Short Communication

Utilization of silkworm litter and pupal waste—an eco-friendly approach for mass production of *Bacillus thuringiensis*


Department of Biotechnology, Vishweshwariah College of Applied Sciences, Gulbarga 585 103, Karnataka, India

**Highlights**

- Use of silkworm litter and pupal waste for mass production of *Bacillus thuringiensis*.
- Analysis of biochemical composition of two substrates.
- Spore count: $3.5 \times 10^{10}$ CFU/g in pupal waste, $3.0 \times 10^{10}$ CFU/g in silkworm litter.
- Process cheaper, simple to run, with substrates available locally and abundantly.
- Better approach for disposal/reuse of wastes, minimizes environmental pollution.

**Abstract**

The objective of the present study was to investigate the utilization of pupal waste and silkworm litter separately as production media for the mass cultivation of the potential biopesticide, *Bacillus thuringiensis* (Bt). Bt is the most successful commercial biopesticide accounting for 90% of all biopesticides sold all over the world. Biochemical analysis of the dry pupal waste revealed to be consisting of 4% carbohydrates, 44.5% proteins and 40% lipids. Similarly the biochemical composition of dry silkworm litter was found to be 4% carbohydrates, 57.5% proteins and 30.5% lipids. *B. thuringiensis* NCIM No. 2159 was mass cultivated in a semi-solid-state fermentation at a pH 7.0 and temperature 32°C. Changes in the pH and biochemical composition of the substrates were evaluated during the course of the fermentation. The reliability of the two substrates as production media was evaluated by determination of growth at regular intervals. Maximum growth was recorded at 96 h incubation showing a spore count in the order of $3.5 \times 10^{10}$ and $3.0 \times 10^{10}$ CFU/g in pupal waste and silkworm litter respectively.

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**1. Introduction**

India is the world’s second largest producer of raw silk (18,500 MT/yr). The silkworm *Bombyx Mori* is reared throughout the year. Enormous quantity of pupal waste and silkworm litter are produced per year as a by-product of sericulture industry. For 1 Kg of raw silk 8.014 Kg wet and 2 Kg of dry pupa is produced. It has been estimated that the silkworm digests only 55% of the ingested leaf and the rest comes out as litter. Karnataka is one of the leading states where sericulture is practiced. There is accumulation of huge quantities of sericultural wastes but utilization of by-products has not kept pace with the strides the country has achieved in sericultural fronts over the years. Such by-products which are presently discarded as waste can be put to better use for financial gains and generation of value-based products. In adopting any technology it is important to consider the waste utilization aspect so that better returns are obtained and the pollution of the environment is minimized. The silkworm litter is presently used as fodder and as compost and the pupal waste is utilized in oil extraction, biogas production, mushroom cultivation (Sharma and Madan, 1992).

Bt is a Gram-positive, spore-forming soil bacterium that produces parasporal crystal inclusions which are toxic to the larval forms of a wide variety of insects including nematodes. Bt has been isolated from different environments including soil (Martin and Travers, 1989), stored grains (Meadows et al., 1992) and sericulture environments (Xavier et al., 2007). Over 90 species of naturally occurring, insect specific (Entomopathogenic) bacteria have been isolated from insects, plants and the soil, but only a few have been studied intensively. Much attention has been given to Bt sold under various trade names like Dipel®, Javelin®, Thuricide®, Bactospeine®, Bactospore®, SOK-Bt®, Caterpillar Killer®, Worm Attack®, and SOK-Bt®. The Bt biopesticide preparations are based on endotoxin proteins accumulated as parasporal crystals produced by the bacterial cells. Various agricultural and industrial by-products may be used as...
2. Methods

2.1. Collection and processing of pupal waste and silkworm litter

The pupal waste and silkworm litter were collected from the silkworm cocoon reeling center at Jewargi, Gulbarga, Karnataka. The wet pupal waste was collected immediately after reeling. The silkworm litter of silkworm was collected from the silkworm rearing bed of 5th stage larvae at a silkworm rearing house. The pupal waste was cleaned by removing the palisade layer and other materials attached to it. The substrates were sun-dried for one day, later kept in hot air oven at 80°C till weight consistency was achieved and then ground to fine powder and used for the studies.

2.2. Microorganism

*Bacillus thuringiensis* NCIM No. 2159 was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune. The culture was maintained on nutrient agar and expressed in terms of colony forming units per gram of the substrate (CFU/g).

2.3. Biochemical analysis of the substrates

Total carbohydrate content was determined as per the method of Seifert et al. (1950). An aliquot of the sample was hydrolysed in 4 ml anthrone solution in a boiling water bath for 15 min. After cooling, the carbohydrate concentration was determined spectrophotometrically by absorption at 620 nm using glucose as standard. Protein content was determined as per the method of Lowry et al. (1951) using bovine serum albumin as standard. One milliliter of sample was mixed with 5 ml of freshly prepared alkaline copper sulphate and incubated at room temperature for 20 min. 0.5 ml of Folin Ciocalteu's (1:2) reagent was added and incubated at room temperature for 20 min and the absorbance was measured at 660 nm. An aliquot of the chloroform extracted lipid phase was evaporated and the total lipid measured by reacting with an acid dichromate reagent. The amount of dichromate reduced, determined by change in absorption at 430 nm, is directly proportional to the lipid present (Bragdon, 1951).

3. Results and discussion

The biochemical constituents of the pupal waste and silkworm litter are presented in Table 1. The availability of carbon source can influence the yield of viable cells, spores and toxins in the Bt production process. The growth analysis of Bt in pupal waste and silkworm litter was carried out at every 24 h interval. The maximum growth was recorded at 96 h of incubation in both the substrates (Fig. 1). Sporulation was initiated within 24 h of incubation probably attributed to the low carbohydrate content in both the substrates. There was a sharp increase in the spore concentration up to 96 h of incubation and thereafter it declined. A concentration of $0.4 \times 10^{10}$ CFU/g of the substrates was observed at 24 h of incubation in both the substrates. The spore count was found to increase more than twice by 48 h of incubation showing yields of $1.3 \times 10^{10}$ and $0.9 \times 10^{10}$ CFU/g in pupal waste and silkworm litter respectively. At 96 h of incubation, the highest spore concentration recorded in pupal waste and silkworm litter was in the order of $3.5 \times 10^{10}$ and $3.0 \times 10^{10}$ CFU/g, respectively. Similar studies were conducted by Karthikeyan and Sivakumar (2007) employing pupal waste as substrate for the production of Bt. The authors reported a maximum spore count of $36.9 \times 10^{10}$ VSC/g of the substrate at 96 h of incubation. Adams et al. (2002) reported the mass production of Bt under solid-state fermentation utilizing heat-sterilized broth litter varieties. The authors reported the growth of Bt in methanol extracted litter to $5 \times 10^{10}$ CFU/g litter (dw) and a spore count of $1 \times 10^{10}$ CFU/g litter (dw). Valicente et al. (2012) investigated the growth of Btk HD-1 (Vu et al., 2012). Such relationships indicate that delta-endotoxin concentration can be used as an indicator for monitoring the fermentation.

2.4. Mass production of Bt

Mass production of Bt was carried out as per the modified method of Karthikeyan and Sivakumar (2007). Five hundred grams of substrates were taken separately in Erlenmeyer flasks and the moisture content was adjusted to 75% with distilled water. The initial pH of the media was then adjusted to 7.0 with 1 N NaOH/HCl. Fifty ml of the cell suspension containing $1 \times 10^{9}$ cells/ml was used as an inoculum for 500 g of the substrates. The contents of each flask were transferred equally to sterile Erlenmeyer flasks and the flasks were incubated at 32°C in a humidity chamber. The fermentation was ceased in each flask at an interval of every 24 h and the product was dried in an oven at 60°C for 36 h and ground separately. Samples thus collected were analyzed for the changes in the pH and biochemical constituents of the substrate. Growth analysis of Bt was performed based on Viable Spore Count (VSC) determination. All the experiments were performed independently in triplicates and the results given here are the mean of three values.

VSC determination was performed as per the method of Sella et al. (2009). One gram of the fermented substrate was mixed with 100 ml of 0.02 M calcium acetate solution with Tween 80 (0.01%). The resulting spore suspension was filtered, washed, centrifuged and subjected to a heat shock (80°C, 10 min) and was stored at 4°C. VSC was done by standard plate count method on Nutrient agar and expressed in terms of colony forming units per gram of the substrate (CFU/g).

Table 1: Biochemical composition of pupal waste and silkworm litter.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Constituents</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pupal waste</td>
<td>Silkworm litter</td>
</tr>
<tr>
<td>1</td>
<td>Total carbohydrate</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>Protein</td>
<td>44.9</td>
</tr>
<tr>
<td>3</td>
<td>Lipids</td>
<td>40.0</td>
</tr>
<tr>
<td>4</td>
<td>Others</td>
<td>11.0</td>
</tr>
</tbody>
</table>

et al. (2010) employed synthetic media in their studies and reported the highest number of viable spores in the order of $2 \times 10^8$ CFU/ml within 96 h of incubation. The level of spore count obtained in the present study is comparatively higher than those reported by Valicente et al. (2010) and Adams et al. (2002). Zhuang et al. (2011) reported a cost-effective method to produce Bacillus thuringiensis subsp. kurstaki (Btk) based biopesticides using wastewater sludge as raw-materials under solid-state fermentation (SSF). More than $10^{10}$ CFU/g viable cells of Btk were obtained using sludge or its mixture with agricultural wastes. The mixture of sludge and wheat bran gave the highest yield of viable cells ($5.98 \times 10^{10}$ CFU/g).

Variations in the pH were observed (Fig. 1) at every 24 h interval. The gradual increase in the pH indicated the utilization of the available proteins by the bacteria as the sole source of carbon; resulting in the accumulation of ammonia in the medium, probably due to deamination of the amino acids. The gradual increase in the pH of the media corresponded to the decrease in the protein content during the course of the fermentation. The pH changes however did not adversely affect the growth of the organism. Similar observations have been made by Karthikeyan and Sivakumar (2007). Valicente et al. (2010) employed three different synthetic media in their studies and reported that all the three media showed pH variation during the fermentation process. Medium 1 and 2 showed a tendency to shift toward a basic pH and medium 3 to an acidic pH. The fermentations of the different isolates of Bt, regardless of subspecies, have some general characteristics in common. They all use sugar (usually glucose, molasses, or starch), producing acid during the fermentation. In general, they have similar requirements for proteins or protein hydrolysates, can use ammonium salts, and respond similarly to minerals. However, the individual isolates are unique entities, and a particular medium that may support good growth or toxin production by one isolate may be less satisfactory for another.

Total carbohydrates, lipids and protein changes in the pupal waste and silkworm litter were recorded at every 24 h intervals. As the fermentation proceeded there was a gradual decrease in the protein and lipid content of the substrates, however, no profound changes in the total carbohydrate content were recorded. Within 24 h of incubation, the protein content of the pupal waste was sharply reduced from 44.9% to 40%. Similar effect was observed in case of silkworm litter lowering the protein content from 57.5% to 55%, suggesting the utilization of protein by Bt as the sole carbon source. At 120 h of incubation, the protein content was reduced to 21% and 32% in case of pupal waste and silkworm litter respectively. There was a significant decrease in the lipid content of both the substrates throughout the fermentation process. The lipid content was lowered to 27% and 18% in pupal waste and silkworm litter, respectively, at 120 h of incubation. Mass cultivation studies revealed that pupal waste is a better substrate for Bt cultivation than silkworm litter.

There is tremendous scope of converting sericulture by-products into value added products like biopesticides. Separation of litter and pupal waste is performed by the use of machines which makes the process easier and simple. The litter separator machine is very effective in separating leftover leaves and litter. The cocoon deflossing machine can defloss 50–60 kg cocoons per hour and hand deflossing machine can defloss about 15 kg cocoons per hour. The process employed in the present study is simple, affordable and sustainable.

4. Conclusions

Present study concludes that mass production of Bt employing seriwastes is an excellent method. To the best of our knowledge, this is the first report on utilization of silkworm litter for mass production of Bt. Since litter is generated in enormous quantities; its usage for cultivation of Bt is an economical approach. Use of such substrates represents a better alternative for disposal and/or recycling wastes and minimizes pollution. The study promises to introduce such raw-materials with added advantages of improving economy by adding value to waste. Future prospects in this direction shall include optimization studies and development of Bt formulations.

References