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Simple and Cheap Media for Culturing *Bacillus thuringiensis kurstakii* and *Bacillus thuringiensis aizawai* used against the Cotton Leaf worm, *Spodoptera littoralis* (Boisd.)

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ABSTRACT

This study aims to produce a simple and low- cost medium for production of the protein crystal delta- endotoxin from *Bacillus thuringiensis kurstaki* and *Bacillus thuringiensis aizawai*, and to evaluate the effectiveness of these crystal towards the 1st instar of the cotton leaf worm, *Spodoptera littoralis* larvae. The results indicated that the use of potato .and chick – pea media, induced good growth and sporulation. These growth and sporulation were very similar to that of the usual nutrient broth medium (peptone+beef extract+glycerol). Larvicidal effects of the delta-endotoxin produced from the four culture media (chick- pea, chick-pea + sucrose, potato, potato + sucrose) were statistically similar to that produced from nutrient broth medium. Chick- pea medium is recommended for *B. t.* production because of its advantages as cheap medium (1L. costs 0.2 LE. in comparison with nutrient broth where 1L.costs 6.78 LE.), easy in preparation and available at the local market.

Key Words: *Bacillus thuringiensis*, *Spodoptera littoralis*, growth, sporulation, bioassay, protein analysis, media.

INTRODUCTION

Insecticidal activity of *Bacillus thuringiensis (B.t.)* is based on spores and crystals. The crystals are synthesized concomitantly with sporulation, and are composed of proteins named ĉ-endotoxin. *Bacillus thuringiensis* var. *kurstakii* and var. *aizawai* are the most effective microbial control agents against lepidopteran larvae such as the cotton leaf worm, *Spodoptera littoralis* (Boisd.). They synthesize intracellular crystal inclusions containing multiple protein components with molecular weights of 134 and 60 Kda (Hofte and Whiteley 1989). Crystals are most responsible for the toxicity and spores are not directly involved, but a qualitative effect on mortality can be assessed. Indeed, large quantities of spores with high insecticidal activity are required for practical applications. This means that when producing *B. t.* as bioinseticide, a high spore count is not sufficient to ensure toxicity, and it is necessary to reach high ĉ -endotoxin titers (Zouari *et al.* 1998).

One of the most underreported aspects of *Bt* is that of production and formulation, although there are certain works in connection with *B. t.* growth on several synthetic or complex media (Pearson and Ward 1988. Avignone and Mignone 1993). Indication that media supporting high vegetative cell growth may not be adequate for sporulation (Sikdar 1991and Drehval *et al.* 2003). They also reported that vigorous vegetative growth was not always followed by good sporulation.

The cost to grow and to produce *B. thuringiensis kurstaki* or *B. thuringiensis aizawai* formulations through existing fermentation technology (using a bio fermenter) is extremely high. Therefore, the use of these biopesticides has limitations, Obeta and Okafor (1984) formulated five media using the seeds of legumes, dried cow blood and mineral salts and assessed the production of the insecticidal toxins of *B. thuringiensis israelensis* which were effective against *Aedes, Anopheles* and *Culex* species of mosquitoes. Similarly, other media containing fishmeal, soyabean and corn steep liquor have also been reported for the production of *B. sphaericus* and *B. t. iseraelensis* have also been reported (Salama *e.t al.* 1983, and Kumar *et. al.* 2000). Poopathi *et al.* (2002) suggested that potato-based culture media are more economical for the industrial production of *B. sphaericus* and *B. t. israelensis.*

In the present study, simple and cheap several media based predominantly on locally available substrate in Egypt were assessed for the production of *B. t. kurstaki* and *B. t. aizawai*, to control *S. littoralis.*

MATERIALS AND METHODS

Bacterial culture medium:

I-The standard laboratory culture broth (nutrient broth) used as reference medium in the present study, included peptone and meat extract.

II- Potatoes were purchased (150gm) from the local market, their skin peel off, then cut in to small pieces and boiled in tap water (500 ml) for 15-20min (till the potatoes became soft). After cooling, potatoes were mashed thoroughly by hand and filtered through a muslin cloth. The resulting potatoes extract was made up to 1 liter with tap water. The extract was dispensed separately into two conical flasks. Common sugar (sucrose 0.5%w/v) was added in flask.

III- Chick-pea (*Cicer arietinum*) (50gm) were steeped in tap water (500 ml) for 60 min, then boiled for 30 min. The chick-pea steep liquor was mashed. It was made up to 1 liter and dispensed separately into two flasks. The common sugar (sucrose 0.5% w/v) was added to one of the two flasks. All the culture media were autoclaved at 120 °C for 20 min.

A small amount (0.5 gm) of *B. t.kurstaki* and *B. t. aizawai* were inoculated separately in 2 ml of nutrient broth medium and allowed to grow for 8h. at 37°C. A small volume of the precultures (200µl each) were inoculated into the six different media.

The cultures were allowed to grow under constant agitation (200 rev/min) and at 30°C. Culture samples (2ml) were drawn from each culture medium at 12h intervals from zero to 72h. The culture turbidity were measured using spectrophotometer (at 650 nm optical density), and were also examined microscopically for sporulation and production of crystalline inclusion. As soon as the cultures were fully sporulated (48 h), they

were harvested by centrifugation (6000 rpm for 10 min). The spore / crystal mixtures were thoroughly washed three times with 0.1M Nacl solution. Finally the pellets were stored at -20°C until further use.

SDS-PAGE

A total of 5ug protein equivalent samples from *B. t. kurstaki* and *B..t aizawai* cultures grown in different media was incubated with an equal volume of protein sample buffer and boiled for 5 min and separated on sodium dodecylsulfte- polyacrylamide gel electrophoresis (SDS-PAGE) according to Lammeli (1970). The protein bands were stained with Coommasie Brilliant Blue R 250.

Bioassays

The bioassay technique was performed according to Kalfon and De Barjac (1985), with some modifications. Twenty 1st instar larvae of *S. littoralis* were transferred to Petri dishes contain 25ml of the insect media supplemented with different dilutions of bacterial cell suspension. Using a cell density of (1×10^7 cell/ml) as a reference stock., the dilutions used were 1, 0.75 and 0 .5. Counts of survived larvae were scored after 72 and 144 hours. Three replicates were raised for each bacterial cell suspension and probit line was plotted of probit paper in order to estimate the LC_{50} in each case.

RESULTS AND DISCUSSION

1- Growth and sporulation

The production of *B. t. kurstaki* and *B. t. aizawai* spore/crystal Mixtures in the different culture media (potato, potato + sucrose, chick pea and chick pea +sucrose) was measured and compared with the toxin production in a conventional laboratory medium (nutrient broth). The present data indicated that with increasing culture time, culture density increased and reached a plateau in the range 1.5–2.0 (measured by optical at 650nm) (Figures 1 and 2). such differences were proved by Dipak and Shethna (1999) and Poopathi *et al.* (2003). They found that *B. t. israelensis* in various test media and in the standard medium had reached a plateau in the range of 1 – 1.5.

2- Microscopic observations

The microscopic observations showed clearly that after 72 h , the growth and the sporulation of the subject two *B. t.* varieties (*kurstaki* and *aizawai*) in chick-pea medium was as good as that of nutrient broth (standard medium). Appreciable levels of growth and sporulation were repeated four times and the same results were obtained. Poopathi et al. (2003) showed that microscopic observation on *B. t. israelansis* spores and crystals, recorded from standard and potato media after 72h, indicated that sporulation in potato medium was as good as that of the standard one.

3- SDS-PAGE

The total protein profiles of broth *B. t. kurstaki* and *B. t. aizawai* produced from the standard and the tested culture media were analyzed by SDS-PAGE, and the results were represented in Figure (3a and b). The major polypeptides present in the parasporal crystal proteins of both *B. t. kurstaki and B. t.aizawai* were clear and conspicuous, there was no variation in the protein patterns of the toxins produced from the standard and test media, The protein profiles as toxins in the *B. t. kurstaki and B.t. aizawai* were consistent with larvicidal activity in the laboratory bioassay experiments.

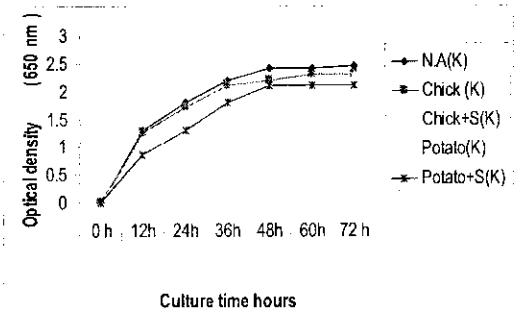

Figure (1): Growth pattern of *B. thuringiensis kurstaki* in different culture media.

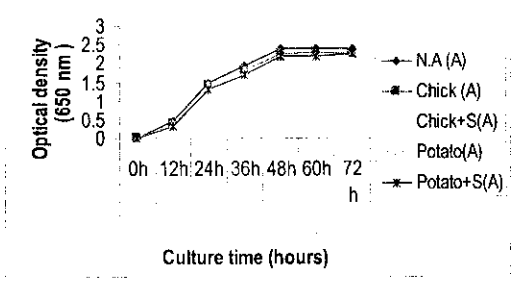

Figure (2): Growth pattern of *Bacillus thuringiensis aizawai* in different culture media.

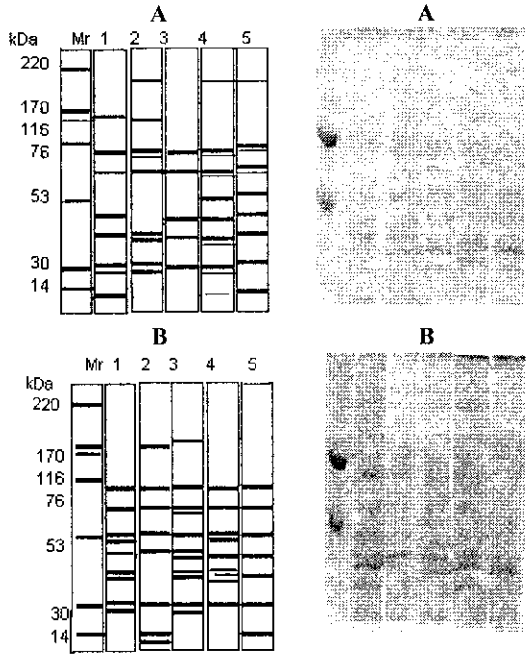

Figure (3): Diagrammatic representation of soluble protein electrophoretic patterns of *B. thuringiensis kurstaki* (1) Nutrient broth, (2) chick-pea, (3) chick-pea with sucrose, (4) potato, (5) potato with sucrose media after 72h of culture growth.

4- Larvicidal activity of *B. t.* tested varieties

The .larvicidal activity of both *B. tkurstaki and B. t. aizawad* toxins produced by the standard medium (nutrient broth) and the other four test culture media , was laboratory bioassaied. The comparative toxicity based on the LC_{50} values, of the two tested varieties of *B. t.* cultured on the five subject media, against the first-instar larvae of *S. littoralis,* is shown in Table (1). The LC_{50} value of *B. tkurstaki* cultured on nutrient broth, was statistically similar to those of *B. t. kurstaki* produced in chick pea, and potato + sucrose broth.

Table (1) shows the LC_{50} values, at 72 hours post treatment of *B. t. aizawai* (cultured on both potato and potato+sucrose broths) for *S. littoralis* first instar larvae were the same and similar to the corresponding values for *B. t. kurstaki.* On the other hand, the total production cost of one liter of each *B. t.* tested variety was too much low and ranged from 0.2 to 0.31L.E compared to the standard medium (Table 1). The use of several less expensive alternative *B. t.* culture media was previously reported by several authors (Poopathi and Kumar, 2003 and Devi *et al.* 2005).

Table (1): Efficacy of two *B. thuringiensis* varieties (based on both the LC_{50} and the total production costs of one liter *B. t.* broth) cultured on five culture media as microbial control agents for *S. littoralis* larvae.

Culture media	LC_{50} (ml/15ml rearing diet) at 72hr post treatment		Total production cost of one liter--
	B. t. kurstaki	*B. t. aizawai*	*B.t.* broth (in L.E)
Nutrient broth	0.232	0.287	6.87
Chick- pea broth	0.232	0.287	0.2
Chick – pea + Sucrose broth	0.181	0.279	0.21
Potato broth	0.232	0.232	0.3
Potato + sucrose broth	0.232	0.232	0.31

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