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Synthesis of prebiotic galactooligosaccharides from lactose using bifidobacterial β-galactosidase (BbgIV) immobilised on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose

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ABSTRACT

The bifidobacterial β-galactosidase BbgIV was immobilised on DEAE-Cellulose and Q-Sepharose via ionic binding and on amino-ethyl- and glyoxal-agarose via covalent attachment, and was then used to catalyse the synthesis of galactooligosaccharides (GOS). The immobilisation yield exceeded 90% using ionic binding, while it was low using amino-ethyl agarose (25–28%) and very low using glyoxal agarose (<3%). This was due to the mild conditions and absence of chemical reagents in ionic binding, compared to covalent attachment. The maximum GOS yield obtained using DEAE-Cellulose and Q-Sepharose was similar to that obtained using free BbgIV (49–53%), indicating the absence of diffusion limitation and mass transfer issues. For amino-ethyl agarose, however, the GOS yield obtained was lower (42–44%) compared to that obtained using free BbgIV. All the supports tried significantly ($P < 0.05$) increased the BbgIV operational stability and the GOS synthesis productivity up to 55 °C. Besides, six successive GOS synthesis batches were performed using BbgIV immobilised on Q-Sepharose; all resulted in similar GOS yields, indicating the possibility of developing a robust synthesis process. Overall, the GOS synthesis operation performance using BbgIV was improved by immobilising the enzyme onto solid supports, in particular on Q-Sepharose.

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1. Introduction

The synthesis of prebiotic galactooligosaccharides (GOS) is a process carried out by β-galactosidases (EC 3.2.1.23) using lactose as a substrate [1,2]. It is based on the transgalactosylation activity of the enzyme, which transfers the galactosyl moiety of lactose into acceptor molecules containing hydroxyl groups rather than water. This process yields a mixture of transgalactosylated oligosaccharides with different degrees of polymerisation (DP) and different glycosidic linkages, which find application as functional food ingredients [3,4]. From an industrial perspective, the two major bottlenecks in the GOS synthesis process are the production of the biocatalyst and its operational stability. The latter depends on the intrinsic properties of the enzyme and the synthesis temperature. It is known that transgalactosylation is favoured at elevated temperatures as very concentrated lactose solutions can be used

and high reaction rates can be achieved; hence, a high GOS yield can be obtained [2,4,5]. However, stability of the β-galactosidase used at high temperatures is a major concern, which may limit the potential of developing a robust GOS synthesis process on an industrial scale.

Immobilisation is one of the procedures used to enhance the stability of β-galactosidases during GOS synthesis. It is defined as a process which converts the enzyme into a form that is physically confined in a certain defined region of space, which hinders its mobility but retains its catalytic activity [6]. Immobilisation techniques can be divided into inclusion, carrier-binding (i.e. ionic binding, physical adsorption, hydrophobic interaction and van der Waals forces) and cross linking. These techniques have been used to immobilise β-galactosidases of different origins for their use in GOS synthesis. Examples are the entrapment of *Penicillium expansum* F3 β-galactosidase in calcium alginate beads [7], the covalent binding of *Kluyveromyces lactis* β-galactosidase to cellulose acetate membrane surfaces [8], the covalent attachment of *Aspergillus oryzae* β-galactosidase to glyoxal agarose beads [9], the non covalent binding of a β-galactosidase from *Bullera singularis* ATCC 24193 to

chitopearl BCW 3510 beads [10] and the use of glutaraldehyde to immobilise *A. oryzae* β-galactosidase on magnetised polysiloxane coated with polyaniline [11]. The major advantages reported for the immobilisation of β-galactosidases, including the above examples, are the ability to use the enzyme repeatedly in a wide variety of reactors, stop the reaction by easily removing the enzyme from the reaction medium, prevent the contamination of products by the enzyme, increase the enzyme stability and improve the process productivity and robustness [7,12–16].

The interest in using bifidobacterial β-galactosidases for GOS synthesis has just recently increased. Therefore, only a little is known about the characteristics of bifidobacterial β-galactosidases after immobilisation and the extent to which these enzymes can be used, in their immobilised form, for GOS synthesis. Based on the fact that the β-galactosidase BbgIV form *Bifidobacterium bifidum* NCIMB 41171 has recently received a lot of industrial attention for GOS synthesis [2] and the fact that this enzyme has been produced in levels suitable for industrial applications [17], the aim of this study was to immobilise BbgIV on several supports and assess the potential of using the immobilised enzyme for GOS synthesis; this is the first study which reports the use of immobilised BbgIV in GOS synthesis. The rationale is that immobilisation will improve the process productivity and robustness, and decrease the process cost by increasing the enzyme operational stability. For this purpose, BbgIV was expressed in *Escherichia coli* DH5α, purified and then immobilised via ionic binding using DEAE-Cellulose and Q-Sepharose, and via covalent attachment using glyoxal- and amino-ethyl-agarose. DEAE-Cellulose was selected because cellulose is the most widely abundant biodegradable biopolymer and has been successfully used to immobilise a variety of other enzymes. Q-Sepharose was selected based on the observation that this resin showed a high enzyme loading capacity during BbgIV purification, performed using HiTrap Q HP column, which contains Q-Sepharose as a stationary phase. Multipoint covalent attachment on glyoxal- and amino-ethyl-agarose was chosen due to the simplicity of the binding procedure. The criteria examined for assessing the performance of the immobilised enzyme were the immobilisation yield, enzyme characteristics after immobilisation, enzyme loading capacity, GOS yield and profile, BbgIV operational stability and process productivity.

2. Materials and methods

2.1. Materials

All chemicals were of the highest purity from Sigma Aldrich (Dorset, UK), unless otherwise stated. Tryptone, yeast extract and bacteriological agar were from Oxoid (Basingstoke, UK). The *E. coli* DH5α competent cells (Genotype: F-φ80lacZΔM15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rk−, mk+) phoA supE44 thi-1 gyrA96 relA1 λ−) were from Life Technologies (Paisley, UK). Diethyl amino-ethyl cellulose (DEAE-Cellulose) was from E. Merck (Darmstadt, Germany). Q-Sepharose and the columns used for BbgIV purification were from GE Healthcare (Little Chalfont, UK). The glyoxal- and amino-ethyl-agarose beads were from Web Scientific (Cheshire, UK). All Vivaspin centrifugal concentrators (30,000 MWCO) were from Sartorius Stedim Biotech (Surrey, UK).

2.2. Production of BbgIV

A single colony from a freshly prepared sub-culture of *E. coli* DH5α cells, harbouring the plasmid pBL-2-K2, which was constructed from pBluescript SK (+) (accession number X52324) (Stratagene, USA) and carried the gene encoding for BbgIV [18], was transferred into 10 mL Luria Bertani (LB) medium with pH

7 (containing 100 μgmL^{-1} ampicillin) and grown for 8 h at 37 °C and at 200 rpm. An aliquot of this culture was used to prepare the inoculum (200 mL of LB medium supplemented with 100 μgmL^{-1} ampicillin and incubated for 10 h at 37 °C and at 200 rpm). The *E. coli* cells were harvested by centrifugation (8000 × *g*, 15 min, 4 °C), suspended in sterilised phosphate buffer saline (PBS) at pH 7.0 and used to inoculate a 5 L bioreactor (Applikon, Gloucestershire, UK), at a starting OD$_{600 nm}$ of 0.1 and a working volume of 4 L. The fermentation medium consisted of 10 gL^{-1} yeast extract, 10 gL^{-1} glycerol, 10 gL^{-1} tryptone, 5 gL^{-1} sodium chloride, 4 gL^{-1} ammonium sulfate, 11.4 gL^{-1} disodium hydrogen phosphate and 2.4 gL^{-1} sodium dihydrogen phosphate (pH 7.0) [17]. After autoclaving, magnesium sulfate, iron chloride, thiamine and ampicillin were filter-sterilised and added to the medium at final concentrations of 0.5, 0.025, 0.01 and 0.1 gL^{-1}, respectively. The fermentation was carried out at 37 °C using an aeration rate of 1 vvm (gas volume flow per unit of liquid volume per minute). The dissolved oxygen level was left to drop to 0–2% during the fermentation as previously described by Osman et al. [17].

2.3. Purification of BbgIV

Aliquots of the *E. coli* cells were harvested after 24 h by centrifugation (8000 × *g*, 4 °C, 15 min) and washed with PBS (pH 7.0). The cells were suspended in sodium phosphate buffer (50 mM, pH 6.8) and sonicated at 4 °C (3 min at 24 amplitude microns) using a Soniprep 150 (SANYO Gallenkamp PLS, UK). The sonicated cell suspension was centrifuged (15,000 × *g*, 4 °C, 30 min) and the supernatant was concentrated using Vivaspin 20 concentrators. BbgIV was partially purified from the concentrated supernatant (crude extract) by anion exchange chromatography, using HiTrap Q HP column at a flow rate of 2.5 mL min^{-1}. The start buffer was 50 mM sodium phosphate (pH 7.4), whereas a linear gradient of 50 mM sodium phosphate (pH 7.4) containing 1 M sodium chloride was used as the elution buffer. BbgIV active fractions were pooled, concentrated and then desalted and buffer exchanged 4 times with sodium phosphate buffer (5 mM, pH 6.8) using Vivaspin 500 concentrators. BbgIV was then further purified by size exclusion chromatography, using Superdex 200 HR 10/30 column at a flow rate of 0.2 mL min^{-1}. The elution buffer consisted of 50 mM sodium phosphate, 50 mM sodium chloride, 1 mM magnesium chloride and 1 mM DTT (pH 6.5). Active fractions of BbgIV were pooled, concentrated and then desalted and buffer exchanged 4 times with sodium phosphate buffer (5 mM, pH 6.8), containing 1 mM magnesium chloride, using Vivaspin 500 concentrators. The purification yield was expressed as the relative activity of BbgIV, recovered after each purification step, based on the total BbgIV activity in the crude extract. All purification steps were performed using an AKTA FPLC system (GE healthcare Ltd, UK).

2.4. Ionic binding of BbgIV using DEAE-Cellulose and Q-Sepharose

Aliquots of DEAE-Cellulose (0.5 g in 20 mL distilled water) and Q-Sepharose (10 mL in 20% ethanol) suspensions were placed separately on a glass filter connected to a Buchner funnel and washed with distilled water under vacuum conditions. The immobilisation of BbgIV on these resins was performed at pH 5, 6, 7, 8 and 9 to evaluate the optimum pH of immobilisation. For pH 5, a citrate-phosphate buffer (50 mM) was used. For pH 6, 7 and 8, a sodium phosphate buffer (50 mM) was used and for pH 9 a sodium carbonate-bicarbonate buffer (50 mM) was used. Initially, the resins were equilibrated with these buffer solutions. Then, the equilibrated resins (each time 0.2 g in 20 mL buffer) were mixed, for 1 h at room temperature and at 50 rpm, with freshly prepared BbgIV at a ratio of 1600 units of β-galactosidase activity (U) per g

resin, measured as described in Section 2.6. The enzyme-resin suspension was then placed on a Buchner funnel and washed with the same buffer used for equilibration until no enzymatic activity was found in the filtrate and a stable activity was found on the resins. Different initial loadings of enzymatic activity were used to assess the maximum capacity of each resin for immobilising BbgIV, at the optimum pH of immobilisation. These were 400, 1600, 2800, 4000, 8000, 16,000, 40,000 and 80,000 U per g resin. The enzymatic activity and the protein content were measured for the initial enzyme solutions, the resins and the filtrates as described in Section 2.6. The immobilisation yield of BbgIV was presented as:

$$Yi = \frac{\text{Units of } \beta - \text{galactosidase activity observed after immobilisation per g resin}}{\text{Initial units of } \beta - \text{galactosidase activity added per g resin}} \times 100 \tag{1}$$

2.5. Covalent attachment of BbgIV using glyoxal- and amino-ethyl agarose beads

The glyoxal agarose beads (low density glyoxal 4BCL, high density glyoxal 4BCL, low density glyoxal 6BCL, high density glyoxal 6BCL and very high density glyoxal 6BCL) were washed with distilled water and equilibrated with sodium carbonate-bicarbonate buffer (50 mM, pH 10). The equilibrated beads (each time 0.3 g in 40 mL buffer containing 10% glycerol and 0.5 g galactose as protecting agents) were mixed, at room temperature and at 50 rpm, with freshly prepared BbgIV at a ratio of 1600 U per g resin, measured as described in Section 2.6. Aliquots of the enzyme-resin suspension were withdrawn after 1, 2, 3, 5 and 10 h, mixed with 5 mg sodium borohydride and stirred for 30 min at room temperature. Then, the beads were washed with sodium phosphate buffer (25 mM, pH 7.0) to eliminate any excess borohydride, followed by distilled water.

The amino-ethyl agarose beads (low density amino-ethyl 6BCL and very low density amino-ethyl 4BCL) were washed and equilibrated with distilled water. Then, the equilibrated beads (each time 0.3 g in 40 mL distilled water containing 0.8 g of 1-(3-dymethylaminopropyl)-3-ethylcarbodiimide hydrochloride (CDI)) were mixed, for 3 h at room temperature and at 50 rpm, with freshly prepared BbgIV at a ratio of 1600 U per g resin, measured as described in Section 2.6. Aliquots of the enzyme-resin suspension were withdrawn every hour and the beads were washed with distilled water, then with 0.1 M sodium chloride and finally with distilled water. The immobilisation yield for both types of agarose beads was measured as in Eq. (1). Also, different initial loadings of enzymatic activity, similar to those described in Section 2.4, were used to assess the maximum capacity of each resin for immobilising BbgIV.

2.6. β-Galactosidase activity assay

The β-galactosidase reaction was initiated by adding 80 μL of BbgIV (either in its free or suspended immobilised form) to 910 μL of lactose (10 mM final concentration prepared in 50 mM sodium phosphate buffer pH 6.8) and 10 μL of magnesium chloride (50 mM). The mixture was incubated for 10 min at 40 °C with shaking every 2 min. The reaction was stopped by heating at 90 °C for 10 min. From this mixture, 200 μL were added to 400 μL of glucose oxidase/peroxidase solution (provided in the glucose oxidase/peroxidase kit of Sigma Aldrich) and incubated at 37 °C for 30 min. Then, 400 μL of 12 N sulfuric acid were added and the absorbance was measured at 540 nm against a suitable blank. One unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 μmol of glucose per min under the assay conditions. The protein content was measured by the Bradford method [19] using bovine serum albumin (BSA) as the standard. The enzyme specific activity was defined as U per mg protein. All measurements were carried out in triplicate.

2.7. Temperature and pH optima, kinetic parameters and thermodynamic properties of BbgIV

The effect of pH on BbgIV activity was studied in a pH range of 5–9 using phosphate-citrate buffer for pH 5 and 5.4, sodium phosphate buffer for pH 5.8–8 (0.2 pH unit intervals) and sodium carbonate-bicarbonate buffer for pH 9. Also, the effect of temperature on BbgIV activity was studied in a temperature range of 30–70 °C (4 °C intervals from 30 to 46 °C and 62 to 70 °C, and 2 °C intervals from 46 to 62 °C). Kinetic parameters were estimated using lactose (0.01–70 mM) as a substrate at the optimum conditions of pH and temperature. The enzymatic assay was carried out according to Section 2.6. Non-linear regression, performed by Graph Pad Prism (La Jolla, USA), was used to estimate the steady state kinetic parameters (K_m, V_{max} and K_{cat}) under the assumption that each subunit of BbgIV had one active site. The activation energy (Ea) was determined from the plot between Ln K and $1/T$ using the Arrhenius equation, where the slope is $-Ea/R$ (K is the reaction rate constant, T is the temperature in Kelvin (K) and R is the universal gas constant). The activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) were calculated from the plot between Ln K/T and $1/T$ using the Eyring equation, where the slope is $-\Delta H^{\ddagger}/R$ and the intercept at $1/T = 0$ is $(\Delta S^{\ddagger}/R) + 23.76$ (23.76 is the result of Ln K_B/h where K_B is the Boltzmann constant and h is the Planck constant). Thermodynamic parameters were measured in a temperature range of 30–50 °C at 5 °C intervals. All measurements were performed in triplicate.

2.8. Galactooligosaccharides (GOS) synthesis and analysis

GOS synthesis was conducted batch-wise in duplicate in 500 mL Duran bottles, at 150 rpm and at 45, 55 and 65 °C, using the free and immobilised BbgIV on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose. Immobilisation of BbgIV on DEAE-Cellulose and Q-Sepharose was performed at pH 6.5 when the immobilised enzyme was used for GOS synthesis. Filter-sterilised lactose (43% w/w in 0.05 M sodium phosphate buffer pH 6.5) was used as the substrate. The synthesis was initiated by adding 10 U per g of lactose. Samples were collected after 0.5, 1, 2, 4, 8, 12, 16, 20 and 24 h of synthesis. The percentage lactose conversion (C) and the GOS yield (Y_p) were estimated according to the following equations:

$$C = \frac{[\text{Lac}]c}{[\text{Lac}]i} \times 100 \tag{2}$$

$$Y_p = \frac{\text{GOS produced}(g)}{\text{Total carbohydrates}(g)} \times 100 \tag{3}$$

where $[\text{Lac}]i$ and $[\text{Lac}]c$ are the molar concentrations of lactose at time 0 h and the lactose catalysed during the reaction, respectively. The enzymatic activity was measured during GOS synthesis for the free and immobilised BbgIV. The BbgIV operational stability was expressed as the operational half life ($T_{1/2}$), which was calculated by plotting the natural logarithm of U during GOS synthesis versus time. Carbohydrate analysis was conducted, in duplicate, by high performance liquid chromatography (HPLC) and high performance anion exchange chromatography coupled with pulsed amperometric detector (HPAEC-PAD). The HPLC system consisted of a G1310A isocratic pump (Agilent Technologies), a Shodex RI-71 refractive index detector (Kawasaki, Japan) and a Rezex RCM-Monosaccharide Ca^{2+} (8%) column (300 mm × 7.8 mm)

supplied by Phenomenex (Macclesfield, Cheshire, UK). The column was maintained at 84 °C and HPLC grade water was used as the mobile phase at a flow rate of 0.5 mL min^{-1}. Quantitative determination 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 HPAEC-PAD system consisted of a GS50 gradient pump, an ED50 electrochemical detector with a gold working electrode, an LC25 chromatography oven and an AS50 autosampler (Dionex Corp., Surrey, UK). Separation was performed using a pellicular anion-exchange resin based column, CarboPac PA-1 analytical (4 mm × 250 mm), connected to a CarboPac PA1 Guard (4 mm × 50 mm) (Dionex Corp., Surrey, UK). The column was maintained at 25 °C and the elution was performed at a flow rate of 1 mL min^{-1} using gradient concentrations of sodium hydroxide and sodium acetate solutions. The HPAEC-PAD method was used to quantify lactose, which was coeluted with other disaccharides in the same peak using the HPLC method.

BbgIV immobilised on Q-Sepharose was also used to conduct GOS synthesis, at 55 °C and at pH 6.5, for 6 consecutive batches using 10 U per g of lactose in the first batch. The resins were collected after obtaining the maximum GOS yield in each synthesis batch, washed with sodium phosphate buffer (50 mM, pH 6.5), assayed for the residual enzymatic activity and then used in the next synthesis batch. The productivity of GOS was expressed as the total amount of GOS (in grams) produced per litre per hour.

2.9. Scanning electron microscopy (SEM)

The resins were placed on SEM metal studs with double sided tape and coated with a thin layer of gold (10 nm thick) using an Edwards Sputter Coater S150B at a reduced pressure of 0.1 mbar and a current of 15 mA for 2 min. These gold coated samples were then observed using an FEI Quanta FEG 600 Environmental Scanning Electron Microscopy (Hillsboro, Oregon, USA) operated at a voltage of 20 KeV (kilo-electronvolts) under high vacuum mode.

2.10. Statistical analysis

All data were analysed by analysis of variance (ANOVA) using Minitab (Release 15, State College, PA, USA). Significant differences were defined at $P < 0.05$.

3. Results and discussion

3.1. Production and purification of BbgIV

The yield of BbgIV produced in *E. coli* DH5α was 20–25% of the total *E. coli* soluble proteins; this represented 71–75 mg BbgIV per g dry cell weight. The enzyme was purified from the *E. coli* crude extract almost to homogeneity and a purity of 90–93% was achieved after a two-step purification scheme including anion exchange and size-exclusion chromatography (Fig. 1). The purification yield was 75–78 and 55–57% after anion-exchange and size-exclusion
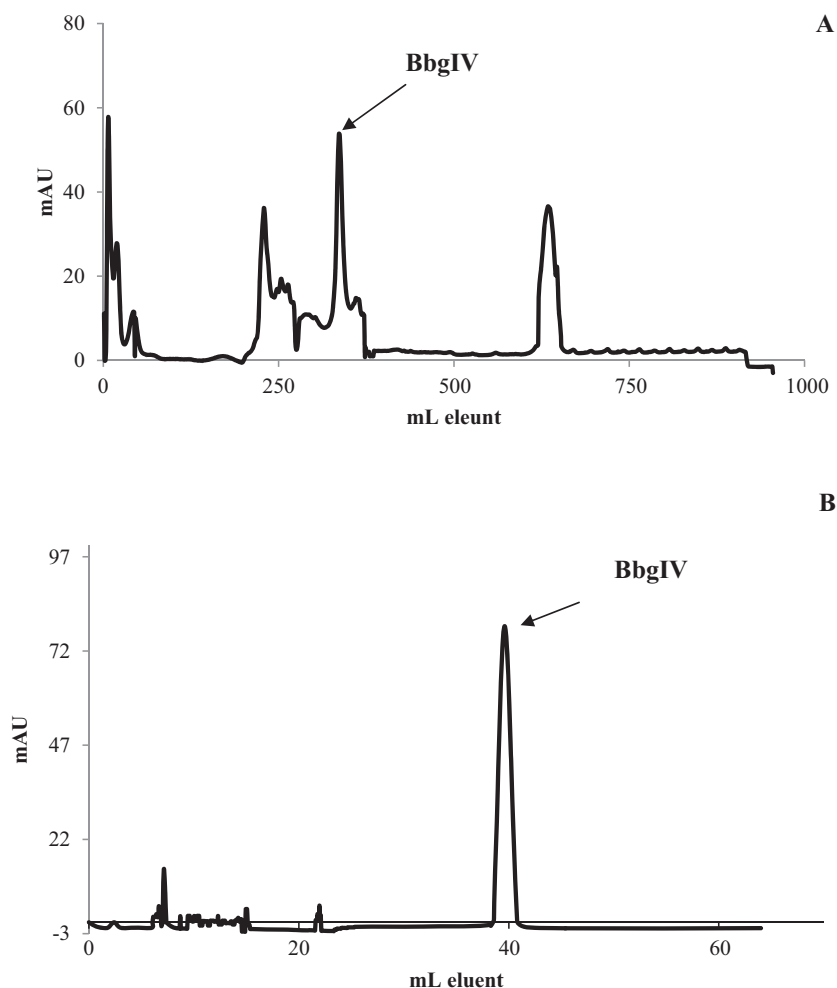

Fig. 1. AKTA FPLC chromatograms of BbgIV following anion exchange (A) and size exclusion purification (B).

Table 1
Immobilisation yield (Y_i) of BbgIV on glyoxal- and amino-ethyl-agarose beads using 1600 U per g resins. Immobilisation was performed at pH 10 for the glyoxal agarose beads.

Resin	1 h	2 h	3 h	5 h	10 h
High density glyoxal agarose 6BCL	0.85 ± 0.02	1.65 ± 0.05	1.82 ± 0.03	1.94 ± 0.02	1.22 ± 0.10
Low density glyoxal agarose-4BCL	1.36 ± 0.10	2.20 ± 0.15	2.40 ± 0.06	2.10 ± 0.04	1.73 ± 0.02
Very high density glyoxal agarose-6BCL	0.84 ± 0.05	1.80 ± 0.08	2.30 ± 0.04	2.60 ± 0.09	2.20 ± 0.40
Low density glyoxal agarose-6BCL	1.13 ± 0.01	1.24 ± 0.02	1.45 ± 0.03	1.53 ± 0.05	0.64 ± 0.04
High density glyoxal agarose-4BCL	1.30 ± 0.02	1.46 ± 0.03	1.69 ± 0.01	1.88 ± 0.04	1.58 ± 0.01
Low density amino-ethyl agarose-6BCL	16.8 ± 0.20	23.6 ± 0.60	26.4 ± 0.40	/	/
Very low density amino-ethyl agarose 4BCL	15.1 ± 0.30	18.9 ± 0.20	21.3 ± 0.70	/	/

Table 2
Immobilisation yield (Y_i) of BbgIV and the corresponding observed units of activity on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose as a function of different initial loadings of enzymatic activity.

| Initial loadings of enzymatic activity (U per g resin)	Amino-ethyl agarose		DEAE-Cellulose		Q-Sepharose	
	Y_i	Observed U per g resin	Y_i	Observed U per g resin	Y_i	Observed U per g resin
400	26.8 ± 0.5	107 ± 1	96.5 ± 0.1	385 ± 4	96.5 ± 1.6	386 ± 7
1600	26.4 ± 0.4	422 ± 2	94.9 ± 1.4	1518 ± 22	92.2 ± 0.8	1475 ± 12
2800	25.6 ± 0.9	717 ± 7	94.3 ± 1.1	2640 ± 29	90.4 ± 1.1	2531 ± 28
4000	18.6 ± 0.6	743 ± 5	67.5 ± 0.8	2760 ± 23	87.8 ± 0.7	3512 ± 28
8000	9.90 ± 0.4	792 ± 4	34.5 ± 1.3	2700 ± 34	82.1 ± 0.8	6568 ± 53
16,000	4.90 ± 0.2	784 ± 2	16.8 ± 0.6	2688 ± 16	80.1 ± 1.1	12,815 ± 153
40,000	/	/	/	/	78.2 ± 0.9	31,280 ± 281
80,000	/	/	/	/	38.5 ± 1.1	30,800 ± 338

chromatography, respectively. The BbgIV specific activity in the crude extract was 240 ± 5 U per mg protein; this increased to 1072 ± 36 and 1512 ± 27 U per mg protein after anion exchange and size exclusion, respectively. The partially purified BbgIV after anion exchange chromatography was used in its free form and after immobilisation for GOS synthesis, while the pure BbgIV after size exclusion chromatography was used in its free form and after immobilisation for estimating the pH and temperature optima, kinetic parameters and thermodynamic properties.

3.2. Covalent attachment of BbgIV to glyoxal- and amino-ethyl-agarose beads

Glyoxal activated agarose covalently binds to lysine amino groups, while amino-ethyl activated agarose covalently binds to acidic amino acids like aspartic and glutamic acids. Regarding glyoxal agarose, the immobilisation yield (Y_i) was very low and did not exceed 3% for all types of beads at pH 10 (Table 1). Also, no increase in Y_i was observed when the immobilisation was conducted at lower pH values, such as pH 8 and 9 (data not shown). The low Y_i values obtained using glyoxal agarose were most likely due to the presence of only 21 lysine residues out of 1052 amino acids in each monomer of BbgIV (see enzyme sequence with accession number ABE00939 in NCBI database). Moreover, the location of these residues is not known yet. Typically, surface-located lysine residues are expected to initiate more covalent bonds with glyoxal agarose, compared to other locations. Another likely reason was the incubation of BbgIV at high pH value (pH 10), typically used to favour a stable covalent bond formation with glyoxal agarose. This most likely resulted in unfavourable distortions and losses in the enzyme stability, despite the use of protecting agents. Due to the very low Y_i values (Table 1), glyoxal agarose beads were not further used for BbgIV immobilisation. The immobilisation yield of other enzymes, including β-galactosidases, on glyoxal agarose was reported by other researchers to be higher than what was found in this study. The immobilisation yield of *A. oryzae* β-galactosidase, for instance, was 42.7% [9]. Other enzymes showed immobilisation yields even higher than 50% [20]. However, different enzymes vary

in the sequence and location of their amino acids; thus, they show different immobilisation yields on glyoxal agarose.

For amino-ethyl agarose, the maximum Y_i value was obtained after 3 h of incubation and was significantly ($P < 0.05$) higher compared to glyoxal agarose (Table 1). Incubating BbgIV with amino-ethyl agarose for more than 3 h was not carried out, as the coupling agent (CDI) used for immobilisation is known to decompose after 3 h, according to the manufacturer. Based on the values of Y_i in Table 1, the low density amino-ethyl agarose 6BCL was selected to assess its maximum capacity for immobilising BbgIV. The maximum activity observed on the beads, i.e. 792 ± 4 U per g resin, was obtained using the initial enzymatic loading of 8000 U per g resin, which represented a Y_i value of only $9.9 \pm 0.4\%$ (Table 2). Due to this low Y_i value, another initial enzymatic loading, i.e. 2800 U per g resin, was selected to immobilise BbgIV on low density amino-ethyl agarose for GOS synthesis. In this case, 717 ± 7 U (2.6 mg protein) per g resin were observed, which represented an immobilisation yield of $25.6 \pm 0.9\%$; both values were close to the maximum obtained for these two parameters (Table 2). The use of amino-ethyl agarose resulted in obtaining higher immobilisation yields compared to glyoxal agarose, as 100 aspartic acid and 71 glutamic acid residues, out of 1052 amino acids, are present in each monomer of BbgIV. This most probably increased the likelihood of covalent bond formation between the enzyme and amino-ethyl groups compared to glyoxal groups. Another reason was the use of distilled water as a medium for immobilisation, and therefore harsh alkaline conditions were avoided. Despite this, Y_i did not exceed 28% (Table 2). Low immobilisation yields, concomitant with enzymatic activity losses, are common observations in covalent binding type of immobilisation. This is usually due to the fact that covalent binding might involve functional groups that are near the enzyme active site and the fact that exposure to chemical reagents (carbodiimide in the case of amino-ethyl agarose) might negatively affect the active site. Low immobilisation yields were also reported for other β-galactosidases immobilised via covalent binding. For instance, the immobilisation yield of *A. oryzae* β-galactosidase covalently bound to cotton cloth activated with tosyl chloride was 30% [12], while it was only 18.5 and 10% when the same enzyme was covalently bound to chitosan [21]
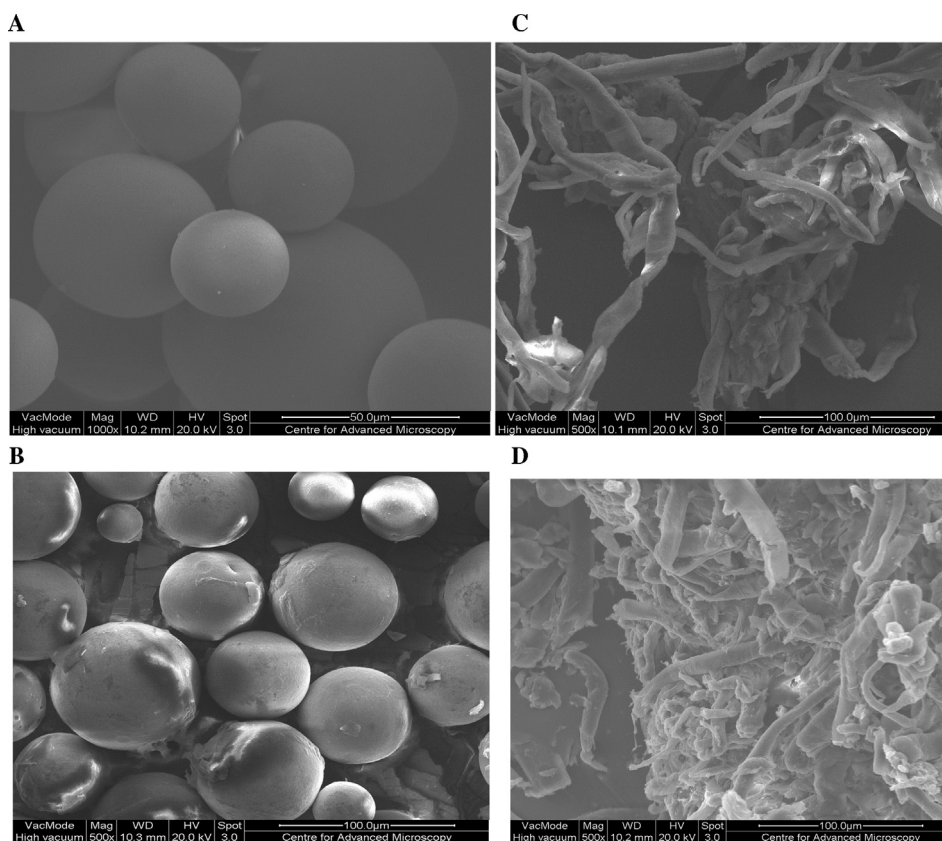
Fig. 2. Scanning electron micrography (SEM) showing the microstructure of Q-Sepharose before (A) and after (B) BbgIV immobilisation, and DEAE-Cellulose before (C) and after (D) BbgIV immobilisation.

and cotton cloth activated with polyethyleneimine-glutaraldehyde [22], respectively.

3.3. Ionic binding of BbgIV using DEAE-Cellulose and Q-Sepharose

Immobilisation via ionic binding is a simple method, which involves binding two oppositely charged ions via ionic interaction. Enzymes are negatively charged at pH values above their isoelectric points and therefore positively charged ion exchangers can be used for their immobilisation. The effect of pH on the immobilisation yield, using DEAE-Cellulose and Q-Sepharose, was evident due to the mode of immobilisation. The maximum Yi was obtained at pH 6 for DEAE-Cellulose and at pH 7 for Q-Sepharose. At pH 5, 7, 8 and 9, Yi was 36–38, 76–78, 69–71 and 58–60%, respectively, of the maximum yield obtained for DEAE-Cellulose. Also, at pH 5, 6, 8 and 9, Yi was 53–56, 79–83, 88–90 and 66–67%, respectively, of the maximum yield obtained for Q-Sepharose.

For both resins, the enzymatic activity observed on the beads increased as a function of the initial enzymatic loading until a maximum was obtained and was then followed by a stable level of activity (Table 2). For DEAE-Cellulose, the maximum activity, i.e. 2760 ± 23 U per g resin, was obtained using the initial enzymatic loading of 4000 U per g resin, which corresponded to a Yi value of $67.5 \pm 0.8\%$. However, 2800 U per g resin were selected as the initial enzymatic loading to immobilise BbgIV on DEAE-Cellulose for GOS synthesis, as the corresponding Yi was significantly ($P < 0.05$) higher, i.e. $94.3 \pm 1.1\%$, and the activity observed on the resin, i.e. 2640 ± 29 U (9.8 mg protein) per g resin, was close to the maximum (Table 2). For Q-Sepharose, the maximum activity, i.e., $31,280 \pm 281$ U (114 mg protein) per g resin, was obtained using the initial enzymatic loading of 40000 U per g resin and was significantly ($P < 0.05$) higher than those obtained using lower initial

enzymatic loadings. The corresponding Yi value was also reasonable, i.e. 78.2 ± 0.9 (Table 2). Therefore, 40,000 U per g resin were selected as the initial enzymatic loading to immobilise BbgIV on Q-Sepharose for GOS synthesis.

Of great importance is the difference in the microstructure and surface morphology between both resins. This leads to understanding the spatial arrangement of both resins and their ionic interaction with BbgIV. Q-Sepharose is a well-defined spherically beaded set of particles, with a diameter of 45–160 μm (Fig. 2). The matrix is a highly cross-linked agarose (6%) to which charged groups, in the form of quaternary ammonium (strong ion exchanger), are attached. The DEAE-Cellulose used, however, is a fibrous material with a very compact web-like structure (Fig. 2) that is derivatised with di-ethyl amino-ethyl tertiary amine functional groups (weak ion exchanger). These differences in the support material, the nature of the charged groups, the geometry of the supports and the total numbers and availability of the charged groups determine the type and strength of the ion exchanger as well as its binding capacity. Therefore, the fact that Q-Sepharose was able to bind 11 times more active enzyme than DEAE-Cellulose (Table 2) is well understood. Obtaining a high loading capacity of active BbgIV on Q-Sepharose is very desirable, as this leads to achieving high conversion efficiency per unit mass of the resin.

In addition, the immobilisation yield and the BbgIV activity on the resins using ionic binding were significantly ($P < 0.05$) higher compared to covalent attachment (Table 2). This was most likely due to the simplicity, mild conditions and absence of chemical reagents during the ionic binding procedure. Despite this, it is not always true that non covalent attachment leads to high immobilisation yields and better enzymatic activity, compared to covalent attachment. For instance, the immobilisation yield of A. oryzae β-galactosidase was more than 70% using covalent attachment on

chitosan granules activated with glutaraldehyde [9], while it was only 2% for the same enzyme using adsorption on celite [21].

3.4. Temperature and pH optima, kinetic parameters and thermodynamic properties of free and immobilised BbgIV

The extent to which changes in the pH and temperature optima of β-galactosidases take place, as a result of immobilisation, is based on the source, sequence and structure of the used enzymes as well as the supports and techniques used during immobilisation. In the case of BbgIV, the pH and temperature optima were not affected by immobilising the enzyme on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose. The optimum pH of activity for the free enzyme was 6.4. After immobilisation on Q-Sepharose and amino-ethyl agarose, the optimum pH of activity remained constant, i.e. 6.4, while it increased to 6.6 after immobilisation on DEAE-Cellulose. In addition, the optimum temperature of activity for the free enzyme was 54 °C. After immobilisation on DEAE-Cellulose, the optimum temperature remained constant, while it decreased to 52 °C after immobilisation on Q-Sepharose and amino-ethyl agarose. In several other studies conducted with β-galactosidases, the pH and temperature optima were not altered by immobilisation, as in the case of *A. oryzae* β-galactosidase immobilised on fibres of alginate and gelatine hardened with glutaraldehyde [23], *A. aculeatus* β-galactosidase immobilised on Eupergit C [24] and *K. lactis* β-galactosidase immobilised on polysiloxane-polyvinyl alcohol magnetic composite [25]. On the other hand, a decrease in the pH optimum from 4.5 to 3 was observed for *A. oryzae* β-galactosidase when cross linked enzyme aggregates were produced using glutaraldehyde [21]. A similar shift in the pH optimum from 7 to 6 and the temperature optimum from 35 to 50 °C was also reported for *K. Fragilis* β-galactosidase upon its immobilisation on silanised glass modified by glutaraldehyde [26].

For kinetic parameters, K_m increased from 2.08 ± 0.2 mM in the case of free BbgIV to 2.41 ± 0.2, 2.51 ± 0.2 and 2.81 ± 0.2 mM after immobilisation on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose, respectively; a significant ($P < 0.05$) increase in K_m took place only after immobilisation on amino-ethyl agarose. The increase in K_m after immobilisation is usually due to conformational, steric, diffusional and mass transfer effects taking place at the microenvironment of the enzyme. Several researchers have also found that K_m increased significantly upon immobilisation. Examples, in this regard, are the 2.3 fold increase in K_m when *A. oryzae* β-galactosidase was immobilised on concanavalin A layered celite 545 [28], the 2 fold increase in K_m after immobilising *A. aculeatus* β-galactosidase on Eupergit C [24] and the 2.5 fold increase in K_m upon immobilising *A. oryzae* β-galactosidase on chitosan [21]. Other researchers, on the other hand, reported only minor changes in the K_m of β-galactosidases upon immobilisation such as Tanriseven et al. [23] who observed an increase in K_m from 42 to 51 mM after immobilising *A. oryzae* β-galactosidase on fibres of alginate and gelatine hardened with glutaraldehyde, and Lu et al. [27] who did not report any change in the K_m of the β-galactosidase (CBD-BgaL3) from *Lactobacillus bulgaricus* L3 upon its immobilisation on microcrystalline cellulose.

It is also noteworthy to state that V_{max} increased from 700.6 ± 31.1 U per mg protein in the case of free BbgIV to 818.5 ± 47.2, 923.4 ± 43.5 and 732.8 ± 27.4 U per mg protein after immobilisation on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose, respectively. Consequently, K_{cat} increased from 1377 s^{-1} in the case of free BbgIV to 1610, 1816 and 1441 s^{-1} after immobilisation on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose, respectively. The observed increase in V_{max} and K_{cat} is very desirable, suggesting that the enzyme active site was still easily accessible by the substrate after immobilisation. In several other

studies, however, immobilisation of β-galactosidases resulted in a decrease in V_{max}; the extent of which depended on the enzyme source as well as the immobilisation supports and techniques [11,27–29]. As a result of the measured kinetic parameters, the catalytic efficiency (K_{cat}/K_m) remained almost constant when BbgIV was immobilised on DEAE-Cellulose, i.e. 6.6×10^5 M^{-1}.s^{-1} for free BbgIV and 6.4×10^5 M^{-1}.s^{-1} after immobilisation on DEAE-Cellulose, while it increased to 7.5×10^5 M^{-1}.s^{-1} after immobilisation on Q-Sepharose and decreased to 5.1×10^5 M^{-1}.s^{-1} after immobilisation on amino-ethyl agarose.

In terms of thermodynamic properties, the activation energy (Ea), activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) for free BbgIV were 19.4 kJ. mol^{-1}, 16.9 kJ mol^{-1} and -1.9 J K^{-1} mol^{-1}, respectively. When BbgIV was immobilised on DEAE-Cellulose, Ea, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ increased to 23.7 kJ mol^{-1}, 21.1 kJ mol^{-1} and -1.7 J K^{-1} mol^{-1}, respectively. Likewise, Ea, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ increased to 27.9 kJ mol^{-1}, 25.3 kJ mol^{-1} and -1.5 J K^{-1} mol^{-1}, respectively, after immobilisation on Q-Sepharose. Covalent attachment of BbgIV to amino-ethyl agarose had similar effects, i.e. Ea, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ increased to 24.3 kJ mol^{-1}, 21.7 kJ mol^{-1} and -1.7 J K^{-1} mol^{-1}, respectively. The values of activation energy and activation enthalpy are within the typical range of 15–150 kJ mol^{-1}, and the observed increase in Ea and $\Delta H^{\ddagger}$ upon immobilisation indicated that the energy needs of lactose catalysis by BbgIV slightly increased after immobilisation. Moreover, no differences were found in the activation entropy between the free and immobilised BbgIV. The low negative values of $\Delta S^{\ddagger}$ indicated that the transition state had slightly more ordered and rigid structure than the reactants in the ground state. Based on the very low values of activation entropy compared to activation enthalpy, the activation barrier of lactose catalysis by BbgIV is mainly enthalpic in nature with a negligible entropic contribution. The thermodynamic properties of free and immobilised β-galactosidases were also measured by few other researchers. For instance, Neri et al. [25] found no significant difference in Ea between free and immobilised β-galactosidase from *K. Lactis*, while Tu et al. [30] reported a significant increase in Ea from 41.6 to 71 kJ mol^{-1} after immobilising *Cicer arietinum* β-galactosidase on the resin D202.

3.5. GOS synthesis using free and immobilised BbgIV

Development of the GOS synthesis curve, as a function of time, was similar between free and immobilised BbgIV (Fig. 3). Each curve consisted of three distinctive phases, i.e. synthesis, equilibrium and hydrolysis. In the synthesis phase, the GOS yield increased significantly ($P < 0.05$) as a function of time until a maximum was obtained at different time points. This was followed by the equilibrium phase where GOS yield remained constant, indicating a balanced state between GOS synthesis and hydrolysis. Then, a decrease in GOS yield took place, as hydrolysis became more pronounced than synthesis (Fig. 3).

The maximum GOS yield (Yp) and the duration and extent of each phase depended on the type of BbgIV and the synthesis temperature. For free BbgIV, the maximum Yp (49–53%) (Fig. 3) was obtained at 83–89% lactose conversion (Fig. 4) after 20, 16 and 8 h at 45, 55 and 65 °C, respectively. When BbgIV was immobilised on DEAE-Cellulose, the maximum Yp (49–51%) (Fig. 3) was obtained at 85–88% lactose conversion (Fig. 4) after 12 and 8 h at 45 and 55 °C, respectively. At 65 °C, however, lactose was not fully catalysed, i.e. only 55.9–56.5% of lactose was converted after 20–24 h (Fig. 4), due to a significant ($P < 0.05$) deactivation of BbgIV (Fig. 5) and therefore a GOS yield of only 34.5–35.5% was obtained after 20–24 h (Fig. 3). For BbgIV immobilised on Q-Sepharose, the maximum Yp (49–53%) (Fig. 3) was obtained at 83–88% lactose conversion (Fig. 4) just after 2 h at 45 and 55 °C and after 20 h at 65 °C. The delay in obtaining the maximum Yp at 65 °C was also due to the deactivation of BbgIV
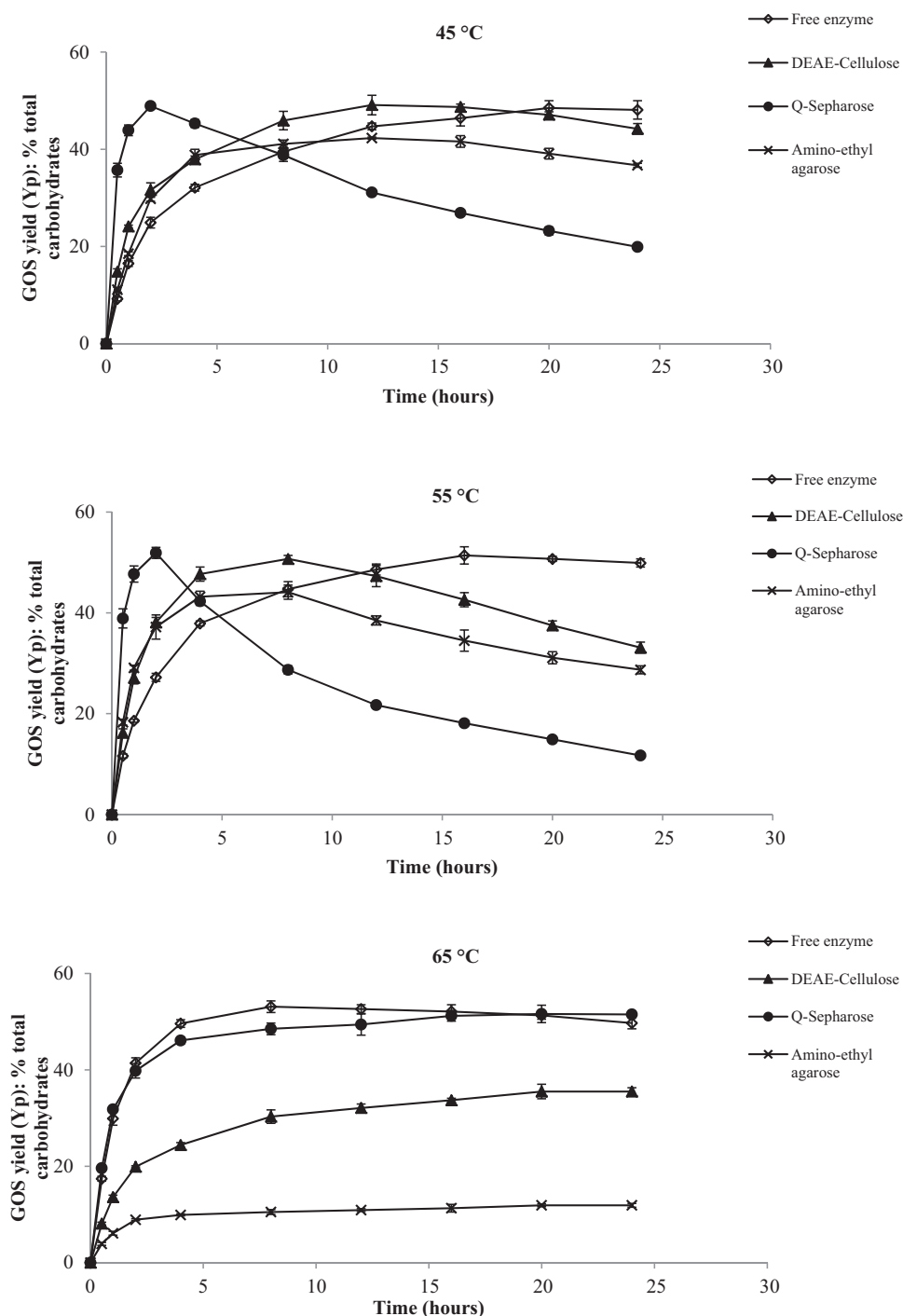
Fig. 3. Time course of GOS synthesis using free and immobilised BbgIV on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose at 45, 55 and 65 °C.

(Fig. 5). Despite this, the residual activity was most likely adequate to catalyse the synthesis of GOS; lactose conversion in this case was $84.4 \pm 1.9\%$ after 24 h (Fig. 4). In the case of BbgIV immobilised on amino-ethyl agarose, the maximum Yp (42–44%) (Fig. 3) was obtained at 88–92% lactose conversion (Fig. 4) after 12 and 8 h at 45 and 55 °C, respectively. At 65 °C, there was a significant ($P < 0.05$) deactivation of BbgIV (Fig. 5); thus, lactose was barely converted, i.e. only $25.4 \pm 1.5\%$ after 24 h (Fig. 4), and a very low GOS yield, i.e. $11.9 \pm 1.2\%$, was obtained (Fig. 3).

It is noteworthy to state that the maximum Yp obtained at 45 and 55 °C using BbgIV immobilised on DEAE-Cellulose and Q-Sepharose was similar to that obtained using the free enzyme (Fig. 3),

further confirming the fact that diffusion limitation and mass transfer issues were not observed upon immobilising BbgIV via ionic binding. In accordance with these results, other researchers confirmed that free and immobilised β-galactosidases produced similar GOS yields. For instance, Huerta et al. [9] found that the GOS yield produced using *A. oryzae* β-galactosidase immobilised on amino-epoxy sepabeads (39.4–46.4%) and glyoxal agarose 6BCL (46.6–46.7%) was similar to the yield produced using the free enzyme (36.1–46.1%). Albayrak et al. [12] also obtained similar GOS yields, i.e. 21–26%, when *A. oryzae* β-galactosidase was used in its free form and after immobilisation on cotton cloth activated with tosyl chloride.
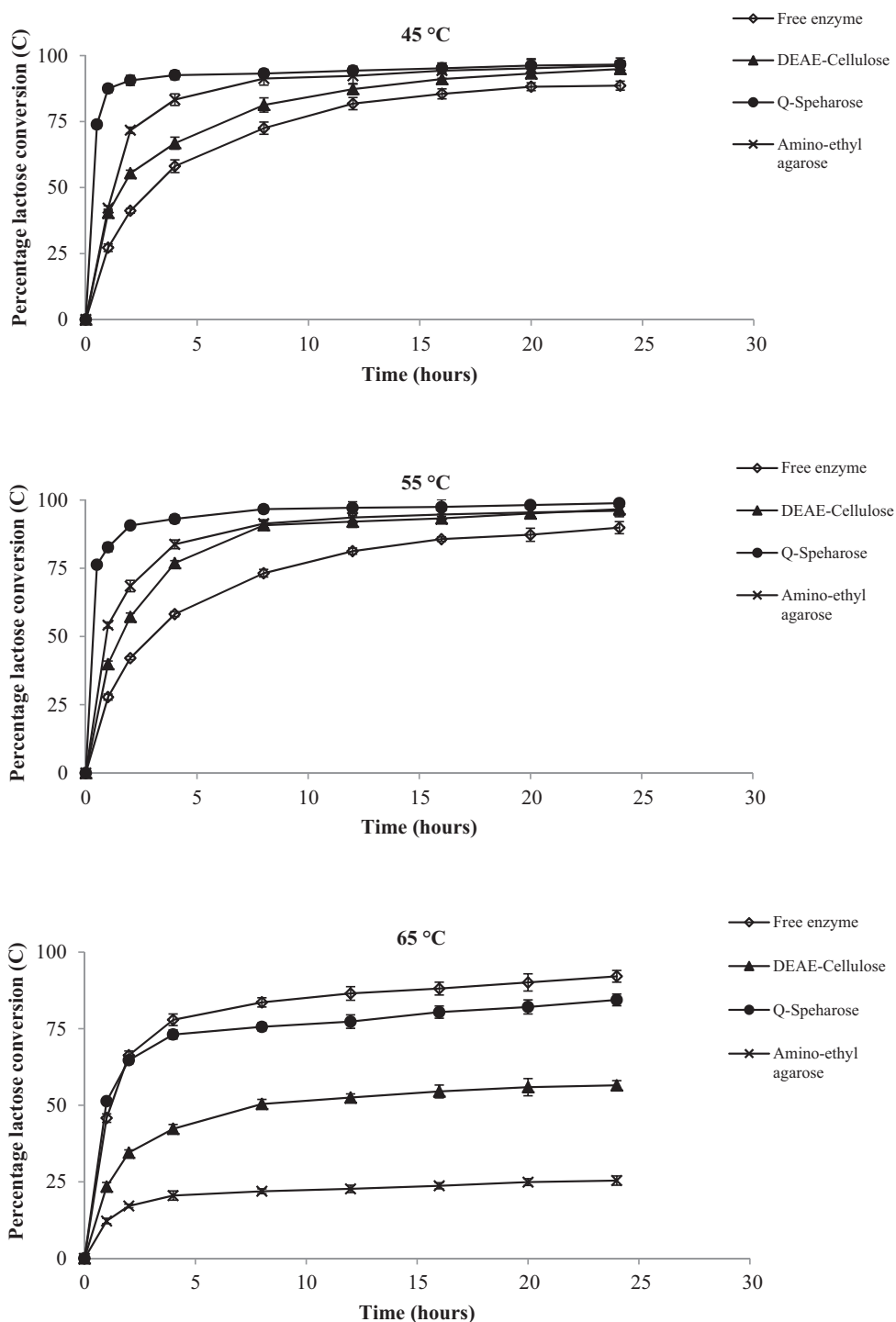
Fig. 4. Time course of lactose conversion (C) using free and immobilised BbgIV on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose at 45, 55 and 65 °C.

On the other hand, it is known that diffusion limitation and mass transfer issues have an impact not only on the reaction rate but also on the type of products formed, especially in the case of competing reactions with varying substrate sizes. This is of particular importance in GOS synthesis reactions, which involve several substrates and two competing pathways; one is transgalactosylation which leads to the formation of GOS and the other is hydrolysis leading to the formation of the hydrolytic products galactose and glucose [4]. Therefore, the GOS yield and the type of GOS products formed might be affected by diffusion limitation and mass transfer issues.

An example of such effects in this study was observed when BbgIV was immobilised on amino-ethyl agarose via covalent binding, as a lower maximum GOS yield, i.e. 42–44%, was obtained at 45 and 55 °C compared to that obtained using the free enzyme (Fig. 3). This indicated that BbgIV immobilised on amino-ethyl agarose preferred more hydrolysis and less transgalactosylation compared to the free enzyme, most likely due to events taking place at the enzyme microenvironment such as mass transfer resistance and diffusion limitations imposed by the covalent binding of aspartic and glutamic acid residues present in BbgIV to the amino-ethyl
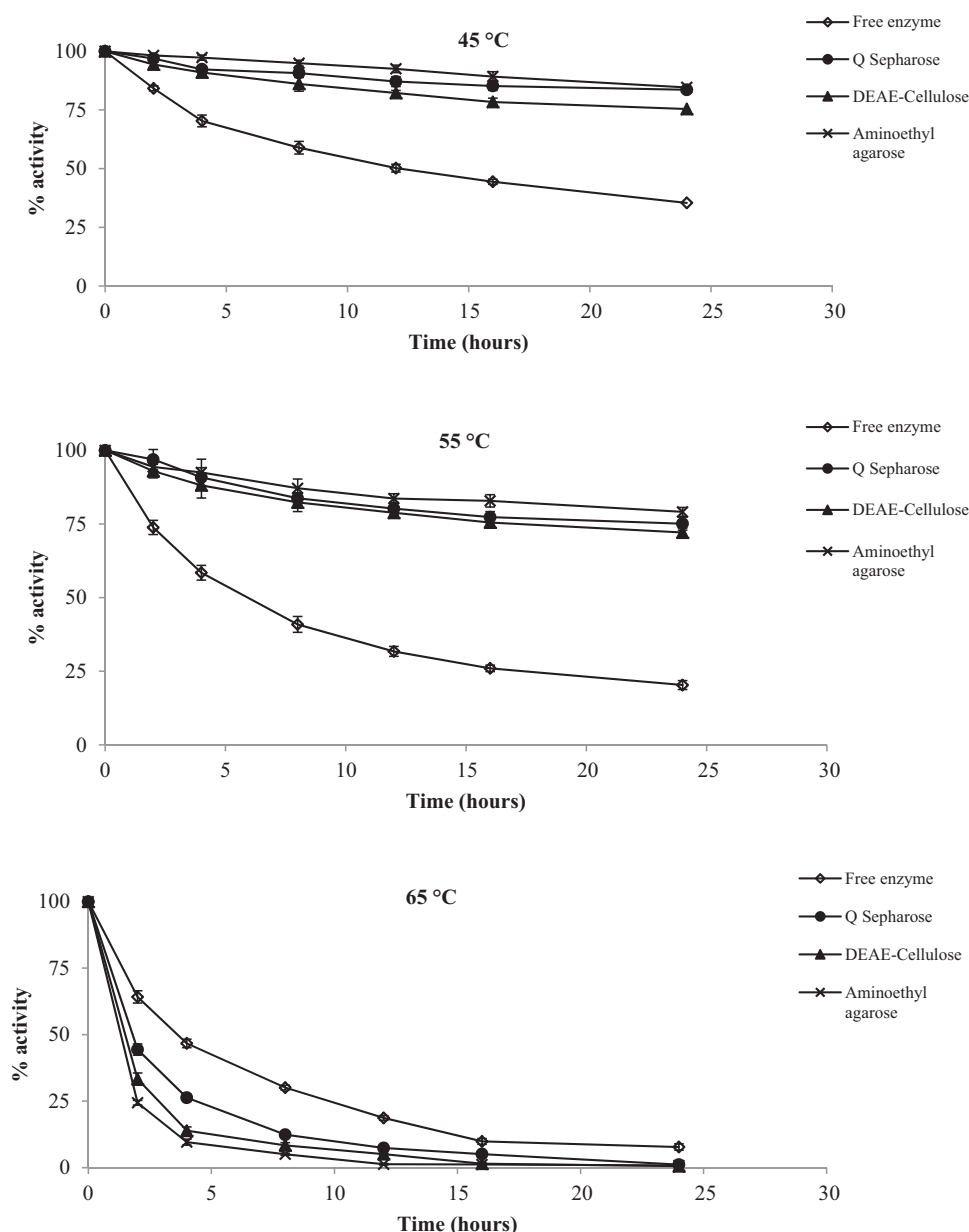
Fig. 5. Operational stability of free and immobilised BbgIV on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose at 45, 55 and 65 °C.

groups on the resin. Several other researchers also pointed out the occurrence of such a phenomenon and the obtainment of low GOS yields using immobilised β-galactosidases [31–33].

The produced GOS profile consisted of di-, tri-, tetra- and pentasaccharides (Table 3). No significant differences ($P > 0.05$) were found in the GOS profile between the free and immobilised BbgIV. However, the final GOS mixture obtained using BbgIV immobilised on amino-ethyl agarose at 45 and 55 °C contained slightly less trisaccharides compared to the mixture obtained using other forms of BbgIV (Table 3). Besides, the disaccharide content in the final GOS mixture obtained at 65 °C using BbgIV immobilised on DEAE-Cellulose was significantly ($P < 0.05$) higher than that obtained using other forms of BbgIV (Table 3); however, this difference should not be considered as lactose conversion was incomplete at 65 °C using BbgIV immobilised on DEAE-Cellulose (Fig. 4). In other studies, the GOS profile obtained was more clearly affected when an immobilised β-galactosidase was used instead of the free enzyme. For instance, Huerta et al. [9] reported an increase

in trisaccharides from 70 to 77–80% (percent of GOS mixture), a decrease in tetrasaccharides from 20 to 15–17% and a decrease in pentasaccharides from 9 to 4–4.8%, when a β-galactosidase from *A. oryzae* was used in its immobilised form instead of the free enzyme.

3.6. Operational stability of BbgIV

One of the most practically important criteria of industrial enzymes is their operational stability during catalysis. Immobilisation has been always documented to improve the operational stability of β-galactosidases from different sources such as *A. oryzae* [11,28], *L. bulgaricus* [27], *K. lactis* [25] and *K. fragilis* [26]. Immobilisation is known to protect enzymes from the damaging effects of the surrounding environment and increase their operational stability by reducing their structural mobility and increasing their rigidity in the newly formed enzyme-support complex. Immobilisation of BbgIV significantly ($P < 0.05$) increased the enzyme operational stability at 45 and 55 °C compared to

Table 3
Maximum GOS yield, profile of GOS mixture (% of oligosaccharides of different degrees of polymerisation (DP) in the final GOS mixture obtained at the time point of the maximum GOS yield), synthesis productivity and operational half life ($T_{1/2}$) of BbgIV during GOS synthesis at 45, 55 and 65 °C using free and immobilised BbgIV on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose.

Parameter	45 °C				55 °C				65 °C			
	Free BbgIV	DEAE-Cellulose	Q-Sepharose	Amino-ethyl agarose	Free BbgIV	DEAE-Cellulose	Q-Sepharose	Amino-ethyl agarose	Free BbgIV	DEAE-Cellulose	Q-Sepharose	Amino-ethyl agarose
Yp	48.5 ± 1.6	49.1 ± 2.0	48.9 ± 0.6	42.3 ± 0.2	51.4 ± 1.7	50.7 ± 0.7	51.9 ± 1.1	44.1 ± 1.4	53.1 ± 1.2	35.5 ± 1.5	51.6 ± 1.8	11.9 ± 0.4
DP 2 (% of total GOS)	57.2 ± 1.1	58.6 ± 0.9	59.1 ± 0.9	61.7 ± 1.2	55.6 ± 0.7	57.3 ± 1.1	56.5 ± 0.8	57.9 ± 0.1	52.1 ± 0.5	60.1 ± 1.4	50.4 ± 0.7	53.1 ± 0.8
DP 3 (% of total GOS)	35.6 ± 0.9	35.5 ± 0.4	35.4 ± 0.7	31.7 ± 0.6	37.3 ± 0.6	36.3 ± 0.6	36.2 ± 0.5	32.8 ± 0.8	36.8 ± 0.7	32.3 ± 0.4	40.1 ± 0.5	36.1 ± 0.9
DP ≥ 4 (% of total GOS)	7.2 ± 0.3	6.90 ± 0.2	6.50 ± 0.2	7.6 ± 0.1	8.10 ± 0.2	7.40 ± 0.3	8.30 ± 0.1	9.30 ± 0.4	10.1 ± 0.2	7.60 ± 0.2	9.50 ± 0.4	10.8 ± 0.3
Productivity g L⁻¹ h⁻¹	13.4 ± 0.9	22.6 ± 0.8	135.2 ± 4.2	19.5 ± 0.6	17.7 ± 0.8	35.1 ± 1	143.5 ± 4.6	30.5 ± 0.5	36.6 ± 1.3	9.80 ± 0.5	14.3 ± 0.2	3.3 ± 0.2
$T_{1/2}$ (h)	12.9 ± 0.9	56.9 ± 1.2	90.1 ± 1.6	100.3 ± 1.5	7.30 ± 0.6	48.7 ± 1.7	52.8 ± 1.1	71.2 ± 2.3	4.2 ± 0.4	0.80 ± 0.1	1.40 ± 0.2	0.6 ± 0.1

free BbgIV (Fig. 5). More specifically, while free BbgIV retained 35.4 ± 1.1% of its initial activity after 24 h at 45 °C, BbgIV immobilised on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose retained 75.4 ± 1.6, 83.6 ± 1.9 and 84.6 ± 2.1% of its initial activity, respectively, under the same conditions (Fig. 5). Likewise, BbgIV immobilised on DEAE-Cellulose, Q-Sepharose and amino-ethyl agarose retained 72.1 ± 1.5, 75.1 ± 2.2 and 79.1 ± 1.5% of its initial activity, respectively, after 24 h at 55 °C, while free BbgIV retained only 20.3 ± 1.5% of its initial activity, under the same conditions (Fig. 5).

As the operational stability of BbgIV at 45 and 55 °C increased as a result of immobilisation, the maximum Yp was obtained at shorter reaction times compared to that obtained using free BbgIV (Fig. 3). This was due to the presence of higher enzymatic activity per unit mass of lactose in the case of immobilised BbgIV, compared to the free enzyme. Of great interest was the immobilisation of BbgIV on Q-Sepharose, as the maximum Yp was obtained just after 2 h of synthesis at 45 and 55 °C, which also increased the productivity of GOS synthesis to > 135 g L⁻¹ h⁻¹ (Table 3). In addition to the effect of improved operational stability of BbgIV after immobilisation on Q-Sepharose, the fact that the maximum Yp was obtained just after 2 h of synthesis at 45 and 55 °C might be also due to the increase in the catalytic efficiency of BbgIV after immobilisation on Q-Sepharose, as discussed previously. Moreover, it might be also due to the fact that, upon immobilisation on Q-Sepharose, events such as positive partition effects and improved conformational flexibility took place in the microenvironment of BbgIV. Such effects are of particular importance for kinetically controlled synthesis reactions, as in the case of GOS synthesis, and can speed up the enzyme activity when supersaturated lactose solutions are used as both the substrate and acceptors of the galactosyl moieties. Few other studies, in this regard, have observed similar improvements in the enzyme activity and functionality upon immobilisation, such as the epoxy hydrolase adsorbed on DEAE-Cellulose [34] and the lipase-lipid complex entrapped in n-vinyl-2-pyrrolidone gel matrix [35].

In contrast to 45 and 55 °C, the immobilised BbgIV at 65 °C did not retain its activity compared to its free form (Fig. 5). Therefore, lactose was not fully catalysed, particularly for DEAE-Cellulose and amino-ethyl agarose (Fig. 4), and hence low GOS yields were obtained, as previously shown (Fig. 3). The low operational stability of immobilised BbgIV at 65 °C was most likely due to accelerated Maillard reactions, compared to lower temperatures. At 65 °C, a very dark brown colour was developed in the reaction medium, which indicated that Maillard reactions took place between the reducing sugars (very high concentration in the reaction medium) and the amino groups of the enzyme and particularly the amino groups found on the supports (all three supports contain numerous amino groups). In this context, it has been already suggested that Maillard glycation of enzymes takes place during oligosaccharide synthesis and can have a significant impact on the activity of the used enzyme [36]. Overall, a several fold increase in the operational half life of BbgIV ($T_{1/2}$) was observed at 45 and 55 °C upon immobilisation (Table 3), suggesting that immobilised BbgIV can be used to conduct GOS synthesis for several batches at temperatures up to 55 °C.

3.7. Repeated use of BbgIV immobilised on Q-Sepharose in GOS synthesis

BbgIV immobilised on Q-Sepharose was selected to carry out GOS synthesis in 6 consecutive batches at 55 °C, due to the high capacity of Q-Sepharose to immobilise BbgIV (Table 2), the high GOS yield obtained (Fig. 3), the improved operational stability of the enzyme (Fig. 5) and the fact that the maximum Yp was obtained in just 2 h after the start of the synthesis (Fig. 3). The maximum Yp obtained was similar in all the 6 synthesis batches, i.e. 49–53%. In

the first two batches, the maximum *Yp* was obtained just after 2 h of synthesis, while it was obtained after 3 h in the third batch. For batches 4 and 5, the maximum *Yp* was obtained after 4 h, whereas it took 5 h in the last batch to obtain the maximum *Yp*. Moreover, the loss in BbgIV activity took place gradually during the 6 successive synthesis batches. The loss in the enzymatic activity was 4.8 ± 0.3, 11.5 ± 0.6, 16.8 ± 0.9, 21.7 ± 0.8, 28.6 ± 0.5 and $32.5 \pm 1.1\%$ after the first, second, third, fourth, fifth and last synthesis batch, respectively. Other researchers have also demonstrated that immobilised β-galactosidases can be used in several repeated batches for GOS synthesis. For instance, *L. bulgaricus* β-galactosidase immobilised on microcrystalline cellulose was used for 20 repeated batches at 45 °C for GOS synthesis; the GOS yield obtained was >40% and the immobilised enzyme retained >85% of its initial activity [27]. Moreover, *A. oryzae* β-galactosidase immobilised on polysiloxane coated with polyaniline was used for 10 batches at 25 °C and retained 87% of its initial activity [11].

Despite the partial loss in the activity of immobilised BbgIV during the repeated batches of GOS synthesis, it should be kept in mind that the repeated use of immobilised BbgIV was performed at 55 °C which is much higher than most of the temperatures used to carry out repeated GOS synthesis in the literature. At this high temperature, BbgIV immobilised on Q-Sepharose was able to carry out GOS synthesis with a yield of 49–53% and retain 67.5% of its activity after 6 repeated uses. This indicated that immobilisation is very likely to have a significant impact on the cost of GOS synthesis using BbgIV.

4. Conclusion

This is the first work that demonstrates the potential of using immobilised BbgIV, a β-galactosidase from *B. bifidum* NCIMB 41171, to catalyse the synthesis of prebiotic GOS. Of the different supports tried, Q-Sepharose was particularly interesting due to its high enzyme loading capacity and the fast lactose conversion obtained using this support. Therefore, a robust process, including six repeated GOS synthesis batches, was developed using BbgIV immobilised on Q-Sepharose. As this enzyme has been recently produced and used on an industrial scale, the results of this work are expected to improve the productivity and robustness of the GOS synthesis process from an industrial perspective.

Acknowledgments

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