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High Yield Production of a Soluble Bifidobacterial β-Galactosidase (BbgIV) in *E. coli* DH5α with Improved Catalytic Efficiency for the Synthesis of Prebiotic Galactooligosaccharides

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ABSTRACT: The bifidobacterial β-galactosidase (BbgIV) was produced in *E. coli* DH5α at 37 and 30 °C in a 5 L bioreactor under varied conditions of dissolved oxygen (dO2) and pH. The yield of soluble BbgIV was significantly ($P < 0.05$) increased once the dO2 dropped to 0−2% and remained at such low values during the exponential phase. Limited dO2 significantly ($P < 0.05$) increased the plasmid copy number and decreased the cells growth rate. Consequently, the BbgIV yield increased to its maximum (71−75 mg per g dry cell weight), which represented 20−25% of the total soluble proteins in the cells. In addition, the specific activity and catalytic efficiency of BbgIV were significantly ($P < 0.05$) enhanced under limited dO2 conditions. This was concomitant with a change in the enzyme secondary structure, suggesting a link between the enzyme structure and function. The knowledge generated from this work is very important for producing BbgIV as a biocatalyst for the development of a cost-effective process for the synthesis of prebiotic galactooligosaccharides from lactose.

KEYWORDS: *β-galactosidase, Bifidobacterium, protein expression, dissolved oxygen, prebiotic galactooligosaccharides, specific activity*

INTRODUCTION

β-Galactosidases (EC 3.2.1.23) are ubiquitous enzymes in nature. According to the International Union of Biochemistry and Molecular Biology (IUBMB), β-galactosidases catalyze the hydrolysis of terminal nonreducing β-D-galactose residues in β-D-galactosides. One of the preferred substrates for this enzyme is the disaccharide lactose. The application of β-galactosidase in the hydrolysis of lactose into its monosaccharide components, glucose and galactose, is a well-established industrial process which increases the sweetness of food products containing lactose and helps making dairy nutrients available to lactose intolerant individuals.[1] More recently, the use of β-galactosidase to catalyze the synthesis of galactooligosaccharides (GOS) from lactose through transgalactosylation reactions has become a process of great interest, due to the emerged health benefits associated with the consumption of prebiotic GOS as functional food ingredients.[2,3] β-Galactosidases from various sources such as *Lactobacillus*, *Bifidobacterium*, *Aspergillus* and *Kluyveromyces* species have been used as either whole cell or single enzyme biocatalysts to conduct GOS synthesis reactions. Compared to whole cells, the use of free β-galactosidases usually increases GOS synthesis reaction rates and leads to obtaining the maximum GOS yield in shorter reaction times.[4] Additionally, the use of free β-galactosidases ensures that transgalactosylation can be better controlled compared to the use of whole cells, especially when whole cells contain multiple β-galactosidases which differ in their biochemical characteristics and their ability to conduct GOS synthesis.[4,5] However, the use of native β-galactosidases as biocatalysts in GOS synthesis requires the isolation of the enzyme either from the producing micro-organism or from the culture medium. In both cases, the isolation process is tedious and costly as bacterial β-galactosidases, in particular, are naturally found in low quantities. This represents the major hurdle in using free β-galactosidases as biocatalysts in GOS synthesis reactions. Recombinant DNA technology offers the possibility to produce high yields of the β-galactosidase of interest using suitable expression hosts, such as *Escherichia coli*. Compared to native β-galactosidases, the use of recombinant β-galactosidases offers various advantages such as the large scale production, the ease of purification due to high expression yields and the possible improvement in enzyme activity and stability.[6] In addition to the expression yield of the β-galactosidase of interest, the solubility and the enzyme characteristics such as its activity, stability and catalytic efficiency are additional important parameters that influence the potential of using recombinant β-galactosidases as biocatalysts in GOS synthesis reactions. These parameters are likely influenced by a variety of factors related to the used vector, the host strain, the host-vector relationship, the folding of the expressed enzyme and the fermentation conditions such as pH, temperature, aeration rate, dissolved oxygen and type of cultivation.[7−11]

From our previous research, it was demonstrated that GOS can be efficiently synthesized using whole cells of *Bifidobacterium bifidum* NCIMB 41171.[12−14] It was also reported that this bacterial strain contains four β-galactosidases, i.e., BbgI, BbgII, BbgIII and BbgIV,[15] of which BbgIV was shown to be the best enzyme that converts lactose into GOS.[4,16] Consequently, increased industrial attention has been given to the production

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of soluble and active BbgIV in high yields in order to use it as the biocatalyst instead of the whole *Bifidobacterium* cells,[4,12,13] currently used for the commercial production of GOS. Therefore, the aim of this study was to develop an industrially relevant fermentation process for producing high yields of soluble BbgIV with high catalytic efficiency, and then use it as the biocatalyst for GOS synthesis. To achieve this, the plasmid pBL-2-K2, harboring the gene which encodes for BbgIV,[5] was transformed into chemically competent *E. coli* DH5α and the cells were grown in a 5 L bioreactor at 37 and 30 °C. The fermentation conditions such as pH and dissolved oxygen were varied in an attempt to mimic shake flask fermentations. The bioprocess parameters measured were the cell density, the maximum specific growth rate, the plasmid copy number and segregational stability, the expression yield of soluble and insoluble BbgIV and the BbgIV specific activity. Moreover, the changes in the BbgIV secondary structure, as a result of the different fermentation conditions, were evaluated and the influence of these changes on the BbgIV catalytic efficiency and the subsequent GOS synthesis process were examined.

MATERIALS AND METHODS

Materials. All chemicals, including those in the glucose oxidase/peroxidase kit, were of the highest purity from Sigma Aldrich (Dorset, UK). Tryptone, yeast extract and bacteriological agar were from Oxoid (Basingstoke, UK). The NuPage Bis-Tris gels, SDS-PAGE reagents, protein marker (Mark 12), pUC19 control DNA and the *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 from Invitrogen (Paisley, UK). The QiAprep Spin Miniprep kit was from QIAGEN (West Sussex, UK) and the 1 kb DNA ladder was from Promega (Southampton, UK).

Plasmid Transformation and Inoculum Preparation. The plasmid pBL-2-K2, which carried the gene encoding for BbgIV, was previously constructed from pBluescript SK (+) (accession number X52324) (Stratagene, La Jolla, CA).[5] All the steps used to construct the plasmid are thoroughly described by Goulas et al. (2009a).[5] The plasmid was then transformed into chemically competent *E. coli* DH5α cells according to the manufacturer's instruction. 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-beta-D-galactopyranoside (X-gal) and 100 μg/mL of ampicillin. These clones were subcultured on LB agar plates containing 100 μg/mL ampicillin and grown for 16 h at 37 °C. A single colony was used to inoculate 10 mL modified LB medium of pH 7.0 (10 g/L tryptone, 10 g/L yeast extract, 10 g/L glycerol, 5 g/L sodium chloride, 11.4 g/L disodium hydrogen phosphate, 2.4 g/L sodium dihydrogen phosphate and 100 mg/L ampicillin). The culture was incubated for 8 h (37 °C, 200 rpm). An aliquot of this culture was used to inoculate 200 mL of the modified LB medium described above, which was incubated for 10 h (37 °C, 200 rpm) and used to inoculate the 5 L bioreactor.

Production of BbgIV in a 5 L Bench-top Bioreactor. The *E. coli* DH5α cells of the inoculation culture were separated by centrifugation (8000× *g*, 15 min, 4 °C), suspended in sterilized phosphate buffer saline (PBS) (pH 7.0, containing 8 g/L NaCl) and used to inoculate a 5 L bioreactor (Applikon, Tewkesbury, Gloucestershire, UK), with a working volume of 4 L and a starting $OD_{600\ nm}$ of 0.1. The fermentation medium (pH 7.0) was 10 g/L tryptone, 10 g/L yeast extract, 10 g/L glycerol, 5 g/L sodium chloride, 4 g/L ammonium sulfate, 11.4 g/L disodium hydrogen phosphate and 2.4 g/L sodium dihydrogen phosphate. Following autoclaving, magnesium sulfate, iron chloride, thiamine and ampicillin were filter-sterilized and added to the medium at a final concentration of 0.5, 0.025, 0.01, and 0.1 g/L, respectively. A mixture of trace elements stock solution was also filter-sterilized and added to the fermentation medium at a final concentration of: 11.5 mg/L calcium chloride dihydrate, 0.3 mg/L aluminum chloride hexa-hydrate, 7 mg/L zinc-sulfate hepta-hydrate, 1.3 mg/L cobalt chloride hexa-hydrate, 12.4 mg/L copper sulfate monohydrate, 0.008 mg/L boric acid, 11.4 mg/L manganese chloride tetra-hydrate, 0.008 mg/L nickel chloride hexa-hydrate and 1.8 mg/L sodium molbydate. The aeration rate was 1 vvm (gas volume flow per unit of liquid volume per minute) in all the fermentations. Eight duplicate fermentations were performed in total; four at 30 °C and four at 37 °C. At each temperature, two fermentations were carried out at pH 7.0 and two fermentations were performed without pH control. At each pH two fermentations were conducted; one at ∼20% dissolved oxygen (dO2) and one at limited dissolved oxygen, that is, 0−2% dO2. The dO2 was adjusted at ∼20% by automatically controlling the agitation speed, and at 0 − 2% by keeping the agitation speed constant at 300 rpm throughout the fermentations. The dO2, temperature, agitation speed and pH data were collected using a data logging software (BioExpert, Applikon, Tewkesbury, Gloucestershire, UK). Samples were withdrawn, in duplicate, every hour for measuring, in triplicate, the optical density (OD), the cell concentration (cfu/mL) and the dry cell weight (DCW), and every four hours for estimating, in triplicate, the β-galactosidase activity, the expression yield of soluble and insoluble BbgIV and the plasmid copy number and segregational stability. The cell concentration (cfu/mL) was estimated by plating serial dilutions of the cells in sterilized PBS (pH 7.0), incubating the LB agar plates (supplemented with 100 μg/mL ampicillin) at 37 °C for 16 h, and then counting the colonies on the plates. The maximum specific growth rate (μ_{max}) was calculated by fitting the curves (Ln cfu/mL vs time) to a sigmoid model using the Microsoft Excel add in function DMFit V.2.1 [17] available at http://www.ifr.ac.uk/safety/DMfit/default.html.

Preparation of Bacterial Cell Extracts and Determination of the Expression Yield of BbgIV. Aliquots of the *E. coli* cells were harvested from the bioreactor by centrifugation (8000× *g*, 4 °C, 15 min), washed twice with PBS (pH 7.0), suspended in sodium phosphate buffer (50 mM, pH 6.8) and then sonicated at 4 °C (three times for 1 min at 26 amplitude micrometers) using a Soniprep 150 (SANYO Gallenkamp PLS, UK). The resultant cell extract was centrifuged (15000× *g*, 4 °C, 30 min) to separate the soluble (crude preparation) and insoluble fractions. The expressed BbgIV in both fractions was quantified by SDS-PAGE using NuPage Novex 4−12% Bis−Tris gels (pH 7.0) in X cell Sure Lock Mini-cell electrophoresis system (Invitrogen, Paisley, UK). The operation conditions were 200 V constant voltage and 50 min run time using 3-(N-morpholino) propanesulfonic acid (MOPS) (2.5 mM MOPS, 2.5 mM Tris base, 0.005% sodium dodecyl sulfate (SDS), 0.05 mM ethylenediaminetetra-acetic acid (EDTA), pH 7.7) as a running buffer. Quantification of the BbgIV bands was conducted using bovine serum albumin (BSA) as the external standard, and myosin from rabbit muscle as the internal standard, used in each lane. Myosin was chosen because it appeared as a single band and did not interfere with any of the *E. coli* protein bands. The gel images were analyzed using GelCompare II software (Applied-Maths NV, Belgium), which converted the band density into a chromatogram-like peak for which the area was calculated. Determinations were performed in the linear range, and the coefficient of determination (R^2) between the band density and the external standard was >0.97.

Quantification of Plasmid Copy Number and Evaluation of Plasmid Stability. The plasmid pBL-2-K2 was extracted from the *E. coli* cells during the fermentations using the QiAprep Spin Miniprep kit. The plasmid purity was examined by agarose gel electrophoresis and by measuring the absorbance (A) at 230, 260, and 280 nm. The ratio A_{260}/A_{280} was always about 1.8 ± 0.05, and the ratio A_{260}/A_{230} was always >1.5. Agarose gel was prepared at a concentration of 0.8% in Tris-acetate-EDTA buffer (TEA) (40 mM Tris, 20 mM acetic acid and 1 mM EDTA), containing 5 μg/mL ethidium bromide as a stain. The electrophoresis was carried out in a Gibco BRL horizontal gel electrophoresis apparatus (Invitrogen, Paisley, UK) at 75 V, using TEA as the running buffer. The gel images were recorded under UV light and the quantity of plasmid DNA was measured based on the density of bands, which was analyzed by GelCompare II software (Applied-Maths NV, Belgium) using pUC19 control DNA as the external

Table 1. Expression Yield of BbgIV (mg BbgIV/g DCW) in a Soluble Form under the Standard, the Non pH Controlled and the Limited Dissolved Oxygen Fermentation Conditions[a]

	yield of BbgIV in a soluble form (mg/g DCW)					
time (hours)	37 °C standard (pH 7.0, ~20% dO2)	37 °C (pH ≠ 7, ~20% dO2)	37 °C (pH 7.0, 0−2% dO2)	30 °C standard (pH 7.0, ~20% dO2)	30 °C (pH ≠ 7, ~20% dO2)	30 °C (pH 7.0, 0−2% dO2)
4	3.9 ± 0.08 (~85%)	3.2 ± 0.1 (~84%)	5.4 ± 0.1 (~93%)	5.2 ± 0.1 (~95%)	4.9 ± 0.1 (~94%)	5.5 ± 0.3 (~96%)
8	1.3 ± 0.02 (~65%)	2.2 ± 0.2 (~71%)	11.9 ± 0.2 (~95%)	5.1 ± 0.05 (~97%)	4.8 ± 0.1 (~95%)	8.3 ± 0.2 (~95%)
12	2.1 ± 0.08 (~79%)	2.6 ± 0.1 (~83%)	30.5 ± 0.4 (~98%)	4.9 ± 0.06 (~95%)	4.6 ± 0.2 (~95%)	20.3 ± 0.7 (~98%)
24	2.9 ± 0.02 (~91%)	3.1 ± 0.2 (~90%)	66.6 ± 0.4 (~96%)	5.6 ± 0.1 (~96%)	5.1 ± 0.1 (~94%)	50.6 ± 1.1 (~99%)
36			70.9 ± 1.5 (~95%)			71.8 ± 1.8 (~99%)
48			62.4 ± 1.2 (~95%)			74.9 ± 1.4 (~98%)

[a]DCW stands for dry cell weight. Values in parentheses represent the percentage of soluble BbgIV, measured based on the total expression yield of BbgIV.

standard; R^2 was >0.96. The plasmid copy number was calculated by dividing the number of plasmid DNA templates by the number of *E. coli* cells in which the plasmid DNA templates were obtained. For evaluating the segregational stability of the plasmid DNA, *E. coli* cells obtained at different time points were serially diluted using sterilized PBS (pH 7.0), and then cultured on nonselective (not containing ampicillin) and selective LB agar plates (containing ampicillin). The ratio of *E. coli* colonies harboring the plasmid to the total *E. coli* colonies was used as an indication of the segregational stability of the plasmid during the fermentations.

β-Galactosidase Activity Assay. 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 soluble BbgIV after appropriate dilution with sodium phosphate buffer (0.05 M, pH 6.8), and the mixture was incubated at 40 °C for 10 min. The reaction was terminated by adding 500 μL of sodium carbonate (1 M), and the absorbance was measured at 420 nm. One unit of β-galactosidase 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 4215.4 M^{-1} cm^{-1}. The specific activity of BbgIV was defined as the enzymatic activity units measured per mg of BbgIV.

Purification and Kinetic Parameters of BbgIV. BbgIV was purified from the crude preparation (the soluble fraction of BbgIV) by anion exchange chromatography at a flow rate of 2.5 mL.min^{-1} using 5 mL HiTrap Q column (GE healthcare Ltd., UK), sodium phosphate (50 mM, pH 7.4) as a starting buffer and a linear gradient of sodium phosphate (50 mM, pH 7.4) containing 1 M of sodium chloride as the elution buffer. Active fractions of β-galactosidase were pooled, concentrated using Vivaspin sample concentrators (30000 MWCO, Sartorius Stedim Biotech, Surrey, UK) and then further purified by size exclusion chromatography at a flow rate of 0.2 mL.min^{-1} using Superdex 200 HR 10/30 column (GE healthcare Ltd., UK). The elution buffer was sodium phosphate (50 mM, pH 6.5) containing 50 mM sodium chloride, 1 mM magnesium chloride and 1 mM DTT. β-Galactosidase active fractions were pooled, concentrated and then desalted and buffer exchanged for several times with sodium phosphate (5 mM, pH 6.8) using Vivaspin 500 centrifugal concentrators (30000 MWCO). All purification steps were performed by AKTA FPLC system (GE healthcare Ltd., UK).

The optimum pH of activity was examined in a range of pH 5 − 9 using phosphate-citrate buffer for pH 5−5.4, sodium phosphate buffer for pH 5.8−8 (0.2 pH unit intervals) and sodium carbonate-bicarbonate buffer for pH 9. The optimum temperature of activity was studied in a range of 30−70 °C (4 °C intervals in the range of 30−46 °C and 62−70 °C, and 2 °C intervals for 46−62 °C). Thermostability of the enzyme was also measured at 30, 40, 50, and 60 °C by incubating the pure BbgIV (~0.1 mg·mL^{-1}) in 50 mM sodium phosphate buffer (pH 6.8), containing 1 mM magnesium chloride. Aliquots were withdrawn for up to 48 h and the residual activity was estimated as described previously. Kinetic parameters were estimated for the pure BbgIV, using *o*-NPG and lactose as substrates, at the optimum pH and temperature of activity. The substrate concentration was 0.02 − 50 mM for *o*-NPG and 0.01 − 70 mM for lactose. The assay using *o*-NPG was carried out as described previously. The assay using lactose was conducted based on the glucose oxidase/peroxidase coupled reaction. Briefly, 80 μL of pure BbgIV were added to 910 μL of lactose and 10 μL magnesium chloride. The mixture was incubated for 10 min at the optimum temperature of activity, and the reaction was then stopped by heating at 90 °C for 10 min. From this mixture, 200 μL were added to 400 μL of glucose oxidase/peroxidase solution and incubated at 37 °C for 30 min. Then, 400 μL of 12 N sulphuric acid were added and the absorbance was measured at 540 nm. One unit of activity was defined as the amount of enzyme that liberates 1 μmol of glucose per minute under the assay conditions. The steady state kinetic parameters (K_m, V_{max} and K_{cat}) were calculated by nonlinear regression using Graph Pad Prism (La Jolla, CA), assuming that each subunit of BbgIV had one active site.

Analysis of the Secondary Structure of BbgIV by FT-IR Spectroscopy. The pure BbgIV samples were dried at ~20 °C under nitrogen, suspended in deuterium oxide and left for 60 min at ~20 °C. Then, the FT-IR spectra of BbgIV samples were measured, using deuterium oxide as a blank, by a Thermo Nicolet Nexus 870 Infrared spectrophotometer (Thermo Electron Corp, Cambridge, UK) and a liquid phase FT-IR Omni-cell (Specac) with calcium floride window of 150-μm Teflon spacer. The spectra were collected using a DTGS detector and a KBr beam splitter at ~20 °C in the region of 4000−700 cm^{-1} after purging the spectrophotometer with CO_2 free dry air for 24h. The spectra were recorded in blocks of 128 scans and co-added to obtain the final spectrum, from which the amide I region (1600−1700 cm^{-1}) was Fourier self-deconvoluted using Thermo OMNIC 9 software (Thermo Electron Corp, Cambridge, UK).

Galactooligosaccharides (GOS) Synthesis. GOS synthesis was conducted in duplicate at 55 °C and at 150 rpm using 43% w/w of filter-sterilized lactose as the substrate, dissolved in sodium phosphate buffer (50 mM, pH 6.8). Lactose solution was prepared as described by Osman et al. (2012).[4] GOS synthesis was initiated by adding an amount of BbgIV corresponding to ~40 units of β-galactosidase activity per gram of lactose, measured as described previously using the *o*-NPG assay. Samples were collected from the synthesis solution after 1, 2, 4, 8, 12, 16, 20, and 24 h. The GOS yield (Y_P) was estimated according to the following equation:

$$Y_P = \left(\frac{\text{GOS produced(g)}}{\text{total carbohydrates(g)}} \right) \times 100 \quad (1)$$

The carbohydrate analysis was conducted by high performance liquid chromatography (HPLC) using a Rezex RCM-Monosaccharide Ca^{2+} (8%) column (300 × 7.8 mm) (Phenomenex, Macclesfield, Cheshire, UK) and HPLC grade water as the eluent (0.5 mL.min^{-1} at 84 °C). The carbohydrate analysis was also performed by high performance anion exchange chromatography coupled with pulsed amperometric detector (HPAEC-PAD) using CarboPac PA-1 analytical column (4 × 250 mm) (Dionex Corp, Surrey, UK) and gradient concentrations of sodium hydroxide and sodium acetate solutions (Fischer Scientific,

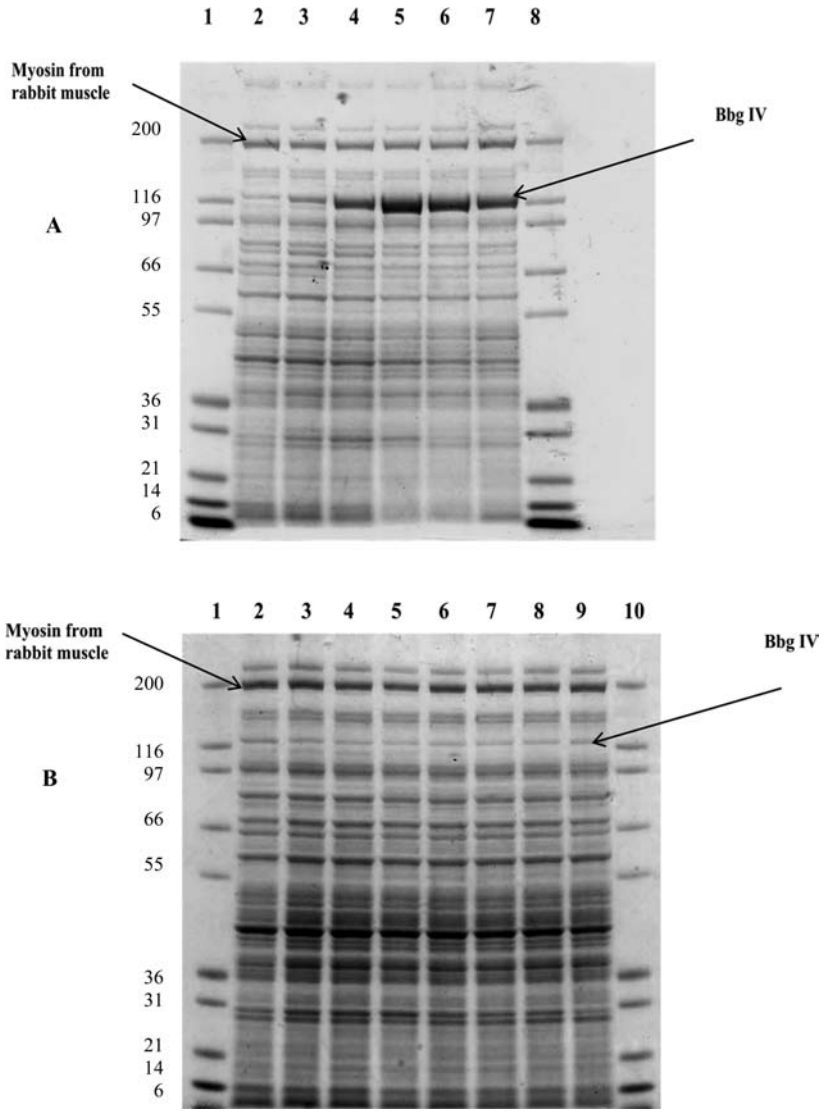

Figure 1. (A) SDS-PAGE of *E. coli* extracts under limited dO2 conditions (37 °C, pH 7, 0−2%). Lanes 1 and 8 represent the protein markers. Lanes 2, 3, 4, 5, 6, and 7 represent the extracts after 4, 8, 12, 24, 36, and 48 h, respectively. (B) SDS-PAGE of *E. coli* extracts under the standard (37 °C, pH 7, ∼20% dO2) and the non pH controlled (37 °C, pH ≠ 7.0, ∼20% dO2) fermentation conditions. Lanes 1 and 10 represent the protein markers. Lanes 2, 3, 4, and 5 represent the extracts after 4, 8, 12, and 24 h, respectively, under the standard conditions. Lanes 6, 7, 8, and 9 represent the extracts after 4, 8, 12, and 24 h, respectively, under the non pH controlled conditions.

Loughborough, UK) as the eluent (1 mL.min^{-1} at 25 °C). The BbgIV activity during GOS synthesis was estimated as previously described. The BbgIV operational stability was expressed as the operational half-life ($T_{1/2}$), which was calculated by plotting the natural logarithm of BbgIV activity during GOS synthesis versus time. The specific GOS productivity was defined as g GOS produced per liter per hour per mg enzyme (g·L^{-1}.h^{-1}.mg^{-1}).

Statistical Analysis. All data were statistically treated by analysis of variance (ANOVA) using Minitab statistical software (Release 15, State College, PA). Significant differences were defined at $P < 0.05$.

RESULTS

Production of BbgIV under Different Fermentation Conditions. The production of BbgIV was initially carried out in the bioreactor at 37 °C under the standard condition (37 °C, pH 7.0, ∼20% dO2). The yield of soluble BbgIV under this condition was significantly ($P < 0.05$) lower than that obtained when preparing the inoculum, that is, in the shake flasks. In the bioreactor, the yield of soluble BbgIV was only 2.9 ± 0.02 mg/g DCW after 24 h (Table 1), while in the shake flasks it was 9.8 ± 0.2 mg/g DCW after 24 h (data not shown). This indicated that almost a 3.5 fold decrease in the yield was obtained in the bioreactor compared to the shake flasks, which was quite surprising taking into account the fact that pH, temperature, stirring, aeration rate and dissolved oxygen were all better controlled in the bioreactor than in the shake flasks. Therefore, in an effort to mimic the shake flask conditions when performing the fermentations in the bioreactor, three strategies were followed. These included the production of BbgIV under non pH controlled conditions, the production of BbgIV under limited dO2 conditions and the production of BbgIV under both non pH controlled and limited dO2 conditions.

The yield of soluble BbgIV under non pH controlled conditions (37 °C, pH ≠ 7, ∼20% dO2) was similar to that obtained under the standard conditions (Table 1). In fact, only a small drop in the pH was observed under the non pH controlled conditions from 7 to 6.4−6.6; this started after ∼2−4 h of the onset of the fermentation until the end of the exponential phase (∼10−12 h), and was followed by an

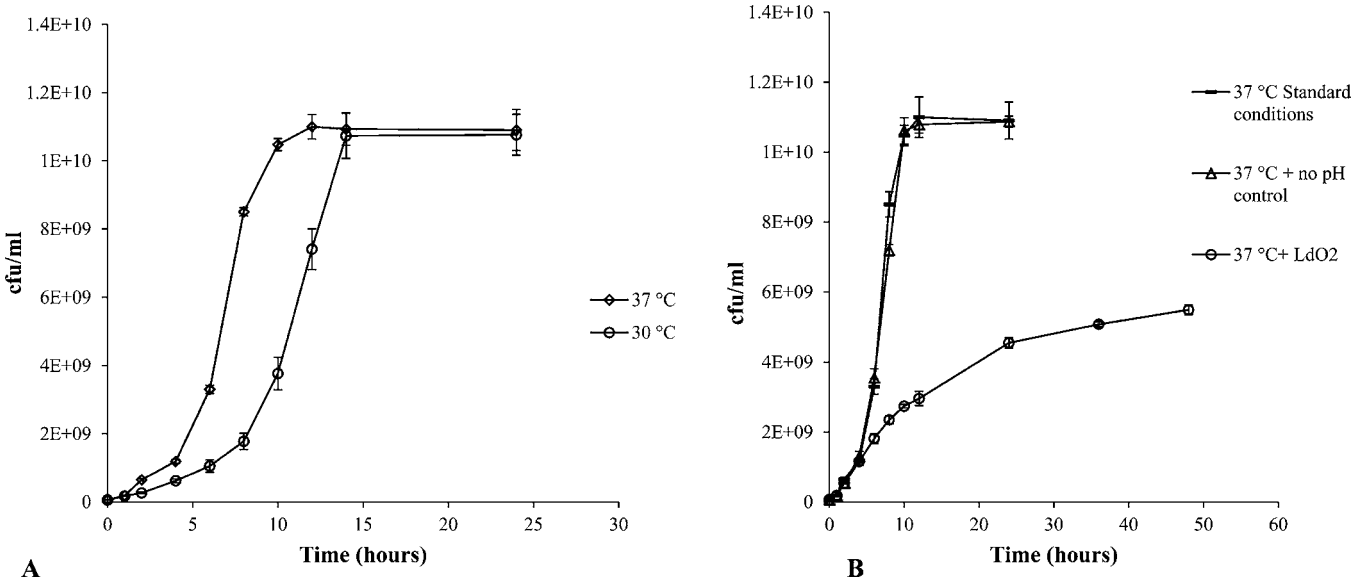

Figure 2. (A) *E. coli* growth under the standard conditions (pH 7, ∼20% dO2) at 30 and 37 °C. (B) *E. coli* growth at 37 °C under the standard (pH 7.0, ∼20% dO2), the non pH controlled (pH ≠ 7.0, ∼20% dO2) and the limited dO2 (pH 7.0, 0−2% dO2) conditions. One $OD_{600\ nm}$ equaled 0.56 mg dry cell weight (DCW) and ∼6 × 10^8 cfu/mL.

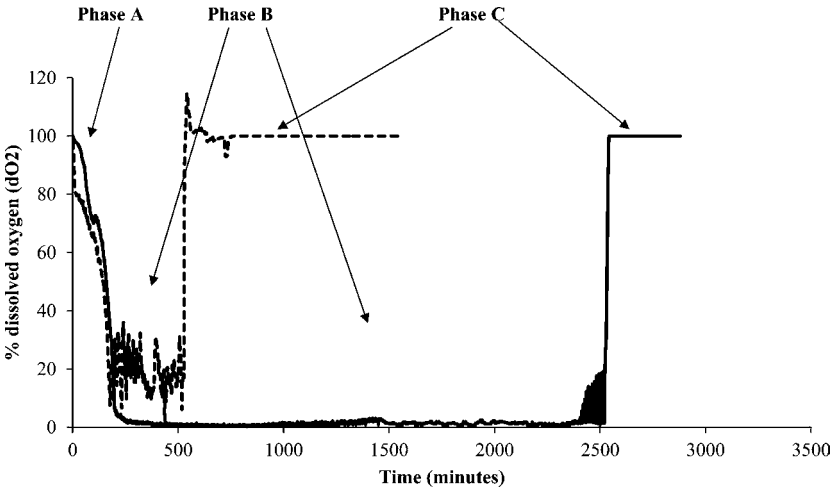

Figure 3. Plot of dissolved oxygen versus time for the fermentations performed at 37 °C under the standard (dashed line) (pH 7.0, ∼20% dO2) and the limited dO2 (straight line) (pH 7.0, 0−2% dO2) conditions.

increase thereafter to pH 7.2−7.4. This little change in the pH neither affected the *E. coli* growth characteristics nor the yield of soluble BbgIV. On the other hand, when limited dO2 conditions (37 °C, pH 7.0, 0−2% dO2) were applied, a significant ($P < 0.05$) increase in the yield of soluble BbgIV was observed (Table 1). The maximum yield of soluble BbgIV under limited dO2 conditions was ∼20−25 times higher than the yield obtained under the standard and the non pH controlled conditions (Table 1). Also, the yield of soluble BbgIV under both non pH controlled and limited dO2 conditions (37 °C, pH ≠ 7, 0−2% dO2) was similar to that obtained under limited dO2 conditions (data not shown). Figure 1 shows the SDS-PAGE of the cell extracts of the fermentations performed at 37 °C. Clearly, the yield of soluble BbgIV started to increase after ∼8−12 h under the limited dO2 conditions compared to the other conditions.

A similar trend to that obtained at 37 °C was observed when the fermentations were performed at 30 °C. More specifically, the yield of soluble BbgIV was low (<6 mg/g DCW) under the standard (30 °C, pH 7.0, ∼20% dO2) and the non pH controlled (30 °C, pH ≠ 7, ∼20% dO2) conditions. Furthermore, dO2 limitation (30 °C, pH 7.0, 0−2% dO2) significantly ($P < 0.05$) increased the yield of soluble BbgIV by ∼12−15 times compared to the yield obtained under the standard and the non pH controlled conditions (Table 1). Also, the fermentation performed under both non pH controlled and limited dO2 conditions (30 °C, pH ≠ 7, 0−2% dO2) gave a similar yield of soluble BbgIV to that obtained under the limited dO2 conditions (data not shown).

Furthermore, the percentage of soluble BbgIV at 37 °C increased to >91% under the limited dO2 conditions, while it varied between 65 and 91% under the standard and the non pH controlled conditions depending on the phase of the fermentation (Table 1). The percentage of soluble BbgIV at 30 °C, however, was >90% under all fermentation conditions (Table 1). It should be also stated that the protease activity was low and similar under all fermentation conditions (data not shown).

Growth Characteristics of *E. coli* Cells under Different Fermentation Conditions. At 37 °C, the *E. coli* DH5α cells reached the same cell concentration (~1.2 × 10^{10} cfu/mL) under the standard (37 °C, pH 7, ~20% dO2) and the non pH controlled (37 °C, pH ≠ 7, ~20% dO2) conditions (Figure 2). At the onset of these fermentations, the cells grew slowly until ~3 h and then entered the exponential phase, which ended at ~10−12 h, after which the stationary phase started (Figure 2). A typical time course of dO2 under these conditions is shown in Figure 3 with three distinctive phases denoted as A, B and C. At inoculation, the dO2 level was ~100%. Then, the cells started to consume the dO2 slowly in phase A (slow growth of the cells up to ~3 h) until it reached ~20%. After this, dO2 was consumed rapidly and concomitantly with a rapid increase in the cell biomass during the exponential phase (Phase B: up to ~10−12 h). This required increasing the agitation speed to maintain the dO2 constant at ~20%. When the carbon source was exhausted, no further consumption of dO2 was observed and the cells stopped growing. This resulted in a sharp increase of dO2 to 100% (Phase C: > 12 h). When limited dO2 conditions were applied (37 °C, pH 7.0, 0−2% dO2), phase A of the growth profile was similar to that obtained under the standard and the non pH controlled conditions. Then, a decreased growth rate in phase B was observed under the limited dO2 conditions compared to the other two conditions, which resulted in a lower final cell concentration (~6 × 10^9 cfu/mL) (Figure 2). The dO2 values in the limited dO2 fermentations dropped to ~20% after 3−4 h (Phase A in Figure 3). Then, it was left to drop further to 0−2% and was maintained at such low values by keeping the agitation speed constant at 300 rpm. Phase B, in this case, lasted up to ~40 h at 37 °C and was followed by phase C where the dO2 consumption ceased. Similar growth characteristics to those obtained at 37 °C were also observed at 30 °C under all fermentation conditions; however, phases A and B lasted slightly longer at 30 °C compared to 37 °C. It is very interesting to highlight the fact that the majority of BbgIV production at 30 and 37 °C took place during the limited dO2 period (0−2% dO2), described by phase B in Figure 3.

The maximum specific growth rate (μ_{max}) of the *E. coli* cells was similar under the standard and the non pH controlled conditions (Table 2). Under these conditions, μ_{max} decreased from 0.52−0.54 to 0.3−0.31 h^{-1} when 30 °C was used instead of 37 °C (Table 2). This decrease in μ_{max} was concomitant with approximately 2 fold increase in the BbgIV yield. When limited dO2 conditions were applied at 37 °C, a decrease in μ_{max} was observed from 0.52−0.54 to 0.22−0.24 h^{-1} (Table 2), which was interestingly concomitant with 20−25 fold increase in the BbgIV yield. Likewise, dO2 limitation at 30 °C resulted in a decrease in μ_{max} from 0.3−0.31 to 0.19−0.2 h^{-1}, which was also simultaneous with 12−15-fold increase in the BbgIV yield.

Table 2. Maximum Specific Growth Rate (μ_{max}) of the *E. coli* Cells under Different Fermentation Conditions, Calculated from the Sigmoid Model of the Microsoft Excel Add-in DMFit V.2.1

temperature (°C)	pH	dO2 (%)	μ_{max} (h^{-1})	R^{2a}
37	7	20	0.54	0.99
		0−2	0.24	0.97
	≠7	20	0.52	0.99
		0−2	0.22	0.96
30	7	20	0.31	0.99
		0−2	0.20	0.96
	≠7	20	0.30	0.98
		0−2	0.19	0.97

[a]Coefficient of determination between the experimental and fitted data.

Plasmid Copy Number under Different Fermentation Conditions. The plasmid copy number was similar under the standard and the non pH controlled conditions (Table 3). Under limited dO2 conditions, however, the plasmid copy number was significantly ($P < 0.05$) higher compared to the other conditions (Table 3). It increased significantly ($P < 0.05$) once the dO2 dropped to 0−2% and remained at such low values (Phase B in Figure 3). The increase in plasmid copy number was concomitant with a significant ($P < 0.05$) increase in the BbgIV yield (~20−25 times at 37 °C and ~12−15 times at 30 °C (Table 1)). Overall, the plasmid copy number increased from 146−156 at the start of the exponential phase (~4 and ~8 h at 37 and 30 °C, respectively) to 593−617 after 24 h and was then reasonably stable until 36 h, after which it decreased slightly to 546−591. This decrease coincided with a slight decrease in the BbgIV yield at 37 °C, a cease in the increase of BbgIV yield at 30 °C and an increase in the dO2 levels at both temperatures. It should be also stated that the *E. coli* DH5α cells showed a plasmid segregational stability of ≥90% under all fermentation conditions (data not shown).

Specific Activity and Kinetic Parameters of BbgIV under Different Fermentation Conditions. The specific activity of BbgIV was similar under the standard and the non pH controlled conditions (Table 4). Under these conditions, a slight increase in the specific activity was observed when 30 °C was used instead of 37 °C (Table 4). Interestingly, dO2 limitations did not only increase the BbgIV yield but also positively affected its specific activity, as this was ~3 times higher under limited dO2 conditions compared to the other conditions (see 24−48 h in Table 4). In order to get a further insight into the fact that the BbgIV specific activity was significantly ($P < 0.05$) higher when produced under limited dO2 conditions compared to the other conditions, BbgIV samples, produced under two distinctive dO2 conditions with two distinctive specific activities, were purified until almost homogeneity (Figure 4) and their kinetic parameters and secondary structures were studied. The first sample (LQ BbgIV) had a specific activity of ~330 units/mg and was produced after 8 h at 37 °C, pH 7 and at ~20% dO2, while the second sample (HQ BbgIV) had a specific activity of ~2390 units/mg and was produced after 24 h at 37 °C, pH 7 and at 0 − 2% dO2 (Table 4). For both HQ BbgIV and LQ BbgIV, the optimum pH of activity was in the range of pH 6.4−7 using *o*-NPG as a substrate and in the range of pH 6.0 − 6.6 using lactose as a substrate (Table 5). Moreover, the optimum temperature of activity for both HQ BbgIV and LQ BbgIV was in the range of 48−54 °C, using *o*-NPG as a substrate, and in the range of 52−56 °C, using lactose as a substrate (Table 5). Also, the thermostability of both HQ BbgIV and LQ BbgIV was similar. The enzyme retained ≥90% of its initial activity at 30 and 40 °C for 48 h in the buffer solution, while at 60 °C it lost >90% of its initial activity just after 20 min. It is therefore concluded that no significant differences exist in the stability and the pH and temperature optima between HQ BbgIV and LQ BbgIV. However, the catalytic efficiency of HQ BbgIV was significantly ($P < 0.05$) higher compared to LQ BbgIV; both K_{cat} and K_{cat}/K_m were ~4 times higher for HQ BbgIV

Table 3. Plasmid Copy Number under the Standard, the Non pH Controlled and the Limited Dissolved Oxygen Fermentation Conditions at 37 and 30 °C

time (hours)	37 °C standard (pH 7.0, ~20% dO2)	37 °C (pH ≠ 7, ~20% dO2)	37 °C (pH 7.0, 0−2% dO2)	30 °C standard (pH 7.0, ~20% dO2)	30 °C (pH ≠ 7, ~20% dO2)	30 °C (pH 7.0, 0−2% dO2)
4	128 ± 6	123 ± 2	146 ± 3	139 ± 2	133 ± 4	125 ± 2
8	78 ± 3	96 ± 3	263 ± 5	123 ± 5	120 ± 8	156 ± 2
12	214 ± 6	203 ± 2	561 ± 2	89 ± 6	94 ± 3	196 ± 3
24	233 ± 7	241 ± 5	617 ± 4	169 ± 6	163 ± 7	593 ± 3
36			586 ± 6			610 ± 14
48			546 ± 9			591 ± 7

Table 4. Specific Activity (Units/mg) of Soluble BbgIV Produced under the Standard, the Non pH Controlled and the Limited Dissolved Oxygen Fermentation Conditions at 37 and 30 °C

time (hours)	37 °C standard (pH 7.0, ~20% dO2)	37 °C (pH ≠ 7, ~20% dO2)	37 °C (pH 7.0, 0−2% dO2)	30 °C standard (pH 7.0, ~20% dO2)	30 °C (pH ≠ 7, ~20% dO2)	30 °C (pH 7.0, 0−2% dO2)
4	642.8 ± 12.5	658.4 ± 14.8	866.8 ± 24.5	827.1 ± 7.6	811.4 ± 14.3	856.9 ± 21.9
8	329.7 ± 14.8	334.6 ± 9.9	1426.9 ± 14.4	851.1 ± 15.7	825.4 ± 18.9	1386.6 ± 12.7
12	695.5 ± 11.1	657.5 ± 21.7	1982.1 ± 19.5	740.7 ± 12.5	729.7 ± 22.3	1613.2 ± 32.1
24	718.9 ± 8.7	702.1 ± 13.2	2392.7 ± 25.3	900.3 ± 24.2	887.1 ± 15.8	1971.5 ± 14.3
36			2310.5 ± 15.5			2374.3 ± 19.6
48			2290.4 ± 32.1			2423.9 ± 22.7

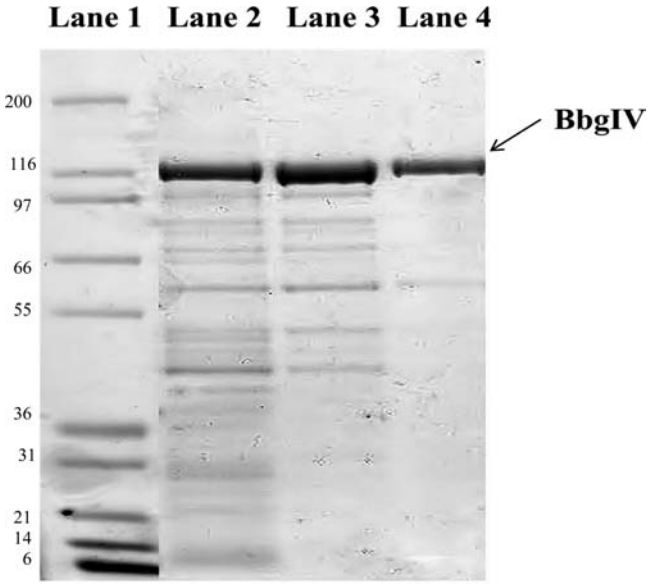

Figure 4. Crude and purified BbgIV. Lane 1 represents the protein marker. Lanes 2, 3, and 4 represent the crude BbgIV, the partially purified BbgIV after ion exchange chromatography and the purified BbgIV after size exclusion chromatography, respectively.

compared to LQ BbgIV using *o*-NPG as a substrate, the K_{cat} for HQ BbgIV was ~3.5 times higher compared to LQ BbgIV using lactose as a substrate and the K_{cat}/K_m for HQ BbgIV was ~5 times higher compared to LQ BbgIV using lactose as a substrate (Table 5).

Secondary Structure of BbgIV. The amide I region (1600−1700 cm^{-1}) of the FT-IR spectra for both HQ BbgIV and LQ BbgIV is presented in Figure 5a. The HQ BbgIV spectrum was slightly different than that of LQ BbgIV. As the FT-IR spectrum in the amide I region is a sum of all the individual components of the protein secondary structure and as the peaks assigned to the individual structural types overlap to give the final amide I peak, it was essential to obtain the deconvoluted FT-IR spectra in order to better observe the differences that possibly exist between HQ BbgIV and LQ BbgIV (Figure 5b). After deconvolution, a clear peak at ~1632 cm^{-1}, characteristic for β-sheets, was observed in HQ BbgIV. This peak was shifted toward ~1643 cm^{-1} in the case of LQ BbgIV, indicating that a higher content of random coils and a lower content of β-sheet were present in LQ BbgIV compared to HQ BbgIV (random coils usually give a clear peak at 1645 ± 4 cm^{-1} in deuterium oxide) (Figure 5b). Also, a clear peak at ~1652 cm^{-1}, characteristic for α-helix, was observed in both HQ BbgIV and LQ BbgIV. However, the intensity of this peak in LQ BbgIV was undoubtedly higher compared to HQ BbgIV, indicating that the α-helix content was most likely higher in LQ BbgIV compared to HQ BbgIV. Other minor peaks observed in LQ BbgIV were the peak at 1678 cm^{-1}, characteristic for β-sheets, and the peak at 1693 cm^{-1}, characteristic for β-turns (Figure 5b). For HQ BbgIV, a minor peak at 1688 cm^{-1}, characteristic for β-turns, was also observed (Figure 5b). The different patterns of the obtained FT-IR spectra suggest that HQ BbgIV differs, to some extent, in its secondary structure from LQ BbgIV and that dissolved oxygen levels during the fermentations most likely had an effect on the secondary

Table 5. Temperature optima, pH optima and kinetic parameters of BbgIV produced at ~20% (LQ BbgIV) and at 0 − 2% (HQ BbgIV) dissolved oxygen in *E. coli* DH5α

Enzyme	Substrate	pH optimum	Temperature optimum (°C)	V_{max} (units/mg)	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m ($M^{-1}.S^{-1}$)
HQ BbgIV	*o*-NPG	6.6	50	3144 ± 57.3	1.86 ± 0.1	6183.2	3.32×10^6
	Lactose	6.4	54	700.6 ± 31.3	2.08 ± 0.1	1377.8	6.6×10^5
LQ BbgIV	*o*-NPG	6.8	52	734.9 ± 16.7	1.68 ± 0.05	1445.3	8.5×10^5
	Lactose	6.4	54	205.7 ± 22.1	3.14 ± 0.2	404.5	1.29×10^5

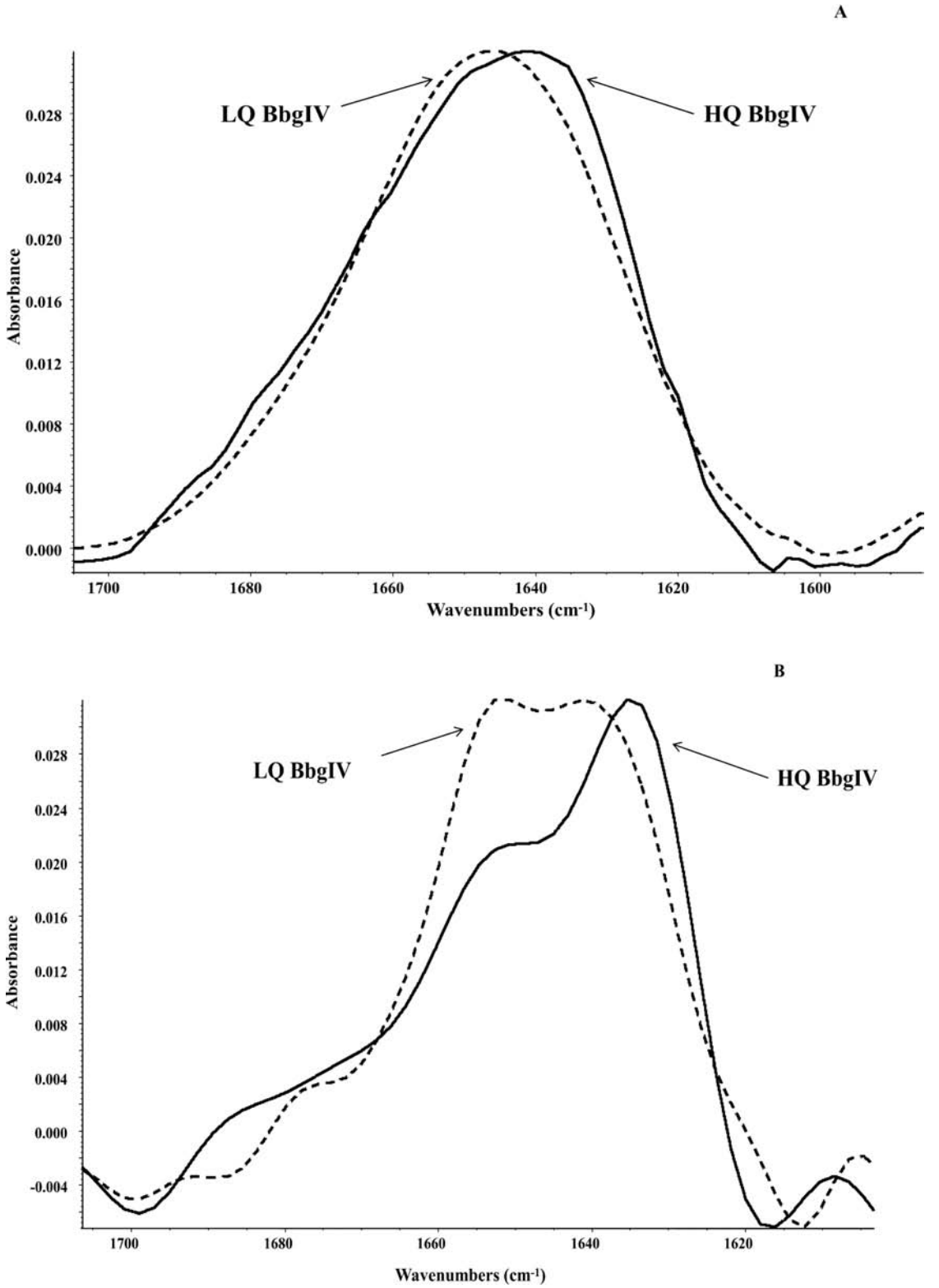

Figure 5. (A) Amide I region of the FT-IR spectra of BbgIV. Straight and dashed lines stand for the enzyme expressed at 0−2% dO2 (HQ BbgIV) and at ∼20% dO2 (LQ BbgIV), respectively. (B) Deconvoluted FT-IR spectra for HQ BbgIV (straight line) and LQ BbgIV (dashed line).

structure, the specific activity and the kinetic parameters of BbgIV.

GOS Synthesis using HQ BbgIV and LQ BbgIV. The synthesis of GOS using HQ BbgIV followed a similar pattern to that obtained using LQ BbgIV in that the maximum GOS yield, the time at which the maximum GOS yield was obtained, the operational stability of BbgIV and the profile of the produced GOS constituents were all the same (Table 6). However, the only difference was that the amount of HQ BbgIV (mg), used for GOS synthesis, was ∼7 times lower than that of LQ BbgIV, which was due to the high catalytic efficiency of HQ BbgIV compared to LQ BbgIV. As a result, the specific productivity of

Table 6. Comparison between HQ BbgIV and LQ BbgIV as Biocatalysts for GOS Synthesis (~ 40 Units of Activity Per Gram Lactose)[a]

criterion	GOS synthesis using HQ BbgIV	GOS synthesis using LQ BbgIV
Maximum GOS yield (Yp)	52.3 ± 1.2	51.6 ± 1.5
% of oligosaccharides of different degrees of polymerization in the final GOS mixture	(54.5 ± 1.5% DP2, 36.7 ± 1.1% DP3 and 8.7 ± 0.5% DP ≥ 4)	(53.9 ± 1.1% DP2, 37.5 ± 0.9% DP3 and 8.4 ± 0.6% DP ≥ 4)
Time at which the maximum GOS was obtained	~16 h	~16 h
Operational half-life ($T_{1/2}$) (hours)	7.1 ± 0.5	7.3 ± 0.4
Amount of enzyme (mg) used as a biocatalyst per 100 g lactose	~1.7	~12
Productivity of GOS synthesis ($g{\cdot}L^{-1}.h^{-1}$)	17.3 ± 0.9	17.1 ± 1.1
Specific productivity of GOS synthesis ($g{\cdot}L^{-1}.h^{-1}.mg^{-1}$)	1.89 ± 0.1	0.27 ± 0.02

[a]DP stands for the degree of polymerization of the obtained oligosaccharides.

the GOS synthesis process significantly ($P < 0.05$) increased when HQ BbgIV was used instead of LQ BbgIV (Table 6), indicating that the use of HQ BbgIV as a biocatalyst in GOS synthesis is more cost-effective compared to LQ BbgIV.

DISCUSSION

The production of recombinant enzymes requires not only a detailed understanding of the host-vector relationship but also a precise control of various other factors such as the fermentation conditions in order to regulate the growth and the activity of the microorganism, and optimize the process yield and the enzyme characteristics. Hence, the aim of this study was to develop a fermentation process, which results in obtaining high yields of a bifidobacterial β-galactosidase (BbgIV) with improved solubility and enhanced enzymatic activity for its use as a biocatalyst in GOS synthesis instead of the whole cells of *Bifidobacterium bifidum* NCIMB 41171, currently used on a commercial scale.

According to the manufacturer, the competent *E. coli* DH5α cells do not require IPTG to induce the expression from the lac promoter even though they express the lac repressor. This is because the copy number of most plasmids, in this host, significantly exceeds the repressor number, and hence IPTG addition does not usually lead to any significant increase in the expression yield. Nevertheless, Goulas et al. (2009a) [5] reported the use of 0.5 mM IPTG for the expression of BbgIV, using *E. coli* DH5α as a host and the same plasmid used in this study as a vector. However, they did not report a detailed determination of the pre- and postinduction expression yields. In contrast to Goulas et al. (2009a),[5] we found that the addition of IPTG (0.05−1 mM) at different induction times (e.g., 4−10 h under the standard conditions) did not result in any significant increase in the expression yield of BbgIV using *E. coli* DH5α cells (data not shown). Alternatively, we found that decreasing the growth rate by performing the fermentations at 30 instead of 37 °C under the standard and the non pH controlled conditions resulted in ~2 fold increase in the BbgIV yield. This effect however could not be extrapolated to lower temperatures (e.g., 23 °C) despite the further decrease in the growth rate (data not shown). In terms of the growth rate, an inverse correlation has been suggested to exist between the yield of recombinant proteins and the growth rate of the producing host strain.[18−24] Despite this, decreasing the growth rate on its own, by only reducing the temperature, did not seem to be the major factor influencing BbgIV yield. A better approach to significantly enhance the BbgIV yield was to increase the plasmid copy number concomitantly with decreasing the growth rate *via* limiting the dissolved oxygen to 0−2% during the exponential phase of the fermentations. Therefore, it was deduced that obtaining high plasmid copy numbers was most likely the principle reason behind the high expression yield of BbgIV under limited dO2 conditions at 30 and 37 °C, while decreasing the growth rate played a secondary role. A concomitant increase in the plasmid copy number with a decrease in the growth rate of bacteria harboring vectors that contain a high copy replication origin, such as the ColE1 replication origin of the plasmid used in this study, can explain the high BbgIV yield obtained under limited dissolved oxygen.[23,25−27] The plasmid copy number determines the gene dosage available for expression.[26] Therefore, high copy numbers are usually associated with increased production of recombinant proteins. Nevertheless, an increase in the plasmid copy number does not always lead to an increase in the gene product;[26,28] most probably due to limitations in the transcription and translation that take place in the host cells.[26,29] This might depend on the physiological state of the cells, as the expression of recombinant proteins requires a threshold level of cellular activity and metabolic energy which, for example, the cells lack in the stationary phase. This was also observed in this study, as BbgIV was not synthesized in significant amounts during the stationary phase (e.g., ≥ 12 h of the fermentations conducted at ~20% dO2 and at 37 °C) although the plasmid copy number increased to >200, in the same example. In contrast to this, when the fermentations were performed under limited dissolved oxygen during the exponential phase where the cells were metabolically active (phase B), the plasmid copy number significantly increased to >500 and the growth rate decreased, and subsequently a significant increase in the BbgIV yield was obtained. The increase in the BbgIV yield under such conditions, which was up to ~25 fold increase as a result of only ~3 fold increase in the plasmid copy number, might be also due to partial alterations in the transcriptomic, proteomic and metabolomic profiles under dissolved oxygen limitations, which subsequently led to increased initiation of the plasmid replication and increased efficiency in the transcription and translation machinery. Such events have been suggested as factors influencing the production of recombinant proteins,[25,30−32] although they were not investigated in this study.

Increasing the expression yield of proteins by decreasing the growth rate and enhancing the plasmid copy number can be achieved not only by changing the medium composition or reducing the temperature,[18,21,26] but also by limiting the dissolved oxygen during fermentation. In a variety of other studies, however, low yields of recombinant proteins were obtained by limiting the dissolved oxygen during the fermentation whether the proteins were expressed in *E. coli*[18,33,34] or in other hosts.[35] For instance, Basar et al. (2010) [36] reported that the growth of *E. coli* DH5α and the

production of a recombinant xylanase were inhibited at 0% dO2, and that a 5 fold increase in the xylanase yield was observed when the dO2 increased from 0 to 10%. Certain other studies, on the other hand, reported similar results to those found in our study. For example, the maximum yield and specific activity of a recombinant penicillin acylase was obtained at 1% dO2 using *E. coli* JM101as the host;[19] the suggested reasons were the reduced growth rate and the increased plasmid copy number, although the latter was not investigated.[19] Furthermore, the yield of a recombinant α-amylase increased by 3.6 fold at 5% dO2 compared to 90% dO2 using *Bacillus subtilis* 1A289 as the host;[37] however, this was due to reduced spore formation and high plasmid stability at 5% dO2.[37]

The high plasmid copy number and the significant increase in the BbgIV yield under limited dO2 conditions imposed a significant metabolic burden on the *E. coli* cells, which eventually reduced the obtained cell biomass. Despite this, the maximum productivity of BbgIV exceeded 12 $mg{\cdot}L^{-1}.h^{-1}$ and 28000 units of $activity.L^{-1}.h^{-1}$. The maximum yield also reached 0.4−0.5 g BbgIV/L culture medium. All these values compare well with those reported for the overexpression of recombinant proteins at levels suitable for industrial production, suggesting that the increased yield and productivity of BbgIV compensated well for the low cell biomass. Therefore, following the approach described in this work might be interesting for producing BbgIV industrially, particularly if fed-batch cultivation is developed based on this work.

Another very important finding was the fact that the specific activity, the maximum reaction velocity, the turnover number and the catalytic efficiency of BbgIV were significantly affected by the dissolved oxygen levels during the fermentations, as indicated by the kinetic parameters V_{max}, K_{cat} and K_{cat}/K_m. Producing BbgIV in a soluble form with high catalytic efficiency, under limited dissolved oxygen conditions, was most likely due to the low specific rate of enzyme synthesis [38] (the translation of BbgIV in the *E. coli* cells), which extended over a long period (Phase B: up to ∼40 h) and presumably allowed for the appropriate folding of the enzyme. Moreover, changes in the BbgIV secondary structure were observed simultaneously with obtaining different catalytic efficiencies of BbgIV. These structural changes of BbgIV from the HQ (produced at 0−2% dO2) to the LQ (produced at ∼20% dO2) type might have been expected, at first glance, to result in a total loss of activity. However, BbgIV retained its activity partially (∼23% of its V_{max} and K_{cat} using *o*-NPG, ∼29% of its V_{max} and K_{cat} using lactose, ∼25% of its K_{cat}/K_m using *o*-NPG and ∼19% of it K_{cat}/K_m using lactose) when expressed at ∼20% dO2 instead of 0−2% dO2 (Table 5). This most likely suggested that either the changes in the secondary structure of the enzyme did not entirely affect the conformation of the active site or that the enzyme, including its active site, had a high degree of flexibility. In this context, it should be mentioned that enzymes are flexible because of the relatively weak noncovalent bonds that define their folded three-dimensional structure. Therefore, enzymes can exist in a range of different conformations, of which one ideal conformation allows the enzyme-catalyzed reaction to proceed at its maximum rate. Consequently, changes in the structure and/or folding of the enzyme highly affect its catalytic efficiency, and the extent of this effect is determined by the degree of retaining the ideal conformation during catalysis. In this study, secondary structural changes were concomitant with obtaining different catalytic efficiencies of the enzyme, indicating that different conformations were most probably obtained; one with high catalytic efficiency (HQ type) and the other one with low catalytic efficiency (LQ type). The fact that the secondary structure, kinetic parameters and catalytic efficiency of BbgIV were influenced by the dissolved oxygen conditions during the fermentations, using *E. coli* DH5α as a host, is a very interesting finding and can have a considerable impact when industrial production of BbgIV is concerned. It highlights the importance of the effects of fermentation conditions on the characteristics of a recombinant enzyme, suggests a link between the structure and the function of BbgIV and emphasizes that, in addition to the expression yield of a recombinant enzyme, other features such as the obtained solubility and catalytic efficiency are critically important for developing a biocatalytic process using a recombinant enzyme.

An immediate impact of the above on the GOS synthesis process, using BbgIV, was evident in that the specific productivity of the GOS synthesis process was significantly enhanced when HQ BbgIV was used instead of LQ BbgIV. Such a finding is very important for developing a cost-effective GOS synthesis process, as it reveals the extent to which the fermentation process can influence the application of BbgIV as a biocatalyst on an industrial scale, after its expression in *E. coli* DH5α. This is the first study describing the production of high yields of a β-galactosidase (BbgIV) from *Bifidobacterium bifidum* NCIMB 41171 in *E. coli* DH5α with high solubility, improved specific activity and enhanced catalytic efficiency under limited dissolved oxygen conditions. The results demonstrate that highly active recombinant BbgIV can be produced at sufficient levels, and can thus be used as the biocatalyst for the synthesis of prebiotic galactooligosaccharides (GOS) in a cost-effective way.

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Notes

The authors declare no competing financial interest.

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