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Initial Study of Production of *Bacillus thuringiensis israelensis* Using Locally Obtained Substrates

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INTISARI

Soesanto – *Produksi percobaan* Bacillus thuringiensis israelensis *menggunakan bahan lokal terasi udang, melase dan tepung kedelai*

Bacillus thuringiensis israelensis diketahui sebagai entomopatogen nyamuk *Aedes aegypti* yang merupakan vektor penyakit demam berdarah.

Telah dilakukan percobaan produksi *Bacillus thuringiensis israelensis* dengan menggunakan medium yang mengandung bahan lokal terasi udang, melase dan tepung kedelai. Hasil uji toksisitas *(bioassay)* terhadap *Final Whole Culture (FWC)* dengan bahan lokal yang murah menunjukkan potensi sebagai agensia bioinsektisida.

Key Words: entomopathogen – *Bacillus thuringiensis* – bioinsecticide – *Aedes aegypti* – toxicity bioassay

INTRODUCTION

Bacillus thuringiensis israelensis is a Gram-positive, spore-forming bacterium that is probably best known as a pathogen of mosquito larvae. The insecticidal agent in these preparations is a parasporal crystalline protein toxin that is produced by the microbe during the sporulation process. The crystal is composed of at least two toxic peptides (mol. wt. = 68 000 and 130 000) and a broadly cytolytic protein (mol. wt. = 23 000) which act in a synergetic manner during toxigenesis (Andrew *et al.*, 1987; Hurley *et. al.*, 1987; Tyrell *et al.*, 1979). The toxic action of the crystals of *B. thuringiensis israelensis* have been limited to the larvae of hematogenous insects, like mosquitoes, and blackflies (Klowden *et al.*, 1985), although cytolytic protein has been shown to be toxic to a wide range of cell from many sources when cultured in vitro. This narrow toxic spectrum has made the use of the microbe for mosquito control very attractive (Andrew *et al.*, 1987).

In Indonesia and other developing countries with tropical climates, there have been massive effort to control mosquito primarily of their role of vectors of human disease. With the growing concern about the accumulation of pesticides in the environment there has been an increased focus on the development of biological control agents as alternatives to chemical control and entomopathogens *B. thuringiensis israelensis* and *Bacillus sphaericus* have received the most attention.

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There has been considerable development regarding large scale fermentation of *B. thuringiensis israelensis,* particularly in the developed countries, such as Japan, the United States, Europe, and the Soviet Union (Bulla *et al.,* 1985).

Although these methods have been quite succesful, they have focused on the use of fermentation materials that are widely available and relatively inexpensive in these countries. The development of more economical media for growth in developing countries is paramount to use of these agents in the developing world. Herein, fermentation of *B. thuringiensis israelensis* using materials locally available in Indonesia is described.

MATERIALS AND METHODS

Microbe and culture conditions

Bacillus thuringiensis israelensis was obtained from Dr. Samuel Singer, Department of Biological Sciences, Western Illinois University, Macomb, IL, USA. The culture was maintained on nutrient yeast extract agar (nutrient agar supplemented with 0.5% yeast extract; NYA) slants and stored at 4°C until ready for use. To start a culture, a fresh NYA slant was inoculated from a stored slant and allow to grow 48 h at approximately 28°C. The growth from this slant was suspended in 10 ml of sterile water (approximately 10^8 cells/ml) and 1 ml of this suspension was transfered to 250 ml Erlenmeyer flasks containing 50 ml of sterile medium; the ingredients for each of the experimental liquid media used are described in TABLE 1. The number of total viable cells (TVC) and total viable spore counts (TVSC) were determined by plating samples on NYA. To determine the TVC in a sample, the cultures were simply diluted and plated. To obtain the TVSC in a sample, the dilution tubes were heated to 60°C for 30 min. prior to plating. Incubation of the plates was at 28°C for 24–48 h. After the media was prepared, the pH was adjusted to 7.0 by the addition of either H_2SO_4, or NaOH (1M) before autoclaving.

TABLE 1. – Composition of media used for fermentation of *Bacillus thuringiensis israelensis.*

Ingredient	Level g/l.													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Salt														
$CaCO_3$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$MgSO_4.7H_2O$	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
$FeSO_4.7H_2O$	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
$ZnSO_4.7H_2O$	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Protein source														
Skim milk	10.00	15.00	20.00	30.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	–	–
Soybean meal	–	–	–	–	–	–	–	–	–	–	–	–	15.00	15.00
Carbon source														
Dextrose	10.00	10.00	10.00	10.00	5.00	10.00	15.00	20.00	10.00	10.00	10.00	10.00	–	–
Molassses	–	–	–	–	–	–	–	–	–	–	–	–	20.00	20.00
Growth factors														
Extract of yeast	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	–	–	–	2.00	–
Fish paste	–	–	–	–	–	–	–	–	–	1.00	2.00	3.00	–	2.00

Soybean seeds, obtained locally, were dried and then ground so the meal passed through a 0.5 mm sieve. Black strap molasses is the principle local source for production of industrial ethanol. The fish paste used is a traditional local fermented food. The product used herein was Trassi Udang Bali Super. The fish, or sometimes shrimps, is mixed with 10% salt soon after harvest, usually while still on the boat. After a few more days, more salt may be added, then the paste is dried to approximately 50% moisture by using sunlight. To complete the paste, the preparation is kneaded and further dried. It is usually mixed with red dyes. Occasionally whole rice and other starches are added. The product herein was purchased in a local market.

Insect Bioassays

Aedes aegypti were obtained from Dr. Sugeng Yuwono at the Laboratory of Parasitology, Gadjah Mada University Faculty of Medicine. Adults were reared in a cage (30 cm × 30 cm × 30 cm) and fed by using interval supplements of 10% glucose. The eggs were removed from an adult reading cage and placed in plastic trays that contained water. After hatching, the larvae were transferred to separate trays at a density of 1 larva per 5 ml of water.

Final whole cultures from each of the fermentation media were diluted 10-fold in deionized water. Twenty-five fourth instar larvae of *A. aegypty* were placed in each dilution and allowed to incubate at room temperature. After 24 h, and again at 48 h, the number of dead and living larvae were counted in each dilution tray, and the dilution LC_{50} was calculated.

RESULTS

Dextrose, skim milk, and yeast extract were initially used as sources of carbon, protein and growth factors as standard "developed country media" (TABLE 1). To determine a base line toxicity with which to measure new media components, the concentration of each in the medium was optimized. The results of these experiments are shown in TABLE 2. The optimum concentration of dextrose was 10 g/l (medium B) and the most favorable concentration of skim milk was 15 g/l, for the growth of *B. thuringiensis* respectively. Under these

TABLE 2. – Total viable cells, total viable spore counts, and mosquiticidal activity of final whole cultures of *Bacillus thuringiensis* grown with various concentration of dextrose and skim milk.

Media	TVC[1] ($\times 10^8$)	TVSC[2] ($\times 10^8$)	LC_{50}[a]	
			24 h	48 h
A	3.9	4.5	4.1×10^{-6}	3.5×10^{-6}
B	9.8	9.3	2.5×10^{-6}	1.4×10^{-6}
C	6.5	4.7	6.1×10^{-6}	5.3×10^{-6}
D	3.9	1.1	1.6×10^{-6}	4.5×10^{-7}
E	3.9	2.6	2.8×10^{-7}	1.0×10^{-6}
F	10.0	8.3	1.4×10^{-6}	1.9×10^{-7}
G	4.6	3.6	3.7×10^{-6}	3.2×10^{-7}
H	3.7	1.5	2.6×10^{-6}	1.8×10^{-6}

1 Total viable cells/ml culture fluid.

2 Total viable spore counts/ml culture fluid.

a The dilution resulting in 50% mortality.

conditions, a final whole culture contained approximately 1×10^9 cfu/ml and a 48 h old culture was toxic to half of the *A. aegypti* third instar larvae in a population when diluted 3.22×10^{-7}.

Yeast extract is expensive in the developing countries, commercially available yeast extract and other such media components may cost four times as much in Indonesia as they would cost in Japan or the United States. Therefore, it was of interest to develop a locally available source of nutrients to replace the yeast extract in the typical medium for fermentation of *B. thuringiensis*. *Trassi udang* is a fermented fish product used as a human food ingredient in Indonesia. It is relatively inexpensive and is widely available. TABLE 3 shows that 2 g of *trassi udang*/l, although resulting in lower levels of viable cells and spores in the culture, results in mosquito larvicidal activity that are even higher than when yeast extract is used (dilution $LC_{50} = 1.1 \times 10^{-7}$).

TABLE 3. – Total viable cells, total viable spore counts, and mosquiticidal activity of final whole cultures of *B. thuringiensis israelensis* grown with various concentrations of *trassi udang* (fermented fish paste).

Media	TVC[1] ($\times 10^8$)	TVSC[2] ($\times 10^8$)	LC_{50}[a]	
			24 h	48 h
I	3.6	3.5	1.4×10^{-6}	1.4×10^{-7}
J	1.5	4.7	1.7×10^{-6}	1.1×10^{-7}
K	1.6	5.2	1.0×10^{-6}	1.1×10^{-7}
L	1.2	3.9	9.2×10^{-7}	2.9×10^{-7}

1 Total viable cells/ml culture fluid.

2 Total viable spore counts/ml culture fluid.

a The dilution resulting in 50% mortality.

Because of the expense associated with the use of dextrose and skim milk in a fermentation it was of interest to find an alternative carbohydrate source in this fermentation. Black strap molasse is the material remaining after recovery of crystalline sugar from concentrated sugar cane extract. Medium M (TABLE 4) shows the results of one such experiment when black strap molasses, soybean meal and yeast extract were used in the fermentation. Interestingly, the TVC (1.7×10^9/ml) and TVSC (1.3×10^9), and toxin production increased LC_{50} at a dilution of 1.05×10^{-9}). Moreover, when *trassi udang* was used as the nutrient source, the TVC increased even further (4.6×10^9), while the TVSC increased only

TABLE 4. – Total viable cells, total viable spore counts, and mosquiticidal activity of final whole cultures of *B. thuringiensis israelensis* grown using black strap molasses, soybean meal, and either yeast extract or *trassi udang*.

Media	TVC[1] ($\times 10^9$)	TVSC[2] ($\times 10^9$)	LC_{50}[a]	
			24 h	48 h
M	1.7	1.3	1.4×10^{-8}	1.0×10^{-9}
N	4.6	1.6	1.7×10^{-8}	5.6×10^{-10}

1 Total viable cells/ml culture fluid.

2 Total viable spore counts/ml culture fluid.

a The dilution resulting in 50% mortality.

slightly. Most importantly, the larvicidal activity of this preparation increased nearly 4–fold when compared to similar fermentations wherein yeast extract was the nutrient source.

DISCUSSION

Dulmage *et al.* (1990) discussed the development of fermentation media for *B. thuringiensis israelensis* in developing countries. This author stressed the need for each area to develop their fermentation based on locally available materials that were economically suitable, either because of their low cost or because they were waste materials. Herein, a medium composed of materials that are readily available in Indonesia is described. Because similar materials are available throughout Southeast Asia this paper should serve as a model for the development of media in this region.

Bulla *et al.* (1985) discussed the importance of using bioassay data, rather than spore or cell counts in determining the activity of insecticidal preparations of *B. thuringiensis*. This principle is demonstrated in the data contained herein. For example, medium B results in a TVSC of 9.3×10^8 spores/ml and a LC_{50} of 1.4×10^{-6}, whereas medium M results in 1.6×10^9 spores/ml, but is toxic to half of the larvae in the population at a dilution of 4.47×10^{-10}. Although the spore count between these two media approximately doubles, the toxicity increases by approximately four orders of magnitude.

The medium described herein provides high levels of toxic product during a typical *B. thuringiensis israelensis* fermentation. Moreover the ingredients are inexpensive and readily available in this country. Development of this medium into a commercially viable product continues in this laboratory.

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