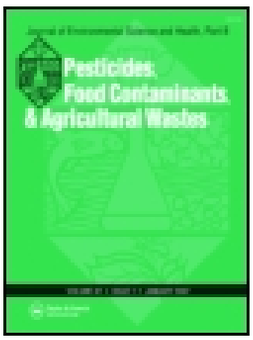

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Alternative low-cost process for large-scale production of *Bacillus thuringiensis* in a simple and novel culture system

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ABSTRACT

An industrial-scale, profitable method for production of the most widely used bioinsecticide, *Bacillus thuringiensis* (*Bt*), is challenging because of its widespread application. The aim of this study is to present a strategy to develop a low-cost, large-scale bioprocess to produce *Bt* H14.

This study was first focused on the design of a culture medium composed of economical and available components, such as glycerol and lysed *Saccharomyces cerevisiae*. The production goal of 1200 ITU was achieved using a medium composed of 20:20 g $L^{-1}$of glycerol:lysed yeast in batch cultures. Efforts were subsequently focused on the design of an appropriate culture system, and an original two-stage culture system was proposed. First, yeast (the primary component of the culture medium) are cultivated using a minimal mineral medium and lysed, and in the second stage, *Bt* is cultivated in the same bioreactor using the lysed yeasts as culture medium (supplemented with a feeding pulse of 10 g L^{-1} glycerol). This system was called fed batch one pot (FOP). A new inoculation strategy is also presented in this study, since these *Bt* cultures were inoculated directly with heat pre-treated spores instead of vegetative bacteria to facilitate the bioprocess. This study was developed from the laboratory to production-scale bioreactors (measuring from 500 mL to 2500 L), and the efficiency of the proposed strategy was evident in LD_{50} tests results, achieving 1796 ITU in large-scale processes. Both the use of non-conventional sources and the process development for biomass production are important for cost-effective production of *Bt*-based insecticides in mosquito control projects.

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Introduction

The production of biological and microbiological entomopathogenic organisms to control agricultural pests and disease-carrying insects is a subject of continuing research. These tools are of great importance to routinely combat and control target areas where insects naturally occur, as well as to counteract emerging outbreaks of endemic diseases due to natural disasters, such as floods.[1] Meanwhile, the use of dangerous and recalcitrant chemical insecticides not only causes severe damage to the environment, since soil, water and air are contaminated, but also leads to insect resistance.[2,3] For these reasons, the use of bioinsecticides to battle pests is preferable, since the active components have no environmental consequences.[4,5]

According to the report "Bioinsecticides Market – Global Trends & Forecast to 2021", statistics indicate that the market for bio-insecticides reached US$ 1.16 billion in 2015. The estimated projected CAGR (Compound Annual Growth Rate) is 18.3% for the 5 years following 2016, reaching 3.18 billion in 2021.[6]

Different *Bacillus thuringiensis* (*Bt*) strains show specific biocidal activity against Lepidoptera, Coleoptera and Diptera,[7] caused by the specificity of the proteic endotoxin produced by each strain, which are encoded by the *cry* genes located on plasmids. Nevertheless, these strains have no entomotoxicity against other organisms, including other insects and mammals.[8–10] *Bt* H14 is particularly used to control populations of *Aedes aegypti*, which is a vector of yellow fever, dengue, chikungunya and the emergent Zika diseases,[11] and it is produced on a large scale to satisfy its demand. In this regard, an extensively investigated issue is the production of *Bt* biomass using economical culture media, formulated with readily available components and even certain agricultural and food wastes.[5,12,14] Several residual products that can be used for biopesticide production are corn sugar, cheese whey, soybean flour, peanut powder, sugarcane molasses, animal manure and even chicken feather waste.[5,15,16] These economical nutrients have been combined in different ways, always pursuing the same goal, which is to produce more and improved entomotoxic crystals and microbial spores at the lowest possible cost. According to recent reports, this goal has been largely achieved at laboratory scales,[4] but when referred to production in large scales to obtain it as a bulk product, there are a limited number of reports.[5,12,14] In developing countries, *Bt*-based products have a social demand, particularly from sectors of the population

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that have few or no economic resources available to pay for the sustainable use of biopesticides.[6]

It is well-documented how the carbon and nitrogen sources influence the entomopathogenic biomass production, the sporulation and the quantity of entomotoxic protein crystals.[13] An additional issue of great importance for proper growth and correct sporulation of *Bt* is the availability of dissolved oxygen, which is related to the volumetric oxygen mass transfer (KLa) of the bioreactor to be used.[5,17]

This paper focuses on the development of a valid resolution to this problem, seeking to produce large amounts of *Bt* biomass with high numbers of spores and entomotoxic crystals using a low-cost medium and a simple culture strategy. We propose the development of a culture medium based on glycerol as the carbon source and using *Saccharomyces cerevisiae* lysate as the major nitrogen and micro and trace elements sources. Regarding the culture system, two different operative strategies were proposed, one operating in pulsed fed batch mode and another that is a novel concept, where *S. cerevisiae* is obtained in the bioreactor where *Bt* would be later produced. This second strategy avoids logistical problems related to procuring large amounts of yeast powder, constituting a valid and practical solution to this issue. This report is organized in terms of different volumes studied (cultures at laboratory scale, pilot scale and production scale) with the intention of explaining technological issues related to each bioreactor, and second, each culture system analyzed is described with the aim of explaining the biological issues, such as the inoculation strategy and the effect of the carbon supply in each case. The results and conclusions are derived from the combination of these studies.

Materials and methods

Strain

The microorganism used for this study was *Bacillus thuringiensis* H14. This microbe was obtained from the cryopreserved strain collection of the Biocatalysis and Biotransformations Laboratory of the National University of Quilmes, which acquired it from the IPS 82 standard lyophilisate of the Pasteur Institute, France.

Culture media

The culture medium Luria Bertani (LB) was prepared as usual with (in g L^{-1}) tryptone 20, yeast extract 10 and NaCl 20. Solid Tryptone Soya Agar (TSA) medium was prepared with 40 g $L^{-1}$of commercial culture medium (Difco). The production of *S. cerevisiae* was performed in a sucrose/urea synthetic culture media composed of (in g L^{-1}) sucrose 20, urea 5, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.05, $CaSO_4$ 0.01, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01 and $ZnSO_4.7H_2O$ 0.01. The Industrial culture medium (IM) was prepared (in g L^{-1}) with soy bean meal 20 and soluble starch 20.[18] The novel culture media proposed in this paper (used for different scale assays) contained variable concentrations of glycerol and commercial food grade *S. cerevisiae* dried powder (GY medium). The compositions of yeast dried powder (per liter of medium) were as follows: carbohydrates 6.8 g, proteins 8.7 g, fiber 4.0 g, sodium 17 mg, thiamine 0.3 mg, riboflavin 0.3 mg, niacin 40 mg, pantothenic acid 1.3 mg, pyridoxine 0.6 mg, folic acid 0.5 mg, biotin 0.02 mg, iron 0.9 mg, zinc 3.9 mg, and magnesium 22.4 mg. The glycerol:yeast concentration ratios (in g L^{-1}) were 10:10, 20:10, 30:10, 20:20, 30:20, 30:30 and 40:20. The initial pH of all culture media was 7.4. All chemicals used were of an analytical grade.

Inoculum preparation

Reactivation of cells from a cryopreserved vial was performed in petri dishes containing TSA medium at 30°C. After a 24-h incubation, individual colonies were transferred to test tubes containing 2.5 mL of sterile physiological solution, homogenized and used as inoculum to obtain a lawn of bacteria in petri dishes (for lab scale cultures) or rectangular bottles used in a side position (300 cm^2 surface, for pilot- and production-scale cultures), both containing TSA medium. These cultures were incubated at 30°C for 96 hours until sporulation was complete.

Spore harvesting in the case of spores obtained in petri dishes was performed using a Drigalski loop and 2 mL of sterile deionized water. Once recovered, the spore solution was transferred to a 15 mL sterile tube and used to inoculate lab scale cultures. For harvesting spores produced in rectangular bottles, 50 mL of sterile deionized water was added, the bottles were incubated (in a side position) for 10 minutes at room temperature and vigorously shaken. Harvested spores were later transferred to 50-mL conical tubes. Counting the spores was performed to establish the volume of the spore concentrate to be used for each fermentation process. In both cases, before being used as an inoculum, each tube was placed in a hot water bath (60°C for 15 minutes) to remove non-sporulating bacteria viability. To inoculate production scale cultures, one-liter Erlenmeyer flasks with a lateral outlet at the base and a negative pressure system were used to ensure the sterility of the process.

Biomass determination

In the case of *Bt* bacteria, CFU mL^{-1} measurements were performed by making serial dilutions of samples in a sterile physiological solution and plating them on TSA dishes using a Drigalski loop. These plates were maintained at 30°C for 24 hours before counting. Measurements were performed in triplicate. *Bt* spores were counted in a Petroff-Hauser chamber using culture samples (conveniently diluted) with a standard optical microscope at 400X magnification. Conversely, *S. cerevisiae* biomass determinations were performed by dry weight measurements. In all cases, to facilitate the observation and differentiation of bacteria, spores and protein crystals were stained with Coomassie brilliant blue,[19] Schaeffer-Fulton and Gram staining of the samples for microscopic observation were performed.

Culture development (different scales)

Laboratory scale in shaken flasks

In the initial stage of exploring the growth and sporulation of *Bt*, cultures were prepared in 500 mL Erlenmeyers containing 100 mL of each medium (LB, IM or GY media). These assays were performed qualitatively to confirm the feasibility of

different culture media and quantitatively to compare the CFU mL^{-1} and spore formation in each case.

The selection of the proper concentration and ratio of glycerol:yeast lysate was performed in 500 mL Erlenmeyers containing 100 mL of culture medium and incubated on an orbital shaker at 30°C during 24 h.

Once the proper medium composition was defined, a batch culture was performed in 20.0 liter Pyrex bottles containing 4.0 L of medium, aerated by compressed sterile air that was controlled by a flow meter to 0.5 VVM. During the process, these bottles were stirred using a magnetic Teflon impeller and a magnetic stirrer. Before the fermentation process, the bottles containing GY media were sterilized in an autoclave for 20 minutes at 121 ± 1°C.

Pilot scale

Cultures on a pilot scale were carried out in a 300 L fermenter, containing 180 L of GY culture medium. This bioreactor was stirred at 100 RPM by a Rushton impeller and is equipped with automatic temperature, pH, pressure and air flow controls and online measurement of dissolved oxygen tension (DOT). It also has a quick coupling system for inoculation and sampling in sterile conditions, using overheated vapor flow. pH was automatically maintained at 7.2 using 1N NaOH. Aeration varied automatically between 0.05 and 0.5 VVM to maintain DOT values above 30% of the maximum dissolved oxygen concentration saturation (C^*). The culture medium was prepared and sterilized by jacket steam in the bioreactor at 121±1°C for 20 minutes.

Production scale cultivation

Production scale experiments were carried out in a 2500 L bioreactor containing 1500 L of GY medium. The bioreactor was stirred at 100 RPM using two Rushton impellers. The bioreactor has temperature and pH automatic controllers and sampling and inoculation system sterilized by steam flow. The bioreactor was aerated through a sparger at an air flow rate of 0.1 VVM and maintained at 1.2 ± 0.6 kg cm^{-2} overpressure so that the DOT was maintained at greater than 30% of C^*. The culture media were prepared into the bioreactor and sterilized by jacket steam at 121 ± 1°C for 20 minutes (retention time).

Culture systems

Figure 1 represents a schematic diagram of the different culture systems developed in this work. The systems were 1) a classic batch system using GY medium; 2) a fed batch system, where a pulse of glycerol is fed to the bioreactor; 3) a system called one pot, a sequential fermentation process; and 4) a fed one pot system, where a pulse of glycerol is added to the one pot system. A detailed explanation of each system is described below.

Batch processes

Batch processes were performed in all four working scales as presented above. Once bioreactors were prepared, they were inoculated with spores (10^5 spores mL^{-1}), which were obtained as described before. Initially, the progress of the process was verified by microscopic observation. The level of sporulation was determined by counting in a Petroff-Hausser chamber at 16, 24, 30, 36 and 48 hours after inoculation. After total carbon depletion, which usually occurs 24 hours after inoculation, cultures were maintained for 24 hours at 20°C such that complete sporulation was achieved (this is considered *Bt* spore maturation).

Pulse-fed-batch processes.

Pulse fed batch processes were performed as described above (500 mL Erlenmeyer flask, 20 L bottles and a 300 L bioreactor). These cultures were initialized as batch processes using GY medium. After 10 hours of exponential growth, cultures were supplemented with sterile glycerol to increase its concentration in the bioreactor by 10 g L^{-1}. The choice of this moment to begin with the addition of glycerol is because after 12 hours of culture, the Phase II or transition to sporulation phase begins.[20] In this study, this transition was verified by

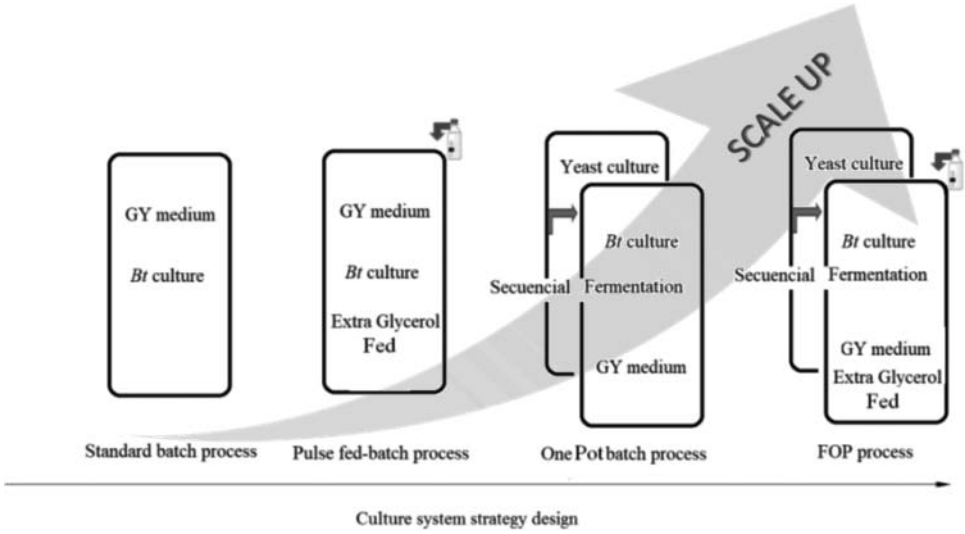

Figure 1. Schematic diagram of different culture systems developed in this work. The bottle represents the glycerol reservoir.

microscopy. This fact is also evidenced by the oxygen consumption rate.[5]

One-pot process

One-pot processes were performed as described above (500 mL Erlenmeyer flask, 20 L bottles and a 300 L bioreactor respective). These processes were similar to the batch process described in the ***Culture system*** section, *Batch processes* but performed in two stages. The first stage consisted of a *S. cerevisiae* culture grown in the sucrose/urea medium described in ***Culture media***. This culture was performed in the same fermenter where the *Bt* culture would subsequently be grown (Erlenmeyers, Pyrex bottles or bioreactors). Regarding the inocula of these *S. cerevisiae* cultures, commercial dry lyophilized yeast previously resuspended in sterile physiological solution was used to achieve 10% of the desired final concentration of yeast. Once inoculated, these cultures were grown at 30°C until sucrose depletion. Finally, these cultures were properly diluted to achieve a concentration of 20 g L^{-1} yeast and heat sterilized in place to obtain a yeast lysate that would be used as a main component of the second stage culture medium. This final heat inactivation process was performed for 15 minutes, after the addition of the other components to the culture medium.

The second stage was a standard proposed batch culture of *Bt*, using the yeast lysate obtained in the first stage. Since GY medium is composed of 20 g L^{-1} of yeast lysate and 20 g L^{-1} glycerol, the latter was added (20 g L^{-1}) to the bioreactor to achieve this composition. A standard batch condition was then replicated; once media and bioreactors were prepared, they were inoculated with the *Bt* spores obtained as described before, to achieve 10^5 spores mL^{-1}. The level of sporulation was determined by counting in a Petroff-Hausser chamber at 16, 24, 30, 36 and 48 hours after inoculation.

Pulse-fed-batch and one-pot combination processes (FOP).

FOP processes were performed in all four work scales presented. These processes are a combination of the systems described as pulse fed batch and one pot. First, a *S. cerevisiae/Bt* one-pot culture was made. After 10 hours of cultivation, extra sterile glycerol was added to achieve a final concentration in the bioreactor of 10 g L^{-1}. The level of sporulation was determined by counting in a Petroff-Hausser chamber at 16, 24, 30, 36 and 48 hours after inoculation.

Entomotoxicity measurement on *Aedes aegypti* – DL_{50}

Laboratory tests were performed using growth stage IV *Aedes aegypti* larvae, generously provided by CEPAVE (UNLP-CONICET). Larvae were kept in suitable 4 L containers with light:dark cycles of 16:8 hours in a 27±1°C growth chamber with 60%–70% relative humidity and ventilation, and the larvae were fed extruded rodent chow powder. For a DL_{50} test, larvae were placed in polypropylene beakers containing 150 mL dechlorinated water (10 larvae per beaker for laboratory scale assays, and 25 larvae per beaker for pilot and production scale assays). Once placed, 50, 100, 150, 200 and 250 μL of each culture were added, in triplicate, to each cup. The tests were carried out in a growth chamber at 27±1°C and 60%–70% relative humidity with continuous light for 24 hours. These results were compared against a negative control, which was performed using sterile GY medium, *S. cerevisiae* dry powder and a cell-free depleted GY broth. A lyophilised IPS82 reference standard was performed as described by Skovmand and Becker.[21]

Statistical analysis

Larval mortality data obtained in DL_{50} assays were tested among different media, culture systems and working scales by a linear model followed by multiple pairwise comparisons of means (Tukey's HSD test) performed with PSPP ver.0.9.0 software.

Results and discussion

Study of media composition

The effects of the composition of the culture medium using variable ratios of glycerol:yeast were studied. It was found that the optimal ratio for the formulation of GY media was 20:20 g L^{-1} of glycerol:yeast for processes of a short duration (Fig. 2). In addition, ratios of 30:20 and 40:20 g L^{-1} of glycerol:yeast seemed to also be appropriate, but these cultures exhibited a longer lag phase, an unusually higher division time, and an inadequate growing synchrony. When these conditions were analyzed in terms of synchrony, it was found that a high increase in glycerol concentration caused an asynchronous culture; i.e., different forms coexisted, such as vegetative bacteria, spores, sporulating bacteria and pre-sporulating bacteria. This issue was an important one, since in the production of a bioinsecticide, the presence of only spores is preferable. However, when concentrations of glycerol were over 40 g L^{-1}, no proper growth of *Bt* was observed. It has been reported that *Bt* growth is probably inhibited in high concentrations of glycerol.[22] In view of these results, it was decided to use a medium composed of 20:20 g L^{-1} of glycerol:yeast, and in the cases that a pulse fed batch was performed, the addition of 10 g L^{-1} of glycerol was chosen.

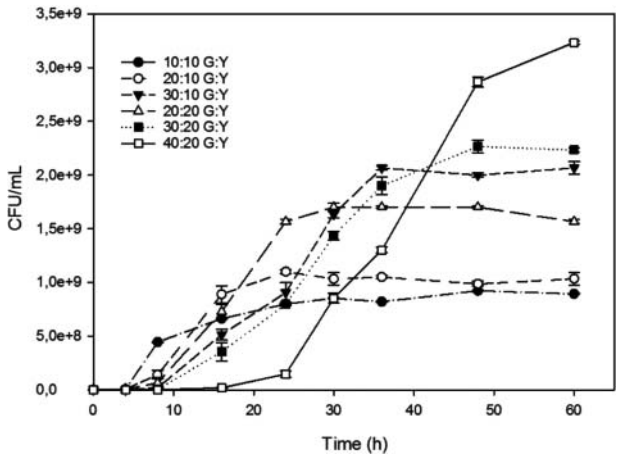

Figure 2. Growth curves obtained in media composed of variable concentrations of glycerol and commercial food grade *Saccharomyces cerevisiae* dried powder (GY medium). The glycerol:yeast concentration ratios (expressed in g L^{-1}) were 10:10 (●); 20:10 (◯); 30:10 (▼); 20:20 (Δ); 30:20 (■); and 40:20 (□), respectively. Initial pH of all culture media was 7.4.

Glycerol, an available commodity for the industrial production of bio inputs, is reported as a convenient substrate to produce high entomotoxicity.[22] Glycerol consumption in *Bacillus* genera results in the generation of organic compounds by the reduction of pyruvate, which is generated by the facilitator protein GlpF. Regarding nitrogen content, it has been previously reported that high initial amino nitrogen in the medium composition is preferable, since it favors the production of biomass and entomotoxicity.[13]

Once the media composition was established, the novel proposed medium was tested again against a classic media used for the production of *Bt*, such as LB and IM. The results obtained showed that GY medium demonstrated similar results to the classic lab media (Fig. 3a). These results were compared in terms of LD_{50} to evaluate the efficacy of the product obtained by using the novel medium proposed in this paper (LB: 1244 ITU; IM: 1164 ITU and GY: 1191 ITU; Fig. 3b). LD_{50} results showed that the efficiency of the product obtained in the new media was equivalent to that obtained in the conventional media. Thus, any of the three media would be useful to produce a larvicide that meets the standard of 1200 ITU.

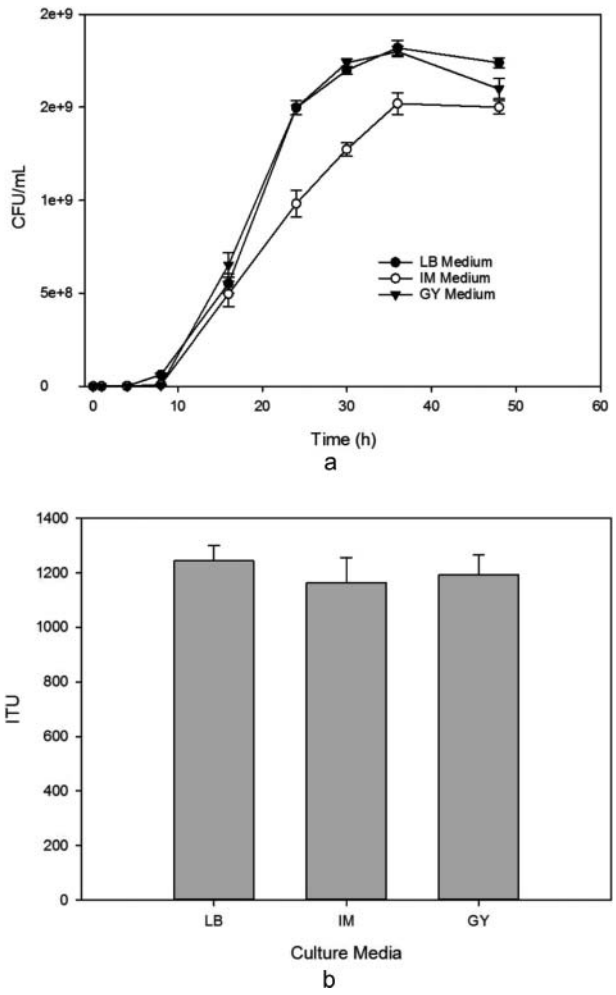

Figure 3. (a) Comparison between different growth curves of *B. thuringensis* in different media (CFU mL^{-1}). LB Medium (●); composed of (in g L^{-1}) tryptone 20, yeast extract 20 and NaCl 20. IM Medium (◯); Industrial culture medium (IM), composed of (in g L^{-1}) soybean meal 20 and starch 20. GY Medium (▼); glycerol: yeast medium (GY), composed of (in g L^{-1}) glycerol 20 and commercial food grade *S. cerevisiae* dried powder 20. (b). Comparison of the three media, evaluated in terms of LD_{50} to determine the actual efficacy of the product obtained using the GY medium.

When considering the productivity achieved in each case, that is, 52 ITU h^{-1}, 49 ITU h^{-1} and 50 ITU h^{-1} for LB, IM and GY media, respectively, it can be seen that in the three cases, the values also support the convenience of using the GY medium, due to its low cost and the fact that it does not lead to asynchrony cultures. From the results of the statistical analysis performed in this study, it can be concluded that there is no significant difference in terms of ITU achieved when different culture media were used (between LB and GY, with p values >0.05). However, IM showed a significant difference with LB and GY ($p < 0.05$), resulting in lower yields with this industrial medium. These results suggest the proper application of the suggested medium, GY, to produce the bio insecticide.

Culture system design

Inoculation

In all cultures developed in this work, inoculations of cultures were performed with heat-pre-treated spores, instead of the traditionally used vegetative bacteria in the exponential growth phase. In previous studies performed in our lab, when inocula was performed using growing bacteria, microscopic preparations of the resulting cultures sometimes showed the coexistence of vegetative bacteria, bacteria in pre-sporulation stage, spores (certain of them giving rise to new vegetative bacteria) and entomotoxic crystals. Nevertheless, preliminary tests showed that the use of heat-pre-treated spores could help to generate synchronized cultures. Furthermore, spore inocula could be prepared and preserved at 4°C in tubes or Erlenmeyer flasks for further use; therefore, it is possible to decouple the generation of the inoculum and the production process, thereby reducing the total sequential steps in large-scale fermentations. In this paper, it was proposed that the generation of spores be used as inocula in large-scale bioreactors using rectangular bottles containing TSA medium. After total sporulation of these cultures, harvesting of spores was a simple step to obtain highly concentrated spore solutions (from 10^9 to 10^{12} spores mL^{-1}). Additionally, it has been tested that spores harvested in distilled water and kept at 4°C for 6 months could be used to start bioprocesses that reach accurate results (data not shown), which could have important technological and economic implications for the process.

It was previously reported that synchrony during culture development is important because the maximal efficiency of the final product is achieved when the end of the fermentation is as close as possible to 100% sporulation. Synchrony of the culture is a main parameter during the production process, since the potential quality of the product is a combination of a biomass quantity and the final sporulation degree: growing bacteria have no biocidal activity against mosquito larvae, since entomotoxic protein crystals are produced during sporulation.[23] Therefore, the proposal of using spores to directly inoculate production scale bioreactors was a simple strategy, and could make the whole process easier in comparison to the classic multistep vegetative inocula in industrial *Bt* production.

Laboratory scale cultures

The cultures were performed in 20-L bottles containing 4 L of GY medium to establish preliminary conditions of growth in the proposed medium and to evaluate the effect of different culture systems on *Bt* production. Regarding the cultures, it was possible to compare the biomass production (bacteria and spores) between different culture systems, such as batch, pulse fed batch, and one pot and FOP, which is a combination of the last two. When a feed step was performed, higher levels of biomass were achieved. Spores produced were directly related to the CFU mL^1 previously obtained in the culture (which also depended on the amount of FCE added) and the multi-step sporulation process kinetic. Since both fed batch and FOP systems were supplemented with glycerol (10 g/L), the total biomass achieved in the cultures was higher than those obtained in the batch and one pot systems (where there was no feeding with a fresh carbon source) with a concomitant increase in the spores' production. The results from Petroff-Hauser counting showed that spore formation did not occur before 12 hours of culture (Figs. 4a, 4b).

In previous studies, the nutritional and aeration requirements of a variety of *Bt* subspecies,[5,12,24,25] as well as the costs associated with these parameters, were established.[5,14] Regarding these challenges, this paper proposes the use of a GY medium, which has been demonstrated to satisfactorily support the generation of the required *Bt* biomass, spores and crystals of δ-endotoxin, the latter of which are used as a larvicide in many applications. ITU obtained in each system were batch: 1241 ITU; fed batch 1561 ITU; one pot 1295 ITU and FOP 1600 ITU. These results were supported by LD_{50} analysis performed at the lab scale (Fig. 4c). As observed, entomotoxicity levels reached in the fed-batch and FOP systems were similar and could be compared to a 1200-ITU commercial standard. These results led us to consider this system was a promising one, since high levels of bioinsecticide could be produced in an inexpensive medium with an easily used culture system.

Pilot scale cultures

The application of bioinsecticides in many fields makes bulk production essential to satisfy that demand. This study will become more valuable if the cost of transporting industrial inputs, such as raw materials for large-scale production, is considered. Higher working volumes and costs related to the transportation of the inputs required for medium preparation can reach an important fraction of the cost of the whole process. For this reason, scaling up the process to a pilot scale and evaluating the most promising culture strategies to produce this bioinsecticide are crucial for the design and optimization of the process.

After the 180-L cultures were performed, they were compared to the biomass production from batch, fed batch, and one pot and FOP, a combination of the last two methods. When a feed step was used, higher levels of biomass were achieved (Figs. 5a, 5b). Thus, the results were consistent with those seen in previous working scale experiments (4-L cultures in 20-L bottles). LD_{50} results obtained at this working scale confirmed the potential of the FOP system, since it obtained 1271 ITU; 1800 ITU; 1188 ITU and 1820 ITU in batch, fed batch, one pot and FOP, respectively. First, these results were adequate to meet the current standards of a bioinsecticide, and they were also higher than those reached in a classic fed-batch system. Second, by using raw materials and this strategy, it was possible to reduce the costs related to the logistics of media components. These results led to the design of the final objective of this work, the production scale culture.

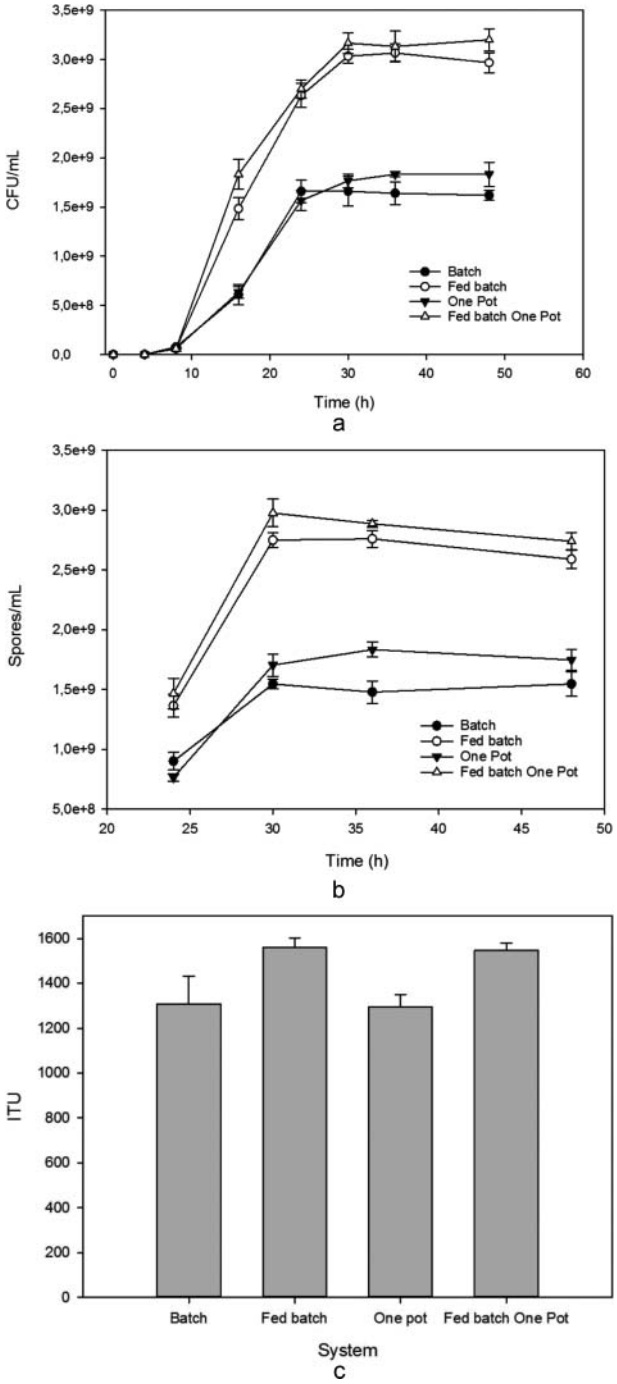

Figure 4. (a) Kinetics of biomass production (CFU mL^{-1}) in 20-liter bottles containing 4 liters of GY medium in different culture systems: batch (●), pulse fed batch (◯), one pot (▼) (yeast lysate is obtained in the same reactor where *Bt* was cultivated) and pulse fed batch combined with one pot (Δ), called FOP. (b). Kinetics of spore production (spores mL^{-1}) in 20-liter bottles containing 4 liters of GY medium in different culture systems: batch (●), pulse fed batch (◯), one pot (▼) and FOP (Δ). (c). LD_{50} analysis performed at lab scale. All assays were performed in triplicate.

Production scale cultures

Once the medium composition and growth parameters of *Bt* were determined, the conditions to initiate a production-scale

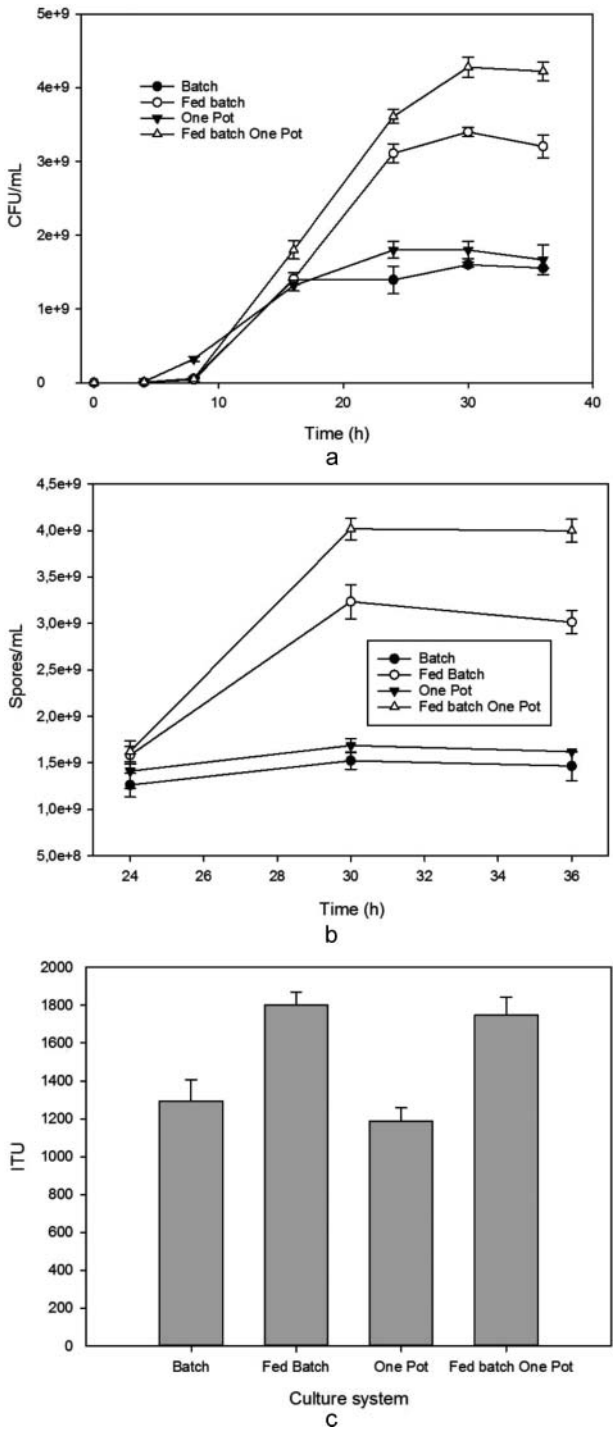

Figure 5. (a). Kinetics of biomass production (CFU mL^{-1}) in 300-liter bioreactor containing 180 liters of GY medium in different culture systems: batch (●), pulse fed batch (◯), one pot (▼) (yeast lysate is obtained in the same reactor where *Bt* was cultivated) and pulse fed batch combined with one pot (Δ), called FOP. (b). Kinetics of spore production (spores mL^{-1}) in 300-liter bioreactor containing 180 liters of GY medium in different culture systems: batch (●), pulse fed batch (◯), one pot (▼) and FOP (Δ). (c). LD_{50} analysis performed at pilot scale. All assays were performed in triplicate.

culture were established. This culture was performed in the FOP system, since it resulted in the most appropriate and convenient production of this commodity (Figs. 6a, 6b). Regarding this culture, the addition of glycerol in the exponential phase resulted in a significant increase in the number of spores (counted microscopically), consistent with the efficiency

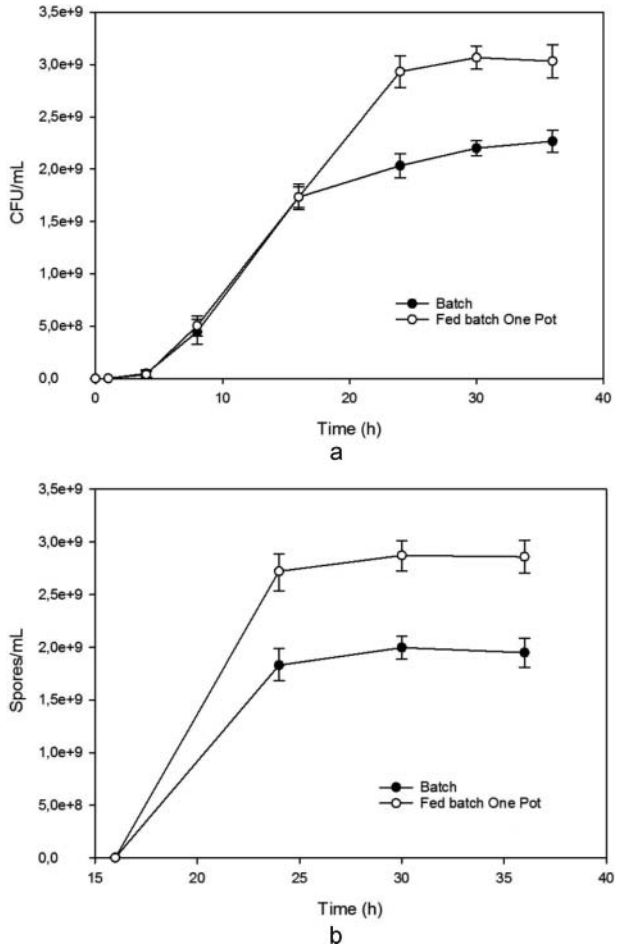

Figure 6. (a). Kinetics of biomass production (CFU mL^{-1}l) in 2500-liter bioreactor containing 1500 liters of GY medium in Batch (●) and pulse fed batch combined with one pot systems (◯), called FOP. (b). Kinetics of spore production (spores mL^{-1}) in 2500-liter bioreactor, containing 1500 liters of GY medium in batch (●) and FOP (◯).

evidenced in LD_{50} tests yields. In the case of batch cultures at this scale, it was possible to obtain 1208 ITU, whereas in the FOP system, a value of 1796 ITU was achieved. When these results were compared to a liquid commercial standard (1200 ITU) (Fig. 7), it was evidenced that the efficiency of the product

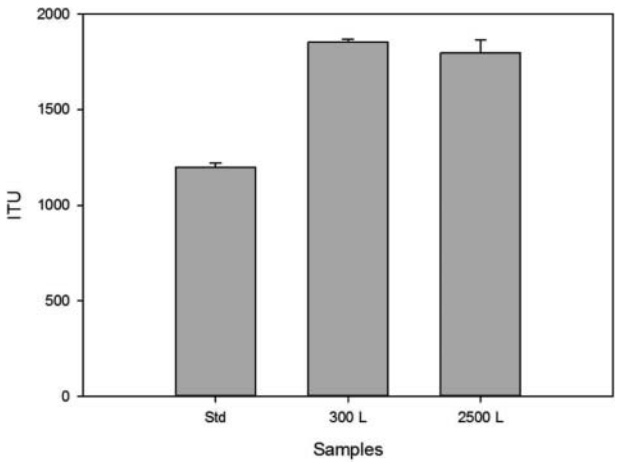

Figure 7. Lethal dose 50 (LD_{50}) analysis performed at pilot and production scales, compared to a standard 1200 ITU bioinsecticide. All assays were performed in triplicate.

Table 1. Comparison between entomotoxicity (ITU, International toxicity units) obtained from different culture strategies and scales and its relationship with standard IPS82.

Culture Volume	Culture system	Efficacy (ITU)	Relative efficacy (ITU/IPS 82 Std)
4 L	Batch	1308	1,09
	Pulse Fed Batch	1561	1,30
	One Pot	1295	1,08
	FOP	1548	1,29
180 L	Batch	1255	1,05
	Pulse Fed Batch	1924	1,60
	One Pot	1188	0,99
	FOP	1780	1,48
1500 L	Batch	1208	1,01
	FOP	1796	1,50

obtained used this culture strategy; i.e., almost 1.5 times higher than that of a commonly used, standard biolarvicide. Glycerol was added 10 hours after the beginning of the culture since, as stated before (*Pulse-fed-batch processes)*, no pre-sporulation was observed before 12 hours of cultivation.

Culture quantitative analysis

Finally, these results were analyzed comparing lethal doses yields and productivity achieved. Table 1 summarizes results obtained from bioassay experiments, for each culture system in every scale studied and also as a comparison with the standard IPS82. When considering productivity, it can be seen that there is a marked difference between the batch and FOP system, (and there was almost no difference upon the volume scale, with p values < 0.05). This effect was probably caused by the increase of carbon content in the fed system, consistent with the higher total biomass achieved in the FOP systems. Conversely, when considering yield values (expressed as ITU gC-1 considering 15.000 ITU mg-1 on IPS 82 standard), these values were similar in both systems, since yields values consider the carbon content in each case.

Conclusions

Bacillus thuringiensis H14 was grown satisfactorily in culture volumes between 100 mL and 1500 L, focusing on the design of an inexpensive method for the production of a bioinsecticide that could meet the standards of formulation in liquid presentations. In this regard, efforts were made to ensure that the cost of the medium was minimized and simplified, including the time and costs related to logistical issues. To accomplish this goal, an alternative method was proposed in this study to generate *S. cerevisiae* (a component of the proposed GY culture medium) *in situ* (i.e., in the same reactor where *Bt* would be produced). It was expected that medium components employed in this study could be replaced in the future by discarded glycerol from the manufacture of biodiesel and *S. cerevisiae* discarded from beer production. Furthermore, another important issue was the usage of spores to directly inoculate a large-scale bioreactor instead of vegetative bacteria, avoiding the previous inoculum fermentations.

These advances could be helpful in the development of a convenient process to obtain Bt in adequate quantities to satisfy governmental mosquito control programs.

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This work is dedicated to the memory of Dr. Carlos F. Mignone, RIP.

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Supplementary Materials

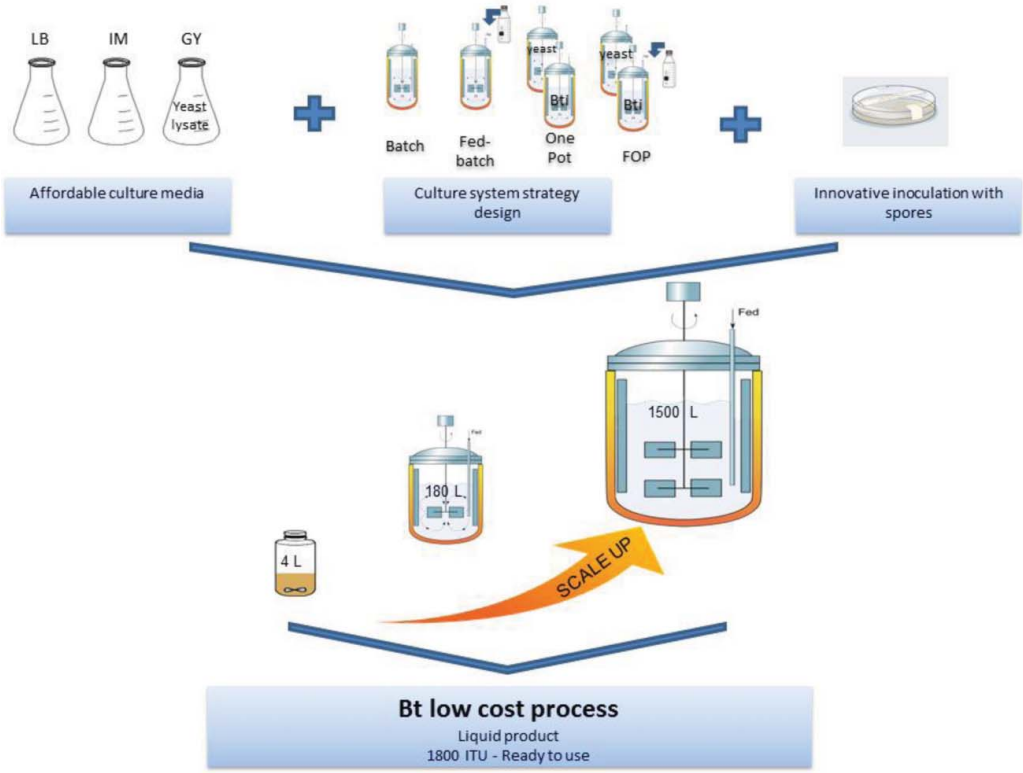

Figure 1S. Graphical abstract