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Oxygen supply in *Bacillus thuringiensis* fermentations: bringing new insights on their impact on sporulation and δ-endotoxin production

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Abstract The growth kinetics, sporulation, and toxicity of *Bacillus thuringiensis* var. *israelensis* were evaluated through the analysis of batch cultures with different dissolved oxygen (DO) profiles. Firstly, DO was maintained constant at 5%, 20%, or 50% throughout fermentation in order to identify the most suitable one to improve the main process parameters. Higher biomass concentration, cell productivity, and cell yield based on glucose were obtained with 50% DO. The higher aeration level also resulted in higher spore counts and markedly improved the toxic activity of the fermentation broth, which was 9-fold greater than that obtained with 5% DO (LC_{50} of 39 and 329 mg/L, respectively). Subsequently, using a two-stage oxygen supply strategy, DO was kept at 50% during the vegetative and transition phases until the maximum cell concentration was achieved. Then, DO was changed to 0%, 5%, 20%, or 100% throughout sporulation and cell lysis phases. The interruption of oxygen supply strongly reduced the spore production and thoroughly repressed the toxin synthesis. On the contrary, when DO was raised to 100% of saturation, toxic activity increased approximately four times (LC_{50} of 8.2 mg/L) in comparison with the mean values reached with lower DO levels, even though spore counts were lower than that from the 50% DO assay. When pure oxygen was used instead of normal air, it was possible to obtain 70% of the total biomass concentration achieved in the air assays; however, cultures did not sporulate and the toxin synthesis was consequently suppressed.

Keywords *Bacillus thuringiensis* var. *israelensis* · Oxygen supply · Sporulation · Toxic activity

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Introduction

Studies on the production of bioinsecticides using different *Bacillus thuringiensis* (Bt) subspecies have been reported in the last two decades. This aerobic spore-forming bacterium produces a variety of plasmid-encoded crystal proteins known as δ-endotoxins or Cry proteins, which exhibit high toxicity against larvae of certain agricultural and forest pests as well as vectors diseases. This toxic activity is highly specific, usually to a particular insect order, and is determined by the type of Cry proteins produced by each Bt subspecies (Baum and Malvar 1995; Schnepf et al. 1998). Crystals produced by *Bacillus thuringiensis* var. *israelensis* (Bti), the most effective subspecies against Diptera, are formed by three Cry toxins (CryIVA, 135 kDa; CryIVB, 128 kDa; and Cry11A, 72 kDa) encoded by the

corresponding *cry* genes *cryIVA*, *cry IVB*, and *cry11A*, respectively (Höfte and Whiteley 1989; Crickmore et al. 1998). The synthesis of Cry proteins is closely linked to the sporulation phase, which occurs when the culture enters the stationary growth phase. The mature spores and crystals are released into the broth by sporangia lysis during the last hours of fermentation and the spores–crystals suspension is recovered to formulate commercial products (Yang and Wang 1998; Aronson 2002). Several Bti formulations have been used worldwide especially for control of mosquitoes implicated in a variety of widespread human diseases, such as yellow fever, dengue, and malaria (Lacey et al. 2001).

Most laboratory-scale studies on Bt fermentation have been carried out using submerged cultures in batch mode. The main focus has been the optimization of process variables that lead to improvements in growth, sporulation, and toxin synthesis. Several culture media formulations, frequently based on low-cost substrates, have been proposed with this same purpose (Liu and Tzeng 2000; Saksinchai et al. 2001; Yezza et al. 2005; Ghribi et al. 2007b). Additionally, there is evidence that process performance can be affected by the Bt subspecies or strain employed (Farrera et al. 1998). Therefore, and considering the biotechnological importance of this bacterium, it is not surprising that Bt fermentation is still of great scientific interest.

Among the operational conditions, oxygen supply has been shown to be decisive in Bt culture, independently of the subspecies. In two of first studies on this subject, Pearson and Ward (1988) and Abdell-Hameed et al. (1991) pointed out the need for high aeration rates so as to reach appropriate Bti development. Later, two other papers (Avignone-Rossa et al. 1992; Kraemer-Schafhalter and Moser 1996) analyzed the growth kinetics of this subspecies under controlled oxygen levels in the fermentation broth, linking it with sporulation and toxin synthesis. Avignone-Rossa et al. (1992) employed different oxygen transfer rates (OTR), by fixing air flow rate and varying agitation speed, to obtain limiting and non-limiting oxygen conditions. Maximum biomass, spore count, and toxin production were constant in non-limited cultures (OTR 14.9–48 mmol O_2/L/h), but under oxygen limitation (OTR 14.9–48 mmol O_2/L/h), besides lower biomass, a significant decrease in spore count and toxin concentrations was observed. Kraemer-Schafhalter and Moser (1996) also utilized variable agitation and fixed aeration in order to obtain different dissolved oxygen concentrations (DO) in the fermentation broth, which were maintained constant all over the cultivation time. The lowest biological activity was achieved in the culture with the highest level of dissolved oxygen (70%), whereas the optimum was reached with DO 26%. By using different combinations of aeration and stirring speeds, Maldonado-Blanco et al. (2003) also reported that operational conditions that ensured a DO concentration at or above 26% in most of the time led to more toxic Bti products against *Aedes aegypti* larvae. By employing a similar strategy, Abdell-Hameed (2001) showed, nevertheless, that agitation rate, instead of the aeration rate, had the most significant effect on toxin yield by Bti.

More recently, attempts have been made to improve Bt insecticides production based on phase-specific oxygen optimization, as already tested for other fermentation variables. In order to evaluate the impact of aeration conditions on sporulation, as an isolated phase of Bti fermentation, Sarrafzadeh and Navarro (2006) performed fed-batch cultures under constant DO concentration of 20% for 24 h, and sustained them in batch mode for additionally 24 h at different OTR. The highest sporulation rate (100%) was observed in the absence of oxygen, while the lowest rate (48%) occurred with saturated oxygenation, the results with intermediate OTR being closer to those achieved using interrupted aeration. In spite of the significant inhibition of sporulation, the highest toxicity was detected at the oxygen saturation condition. Using another approach, Ghribi et al. (2007a) assessed the development of *B. t.* var. *kurstaki* (Btk) cultures under various aeration profiles by regulating DO to specific levels in two independent phases. By keeping DO concentrations of 60% or 70% during the first 6 h, with a subsequent drop to 40% throughout the rest of the fermentation, the authors observed lower spore production but higher δ-endotoxin synthesis. With higher DO values, spore counts were increased, but toxin yields were largely reduced.

In view of all results presented above, it seems clear that numerous variables interact and influence sporulation and toxin production in Bt fermentations under variable oxygen levels. As such, the aim of this work was to contribute to the knowledge on the role of oxygen in this process by assessing the global behavior of Bti cultures carried out at constant and/or variable DO levels. Taking into account that it is quite difficult to optimize simultaneously the complex processes of growth, sporulation, and δ-endotoxin synthesis to obtain the best bioinsecticide production using this bacterium, our data present technical alternatives to reasonably achieve these objectives based on adequate oxygen level profiles throughout the fermentation run.

Materials and methods

Microorganism, medium, and inoculum preparation

International standard strain of *B. thuringiensis* var. *israelensis* (IPS-82) was obtained from Institut Pasteur, Paris, France. The strain was maintained as a sporulated culture on nutrient agar at 4 °C. Modified GYS medium (Rogoff and Yousten 1969) was used for inoculum production and

fermentation assays. The medium contained (in g/L): glucose, 30.0; yeast extract, 12.0; $(NH_4)_2SO_4$, 3.0; $CaCl_2{\cdot}2H_2O$, 0.12; $MgSO_4{\cdot}7H_2O$, 1.5; $MnSO_4{\cdot}H_2O$, 0.09; K_2HPO_4, 1.5; KH_2PO_4, 1.5. Concentrated glucose and all salts solutions were prepared and sterilized separately and added to a previous sterilized yeast extract solution before inoculation.

Cultures conditions

For inoculum preparation, five 125-mL Erlenmeyer flasks containing 25 mL GYS medium each were inoculated with a loopful of sporulated culture and statically incubated for 15 h at 30 °C. The whole content of the flasks was transferred to a 500-mL Erlenmeyer flask and an aliquot of this culture, corresponding to 5% of the initial volume of fermentation medium, was used to inoculate the bioreactor medium. Fermentation assays were carried out in batch mode in a 2-L Biostat B fully controlled bioreactor (B. Braun Biotech, Germany) with 1.5 L of culture medium. The pH was continuously maintained at 6.5 with automatic addition of 5 M KOH and the temperature was kept at 30 °C. Foaming was control (led by the use of antifoam (Fluent Cane 114, Dow Chemical Company). Dissolved oxygen concentrations (DO) were kept constant at different values throughout fermentation (see details in "Results" section) by variation of the agitation speed and the air supply. The following strategy was employed: just before the inoculation, nitrogen gas was introduced in the culture medium in order to expunge part of the oxygen until obtaining the desirable DO concentration. In the first 2 h, when the oxygen demand was very low, a low agitation (between 20 and 180 rpm) was applied and controlled automatically at the settled values. From this point on, the agitation speed was directly operated by the DO controller of the control unit and varied automatically within a predefined range (200–1,100 rpm). An initial air flow rate of 0.66 vvm was applied, but from the end of *lag* phase until the maximum cell concentration was reached, the air supply was gradually increased using a rotameter. The air flow rate values (1 to 2 vvm) and the frequency they were applied varied with the required DO level. At the later growth phases, the airflow rates were reduced since the respiratory metabolism was very low. When a DO of 100% was required, a low flow rate (0.2 vvm) of pure oxygen was injected into the bioreactor vessel together with the inlet air. The fermentation runs were stopped when approximately 50% of the sporulated cells were lysed.

Analytical methods

During the vegetative growth phase, defined as phase I in this work (see details in "Results" section), cell growth was monitored by measuring optical density (OD) of the diluted fermentation broth at 610 nm in a spectrophotometer (Micronal B582, Brazil). OD values were converted into concentration (g dry weight/L) using the equation from a calibration curve. For all subsequent samples, biomass concentration was determined by dry weight measurement since the flocculation of culture, which characterizes the transition and sporulation phases (phases II and III, respectively), hinders the biomass quantification by turbidimetry. Hence, a predefined volume of fermentation broth was centrifuged at $2{,}100\times g$ for 20 min (EPPENDORF 5804 R), washed three times and the pellet dried at 85 °C for 24 h. Sporulation development and cell lysis were monitored by analyzing fresh fermentation broth preparations in a phase contrast microscope (JENA, Jenamed 2). Spore counts were performed by diluting broth samples in saline solution, followed by vigorous mixing and direct microscopic counting in a Neubauer chamber. For each sample, two independent dilutions were prepared to obtain between 30 and 60 spore counts per field, resulting in a total of 16 fields counted. Both lysed and unlysed sporulated cells were counted as spores. Toxicity was determined by bioassays against early fourth instars larvae of *Aedes aegypti* according to Misch *et al*. (1992), using spore-crystal suspensions prepared as described in Poopathi and Abidha (2007) with slight modifications. The mortality data were recorded after 24 h and the values of 50% lethal concentration (LC_{50}) were obtained by probit analysis. Glucose was assayed by HPLC (Waters, Milford, MA, USA) with RI detector (model 2414), using a Biorad Aminex HPX-87H column (300 mm × 7.8 mm) at 45 °C, with 0.005 M sulfuric acid as eluent, at a flow rate of 0.6 mL/min. Dissolved oxygen was continuously measured using a polarographic electrode (Mettler Toledo, InPro 6100) connected to a digital control unit. The electrode was calibrated in fermentation medium just before the inoculation at 30 °C and initial settled aeration/agitation conditions. The results related to biomass concentration, spore counts, and toxicity represent the average of two determinations from two independent experiments for each culture condition.

Estimation of biomass yield, cell productivity, and rate coefficients

The biomass yield based on glucose ($Y_{x/s}$, g/g) was calculated as the ratio of the biomass produced (g dry weight/L) to the glucose consumed (g/L) until $t_{X\mathrm{m}}$ (h), which corresponded to the time to achieve the maximum cell concentration (X_m). Cell productivity (p_x) was calculated as the ratio of the biomass produced to elapsed time until $t_{X\mathrm{m}}$. The maximum specific growth rates (μ_m) were calculated from linear regressions of natural logarithm of biomass concentrations versus time. The angular coefficient of the linear trend corresponded to the μ_m value.

Results

In a previous work (Berbert-Molina et al. 2008), we proposed the division of the cell growth of *B. thuringiensis* var. *israelensis* (Bti) in four distinct phases, based on morphological and physiological changes observed in batch fermentations carried out under different glucose concentrations and unlimited oxygen supply (DO≥35% saturation). These phases were named as follows: phase I, vegetative growth; phase II, transition to sporulation; phase III, sporulation; and phase IV, cell lysis. The main characteristics exhibited by cultures within each phase were well detailed and allowed the identification of the temporal sequence of the main events related to its overall metabolic behavior. Herein we describe a set of new assays in which different DO levels were employed along these fermentation phases, in order to analyze their impact on the kinetics of bacterial growth and mainly on sporulation and toxicity. The specific features of each fermentation phase are highlighted throughout the text.

Growth, sporulation, and toxicity under constant dissolved oxygen levels during all fermentation phases

In a first group of batch runs, DO concentrations were kept constant at 5%, 20%, or 50% throughout fermentation. The aim was to establish the time required to achieve maximum cell concentration, which characterizes the end of phase II, as well to choose the best one for the toxin production. The general fermentation parameters, spore counts, and the toxic activity are shown in Table 1. The corresponding profiles of cell growth and substrate consumption are reported in Fig. 1. Figure 2 shows the speed agitation, airflow rates, and dissolved oxygen (DO) variations throughout the fermentation, corroborating the effectiveness of the strategy employed to DO control. As can be seen, a slight increase in maximum cell concentration (X_m) was achieved by increasing oxygen supplies. Maximum X_m variation was 16%, between concentrations achieved in the lowest and the highest DO conditions. Regarding the time required to reach such concentrations (t_{Xm}), no difference was observed between values achieved using DO 5% or 20% (14 h for both); however, when DO was set at 50% of saturation, t_{Xm} was shortened by 2 h. In this circumstance, increasing cell productivities (p_x) were calculated, with the maximum value of 1.23 g/L/h in the 50% DO assay. Cell yields based on glucose ($Y_{x/s}$) were identical for the two lowest DO concentrations, but somewhat higher (15%) for the highest one. As can be observed (Table 1; Fig. 1), in neither case total glucose consumption took place during cellular growth (phases I and II), probably due to the depletion of yeast extract in fermentation broth, as previously demonstrated (Berbert-Molina et al. 2008). The highest $Y_{x/s}$ value (0.53 g/g) was obtained with 50% DO.

Table 1 Results obtained in *Bacillus thuringiensis* var. *israelensis* batch fermentations carried out at constant dissolved oxygen (DO) concentrations

	DO (%)		
	5	20	50
t_{Xm} (h)	14	14	12
t_f (h)	27	26	26.5
X_m (g/L)	12.4^{c}	13.4^{b}	14.8^{a}
ΔS_{Xm} (g/L)	26.8	29.2	27.7
$Y_{x/s}$ (g/g)	0.46^{b}	0.46^{b}	0.53^{a}
p_x (g/L.h)	0.89^{b}	0.96^{b}	1.23^{a}
μ_m (h^{-1})	0.99^{a}	1.02^{a}	1.01^{a}
Spores (ufc/mL)	3.1×10^{9c}	3.4×10^{9b}	3.8×10^{9a}
LC_{50} ($mg.L^{-1}$)	325.2^{c}	104.5^{b}	39.2^{a}

Analysis of variance with Tukey's multiple range tests. Values in the same row that are not preceded by the same upper case letters are significantly different ($p<0.05$). t_{Xm} time to achieve the maximum cell concentration; t_f fermentation time; X_m maximum cell concentration; ΔS_{Xm} glucose consumption until t_{Xm}; $Y_{x/s}$ biomass yield based on glucose, calculated at t_{Xm}; p_x cell productivity, calculated at t_{Xm}; μ_m maximum specific growth rate; LC_{50} lethal concentration inducing 50% mortality

In all cases, growth patterns were characterized by comparable *lag* phases followed by exponential growth phases with similar values of maximum specific growth rates (μ_m). This affords to assure that even the lowest DO employed (5%) was over the critical DO value for the microorganism, in the process conditions employed. Such a critical dissolved oxygen concentration (C_{crit}) is consistent with that reported for Btk (Moraes et al. 1980). Furthermore, these high μ_m values (around 1 h^{-1}) showed that the cultures were not under any other nutritional or environmental limitation, including oxygen, during the first 4–5 h of fermentation, the time they were calculated. Similar or even smaller μ_m values were previously described for other Bt strains under culture conditions that were not considered limiting for growth (Flores et al. 1997; Rowe et al. 2003; Yezza et al. 2005; López-Y-López and De La Torre 2005). For all conditions evaluated, it was also observed that the exponential phase was followed by a period of non-exponential growth, where cell growth curves presented a linear profile. Such a trend was more pronounced for the 50% DO assay (Fig. 1c), in which it was extended from about 5.5 to 8.2 h. The level of oxygen in the culture medium also affected instantaneous growth rates (dX/dt), whose values were 2.8, 3.2, and 3.8 g/L/h for DO 5%, 20%, and 50% (data not shown), respectively. The influence of dissolved oxygen concentrations on growth rates of Bti has already been mentioned earlier, but their values were not described (Kraemer-Schafhalter and Moser 1996). The linear growth profile was also evidenced by other Bt strains growing in different conditions (Vallejo et al. 1999; Rowe et al. 2003; López-y-López and De La

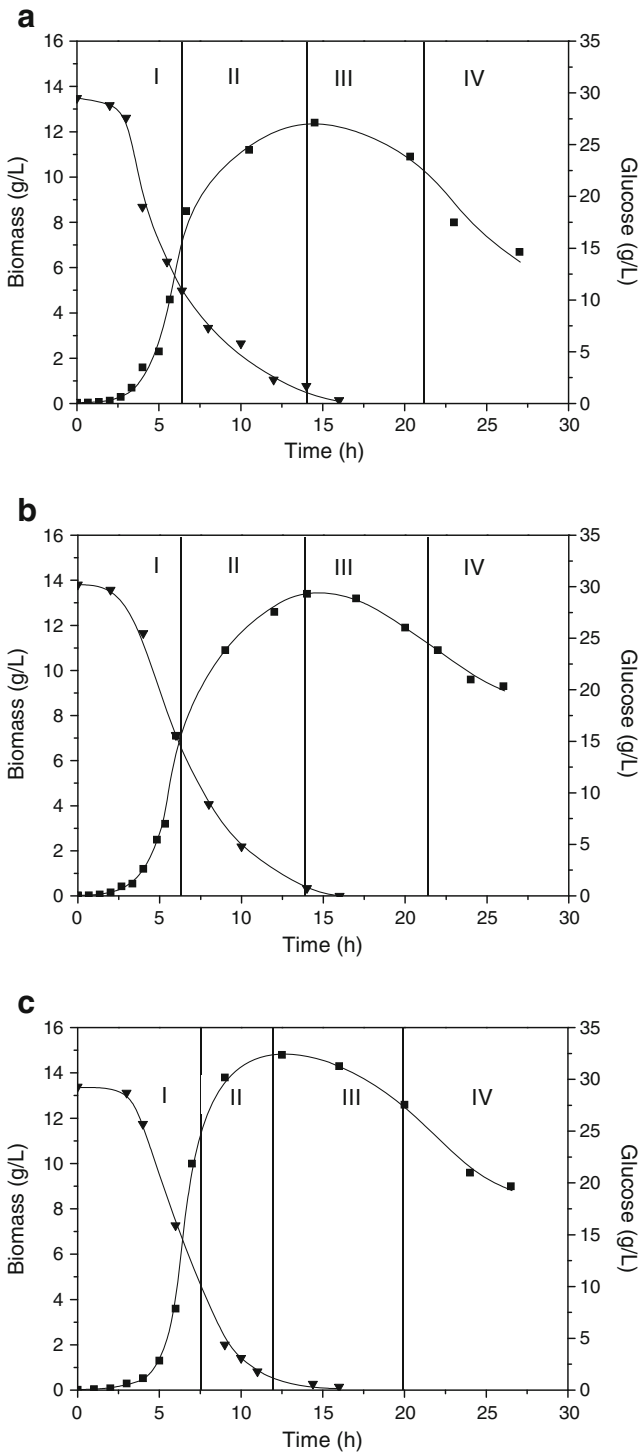

Fig. 1 Biomass (*filled squares*) and glucose (*filled down-pointing triangles*) concentrations in *Bacillus thuringiensis* var. *israelensis* batch fermentations carried out at constant dissolved oxygen concentrations of 5% (**a**), 20% (**b**), and 50% (**c**)

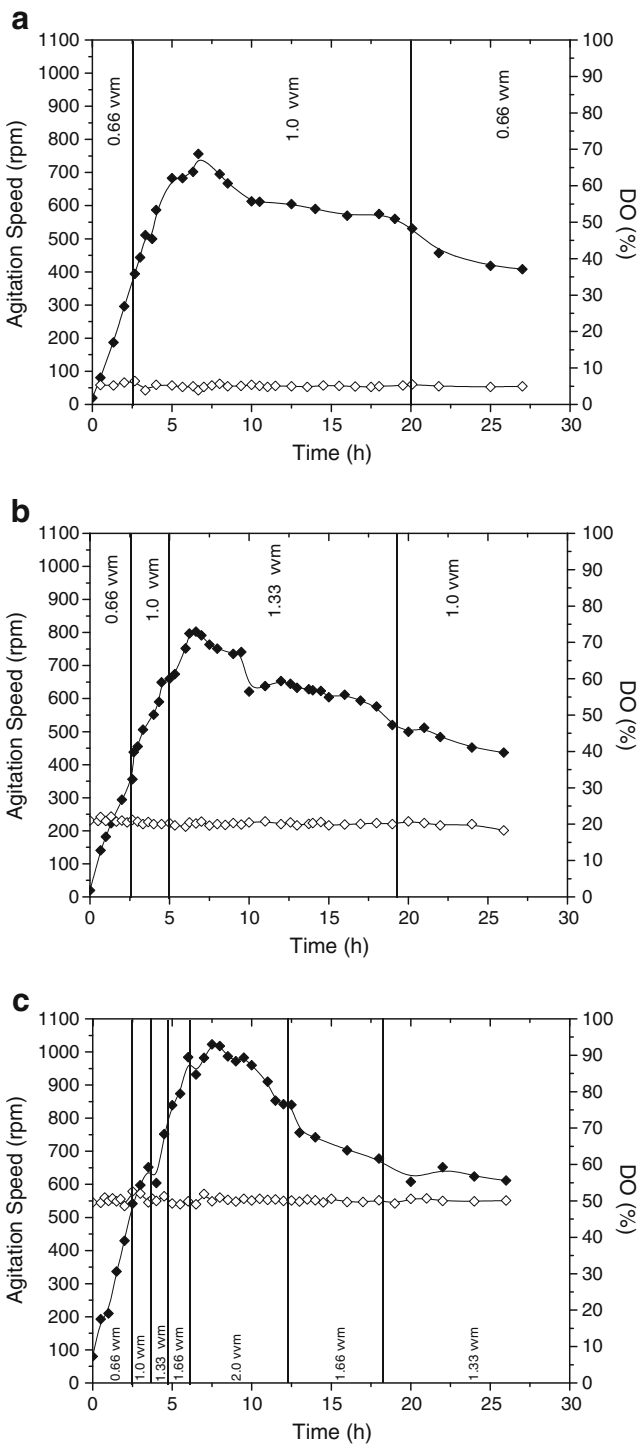

Fig. 2 Agitation speed (*filled diamonds*), airflow rates (values and intervals), and dissolved oxygen (DO) (*open diamonds*) variations throughout the fermentation runs in the 5% (**a**), 20% (**b**), and 50% (**c**) DO assays

Torre 2005), indicating that this is a common specific characteristic of this bacterial species, as observed with *Bacillus cereus* (de Vries et al. 2004), which is genetically very closely related to Bt (Helgason et al. 2000).

Concerning morphological features, cells showed high motility from the end of the *lag* phase onward, independently of DO levels. A few hours later, this event was followed by bacteria aggregation and clump formation, with

concomitant loss of motility. Such a trend, which defines precisely the end of the phase I or the beginning of the phase II (Berbert-Molina et al. 2008), occurred about 1.5 h later (at 7.6 h) in the 50% DO assay. On the other hand, the maximum cell concentration was reached at 12 h ($t_{X\mathrm{m}}$), which represented a shortening in transition phase (phase II) and an early start of sporulation.

The spore production was also improved with increasing DO levels (Table 1). The last two growth phases, whose main characteristics are the intense sporulation (phase III) and advanced cell lysis (phase IV) lasted longer with DO of 50%. During this period, all growth curves presented a short stationary phase followed by decreasing profile due to the reduction of cell mass caused by cells lysis.

The results showed a strong effect of oxygen supply on δ-endotoxin production. The higher the DO concentration the greater the toxicity, with almost 9-fold difference between the LC_{50} obtained with 5% and 50% DO. The difference dropped to 2.7 times when DO was raised from 20% to 50%, demonstrating that the oxygen requirement to support toxin synthesis in such cell concentrations was close to 50%. The results revealed also that oxygen levels exerted a much more pronounced effect on toxin synthesis than on spore formation.

Earlier works demonstrated that in Bt cultures oxygen uptake rates are very high during the vegetative growth, falling drastically after the beginning of the transition phase and maintaining a decreasing profile until the end (Rowe et al. 2003; Yezza et al. 2005; Berbert-Molina et al. 2008; Amicarelli et al. 2010). Indeed, some authors verified that lower aeration rates in the sporulation phase, in relation to those employed in the earlier stages of growth, led to a greater toxicity in Btk cultures (Ghribi et al. 2007a). So, the aim of next step of this work was to investigate how a change in DO concentrations in the course of the fermentation could affect Bti cultures. Taking into account that dissolved oxygen concentrations of 50% were able to support adequate cell growth in the medium employed, this concentration was chose for the first two phases of the fermentation runs.

Growth, sporulation, and toxicity under two-stage oxygen supply strategy

Four novel process conditions were tested in order to evaluate overall culture performance under variable DO concentrations throughout the fermentation phases. In all, dissolved oxygen was maintained constant at 50% until 12 h of fermentation, identified as the time needed to obtain the maximum cell concentration in the early assays. From this point on, aeration and/or agitation rates were shifted to keep DO levels above and below 50% along phases III and IV, when sporulation and toxin synthesis take place. In the two extreme conditions, DO concentration was reduced to 0% (50–0% DO assay) by an immediate interruption of air supply or, oppositely, it was incremented to 100% (50–100% DO assay) by increasing the oxygen level in the inlet gas. The two other concentrations tested in the first growth phases, 5% and 20%, were also investigated (50–5% and 50–20% DO assays, respectively).

As expected, there was no significant difference ($p<0.05$) between the maximum cell concentrations (X_{m}) obtained in each experimental condition (Table 2). Likewise, the mean X_{m} value was not different from that obtained with constant 50% DO in all phases. However, except for the 50–0% DO assay, the time required to achieve these cell concentrations ($t_{X\mathrm{m}}$) was extended by 1 h or more, resulting in a slight difference in cell productivities. Nevertheless, the mean p_x value did not differ ($p<0.05$) from that obtained with constant 50% DO. Kinetic growth profiles were also very similar until $t_{X\mathrm{m}}$ (Fig. 3), with identical μ_{m} values (Table 2). However, in phases III and IV the growth curve of the 50–100% DO assay diverged from all others, without the characteristic decreasing profile caused by sporangia lysis (Fig. 3b). The 50–0% (Fig. 3a) and 50–5% (not shown) DO assays exhibited a decrease in glucose uptake rate from $t_{X\mathrm{m}}$ onwards, with glucose depletion occurring later than that observed for the other conditions. As all cultures were under the same DO condition during vegetative growth, $Y_{x/s}$ values were not affected (Table 2).

In spite of similar X_{m} values, DO levels in phases III and IV had a significant effect on sporulation (Table 2). The impact was noteworthy when DO concentration was reduced to 0% after $t_{X\mathrm{m}}$ (50–0% DO assay; Fig. 3a), in which spores count was as low as 1.97×10^4 ufc/mL (Table 2). The higher DO concentrations, greater was the spore counts when 5%, 20%, or 50% DO level was applied (Table 2). In the 50–100% DO assay, the presence of pure oxygen in the inlet gas negatively affected spore counts, which was 40% lower than that of the 50% DO assay. Regarding cell lysis, the lower the DO level in phases III and IV, the higher the number of free spores (data not shown). In the 50–100% DO assay, the ratio vegetative/sporulated cells remained similar until the end, which explains the plateau in the growth curve. Large differences were observed in toxin production under the different aeration conditions in the last two phases (Table 2). No toxic activity was exhibited when oxygen supply was interrupted, although a portion of the population had sporulated. No significant difference ($p<0.05$) was observed in LC_{50} when DO was changed to 5%, 20%, or 50%, even though spore concentrations increased. On the other hand, with 100% DO a LC_{50} of 8.2 mg/L was found, which represented a 4-fold higher toxicity than the mean LC_{50} value obtained with 5%, 20%, or 50% DO.

Table 2 Results obtained in *Bacillus thuringiensis* var. *israelensis* batch fermentations carried out at different dissolved oxygen (DO) profiles throughout fermentation run

	DO (%)					
	50–0*	50–5*	50–20*	50–100*	20–50*	50**
t_{Xm} (h)	12.1	12.8	13.8	13.0	15	12
t_f (h)	25.7	25	22.2	23.5	23	26.5
X_m (g/L)	14.9^{a}	15.2^{a}	15.4^{a}	15.0^{a}	13.3^{b}	14.8
ΔS_{Xm} (g/L)	26.4	29.8	32.2	26.7	29.9	27.7
$Y_{x/s}$ (g/g)	0.56^{a}	0.52^{ab}	0.49^{b}	0.56^{a}	0.44^{c}	0.53
p_x (g/L.h)	1.23^{a}	1.19^{a}	1.12^{b}	1.15^{a}	0.89^{b}	1.23
μ_m (h^{-1})	1.10^{a}	1.13^{a}	1.12^{a}	1.13^{a}	1.18^{a}	1.01
Spores (ufc/mL)	1.97×10^{4e}	1.7×10^{9d}	2.1×10^{9b}	2.7×10^{9a}	2.0×10^{9}	3.8×10^{9}
LC_{50} ($mg.L^{-1}$)	ND	33.2^{b}	34.9^{b}	8.2^{c}	54.5^{a}	39.2

Analysis of variance with Tukey's multiple range tests. For the four first columns, values in the same row that are not preceded by the same upper case letters are significantly different ($p<0.05$). The values of the fifth column were statistically compared with the mean values of the four previous columns. t_{Xm} time to achieve the maximum cell concentration; t_f fermentation time; X_m maximum cell concentration; ΔS_{Xm} glucose consumption until t_{Xm}; $Y_{x/s}$ biomass yield based on glucose, calculated at t_{Xm}; p_x cell productivity, calculated at t_{Xm}; μ_m maximum specific growth rate; LC_{50} lethal concentration inducing 50% mortality; *ND* not detected

*The first value represents DO at phases I and II and the second value represents DO at phases III and IV

**Results from the 50% DO assay (statistics are shown in Table 1)

A more detailed analysis also showed that the spore count reached in the 50–20% DO assay (Table 2) was 62% lower than that of the 20% DO assay (Table 1). The same behavior was observed when the results of the 50–5% DO were compared to those of the 5% DO assays, where the difference was 82%. A similar trend was observed in toxin production since the LC_{50} values obtained in the 50–20% and 50–5% DO assays were 3-fold and 10-fold higher than those of the 20% and 5% DO, respectively.

It could be asked whether low LC_{50} and high spore counts found in the 50% DO assay were due to the maintenance of 50% DO in phases I and II or, on the contrary, in phases III and IV. To elucidate this question, another experiment was performed in which DO was maintained at 20% until t_{Xm} and then increased to 50% until the end (20–50% DO assay). As expected, the general fermentation parameters (Table 2) did not differ from those obtained in the 20% DO assay (Table 1). The kinetics of growth and glucose uptake profiles were also very similar (data not shown). But spore counts were significantly lower, while toxic activity was markedly improved (Table 2).

Growth, sporulation, and toxicity under two-stage oxygen supply strategy using pure oxygen

In order to investigate the real effect of pure oxygen on Bti fermentation and to clarify the results obtained in the 50–100% DO assay, the 50–5%, 50–20%, and 50–100% conditions were replicated, replacing the inlet air with pure oxygen. As shown, such approach had no influence on maximum specific growth rates (Table 3), which were also very similar to those of the aerated cultures, indicating the absence of growth inhibition by pure oxygen in the first hours. Analogous *lag* phases, followed by the characteristic linear profiles, were also observed (Fig. 4). However, after the shift in DO concentration, growth curves showed clear differences. There was a reduction in growth rates, followed by a small plateau and a new increment in cell concentration. In the case of the 50–5% DO (Fig. 4a) and 50–20% DO (Fig. 4b), when this diauxic behavior was more pronounced, a reduction in glucose uptake rate was also observed. However, except for the 50–5% DO, total glucose consumption took place until maximum biomass concentrations were reached (t_{Xm}). Even so, quite similar total cell concentrations (X_m) and comparable $Y_{x/s}$ values were achieved. The t_{Xm} values, in turn, diminished with increasing DO in the last phases, occurring at 16.5 h with DO 100% (Table 3), which resulted in a slight increase in cell productivity. Compared to the mean values obtained in the original experiments (air instead of pure oxygen), these differences were, however, very significant. The t_{Xm} values increased by more than 4 h, while X_m, $Y_{x/s}$, and p_x values were reduced by about 30%, 28%, and 60%, respectively.

Besides the features highlighted, the cultures performed with pure oxygen showed other metabolic dissimilarities. The characteristic acetic acid production

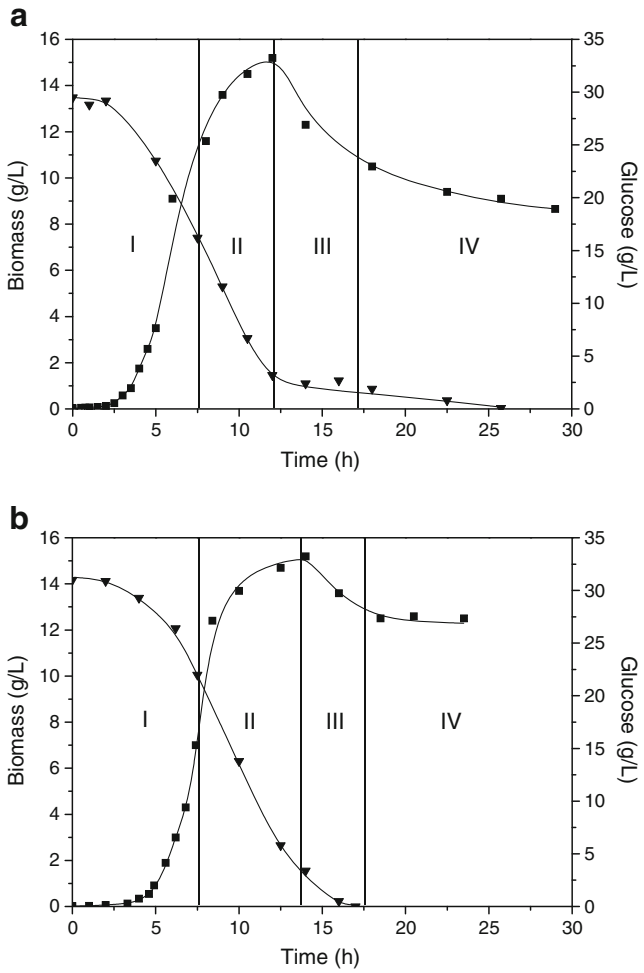

Fig. 3 Biomass (*filled squares*) and glucose (*filled down-pointing triangles*) concentrations in *Bacillus thuringiensis* var. *israelensis* batch fermentations carried out with 50% dissolved oxygen (DO) in the vegetative growth phase (phase I) and transition phase (phase II), and different DO in the sporulation phase (phase III) and cell lysis phase (phase IV). **a** 50–0%; **b** 50–100%. The first value represents DO at phases I and II and the second value represents DO at phases III and IV

was not observed, which was monitored by the amount of alkali for pH control. No KOH was utilized to maintain the settled pH value of 6.5, in contrast with that observed in the fermentation runs performed with air. Morphologically, the cells presented greater diameters and there was no sign of inclusions. The clumps, which began to appear after 8 h, reached much larger sizes and, contrary to the typical behavior, they did not disappear in the last fermentation stages. All these events resulted, ultimately, in a strong effect on the bacterial cellular cycle. Cultures were not sporulated and no biological activity was exhibited at the end of fermentation (Table 3). Because of the lack of sporulation, none of the growth curves presented the characteristic decreasing profile in phases III and IV.

Table 3 Results obtained in *Bacillus thuringiensis* var. *israelensis* batch fermentations carried out at different dissolved oxygen (DO) profiles throughout fermentation run, using pure oxygen instead of normal air

	DO (%)		
	50–5*	50–20*	50–100*
t_{Xm} (h)	18.5	18	16.5
t_f (h)	27.5	27	26.5
X_m (g/L)	10.8a	10.3a	10.6a
ΔS_{Xm} (g/L)	27.4	27.4	29.5
$Y_{x/s}$ (g/g)	0.39a	0.38a	0.36a
p_x (g/L.h)	0.58b	0.57b	0.64a
μ_m (h^{-1})	1.2	1.2	1.1
Spores (ufc/mL)	–	–	–
LC_{50} ($mg.L^{-1}$)	ND	ND	ND

Analysis of variance with Tukey's multiple range tests. Values in the same row that are not preceded by the same upper case letters are significantly different ($p<0.05$). t_{Xm} time to achieve the maximum cell concentration; t_f fermentation time; X_m maximum cell concentration; ΔS_{Xm} glucose consumption until t_{Xm}; $Y_{x/s}$ biomass yield based on glucose, calculated at t_{Xm}; p_x cell productivity, calculated at t_{Xm}; μ_m maximum specific growth rate; LC_{50} lethal concentration inducing 50% mortality; *ND* not detected

*The first value represents DO at phases I and II and the second value represents DO at phases III and IV

Discussion

Growth, sporulation, and toxicity under constant dissolved oxygen levels during all fermentation phases

The first set of experiments showed that the best biomass concentration, cell productivity, and cell yields based on glucose were obtained with 50% DO (Table 1). Avignone-Rossa et al. (1992) found smaller cell concentrations and $Y_{x/s}$ in Bti cultures under limited oxygen conditions (OTR 3.1 and 7.2 mmol O_2/L/h), as compared to those of non-limited concentrations (OTR 14.9–48 mmol O_2/L/h). Other authors reported greater differences in X_m and in $Y_{x/s}$ values in Bti fermentations at constant DO of 5% instead of 26%, 50%, or 70% DO (Kraemer-Schafhalter and Moser 1996). Compared to our own results (Berbert-Molina et al. 2008), $Y_{x/s}$ achieved in the DO 50% assay is similar to those of the cultures in which DO was maintained at or above 35% along most of the fermentation time, using the same medium and the same initial glucose concentration (30 g/L).

Higher DO not only led to higher growth rates in phases I and II but also prolonged phase I and a reduced phase II (Fig. 1). Bt cultures have maximal oxygen demand during vegetative to transient growth phases (Rowe et al. 2003; Berbert-Molina et al. 2008) and higher oxygen uptake rates (OUR) are observed with higher DO (Avignone-Rossa et al. 1992; Kraemer-Schafhalter and Moser 1996). The

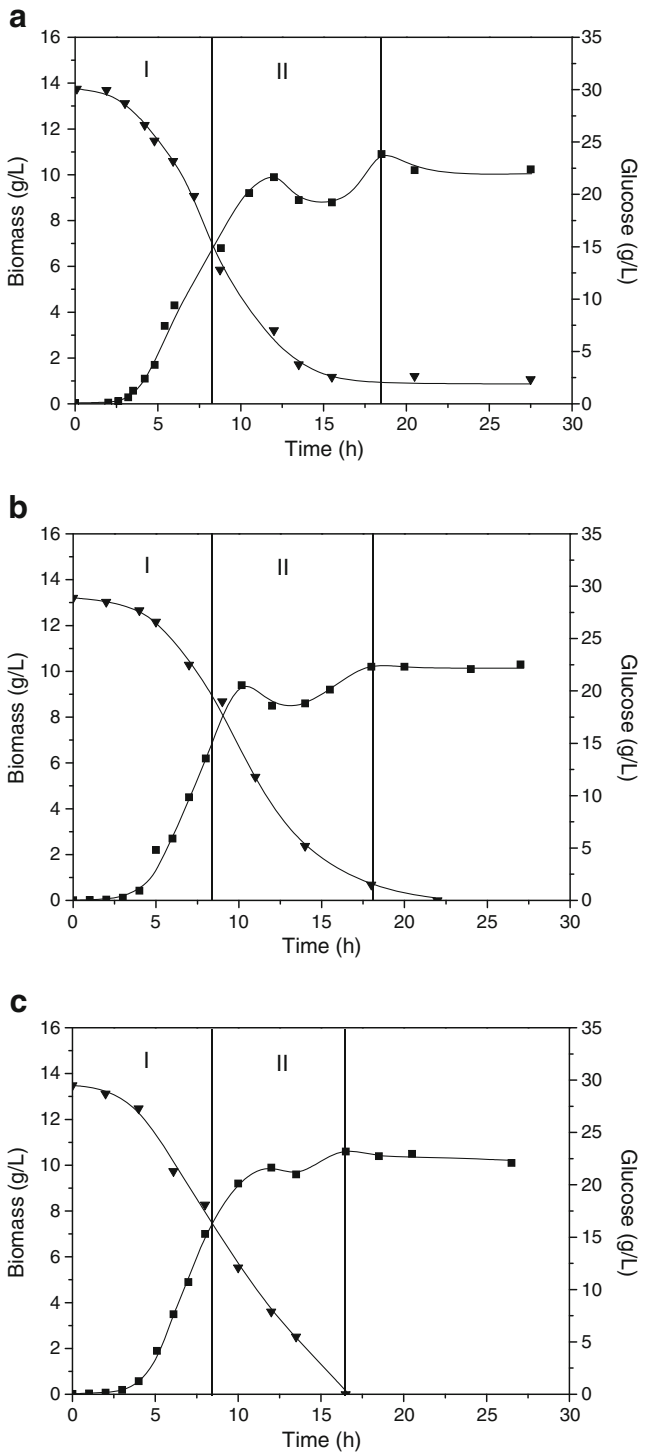

Fig. 4 Biomass (*filled squares*) and glucose (*filled down-pointing triangles*) concentrations in *Bacillus thuringiensis* var. *israelensis* batch fermentations carried out with 50% dissolved oxygen (DO) in the vegetative growth phase (phase I) and transition phase (phase II), and different DO in the sporulation phase (phase III) and cell lysis phase (phase IV) using pure oxygen instead of normal air. **a** 50–5%; **b** 50–20%; **c** 50–100%. The first value represents DO at phases I and II and the second value represents DO at phases III and IV

maximum OUR value coincides with the start of cell aggregation, which is precisely the event that marks the end of phase I (Berbert-Molina et al. 2008). As this event occurred later in the 50% DO assay, the strong respiratory metabolism was extended for a longer period, allowing better utilization of nitrogen sources from yeast extract. According to Kraemer-Schafhalter and Moser (1996), the uptake of most amino acids from yeast extract ceases when clumps begin to form. Cell motility, another important indicator of cellular activity during bacterial growth, could also corroborate the higher performance of cultures at 50% DO. As observed by Chen et al. (2003), motile intensity (mean specific kinetic energy) of *Bacillus thuringiensis* var. *darmstadiensis* was substantially higher in culture with DO of 60% as compared to those obtained with 5% or 20%.

Higher DO level also led to a positive impact on spore production (Table 1), which can be explained by increasing growth rates with the enhancement of DO concentrations. Fast growing cells carry higher energy, which leads to a higher sporulation (Yezza et al. 2005). Quantitatively, the differences in spore counts are in agreement with those verified for Bti by Kraemer-Schafhalter and Moser (1996) using similar DO levels, but diverged from those obtained by Avignone-Rossa et al. (1992), who found analogous spore counts with different DO values. For Btk, Flores et al. (1997) demonstrated that the spore counts were a linear function of $k_L a$ (volumetric oxygen transfer coefficient) ranging from 180 to 570 h^{-1}, while Ghribi et al. (2007a, b) found that the higher the DO concentrations, the higher the spore concentrations.

Increasing DO concentrations markedly improved the toxic activities (Table 1). These results contrast drastically with those of Kraemer-Schafhalter and Moser (1996), who found the lowest toxicity in the culture with the highest DO level (70%) and the optimum with 20–30% DO. Likewise, our data do not agree with those obtained by Avignone-Rossa et al. (1992), who verified very similar levels of toxin even with DO above 70%. Maldonado-Blanco et al. (2003) also reported the highest toxic activities with moderate oxygen concentrations (26%). In contrast, Ghribi et al. (2007a) observed that DO concentrations of 40% or 60% throughout Btk fermentations led to higher toxin production (up to 45%) than those achieved with DO 80%. Yezza et al. (2005) also reported that toxicity was significantly reduced with DO levels below 20–24% in Btk cultures. In our case, the increase in toxicity with 50% DO may be due, among other factors, to prolongation of phases III and IV, which may have led to proper maturation of the spores and allowed sufficient time for Cry proteins formation. Among the different Bt strains, the stage of sporulation in which the different *cry* genes are transcribed is variable, as well as the mother cell forms of RNA polymerase utilized in this process. Likewise, the individual *cry* genes can be transcribed at different rates, resulting in unequal amounts of

protoxins in the inclusions bodies, and several factors appear to be involved in this regulation (Aronson 2002). Sarrafzadeh et al. (2005) clearly demonstrated that in Bti cultures the relative concentration of each Cry protein changes over time. The 65-kDa protein was the first to be synthesized, when sporulation was still low, whereas the 25- and 130-kDa proteins appeared when sporulation rate was at about 40%.

Growth, sporulation, and toxicity under two-stage oxygen supply strategy

Since oxygen requirement of Bt varies with different stages of the cellular cycle (Rowe et al. 2003; Yezza et al. 2005; Berbert-Molina et al. 2008), attempts were made to identify optimal dissolved oxygen profiles to achieve higher toxin levels. An aeration level equivalent to 50% of saturation was applied during fermentation phases I and II. Afterwards, it was changed to 0%, 5%, 20%, or 100% along phases III and IV. The highest DO concentration (100%) had a strong negative impact on spore production, but toxic activity increased by four times in comparison with values reached with lower DO levels (5%, 20%, or 50%) during phases III and IV (Table 2). These results are in agreement with the reports given by Sarrafzadeh and Navarro (2006), who found lower spore counts and higher toxicity when the sporulation phase of Bti cultures were carried out under saturated oxygen condition. The authors also report that in such a situation mature spores and vegetative cells were the only two populations present at the end of culture, meaning that immature spores were completely absent, a finding also observed here.

Compared to the 100% DO, the interruption of oxygen supply immediately after the end of the growth phase (50–0% DO assay) imposed an even more severe restriction to the sporulation process and a total repression of toxin production (Table 2). It seems that the dissolved oxygen remaining at the beginning of the stationary phase was not enough to provide energy to sustain spore formation by such a population of cells. Likewise, this was apparently insufficient to supply the requirements of sporulated cells to perform the adequate synthesis of Cry proteins. Or else, although Cry protein synthesis initiated when there was available oxygen, the interruption of aeration seemed to trigger the inhibition of the process, hampering the adequate production of individual toxic proteins or their assembly to form the crystals. By applying an identical approach, Avignone-Rossa et al. (1992) obtained spore counts similar to those reached in non-limited cultures; however, toxin production was not thoroughly suppressed. Sarrafzadeh and Navarro (2006) found that the interruption of air supply in the course of the fermentation led to a total sporulation of the culture and slightly lower toxicity than that observed in non-limiting conditions. However, the aeration was interrupted after 24 h of fed-batch cultures performed without oxygen limitation, when a considerable portion (40%) of the population had already started sporulation. So, since sporulation and/or toxin synthesis had been triggered in part of the cells, both processes finished, even in the absence of oxygen.

The reduction of DO to 5% or 20% during phases III and IV (50–5% and 50–20% DO, respectively) led to lower spore counts, but significantly higher toxicity (Table 2) than those observed when these same DO levels were maintained throughout the fermentation (5% and 20% DO assays, respectively) (Table 1). These findings clearly indicate that oxygen levels applied during vegetative and transition phases play a decisive role in defining the extent of sporulation, in contrast with toxin production process, which appears to be dependent not only on the oxygen supply in phases I and II but also on that of the phases III and IV. The results obtained in the 20–50% DO assay afforded a more consistent analysis of this subject. Compared to 50–20% DO assay, spore counts were markedly reduced, while the toxic activity was significantly enhanced (Table 2). So, since oxygen concentrations above the critical value are used in the first two phases, it was possible to increase toxicity by increasing oxygen levels after achieving maximum biomass. The oxygen level to be employed during vegetative phases will be defined, ultimately, by total cell concentration.

A DO-shift strategy was previously utilized for Btk fermentation by Ghribi et al. (2007a, b), who found highest toxin production when DO of 60–70% was applied during the first 6 h and from this point controlled at 40%. However, their protocol differs substantially from ours since the shift in oxygen supply was done when cells exhibited intense vegetative growth. Using the same strain of Bti employed in this work in a semicontinuous combined process, da Silva et al. (2011) achieved a much higher toxicity when the final phase of sporulation was performed in a second unaerated bioreactor. The fact that aeration was suppressed in the stage of spore's maturation may explain the difference in relation to our 50–0% DO assay, which did not show any toxicity.

The Bt responses to different dissolved oxygen profiles surely cannot be detached from their innate capacity to produce proteases during the cellular cycle. As reviewed by Brar et al. (2007), the proteinaceous components used in spore and crystal formation are generated by extracellular and intracellular proteases secreted in the first growth phases and during the stationary phase, respectively. So, the environmental factors that affect protease production, which include dissolved oxygen tension (Hameed et al. 1999), could affect indirectly the overall performance of Bt for spore and toxin production.

The data obtained in the two experimental groups confirm that, in terms of oxygen availability, the best aeration conditions for Bti sporulation do not coincide with the toxin

synthesis optimum, as previously pointed out in the literature. Considering that the later process is essential in the cost-effective production of bioinsecticides using this bacterium, the conditions that lead to the highest toxic activity should be adopted.

Growth, sporulation, and toxicity under two-stage oxygen supply strategy using pure oxygen

Attempts to grow Bti under pure oxygen have not been previously reported. Here, the general growth parameters obtained when pure oxygen was used instead of normal air in fermentation runs were particularly interesting. Despite the toxic effect that excessive oxygen can potentially exert on bacteria (Cabiscol et al. 2000), some cultures were able to metabolize all available glucose and to produce almost 70% of the mean biomass reached with air. However, the sporulation processes was thoroughly inhibited and toxin synthesis, a process intrinsically associated to sporulation, was consequently suppressed (Table 3). It seems that although cultures were able to partially remove or inactivate radical oxygen species and produce relatively high biomass, the concentration of the oxidizing species exceeded the capacity of cells to confront them. Oxidative stress may have affected the formation of proteins involved in the sporulation. Either the expression of the *spo* genes may have been affected or some proteins may have been irreversibly damaged, leading to the concomitant inhibition of the δ-endotoxins production. Thus, taking into account that this event is decisive in the industrial production of bioinsecticides with Bti, the use of pure oxygen throughout fermentation should not be adopted. On the other hand, as shown by the results of the 50–100% DO assay (Table 2), the processes were effectively improved by partial enrichment of the air with pure oxygen during the sporulation phase. In this scenario, the molecular events that explain the enhanced toxicity observed will be the subject for further studies by our group.

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