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ORIGINAL RESEARCH

Influence of nutrient feeding variations on AbrB accumulation, sporulation and *cry1Ac* expression during fed-batch cultures of *Bacillus thuringiensis*

Influencia de la variación de nutrientes en la alimentación sobre la acumulación de AbrB, esporulación y expresión de *cry1Ac* durante cultivos por lote alimentado de *Bacillus thuringiensis*

Víctor Eric López y López[1], Shirlley Elizabeth Martínez Tolibia[1], Adrián Díaz Pacheco[2], Astrid Magdalena Lozano Goné[3], Pável Sierra Martínez[4], Jabel Dinorín Téllez Girón[1,5]*

[1]Centro de Investigación en Biotecnología Aplicada del Instituto Politécnico Nacional, Carretera Estatal Santa Inés Tecuexcomac-Tepetitla, km 1.5, Tepetitla de Lardizábal, Tlaxcala, C.P. 90700, Mexico. [2]Unidad Profesional Interdisciplinaria de Ingeniería Campus Tlaxcala del Instituto Politécnico Nacional, Guillermo Valle 11, Col. Tlaxcala Centro, Tlaxcala, Tlaxcala, C.P. 90000, Mexico. [3]ALTECSA, Cd. Industrial Xicoténcatl II, Av. 15 de agosto lote 3 y 4, Huamantla, Tlaxcala, C.P. 90500, Mexico. [4]Universidad Autónoma de Guerrero. Av. Lázaro Cárdenas s/n, La Haciendita, Ciudad Universitaria Sur; Chilpancingo, Guerrero, C.P. 39087, Mexico.
[5]Unidad Profesional Interdisciplinaria de Ingeniería Campus Guanajuato del Instituto Politécnico Nacional, Av. Mineral de Valenciana 200-Interior, Col. Fraccionamiento Industrial Puerto, Silao, Guanajuato, C.P. 36275, Mexico.

*Corresponding author
E-mail address: jdinorintg@ipn.mx (J. Dinorín-Téllez-Girón).

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ABSTRACT

The biological control agent *Bacillus thuringiensis* (Bt) coordinates metabolic and gene regulation functions to enter to the differentiation process for the synthesis of spores and Cry toxin proteins. The master regulator for sporulation initiation is Spo0A, while AbrB regulator coordinate cellular functions during vegetative and transition growth. To

understand how differentiation is modified during fed-batch cultures (FBC) of Bt under variable nutrient conditions, we conducted fermentations with two different nutrient concentration and feeding times using an industrial medium. Since sporulation efficiency has been lower in FBCs than in batch cultures, AbrB was monitored by Western blot during these cultures. Kinetic parameters were used to stablish timing of cell differentiation process, and fermentations were monitored on-line using impedance spectroscopy. AbrB was accumulated during the entire fed-batch culture, when a medium with the highest concentration of carbon and nitrogen sources (FBC1) were fed. Under these conditions, sporulation efficiency and *cry1Ac* expression were negatively affected, compared to the wild-type strain. Conversely, when the feeding concentration was only half of FBC1, AbrB accumulates in minor proportion, and sporulation efficiency and *cry1Ac* expression increased (FBC2). These results suggest that variable nutrient conditions can cause an imbalance in the accumulation of transition state regulators such as AbrB during FBCs, which can be detrimental to produce spores and Cry proteins, that are the main Bt products for biological control of agricultural pests.

Keywords: AbrB, *Bacillus thuringiensis*, cell differentiation, dielectric spectroscopy, Fed-batch culture.

RESUMEN

El agente de control biológico *Bacillus thuringiensis* (Bt) coordina funciones metabólicas y de regulación génica para entrar al proceso de diferenciación para la síntesis de esporas y proteínas Cry. El regulador maestro de inicio de la esporulación es Spo0A, mientras que el regulador AbrB coordina funciones celulares durante el crecimiento vegetativo y de transición. Para comprender cómo se modifica la diferenciación durante cultivos por lote alimentado (FBC) de Bt bajo condiciones variables de nutrientes, se realizaron fermentaciones con dos diferentes concentraciones de nutrientes y tiempos de alimentación utilizando un medio industrial. Los parámetros cinéticos fueron utilizados para establecer el tiempo del proceso de diferenciación, y las fermentaciones se monitorearon en línea usando espectroscopia de impedancia. AbrB se acumuló durante la mayor parte del cultivo cuando se alimentó un mayor contenido de carbono y nitrógeno (FBC1). Bajo estas condiciones, la eficiencia de esporulación y expresión de *cry1Ac* fueron afectadas negativamente, comparadas con la cepa parental. Por el contrario, cuando el contenido de nutrientes en la alimentación fue la mitad del alimentado en FBC1, AbrB se acumuló en menor proporción, y la eficiencia de esporulación y expresión de *cry1Ac* incrementaron (FBC2). Estos resultados sugieren que las condiciones variables de nutrientes pueden causar un desbalance en la acumulación de reguladores de estado de transición como AbrB durante FBCs, lo cual puede ser perjudicial para la producción de esporas y proteinas Cry, que son los principales productos de Bt para el control biológico de pestes agrícolas.

Palabras clave: AbrB*, Bacillus thuringiensis*, diferenciación celular, espectroscopia dieléctrica, Lote-alimentado.

1. INTRODUCTION

Bacillus thuringiensis (Bt) represents around 90% of the global biopesticide market (Jallouli et al., 2020), which means about $3 billion worldwide (Damalas & Koutroubas 2018). During sporulation, Bt synthetizes parasporal inclusions named Cry proteins, which are specifically toxic to different insect orders such as Lepidoptera, Coleoptera, Diptera and Hymenoptera (Bravo et al., 2007), and some phytopathogenic nematodes, yet also spores contribute to the insecticidal activity. Therefore, the increase of spore and Cry protein yields, and volumetric productivity is a challenge to improve the Bt production since the past decades. Some of the strategies followed to produce higher spore counts or higher Cry protein concentrations are the fed-batch (FBCs) or continuous cultures. However, the batch culture is the preferred cultivation method (López y López et al. 2000). In order to match nutrient demand with nutrient availability, the fed composition used in experiments of Bt fed-batch cultures, generally contain a concentrated version of the media culture used in batch experiments or concentrated carbon and nitrogen sources, and salts components. Despite in FBCs high cell densities have been reached, spore efficiencies are still lower as well as Cry yields compared to batch cultures (Arcas et al., 1987; Kang et al., 1992; Liu et al., 1994; Mignone & Avignone Rossa 1993), and new strategies have not been proposed. Thus, a deeper knowledge of the differentiation process of sporulation is needed for improving this process.

This biotechnological drawback can be explained by the genetic coordination and metabolic functions to synthetize the spore and Cry proteins which starts at the transition state (Ben *et al*., 2017). Transition state regulators work integrating metabolic and environmental information available in cells, modulating the gene expression, canalizing the cells through an appropriate route to ensure that nutrient and energy resources can be destined to accomplish their functions (Strauch and Hoch, 1993; Phillips and Strauch, 2002). The intricated network of regulation involves a shared effect between transition state regulators (AbrB, Hpr, SinI) and global regulators such as CodY, plcR, NpR and Spo0A, that contribute to the diversity of cell differentiation behaviors (Banse et al., 2008, De Jong et al. 2010, Rejeb et al. 2017). In this context, the regulator protein AbrB is one of the main transition regulators that can act as an activator or repressor of the expression of more than 100 post-exponential-phase genes with biological functions such as biofilm formation, antibiotic and extracellular enzymes production, motility, development of competence and sporulation (Chumsakul *et al*., 2011). Previous works have determined that maximum accumulation of AbrB protein occurred at the beginning of exponential growth in *Bacilllus*, at a short period between the lag and exponential phases, then declines until undetectable levels are reached before the mid-exponential phase (O'Reilly and Devine, 1997, Lozano Goné et al. 2014). Moreover, the intertwined effects of AbrB with Spo0A and other global regulators can affect the cell differentiation process. In addition, there are extracellular signals that in response to environmental changes act by phosphorylating the regulatory proteins of specific differentiation programs such as Spo0A, DegU and ComA. This process is known as Quorum sensing and, for the specific case of sporulation, it is activated in response to low intracellular

concentrations of key metabolites such as ATP or GTP, which is a direct consequence of a limitation of nutrients or oxygen (López and Kolter, 2010). In *B. subtilis*, the expression of nutrient starvation or limitation signaling molecules ComX and CSF are regulated by sigma H (Lazazzera, 2000). On the other hand, with an excess of glucose, it was observed that *spo0A* transcription was decreased in *B. subtilis* (Mirouze et al. 2011), which could be related to the low yields of sporulation in FBCs. In addition, it has been reported a role performed by AbrB to regulate the expression of virulence in strains of the *B. cereus* group such as the cereulide (ces) synthetase genes in *B. cereus* (Lücking et al. 2009) and the anthrax toxin genes *pagA, lef* and *cya* in *B. anthracis* (Saile and Koehler, 2002). However, in *B. thuringiensis* the AbrB role in *cry* expression has not been well addressed, neither described at bioreactor conditions nor different cultivation methods than batch cultures.

The characterization of the variable effects of AbrB levels has been described in *B. subtilis* at flask level (Perego *et al*., 1988, Bobay *et al*., 2006, Chumsakul *et al*., 2011, O'Reilly and Devine, 1997), and its identification at bioreactor level in Bt has been developed during batch cultures (Lozano et al., 2014). However, their effects on sporulation and *cry* gene expression have not been described in FBCs. In this sense, there is no information regarding how cell differentiation can impact the Bt bioprocess, just information about nutrient limitation is reported to induce higher sporulation rates during fed-batch operations (Klausmann et al. 2021); however molecular mechanisms are still poorly described. In this report, we present the first evidence of accumulation of AbrB during Bt fed-batch cultures, which depending on the level reached it can impact the spore counts, affecting also *cry1Ac* gene expression. Due to the observed AbrB implication on regulation, the online monitoring of FBCs by impedance dielectric spectroscopy is also presented, as an additional support to follow-up the cell differentiation process.

2. MATERIALS AND METHODS

2.1. Microorganism

Bacillus thuringiensis (Bt) strain HD73 pHT1kAc, derived from Bt var. kurstaki HD73 (ATCC-35866) was kindly provided by Mayra de la Torre. This transformed strain was constructed by Sierra-Martínez et al. (2004) and has a 1000-bp fragment upstream from the *cry1Ac* promoter derived from the wild type Bt HD73, fused to *lacZ* to serve as reporter gene into plasmid pHT304-18Z (Agaisse & Lereclus 1994).

2.2. Media and culture conditions

FBCs were performed using the clean medium with a carbon:nitrogen ratio of 7 for both the batch and nutrient feeding stages (López y López & de la Torre 2005). The clean medium used for the batch stage comprised (g/l): glucose, 12; soy peptone, 12; yeast extract, 2.59; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4$, 0.04; $ZnSO_4 \cdot 7H2O$, 0.0058; $CuSO_4 \cdot 5H_2O$, 0.0075; KCl 3.0; $FeSO_4 \cdot 7H_2O$, 0.00135; $CoCl_2 \cdot 6H_2O$, 0.03 and Mazu DF antifoaming agent, 1 (ml/l). The composition of the feed for Fed-batch culture 1 (FBC1) was (g/l): glucose, 300; soy peptone, 300; and yeast extract, 64.75; while the feed for Fed-batch

culture 2 (FBC2) had a half of the same nutrients of FBC1. The salt concentration in both fed-batch experiments was duplicated in the feeding. All reagents and media components were purchased from Bioxon, Meyer and Fermont. Fermentation cultures were performed in a 7-l Biostat APlus reactor (Sartorius), under the following operating conditions: 3-l initial volume, 600 rpm stirrer speed, 30°C, and aeration rate 1 vvm. During fermentations, antifoaming agent was added as required and pH was controlled automatically at 7.2±0.2 with NaOH (5 M) and H_3PO_4 (0.67 M). Dissolved oxygen (DO) was controlled by increasing the stirrer speed (between 600 and 1300 rpm) and aeration rate (between 1.0 and 1.8 vvm). The reactor was seeded with 5% (v/v) for the one-stage log-phase inoculum prepared from a spore disk with 10^7 spores in Farrera medium (López y López & de la Torre 2005) and incubated at 30ºC and 200 rpm for 14 h. The rate of feeding was exponential according to the equation:

$$F = \frac{q_s x_0 V_0 e^{\mu t}}{S_0 - S} \quad (1)$$

Where:
F, feeding (l/h)
q_s, specific rate of substrate consumption ($g_s/g_x h$)
x_o, initial concentration of biomass at t_0 (g/l)
V_0, initial volume at t_0 (l)
μ, specific growth rate (h^{-1})
t, time (h)
s_0, substrate concentration in the feed (g/l)
s, substrate concentration in reactor (g/l)

q_s and μ used in the equation were obtained from the experimental data of Lozano et al. (2014). The feeding for FBC1 started at 2 h, from the beginning of batch culture while feeding for FBC2 started at 1 h from the batch culture stage. The experimental cell growth data were adjusted to an exponential model (Eq. 2):

$$x = x_0 e^{\mu t} \quad (2)$$

The beginning of the transition phase was assumed when the specific growth rate decreases. It was assumed that the onset of sporulation was two hours prior to the detection of β-galactosidase activity (Agaisse & Lereclus 1995; López y López & de la Torre 2005; Schnepf *et al*., 1998).

2.3. Analytical methods

Bacilli and spores were counted by quadruplicate, diluting culture samples in saline solution, followed by vigorous mixing to avoid cell clumping and direct microscopic visualization in a Neubauer chamber (Dinorín *et al*., 2015; Farrera *et al*., 1998). Sporulation efficiency was calculated as the ratio of free spores (thermoresistant spores obtained from incubation 10 min, 85°C) at the end of the fed batch: bacilli cells at the

onset of sporulation multiplying by 100 (López y López & de la Torre 2005). The biomass in g/l was calculated multiplying bacilli counts by the unitary dry weight of a bacillus cell (2.3 picograms) (Rodríguez & de la Torre 1996). Residual glucose was analyzed through the glucose oxidase method in a biochemical analyzer (YSI 2700 Select). Carbon dioxide (CO_2) levels in the reactor gas exit was monitored in real time using an infrared analyzer (Carbon dioxide analyzer Mod. 906, Quantek Instruments). Sartorius PC-Panel µDCU 6.34 was used for dissolved oxygen (DO), pH and temperature measurement-control.

2.4. Determination of *cry1Ac* expression

Expression of *cry1Ac* gene was determined by β-galactosidase assay (López y López & de la Torre 2005; Dinorín *et al.*, 2015), where 34 samples were taken at determined times in both fermentations (t_0 – t_{34}). To achieve this, samples of 1-ml were centrifuged (12,800 *g*, 4°C, 7 min) and supernatants were discarded. Each cell pellet was washed twice and resuspended in 1-ml of 0.1M sodium phosphate buffer (pH 7.5). The reaction mixture per sample had the following composition: 3 µl 100x Mg solution, 66 µl orto-nitrophenyl-*β*-D-galactopyranoside (ONPG, *Sigma Aldrich*), 30 µl cellular suspension and 201 µl 0.1M sodium phosphate buffer (pH 7.5). The mixtures were incubated for 30 min at 30°C and the reaction was stopped by the addition of 500 µl 1M Na_2CO_3, and then centrifuged (12,800 *g*, 4°C, 7 min). The analysis was performed by UV-Vis spectroscopy at 420 nm. One unit of β-galactosidase (Miller Unit, MU) was defined as the enzyme quantity hydrolyzed by 1 µmol ONPG per minute. This assay was done by duplicate.

2.5. Western blot of AbrB

Samples obtained along fed-batch cultures were taken at different times of fermentations, centrifuged and resuspended in in 1-ml of cell lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5; 2 mM EDTA) according to the conditions reported by Lozano *et al*. (2014) The presence or absence of AbrB protein was detected (discriminated) by observation of Western blot membranes, and a semi-quantitative analysis from the obtained images was carried out with the free available software ImageJ to obtain densitometric profiles to compare AbrB accumulation.

2.6. Impedance measurements

The impedance parameters Reactance (R) and Resistance (X) were obtained by the Hioki 3532-50 LCR HiTester impedance meter, by applying both a fixed current of 20 mA (milliamps) and 5 V (volts) at 1.225 MHz. The impedance probes were configured in accordance with Dinorín *et al.* (2015).

2.7. Statistical analysis

The statistical software Minitab 16 (Minitab Inc.) was used to perform the analysis of variance (ANOVA) and the least significant difference (LSD) multiple comparison Fisher's test. The confidence level used for ANOVA tests was 95%.

3. RESULTS

3.1 FBC1- high fed concentration

Growth and kinetic parameters from FBC1 are shown in Figure 1 and 2, where the feeding started at 2 h and stopped at 9 h (Figure 1b). Two μ values were determined during growth, the first $\mu^{I} = 0.78\ h^{-1}$ from 0 to 6 h, whereas the second was $\mu^{II} = 0.087\ h^{-1}$ calculated between 6 and 11 h (Table 1). A reduction in μ indicates the establishment of the transition state during feeding. At the first exponential growth, glucose was kept in a range between 9 g/l and 15 g/l, but at the transition phase, glucose was accumulated up to 39.2 g/l at 9 h, when the feeding was finished (Figure 1b shaded area). After, it was consumed during the transition and part of the sporulation phase being exhausted at 16 h (Figure 1b). The minimum DO level was 4.4% and the maximum CO_2 production (1.99%) occurred during the transition state at 9 h (Figure 1b and 2a). The *cry1Ac* expression was first detected at 14 h, consequently since transcription of Cry toxins occurred usually 2-h after the onset of sporulation phase (Agaissse & Lereclus 1995, Schnepf et al., 1998), then the established sporulation stage was set-up at 12 h where glucose and DO were 17.4 g/l and 22.3% respectively (López y López & de la Torre 2005; Dinorín *et al*., 2015), and *cry1Ac* expression reached a maximum of 4531 MU at 24 h (Figure 1a, Table 1). Despite of the stationary phase lasted 14 h, few spores were released and sporulation efficiency was only 2%, suggesting that sporulation started but the process was not completed. In terms of dielectric properties, Figure 2b shows the resistance and reactance profiles as a tool for monitoring differentiation process in real time. As observed, the resistance decreased abruptly to 3.5 ohms as the feed occurred and continued decreasing to a final value of 3.2 ohms. Regarding the reactance, it reached a maximum value of 2.9 ohms at 4h and a minimum value of 2.3 ohms at 11 h. However, the profile presents staggered increases after 12 h.

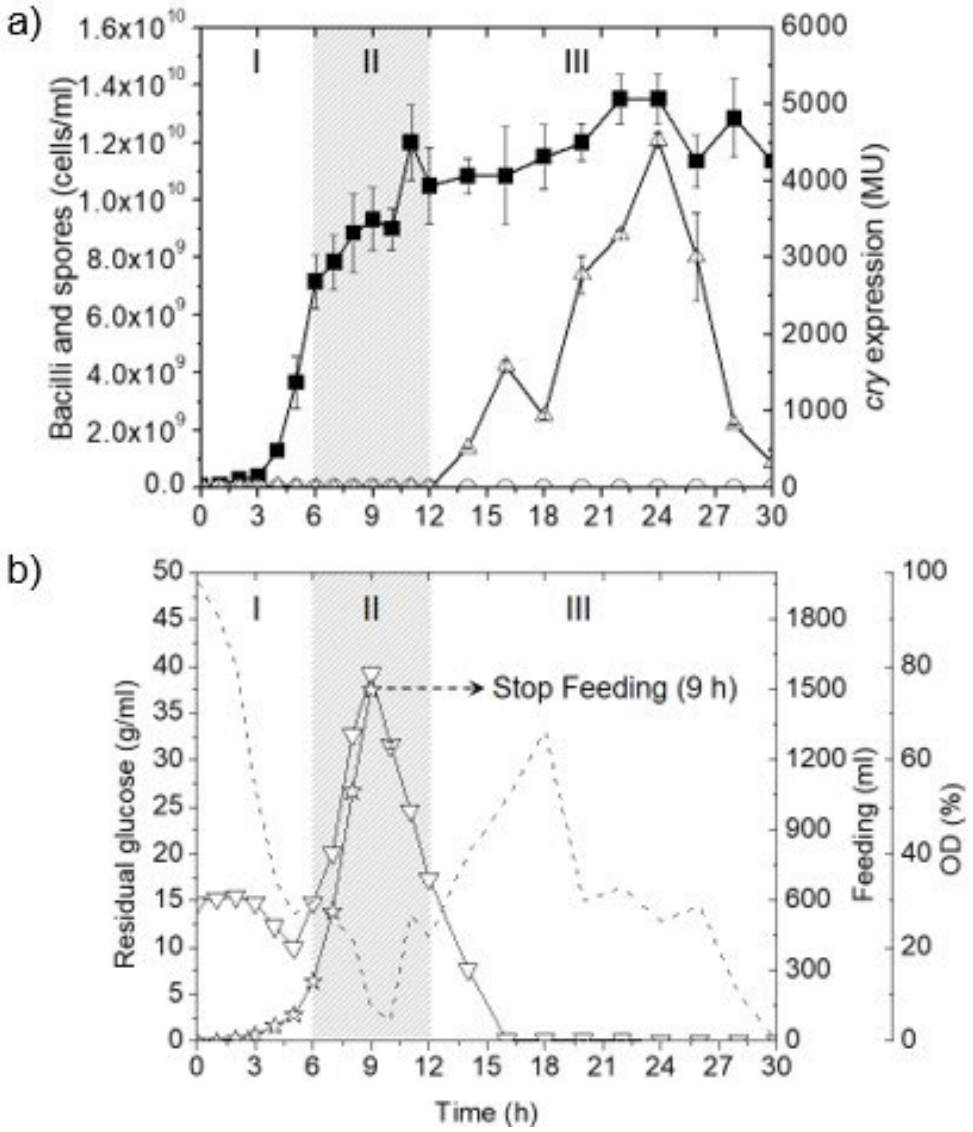

Figure 1. Time course of *Bt* HD73 pHT1kAc FBC1. Shaded area represents the transition state. Growth phases are represented as: exponential (I), transition (II) and sporulation phase (III). Graphs symbols are: **a)** Profiles of bacilli (filled squares), spores (circles) and *cry*1Ac expression (triangles); **b)** profiles of dissolved oxygen (dotted line), residual glucose (inverted triangles) and nutrient feeding (star). Numbers correspond to the sampling times (h) of fermentation.

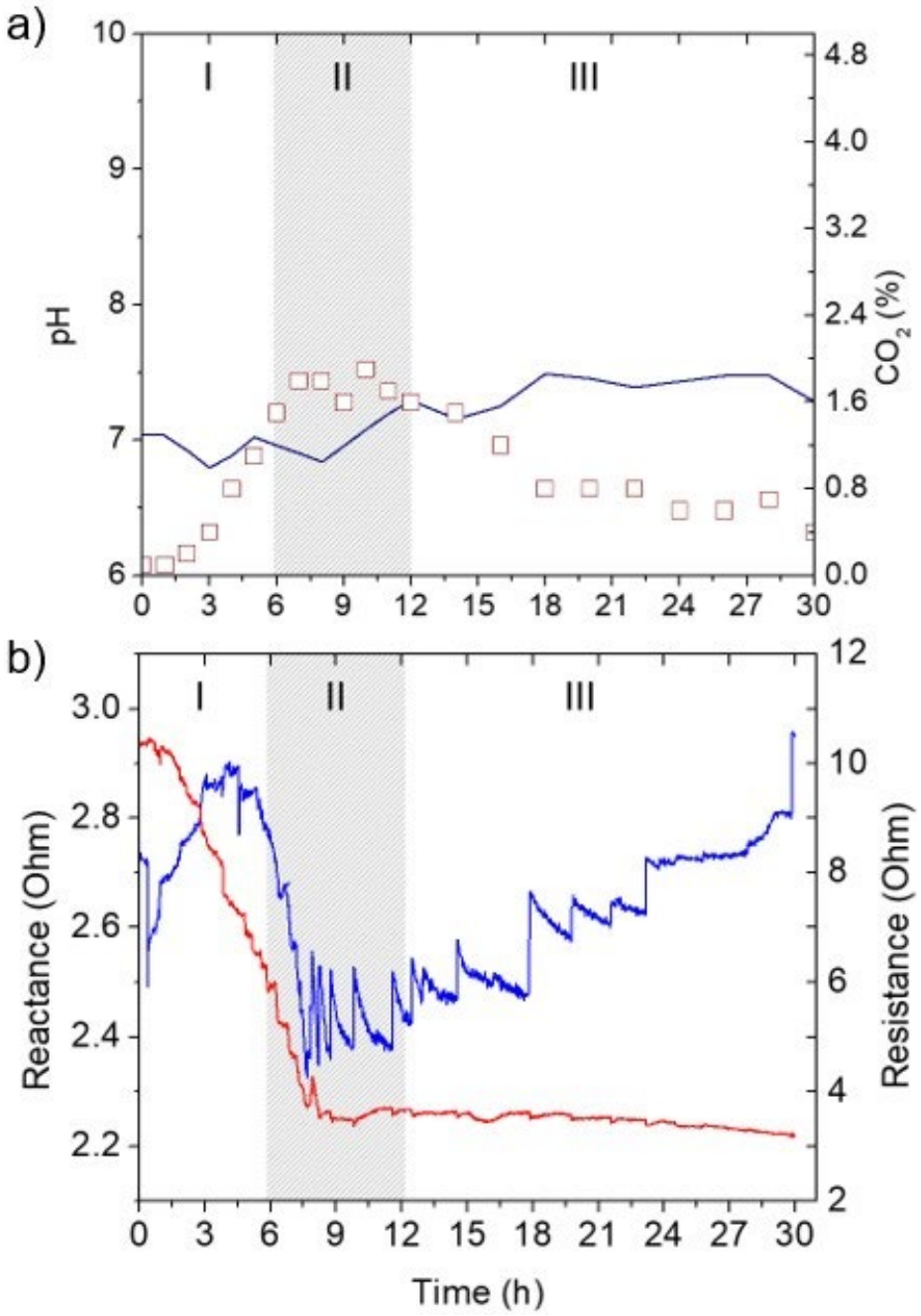

Figure 2. Time course of *Bt* HD73 pHT1kAc FBC1. Shaded area represents the transition state. Growth phases are represented as: exponential (I), transition (II) and sporulation phase (III). Graphs symbols are: **a)** pH (line) and CO_2 (squares) profiles and **b)** Dielectric properties: Reactance (blue line) and Resistance (red line). Numbers correspond to the sampling times (h) of fermentation.

Table 1. Kinetic parameters obtained in FBCs of Bt pHT1kAc.

	FBC1	FBC2
$\mu^{I)}$ (h^{-1})	0.78 (R^2=0.98)	0.66 (R^2=0.98)
$\mu^{II)}$ (h^{-1})	0.087 (R^2=0.86)	0.1342 (R^2=0.78)
Bacilli counts (cell/ml)		
Culture start (t_0)	$8x10^7$ ($\pm 1.08x10^7$)^A	$8.67x10^7$ ($\pm 6.45x10^6$)^A
At change in μ	$7.17x10^9$ ($\pm 9.13x10^8$)^A	$8.67x10^9$ ($\pm 9.13x10^8$)^A
At sporulation phase onset	$1.05x10^{10}$ ($\pm 1.32x10^9$)^B	$1.6x10^{10}$ ($\pm 1.49x10^9$)^A
Onset of transition phase (h)	6	7
Sporulation efficiency at the end of the culture (%)	2	100
Maximum *cry* expression (MU)	4531 (± 100)^B (at 24 h)	9233 (± 295)^A (at 20 h)
Glucose at μ change (g/l)	14.80	5.04

Values calculated with the bacilli counts determined at $^{I)}$ the onset of transition phase, $^{II)}$ transition phase, determined at the onset of sporulation phase and the final spore count. A and B indicate the grouping according to Fisher's post-hoc test ($p<0.05$). A corresponds to the group with the highest mean value, B and C are in descending order, respectively.

3.2 FBC2 - low fed concentration

In FBC2, two values of μ were determined as in FBC1, the first μ^I =0.66 h^{-1} from 0 to 7 h (exponential phase) and the second μ^{II} = 0.1342 h^{-1} calculated between 7 and 12 h (transition phase) (Figure 3a shaded area). During the first exponential growth, glucose was maintained in a range of 13.7 g/l to 5.04 g/l. Then, at the transition phase, glucose was accumulated up to 35.8 g/l at 9 h (Figure 3b), then it was totally consumed at 12 h, with the sporulation set up. Maximum CO_2 production (2.5%) occurred at 11 h during the transition state (Figure 4a), whereas DO level (Figure 3b) diminished during the exponential growth and remained below 40% throughout the entire fermentation, despite the increase of agitation speed and aeration rate. Stationary phase initiated at 12 h, while *cry* promoter activity was first detected at 14 h reaching a maximum of 9233 MU at 20 h (Figure 3a, Table 1), that was two-fold higher than the calculated for FBC1. Spores release occurred between 26 and 28 h, reaching 100 % sporulation efficiency at the end of the culture (Figure 3a). According to the Fisher's grouping test (Table 1), there are no differences in the concentration of bacilli for both cultures at 0-h and at the time of μ change. However, there are significant differences in the concentration of bacilli at the time of sporulation phase onset, and at the maximum *cry1Ac* expression determined

by β-galactosidase assay. Regarding dielectric properties, Figure 4b shows that resistance value decreased to 3.1 ohms once 9 h is reached, decreasing more until a final value of 2.6 ohms. Contrarily from the observations for FBC1, the reactance profile of FBC2 showed changes around 2.6 ohms, and this parameter presented the higher differences between both cultures.

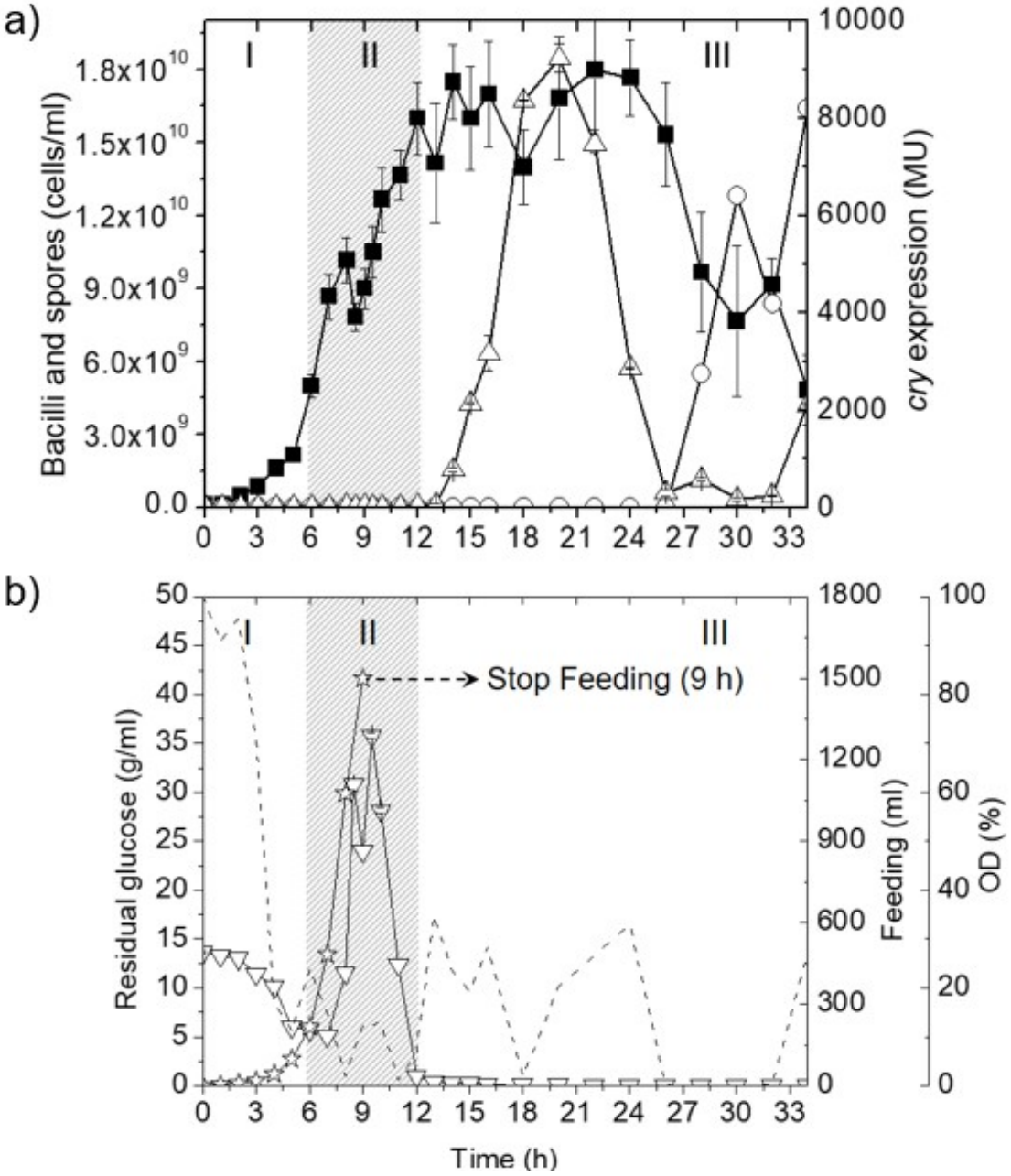

Figure 3. Time course of *Bt* HD73 pHT1kAc FBC2. Shaded area represents the transition state. Growth phases are represented as: exponential (I), transition (II) and sporulation phase (III). Graphs symbols are: **a)** Profiles of bacilli (filled squares), spores (circles) and *cry*1Ac expression (triangles); **b)** profiles of dissolved oxygen (dotted line),

residual glucose (inverted triangles) and nutrient feeding (star). Numbers correspond to the sampling times (h) of fermentation.

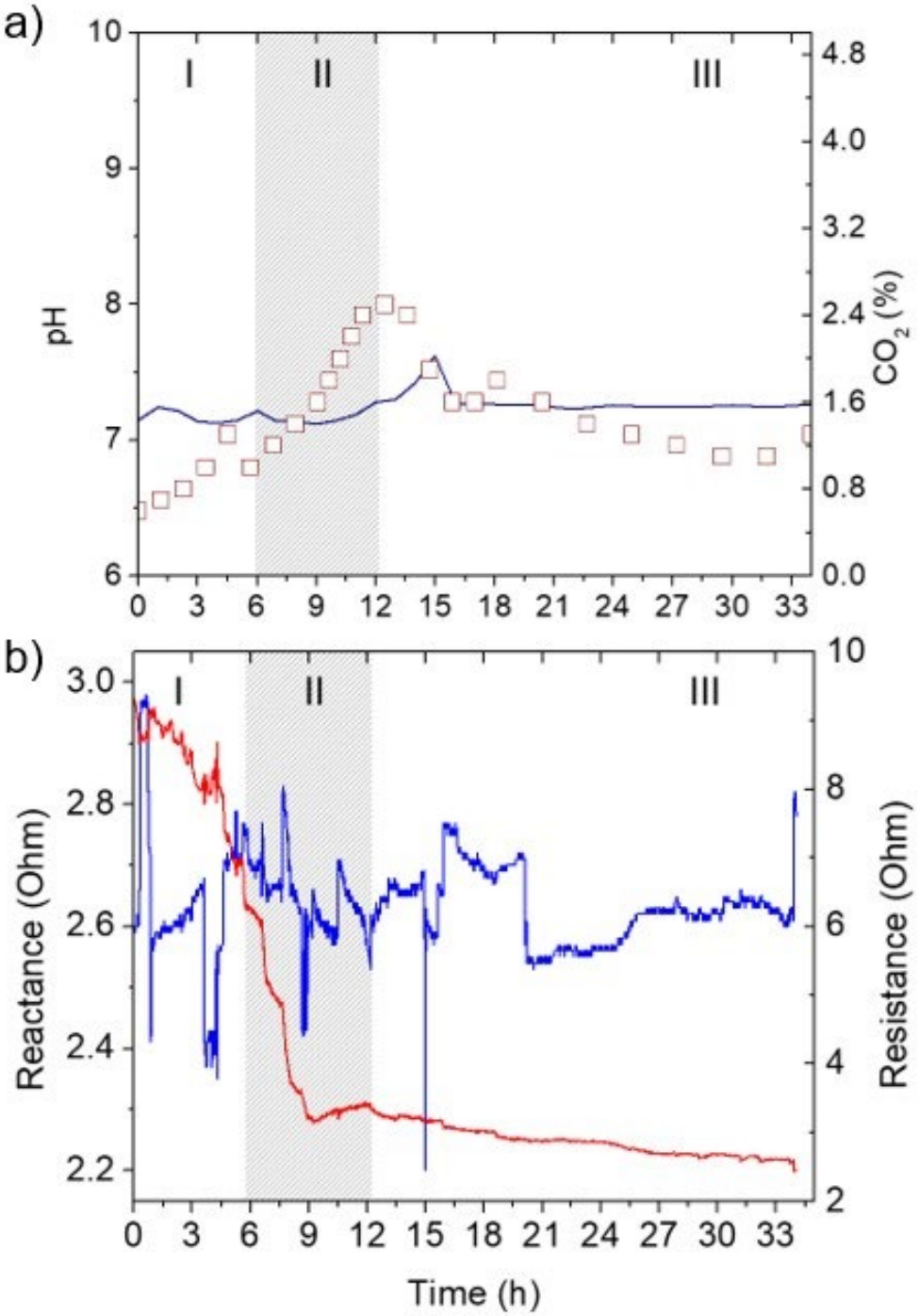

Figure 4. Time course of *Bt* HD73 pHT1kAc FBC2. Shaded area represents the transition state. Growth phases are represented as: exponential (I), transition (II) and sporulation phase (III). Graphs symbols are: **a)** pH (line) and CO_2 (squares) profiles and **b)** Dielectric properties: Reactance (blue line) and Resistance (red line). Numbers correspond to the sampling times (h) of fermentation.

3.3 Accumulation levels of AbrB transition regulator from Fed-batch Cultures

AbrB accumulation was evaluated through Western blot as a function of time (Figure 5), in order to monitor how is its expression pattern, and how nutrient variations can affect AbrB expression levels. In both fed-batch culture experiments, it was observed that AbrB was accumulated since the beginning of the culture and this accumulation remained during the exponential (green arrow), transition phase (yellow arrow) and even at the stationary phase (red arrow).

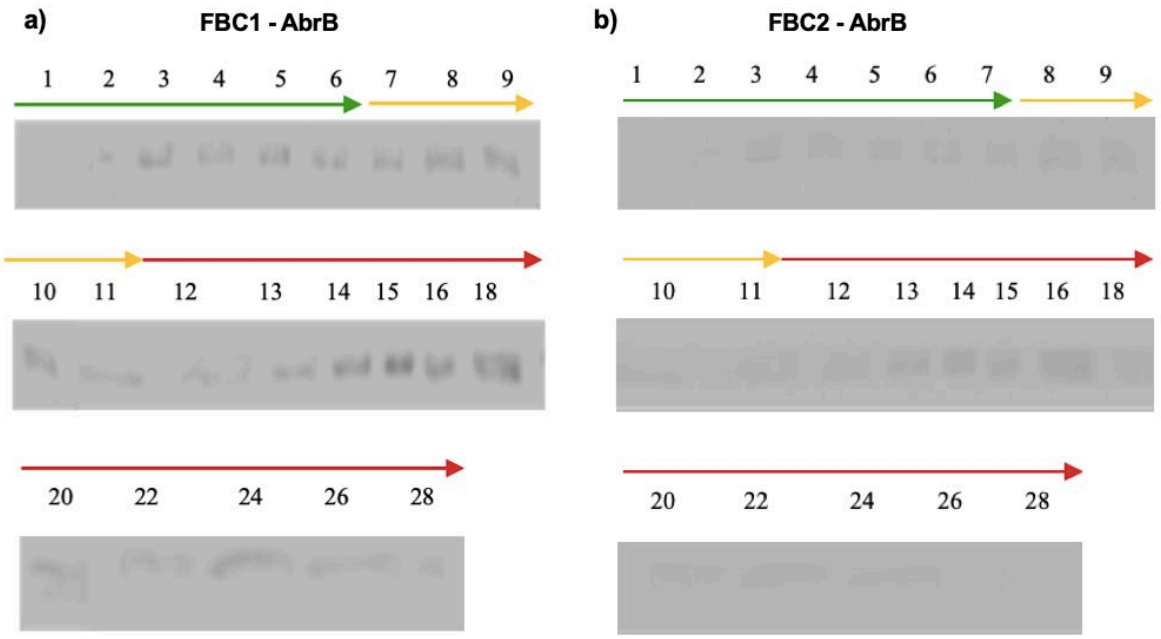

Figure 5. Accumulation levels of AbrB from Fed-batch Cultures **a)** FBC1 and **b)** FBC2 observed from Westernblot assays. Numbers correspond to the sampling times (h) of fermentation.

The low sporulation efficiency achieved in FBC1 could be due to the presence of a higher and maintained AbrB accumulation at the transition, even notorious at stationary phase compared to FBC2 (Figure 5a). In contrast, AbrB was accumulated differentially from 0 to 18 h in FBC2, afterwards AbrB levels were decreased as observed in Figure 5b. This is an important fact since the sporulation synthesis is reported to last 8 h in batch cultures (López y López & de la Torre 2005), that was correlated to the FBC2, which had enough time to achieve a sporulation efficiency (100%), compared to FBC1, which delayed the spore maturation and release. A densitometric analysis to compare AbrB accumulation levels as a function of time is presented in Figure 6, where it is evident that levels were higher in FBC1 and were maintained even after 24 h of cultivation. Since *abrB* expression is controlled by several mechanisms, for example the master regulator Spo0A~P, and this regulator control the sporulation start up, the

concentration of AbrB should be low if most of the bacilli enter into sporulation (O'Reilly & Devine 1997; Lozano *et al.*, 2014).

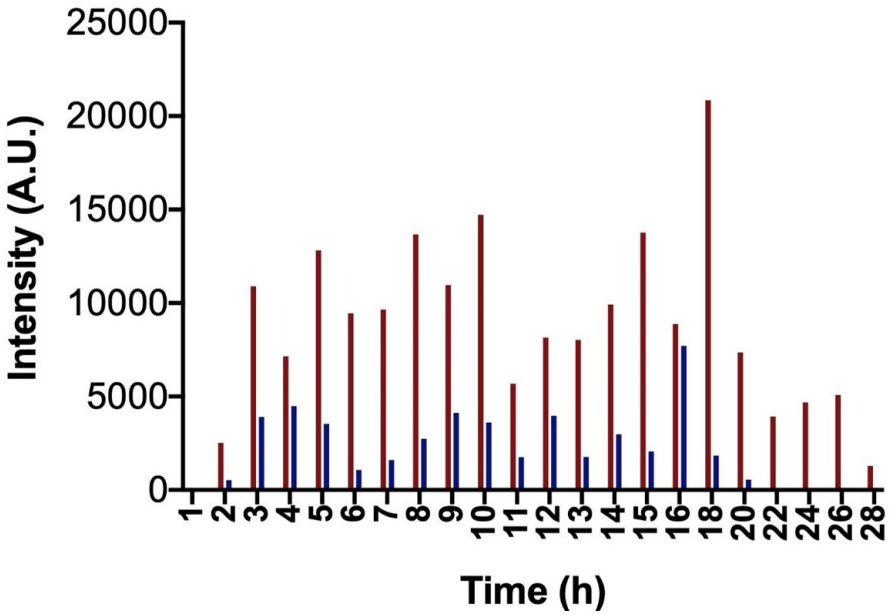

Figure 6. Accumulation levels of AbrB from Fed-batch Cultures. Semi-quantitative analysis of AbrB levels obtained from a densitometric evaluation. FBC1 (red), FBC2 (blue) (*Image J software*).

4. DISCUSSION

During batch cultures of *B. subtilis* at flask level, O'Reilly & Devine (1997) reported that the maximal accumulation of AbrB occurred at the middle exponential growth and decreased during the transition phase, being null at stationary phase. On the other hand, in batch cultures at reactor scale with industrial media Lozano *et al.* (2014) reported that AbrB accumulation occurred from the beginning of the culture and was maximal during early exponential growth. Thereafter, AbrB decreased when no nutrient limitations exist during transition phase, and it was undetectable at the stationary phase, achieving a sporulation efficiency of 72% in the same media used in this work, and 65% in Farrera medium (Lozano *et al.,* 2014). According to our experiments, during fed-batch cultures of *B. thuringiensis* the AbrB accumulation started at the early exponential stage of the culture (t_2) and continued at the transition phase and even during the stationary phase, being more evident in FBC1. From these observations, we conclude that a higher nutrient fed concentration and a difference in the starting time of feeding (FBC1: 2 h, and FBC2 :1h after the batch stage) were able to influence the accumulation of the transition state regulator AbrB. As a result, an increased and maintained accumulation of AbrB during FBCs impacts directly on the sporulation development, being detrimental to obtain high spore counts, affecting as well the *cry1Ac* gene expression. Many efforts have been made to reach higher amounts of Bt based bioinsecticides with FBC, which implies not only obtaining high cell counts, but also cells that are able to synthetize more spores and Cry proteins. This would be a robust production process of Bt. However, the

higher number of cells achieved using FBCs did not necessarily correspond to higher quantities of spores or higher toxicities (Kang *et al*.,1992; Mignone & Avignone-Rossa 1993; Jong *et al.,* 1995; Liu *et al.,* 1994). This is evidenced in Table 2, where data from different works was processed and efficiencies of sporulation were calculated and compared to our results. As we can see although high spore counts can be achieved, sporulation efficiency was not high enough. For instance, the highest sporulation obtained (Liu *et al.,* 1994) was 64% with 53.7 g/L biomass, whereas only 39% sporulation efficiency was obtained (Kang *et al.,* 1992) with 72.6 g/L biomass. The other reports from Table 2 describe variable efficiencies that are probably correlated to the feed strategy, which would denote the importance of metabolic and regulatory pathways. In terms of productivity, this information suggests that not all nutrient sources are focused on production of a cell that in turn produces a spore or Cry proteins. For example, not all the glucose supplied in the feeding is entirely metabolized, resulting in the production of organic acids, as reported during fed-batch cultures of *Bacillus thuringiensis* subsp. *darmstadiensis* (Wen et al., 2007), and the accumulation of citrate and butyrate also observed during FBCs of BtpHTcry1A2 (López y López & de la Torre 2005). Hence, it becomes essential to understand how are regulated the circuits for the development of spores and how it is affected under different feeding conditions at bioreactor level.

Table 2. Comparison of FBCs of Bt.

Author	Biomass (g/l)	Spores (spores/ml)	Bacilli (bacilli/ml)	Sporulation efficiency (%)	Feed strategy
Arcas *et al*. (1987)	56.4	$1.12x10^{10}$	$2.45x10^{10}$*	45	constant
	36	$4.0x10^{9}$	$1.57x10^{10}$*	25	constant
Kang *et al*. (1992)	49	ND	$2.13x10^{10}$*	ND	constant
	72.6	$1.25x10^{10}$	$3.13x10^{10}$*	39	intermittent
Liu *et al*. (1994)	53.7	$1.45x10^{10}$	$2.33x10^{10}$*	64	exponentially
FBC1 (our study)	24.1†	$2.1x10^{8}$	$1.05x10^{10}$	2	exponentially
FBC2 (our study)	36.8†	$1.6x10^{10}$	$1.6x10^{10}$	100	exponentially

ND = Not detected

† Biomass was calculated from bacilli counts and multiplied by the unitary cell dry weight of 2.3 picograms (Rodríguez & de la Torre 1996).
* Bacilli were calculated from the biomass divided by the unitary cell dry weight of 2.3 picograms and 1000 ml (Rodríguez & de la Torre 1996).

In this context, there are just few studies describing FBCs of Bt that have reported and monitored sporulation efficiencies. López y López & de la Torre (2005) reported four FBCs experiments with shot-wise fed, where sporulation efficiencies of 1, 5, 46 and 76 % were related to *cry* gene expression levels of 359, 419, 489 and 589 MU, respectively. Amin et al. (2008) also reported a sporulation efficiency of 98 % ($2.31x10^{12}$ spores/ml) with exponential feed, and 20 $g \cdot l^{-1}$ of toxin production determined by the Lowry method. As mentioned early, in most of the cases to obtain a successful FBC of Bt, higher spore counts and Cry protein must be achieved; however, it has not been explored whether the transcriptional regulation exerted by the main global regulators have a greater influence on this process, as well as nutrimental conditions. Our results suggest that both feed concentration and the start feeding are key points to modulate the accumulation of signals and activity of transition state regulators such as AbrB during Bt FBCs, which are important to consider and are not commonly described under bioreactor conditions. One of the main findings from this work is that cultures with high nutrients concentration (FBC1) presented higher levels of AbrB accumulation since the beginning of the FBC (Fig. 5a and 5c) and it remained during the exponential (I), transition phase (II) and even at the stationary phase (III). The scarce sporulation determined in FBC1 could be attributed to the presence of a high amount of AbrB that triggers an imbalance for spore development. It is noteworthy to clarify that AbrB is not the only regulator protein that is involved in changes at the stationary phase, but it is one of the most relevant acting as a direct repressor of *sigH*, which is partially responsible not only for the transcription of *spo0A,* but also for the expression of genes needed (*kinA* and *spo0F*) or influenced (*phr* genes) for phosphorylation of Spo0A (De Hoon et al. 2010). Moreover, it has been previously shown that mutations on *spo0A* can result in increased levels of AbrB which can repress sporulation in *B. subtilis* (Hahn et al., 1995). Likewise, δ-endotoxins synthesis is in most cases closely coupled to sporulation in Bt, as in the case of *cry1Ac* (Aronson, 2002; Deng *et al*., 2014). This coincides with our present work, where an accumulation of AbrB triggers the repression of sporulation in FBCs, affecting in a lesser extent the *cry1Ac* expression. This information suggests a differential regulation of AbrB on sporulation and *cry* gene expression. Low-expression levels of cry have been observed by reverse transcription-PCR and Western blot analysis in non-sporulating cells (*spo0A* mutants) of Bt (Yang et al., 2012), as we observed for FBC1. The difference in the accumulation of AbrB is also evidenced by the Reactance profiles obtained from impedance spectroscopy. The Reactance (inverse of capacitance) could be related to functionality, integrity, and composition of the cell membrane (Denneman et al., 2020). The changes in the membrane may be associated with the repression exerted by AbrB on *aprE*, which codes for the main extracellular protease, in addition to other genes involved in membrane transport (Liu et al. 2020). This information suggests that the membrane could undergo more changes when the presence of the transition state regulator AbrB is maintained throughout the culture as in FBC1, where changes in Reactance were

more evident (Figs. 2b and 4b). This difference in the Reactance profiles coincides with the difference in the behavior of Bt under two different culture strategies.
On the other hand, the online monitoring by impedance spectroscopy showed consistent changes to different growth phases, which results important for monitoring specific cell behavior with the aim of improving the characterization of cell differentiation process. Resistance profile has previously been related to production of extracellular metabolites such as organic acids (Díaz et al., 2021; Díaz et al., 2022; Chabchoub & Nakkach 2018). Additionally, the constant addition of medium during feeding, which is essentially a conductive solution, could explain the declining Resistance profile obtained during the first 9 h in both cultures. In the case of FBC2, a value lower than 2.6 Ohms was reached, which was consistent with a higher Bt concentration. At 1.225 MHz Resistance and Reactance manifest more propitious behavior in relation to the growth phases, due to changes in their signal influenced by morphological and physiological cellular changes (Dinorín et al., 2015).
Since the *Bacillus* genus is widely used for large-scale production of spores and toxins, and Bt fermentations are mainly focused on Cry toxin optimization, it results important to determine what is happening in processes with low spore and Cry production yields, which can be attributed to metabolic and genetic regulation issues. This work provided valuable information about the accumulation of the AbrB transition state regulator under different FBC conditions, which is related to differences on the sporulation development and variations in the level of *cry1Ac* gene expression. Research efforts are required to highlight the importance of transition state regulators, such as AbrB, for the development of processes focused on commercial products obtained from the *Bacillus* genus, one of the most important in the biotechnological industry.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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