Increasing the production of a recombinant bifidobacterial β-galactosidase (BbgIV) in *E. coli* DH5α under limited dissolved oxygen conditions

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Abstract

The production of a recombinant bifidobacterial β-galactosidase (BbgIV) in *E. coli* DH5α was performed under different fermentation conditions. Limited dissolved oxygen increased the yield of the enzyme by 25 and 14 folds at 37 and 30 °C, respectively, compared to the fermentations conducted at 20 % dissolved oxygen. BbgIV constituted about 20 – 25 % of the total soluble cell proteins at its maximum yield (75.85 mg BbgIV/ g dry cell weight); this was obtained at 30 °C, after 48 hours of fermentation. Also, limited dissolved oxygen significantly (P < 0.05) increased the specific activity of the obtained BbgIV, as a three-fold increase was observed compared to the fermentations conducted at 20 % dissolved oxygen. These results were mainly attributed to the significant (P < 0.05) increase in the plasmid copy number and to the decrease in the growth rate observed under the limited dissolved oxygen conditions. Additionally, the % of soluble BbgIV and the process productivity were improved under such conditions. All the above features indicate that the production process of BbgIV using limited dissolved oxygen conditions is comparable to the processes reported in the literature for the over-expression of recombinant proteins on an industrial scale.

Keywords: β-galactosidase, *Bifidobacterium*, protein expression, dissolved oxygen, yield, fermentation
1. 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 catalyse the hydrolysis of terminal non-reducing 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 (Gänzle et al., 2008). More recently, the use of β-galactosidase to catalyse 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 GOS as functional food ingredients (Macfarlane et al., 2006; Macfarlane et al., 2008). However, the extent to which this enzyme catalyses the synthesis of GOS depends on its origin and on its biochemical characteristics, as β-galactosidases from various sources were shown to have different capabilities to produce GOS (Rabiu et al., 2001; Reuter et al., 1999). In this respect, it has recently been shown that GOS can be synthesised efficiently using whole cells of *Bifidobacterium bifidum* NCIMB 41171 as the biocatalyst (Goulas et al., 2007a; Osman et al., 2010; Tzortzis et al., 2005). It is also known that this bacterial strain contains four β-galactosidases, i.e. BbgI, BbgII, BbgIII and BbgIV (Goulas et al., 2007b), of which BbgIV was cited to be the best enzyme that efficiently converts lactose into GOS (Goulas et al., 2009). Therefore, increased industrial attention has been paid to the production of BbgIV in high yields, which will most likely lead to the use of this enzyme as the biocatalyst in the current industrial process, which uses the whole cells of *Bifidobacterium bifidum* NCIMB 41171.

Generally, bacterial β-galactosidases are found intracellularly and are expressed in low levels. Therefore, their heterologous expression is highly desirable in order to produce them on a large scale. In fact, *Escherichia coli* is the most widely used expression host for heterologous protein
production. This is due to the fact that it grows rapidly and yields high cell density using inexpensive substrates, and hence results in a lower process cost compared to other hosts. Additionally, it has a well characterised genetics, and various mutant host strains are available with the potential of using a large number of cloning vectors (Baneyx et al., 1999; Sorensen et al., 2005).

Despite the above facts, it is not assured that recombinant proteins will be produced in *E. coli* in high yields and in a biologically active form. The expression yield and the biological activity of recombinant proteins are influenced by a number of factors, which include the plasmid copy number, plasmid stability, promoters’ type and strength, transcription efficiency, mRNA stability, translational efficiency, *E. coli* host strain, folding of the expressed protein, proteolysis, composition of the growth medium, and fermentation conditions, such as pH, temperature, aeration rate, dissolved oxygen, and type of cultivation, (Bailey et al., 1993; Baneyx et al., 1999; Hoffmann et al., 2004; Sorensen et al., 2005).

Taking the above into account, the aim of this study was to produce high yields of a biologically active bifidobacterial BbgIV in *E. coli* DH5α. To achieve this, the plasmid pBL-2-K2, previously constructed from pBluescript SK (+), which carried the gene encoding for BbgIV (Goulas et al., 2009), was transformed into chemically competent *E. coli* DH5α. The cells were grown in a 5 litre bioreactor under different conditions of pH, temperature, and dissolved oxygen. The bioprocess criteria measured were the cell density, the specific growth rate, the expression yield of the recombinant β-galactosidase in both soluble and insoluble forms, the protease activity, the specific activity of the expressed enzyme, the productivity of the fermentation process, the plasmid copy number, and the plasmid segregational stability.

2. Materials and methods

2.1 Materials
All chemicals were purchased from Sigma Aldrich (Dorset, UK) and were of the highest purity unless otherwise stated. Tryptone, yeast extract, and bacteriological agar were from Oxoid (Basingstoke, UK). The NuPage Bis-Tris gels, the SDS-PAGE reagents, the pUC19 control DNA, which was used to confirm the transformation efficiency, and 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 Invitrogen (Paisley, UK). The QiAprep Spin Miniprep kit from QIAGEN (West Sussex, UK), the 1 kb DNA ladder from Promega (Southampton, UK), and the pUC19 control DNA from Invitrogen (Paisley, UK) were used for plasmid copy number determination.

### 2.2 Plasmids transformation and cell bank preparation

The plasmid pBL-2-K2, which was constructed from pBluescript SK (+) (accession number X52324) (Stratagene, USA) and carried the gene encoding for BbgIV (Goulas et al., 2009), was transformed into chemically competent *E. coli* DH5α cells, as instructed by the competent cells manufacturer. 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 supplemented with 100 µg/ml of ampicillin. For the preparation of the cell bank, the *E. coli* cells were grown in Luria Bertani (LB) medium, supplemented with 100 µg/ml of ampicillin, at 37 °C for 7 hours. The cultures were then diluted with glycerol at a final concentration of 30 % v/v. Aliquots of 1 ml were stored in 1.8 ml cryovials (Microbank™ Pro-Lab Diagnostics® South Wirral, UK) at -80 °C.

### 2.3 Inoculum preparation

Aliquots of stock cultures of the *E. coli* cells were sub-cultured on LB agar plates containing 100 µg/ml of ampicillin, and were grown at 37 °C for 16 hours. Then, a single colony was transferred into 10 ml modified LB medium (10 g/l tryptone, 10 g/l yeast extract, 10 g/l glycerol, 5 g/l sodium...
chloride, 11.36 g/l disodium hydrogen phosphate, 2.39 g/l sodium dihydrogen phosphate) supplemented with 100 µg/ml of ampicillin at a pH of 7.0 ± 0.05, and grown for 8 hours at 37 °C and at 200 rpm. An aliquot of this culture was used to inoculate 200 ml of the modified LB medium described above, which was incubated at 37 °C and at 200 rpm for 10 hours, and subsequently used to inoculate the 5 l bioreactor.

2.4 Production of BbgIV in a 5L bench-top bioreactor

The E.coli DH5α cells of the inoculum preparation were separated by centrifugation at 8000 g for 15 minutes at 4 °C, and then suspended in sterilised phosphate buffer saline (PBS) at pH 7.0 ± 0.05. An appropriate volume of this suspension was used to inoculate a 5 l bioreactor (Applikon, Tewkesbury, Gloucestershire, UK), at a starting OD₆₀₀nm of 0.1. The fermentation medium consisted of: 10 g/l tryptone, 10 g/l yeast extract, 10 g/l glycerol, 5 g/l sodium chloride, 4 g/l ammonium sulphate, 11.36 g/l disodium hydrogen phosphate, 2.39 g/l sodium dihydrogen phosphate, pH of 7.0 ± 0.05. Following autoclaving, magnesium sulphate, iron chloride, and thiamine were all filter-sterilised and added to the medium at a final concentration of 0.5, 0.025, 0.01 g/l, respectively. A filter-sterilised ampicillin solution was also added at a final concentration of 100 µg/ml. Finally, a mixture of trace elements stock solution was filter-sterilised and added to the bioreactor at a final concentration of: 11.5 mg/l calcium chloride dihydrate, 0.32 mg/l aluminium chloride hexa-hydrate, 7.04 mg/l zinc-sulphate hepta-hydrate, 1.28 mg/l cobalt chloride hexa-hydrate, 12.4 mg/l copper sulphate mono-hydrate, 0.008 mg/l boric acid, 11.36 mg/l manganese chloride tetra-hydrate, 0.008 mg/l nickel chloride hexa-hydrate and 1.76 mg/l sodium molybdate. The aeration rate in all the fermentation experiments was fixed at 4 vvm (gas volume flow per unit of liquid volume per minute). Three different temperatures were used for the fermentations. These were 23, 30 and 37 °C. The pH value was controlled at 7.0 ± 0.05 in the fermentations conducted at the above temperatures with automatic addition of 5 M HCl and 5 M NaOH. Also, fermentations were conducted at the above temperatures without pH control. The
dissolved oxygen (dO2) was regulated in the fermentations carried out at the above temperatures (with and without pH control) at 20% by controlling the agitation speed. Moreover, the fermentations at the above temperatures (with and without pH control) were also performed at about 0 – 1% dO2; this was achieved by keeping the agitation speed constant at 300 rpm all throughout the fermentations. Overall, twelve fermentation experiments were carried out, all in duplicate. The dissolved oxygen (dO2), temperature, agitation speed and pH data were collected using a data logging software (BioExpert, Applikon, Tewkesbury, Gloucestershire, UK). The specific growth rate was calculated by fitting the curves (Log cfu/ml vs. time) to a sigmoid model using the Microsoft Excel add in DMFit V.2.1 (Barayni and Roberts, 1994) (available at http://www.ifr.ac.uk/safety/DMfit/default.html). Additionally, samples were withdrawn every hour for optical density (OD) values and dry cell weight (DCW) measurements, and every four hours for β-galactosidase activity, protease activity, quantification of the expression yield of the soluble and the insoluble forms of BbgIV, and determination of the plasmid copy number and plasmid stability.

2.5 Preparation of bacterial cell extracts

Bacterial cell extracts were prepared after harvesting the E. coli cells by centrifugation at 8000 g, and at 4°C for 15 minutes. The bacterial cells were then washed twice with PBS at a pH of 7.0 ± 0.05, and suspended in 15 ml sodium phosphate buffer (pH = 6.8, 50 mM), containing a mixture of protease inhibitors at the following final concentrations: 1 mM AEBSF, 2 mM EDTA, 0.04 mM bestatin, 0.006 mM pepstatin A, and 0.006 mM E-64. The cell suspension was then sonicated at 4°C (three times for 60 s, each time at 26 amplitude microns) using a Soniprep 150 (SANYO Gallenkamp PLS, UK). The resultant cell solution was centrifuged at 15000 g and at 4°C for 30 minutes to separate the soluble fraction of BbgIV from the insoluble fraction.

2.6 Determination of the expression yield of Bbg IV
The expressed BbgIV in both soluble and insoluble forms was quantified by SDS-PAGE. Both fractions were prepared as described in section 2.5, and electrophoresed using NuPage\textsuperscript{R} Novex\textsuperscript{R} 4–12 % Bis–Tris gels with a pH of 7.0 ± 0.05 in X cell Sure Lock\textsuperscript{TM} Mini-cell electrophoresis system from Invitrogen (Paisley, UK). The running buffer used was MOPS (2.5 mM MOPS, 2.5 mM Tris base, 0.005 % SDS, 0.05 mM EDTA) with a pH of 7.7 ± 0.05. The operation conditions were 200 V constant voltage and 50 minutes run time. Quantification of BbgIV bands was conducted using known quantities of bovine serum albumin (BSA) as the external standard, and a fixed quantity of myosin from rabbit muscle as the internal standard, in each lane. This protein was chosen as the internal standard, because it appeared as a single band and did not interfere with any of the \textit{E. coli} protein bands. The gel images were analysed for their area density using GelCompare II software (Applied-Maths NV, Belgium), which converted the density of each band into a chromatogram-like peak, for which the area was calculated. Determinations were always done within the linear range, and the area density was linked to the quantity of the expressed enzyme. The coefficient of determination ($R^2$) between the area density and the quantity of the external standard was $> 0.97$.

\textbf{2.6 \textit{β}-Galactosidase activity assay}

The reaction mixture consisted of 250 µl of \textit{o}-nitrophenol-\textit{β}-galactoside (\textit{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 the addition of 40 µl of the soluble fraction of BbgIV after appropriate dilution with sodium phosphate buffer (0.05 M, pH = 6.8), and the mixture incubated at 40 °C for 10 minutes. The reaction was terminated by the addition of 500 µl of sodium carbonate (1 M). The absorbance was immediately measured at 420 nm against a suitable blank. One unit of \textit{β}-galactosidase activity was defined as the amount of enzyme that liberates 1 µmol of \textit{o}-nitrophenol (\textit{o}-NP) per minute, under the above mentioned conditions. The molar extinction coefficient under these conditions was 4215.4 M$^{-1}$ cm$^{-1}$. The protein concentration was measured using the Bradford
method (Bradford, 1976) with bovine serum albumin (BSA) as the standard. All enzymatic assay measurements were carried out in triplicate.

2.7 Protease activity assay

The reaction was initiated by adding 200 µl of the bacterial cell extract to 400 µl of casein solution (0.65 % w/w) and the mixture incubated at 37 °C for 15 minutes. The bacterial cell extract was prepared as in section 2.5, but without adding the protease inhibitors. Following this, 400 µl of trichloroacetic acid (TCA) solution (110 mM) were added and the mixture was further incubated for 30 minutes, after which it was centrifuged at 13000 g. From this new supernatant, 350 µl were added to 500 µl sodium carbonate solution (1M) and 150 µl Folin-Ciocalteu F-C reagent (0.5 N). This mixture was incubated again at 37 °C for 30 minutes, centrifuged at 13000 g, and then the absorbance was measured at 660 nm against a suitable blank. One unit of protease activity was defined as the amount of enzyme that liberates 1 nmol of tyrosine per minute, under the above mentioned conditions. The molar extinction coefficient under these conditions was 11400 M⁻¹ cm⁻¹.

The protein concentration was measured using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard. All enzymatic assay measurements were carried out in triplicate.

2.8 Quantification of plasmid copy number and evaluation of plasmid stability

The plasmid pBL-2-K2 was extracted from the E. coli cells at various time points during the fermentations using the QiAprep Spin Miniprep kit. The plasmid purity was examined using agarose gel electrophoresis and by measuring the absorbance (A) at 230, 260, and 280 nm. The ratio A₂₆₀/A₂₈₀ was always about 1.8 ± 0.05, and the ratio A₂₆₀/A₂₃₀ was always > 1.5. Agarose gel electrophoresis was also used for plasmid quantification. 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 agarose gel electrophoresis was also used for plasmid quantification. 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
Gibco BRL horizontal gel electrophoresis apparatus (Invitrogen, Paisley, UK) at 75 V, using (TEA) as a running buffer at the above concentration. The gel images were recorded under UV light, and analysed for their area density using GelCompare II software (Applied-Maths NV, Belgium). Determinations were always performed within the linear range; the area density was linked to the quantity of the plasmid DNA using known quantities of pUC19 control DNA as an external standard. The coefficient of determination (R²) between the area density and the concentration of the external standard was > 0.96. The plasmid copy number was calculated by dividing the number of plasmid DNA templates by the number of E. coli DH5α 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 sterilised PBS at a pH of 7.0 ± 0.05, and then were cultured on non selective LB agar plates (not containing ampicillin), from which the colonies were transferred using the sterilised stamp method into selective LB agar plates (containing ampicillin). The ratio of E. coli colonies harbouring the plasmid to the total E. coli colonies was used as an indication of the segregational stability of the plasmid during the fermentations. All the measurements were performed in triplicate.

3. Results

3.1 Effect of fermentation conditions on the production of BbgIV

The production of BbgIV was firstly carried out in the bioreactor at 37 °C, pH 7.0 ± 0.05, and at 20% dO2. The yield of soluble BbgIV obtained under this standard condition was significantly (P < 0.05) lower than that obtained when preparing the inoculum, i.e., in the shake flasks. In the bioreactor, the yield of soluble BbgIV reached only 2.89 ± 0.02 mg/g DCW after 24 hours, while it was 9.82 ± 0.24 mg/g DCW in the shake flasks (Table 1), after 24 hours. This indicated that almost a 3 fold decrease in 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 fermentation process 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 (0 – 1 %), and the
production of BbgIV under both non pH controlled and limited dO2 conditions. Conducting the
production of BbgIV under non pH controlled conditions gave a similar yield (3.01 ± 0.15 mg/g
DCW after 24 hours) to that obtained under the standard conditions (2.89 ± 0.02 mg/g DCW after
24 hours), as seen in Table 1. However, when the production was carried out under limited dO2 (0 –
1 %), a significant (P < 0.05) increase in the yield of soluble BbgIV was observed (Table 1). The
maximum yield of soluble BbgIV, obtained under limited dO2 at 37 °C, was 70.93 ± 1.5 mg/g
DCW, after 36 hours of fermentation (Table 1), which represented a 25 fold increase compared to
the standard condition. The production of BbgIV following the third strategy gave a similar yield to
that obtained following the second strategy (data not shown). Figure 1 shows the SDS-PAGE of E.
coli DH5α extracts of the fermentations performed at 37 °C under the standard, the non pH
controlled, and the limited dO2 (0 – 1 %) conditions. It is clear that the expression yield of BbgIV
increased significantly (P < 0.05) after 12 hours under the limited dO2 compared to the other two
conditions. In terms of the temperature effects, the fermentations conducted at 30 °C followed the
same trend as those performed at 37 °C. More specifically, dO2 limitation significantly (P < 0.05)
increased the yield of soluble BbgIV from 5.57 ± 0.1 mg/g DCW under the standard conditions (30
°C, pH 7.0 ± 0.05, 20 % dO2, and 24 hours) to 75. 85 ± 1.4 mg/g DCW under limited dO2 (0 – 1
%) after 48 hours, which corresponded to a 14 fold increase (Table 1). However, this was not the
case when the production of BbgIV was conducted at 23 °C, as the yield of soluble BbgIV was low
under both limited dO2 and 20 % dO2 conditions (Table 1). In the case of insoluble BbgIV, a
significant (P < 0.05) increase in the yield was obtained at 37 °C under limited dO2 compared to all
other conditions carried out at 37 °C, as shown in Table 1 (5.88 ± 0.55 mg/g DCW after 36 hours
under limited dO2 compared to < 1 mg/g DCW under all other conditions). When the other
temperatures were tested, the yield of insoluble BbgIV was low whether or not dO2 was limited (Table 1).

The percentage (%) of soluble BbgIV, calculated based on the total expression yield of BbgIV, also changed depending on the fermentation conditions. The % of soluble BbgIV at 37 °C under the standard and the non pH controlled conditions varied between 65 and 91 %, depending on the phase of the fermentation (Table 2). Only limited dO2 at 37 °C improved the % of soluble BbgIV to > 91 % (Table 2). In contrast to this, the fermentations performed at 30 °C showed a higher than 90 % soluble BbgIV under limited dO2 conditions and at 20 % dO2 (Table 2). In the case of 23 °C, the % of soluble BbgIV was similar under both conditions (~ 95 – 98 %) (Table 2).

The protease activity of the cells did not change significantly with the different fermentation conditions, indicating that the increase in the yield of BbgIV obtained by limiting the dissolved oxygen was not due to a reduced protease activity. Generally, the protease activity of the cells was low, ranging from 2.4 to 4.2 units/mg DCW under the standard and the non pH controlled conditions, and from 2.3 to 3.9 units/mg DCW under the limited dO2 conditions, at 37 °C. A slightly lower protease activity was observed when the fermentations were conducted at 30 and 23 °C (data not shown).

3.2 Growth characteristics of the different fermentations
The *E. coli* DH5α cells reached the same density when the fermentations were conducted at 37, 30, and 23 °C under the standard conditions (OD$_{600nm}$ ~ 19) (Figure 2a). The only difference was that the cells took longer time to reach their final density as the temperature decreased, due to a longer lag phase at low temperatures.
The growth curve characteristics at 37 °C were similar for both the standard and the non pH controlled fermentations. The lag phase of these fermentations lasted for about 3 hours and was followed by the exponential phase, which ended at about 12 hours, after which the *E. coli* cells entered the stationary phase (Figure 2b). The areas A, B, and C in Figure 3, which show the dO2 levels, matched well with the lag, exponential, and stationary phases, respectively. The fermentation performed under limited dO2 was similar to the above fermentations for up to 3 – 4 hours; after this period the cells showed a decreased growth compared to the standard and the non pH controlled fermentations (Figure 2b). This resulted in a lower final cell density in the limited dO2 fermentation (OD₆₀₀nm ~ 10 at 37 °C) compared to the standard and the non pH controlled fermentations (OD₆₀₀nm ~ 19). In all the fermentations performed at 37 °C, the dO2 values started from 100 % at the onset of the fermentations and then dropped to ~ 20 % after 3 – 4 hours (area A in Figures 3 and 4). After this period, the dO2 was controlled to about 20 % in all fermentations (area B in Figure 3) by increasing the agitation speed, except for the limited dO2 fermentation where the dissolved oxygen was left to drop until 0 – 1 % (area B in Figure 4) by keeping the agitation speed constant at 300 rpm. This resulted in a lower specific growth rate for the limited dO2 fermentation (µ = 0.236 h⁻¹), compared to the standard fermentation (µ = 0.286 h⁻¹). The drop in the dO2 to 0 – 1 %, and the extended period of dO2 limitation, i.e. from 4 to 40 hours (area B, Figure 4), along with the decrease in the specific growth rate, induced the production and the significant (P < 0.05) accumulation of BbgIV (Table 1). It is interesting to emphasise that the significant production of BbgIV took place during the limited dO2 period, described by area B in Figure 4. During the stationary phase, The *E. coli* cells did not seem to require oxygen (area C in Figures 3 and 4), and therefore the dissolved oxygen increased to high values, even under mild agitation conditions; this was accompanied with a cease in BbgIV production. The fermentations conducted at 30 °C followed exactly the same pattern as those performed at 37 °C, with a specific growth rate (µ) of 0.167 h⁻¹ under the standard conditions (pH = 7.0 ± 0.05, and 20 % dO2), and a specific growth rate (µ) of 0.127 h⁻¹ under limited dO2 conditions (0 – 1 %). Also, the production of BbgIV took place...
mainly during the limited dO2 period in the exponential phase (area B). The only difference was that at 30 °C areas A, B, and C were longer than at 37 °C. For the fermentations carried out at 23 °C, there was no distinctive difference between the standard and the limited dO2 fermentations, as the specific growth rate was 0.058 h⁻¹ in the former and 0.054 h⁻¹ in the latter. Also, no increase in the yield of BbgIV was observed even when dO2 was limited.

3.3 Effect of fermentation conditions on the plasmid copy number

The plasmid copy number obtained at 37 °C followed the same trend for the standard and the non pH controlled fermentations. Under these conditions, the plasmid copy number gradually decreased from 116 – 128 at the end of the lag phase (~ 4 hours) to 70 – 113 around the mid exponential phase (~ 8 hours); this decrease was associated with an increase in the growth rate and a decrease in the yield of BbgIV. It then increased again during the stationary phase (> 12 hours) to 226 – 241 (Table 3). Despite this increase, the yield of BbgIV remained unchanged during the stationary phase (Table 1). Under limited dO2 conditions, however, the plasmid copy number was significantly (P < 0.05) higher than it was under the above two conditions (Table 3). In the limited dO2 fermentation, the plasmid copy number increased sharply (Table 3), once the dO2 dropped to 0 – 1 % and remained at such low value during the period of oxygen limitation (area B in Figure 4). The increase in the plasmid copy number under these conditions initiated the production and the accumulation of BbgIV. Overall, the plasmid copy number increased from 126 at the end of the lag phase (~ 4 hours) to 617 after 24 hours. It was then reasonably stable until 36 hours, after which it decreased gradually to 506 after 48 hours. The decrease in the plasmid copy number after 36 hours coincided with a decrease in the yield of BbgIV and with an increase in the dO2 in the fermentation medium, although the aeration rate and the stirring were kept constant. The same trend was observed for the fermentation carried out at 30 °C, i.e. a significant difference in the plasmid copy number was observed between the standard and the limited dO2 fermentations (Table 3). However, at 23 °C, no differences were found in the plasmid copy number between the standard and the limited dO2
fermentation (Table 3). It should also be stated that the *E. coli* DH5α cells showed segregational plasmid stability above 90 – 95% for all the fermentation conditions studied (data not shown).

### 3.4 Effect of limited dissolved oxygen on the specific activity of the Bbg IV

At 37 °C, the specific activity of BbgIV was similar under the standard and the non pH controlled conditions. Table 4 clearly shows that the specific activity (units/mg) under these conditions dropped from about 630 – 659 at the end of the lag phase (~ 4 hours) to 309 – 335 during the mid of the exponential phase (~8 hours). Then, it increased again to 624 – 696 and remained fairly constant during the stationary phase (> 12 hours). It is interesting to note that the drop in the specific activity of BbgIV during the exponential phase, where the cell growth was at its highest, was simultaneous with a drop in the yield of BbgIV and a decrease in the plasmid copy number.

The limitation in dO2 did not only increase the yield of BbgIV, but also positively affected its specific activity, which reached a value of 2311 – 2393 between 24 and 48 hours, which is 3 times higher than the maximum value obtained under the rest of the fermentations at 37 °C (Table 4). The same pattern was observed for the fermentations conducted at 30 °C (Table 4). However, the fermentations performed at 23 °C showed just a slightly higher specific activity under dO2 limitation compared to the other conditions (Table 4).

### 3.5 Effect of the fermentation conditions on BbgIV productivity

The cell density, the obtained yield of BbgIV (mg BbgIV/ g DCW), and the time at which the yield was obtained were the three factors that determined the productivity of the BbgIV production process. The maximum productivity (2.11 – 2.64 mg.l⁻¹.h⁻¹) of the fermentations conducted under the standard and the non pH controlled conditions at 37 °C was obtained at 12 hours (Table 5). Further incubation resulted in a lower productivity, as both the yield of BbgIV and the cell density did not increase significantly. However, the fermentations conducted at 37 °C under limited dO2
conditions showed a maximum productivity (\( \sim 12.31 \text{mg.L}^{-1}\text{.h}^{-1} \)) at 24 hours, which meant that almost a 4.6 fold increase in productivity was achieved under dO2 limitation. Although the BbgIV yield increased at 36 hours compared to 24 hours, the productivity slightly decreased, due to the longer incubation time and the slight increase in the cell density. Clearly, the productivity at 48 hours decreased due to the same reasons. The same trend was observed for 30 °C, but with the maximum productivity (\( \sim 9.92 \text{mg.L}^{-1}\text{.h}^{-1} \)) obtained at 36 hours under limited dO2 (Table 5). At 23 °C, the productivity was the same for both conditions (Table 5).

Despite the 25 and the 14 fold increase in the yield of BbgIV under dO2 limitation at 37 and 30 °C, respectively, compared to all other conditions (Table 1), the productivity only increased by 4 – 5 fold (Table 5). This was due to the low E. coli cell density, which was the only drawback of the limited dO2 fermentation. However, because the enzymatic activity of the expressed BbgIV was as important as the yield, the productivity was also measured as KU.L^{-1}.h^{-1}. Taking this new measurement into account, the fermentations conducted under limited dO2 at 37 °C showed about a 19 – 27 fold increase in the productivity compared to all the other conditions (Table 5), with a maximum productivity of about 28 KU.L^{-1}.h^{-1} obtained under limited dO2. Likewise, the productivity of the 30 °C fermentation performed under limited dO2 was about 11 times higher than it was under the other conditions (Table 5). In fact, this was caused by the enhanced specific activity of BbgIV when expressed under limited dO2 (Table 4). Overall, the productivity of BbgIV was far superior under limited dO2 at 37 and 30 °C compared to the other fermentation conditions.

The fermentations performed at 23 °C showed no difference in their productivity whether or not the dO2 was limited.

4. Discussion

The production of recombinant proteins requires not only a detailed understanding of host-vector relationship but also a precise control of various factors, including the fermentation medium and
conditions such as temperature, pH, type of cultivation, aeration rate, and dissolved oxygen, in order to regulate the growth and the activity of the microorganism, and to optimise the process yield and productivity. Hence, the aim of this study was to produce a biologically active bifidobacterial β-galactosidase, namely BbgIV, using *E. coli* DH5α as the expression host, and considering the above mentioned factors. The BbgIV gene was previously cloned into a pBL-2-K2 plasmid, constructed from pBluescript SK (+) plasmid, the expression of which was controlled by the lac promoter (Goulas et al., 2009). The plasmid was transformed into the chemically competent *E. coli* DH5α cells and the transformed cells were grown under different conditions of pH, temperature, and dissolved oxygen.

According to the competent cells manufacturer, *E. coli* DH5α cells are mainly designed to amplify plasmids up to a high copy number and are not designed for the expression of recombinant proteins in high yields. These cells do not require IPTG to induce the expression from the lac promoter even though the strain expresses the lac repressor. This is because the copy number of most plasmids significantly exceeds the repressor number in the cells, and hence the addition of IPTG does not usually lead to any significant increase in the expression yield. Despite this, Goulas et al. (2009) cited the use of 0.5 mM IPTG to induce the expression of BbgIV, using *E. coli* DH5α as a host and the same plasmid we used as a vector, although they did not report a detailed determination of the pre- and the post-induction yields. In contrast to Goulas et al. (2009), we found that IPTG addition did not result in any significant increase in the expression yield of BbgIV using *E. coli* DH5α (data not shown). The increase in the BbgIV yield in our study seems to be achieved by decreasing the specific growth rate of the *E. coli* cells and by increasing the plasmid copy number as a result of limiting the dissolved oxygen.

Firstly, three temperatures (37, 30, and 23 °C), resulting in three distinctive cell growth rates (0.287, 0.167, and 0.057 h⁻¹), were used for the production of BbgIV. Only a 1.5 – 2 fold increase in the
BbgIV yield was achieved following this strategy. Secondly, a limited dissolved oxygen approach was followed at each studied temperature, where the dO2 was controlled at 0 – 1 % during the exponential phase. This resulted in a significant increase in the BbgIV yield, and was also simultaneous with a decrease in the growth rate. The inverse correlation between the yield of recombinant proteins and the growth rate of the producing host strain is well documented in the literature (Chao et al., 2010; De Léon et al., 2003; Kennedy et al., 2003; Ramirez et al., 1994; Satyagal et al., 1989; Seo et al., 1985; Sunley et al., 2010). However, the reduction in the growth rate upon limiting the dissolved oxygen, in our study, was just from 0.286 h⁻¹ to 0.236 h⁻¹ at 37 °C for example, which did not seem to be the major factor for the significant increase in the yield of the expressed BbgIV. Therefore, it appeared that the high plasmid copy number obtained upon limiting the dissolved oxygen was the main factor that influenced the expression yield of BbgIV, and was simultaneous with a decrease in the growth rate. A concomitant increase in the plasmid copy number with a decrease in the growth rate of bacteria harbouring vectors that contain a high copy replication origin (Carnes et al., 2006; Friehs et al., 2004; Prather et al., 2003; Seo et al., 1985), such as the CoIE1 replication origin of the plasmid used in this study, can most probably explain the high BbgIV yield obtained under limited dissolved oxygen. The plasmid copy number determines the gene dosage available for expression (Friehs et al., 2004). Therefore, high copy numbers are associated with increased production of recombinant proteins, which was clearly evident in our study. However, in some cases, an increase in the plasmid copy number does not lead to an increase in the gene product (Friehs et al., 2004; Nacken et al., 1996), most probably due to limitations in the transcription and the translation that take place in the host cells (Friehs et al., 2004; Kim et al., 1991), which might depend on the physiological state of the cells as the expression of recombinant proteins necessitates a threshold level of cellular activity and metabolic energy, which for example the cells lack in the stationary phase. This was clearly evident in our case, as BbgIV was not synthesised in significant amounts during the stationary phase (≥ 12 hours) of the fermentations conducted at 20 % dO2, although the plasmid copy number increased above 200. Instead, by
performing the fermentations under limited dissolved oxygen (0 – 1 %) during the phase where the
cells were metabolically active (between 4 and 40 hours, when oxygen consumption was required),
the plasmid copy number increased (> 500) and the growth rate simultaneously decreased compared
to the standard fermentation; this eventually resulted in a significant increase in the BbgIV yield.
The increase in the BbgIV yield under limited dissolved oxygen might be also due to the fact that
limitations in the dissolved oxygen changed the metabolism of the cells and hence the
transcriptomic, metabolomic, and proteomic profile was partially altered. Such an alteration might
have led to increased initiation of the plasmid replication, and an increased efficiency in the
translation machinery. Such alterations have been also mentioned as factors influencing the
production of recombinant proteins (Birnbaum et al., 1991; Carnes et al., 2006; Haddadin et al.,
2005; Rozkov et al., 2004)
The most widely used approach to increase the plasmid copy number and reduce the growth rate is
either by changing the media composition (limiting carbon or nitrogen sources) or by changing the
temperature (Chao et al., 2001; Friehs at al., 2004; Ramirez et al., 1994). However, such alterations
in the plasmid copy number and the growth rate might also be achieved by limiting the dissolved
oxygen concentration. Despite this link, the yield of several recombinant proteins was reported to
decrease when the cells were left under limited dissolved oxygen (Bhattacharya et al., 1997; Chao
et al., 2001; Li et al., 1992; Mayer et al., 1999). For instance, Jin et al. (2010) cited a 16 fold
decrease in the yield of porcine interferone-α at 20 °C using recombinant Pichia pastoris for a
process under non dO2 controlled compared to a process with 50 % dO2. Also, Basar et al. (2010)
found that the growth of E. coli DH5α and the production of a recombinant xylanase were inhibited
at 0 % dO2, and the xylanase yield increased 5 times when the dO2 increased from 0 to 10 %.
Moreover, Lee at al. (2003) showed that a 3 fold increase in the yield of elastase inhibitory peptides
was achieved when the dissolved oxygen increased from 10 to 30 %. In contrast to the above
studies, our work indicated that dO2 limitation could significantly increase the yield of a
recombinant β-galactosidase, up to 20 – 25 % of the total soluble cell proteins. Few other studies are in line with ours. For example, the maximum yield and specific activity of a recombinant penicillin acylase was obtained at 1 % dO2 using *E. coli* JM101 as the host under the control of the *LacZ* gene promoter (De Léon et al., 2003). 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 expression host (Park et al., 1997). Some reports also suggested that certain recombinant proteins were produced at higher yields by *Pichia pastoris* in oxygen limited fermentation processes (Charoenart et al., 2005; Hellwig et al., 2001; Trentmanne et al., 2004).

The increase in the yield of a recombinant protein, as a result of an increased plasmid copy number and a decreased growth rate upon limiting the dissolved oxygen, would be an important strategy to follow for producing recombinant proteins if the increase in the yield compensates for losses in productivity caused by the lower biomass yields usually obtained under limited dissolved oxygen conditions. In our study, the high plasmid copy number and the significant increase in the synthesis of the recombinant enzyme under dO2 limitation imposed a significant metabolic burden on the *E. coli* cells, which eventually led to a low biomass yield. Such a correlation between an increased plasmid copy number, an increased recombinant protein production and the growth of the host cell is widely cited in the literature (Carnes et al., 2006; Friehs et al., 2004). Despite this, our process for the production of the recombinant BbgIV showed a maximum productivity exceeding 27 KU.l^{-1}.h^{-1} and a maximum yield reaching 0.4 – 0.5 g BbgIV /L culture medium, which are comparable to those values already reported in the literature for the over-expression of recombinant proteins.

Other important aspects of the process for the expression of BbgIV were that almost all of the BbgIV was obtained in a soluble and a biologically active form, the fact that limited dissolved oxygen significantly increased the specific activity of the expressed enzyme, the low levels of protease activity in the host cell, and the high segregational stability of the plasmid. All of the above
are key features when considering the expression of a recombinant enzyme on an industrial scale. The main reason for the fact that BbgIV was obtained in a soluble and a biologically active form was most likely the slow specific rate of enzyme synthesis (the translation of BbgIV in the E. coli cells), which extended over a long period during the fermentation (from 4 to about 40 hours at 37 °C) under dO2 limitation. This probably allowed an appropriate folding of the enzyme, which resulted in a higher specific activity than what was obtained at 20 % dO2. Usually, the rapid accumulation of recombinant proteins intracellularly leads to a higher probability of protein aggregation. This aggregation can be overcome by favouring a slower rate of recombinant protein production (Swartz et al., 2001). This is exactly what was noticed in our case.

Conclusion

A significant increase in the yield and the productivity of a recombinant bifidobacterial (BbgIV) was obtained under dissolved oxygen limitation (0 – 1%) using E. coli DH5α as the host. Such a condition also improved the solubility of the recombinant enzyme and significantly increased its specific activity. The features of this production process fulfil the criteria for the industrial production of recombinant enzymes.

Acknowledgments

The authors wish to thank EPSRC and Clasado Ltd for the financial support of this work.

References


**Figure captions**

**Figure 1.** (A) SDS-PAGE of *E. coli* DH5α extracts expressing BbgIV from the 37 °C fermentations performed under limited dO2 (0 – 1 %). Lanes 1 and 8 represent the protein markers. Lanes 2, 3, 4, 5, 6, and 7 represent the *E. coli* extracts after 4, 8, 12, 24, 36, and 48 hours, respectively. (B) SDS-PAGE of *E. coli* DH5α extracts expressing BbgIV from the 37 °C fermentations performed under the standard and the non pH controlled conditions. Lanes 1 and 10 represent the protein markers. Lanes 2, 3, 4, and 5 represent the *E. coli* extracts after 4, 8, 12, and 24 hours, respectively, of the fermentations conducted under the standard conditions. Lanes 6, 7, 8, and 9 represent the *E. coli* extracts after 4, 8, 12, and 24 hours, respectively, of the fermentations conducted under the non pH controlled conditions. Myosin from rabbit muscle was the internal standard in each lane.

**Figure 2.** Growth of *E. coli* DH5α under different conditions. One OD_{600nm} equals 0.56 mg dry cell weight (DCW). Figure A shows the growth under the standard conditions (20 % dO2, pH = 7 ± 0.05) at 37, 30, and 23 °C. Figure B shows the growth at 37 °C under the standard condition (37 °C, pH = 7.0 ± 0.05, and 20 % dO2), the non pH controlled condition (37 °C, pH ≠ 7.0 ± 0.05, and 20 % dO2), and the limited dissolved oxygen conditions (37 °C, pH = 7.0 ± 0.05, and 0 – 1 % dO2).

**Figure 3.** Online plot of dissolved oxygen with time for the fermentation at 37 °C under standard conditions (pH = 7.0 ± 0.05, and 20 % dO2).

**Figure 4.** Online plot of dissolved oxygen with time for the fermentation at 37 °C under limited dissolved oxygen (pH = 7.0 ± 0.05, and 0 – 1 % dO2).
Table 1. Expression yield of BbgIV (mg Bbg IV/ g DCW) in both soluble and insoluble forms under different fermentation conditions. St, pH ≠ 7, and LdO2 stand for the standard, the non pH controlled, and the limited dissolved oxygen fermentations, respectively.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>37 °C st</th>
<th>37 °C (pH ≠ 7)</th>
<th>37 °C (LdO2)</th>
<th>30 °C st</th>
<th>30 °C (LdO2)</th>
<th>Time (Hours)</th>
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<td>5.39 ± 0.03</td>
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<tr>
<td>8</td>
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<td>2.15 ± 0.1</td>
<td>11.92 ± 0.24</td>
<td>5.03 ± 0.05</td>
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<td>28</td>
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<tr>
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<td>4.93 ± 0.06</td>
<td>18.28 ± 0.7</td>
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<td>48.61 ± 1.1</td>
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</tr>
<tr>
<td>36</td>
<td>/</td>
<td>/</td>
<td>70.93 ± 1.5</td>
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<td>/</td>
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<table>
<thead>
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<th>Time (Hours)</th>
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<th>37 °C (LdO2)</th>
<th>30 °C st</th>
<th>30 °C (LdO2)</th>
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<td>0.06 ± 0.03</td>
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<td>0.68 ± 0.1</td>
<td>0.89 ± 0.09</td>
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<td>5.43 ± 0.36</td>
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<td>0.59 ± 0.06</td>
<td>72</td>
<td>/</td>
<td>0.21 ± 0.03</td>
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Table 2. % of Bbg IV expressed in a soluble form. St, pH ≠ 7, and LdO2 stand for the standard, the non pH controlled, and the limited dissolved oxygen fermentations, respectively.

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<tr>
<th>Time (hours)</th>
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<th>37 °C (LdO2)</th>
<th>30 °C st</th>
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Table 3. Plasmid copy number under different fermentation conditions. St, pH ≠ 7, and LdO2 stand for the standard, the non pH controlled, and the limited dissolved oxygen fermentations, respectively.

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Table 4. Specific activity of the expressed Bbg IV (Units/mg). St, pH ≠ 7, and LdO2 stand for the standard, the non pH controlled, and the limited dissolved oxygen fermentations, respectively.

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<tr>
<th>Time (hours)</th>
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<td>1314.7 ± 11.9</td>
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<td>900.3 ± 24.2</td>
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<td>2423.9 ± 22.7</td>
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<td>/</td>
<td>1006.4 ± 24.8</td>
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Table 5. Productivity of Bbg IV. St, pH ≠ 7, and LdO2 stand for the standard, the non pH controlled, and the limited dissolved oxygen fermentations, respectively. KU stands for kilo units of activity (1000 units of activity).

<table>
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<th>37 °C (LdO2)</th>
<th>30 °C st</th>
<th>30 °C (LdO2)</th>
<th>Time (hours)</th>
<th>23 °C st</th>
<th>23 °C (LdO2)</th>
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<td>12.31 ± 0.35</td>
<td>2.43 ± 0.09</td>
<td>9.48 ± 0.6</td>
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<td>0.73 ± 0.09</td>
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<td>9.92 ± 0.8</td>
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<td>9.43 ± 0.5</td>
<td>72</td>
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Figure 1.
Figure 2.
Figure 3.
Area A
Area B
Area C

Figure 4.