# Cost effective production of *Bacillus thuringiensis aizawai* and their application against *Spodoptera litura*

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***Abstract*—** *Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae) was prevalent in many species of aquatic plants grown in Green Farms. This study was carried out to understand the production of Bacillus thuringiensis aizawai (Bta) using cost effective method to manage the pest biologically. Bta is being widely used in pest control programs. However, the production of this Bta is expensive due to the high cost of the production medium. In this study, an attempt has made to develop a cost-effective medium, based on a locally available raw materials namely coconut water which is available in plenty as waste product from coconut oil industry, coconut poonac, rice bran and coir dust. A standard conventional Luria Bertani medium was included in the assay for comparison. Media were assessed for the growth, sporulation and production of insecticidal properties of Bta. Coconut poonac extract and coconut water media produced higher spores than compare with conventional LB medium. Maximum spore count of 25.0×1013 spores/mL was obtained with a 72 h old culture of this bacterium grown in coconut poonac extract. Larvicidal activity (LC50) of 8 ×106 spores/mL (coconut poonac extract) against early second-instar larvae of S.litura were obtained. This is almost similar to that obtained with LB (9 × 106 spores/mL) medium. Hence, coconut based culture medium is economical for the production of Bta and compared favorably with the standard. Cost-effective analyses have revealed that production of Bta from test media is highly economical. The cost of production of Bta with local media was significantly reduced by 88-293 fold. The use of nonconventional sources has yielded a new knowledge in this area as the process development aspects of small scale production have been neglected as an area of research. These studies are very important from the point of media optimization for economic production of Bta based agents in pest management programs.*

***Keywords*— Spodoptera litura, Bta, cost effective media, spores, small scale production.**

## I. INTRODUCTION

Green Farms Ltd., being one of the biggest exports oriented floriculture industry in Sri Lanka. Recently larvae of *S.litura* emerged as a problem in one of the aquatic plant *Ludwigia repens*. This larva is polyphagous, larval stages caused severe damage on the other variety of aquatic plants. The occurrence of the larvae of *S.litura* on aquatic plants as reported first time in the farm and being new it caused significant damage to the plant resulting to an economic loss of the export section.

*S. litura* also becomes resistant to many commonly used insecticides, resulting in failure of effective controls (Ahmad et al., 2007). *Bacillus thuringiensis* (*Bt*) is the most successful commercial biopesticide in the biological control market (Glare and Callaghan, 2000) accounting for 90% of all biopesticides sold all over the world. This bacterium is characterized by its ability to produce crystalline inclusions proteins have a great potential to control a great number of pest insects belonging to the order Lepidoptera, Diptera and Coleoptera (Vidyarthi et al., 2002).

Cost to grow and produce *Bt*, through highly refined laboratory bacterial culture medium, is comparatively high than other bio agents. The cost of *Bt* production depends on many factors; however, the raw material cost is one of the most important criteria, which comprises more than 70% of the overall production cost (Ejiofor, 1991). In order to encourage the commercial production of biopesticides, utilization of less expensive raw material is advisable (Mummigatti and Raghunathan, 1990).

## II. METHODOLOGY

Micro-organism and materials *Bacillus thuringiensis* subsp. *aizawai* ABTS-1857 under the trade name of Xentari obtained from the microbiology lab, Green farms limited, Marawila was used in the present study. The strain was maintained on Yeast Extract Agar Medium (YEAM) plate containing 5 g/L peptic digest of animal tissue, 3 g/L yeast extract and 15 g/L agar. It was stored at 4°C throughout the study. Media preparation Liquid media were used for the experiment while LB medium was used as reference medium.

**TABLE 1**
**MEDIA PREPARATION**

| Media | Concentration |
|---|---|
| Luria Bertani, LB (Reference media) | Peptone 20g, yeast extracts 10g and NaCl 20g in 1L distilled water (Poopathi and Archana, 2012) |
| Coconut water | Fresh liquid |
| Coconut poonac | 50g of poonac was boiled in 1L water for 15 minutes |
| Rice bran | 50g of bran was soaked in 1L water for 2 hours |
| Coir dust extract | 50g of coir dust was boiled in water for 15 minutes |

All media were filtered through plastic strainer and pH was adjusted to 7.8 with 10% NaOH. 100 ml of each filtered extract was dispensed separately into 180 ml flat bottles and closed with cotton wool. From the above test, the medium which showed maximum *Bta* biomass production was selected for further studies.

### 2.1 Sterilization of media

All culture media were autoclaved at 121°C, for 15 minutes.

### 2.2 Production in flatten flasks, Growth conditions

First stage seed was prepared by inoculating 10 mL of nutrient broth with one loop full of cells from YEAM and incubating on a rotary shaker at 30°C, 180 rpm for a period of 6 h. The seed thus prepared was transferred to 100mL medium in 180mL flat bottles (8 bottles per medium) at 1% level (v/v) and the bottles were incubated on an incubator at 30°C for a period of 96 hours. 2 bottles each were removed at 24 h intervals and used for assessment of sporulation and larvicidal activity. The pH was measured using digital pH meter. The bacterial stages (vegetative to sporulative stage) were also examined by Oil emersion 1000 LED microscope.

### 2.3 Microscopic studies

Simple staining procedure was used to stain microbial cells. Spores of *Bacillus* species do not stain, and they may be seen as unstained bodies within bacterial cells stained with methylene blue. Smears of *Bacillus* isolates were prepared and they were fixed by heat. The bacterial smears were then flooded with methylene blue. Staining lasted for 5 min. Finally, destaining was performed by washing under the tap water and stained bacterial colonies were observed under an oil emersion objective.

In addition, endospore staining with malatchite green was performed for a better observation of *Bacillus* spores. This staining procedure involved primary staining with malatchite green for 5 min and steam heat to drive the stain into spores. This stain was retained by endospores but washed out of the rest cells with water. Cells were then counterstained with the red dye safranin. The spores appeared green and cells appeared red after staining by this procedure (Tuba, 2002).

### 2.4 Spore count

For spore count culture samples were heat treated at 80°C for 15 minutes and serially diluted. The appropriately diluted samples were plated on Yeast Extract Agar medium plates and incubated overnight at 30°C to form fully developed colonies. Fully developed colonies were counted and expressed in colony forming units per mL (cfu/mL).

### 2.5 Measurement of pH change

The pH of all media was adjusted to 7.8 with NaOH before sterilization, and was measured at regular intervals during 96 hours of fermentation process. Two replicates were used each one was measured three times.

### 2.6 Cost analysis

Cost to prepare 1 l of each medium was calculated. For the comparison, preparation cost in 1L of the LB medium was used.

### 2.7 Toxicity assessment of *Bacillus thuringiensis aizawai* on *S. litura* (In vitro study) Larvacidal activity

The spore crystal complex produced in different media was assayed against second instar larvae of laboratory-reared *S.litura*. Water primrose (*Ludgwia* spp) leaves were cut and washed once with 0.5% sodium hypochlorite and two times with distilled water. Test solutions were sprayed on both side of leaves. They were allowed to surface-dry on a paper towel and then placed into Petri-dishes containing moistened filter papers to avoid desiccation of leaves. Larval mortality was scored after 24 h and corrected for control mortality, using Abbott's formula (Abbott, 1925). The experiment was done at the temperature of

30±1°C and 75% Relative humidity. Each bioassay included 6 treatments of 2 replicates each, along with the appropriate control. Larval mortality was scored up to 4 days after larvae infection to check pathogenicity. Each treatment was replicated 2 times, along with an untreated control under complete randomized design. Probit regression analysis (Finney, 1971) was carried out to calculate LC50 and LC90 values as well as their 95% fiducial limits.

**TABLE 2**
**CONCENTRATION OF *Bta* ON MASS PRODUCTION OF DIFFERENT MEDIA USED TO CONTROL THE SECOND INSTAR LARVAE OF *S.litura***

| Treatments | Concentration |
|---|---|
| T1 | $10^6$ spores/mL |
| T2 | $10^7$ spores/mL |
| T3 | $10^8$ spores/mL |
| T4 | $10^9$ spores/mL |
| T5 | $10^{10}$ spores/mL |
| T6 | $10^{11}$ spores/mL |
| T7 (Control) | |

### 2.8 Statistical analysis

All the experiments were designed according to complete randomized design (CRD) and obtained data were statistically analyzed using SAS package and the significance among the treatments were determined according to Dunnett mean separation test at 95% of confidence Interval.

## III. RESULTS AND DISCUSSION

### 3.1 pH change during 96 hours of fermentation

Results showed that the pH varied among the five media during 96 hours of fermentation, and since there was no pH control during the experiments, a pH fluctuation was observed (Figure 1).

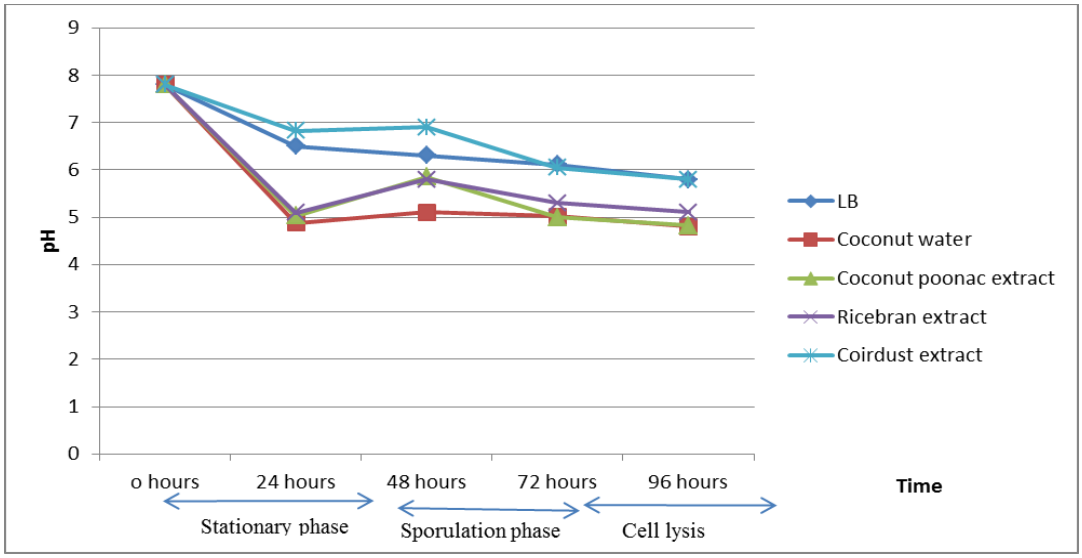



**FIGURE 1: pH change on different media during 96 hours of fermentation**

LB and coir dust extract media slowly decreased the pH in the first 24 hours, and reaching a minimum pH value of 5.8 within 96 hours of fermentation. Coconut water, coconut poonac extract and rice bran media maintained a very low pH (between 4.8 to 5.8) after 24 hours until the end of fermentation process (Figure 1). Tirado-Montiel *et al*. (2001) stated that this fact is probably due to the utilization of the carbohydrates before the sporulation phase.

Dingman & Stahly (1983) stated that the decrease in pH is due to acid accumulation that results from catabolism of glucose, and this bioassay was added to the media.

### 3.2 Spore count

**TABLE 3**
**SPORE COUNT OF *Bta* ON DIFFERENT MEDIA AFTER 24, 48, 72, 96 HOURS OF INCUBATION**

| Locally available agro industrial by product substrates | Mean spore count $10^{13}$ per mL* | | | |
|---|---|---|---|---|
| | After 24 hours | 48 hours | 72 hours | 96 hours |
| LB (Reference medium) | 9.07 | 8.70 | 18.17 | 11.27 |
| Coconut water | 7.23 | 13.27 | 22.10 | 18.23 |
| Coconut poonac extract | 8.40 | 15.30 | 25.70 | 20.13 |
| Ricebran extract | 1.97 | 4.23 | 9.30 | 5.27 |
| Coirdust extract | 2.87 | 5.77 | 11.23 | 7.50 |
| Control | 0.00 | 0.00 | 0.00 | 0.00 |

The growth and sporulation of *Bta* in locally available agro industrial by product substrates are shown in table 10. In all five media, the highest number of viable spores was reached after 72 hours of growth. According to this study the highest spore count was recorded after 72 hours of fermentation in coconut poonac extract as $25.70 \times 10^{13}$/mL. Next highest spores count was observed in coconut water was $22.10 \times 10^{13}$/mL, followed by LB ($18.17 \times 10^{13}$/mL) and coir dust extract ($11.23 \times 10^{13}$ /mL).

Lowest spore count was recorded in rice bran extract as $1.97 \times 10^{13}$/mL after 24 hours of fermentation. Spore production was increased until 72 hours fermentation then amount of spore decreased after 72 hours fermentation.

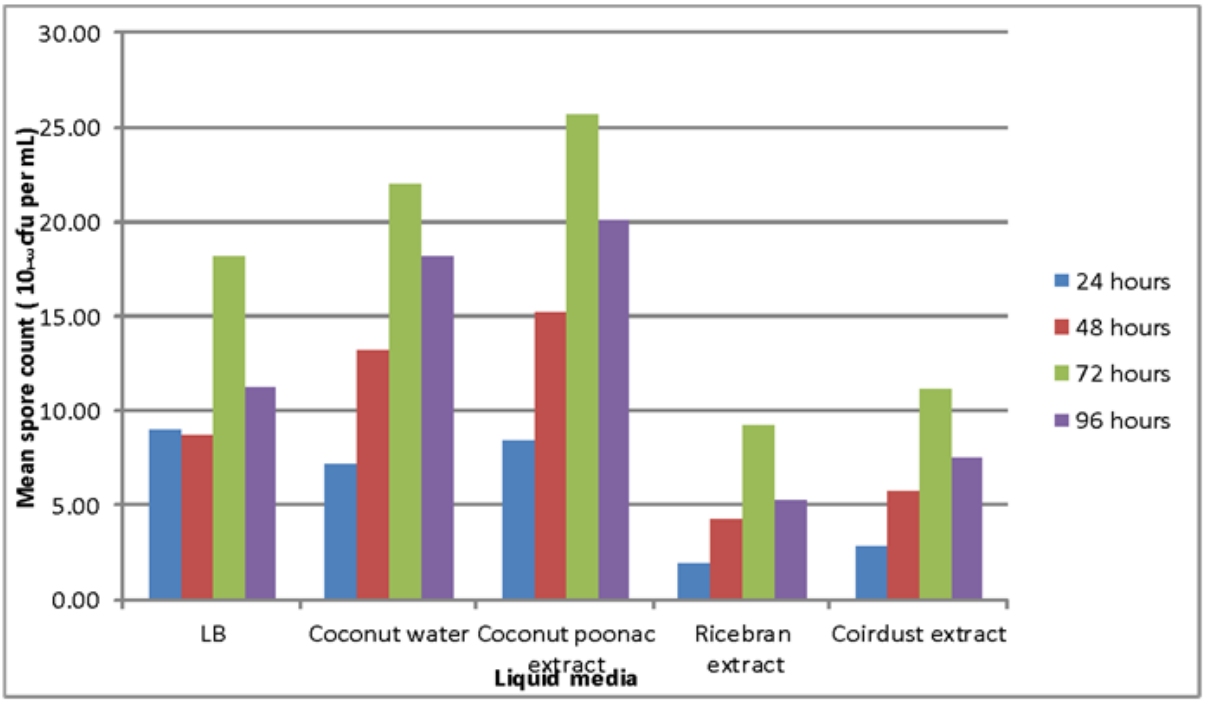
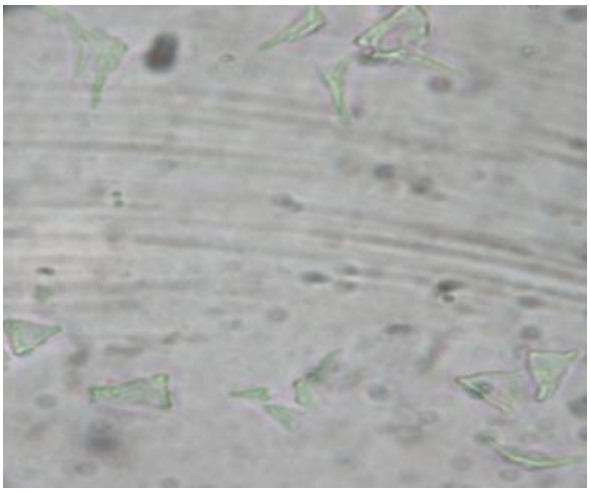


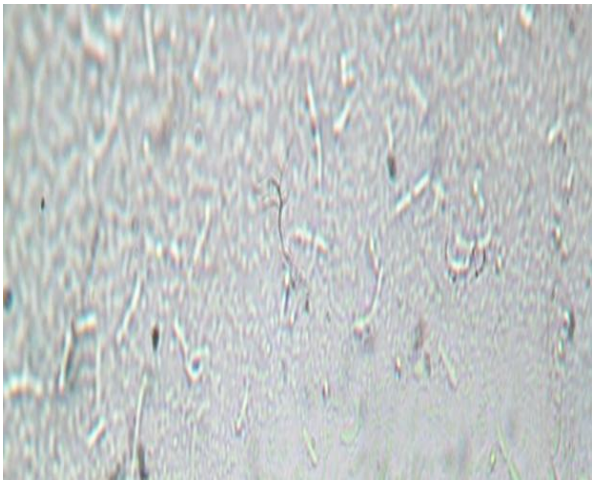

**FIGURE 2: Mean spore concentration produced by different media**

### 3.3 Microscopic observation

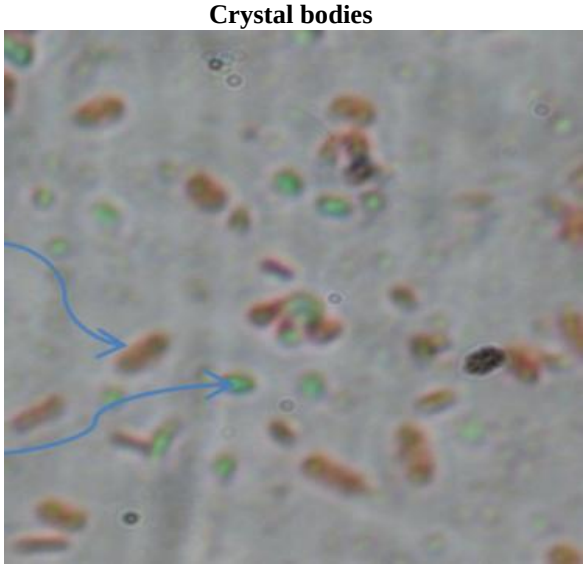
Gram positive, produce ellipsoidal spores that do not distend the sporangium was observed. In the endospore staining with malatchite green, vegetative cells appeared as red and spores appeared as green.



**Crystal bodies**



**vegetative cells**



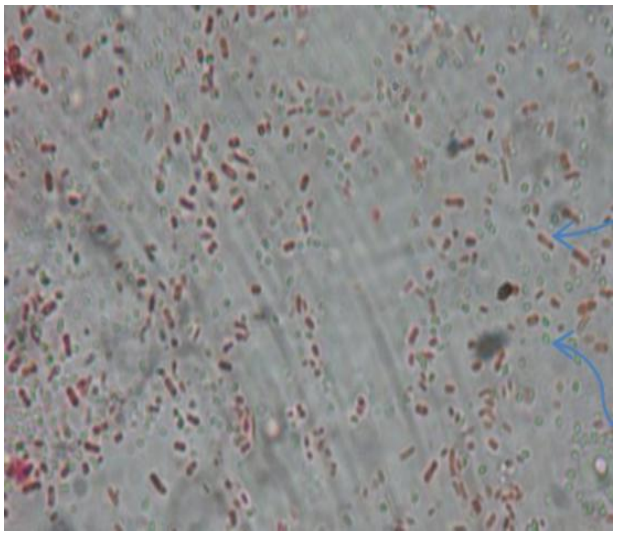


**FIGURE 3: Red - vegetative cells Green- spores**

### 3.4 Larvacidal activity

**TABLE 4**
**MEAN MORTALITY PERCENTAGES OF LARVAE OF *S.litura* WITH DIFFERENT CONCENTRATION OF *Bta* SUSPENSION IN STANDARD LB MEDIA**

| Concentration | Mean mortality Percentage* | | | |
|---|---|---|---|---|
| | 1DAT | 2DAT | 3DAT | 4DAT |
| (T1) $10^6$ spores/mL | $27.08^f$ | $42.71^e$ | $58.33^e$ | $66.66^c$ |
| (T2) $10^7$ spores/mL | $56.25^e$ | $76.04^d$ | $83.33^d$ | $90.62^b$ |
| (T3) $10^8$ spores/mL | $70.83^d$ | $83.33^c$ | $92.71^c$ | $98.96^a$ |
| (T4) $10^9$ spores/mL | $81.25^c$ | $88.54^b$ | $96.87^b$ | $100.00^a$ |
| (T5) $10^{10}$ spores/mL | $90.62^b$ | $100.00^a$ | $100.00^a$ | $100.00^a$ |
| (T6) $10^{11}$ spores/mL | $98.96^a$ | $100.00^a$ | $100.00^a$ | $100.00^a$ |
| (T7) Control | $0.00^g$ | $0.00^f$ | $0.00^f$ | $0.00^d$ |

***All the values with the means of three replicates***

Figures having same letters in a column indicate that the values are not significantly different at 0.05 α.

**TABLE 5**
**MEAN MORTALITY PERCENTAGES OF LARVAE OF *S.litura* WITH DIFFERENT CONCENTRATION OF *Bta* SUSPENSION IN COCONUT POONAC EXTRACT MEDIA**

| Concentration | Mean mortality Percentage* | | | |
|---|---|---|---|---|
| | **24 h** | **48 h** | **72 h** | **96 h** |
| (T1) $10^6$ spores/mL | $27.77^f$ | $46.62^e$ | $57.77^d$ | $66.94^c$ |
| (T2) $10^7$ spores/mL | $54.44^e$ | $69.50^d$ | $78.89^c$ | $85.55^b$ |
| (T3) $10^8$ spores/mL | $73.33^d$ | $84.44^c$ | $93.33^b$ | $100.00^a$ |
| (T4) $10^9$ spores/mL | $84.44^c$ | $95.69^b$ | $100.00^a$ | $100.00^a$ |
| (T5) $10^{10}$ spores/mL | $93.33^b$ | $100.00^a$ | $100.00^a$ | $100.00^a$ |
| (T6) $10^{11}$ spores/mL | $100.00^a$ | $100.00^a$ | $100.00^a$ | $100.00^a$ |
| (T7) Control | $0.00^g$ | $0.00^f$ | $0.00^e$ | $0.00^d$ |

***All the values with the means of three replicates***

Figures having same letters in a column indicate that the values are not significantly different at 0.05.

Paralysis was observed in larvae within 2 h at a $10^{11}$ spores/mL concentration of each bacterial media. The effects on low concentrations were less severe, at the low concentration; the larvae merely gathered at the top center of the assay cup and stopped feeding.Maximum numbers of dead larvae were recorded in all the *B.thuringiensis subsp. aizawai* media at concentration $10^9$ spores/mL after 48 h of treatment.

The concentrations required to kill 50% of the larvae (LC50) coconut poonac extract (8.1 × 106 spores/mL) indicated that the two locally produced *Bta* medium compared favorably with conventional LB (LB (9 × 106 spores/mL) in the *S.litura* assays.

### 3.5 Cost analysis

As summarized in Table 3, the amount of coconut poonac, coir dust and rice bran required to prepare 1 l culture of these media was 50.0 g, which costs Rs 2.5, 5.0, 1.5 respectively. Coconut water is a plenty of wastage in oil industry. In comparison, preparation of 1 l of the LB medium costs Rs.440.0. Hence local media such as coconut poonac, coir dust and rice bran were found to be 176 times, 88 times and 293 times less expensive than the conventional medium respectively. Thus the use of an agro-industrial by-product-based medium is highly economical for large-scale industrial production of this entomopathogenic *Bta*.

**TABLE 6**
**COST TO PREPARE 10 L MEDIA**

| Media | Price (Rs) |
|---|---|
| Coconut water | 40 |
| Coconut poonac | 25 |
| Coir dust | 50 |
| LB | 4400 |
| Rice bran | 15 |

## IV. CONCLUSION

*Bta* was successfully grown on all selected locally available media such as coconut water, coconut poonac extract, ricebran extract and coirdust extract. Coconut water and coconut poonac extract media was produced higher spores compare to standard LB media.

The highest number of viable spores was reached after 72 hours of growth. According to this study, the highest spore count was recorded after 72 hours of fermentation in coconut poonac extract as $25.70 \times 10^{13}$/mL. Next highest spores count was

observed in coconut water was 22.10×10 $^{13}$ /mL, followed by LB (18.17 ×10 $^{13}$ /mL) and coir dust extract (11.23 ×10 $^{13}$ /mL). Lowest spore count was recorded in rice bran extract as 1.97×10 $^{13}$ /mL after 24 hours of fermentation.

The concentrations required to kill 50% of the larvae (LC50) coconut poonac extract (8.1 × 106 spores/mL) indicated that locally produced *Bta* media compared favorably with conventional LB (9 × 106 spores/mL) in the S.litura assays.

Cost-effective analyses have revealed that production of *Bta* from test media is highly economical. The cost of production of *Bta* with local media was significantly reduced by 88 to 293 fold.

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