CULTIVATION OF Bacillus thuringiensis var. israelensis H14 IN BIOREACTOR FOR BIOLOGICAL CONTROL OF Aedes aegypti LARVAE

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ABSTRACT – The aim of this study was the cultivation of Bacillus thuringiensis var. israelensis (Bti) H14 for the biological control of Aedes aegypti. Bti cultures were cultivated in a bioreactor, and the growth kinetics, toxin concentration and larvicidal activity were evaluated. The results showed that the bacterial fermentates produced on average 0.62 g/L of toxin (LC₅₀ 2.25 mg/L and LC₉₀ 3.82 mg/L). Upon concentration of the biomass, the toxin yield was almost three-fold higher (1.64 g/L), also resulting in an increase in the bioactivity (LC₅₀ 0.46 mg/L and LC₉₀ 0.84 mg/L). The fermentation conditions employed were suitable for the cultivation of Bti, generating batches with high concentration of cells, short time required for sporulation and adequate production of toxins with confirmed biolarvicidal activity.

1. INTRODUCTION

Zika virus fever is an acute viral disease that was first reported in Brazil in 2015 (Campos et al., 2015) and has been associated with increased cases of microcephaly (Nunes et al., 2016) and neurological manifestations (Blázquez and Saiz, 2016). The Zika virus (ZIKV) is mainly transmitted by the mosquito Aedes aegypti, whose control has been traditionally based on chemical insecticides that target the nervous system (Cunha et al., 2005). With the emergence of resistant populations in the late 1990s (Braga and Valle, 2007), alternative methods have been investigated, with emphasis on biological control.

Among the several microorganisms used to this end, Bacillus species are known for their ability to form protein crystals with highly specific insecticidal action. For instance, B. thuringiensis strains possess high activity against larvae of aquatic dipterans, mainly Culicidae and Simulidae (De Barjac, 1978). The crystals are liberated in the environment and ingested by mosquito larvae in the form of protoxins, which are then cleaved and activated into toxins in the insect gut by the action of alkaline intestinal pH and proteases. Finally, the activated toxin causes the lysis of epithelial cells and the death of the larvae (Aronson et al., 1986; Charles, 1983). In this context, the aim of this study was to produce concentrated
biomass of *B. thuringiensis* var. *israelensis* (Bti) H14 using a bioreactor, as well as to evaluate its growth, protein crystal concentration and toxicity towards *A. aegypti* larvae.

### 2. METHODS

First, stock cultures for standardization of inocula were prepared as a concentrated suspension of spores (9 x 10⁸ CFU/mL), which was separated into aliquots of 20 μL to impregnate pre-sterilized Ø7 mm filter paper disks, followed by oven drying at 35°C for 24 hours and storage at 4°C. For each fermentation, the inoculum was prepared in two 500 mL Erlenmeyer flasks containing 75 mL of medium (g/L: meat peptone 7.5, KH₂PO₄ 6.8, MgSO₄.7H₂O 0.123, MnSO₄.7H₂O 0.0017, ZnSO₄.7H₂O 0.014, Fe₂(SO₄)₃ 0.02, CaCl₂.2H₂O 0.147 and Glucose 10, with initial pH adjusted to 7.4.) and one disk of Bti spore suspension. The flasks were incubated at 200 rpm for 15 hours at 30°C.

Batch culture fermentations were conducted in triplicate in a bioreactor (Tec-Bio-V 4.5 L, Tecnal, SP, Brazil) with 4.5 L nominal capacity and 3 L medium volume, under the following operational conditions: 300 rpm for 72 hours at 30°C, with a minimum oxygen rate of 40%, inoculum concentration of 5% (v/v) and the use of the impellers Smith-CD6 (top) and Rushton (bottom). The variables pH and dissolved oxygen concentration were monitored along the fermentation process with the bioreactor integrated electrodes, while the growth kinetics was measured via absorbance at 600 nm (GENESYS 10S UV-Vis, Thermo Scientific, SP, Brazil) by sampling the cultures every 30 minutes for 24 hours. At the end of the fermentation, the cells were separated by flocculation and sedimentation as described by Luna (2004). Quantification of total cells and spores was performed in nutrient agar medium (g/L: meat peptone 5, yeast extract 1.5, meat extract 1.5, NaCl 5, agar 15, pH 7.4) for different dilutions of both the fermented broth and the biomass after incubation at 30°C for 18 hours. For the spore count, the samples were previously subjected to thermal lysis at 80°C for 12 minutes to eliminate vegetative cells.

After 72 hours of incubation, aliquots of both the fermented broth and the biomass were subjected to separation of toxin crystals according to Mounsef *et al.* (2014) and quantified by the method of Bradford (1976) using BSA as the standard. The samples were then used in bioassays against *A. aegypti* L₄ larvae of the colony Rec-L from Centro de Pesquisa Aggeu Magalhães according to the method described by De Barjac and Larget-Thiéry (1984). The mortality data was analyzed by linear log-probit regression using the SPSS Statistics 8.0 program for Windows (1997) to estimate the values of lethal concentrations to 50% (LC₅₀) and 90% (LC₉₀) of the larvae.

### 3. RESULTS AND DISCUSSION

In the tested conditions, Bti displayed exponential growth during the first six hours of incubation with no lag phase observed, which was confirmed by both the pH and dissolved oxygen profiles (Figure 1).
Figure 1 – Growth kinetics (left) of Bti under batch bioreactor cultures for 24 hours of fermentation, with pH (upper right) and dissolved oxygen (lower right) profiles after 72 hours.

The average total cell and spore concentration of samples were 9.9 x 10^8 and 6.6 x 10^8 CFU/mL for the fermented broth and 5.4 x 10^9 and 2.9 x 10^9 CFU/mL for the concentrated biomass, respectively (Table 1). These results show that the produced batches had a high level of sporulation, indicated by the proximity of the total cells and spores counts. As expected, the concentration of biomass increased the cellular counts, in this case, by one order of magnitude. The mean toxin yield in the concentrated biomass (1.64 g/L) was almost three-fold higher than in the fermented broth (0.62 g/L). The biomass also showed the highest toxicity levels (LC50 0.46 mg/L and LC90 0.84 mg/L), with an increase of almost five-fold compared to the fermented broth (LC50 2.25 mg/L and LC90 3.82 mg/L) (Table 1). The conditions employed in this study have yielded better results when compared to similar works described in the literature (for instance, Elsayed et al., 2014).

Table 1 – Cellular concentration, quantification of proteins and toxicity of Bti batches

| Analysis    | Fermented broth | Concentrated biomass |
|-------------|-----------------|----------------------|
|             | Batch-1 | Batch-2 | Batch-3 | Batch-1 | Batch-2 | Batch-3 |
| **Total cells** (CFU/mL) | 8.0E+08 | 8.6E+08 | 1.3E+09 | 2.3E+09 | 5.8E+09 | 8.0E+09 |
| **Spores** (CFU/mL) | 6.7E+08 | 8.1E+08 | 5.0E+08 | 3.0E+09 | 2.8E+09 | 3.0E+09 |
| **Toxin** (g/L) | 0.78 | 0.31 | 0.78 | 1.97 | 1.54 | 1.40 |
| **LC50** (mg/L) | 2.25 | 1.90 | 1.95 | 0.46 | - | - |
| **LC90** (mg/L) | 3.82 | 3.03 | 3.09 | 0.84 | - | - |

4. CONCLUSIONS

Our results suggest that the fermentation conditions employed were suitable for the
cultivation of *B. thuringiensis* var. *israelensis* H14, generating batches with high concentration of cells, short time required for sporulation and adequate production of toxins with confirmed biolarvicidal activity against *A. aegypti* larvae.

5. REFERENCES

ARONSON AI, BECKMAN W, DUNN P, *Bacillus thuringiensis* and related insect pathogens. *Microbiological Reviews*, v. 50, n. 1, p. 1-24, 1986.

BLÁZQUEZ A-B, SAIZ J-C, Neurological manifestations of Zika virus infection. *World Journal of Virology*, v. 5, n. 4, p. 135-143, 2016. DOI: 10.5501/wjv.v5.i4.135.

BRADFORD MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, v. 72, n. 1-2, p. 248-254, 1976. DOI: 10.1016/0003-2697(76)90527-3.

BRAGA IA, VALLE D, *Aedes aegypti*: histórico do controle no Brasil. *Epidemiologia e Servicos de Saude*, v. 16, p. 113-118, 2007. DOI: 10.5123/S1679-49742007000200006.

CAMPOS GS, BANDEIRA AC, SARDI SI, Zika virus outbreak, Bahia, Brazil. *Emerging Infectious Diseases*, v. 21, n. 10, p. 1885-1886, 2015. DOI: 10.3201/eid2110.150847.

CHARLES J-F, Action de la endotoxine de *Bacillus thuringiensis* var. *israelensis* sur cultures de cellules de *Aedes aegypti* L. en microcopie eletronique. *Annals of Microbiology*, v. 134A, p. 365-381, 1983.

CUNHA MP, LIMA JBP, BROGDON WG, MOYA GE, VALLE D, Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* (Diptera: Culicidae) populations collected between 2001 and 2003. *Memórias do Instituto Oswaldo Cruz*, v. 100, p. 441-444, 2005.

DE BARJAC H, Une nouvelle variété de *Bacillus thuringiensis* très toxique pour les moustiques: *B. thuringiensis* var. *israelensis* sérotype H14. *Comptes Rendus des Seances de L'Academie des Sciences*, v. 286, p. 297-314, 1978.

DE BARJAC H, LARGET-THIÉRY I, Characteristics of IPS-82 as standard for biological assay of *Bacillus thuringiensis* H-14 preparations. *WHO Mimeoograph Document*, *VBC/84.892*, Geneva, Switzerland, 1984.

ELSAYED EA, OTHMAN NZ, MALEK R, AWAD HM, WU K, AZIZ R, WADAAN MA, ENSHASY HAE, Bioprocess development for high cell mass and endospore production by *Bacillus thuringiensis* var *israelensis* in semi-industrial scale. *Journal of Pure and Applied Microbiology*, v. 8, n. 4, p. 2773-2783, 2014.

LUNA CL, Avaliação de técnicas de separação fluido-sólido na produção de bioinseticidas a partir de *Bacillus sphaericus* e *Bacillus thuringiensis* var. *israelensis*. 2004. 202 f. Doctorate (Programa de Engenharia Química) - Instituto Alberto Luiz Coimbra de Pós-Graduação e Pesquisa de Engenharia (Coppe), Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2004.

MOUNSEF JR, SALAMEH D, AWAD MK, CHAMY L, BRANDAM C, LTEIF R, A simple method for the separation of *Bacillus thuringiensis* spores and crystals. *Journal of Microbiological Methods*, v. 107, p. 147-149, 2014. DOI: 10.1016/j.mimet.2014.10.003.

NUNES ML, CARLINI CR, MARINOWIC D, KALIL NETO F, FIORI HH, SCOTTA MC, ZANELLA PLÁ, SODER RB, COSTA JCD, Microcephaly and Zika virus: a clinical and epidemiological analysis of the current outbreak in Brazil. *Jornal de Pediatria*, v. 92, n. 3, p. 230-240, 2016. DOI: 10.1016/j.jped.2016.02.009.