0.4	11.6 ± 0.1	78.8 ± 1.2	4.7 ± 0.1	ND	95.3 ± 0.4	ND	ND	100
16	9.4 ± 0.2	16.1 ± 0.3	74.5 ± 0.4	7.6 ± 0.2	10.1 ± 0.1	82.3 ± 0.6	ND	ND	100	ND	ND	ND
24	9.6 ± 0.2	15.9 ± 0.1	74.5 ± 0.5	5.7 ± 0.1	9.5 ± 0.2	84.8 ± 0.9	ND	ND	100	ND	ND	ND

[a]ND stands for not detected.

continuous decrease in their stability as the temperature increased from 40 to 75 °C (Table 3). In fact, such a significant decrease in the operational stability could have negatively affected the GOS yield at such high temperatures. The reason this did not happen was probably the fact that when BbgIII and BbgIV were used at 60 and 65 °C, the rate of lactose conversion (inversely related to the time required for the conversion of 50% of the initial lactose, expressed as TL_{50}) was much higher than it was at 40 °C (Table 4). Additionally, the rates of lactose conversion at 60 and 65 °C were higher in the case of BbgIII and BbgIV compared to BbgI and BbgII (Table 4). All of the above ensured that the majority of the lactose was used in the reaction before BbgIII and BbgIV lost their activities, as a result of prolonged incubation. Another interesting finding was that the TL_{50} values obtained using whole cells were significantly higher, in most of the studied temperatures, than those obtained using the individual β-galactosidases, indicating a slower rate of lactose conversion using the whole cells. This is most likely due to the fact that whole cells create issues with the accessibility of the substrate to the enzymes, compared to the use of free enzymes in solution.[26]

It should be also pointed out that the maximum GOS yields were obtained at different times under different conditions of temperature. In the case of BbgIV, the maximum GOS yield was obtained at about 8 h of synthesis at 65 °C, whereas it was obtained after 16−20 h at 40 °C. This was a clear indication of the faster rate of lactose conversion at 65 °C compared to 40 °C. This fact significantly improved the productivity of the process at 65 °C compared to 40 °C (Figure 4). For example, the productivity using BbgIV was 35.1 g L^{-1} h^{-1} at 65 °C compared to 11.3 g L^{-1} h^{-1} at 40 °C, which represented approximately a 3-fold increase in productivity at 65 °C compared to 40 °C. This trend was observed for all biocatalysts (Figure 4).

4. DISCUSSION

Conducting GOS synthesis at high temperatures offers many advantages from an industrial perspective. Therefore, understanding the temperature effects on the operational stability of the biocatalyst and consequently on GOS yield is crucial for improving the existing industrial production process, which uses whole *Bifidobacterium* cells as the biocatalyst, and for developing novel ones, using free enzymes. Considering the fact that the *Bifidobacterium* cells used in this study contain four β-galactosidases, the aim of this work was to elucidate the reasons behind the high GOS yield at elevated temperatures using the whole cells, to understand the contribution of each enzyme to GOS synthesis at elevated temperatures, and to assess the operational stability of each of the β-galactosidases during GOS synthesis. Therefore, GOS synthesis was conducted at different temperatures using whole *Bifidobacterium* cells and the four enzymes individually, after being expressed in *E. coli*.

Under isothermal conditions, the solubility of lactose is low compared to that of other disaccharides. According to Machado et al.,[31] the solubility of lactose, expressed as grams of lactose in 100 g of water, is 18.9% at 25 °C, 25.2% at 40 °C, and 37.2% at 60 °C. At first glance, this seems to constrain GOS synthesis, due to the fact that GOS synthesis requires highly concentrated lactose solutions which favor transgalactosylation. However, this issue can be simply overcome by preparing supersaturated lactose solutions at high temperatures (85−90 °C), followed by rapid cooling to the synthesis temperature. This reasonably

Table 3. Operational Half-Lives ($t_{1/2}$) of *Bifidobacterium bifidum* NCIMB 41171 Cells, BbgI, BbgII, BbgIII, and BbgIV, for GOS Synthesis Reactions Taking Place at Various Temperatures[a]

	$t_{1/2}$ (h)				
temperature (°C)	whole cells	BbgI	BbgII	BbgIII	BbgIV
40	17.8 ± 0.6	12.1 ± 0.4	13.1 ± 0.2	15.2 ± 0.6	14.9 ± 0.4
45	14.3 ± 0.3	8.9 ± 0.4	7.3 ± 0.5	12.6 ± 0.3	12.3 ± 0.7
50	8.8 ± 0.7	4.3 ± 0.3	3.1 ± 0.5	9.1 ± 0.1	9.9 ± 0.4
55	5.7 ± 0.2	3.6 ± 0.1	1.1 ± 0.05	6.4 ± 0.2	7.2 ± 0.7
60	3.3 ± 0.2	2.2 ± 0.06	0.56 ± 0.02	4.1 ± 0.07	5.1 ± 0.7
65	2.2 ± 0.1	1.5 ± 0.04	0.33 ± 0.04	2.7 ± 0.1	3.9 ± 0.3
70	0.92 ± 0.1	0.51 ± 0.07	ND	0.86 ± 0.05	2.1 ± 0.08
75	0.29 ± 0.06	ND	ND	0.34 ± 0.1	1.1 ± 0.06

[a]ND stands for not detected.

Table 4. Time Required for the Conversion of 50% of the Initial Lactose (TL_{50}) Using *Bifidobacterium bifidum* NCIMB 41171 Cells, BbgI, BbgII, BbgIII, and BbgIV, for GOS Synthesis Reactions Taking Place at Various Temperatures[a]

	TL_{50} (h)				
temperature (°C)	whole cell biocatalysts	BbgI	BbgII	BbgIII	BbgIV
40	8.34 ± 0.13	2.65 ± 0.22	2.28 ± 0.23	3.83 ± 0.07	4.65 ± 0.18
45	7.92 ± 0.24	1.94 ± 0.14	1.37 ± 0.04	3.21 ± 0.12	4.04 ± 0.07
50	7.10 ± 0.11	1.57 ± 0.11	1.45 ± 0.01	2.55 ± 0.05	3.28 ± 0.08
55	5.97 ± 0.06	1.28 ± 0.13	1.64 ± 0.12	2.06 ± 0.09	2.27 ± 0.1
60	4.86 ± 0.05	2.43 ± 0.09	3.86 ± 0.17	1.23 ± 0.1	1.56 ± 0.03
65	4.43 ± 0.08	3.39 ± 0.13	13.12 ± 0.33	1.49 ± 0.04	1.46 ± 0.04
70	7.76 ± 0.12	44.63 ± 0.76	224.3 ± 2.4	5.25 ± 0.08	4.19 ± 0.16
75	45.86 ± 0.32	192.19 ± 3.3	739.8 ± 11.9	53.91 ± 1.4	27.46 ± 0.9

[a]ND stands for not detected.

Table 5. Percent of Transgalactosylated Oligosaccharides of Different Degrees of Polymerization (DP) in the Final GOS Mixture, Obtained Using the Five Biocatalysts[a]

type of biocatalyst	DP 2	DP 3	DP ≥ 4
whole-cells	49.1 ± 1.4	38.3 ± 1.1	12.3 ± 0.8
BbgI	55.2 ± 2.1	35.1 ± 1.5	9.7 ± 0.3
BbgII	46.2 ± 1.6	41.8 ± 1.3	11.9 ± 0.5
BbgIII	50.6 ± 1.5	36.1 ± 1.7	13.1 ± 0.6
BbgIV	51.5 ± 1.1	37.7 ± 1.3	10.9 ± 0.4

[a]The GOS mixtures presented are the maximum GOS obtained at 55 °C for BbgI and BbgII and at 65 °C using the whole cell biocatalyst, BbgIII, and BbgIV.

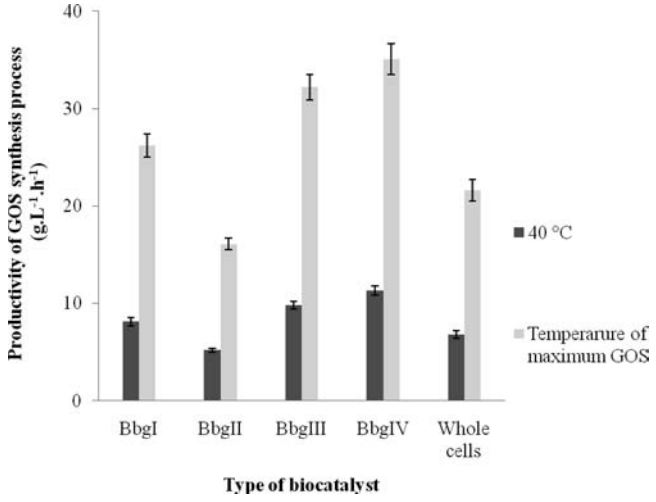

Figure 4. Productivity of GOS synthesis using *Bifidobacterium bifidum* NCIMB 41171 cells and crude BbgI, BbgII, BbgIII, and BbgIV from 43% w/w lactose. The productivity is presented at two different temperatures, at 40 °C and at the temperature at which the maximum GOS was obtained for each biocatalyst (55 °C for BbgI and BbgII; 65 °C for whole cells, BbgIII, and BbgIV).

delays the formation of lactose crystal nuclei. Once the supersaturated lactose solution is at the synthesis temperature, GOS synthesis can be immediately started. The conversion of lactose into glucose, galactose, and GOS avoids the crystallization of the remaining lactose in the synthesis solution. Preparing the lactose solution using the above methodology is very important if the aim is to study the effect of temperature at a fixed lactose concentration. This has been a common practice by many researchers who studied GOS synthesis using supersaturated lactose solutions at temperatures ≤50 °C, such as Goulas et al.,[27] who used 450−500 mg/mL lactose for GOS synthesis at 40 °C, and Cruz et al.,[32] who used 60% w/v lactose for GOS synthesis at 50 °C. In the study presented here, a fixed lactose concentration (43% w/w) was used in all of the synthesis reactions with the temperature being the only variable that was changed in the experiments.

It is interesting to highlight the fact that the GOS yield obtained using BbgIII, BbgIV, and whole *Bifidobacterium* cells was at its maximum at 65 °C. Optimum GOS yields at such elevated temperatures have been mentioned by other studies using different microorganisms. More specifically, Cardelle-Cobas et al.[33] reported an optimum transgalactosylation at 60 °C using lactulose as the substrate and the commercial enzymatic preparation Pectinex Ultra SP-L, derived from *Aspergillus aculeatus*, as the biocatalyst. The same research group showed that 55−60 °C was the optimum temperature for the activity of the same enzymatic preparation using lactose as a substrate.[34] Additionally, Kim et al.[35] pointed out that an

optimum transgalactosylation at 80 °C for the production of lactulose was observed using a thermostable β-galactosidase from *Sulfolobus solfataricus*.

On the basis of the GOS yield and the operational stability, BbgIV was the best enzyme for GOS synthesis at elevated temperatures among the biocatalysts studied. Maximum GOS yields of 54.8% (percent of total carbohydrates) and 63.9% (percent of lactose converted to GOS) were obtained using BbgIV at 65 °C, from 43% w/w lactose. The yield obtained using BbgIV at elevated temperatures compares well with the yield obtained using other microbial β-galactosidases at high temperatures. For instance, GOS yields of 40, 47.7, and 35.5% (percent of total carbohydrates) were obtained using a β-galactosidase from *Thermus* sp.Z-1 at 70 °C,[36] *S. solfataricus* at 65 °C,[37] and *Aspergillus oryzae* at 60 °C,[37] respectively; the initial lactose concentration was 300 g/L for *Thermus* sp.Z-1 and 342 g/L for both *S. solfataricus* and *A. oryzae*. Moreover, the use of toluene-treated cells of *Rhodotorula minuta* IFO 879 at 60 °C[38] and a β-galactosidase from *Sterigmatomyces elviae* CBD 8119 at 60 °C[39] resulted in GOS yields of 38 and 39% (percent of lactose converted to GOS) from 200 mg/mL lactose.

The use of *Lactobacillus* and *Bifidobacterium* β-galactosidases for GOS synthesis has usually been shown to take place at temperatures ≤55 °C. GOS yields of 36 and 41% (percent of total carbohydrates) were obtained using a β-galactosidase of *L. reuteri* at 37 °C[21] and *L. sakei* LB790 at 37 °C,[22] from 205 and 215 g/L lactose, respectively. Moreover, Rabiu et al.[15] reported yields of 43.8, 37. 6, 47.6, 26.8, and 43.1% (percent of total carbohydrates) using β-galactosidases from *Bifidobacterium angulatum*, *Bifidobacterium bifidum* BB-12, *Bifidobacterium infantis* DSM-20088, *Bifidobacterium pseudolongum* DSM-20099, and *Bificobacterium adolescentis* ANB-7 respectively, from 30% w/w lactose at 55 °C. Also, a β-galactosidase from *B. longum* BCRC 15708 resulted in a yield of 32.5% (pecent of total carbohydrates) from 40% lactose at 45 °C.[25] Previous studies using *B. bifidum* NCIMB 41171 or any of its enzymes for GOS synthesis ignored the effect of temperature on the GOS yield.[27,29] Goulas et al.[27] and Goulas et al.[40] produced 43 and 41% of GOS (percent of lactose converted to GOS) at 40 °C, respectively. The former used 450 mg/mL lactose, whereas the latter used 400 mg/mL lactose; however, the authors did not try temperatures higher than 40 °C. Only Osman et al.[18] demonstrated that increasing the temperature from 40 to 60 °C positively affected GOS synthesis; however, they used only the whole cells of *B. bifidum* NCIMB 41171 as the biocatalyst. Additionally, Goulas et al.[40] reported a yield of 47% of GOS (percent of lactose converted to GOS) using BbgIV, at 40 °C, and 400 mg/mL lactose. In the current study, we obtained a yield of 63.9% GOS at 65 °C (percent of lactose converted to GOS) using BbgIV and 43% w/w lactose. To our knowledge, this is the first time a bifidobacterial β-galactosidase (BbgIV from *B. bifidum* NCIMB 41171) has been cited to synthesize GOS efficiently at 65 °C.

It is widely accepted that β-galactosidases from thermophilic microorganisms are appropriate biocatalysts for GOS synthesis at elevated temperatures, provided they possess a high transgalactosylation activity. In this context, it should be stated that neither *B. bifidum* NCIMB 41171 is a thermophilic microorganism nor were any of its four β-galactosidases previously reported to be a thermophilic enzyme. According to Goulas et al.,[29] the optimum temperature of activity toward lactose was 40 °C for BbgI, BbgII, and BbgIII and 50 °C for BbgIV. Our study, on the other hand, found that the optimum temperature for GOS synthesis was 65 °C using BbgIII, BbgIV, and whole cells. We also found that during GOS synthesis at elevated temperatures, BbgIII and BbgIV were more temperature stable, compared to BbgI and BbgII, which were more susceptible to temperature inactivation, whereas Goulas et al.[29] showed that BbgII and BbgIII retained only 10% of their initial activity at 50 °C for 3 h in comparison to BbgI and BbgIV, which retained 60% of their initial activity at 50 °C for 3 h. One possible reason for the differences between the current findings and those previously found could be the fact that Goulas et al.[29] studied the stability of the enzymes in buffer solutions in the absence of substrate, whereas the present work examined the operational stability of these enzymes in the presence of substrate, that is, during GOS synthesis. It can be therefore argued that our data are more relevant from a production point of view. The fact that BbgIII and BbgIV were able to efficiently perform GOS synthesis at elevated temperatures is very interesting, although their thermal properties are not comparable with thermophilic enzymes, previously reported to carry out GOS synthesis at high temperatures, such as the β-galactosidase from *R. minuta* IFO 879, which has an optimum temperature of activity at 70 °C,[37] and the β-galactosidase from *S. elviae* CBD 8119, which has an optimum temperature of activity at 85 °C.[38]

The significant increase observed in the reaction rate at 60 and 65 °C compared to that at lower temperatures ensured the conversion of the majority of lactose before the remaining activities of BbgIII and BbgIV were inadequate to carry out the lactose conversion. The effect of temperature on reaction rates is well-known. A similar finding to ours was cited by Hung et al.,[41] who observed accelerated lactose hydrolysis as well as GOS synthesis when the temperature increased from 30 to 60 °C using a β-galactosidase from *B. infantis* HL 96. Other researchers, on the other hand, such as Hsu et al.,[25] noted that temperatures >55 °C resulted in a sharp decline in the GOS yield using β-galactosidase from *B. longum* BCRC 15708. Therefore, the effect of temperature varies on the basis of the source of the β-galactosidase. In our study, the increased rate of lactose conversion at elevated temperature resulted in a higher productivity compared to low temperatures. In the case of BbgIV, the productivity was 35.1 g L^{-1} h^{-1} at 65 °C. This is much higher than the productivity obtained using other β-galactosidases; for instance, the GOS productivities using β-galactosidases from *Bullera singularis*,[42] *B. infantis* HL96,[41] *A. oryzae*,[43] and *Thermotoga maritima*[44] were 4.8, 12.7, 24.3, and 18.2 g L^{-1} h^{-1}, respectively.

This research showed that BbgIV is the most important β-galactosidase present in *B. bifidum* NCIMB 41171 and can be used for GOS synthesis at high temperatures. In contrast, BbgI and BbgII are not suitable for GOS synthesis at temperatures >55 °C.

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