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Selection of *Bacillus thuringiensis* Berliner strains to control *Aedes aegypti* Linnaeus

André Luiz de Almeida Melo[1,*], André Luís Lopes da Silva[2], Magda Clara Vieira da Costa-Ribeiro[3], Mitiyo Fukuda Miyaoka[2], Vanete Thomas Soccol[2], Carlos Ricardo Soccol[2]

ABSTRACT

The present study aimed to select strains of Bacillus thuringiensis with insecticidal activity against Aedes aegypti. It was tested sixteen strains of bacteria, isolated from Paraná state, Brazil, that were used in laboratory assays with mosquito larvae. Tests were carried out in two stages, first one to select the most efficient strains (screening) and second to estimate LC_{50}. The protein profile of the highest toxicity of strain was obtained by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The best performance of larval mortality was produced by BR-01 strain, which 96.7% mortality rate, significantly higher than others. In the second part, there was obtained a LC_{50} of 9.07 $\mu L.L^{-1}$ fermented extract. The protein profile revealed many peptides between 15 and 140kDa, similar to that reported in Bacillus thuringiensis ser. israelensis strains which high toxicity to mosquito species.

Keywords: *Vector control, bioassay, endotoxin, SDS-PAGE.*

Seleção de cepas de *Bacillus thuringiensis* Berliner para o controle de *Aedes aegypti* Linnaeus

RESUMO

O presente trabalho buscou selecionar cepas de *Bacillus thuringiensis* com atividade inseticida contra *Aedes aegypti*. Foram testadas 16 estirpes da bactéria provenientes do estado do Paraná, Brasil, que foram utilizadas em ensaios laboratoriais com larvas do culicídeos. Os testes foram realizados em duas etapas, a primeira com a seleção de cepas e a segunda para estimar a CL_{50} das cepas com melhores resultados. O perfil protéico do isolado de maior toxicidade foi obtido por SDS-PAGE (dodecil sulfato de sódio – eletroforese em gel de poliacrilamida). O melhor resultado de mortalidade foi produzido com a cepa BR-01, que produziu 96,7% de mortalidade de *A. aegypti*, valor significativamente superior aos demais. Na segunda fase, obteve-se CL_{50} de 9,07 $\mu L.L^{-1}$ de extrato fermentado. O perfil protéico revelou peptídeos entre 15 e 140kDa, resultado semelhante ao relatado nas cepas de *Bacillus thuringiensis* var. *israelensis* de maior toxicidade às espécies de culicídeos.

Palavras-chave: Controle de vetores, bioensaio, endotoxina, SDS-PAGE.

*Author for correspondence.
[1]*Doctoral Student in Bioprocess Engineering and Biotechnology, Bioprocess Engineering and Biotechnology Division, Federal University of Parana, Curitiba – PR, Brazil, *andremelo101@gmail.com*
[2]*Bioprocess Engineering and Biotechnology Division, Federal University of Parana, Curitiba – PR, Brazil, clonageinvitro@yahoo.com.br; mitiyo_miyaoka@hotmail.com; vanetesoccol@gmail.com; soccol@ufpr.br*
[3]*Basic Pathology Department, Federal University of Parana, Curitiba – PR, Brazil, magdacostaribeiro@gmail.com*

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INTRODUCTION

The *Aedes aegypti* Linnaeus is an insect extremely anthropophilic and is one of the main species of medical interest, related to the occurrence of several pathogens worldwide. Endemic in tropical and subtropical areas in the four continents, the mosquito is widely adapted to urban environments, invading cities and human settlements. The insect is the major responsible to transmit dengue, urban yellow fever and other arboviroses. Its occurrence is usually related to the periods of summer rains until early drought during the winter, period that the diseases occurrence is increased (Barreto and Teixeira, 2008). In many countries, the presence of *Aedes aegypti* is constantly monitored as a control measure, mainly where dengue has high endemicity.

From the understanding of the role of vector-borne insects Culicidae, strategies for combating diseases began to focus on vector control. The development of synthetic insecticides in the decade of 30, organophosphates and organochlorines, gave new impetus to combat mosquito populations. Thus, successful campaigns to dengue and yellow fever combat in the decade of 50 eliminated the presence of *A. aegypti* in many parts of the globe (Consoli and Lourenço-de-Oliveira, 1994). However, after few decades the indiscriminate use of these substances has proved extremely harmful to humans and the environment.

The insect resistance to chemical pesticides had been noticed, indicating that the combat strategies need to change. In this context, the research for alternative and less harmful pest control substances had started. The endotoxin produced by bacteria had shown a great potential, with efficiency and environmental safety. Produced with specificity to the target organism, the bio-insecticides are not accumulate in the food chain, and harmless to humans and other animals (Rabinovitch et al., 1998).

Bacillus thuringiensis (Bt) is a gram-positive spore-forming bacteria with aerobic growth, known as the main microorganisms used in the formulation of biological insecticides (Federici et al., 2010). The action mechanism is associated with bacterial endotoxin, that is activated in the larvae gut, and then disintegrates forming Cry proteins. Molecular interactions between these peptides and epithelial receptors trigger a cytopathic response, leading to larval death (Bravo et al., 2007). Frequently found in soil samples, the strains of *B. thuringiensis* are isolated by simple techniques with selective media and morphological analysis. Meanwhile, the toxicity can vary significantly according to polymorphism, increasing or decreasing the efficiency and specificity to the target organism. Thus, biological assays are essential in determining the strain of *B. thuringiensis* suitable for the formulation of biological insecticides

This study aimed to evaluate the toxicity of *B. thuringiensis* strains forward to the 3rd instar larvae of *Aedes aegypti* using laboratory bioassays. The LC_{50} was estimated to the best strain; and its protein profiles determinate by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

MATERIAL AND METHODS

Bioinsecticide production

Sixteen strains of *Bacillus thuringiensis* isolated from Paraná State, Brazil, and stored in Biotechnological and Bioprocess Laboratory (LPB-1) of the Federal University of Paraná (UFPR) were used to produce the bioinsecticide. The reactivation of the bacteria was carried out with the inoculation of 2.0 to 5.0 x 10^5CFU.mL^{-1} into 50 mL Luria Bertani medium (Sambrook et al., 1989) in Erlenmeyer flasks, previously sterilized. The fermentation was carried in rotatory shaker at 120rpm at 30º C for 72h, and stored in refrigerator at 4º C until use.

Mosquitoes

The strain Rockfeller of *Aedes aegypti* maintained in the Molecular Parasitology Laboratory at the Federal University of Paraná, Brazil was used to bioassays. In order to obtain the larvae of mosquitoes, the adults were kept in cages (25cm x 25cm) covered with mesh and maintained in a chamber at 28ºC (Melo et al., 2011). The adults were fed with an apple slice approximately 2cm^2 thick and 4cm long, replaced weekly. The blood meal was placed weekly, using newborn mice. The oviposition was done on filter papers inserted inside recipients with water (6cm x 6cm). The larvae were maintained in plastic trays (10cm x 25cm) with approximately 400mL of dechlorinated water, changed every three days. They were fed with fish food (Alcon Pet®, Goldfish) *ad libitum*.

Bioassays

The bioassays were performed in the same laboratory, based on the model recommended by WHO for experiments with the bacteria (WHO, 2005). The biological tests were carried out in two steps: first for screening (to select the best strains) and second for estimating toxicity values (LC_{50}). In the first part, we used 100µL bacterial culture added to a flask with 20 third instar larvae in 100 mL dechlorinated water. The tests were carried out in triplicate and three containers not received the product (negative control). After 24 hours, the counting of the number of living larvae was made and to estimate the mortality rate (%). If the negative control mortality was less than 20%, the tests were canceled and redone. The mortality values were corrected using Abbot's formula (1925).

Abbott's formula

$$\text{Larvae Mortality rate (\%)} = 100.(X - Y)/X$$

x = rate of living larvae in negative control; y = rate of living larvae in treatment.

In the second part, the strain that had more significant mortality (above 50%) was applied at five different dilutions (1, 5, 10, 15 and 25 $\mu L.L^{-1}$ fermented extract) under the same conditions of the previous experiment. The results were used to estimate of LC_{50} against *A. aegypti*.

Statistical analysis

All experiments were performed in triplicate. Mortality rate values from the first experiment (screening) were transformed to arcsine $\sqrt{x/100}$ and submitted to analysis of variance (ANOVA) and the means were compared by the Scott-Knott test, both at $P<0.05$. The estimation of LC_{50} and the confidence intervals (95%) were calculated by the Trimmed Spearman-Karber method, using the software TSK, version 1.5 (USEPA, 1990).

SDS-PAGE

The protein profile of the strain with highest mortality rate was determinate. The biomass had suffered the reaction of sodium dodecyl sulfate and it was analyzed by gel electrophoresis in 15% polyacrylamide (Laemmli, 1970). After fractionation, the gel was stained for 10 minutes in coomassie blue solution and then placed in a solution of 7% acetic acid for removal of excess dye, until the protein fractions is sharp present. As reference molecular weight, we used the precision pre-stained molecular weight markers (BioRad).

RESULTS AND DISCUSSION

In the first experiment (screening), it was observed diversified results varying from 0 to 96.7% mortality among the strains of *B. thuringiensis* (Table 1). Eleven strains were innocuous, does not produce any mortality of larvae. Four were partially effective, with reduced toxicities, ranging from 16.7 to 48.3%. The best performance was produced by strain BR-01, which 96.7% mortality rate of *A. aegypti* larvae. This result was significantly higher than other strains, qualifying the strain for subsequent tests.

Table 1. Mortality of larvae of *Aedes aegypti* produced by different strains of *Bacillus thuringiensis* isolated from the state of Paraná, Brazil, at 1000 $\mu L.L^{-1}$.

Strain	Mortality (%)
Negative control	0 d[1]
BR-01	96.7 a
BR-02	21.7 c
BR-03	16.7 c
BR-04	0 d
BR-07	0 d
BR-08	0 d
BR-09	31.7 b
BR-10	0 d
BR-11	0 d
BR-12	0 d
BR-13	48.3 b
BR-14	0 d
BR-15	0 d
BR-16	0 d
BR-17	0 d
BR-18	0 d

[1] Treatments followed by the same letter do not differ from each other by the Scott-Knott test at $P<0.05$

Mosquito known as Bt susceptible specie, *A. aegypti* was not vulnerable to most isolates of the bacterial strains tested. In spite of contradictory, this result confirms the particular characteristics of *B. thuringiensis*. Although the bacterium is used to control a wide range of invertebrates, the endotoxin of *B. thuringiensis* shows specificity of the target organism. Therefore, strain which has affinity for a particular order, it does not have the same effect on another (Bravo et al. 2007). Knowing the wide variety of organisms potentially targets to Bt, the finding of native high toxicity *Aedes aegypti* can be considered a satisfactory result.

The occurrence of non-toxic strains has been studied lately. According to Benintende et al. (1999), strains of Bt without biological action are more widely distributed than species that produce a toxic effect. Studying four non pathogenic strains of Bt, Roh et al. (2008) observed the presence of protein crystals similar to those found in pathogenic strains, however, with a different shape. Thus, the absence of toxic activity was not due to non-production of the Cry proteins, but not its affinity for the insect species tested. In this study, isolates with low or innocuous activity on *A. aegypti* larvae may show activity to other invertebrate species. But only *in vivo* or *in vitro* tests could confirm this hypothesis.

In the second part, the mortality rate was variable according to the product concentration, ranging from 5 to 95% (Table 2). The LC_{50} for strain BR-01 was 9.07 $\mu L.L^{-1}$ fermented extract, ranging from 6.85 to 12 $\mu L.L^{-1}$ fermented extract (Table 2). The results were satisfactory for small scale fermentations, employing a not optimized culture medium. Knowing the potential of BR-01, fermentations in bioreactor and the use of local raw materials with low cost can surely enhance the performance of mortality of larvae.

Table 2. Lethal concentration (LC_{50}) of fermented extract of *Bacillus thuringiensis* strain BR-01 against *Aedes aegypti* after 24 hours. LC_{50} was estimated using trimmed Spearman-Karber.

Concentration ($\mu L.L^{-1}$)	Mortality (%)	LC_{50} Values ($\mu L.L^{-1}$)	Limits 95% confidence ($\mu L.L^{-1}$)	TSK Trim value (%)
1	5	9.07	6.85-12.0	5.0
5	15			
10	50			
15	75			
25	95			

The analysis of the BR-01 protein profile by SDS-PAGE revealed a large amount of peptides, ranging from 15 to 140kDa as molecular weights (Figure 1). This result was consistent with the spectrum of the greatest toxicity strain, such *Bacillus thuringiensis* var. *israelensis* (Bti). Described as suitable for vector control, strain HD-73 produces Cry and Cyt proteins with high larvicidal action with 130, 70 and 27 kDa (Bravo et al. 2011, Guechicoff et al. 2001). Likewise, the isolated BR-01 showed a profile that contained peptides with molecular weights similar to strains of Bti.

However, the presence of proteins similar to those found in strains of greater toxicity is no guarantee of biological activity. Benintende et al. (1999) studied Bt strains isolated in Argentina and verified by SDS-PAGE the existence of 130, 60 and 40kDa proteins. But in biological assays with Lepidoptera, Coleoptera and Diptera, these strains did not show larvicidal activity, being harmless to organisms. Thus, the arrangement of protein profile analyzes and biological assays results can give a valuable data for understanding of biological control bacteria and their operating mechanisms.

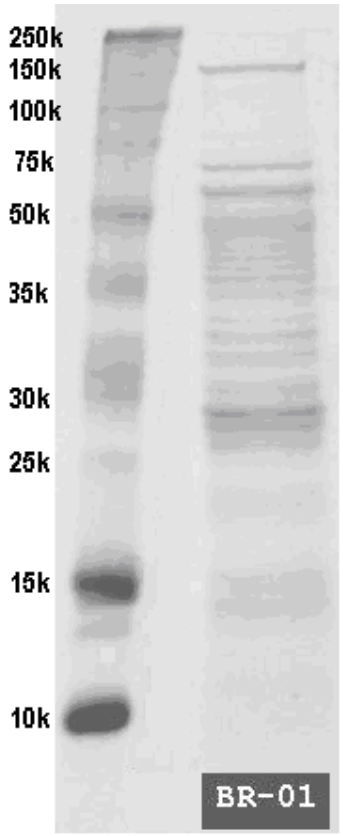

Figure 1: Profile of proteins from *Bacillus thuringiensis* strain BR-01 by SDS-PAGE on 1.5% polyacrylamide gel.

CONCLUSIONS

The BR-01 strain showed the highest toxicity against *A. aegypti* larvae, producing LC_{50} of 9.07 $\mu L.L^{-1}$ fermented extract in a confidence interval from 6.85 to 12 $\mu L.L^{-1}$ fermented extract.

The protein profile has peptides between 15 and 140kDa, similar to that reported with the greatest toxicity Bti strains.

REFERENCES

ABBOTT WS. A method of computing the effectiveness of an insecticide. **Journal of Economic Entomology**, v.18, n. 2, p.265-267, 1925

BARRETO ML, TEIXEIRA MG. Dengue no Brasil: situação epidemiológica e contribuições para uma agenda de pesquisa. **Revista Estudos Avançados**, v. 22, n. 64, p. 53-72, 2008.

BENINTENDE G, LÓPEZ-MEZA J, COZZI J, IBARRA J. Novel nontoxic isolates of *Bacillus thuringiensis*. **Letters in Applied Microbiology**, v. 29, n. 3, p. 151-5, 1999.

BRAVO A, GILL SS, SOBERON M. Mode of action of *Bacillus thuringiensis* toxins and their potential for insect control. **Toxicon**, v. 49, n. 4, p. 423-435, 2007.

BRAVO A, LIKITVIVATANAVONG S, GILL S & SOBERÓN M. *Bacillus thuringiensis*: a story of a successful bioinsecticide. **Insect Biochemistry and Molecular Biology**, v. 41, n. 7, p. 423-431, 2011.

CONSOLI RAGB, LOURENÇO-DE-OLIVEIRA R. **Principais mosquitos de importância sanitária no Brasil.** Editora Fiocruz, Rio de Janeiro, RJ, p. 228, 1994.

FEDERICI BA, PARK HW, BIDESHI DK. Overview of the Basic Biology of *Bacillus thuringiensis* with Emphasis on Genetic Engineering of Bacterial Larvicides for Mosquito Control. **Open Toxinology Journal**, v.3, p. 83-100, 2010.

LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, London. v. 227, n. 5259, p. 680-685, 1970.

GUERCHICOFF A, DELECLUSE A, RUBINSTEIN C P. The *Bacillus thuringiensis* cyt genes for hemolytic endotoxins constitute a gene family. **Applied and Environmental Microbiology**, v. 67, n. 3, p. 1090–1096, 2001.

MELO ALA, SANCHUKI CE, WOICIECHOWSKI AL, THOMAZ-SOCCOL V, SOCCOL CR. Utilização da cama de frango em meio de cultivo de *Bacillus thuringiensis* var. *israelensis* Berliner para o controle de *Aedes aegypti* Linnaeus. **Journal of Biotechnology and Biodiversity**, v. 2, n. 4, p. 1-6, 2011.

RABINOVITCH L, CAVADOS CFC, LIMA MM. Dos *Bacillus* entomopatogênicos, o que se espera? **Biotecnologia, Ciência e Desenvolvimento**, v. 6, n. 6, p. 40-41, 1998.

ROH JY, PARK HW, JIN BR, KIM HS, YU YM, KANG SK. Characterization of novel non-toxic Bacillus thuringiensis isolated from Korea.

Letters in Applied Microbiology, v. 23, n. 4, p. 249-252, 1996.

SAMBROOK J, FRITSCH EF, MANIATIS T. **Molecular Cloning: A Laboratory Manual**, 2nd ed., Cold Spring Harbor Laboratory Press. New York, p. 1626, 1989.

USEPA. Trimmed spearman-karber (TSK) program version 1.5 ecological monitoring research division. **Environmental Monitoring Systems Laboratory**, USEPA, Cincinnati, OH, 1990.

WHO. **Guidelines for laboratory and field testing of mosquito larvicides.** (Document WHO/CDS/WHOPES/GCDPP/13, Geneva, Switzerland, World Health Organization), 2005.

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