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Acta Tropica 105 (2008) 35–38
ACTA TROPICA
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Coconut water as a cheap source for the production of δ endotoxin of *Bacillus thuringiensis* var. *israelensis*, a mosquito control agent

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Received 5 March 2007; received in revised form 29 August 2007; accepted 14 September 2007
Available online 20 September 2007

Abstract

Bacillus thuringiensis var. *israelensis* (*B. t. i.*) is being widely used in mosquito control programs. However, the large-scale production of this bacillus is expensive due to the high cost of the production medium. In this study, we attempted to develop a cost-effective medium, based on a locally available raw material namely coconut water which is available in plenty as waste product from coconut oil industry. The yield of cell mass, sporulation and mosquito larvicidal activity were studied by growing this bacterium in this waste product and in comparison with the conventional medium (NYSM). Cell mass yield of 3.1 g/L, spore count of 3.4×10^{11} spores/mL and mosquito larvicidal activity (LC_{50}) of 14.85 ng/mL (against early fourth-instar larvae of *Aedes aegypti*) were obtained with a 30 h old culture of this bacterium grown in coconut water. This is almost similar to that obtained with NYSM medium. Hence, coconut water-based culture medium is economical for the production of *B. t. i.*

Keywords: *Bacillus thuringiensis*; *Culex quinquefasciatus*; Coconut water; Medium; Toxicity; Mosquito

1. Introduction

Global use of insecticides for mosquito control in recent decades has caused environmental pollution of aqueous ecosystems and has resulted in the development of insecticide resistance in many mosquito species. Biological means of mosquito control based on the entomopathogenic bacteria, *Bacillus thuringiensis* var. *israelensis* (Goldberg and Margalit, 1977) has been advocated for more than 20 years. These bacterial preparations are based on endotoxin proteins accumulated in parasporal crystals produced by the bacterial cells during the sporulation phase. Presently, *B. t. i.* is considered ideal for mosquito control because of its high specificity, lack of toxicity to humans (Sudarani and Balaraman, 1996) and to non-target aquatic organisms found in association with mosquito larvae (Balaraman et al., 1981). Furthermore, the major advantage of this biocide is that risk of development of resistance of mosquitoes to *B. t. i.* based products is very low, due to its multi-toxin complex (Wirth et al., 1998).

However, the high cost of the production medium of bacterial agents is a major factor to be considered when it comes to the large-scale use of biopesticdes. Hence, attempts are being made to arrive at cheaper raw materials for producing this bacterium. Several locally available waste materials such as corn steep liquor, coconut waste, rice bran and molasses have been used for the production of *B. t. i.* (Kumar et al., 2000; Saalma et al., 1983; Lee and Seleena, 1991; Desai and Shethna, 1991). Insecticidal toxins of this bacterium grown in media containing powders of leguminous seeds, supplemented with carbohydrates were found to be effective against *Aedes*, *Anopheles* and *Culex* species (Mummigatti and Raghunathan, 1990; Dregval et al., 1999; Luna et al., 2004; Poopathi and Kumar, 2003; Obeta and Okafor, 1984). In our earlier study, we used locally available raw materials such as soybean flour (*Glycine max*), groundnut cake powder (*Arachis hypogea*) and wheat bran extract (*Triticum aestivum*) for the large-scale production of *B. t. i.* (Prabakaran and Balaraman, 2006). However, these raw materials individually are still not wholesome and need to be supplemented with either protein and/or carbon and mineral source. Coconut water which contains all essential ingredients (Krishnamurthy et al., 1958) supports good growth of several micro-organisms. Therefore, the present study was undertaken to develop a culture medium

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0001-706X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2007.09.002

based on coconut water for the production of *B. t. i.* and the findings are presented in this paper.

2. Materials and methods

2.1. Organism and materials

B. t. i. (VCRC B-17) (Balaraman et al., 1981) obtained from the culture collection of Vector Control Research Centre, Puducherry, India was used in the present study. The strain was maintained on Nutrient Yeast Salt Medium (NYSM) agar slants (Myers and Yousten, 1980).

2.2. Media preparation

Coconut water broth was prepared by adding 1% of stock salt solution (stock salt solution: $MgCl_2$ 20.3 g; $CaCl_2$ 10.2 g; $MnCl_2$ 1.0 g) to 100 mL of coconut water. The pH of the medium was adjusted to 7.2. NYSM broth was used for comparison.

2.3. Growth conditions

First stage seed was prepared by inoculating 10 mL of NYSM broth with one loopful of cells from a slant culture and incubating on a rotary shaker at 30 °C, 180 rpm for a period of 6 h. The seed thus prepared was transferred to 100 mL medium in a 500 mL Erlenmeyer flask (12 flasks per medium) at 2% level (v/v) and the flasks were incubated on a rotary shaker at 30 °C and at 250 rpm for a period of 36 h. Two flasks each were removed at 6 h intervals and used for assessment of cell mass, sporulation and larvicidal activity.

2.4. Determination of cell mass, sporulation and mosquito larvicidal activity

The cultures were centrifuged at 15,000 × *g* for 20 min, supernatant discarded and the cell pellets were washed twice with sterile distilled water and lyophilized. Dry weight was calculated and expressed in g/L. For determining spore count, a small aliquot (1 mL) of the culture sample was heat treated at 80 °C for 15 min, serially diluted and plated on NYSM agar plates. Plates were incubated at 30 °C for 48 h and the developing *B. t. i.* colonies were counted and expressed in spores/mL of culture broth. In order to determine the mosquito larvicidal activity,

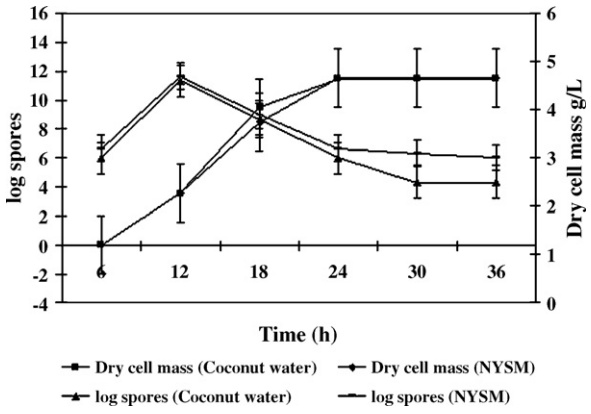

Fig. 1. Dry cell mass and spore count of *Bacillus thuringiensis* var. *israelensis* grown in coconut water and NYSM medium.

the lyophilized cells were assayed against third instar laboratory reared *Aedes aegypti* (strain Bora Bora) larvae. Suspensions of the lyophilized cells were prepared in sterile distilled water and added to 300 mL capacity disposable cups, containing 50 larvae and 250 mL of chlorine free tap water. The cells from each medium was tested at six concentrations in quadruplicate manner along with appropriate controls. Larval mortality was scored after 24 h and corrected for control mortality, using Abbott's formula (Abbott, 1925). The experiment was done three times on different days. The results are expressed in ng/mL. Probit regression analysis (Finney, 1971) was carried out to calculate LC_{50} and LC_{90} values as well as their 95% fiducial limits.

3. Results

The dry weight of the cell mass in coconut water and NYSM broths was 3.1 g/L and 2.5 g/L, respectively, and the maximum spore count obtained was 3.4×10^{11} spores/mL and 3.2×10^{11} spores/mL in the respective media (Fig. 1). The difference in the spore count between the two media was not significant (Student's *t*-test for independent samples, $t = 0.085$, d.f. = 34, $p = 0.93$). Maximum larvicidal activity (LC_{50}) obtained was 14.85 ng/mL and 16.32 ng/mL, respectively, after 30 h of growth (Tables 1 and 2). There was no significant difference between the LC_{50} values of the bacterium grown in the two media (due to overlap of 95% fiducial limits).

The conventional medium and coconut water media did not show any toxin production during the lag (0–6 h) or log phase

Table 1
LC_{50} and LC_{90} values of *Bacillus thuringiensis* var. *israelensis* grown in coconut water medium against early third instar *Aedes aegypti* larvae

Coefficients of probit regression equation[a] ($Y = a + b \log_{10} X$)				LC_{50} (ng/mL) (95% FL)	LC_{90} (ng/mL) (95% FL[b])
Hours	*a*	*b*	X^2		
6	–	–	–	–	–
12	−2.71	1.32	28.90	334.58 (303.68–368.63)	878.05 (740.45–1041.22)
18	−.914	1.46	22.79	57.70 (52.84–63.01)	138.79 (119.59–161.08)
24	0.984	1.47	23.71	15.39 (14.09–16.82)	36.80 (31.38–43.16)
30	1.19	1.41	25.06	14.85 (13.55–16.28)	36.81 (31.47–43.06)
36	1.53	1.27	23.65	15.03 (13.61–16.60)	40.86 (33.69–49.56)

[a] *Y*: probit mortality, $\log_{10} X$ = log dose in ng of spore crystal complex per mL of chlorine free tap water.
[b] FL: fiducial limits.