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Development of a low-cost medium for the culture of *Bacillus thuringiensis* subsp. *israelensis*

Desenvolvimento de um meio de cultura de baixo custo para *Bacillus thuringiensis* subsp. *israelensis*

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Abstract

Bacillus thuringiensis is a Gram-positive spore-forming bacterium that produces a parasporal crystal toxic for many insects. The aim of this study was the development of a low-cost medium for the culture of *B. thuringiensis* subsp. *israelensis* HD 537 to be used in a local insect control program in Brazil. In this study, the evaluated parameters were bacterial growth and toxicity of the cultures against *Aedes aegypti* larvae. The first strategy analised was the effect of salts, glucose, and yeast hydrolizate concentrations in a modified Arcas medium. The maximum toxicity in the bioassays (LC_{50} 2.31 mg.l-1) were obtained by using a medium showing the highest tested concentrations of nitrogen sources and salts (8.00 e 0.47 $g.l^{-1}$, respectively). In the second strategy was used a medium based in a ground *Bombyx mori* pupae. The maximum toxicity (LC_{50} 3.64 $mg.l^{-1}$) was obtained with Bm 100 medium, with 100 $g.l^{-1}$ of ground *B. mori* pupae. Beyond of the significant media differences in LC_{50} values, Bm100 medium was 120 times less expensive than the best Arcas modified medium. Thus, these results confirm the viability of ground *B. mori* pupae for *B. thuringiensis* subsp. *israelensis* growth and crystal production.
Keywords: *Bacillus thuringiensis israelensis*. Bioinsecticide. Insect control. *Bombyx mori*. *Aedes aegypti.*

Resumo

Bacillus thuringiensis é uma bactéria Gram-positiva esporulante que produz um cristal tóxico para muitos insetos. O objetivo deste estudo foi desenvolver um meio de baixo custo para cultivo de *B. thuringiensis* subsp. *israelensis* HD 537, a fim de que este seja utilizado em um programa local de controle de insetos no Brasil. Nesse estudo, os parâmetros avaliados foram crescimento bacteriano e toxicidade das culturas contra larvas de *Aedes aegypti.* Na primeira estratégia analisou-se o efeito das concentrações de sais, glicose e hidrolisado de levedura de um meio Arcas modificado. A toxicidade

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máxima do bioensaio (CL_{50} 2.31 mg. l^{-1}) foi obtida utilizando-se o meio que apresentava as maiores concentrações das fontes de nitrogênio e sais testadas (8.00 e 0.47 $g.l^{-1}$, respectivamente). Em uma segunda estratégia, foi utilizado um meio a base de farinha de pupa de *Bombyx mori*. A toxicidade máxima (CL_{50} 3.64 $mg.l^{-1}$) foi obtida com o meio Bm 100, o qual continha 100 $g.l^{-1}$ de farinha de pupa de *B. mori.* Além das diferenças significativas nos valores de CL_{50}, o meio BM 100 foi 120 vezes mais barato do que o melhor meio Arcas modificado, confirmando a viabilidade da farinha de pupa de *B. mori* para crescimento e produção de cristais de *B. thuringiensis* subsp. *israelensis*.
Palavras-chave: *Bacillus thuringiensis israelensis*. Bioinseticida. Controle de insetos. *Bombyx mori. Aedes aegypti.*

Introduction

Bacillus thuringiensis is an endospore-forming bacterium that produces a crystalline, parasporal body composed of insecticidal crystal proteins known as Cry proteins (CRICKMORE et al., 1998). These crystalline inclusions show varying degrees of specificity toward different insect orders, such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, and Mallophaga (ANGELO et al., 2010).

Due to its extremely specific mode of action and remarkable safety, the use of *B. thuringiensis* products on agricultural and forest crops dates back nearly 40 years (SCHNEPF et al., 1998; VILAS-BÔAS et al., 2007). In addition to an increasing use in agriculture, the discovery of *B. thuringiensi*s subsp. *israelensis* in 1977 resulted in large-scale programs for the control of mosquitoes as *Culex* and *Anopheles*, and black flies (*Simullium* spp.) in several countries, such as Germany, China and West Africa (WORLD HEALTH ORGANIZATION, 2005).

In Brazil, the effective control of insect vectors of diseases such as dengue, malaria, and yellow fever has often been done with synthetic insecticides. However, such products have led to vectors physiological resistance, environmental pollution, and deleterious effects on beneficial insects. As a consequence, there has been interest in *B. thuringiensis* products in recent years in Brazil (FORATTINI et al., 1995; SERUFO et al., 1993).

Research groups have been involved in developing *B. thuringiensis* based products, and at least two companies are currently conducting preliminary tests, Probiom Tecnologia (Campinas, São Paulo) and Bioticom (Recife, Pernambuco). However, only Bthek Biotecnologia Ltda. (Brasília) produces and commercializes a registered formulation (Bt-horus SC®) based on a Brazilian strain of *B. thuringiensis* subsp. *israelensis* to control the dengue and yellow fever vector (*Aedes aegypti*) and black flies (*Simulium* spp.). All other products available in Brazil are imported, at higher prices than the chemical insecticides registered for these pests. This has called for a growing need to develop *B. thuringiensis* subsp. *israelensis* based products to be used in local insect control programs, as previously suggested (ALVES et al., 1997; COUCH; ROSS, 2004; MORRIS; KANAGARATNAM; CONVERSE, 1997; POOPATHI; KUMAR, 2003; PRABAKARAN; BALARAMAN, 2006; VORA; SHETHNA, 1999).

The Universidade Estadual de Londrina, located in the city of Londrina, State of Paraná, Brazil, produces a bioinsecticide based on *B. thuringiensis* subsp. *israelensis*, using a modified Arcas medium because it is straightforward to prepare and has a cost that is consistent with the available work conditions. All the produced bioinsecticide is used for the control of larvae of *Culex spp*. in local

artificial lakes of industrial wastewater and sewage plants. However, in the past few years, the increase in the demand for this product has required the need for optimizing its potency.

This optimization will allow for a reduction in the volume of the product being released in each artificial lake, as well as facilitate production conditions and the later development of formulations. Thus, the present study shows two strategies for the development of a more effective medium for B. thuringiensis production. The first strategy based on medium Arcas, described in the literature (ARCAS; YANTORO; ERTOLA, 1987), and the second strategy based in the use of a complex regional agro-industrial residue, aiming a large-scale production of this bacterium in a cost-effective process.

Materials and Methods

1 Strain and culture media

B. thuringiensis subsp. *israelensis* HD 537 was kindly provided by the Collaboration Centre for Entomopathogenic *Bacillus,* Institut Pasteur, Paris, France. The medium used as standard culture medium was based in Arcas medium (Arcas et al., 1987) and contains ($g.l^{-1}$ of distilled water): 4.00 yeast hydrolysate, 4.50 glucose, 0.10 $MgSO_4.7H_2O$, 0.10 KH_2PO_4, 0.10 K_2HPO_4, 0.03 $MnSO_4.4H_2O$, 0.04 $CaCl_2.2H_2O$, and 0.10 $(NH_4)_2SO_4$. For improvement of this standard medium, some modifications were included and different concentrations were tested (Table 1). All experiments were performed in triplicate.

Table 1 - Culture media compositions used in this work.

Culture Medium	Yeast hydrolisate ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Total of salts ($g.l^{-1}$)
01 (standard medium)	4.00	4.50	0.47
02	4.00	4.50	4.70
03	4.00	9.00	4.70
04	2.00	4.50	4.70
05	8.00	4.50	4.70

Source: authors

A second composition medium was tested, based in ground *B. mori* pupae, these mediums were named Bm50 and Bm100. To produce the medium Bm50; 50 g ground of *B. mori* pupae was added in 100 ml of distilled water, the mixture was boiled and then filtered. In the resulting filtrate, was added ($g.l^{-1}$) KH_2PO_4 0.1, K_2HPO_4 0.1 and glucose 4.5. The medium Bm100 was done in a similar manner to Bm50, however, for the production of the filtrate was used 100 g ground of *B. mori* pupae. The nutritional composition of ground *B. mori* pupae used in both media was determined by LABTEC Laboratory (Campinas, Brazil) and is presented in Table 2. The crude protein was determined by the Kjeldahl method (AOAC, 1997): the lipids by gravimetry (ASSOCIATION OF OFFICIAL

ANALYTICAL CHEMISTS, 1997); amino acids by high-performance liquid chromatography (HPLC) (HAGEN et al., 1989; WHITE et al., 1986), and mineral compounds were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, 2000).

Table 2 - Nutritional composition of ground *B. mori* pupae used for the growth of *B. thuringiensis* subsp. *israelensis* HD 537.

Components	Concentration (%)
Raw Protein	52.66
Ethereal extract	27.17
Alanine	2.82
Arginine	2.73
Aspartic Acid	5.88
Glycine	2.35
Isoleucine	2.18
Leucine	3.75
Glutamic Acid	6.76
Lysine	3.47
Cysteine	0.62
Methionine	1.69
Phenylalanine	2.67
Tyrosine	2.91
Threonine	2.62
Tryptophan	0.67
Proline	2.66
Valine	2.84
Histidine	1.51
Serine	2.65
Calcium (Ca)	0.11
Total Phosphorus (P)	0.69
Potassium (K)	--
Magnesium (Mg)	--
Manganese (Mn)	--
Zinc (Zn)	--
Iron (Fe)	--
Copper (Cu)	--
Cobalt (Co)	--

Source: authors

For all media, the pH was adjusted to 7.2 before sterilization in autoclave at 120 ºC for 20 min. Glucose was sterilized separately and added to the medium at the time of use. Bacterial culture were tested in 500 ml Erlenmeyer flasks containing 50 ml of medium. Precultures were prepared by transferring cells from stock cultures into flasks containing 10 ml of the same medium, and were kept overnight at 30 ºC and 120 rpm on a rotary shaker. Five percent (v/v) of the inoculum was added to the cultures, and these were incubated for 64 h at 30 ºC and 120 rpm on a rotary shaker, until sporulation. Three independent experiments with four replicates each were performed on different days.

2 Analytical methods

All cultures were analyzed for biomass production and amount of viable cells, and bioassayed against *A. aegypti* larvae. Biomass was determined by using the dry weight method, where cells from all sporulated cultures were centrifuged at 10,000 × g for 10 minutes, washed three times with distilled water and dried overnight at 100 ºC.

Viable cells were evaluated by colony count using the Colony Forming Units (CFU) method. Serial dilutions were prepared and 1 ml-aliquots of the appropriate dilutions were spread, in triplicate, on plates with Luria Bertani (LB) agar.

Toxicity assays were performed using third instar *A. aegypti* larvae following the protocol provided by the World Health Organization (WORLD HEALTH ORGANIZATION, 2005). Twenty five larvae used for each assay were placed in containers with 100 ml of distilled water. Each assay was conducted in triplicate, with five assays for each culture medium. Mortality was recorded 24 h after treatment and the lethal concentration inducing 50% larval mortality (LC_{50}) was determined in terms of $mg.l^{-1}$ and calculated by the Probit regression analysis (FINNEY, 1971), using the Polo-PC computer program (RUSSEL et al., 1997).

Average values obtained for LC_{50} and for biomass of each culture were compared by a Student's t test at 0.001 probability level.

Results and Discussion

The purpose of the present study was the development of a low-cost medium for the culture of *B. thuringiensis* subsp. *israelensis* HD 537 to be used to control of *Culex* spp. larvae. Two strategies were used: i) improve production in the Arcas modified medium, which is currently used to cultivate this species; and ii) development of a new culture medium based on ground *B. mori* pupae for cost-effectiveness. In both strategies, bioassays against *A. aegypti* larvae were conducted because this species is reference in the protocol provided by the World Health Organization (WORLD HEALTH ORGANIZATION, 2005).

The ten-fold increase in salt concentration of medium 02 in relation to medium 01 (Table 1), positively influenced bacterial growth and crystal biosynthesis, resulting in 2.16 fold increase in biomass and 1.98 fold reduction in LC_{50} ($p=0.001$ Student's t tests, table 3). These results support Içgen et al. (2002a) and Özkan et al. (2003) concerning the importance of salts in several metabolic pathways, including Cry protein biosynthesis.

No statistically significant difference was observed in LC_{50} values against *A. aegypti* larvae obtained from the two tested glucose concentrations between medium 02 ($4.5\ g.l^{-1}$) and medium 03 ($9.0\ g.l^{-1}$) ($p> 0.001$). However, reduction in concentration of yeast hydrolysate from $4.00\ g.l^{-1}$ (medium 02) to $2.00\ g.l^{-1}$ (medium 04) led to less biomass. This reduction was also observed in crystal biosynthesis, as shown by the toxicity assays against *A. aegypti* larvae, which the obtained LC_{50} values with medium 04 were statistically higher than with medium 02 ($p= 0.001$) (Table 3).

In addition, the increase in yeast hydrolysate concentration from 4.00 g.l-1 (medium 02) to 8.00 g.l-1 (medium 05) enhanced entomopathogenic activity, as shown by the toxicity assays against A. aegypti larvae. In this case, LC_{50} values obtained with medium 05 were significantly lower than with medium 02. Results from medium 05 are consistent with previous studies indicating the importance of the nitrogen source in improving the synthesis of entomopathogenic crystals (IÇGEN et al., 2002b; KHUZHAMSHUKUROV et al., 2001; ÖZKAN et al., 2003; ZOUARI; JAOUA 1999).

Table 3 - Effects of medium composition on the growth of *B. thuringiensis* var. *israelensis* HD 537 and on the entomopathogenic activity against *A. aegypti* larvae, as monitored by bioassays.

Culture Medium[1]	Biomass ($g.l^{-1}$)[2]	LC_{50} ($mg.l^{-1}$)[2,3,4]
01 (reference medium)	0.98 (+/-0.06)[a]	5,99 (5,67 a 6,34)[a]
02	2.12 (+/- 0.02)[b]	3,01 (2,10 a 3,86)[b]
03	2.72 (+/- 0.04)[c]	3,60 (3,19 a 3,77)[b]
04	1.65 (+/- 0.05)[d]	4,21 (3,66 a 4,42)[c]
05	2.31 (+/- 0.02)[e]	2,21 (1,99 a 2,26)[d]

Source: authors

[1] The medium composition is described in Table 1.
[2] Values with same letters in the column
are not statistically different.
[3] The 50% lethal concentration (LC_{50}) was determined in $mg.l^{-1}$.
[4] Range of LC_{50} values were calculated by Probit program.

Thus, a ten-fold increase in salt concentration and two-fold increase in yeast hydrolysate concentration as compared to the reference modified Arcas medium resulted in 2.7 fold higher toxicity and 2.40 fold greater biomass production.

In the second strategy the assays were based in the use of ground *B. mori* pupae for the growth of *B. thuringiensis* subsp. *israelensis* HD 537. Sixty-four hours were required for sporulation of the cultures in media Bm50 and Bm100 under the tested conditions. This was the same time span recorded for reference Arcas modified medium used as standard medium for comparisons (medium 01, Table 1). The number of CFUs obtained from the three media (Bm50, Bm100 and reference Arcas modified - medium 01) did not differ significantly, varying between 1.47 × 108 and 2.49 × 108 $CFU.ml^{-1}$ (Table 4). However, the amount of biomass obtained using medium Bm100 was 1.93 fold higher than in Bm50 medium and 1.45 fold higher than in the reference medium.

Bioassays against *A. aegypti* larvae conducted with Bm100 medium resulted in LC_{50} values

(3.64 $mg.l^{-1}$) significantly lower than bioassays conducted with Bm50 medium and Arcas modified medium (LC_{50} = 8.32 and 5.99, respectively; p= 0.001). Therefore, according to our studies, Bm100 medium had the best performance for the culture of *B. thuringiensis* subsp. *israelensis* HD 537, with higher production of biomass and the best results of LC_{50} in the bioassays. The nutritional analysis of ground *B. mori* pupae revealed that nearly 50% of the dry weight contains raw protein, including 18 of the 20 protein aminoacids (Table 2). Given that the concentration of nitrogen is crucial for regulating toxin biosynthesis of *B. thuringiensis* (IÇGEN et al., 2002b), the large amount of proteins in Bm100 medium provided nitrogen in sufficient concentration to ensure high yields of *B. thuringiensis* subsp. *israelensis* HD 537.

Table 4 - Growth and entomopathogenic activity against *A. aegypti* larvae of *B. thuringiensis* subsp. *israelensis* grown on different culture media.

Measured factors	Culture Medium		
	Reference Arcas	Bm50	Bm100
Biomass ($g.l^{-1}$)	0.98 (+/-0.06)	0.74 (+/- 0.07)	1.43 (+/- 0.06)
CFU (ml)[1]	1.92×10^8	1.95×10^8	1.47×10^8
LC_{50} ($mg.l^{-1}$)[2]	5.99 (5,67 a 6,34)[a]	8.32 (7,36 a 9,84)[b]	3.64 (3,33 a 3,95)[c]

Source: authors
[1] Colony Forming Units. [2] Range of LC_{50} Values with same letters in the line are not statistically different.

In addition, the nutritional composition of Bm100 medium shows that ground *B. mori* pupae contains important ions such as calcium, phosphorus, potassium, magnesium and in lower quantities, also iron, copper and cobalt (Table 2). Calcium and magnesium are important for secondary metabolism. The use of 100 $g.l^{-1}$ of ground *B. mori* pupae provides 0.11 $g.l^{-1}$ and 0,33 $g.l^{-1}$ of calcium and magnesium, respectively, amounts similar to those used in other studies with different culture media (IÇGEN et al., 2002a; ÖZKAN et al., 2003) and in Arcas modified medium 01, used as standard in our study. The lack of appropriate sources of carbon in ground *B. mori* pupae was provide by addition of 4.50 $g.l^{-1}$ glucose to the medium.

One liter of culture medium Bm100 costs US$ 0.025 as compared to US$ 3.00 for Arcas modified medium 05. In spite of the significant differences in LC_{50} values between both media (3.64 and 2.3 $mg.l^{-1}$ for Bm100 and Arcas modified medium 05, respectively), Bm100 was 120 times less expensive that the medium Arcas modified medium 5.

Conclusion

Thus, ground *B. mori* pupae is a promising product to growth and crystal production of *B.*

thuringiensis subsp. *israelensis*, but additional studies are needed on the optimization of fermentation conditions for Bm100 medium, using larger fermenters, and also scaling-up studies. Further research results may confirm the viability of ground *B. mori* pupae for large-scale industrial production of *B. thuringiensis* subsp. *israelensis*.

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