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# Improvement of culture conditions to overproduce β-galactosidase from *Escherichia coli* in *Bacillus subtilis*

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**Abstract** The effect of some culture variables in the production of β-galactosidase from *Escherichia coli* in *Bacillus subtilis* was evaluated. The *lacZ* gene was expressed in *B. subtilis* using the regulatory region of the subtilisin gene *aprE*. The host contained also the *hpr2* and *degU32* mutations, which are known to overexpress the *aprE* gene. We found that, when this overproducing *B. subtilis* strain was grown in mineral medium supplemented with glucose (MMG), β-galactosidase production was partially growth-associated, as 40%–60% of the maximum enzyme activity was produced before the onset of the stationary phase. In contrast, when a complex medium was used, β-galactosidase was produced only at low levels during vegetative growth, whereas it accumulated to high levels during early stationary phase. Compared with the results obtained in complex media, a 20% increase in specific β-galactosidase activity in MMG supplemented with 11.6 g/l glucose was obtained. On the 1-l fermenter scale, a threefold increase in volumetric β-galactosidase activity was obtained when the glucose concentration was varied from 11 g/l to 26 g/l. In addition, glucose feeding during the stationary phase resulted in a twofold increase in volumetric enzyme activity as cellular lysis was prevented. Finally, we showed that oxygen uptake and carbon dioxide evolution rates can be used for on-line determination of the onset of stationary phase, glucose depletion and biomass concentration.

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## Introduction

Bacteria belonging to the *Bacillus* genus have been used for the production of industrial enzymes because of their ability to produce and secrete a large number of useful proteins or metabolites (for a recent review see: Zukowski 1992). The commonest *bacilli* used in industry are *B. licheniformis, B. amyloliquefaciens* and *B. subtilis*. Among them, the best studied from different points of view is *B. subtilis*. Furthermore, because of its GRAS (generally recognized as safe) status, *B. subtilis* is a natural candidate for the production of proteins for the food industry. However, compared with *Escherichia coli* or *S. cerevisiae*, there have been very few reports on the cultivation of recombinant *B. subtilis* at fermenter scale, and on the development of suitable conditions for the production of recombinant proteins. Furthermore, most of the genetically modified *B. subtilis* strains have been evaluated at flask scale where, in contrast to fermenter studies, a strict control on the environmental conditions is not possible.

In this work, we were interested in studying some culture variables, such as the type of medium used, the initial glucose concentration and glucose feeding during stationary phase, to increase the production of β-galactosidase from *E. coli* in *B. subtilis* using batch cultures. For this purpose, we used a strain that carries the *E. coli lacZ* gene under control of the *aprE* promoter from *B. subtilis*, which is a very well characterized regulatory region (Valle and Ferrari 1989; Ferrari et al. 1993). It is well known that, in rich media, this promoter is only expressed during the stationary phase, and that the combination of the *hpr2* and *degU32(Hy)* mutations increases its transcriptional level around 40-fold (Ferrari et al. 1986, 1993). For this reason the producing strain used in this work contained both mutations.

## Materials and methods

### Culture media

A mineral medium supplemented with glucose (MMG) was designed, based on the elementary composition of *B. subtilis* (Dettwiler et al. 1993) and with the mineral salts added to Schaeffer's sporulation medium for *B. subtilis* cultures (Schaeffer et al. 1965). The mineral medium composition was (per liter) 4 g $(NH_4)_2SO_4$, 5.32 g $K_2HPO_4$, 6.4 g $KH_2PO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 5 mg $MnCl_2$, 40 mg $CaCl_2$, and 30 mg $FeSO_4 \cdot 7H_2O$. The initial concentration of glucose was varied, and is specified in Results. When cultures reached a concentration of about 6 g/l dry cell weight, all mineral components were added aseptically, as the nitrogen source became limiting in the culture broth at such biomass concentrations. The initial pH was adjusted to 7.0.

### Strain and cell bank

The genotype of the strain used in this work, designated BB804, was Δ*nprE*, *hpr2*, *aprE*::*lacZ*, $Cm^R$, *degU32(Hy)*, without any auxotrophic requirement. It was constructed in successive steps, starting with the BB80 strain (Δ*nprE*, *glyB, hisA*), the *hpr2* and *degU32* were introduced by transformation using chromosomal DNAs from strains BB82 (Δ*nprE*, *hisA, hpr2*) and BB17 (Δ*nprE*, *degU32*; Olmos et al. 1996). We took advantage of the linkage of the *hpr2* and *degU32* to the *glyB* or *hisA* markers respectively. The strain was grown in MMG containing 10 g/l initial glucose, until an absorbance of 5 was reached (about 12 h culture time). This culture was used immediately to prepare frozen vials containing 1.5% (v/v) glycerol. The vials were stored in liquid nitrogen. We have found that these vials give reproducible results even after 24 months of storage (data not shown).

### Inocula development and shake-flask cultures

A 1-ml sample of the cell bank was thawed steeply (−20 °C, −4 °C, room temperature, and 37 °C, during 5 min for each step) and added to a 500-ml flask with 100 ml MMG containing 10 g/l initial glucose. Inocula were incubated to middle exponential phase (approx. 12–16 h). When cultures reached a biomass concentration of 1.8 g/l, they were used as inocula. All cultures were initiated with 10% inoculum.

To evaluate β-galactosidase production in shake flasks, cultures were performed in 500-ml shake flasks containing 100 ml Schaeffer's medium or MMG. All flasks were incubated at 37 °C and 300 rpm in a G25 Shaker (New Brunswick Inc. New Brunswick, N.J.) When Schaeffer's medium was used, the inoculum was also developed in Schaeffer's medium.

### Fermenter cultures

Cultures were carried out in a baffled stirred-tank fermenter, with a working volume of 1 l (total volume 1.6 l; LSL Biolafitte, Inc. Princeton, N.J.) and equipped with a six-blade Rushton turbine. The temperature was controlled at 37 °C. The pH was maintained at 7.0 by automatic additions of 2M NaOH during exponential growth, and 0.67 M $H_3PO_4$ during the stationary phase. Foam was controlled by automatic additions of food-grade silicone. Oxygen partial pressure was measured with a polarographic oxygen probe (Ingold Electrodes, Inc. Wilminton, Mass.) and was controlled above 20% (with respect to air saturation) through a proportional-integral derivative controller by automatic increases of the impeller speed. Proportional-integral derivative parameters were tuned by Ziegler's method (Lim and Lee 1991) and, after some further empirical adjustments, they were set to a proportional band of 90, an integral time of 500 s, a derivative time of 1 s, and a dead band of 5. An initial impeller speed of 600 rpm and a constant air flow rate of 1 vvm were used. Off-gas analysis for oxygen, carbon dioxide and nitrogen were carried out on-line using a MGA-1200 mass spectrometer (Perkin-Elmer, Pomona, Calif.) The oxygen uptake rate and carbon dioxide evolution rate were calculated on-line from a gas balance over the fermenter volume, assuming a constant composition in the inlet air. Mentor software (LSL Biolafitte Inc. Princeton, N.J.) was used for on-line calculation of $O_2$ uptake and $CO_2$ evolution rates and data logging at 1-min intervals.

### Analytical methods

The cell concentration was measured with a DU-70 spectrophotometer (Beckman. Fullerton, Calif.) as the absorbance at 600 nm ($A_{600}$), and converted to dry cell weight per liter according to predetermined calibration data (1 $A_{600}$ unit = 0.35 g/l). Culture samples of 2 ml and 5 ml, from flasks and fermenter respectively, were taken at intervals and centrifuged at 10 100 *g* for 3 min. Supernatants and pellets were frozen on solid $CO_2$ and stored at −20 °C until the proper determinations were made. The protein concentration was determined on cell pellets by the Lowry method (Lowry et al. 1951). Glucose remaining in the supernatant was measured with a Ektachem DT60 II multiple analyzer (Kodak, Rochester, N.Y.). β-Galactosidase activity was determined at 28 °C in the cell pellets by a modification of the method of Miller (Ferrari et al. 1986), using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate. One unit of the enzyme activity (Miller unit) was defined as the amount of enzyme hydrolyzing 1 nmol ONPG/min at 28 °C. Acetate, propionate, lactate and butanediol levels were determined by HPLC analysis. The separation system consisted of a fast fruit-juice analysis column (7.8 × 150 mm, Waters, Millipore Corp. Milford, Mass.), packed with sulfonated styrene divinylbenzene resin. Elution was carried out isocratically, using 2.5 mM $H_2SO_4$ solution as the mobile phase at 40 °C, and a flow rate of 1.0 ml/min. The compounds were detected with a 410 refractive-index detector (Waters, Millipore Corp. Milford, Mass.).

## Results

### Mineral medium evaluation

Most of the reports on the expression of the *aprE* gene have employed Schaeffer complex medium. However, in addition to the high cost of such medium, its use can mask some important effects of the manipulation of different environmental parameters on cell physiology and, in general, on the whole fermentation process. Therefore, for a good control of the environmental and physiological conditions in *B. subtilis* fermentation processes, the use of a defined mineral medium, supplied with a unique carbon source, e.g. glucose (MMG), is highly desirable (Pierce et al. 1992). On this basis, a medium that could provide all the necessary elements to obtain 5 g cells from 10 g glucose was designed (see Materials and methods). With such a composition, the carbon/nitrogen ratio was 5.5 and the limiting nutrient was carbon.

To evaluate β-galactosidase production under controlled conditions (*i.e.* no oxygen-transfer limitations, and automatic control of pH and dissolved oxygen), the BB804 strain was cultured in MMG supplemented with 11.6 g/l glucose using 1-l batch fermentations Under these conditions, the specific growth rate during the exponential phase was 0.38 $h^{-1}$ (Fig. 1). From the metabolites determined by HPLC, only acetate and low

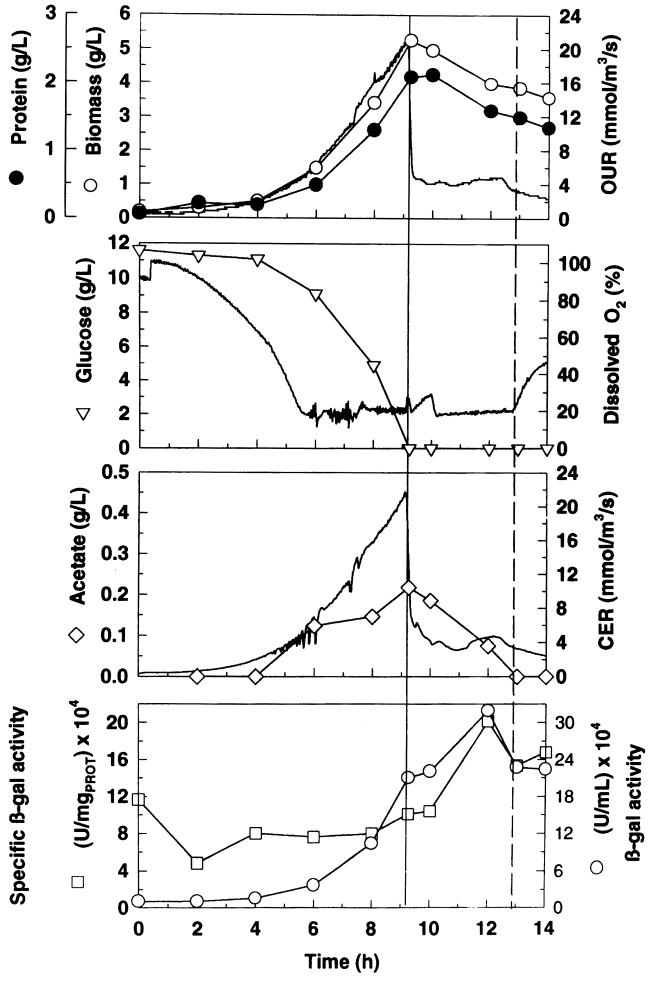


**Fig. 1** Batch culture in a 1-l fermenter in glucose mineral medium. Vertical line at 9 h denotes onset of the stationary phase. Dashed line denotes the time of acetate depletion

quantities of propionate were detected (Fig. 1). However, while acetic acid production reached its maximum at the onset of the stationary phase it was consumed later. The end of the exponential growth occurred upon glucose depletion, and corresponded to the onset of acetic acid consumption, and a characteristic sharp decrease in $O_2$ uptake and $CO_2$ evolution rates. Such a sharp change was used for the on-line assessment of the stationary-phase onset. After acetate depletion, the $O_2$ uptake and $CO_2$ evolution rates steadily decreased, and the oxygen partial pressure increased above 20%.

The β-galactosidase production during exponential growth comprised around 45% of the maximum, which was obtained 3 h after the initiation of the stationary phase (Fig. 1). The maximum specific β-galactosidase activity was $20.1 \times 10^4$ U/$mg_{protein}$. It should be noted that, during the exponential growth phase, the volumetric enzyme activity increased proportionally to biomass concentration. This indicates that, for MMG, enzyme production is partially growth-associated. These results contrast with the well-known behavior of the *aprE* gene, that is, its expression is repressed during vegetative growth in complex medium (Ferrari et al. 1986, 1993). Furthermore, all fermenter cultures showed a high initial specific β-galactosidase activity as the inocula used, originating from exponential cultures grown in MMG, exhibited a high specific enzyme activity .

In all fermenter studies, a good correlation between cell concentration, $O_2$ uptake and $CO_2$ evolution was observed for the exponential growth phase (see Fig. 1). Such a correlation is useful in computerized control strategies, where on-line measurements of biomass can be made without culture sampling. It should be noted that, during the stationary phase, a continuous decrement of biomass was observed (Fig. 1). Since this decrement was also observed at the level of protein measured in cell pellets (see Fig. 1), we attributed it to cellular lysis. This phenomenon was observed during the first 3 h of the stationary phase, with a specific first-order lysis rate of 0.118 $h^{-1}$, which means that 30% of the cells lysed during this period.

## Schaeffer medium evaluation

To corroborate that the *aprE* expression observed during vegetative growth, was not due to a strain property, its behavior was analyzed in Schaeffer's and MMG medium at flask level, and the results are summarized in Fig. 2. Before glucose depletion, two clearly distinctive growth phases were observed for the culture performed with MMG medium at flask scale. During the first 6 h of the culture, a high growth rate of 0.36 $h^{-1}$ was obtained. The second growth phase had a rate of 0.09 $h^{-1}$. The beginning of this second phase coincided with the onset of propionate production and acetate consumption, which, in turn, were probably caused by oxygen-transfer limitations. In Schaeffer's medium, the BB804 strain produced only negligible quantities of β-galactosidase before the onset of stationary phase and reached its maximum value around 3 h after the initiation of the stationary phase. This behavior is typical for *aprE* expression in complex medium (Ferrari et al. 1986). In turn, for MMG medium, the volumetric activity of β-galactosidase increased early during the exponential phase of the culture. Upon glucose depletion,the specific β-galactosidase activity had already reached $12 \times 10^4$ U/$mg_{protein}$, which corresponded to about 55% of the maximum enzymatic activity obtained at the end of the culture ($21 \times 10^4$ U/$mg_{protein}$). Furthermore, the maximum specific β-galactosidase activity obtained in Schaeffer's medium ($16 \times 10^4$ U/$mg_{protein}$) was around 20% less than the maximum specific activity obtained with the MMG (see Figs. 1, 2). These results indicate that medium composition seems to be responsible for the observed *aprE* expression during exponential growth.

## Effect of initial glucose concentration

In previous experiments with the same strain, and in the presence of initial high concentrations of glucose in

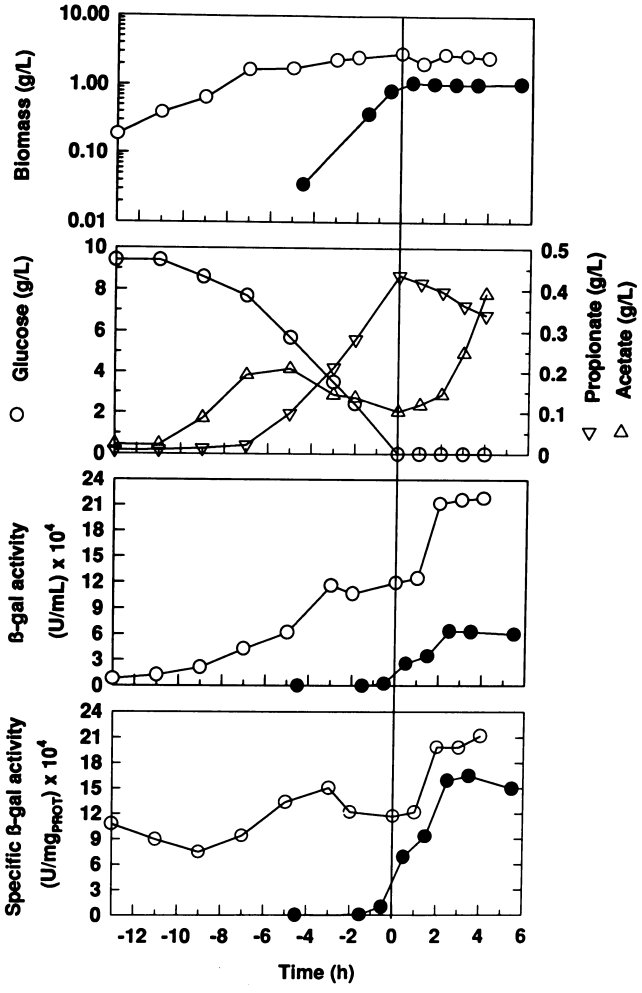


**Fig. 2** Shake-flask cultures in Schaeffer's (closed symbols) and in glucose mineral medium (open symbols). Time 0 and vertical line denote the onset of the stationary phase for both cultures

MMG, acetic acid was never produced above 1 g/l (unpublished observations). On this basis, it was decided to evaluate in more detail whether the initial glucose concentration had an effect on *B. subtilis* physiology and β-galactosidase production. To this end, several batch fermentations were performed, and three initial glucose concentrations were tested: 11, 26 and 30 g/l. The results of these experiments are presented in Table 1. As can be seen, in all conditions, the growth rate and cell yield were essentially the same. Likewise, the specific glucose and oxygen consumption rates remained relatively constant at 0.94 g glucose (g cell dry weight)$^{-1}$h$^{-1}$ and 15 mmol $O_2$ (g cell dry weight)$^{-1}$h$^{-1}$. During exponential growth, cells maintained a constant specific β-galactosidase activity, which was approximately 40%–60% of the maximum activity obtained in the stationary phase (data not shown). Increments in biomass (1.7-fold), and acetic acid production rate (2.5-fold) were observed when the initial glucose concentration was increased from 11 g/l to 30 g/l. However, the maximal β-galactosidase volumetric activity was obtained at an initial glucose concentration of 26 g/l. This value was 3.1-fold higher than that of the culture with 11 g/l of glucose, and 13-fold higher if compared to the culture based on complex medium (see Fig. 2). In this set of experiments, during the first 3 h of the stationary phase, cell lysis was also observed with a specific rate of lysis of 0.08 h$^{-1}$.

## Reduction of cell lysis during the stationary phase

The propensity to autolysis is a well-known property of *B. subtilis*. This is probably due to the combined effect of glucose depletion (Jollife et al. 1981) and the presence of monovalent cations (Svarachorn et al. 1989; Tsuchido 1994), originating from the phosphate salts present in the MMG. Moreover, it has been reported that the addition of an oxidizable carbon source to starved and lysing cultures of *B. subtilis* prevents autolysis (Jollife et al. 1981). For this reason, glucose was fed at the onset of the stationary stage to obtain the maximum biomass for heterologous protein synthesis, and to prevent cellular lysis. The amount of glucose feed needed to maintain cellular integrity was determined from the specific glucose consumption rate during the stationary phase. Such a value corresponds to the maintenance coefficient, which was 0.11 g glucose (g cell dry weight)$^{-1}$h$^{-1}$. Figure 3 shows a culture where, at the onset of the stationary phase, glucose was added according to the calculated maintenance coefficient. As can be seen, no significant lysis was observed, since the cell concentration remained relatively constant at 5.69 ± 0.66 g/l. Such an effect is more evident if the protein content is considered, as it remained at a constant value of 1.86 ± 0.12 g/l. In contrast to the cultures without glucose feeding (see Fig. 1), the biomass

**Table 1** Effect of initial glucose concentration in mineral medium supplemented with glucose on stoichiometric and kinetic parameters on the 1-l fermenter scale

| Initial glucose (g/l) | Specific growth rate[a] ($h^{-1}$) | Biomass yield[a] (g Glc/g dry cell wt) | Maximum biomass[b] (g/l) | Specific acetate production rate[a] [mmol (g dry cell wt)$^{-1}$ $h^{-1}$] | $10^{-4}$ × Maximum β-Galactosidase activity (U/ml) |
|---|---|---|---|---|---|
| 11.2 | 0.44 | 0.465 | 5.21 | 0.33 | 27 |
| 25.9 | 0.40 | 0.425 | 8.96 | 0.82 | 84 |
| 30.6 | 0.43 | 0.468 | 12.81 | 0.85 | 66 |

[a]Values calculated from the exponential-phase data
[b]Maximum biomass obtained at the onset of the stationary phase

and protein concentration steadily declined after glucose depletion, from 5.26 to 3.55 g/l and from 2.08 to 1.34 g/l respectively. Growth, glucose consumption, $O_2$ uptake, $CO_2$ evolution, dissolved oxygen and β-galactosidase patterns during the exponential growth phase were similar to those of other batch cultures. However, after 17 h of cultivation, the maximum volumetric activity obtained was $53 \times 10^4$ U/ml. This level was 96% higher than the one obtained in batch cultures, with a similar initial glucose concentration and without glucose feeding during the stationary phase (see Table 1, line 1). At the end of the culture, the total glucose consumed (*i.e.* initial concentration plus the amount fed during the stationary phase) was 15.1 g (Fig. 3).

Figure 3 also shows that the $O_2$ uptake and $CO_2$ evolution rates sharply decreased after glucose depletion, but partially recovered upon glucose feeding. Upon acetate depletion, at 15 h, both rates decreased and the dissolved oxygen increased steadily. As in batch cultures, the $O_2$ uptake and $CO_2$ evolution rates provided a good indication of biomass concentration during the exponential growth phase.

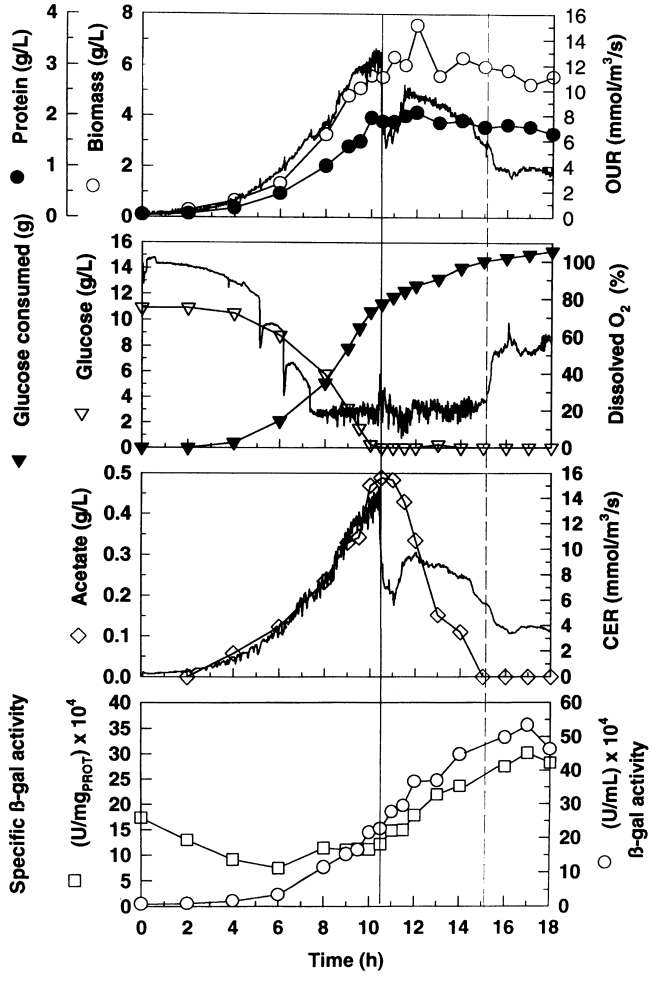


**Fig. 3** Effect of glucose feeding during the stationary phase. Initial glucose concentration during batch cultivation, 10.9 g/l. Vertical line at 10.5 h denotes onset of the stationary phase and beginning of glucose feeding at a constant supply rate of 0.11 g Glc (g dry cell weight)$^{-1}$h$^{-1}$. Dashed-line denotes the time of acetate depletion

## Discussion

The effect of growth conditions on carbon utilization, organic acid by-product formation and gene expression in *B. subtilis* strains using continuous cultures has been documented recently (Snay et al. 1989; Rincón et al. 1994; Vierheller et al. 1995). Furthermore, the importance of asporogenous mutants for fed-batch and continuous fermentation of *B. subtilis* strains has been reported (Pierce et al. 1992; Oh et al. 1995). In this report, the effect on β-galactosidase production, in a recombinant *B. subtilis* strain, of mineral medium, initial glucose concentration and glucose feeding during the stationary phase, was explored. Compared to a production processes based on complex medium, the use of defined medium supplemented with 26 g/l of glucose, allowed a 13-fold increase in the β-galactosidase volumetric activity. Contrary to what has been observed in other *B. subtilis* strains (Park et al. 1992; Vierheller et al. 1995), in the presence of high glucose concentrations, the BB804 strain did not produce excessive amounts of acetic acid that could inhibit growth or protein production. This is probably due to the *degU32(Hy)* mutation. It is known that strains carrying this mutation (originally named *sacU$^h$*), in addition to overexpressing extracellular enzymes, are capable of sporulation in rich medium or in mineral medium containing casein hydrolysate and glucose excess (Kunst et al. 1974).

To our knowledge, this is the first time that the expression of the *aprE* gene has been detected during exponential growth, at such a high level. At this point, no explanation for such a phenomenon is available. However, similar behavior has been observed for the production of the neutral protease in *B. megaterium*. In that case, neutral protease was synthesized throughout the growth cycle in minimal medium while, in complex medium, it was repressed during growth (Priest 1977).

The specific level of β-galactosidase obtained in this work ($20.1 \times 10^4$ U/mg$_{protein}$) is 90-, 28- and 2.7-fold higher than the higher levels reported by Pierce et al. (1992), Park et al. (1992), and Ferrari et al. (1986). We consider that these differences are due to the combination of genetic manipulations as well as to variations in culture conditions.

Finally, $O_2$ uptake and $CO_2$ evolution kinetics allowed the on-line determination of glucose depletion, the onset of the stationary phase, and biomass estimation during exponential growth. Such behavior could be useful for automating the process and establishing control strategies for substrate feeding during fed-batch cultures.

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