Tropical Biomedicine 30(3): 504–515 (2013)

Potential of sugarcane bagasse (agro-industrial waste) for the production of *Bacillus thuringiensis israelensis*

Poopathi, S.*, Mani, C. and Rajeswari, G.
Unit of Microbiology and Immunology, Vector Control Research Centre (Indian Council of Medical Research), Medical complex, Indira Nagar, Pondicherry – 605006, India
*Corresponding author e-mail: Subbiahpoopathi@rediffmail.com
Received 11 November 2012; received in revised form 21 May 2013; accepted 28 May 2013

Abstract. Sugarcane bagasse is a renewable resource that can be used to produce biopesticide for the control of mosquito vectors. In the present study, we demonstrated that cane processed bagasse could be used to produce *Bacillus thuringiensis* serovar *israelensis* (*Bti*) for control of mosquito vectors *viz*: *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. Biochemical studies indicated that the *Bti* spore/crystal toxins produced from the test culture medium (Bagasse, BG + Soybean, SB) are higher than that from the conventional medium (Nutrient Yeast Extract Salt Medium, NYSM). The bacteria produced in these media (NYSM, BG, SB, BG+SB) were bioassayed against the mosquito species and the toxic effect was found to be effective. Cost-effective analysis indicates that the use of BG and SB, as bacterial culture medium, is successful and economical, for production of this mosquito pathogenic bacillus.

INTRODUCTION

It is known that in public health aspect, mosquito vectors cause great trouble to human beings and pose threat to human health, as vectors of diseases like filariasis, malaria, dengue, Japanese encephalitis, chikungunya, West Nile fever etc. The global population at risk of lymphatic filariasis is estimated to be 1307 million people, transmitted primarily by *Culex* sp., *Mansonia* sp and to some extent by *Anopheles* sp (WHO, 2006). About 50 million people are infected every year by dengue viruses transmitted by *Aedes* sp. with about 24,000 deaths (Kroeger *et al.*, 2006). Nearly five hundred million people are estimated to be affected every year by malaria, transmitted by *Anopheles* sp. with more than two million deaths (Suh *et al.*, 2004). Hence, vector control has a direct impact on the reduction of mosquito-borne diseases. Several techniques have been adopted to control these dipteran vectors to reduce vector-borne diseases. Synthetic insecticides have been effectively used during the past several decades for mosquito control operations, but the chemical approach has several demerits, such as the development of insecticide resistance, environmental pollution, bioamplification of organochlorines in food chain and harmful effects to beneficial insects. Therefore, there has been an increased attention, in recent years, in the use of microbial agents like bacterial agents in vector control programmes.

Microbial organism like *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) being highly toxic to dipteran larvae, opened up the possibility of using this as bio-larvicide in mosquito control programmes (de Barjac & Larget-Thiery, 1984; Goldberg & Margalit, 1997). This mosquito pathogenic bacillus has some advantages over conventional insecticides because it has a narrower host spectrum and thus is safer to non-target organisms (including humans) and is more environmental friendly. Physiologically, the

Bti synthesizes intracellular crystal inclusions by sporulation that contains multiple protein components of 134, 125, 67, and 27 kilo Daltons (Sekar, 1986; Hofte & Whitely, 1989; Federici *et al.*, 1990; Wirth *et al.*, 1998). These proteins have been cloned individually and are toxic to mosquito larvae (Sekar & Carlton, 1985; Delecluse *et al.*, 1991, 1993).

Despite the fact that the high efficacy and specificity of *Bti* is valuable in controlling mosquitoes, the cost to grow and produce *Bti*, through a highly refined laboratory bacterial culture medium, is exorbitant. Since the cost of raw materials for *Bti* production comprises > 70% of the overall production cost, the raw material cost is an important factor to achieve reasonable production cost of *Bti* (Ejiofer, 1991). As a result, selection of growth medium or raw material is significant for commercial production of this biopesticide. So as to encourage the commercial production of biopesticide, utilization of less expensive raw material is worthwhile (Mummigatti & Raghunathan, 1990). Several raw materials (industrial and agricultural by-products) have been tested successfully in mosquito control program, as alternative culture media, for the production of *Bti* (Salama *et al.*, 1983; Obeta & Okafor, 1984; Lee & Seleena, 1991; Ventosilla & Guerra, 1997; Kumar *et al.*, 2000; Poopathi *et al.*, 2002; Poopathi, 2005; Poopathi & Abidha, 2008).

In tropical countries of the world, sugarcane (*Saccharum officinarum*) represents a major crop, because of the increasing demand for sugar in the last century, large areas in the tropical and sub-tropical countries all around the world were allotted for sugarcane crops. Low levels of maintenance and good productivity made sugarcane a commercially attractive crop for farmers in these regions. The major product of sugarcane from sugar processing industries is sugar juice by extraction and the main waste-product is considered to be the bagasse which is an environmental menace (Collier & Arora, 1994; Zanzi *et al.*, 1995; Rezende *et al.*, 2011). Recent approaches are in vogue, for utilizing bulk bagasse waste, which includes production of animal feed, enzymes, pharmaceutical and paper (Rainey, 2009; Maed *et al.*, 2011). "Bagasse" is an extremely inhomogeneous material comprising around 30-40% of "pith" fibre, which is derived from the core part of the plant sugarcane and is mainly parenchyma and bast, rind, or stem fibre, which comprises the balance and is largely derived from sclerenchyma (Rodriguez-Vazquez *et al.*, 1992). The chemical composition of bagasse comprises, cellulose 45-55%, hemi-cellulose 20-25%, lignin 18-24% and waxes <1% (Pandey *et al.*, 2000; Covey *et al.*, 2006).

In the present study, we have successfully developed the agro-industrial waste *i.e.* bagasse (sugarcane waste), as a suitable substrate for the production of bacteria (*Bti*). A judicious combination of bagasse (BG) with soybean (SB) to yield an enhanced level of bacterial toxin, than the conventional culture medium (Nutrient Yeast extract Salt Medium, NYSM) was examined. This combination is vital for the reason that, the carbon and nitrogen (N:C) sources are essential for the proliferation of bacteria (Guo *et al.*, 2010; Thomas *et al.*, 2010). BG contains more of carbon whereas soybean contains more of nitrogen as they are the key components for efficient bacterial proliferation. Therefore, the objectives of the present paper are: (1) to demonstrate that a combination of BG + SB could be used as a cheap source of nutrients to produce *Bti* as a biopesticide, (2) to compare the mosquito toxicity levels of *Bti* produced from the test medium, and (3) to assess the cost-effectiveness of the test medium.

MATERIALS AND METHODS

Bacterial strains

Bacillus thuringiensis serovar *israelensis* (IPS-82) (15,000 ITU mg^{-1} against *Aedes aegypti*) was provided by Dr. J.-F. Charles, Institute Pasteur, Paris, France.

Bacterial culture medium

The conventional culture broth (Nutrient Yeast extract Salt Medium, NYSM), used as reference medium in the present study, was

prepared by mixing (g l^{-1}) glucose, 5, peptone, 5, NaCl, 5, beef extract, 3, yeast extract, 0.5, and mineral salt (mole l^{-1}) $MgCl_2$, 0.1, $CaCl_2$, 0.07, $MnCl_2$, 0.008, (pH 7.5). Sugarcane waste *i.e.* bagasse (BG) was collected from sugar processing industries, air-dried and stored at room temperature (30ºC). Soybean powder received from local area was used as supporting agent for bacterial growth.

Bagasse (BG) was boiled (1%) in ordinary tap water for 15 minutes. After cooling, the extract was carefully separated. The soybean (SB) powder was mixed individually with tap water (1%). Both the extract of BG and SB were dispensed separately into Erlenmeyer flasks (capacity: 2 liter) for culturing *Bti*. The plain medium of respective samples was used as control (without *Bti* inoculation). For combination study the BG extract was combined with SB (1:1) and dispensed separately into flasks for culturing *Bti*. The reference medium (NYSM) was also maintained along with. All the bacterial culture media were pH adjusted (7.5) and autoclaved (at 120ºC / 20 lb / in^2 / 20 min). Adequate replicates from respective culture media (3 each) were maintained.

Bacterial inoculation

Bti lyophilized primary powder (5 mg) was inoculated separately in NYSM, BG, SB and BG+SB media (2 ml each) and allowed to grow for 12 h at 30ºC as seed culture. A known volume of these seed cultures (50 µl each) was inoculated into their respective culture media (250 ml). The cultures were allowed to grow under constant agitation (120 rev / min) at room temperature (30ºC) in an orbital shaker. Culture samples (2.5 ml) were drawn from each culture medium at 6 h intervals from 0 to 72 h. The pH and culture turbidity values were measured using a digital pH meter (Genei, India) and SP75 UV-VIS spectrophotometer (Sanyo, UK) respectively. These bacteria were also examined microscopically (Leica, Germany) for occurrence of *Bti* spore/crystal complex.

Bacterial spore/crystal toxin separation

The spore/crystal toxin of *Bti* in the culture media, were harvested by centrifugation process (10,000 x g / 30 min / 4ºC) using SORVALL Evolution RC super speed centrifuge (Kendro, USA), and the culture supernatants were discarded (Payne & Davidson, 1984). The pellets (cell mass) containing spore/crystal complex were thoroughly washed by centrifugation with 0.1 M NaCl and sterile double distilled water (10,000 x g / 15 min / 4ºC). This purified spore/crystal complex (biomass) was weighed and at the end, the spore/crystal toxin complex was subjected to treatment with protease inhibitor (phenyl methyl sulphonyl fluoride, 1mM, Sigma) re-suspended in distilled water and stored at – 20ºC, until further use (Poopathi *et al.*, 2002; Poopathi & Abidha, 2008).

Protein estimation

The intensity of protein in the samples is an estimate of presence of *Bti* toxins (Lecadet *et al.*, 1999; Thiery *et al.*, 1992), which was determined using bovine serum albumin (BSA) as the standard (Lowry *et al.*, 1951). A small quantity of the *Bti* spore/crystal complex was centrifuged (10,000 x g / 30 min / 4ºC) and the pellets were solubilized in alkaline buffer ($NaHCO_3$, 50 mM, dithiothreitol, 10 mM, pH 10) and incubated for 3 h at 30ºC. After centrifugation and extraction, the solubilized protein was quantified using UV-VIS spectrophotometer (optical density at 620 nm).

SDS-PAGE

A total of 10 µg protein equivalent samples (*Bti* spores/crystals) from test and reference media were mixed with equal volumes of sample buffer (β-mercaptoethanol, 0.5M and SDS, 3%), boiled for 5 minutes and separated by electrophoresis on 10% sodium dodecyl sulphate- polyacrylamide gel (SDS-PAGE) unit (Genei, India) (Lammeli, 1970). The protein bands were stained with Coommasie Brilliant Blue R-250 and visualized.

Toxicity assay (Bioassays)

Larvicidal activity of *Bti* samples were tested against early third instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. These larvae were earlier indented from Division of Mosquito Rearing and Colonization (R & C), Vector Control

Research Centre, Puducherry, India. Bioassays for *Bti* were carried out as per WHO recommendations (WHO, 1985). A stock *Bti* solution was prepared (100 mg l^{-1}) and homogenized, using glass beads, in a mechanical shaker. Up to seven dilutions from stock (2-fold) were made to obtain dosages ranging from (mg l^{-1}) 0.001 to 0.05. Bioassays were conducted in disposable wax coated paper cups (350 ml capacity). Test media were prepared by adding appropriate volumes of *Bti* in 300 ml of tap water, into which, 25 early third instar larvae from each of the three mosquito species were introduced separately. Food supplement (dog biscuit and yeast mixture, 2:1) was not given to *Bti* treated larvae, as per WHO recommendation. The bioassays for all three larval species of mosquitoes were replicated three times simultaneously for each toxin dose. The experiments were repeated thrice on different days. Bioassays were conducted at room temperature (30°C) and larval mortality was monitored after 24 h. Control mortality was corrected (Abbott, 1925). Moribund larvae (if any) in the replicates were counted as dead.

Statistical analysis

The growth and sporulation data of *Bti* cultured from the test (BG, SB and BG+SB) and control (NYSM) medium were subjected to students 't' test, to analyze the significance of difference ($P \leq 0.05$). The LC_{50} and LC_{90} values were calculated by regression analysis using SPSS software package ASSAY.

RESULTS

Growth pattern and biomass production

The results obtained from the studies on the growth pattern and the rate of spore production of *Bti* in the experimental (BG, SB and BG+SB) and in the reference culture media (NYSM) are shown in Fig. 1. In all the culture media, after a lag phase of about an hour, there was a rapid multiplication of bacterial cells and maturation of spores. As the culture time increased, there was a corresponding increase in the culture density, as indicated by the optical density at 650 nm which reached an OD of 1.0 to 1.4 (BG+SB). This multiplication process lasted until 48th hour, followed by lysis of the cells, which released the toxins into the culture media. The growth and formation of spore/crystal toxins was found to be high at 72 hours. This indicated that the overall growth and production of *Bti* spore/crystal toxins in the experimental medium (BG+SB) was higher than NYSM, while BG and SB were lower than NYSM.

Further, it is specific to mention that, in all the three experimental culture media, an appreciable amount of spores/crystal toxins were released between 60 and 72 hours of peak growth period (Fig. 1). Microscopic observation showed the complete proliferation of bacteria, (spore/crystal complexes), which indicated the full utilization of carbon and nitrogenous sources present in the experimental media (Figure not shown). The growth characteristics (optical density at 650nm) showed that the range for NYSM was 0.79 to 1.13, on the other hand, for the combination medium (BG+SB), it was 0.95 to 1.43, which indicated that the latter had the maximum bacterial growth rate ($P<0.05$). The growth rate of SB was very close to that of NYSM ($P>0.05$), whereas, BG alone showed a minimum growth. This is due to the lesser degree of sporulation, whereas, a combination strategy of BG with SB exhibited a higher growth rate, in comparison with all other culture media. The biomass production, an indicator of bacterial growth, was also high for the combination medium (43.6 g l^{-1}) than for NYSM (27.5 g l^{-1}) (Fig. 2).

Toxin concentration

Quantitative estimation of protein from bacterial samples, an indicator of toxin production was conducted by standard methods. The toxin (protein) concentration of *Bti* from various media, exhibited the following trend, viz., BG+SB>NYSM>SB>BG (Fig. 3). This showed that the combination medium, BG+SB had the optimum spore/crystal toxin production (1.77g l^{-1}). Further, the overall growth and production of toxins

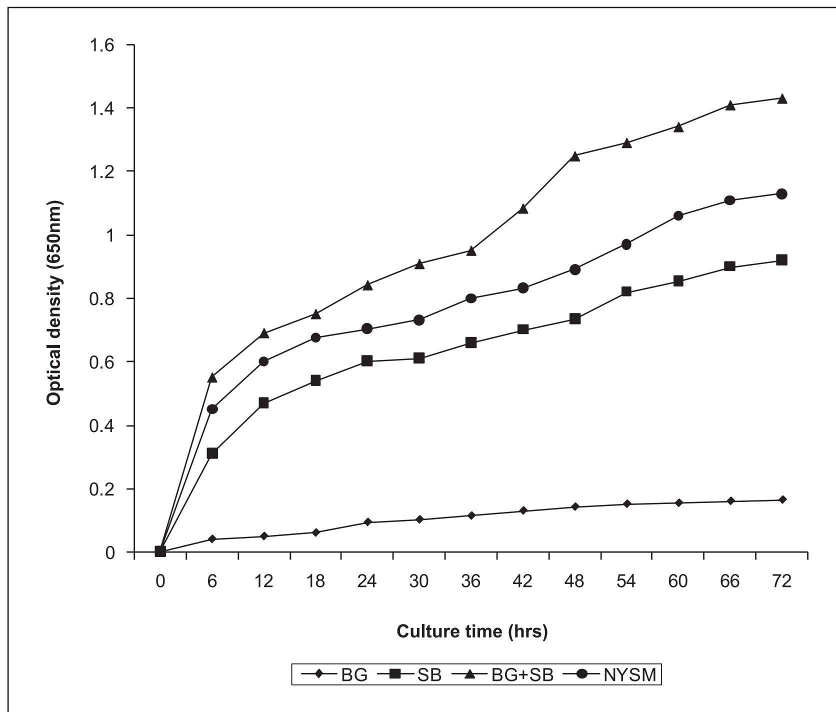


Figure 1. Growth pattern of *Bti* in different culture media

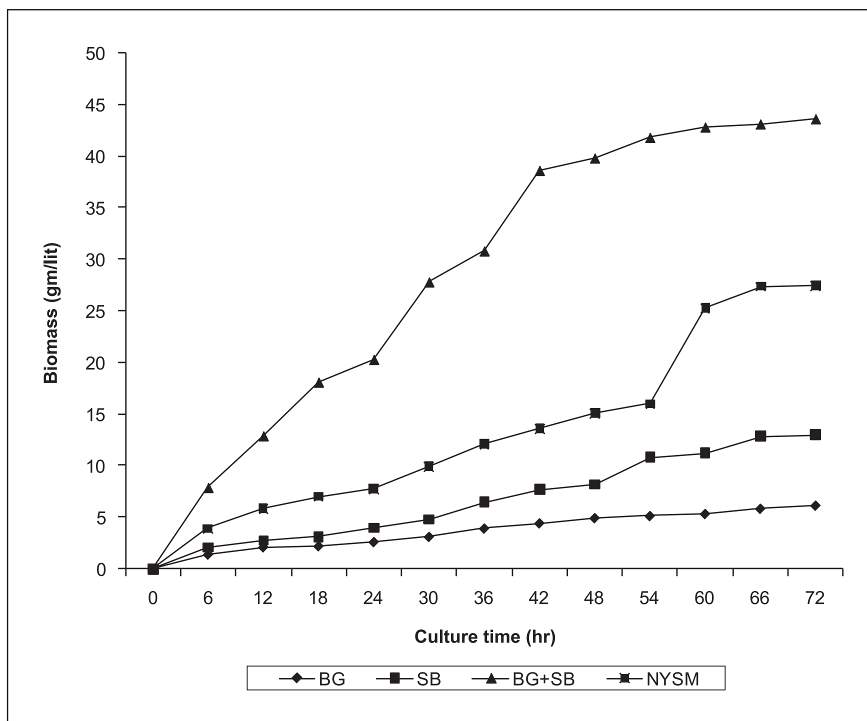


Figure 2. Biomass production of *Bti* produced from different culture media

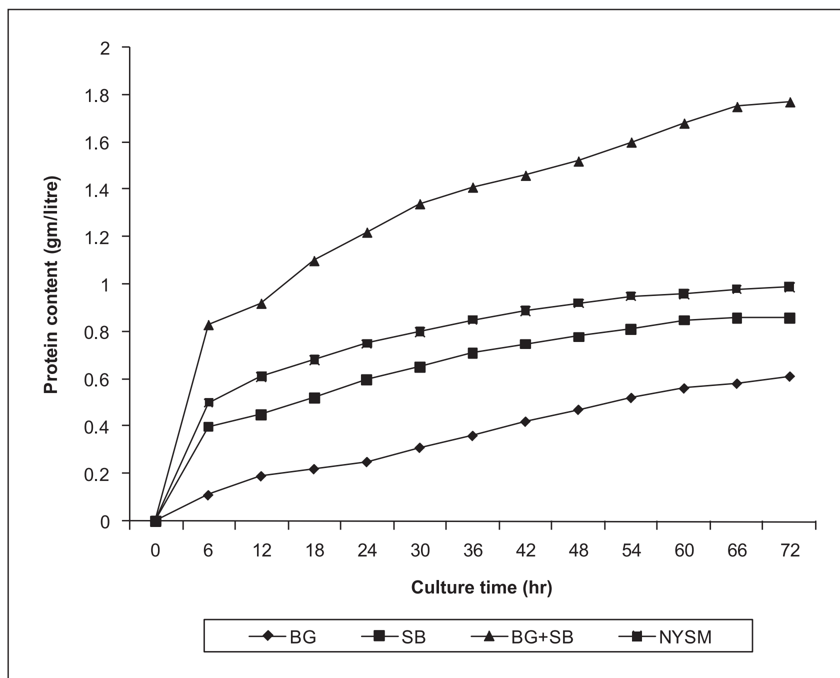
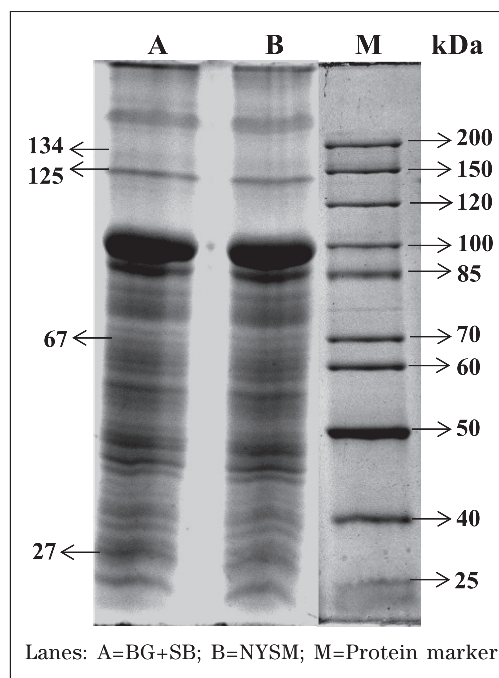


Figure 3. Dynamics of mosquitocidal toxin produced from *Bti*

in the SB medium was statistically similar to that of NYSM, as the mean values were not significantly different (P>0.05).

Protein profiles

The protein profiles of bacterial samples were estimated by SDS-PAGE analysis, which is the standard technique for qualitative determination of proteins. In the present study, the protein profile of *Bti* spore/crystal complex produced from the new (BG+SB) and the reference culture medium (NYSM) were analyzed by 10% SDS-PAGE, using a small quantity of bacterial sample (10 µg) and the results were compared (Fig. 4). The major polypeptides present in the parasporal crystal proteins of *Bti* (*Bti*: 134, 125, 67 and 27 kDa proteins) produced from the test and reference media (BG+SB and NYSM respectively) were clear, conspicuous and identical. The protein profiles, an indicator of *Bti* toxins, were consistent with their larvicidal activity in the laboratory bioassays.



Lanes: A=BG+SB; B=NYSM; M=Protein marker

Figure 4. SDS-PAGE analysis of *Bti* toxins isolated from experimental and control media

Table 1. Toxicity of *Bacillus thuringiensis* serovar *israelensis* (IPS-82) produced from various media against mosquito larvae

Mosquito species	Intercept	Slope ± SE	LC_{50} (mg l^{-1})	LC_{90} (mg l^{-1})	χ^2 (df)
Culex quinquefasciatus					
A	9.08	0.68 ± 0.22	0.004 (0.002–0.006)*	0.024 (0.012–0.047)	7.53 (6)
B	9.16	0.69 ± 0.23	0.003 (0.002-0.004)	0.021 (0.009–0.031)	8.11 (6)
C	9.11	0.70 ± 0.20	0.004 (0.002-0.048)	0.023 (0.015-0.048)	2.56 (6)
D	9.22	0.71 ± 0.20	0.003 (0.002–0.04)	0.019 (0.015-0.031)	2.0 (6)
Anopheles stephensi					
A	6.70	0.70 ± 0.19	0.062 (0.052–0.075)	0.353 (0.253–0.93)	2.58 (6)
B	7.02	0.72 ± 0.21	0.06 (0.047–0.072)	0.38 (0.28–0.69)	1.98 (6)
C	7.13	0.75 ± 0.25	0.08 (0.05–0.085)	0.358 (0.25–0.91)	2.01 (6)
D	7.19	0.76 ± 0.19	0.07 (0.04–0.078)	0.36 (0.23–0.84)	2.30 (6)
Aedes aegypti					
A	7.92	0.59 ± 0.27	0.096 (0.058–1.12)	2.15 (1.41–3.49)	2.33 (6)
B	5.02	0.60 ± 0.19	0.095 (0.051–1.22)	2.98 (1.33–3.50)	2.36 (6)
C	5.16	0.74 ± 0.18	0.88 (0.045–1.20)	2.56 (1.26–5.03)	2.48 (6)
D	4.57	0.81 ± 0.18	0.85 (0.054–1.26)	2.41 (1.46–4.60)	2.58 (6)

Bacillus thuringiensis serovar *israelensis* culture medium: A = Bagasse (BG); B = Soybean (SB); C = GB+SB; D = NYSM.
*95% Fiducial limits of lower and upper at LC_{50} and LC_{90} levels.

Toxicity assays

Protein concentration in the sample is an estimate of toxin production of *Bti*. Larval toxicity assays (bioassays) with mosquito larvae were performed with *Bti* toxins produced from the test and reference culture media. Laboratory reared mosquito larval species of *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* were used for bioassays. The comparative toxicities of *Bti* produced from the culture media were shown in Table 1. The LC_{50} and LC_{90} values for *Bti* from NYSM against *Cx. quinquefasciatus* were 0.0030 and 0.019 (mg l^{-1}). These toxicity values were statistically similar to those of *Bti* produced from all three experimental media (BG+SB, BG, SB), since, fiducial limits (95%) were overlapping. Similar toxic effects were seen with *Bti* from test and reference media, against other mosquito species (*An. stephensi* and *Ae. aegypti*).

DISCUSSION

The above results, revealed that, the new combination medium (BG+SB), resulted in good bacterial growth, sporulation, toxin and biomass production, which indicated that they are suitable substrates for the growth of mosquitocidal bacteria. The entomotoxicity levels of *Bti* (LC_{50} and LC_{90}) between the test and reference culture media in the present study were consistent with each other and within the standard toxicity limit. It is understood that, the toxicity levels (LC_{50} and LC_{90}) of *Bti* using several conventional media (Luria Bertani, Nutrient Broth, Poly-medium, MBS-medium etc) corroborated with the recommended range of lethal concentration levels, as each bacterial strain has its own relative potency with specific titre values compared with the *Bti* standard IPS-82 possessing 15,000 ITU/mg against *Ae. aegypti*.

Cost - analysis studies indicated that the quantity of bagasse and soybean needed to prepare one liter of culture medium was 10 g respectively, which is of negligible value, as they are agro-by products. On the other hand, preparation of one liter of reference culture medium (NYSM), involves a cost of US $4.26. Thus this new combination medium (BG+SB) is economical, owing to its easy availability globally as an environmental, bio-organic and agro-industrial waste.

It is known that industrial biotechnology offers, potential opportunities for economic utilization of agro-industrial residues like sugarcane bagasse which is the major waste of the sugarcane industry. It contains 45-55%,

hemicellulose 20-25%, lignin 18-24% and waxes <1% (Pandey *et al.*, 2000). Due to its abuntant availability, it can serve as an ideal substrate for microbial processes for the production of value-added products. Attempts have been made to produce protein enriched animal feed, enzymes, amino acids, organic acids and compounds of pharmaceutical importance from bagasse substrate protein (Maed *et al.*, 2011). Application of solid state fermentation technology has recently been reported as an attractive method for bio-conversion of bagasse (Bhattacharya *et al.*, 2011). In the present paper, emphasis has been given on development on the production of biopesticide from extract of bagasse for the control of mosquito vectors in public health programme.

Considering the fact that mosquito-borne diseases continue to be a grave global, public health problem, mosquito control is an essential component of disease control which mainly relied on the use of chemical insecticides, inspite of its toxicity to non-target organisms and resistance development. The discovery of biopesticides (*Bti*) over the conventional insecticides revolutionized the mosquito eradication programmes. These biopesticides could also be used in those countries, where synthetic insecticides could not be used against mosquitoes. The high cost of conventional media components to produce these biopesticide on a large scale, makes it imperative to utilize cheap and commonly available biological waste materials, through simple fermentation technology.

In the past, many *Bti* formulations produced from conventional media (LB, NB, NYSM, MBS, BATS, UG and HCT etc.) have been used for mosquito control (de Barjac & Lecadet, 1976; Kalfon *et al.*, 1983; Yousten *et al.*, 1985). As most of them were expensive, this initiated the utilization of cost-effective formulations for biopesticide production. Reports indicated that *Bti* was cultured on formulated media from seeds of legumes (groundnut cake, cow pea of white and black varieties, bambara beans), dried cow blood and mineral salts (Saalma *et al.*, 1983; Obeta & Okafor, 1984). Some other authors have made use of potatoes, coconuts, fishmeal, cornsteep liquor and chicken feathers for the production of bio-pesticides (*Bacillus sphaericus* and *Bti*) (Foda *et al.*, 1983; Salama *et al.*, 1983; Ventosilla & Guerra, 1997; Poopathi *et al.*, 2002; Poopathi & Abidha, 2008; Kuppusamy, 1990, Lee & Seleena, 1991).

The present fermentation technology where we employed agricultural wastes as substratum for mosquitocidal bacteria production indicated that, the combination medium of BG+SB had a higher growth rate, sporulation, toxin and biomass production than the reference medium (NYSM) and it was in the order: BG+SB>NYSM=SB>BG. Quantitative estimation of protein from bacterial sample, an indicator of toxin production was done by standard method (Lowery *et al.*, 1951) and has been recommended by several researchers (Lecadet *et al.*, 1999; Thiery *et al.*, 1992). SDS-PAGE analysis, the standard technique, for qualitative determination of proteins, was followed to visualize the protein profiles of bacterial sample (Tinelli & Bourgouin, 1982; Nicolas & Dossou-Yovo, 1987; Rabinovitch *et al.*, 1995; Silva-Filha *et al.*, 1999). The major polypeptides present in the parasporal crystal proteins of *Bti*, from the test and reference media were identical.

Toxicity assays against disease transmitting mosquito vectors (*Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti*) showed that, the bacterial toxins, produced from all the culture media were effective against the mosquito larvae and their lethal values were within the limit of specific toxicity range (LC_{50} and LC_{90}). These results were in accordance with the earlier report (WHO,1985). The entomotoxicity levels (LC_{50} and LC_{90}) of *Bti* from other conventional media (Luria Bertani, Nutrient Broth, Poly medium, MBS-medium etc), also fall within the lethal dose range (Kalfon *et al.*, 1983; Bourgoung *et al.*, 1984; Charles *et al.*, 1988; Schenkel *et al.*, 1992; Manasherob *et al.*, 1996; Thiery *et al.*, 1997; Kwa *et al.*, 1998; Skovmand *et al.*, 1998). The cost of *Bti* as biopesticide production depends on many factors, however, the raw material cost is one of the most important criteria which comprises >70 % of the overall production

cost (Ejiofer, 1991). Therefore, selection of growth medium or raw material is critical for economical production of these bio-pesticides which depends on the utilization of less expensive raw material (Mummigotti & Raghunathan, 1990). Further, to ensure the quality control in the present study, necessary precautionary measures were adopted as per recommendations (Burges *et al*., 1982).

The findings from the present study permit us to conclude that the combination of bagasse and soybean are suitable substrates, for the production of mosquito pathogenic bacillus (*Bti*). Consequently, this cost–effective technology is more than ever useful, in continents, where mosquito control programmes are ongoing.

Acknowledgements. To Department of Science and Technology, Government of India, for funding (DST F.NO: SR/FTP/LS-A-86/2001), to the Director, Vector Control Research Centre (ICMR), Pondicherry, for permission, to Dr. J-F. Charles, Research Scientist, Bacteries Entomopathogenes, Institute Pasteur, Paris, France for supply of bacterial strain and to Smt. R. Sundarammal, Senior Library Information Officer, VCRC, Pondicherry for supply of literatures.

REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**: 65-267.

Bhattacharya, S., Bhardwaj, S., Das, A. & Anand, S. (2011). Utilization of sugarcane bagasse for solid-waste fermentation and characterization of α-amylase from *Aspergillum flavus* from Muthupettai mangrove, Tamil Nadu. *Australian Journal of Basic Applied Science* **5**: 1012-1021.

Bourgoung, C., Larget-Thiery, I. & de Barjac, H. (1984). Efficacy of dry powders from *Bacillus sphaericus:* RB 80, a potent reference preparation for biological titration. *Journal of Invertebrate Pathology* **44**: 146-150.

Burges, H.D., Krieg, A., Luthy, P. & de Barjac, H. (1982). Guidelines for safety tests and registration of bacterial pesticides. *Entomophaga* **27**: 225-236.

Charles, J.F., Kalfon, A., Bourgouin, C. & de Barjac, H. (1988). *Bacillus sphaericus* asporogenous mutants: morphology, protein pattern and larvicidal activity. *Annals of Institute Pasteur (Microbiology)* **139**: 243-259.

Collier, B.J. & Arora, M.S. (1994). Water pre-treatment and alkaline treatment for extraction of fiber from sugarcane rind. *Clothing and Textiles Research Journal* **14**: 1-6.

Covey, G.R., Thomas, J. & Shore, D. (2006). The potential for bagasse pulping in Australian *Appita Journal* **59**: 17-21.

de Barjac, H. & Lecadet, M.M. (1976). Biochemical determination of *B. thuringiensis* thermostabile, exotoxin, using the inhibition of bacterial RNA polymerases. *C.R. Academic Science D* **282**: 2119-2122.

de Barjac, H. & Larget-Thiery, I. (1984). Characteristics of IPS-82 as standard for biological assay of *Bacillus thuringiensis* H-14 preparations. *WHO Mimeograph Document*, VBC/84892, Geneva, Switzerland.

Delecluse, A., Charles, J.F., Klier, A. & Rapoport, G. (1991). Deletion by *in vivo* recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. *Journal of Bacteriology* **173**: 3374-3381.

Delecluse, A., Poncet, S., Klier, A. & Rapoport, G. (1993). Expression of cryIV A and cryIV B genes independently or in combination in a crystal negative strain of *Bacillus thuringiensis* subsp. *israelensis*. *Applied Environmental Microbiology* **59**: 3922-3927.

Ejiofor, A.O. (1991). Production of *Bacillus thuringiensis* serotype H-14 as bio-insecticide using a mixture of 'spent' brewer's yeast and waste cassava starch as the fermentation medium. *Discovery Innovation* **3**: 85-88.

Federici, B.A., Luthy, P. & Ibarra, J.E. (1990). Parasporal body of *Bacillus thuringiensis* var *israelensis*, structure, protein composition and toxicity. In: Bacterial control of mosquitoes and black flies (Editors, H. de Barjac. & D.J. Sutherland) pp 6-44. Rutgers University Press, New Jersey.

Foda, M.S., Dulmage, H.T. & El-Sharaby, A. (1983). Novel fermentation medium for production of delta-endotoxin from *Bacillus thuringiensis*. *Journal of Invertebrate Pathology* **41**: 8-19.

Goldberg, L.J. & Margalit, J.A. (1977). Bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. *Mosquito News* **37**: 355-358.

Guo, Y., Yan, Q., Jiang, Z., Teng, C. & Wang, X. (2010). Efficient production of lactic acid from sucrose and corncob hydrolysate by a newly isolated *Rhizopus oryzae* GY18. *Indian Journal of Microbiology and Biotechnology* **37**: 1137-1147.

Hofte, H. & Whiteley, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Review*. **53**: 242-255.

Kalfon, A., Larget-Thiery, I., Charles, J.F. & de Barjac. (1983). Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. *European Journal of Applied Microbiology and Biotechnology* **18**: 168-173.

Kroeger, A., Nathan, M.B., Hombach, J., Dayal-Drager, R. & Weber, M.W. (2006). Dengue research and training supported through the World Health Organization. *Annals of Tropical Medicine and Parasitology* **100** Suppl 1: S97-S101.

Kumar, A., Sra, K., Sangodkar, U.M.X. & Sharma, V.P. (2000). Advances in the bio-control of mosquito vectors utilizing *Bacillus sphaericus* and *Bacillus thuringiensis* var. *israelensis*. *Proceedings of National Academy of Sciences (India)* **LXX**: 1-20.

Kuppusamy, M. (1990). Studies based on the production, formulation and by-products of *Bacillus thuringiensis* H-14 and *Bacillus sphaericus* H-5A5B. PhD thesis, Vector Control Research Centre, Pondicherry, India.

Kwa, M.S., de Maagd, R.A., Stiekema, W.J., Vlak, J.M. & Bosch, D. (1998). Toxicity and binding properties of the *Bacillus thuringiensis* delta-endotoxin Cry1C to cultured insect cells. *Journal of Invertebrate Pathology* **71**: 121-127.

Lammeli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lecadet, M.M., Frachon, E., Dumanoir, V.C., Ripouteau, H., Hamon, S. & Laurent, P. (1999). Updating the H-antigen classification of *Bacillus thuringiensis*. *Journal of Applied Microbiology* **86**: 660-672.

Lee, H.L. & Seleena, P. (1991). Fermentation of a Malaysian *Bacillus thuringiensis* serotype H14 isolate, a mosquito microbi-al control agent using local wastes. *Southeast Asian Journal of Tropical Medicine and Public Health* **22**: 108-112.

Lowry, O.H., Rosebrough, N.J., Far, A.L. & Randall, R.L. (1951). Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry* **193**: 265-275.

Maed, R.N., Serpa, V.I., Rocha, V.A.L., Santa, L.M.M., Castro, A.M. & Driemeier, C.E. (2011). Enzymatic hydrolysis of pretreated sugarcane bagasse using *Pencillium funiculosum* and *Trichoderme harzianum cellulases*. *Process Biochemistry* **46**: 1196-1201.

Manasherob, R., Ben-Dov, E., Margalit, J., Zaritsky, A. & Barak, Z. (1996). Raising activity of *Bacillus thuringiensis* var. *israelensis* against *Anopheles stephens*i larvae by encapsulation in *Tetrahymena pyriformis (Hymenostomatida: Tetrahymenidae)*. *Journal of American Mosquito Control Association* **12**: 627-631.

Mummigatti, S.G. & Raghunathan, N. (1990). Influence of media composition on the production of δ-endotoxin by *Bacillus thuringiensis* var. *thuringiensis*. *Journal of Invertebrate Pathology* **55**: 147-151.

Nicolas, L. & Dossou-Yovo, J. (1987). Differential effects of *Bacillus sphaericus* strain 2362 on *Culex quinquefasciatus* and its competitor *Culex cinereus* in West Africa. *Medical and Veterinary Entomology* **1**: 23-27.

Obeta, J.N. & Okafor, N. (1984). Medium for the production of primary powder of *Bacillus thuringiensis* subsp. *israelensis*. *Applied Environmental Microbiology* **47**: 863-867.

Pandey, A., Soccol, C.R., Nigam, P., Brand, D., Mohan, R. & Roussos, S. (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal* **6**: 153-162.

Payne, J.M. & Davidson, E.W. (1984). Insecticidal activity of crystalline parasporal inclusions and other components of *Bacillus sphaericus* 1593 spore complex. *Journal of Invertebrate Pathology* **43**: 383-388.

Poopathi, S., Nielsen Le-Roux, C. & Charles, J.-F. (2002). Alternative methods for preservation of mosquito larvae to study binding mechanism of *Bacillus sphaericus* toxin. *Journal of Invertebrate Pathology* **79**: 132-134.

Poopathi, S. & Abidha, S. (2008). Biodegradation of poultry waste for the production of mosquitocidal toxins. *International Journal of Biodeterioration and Biodegradation* **62**: 479-482.

Poopathi, S. (2005). Microbial fermentation process from bird feather for the production of bio-pesticides Indian Patent Application No 319/Del/2005.

Rabinovitch, L., de Jesus, F.F., Cavados, C.F., Zahner, V., Momen, H. & de Silva, M.H. (1995). *Bacillus thuringiensis* subsp. *oswaldocruzi* and *Bacillus thuringiensis* subsp. *brasiliensis*, two novel Brazilian strains which determine new serotype H38 and H39, respectively. *Memorias Instituto Oswaldo Cruz* **90**: 649-651.

Rainey, A. (2009). A study into the permeability and compressibility properties of baggase pulp. PhD Thesis, Queensland University of Technology.

Rezende, C.A., de Lima, M.A., Maziero, P., Deazevedo, E.R., Garcia, W. & Polikarpov, I. (2011). Chemical and morphological characterization of sugarcane bagasse submitted to a delignification process for enhanced enzymatic digestibility. *Biotechnology for Biofuels* **4**: 54 doi:10.1186/1754-6834-4-54.

Rodriguez-Vazquez, R., Villanueva-Ventura, G. & Rios-Leal, E. (1992). Sugarcane bagasse pith dry pretreatment for single cell protein production *Bioresource Technology* **39**: 17-22.

Saalma, H.S., Foda, M.S., Dulmage, H.T. & al-Sharaby, A. (1983). Novel fermentation medium for production of delta-endotoxin from *Bacillus thuringiensis*. *Journal of Invertebrate Pathology* **41**: 8-19.

Schenkel, R.G., Nicolas, L., Frachon, E. & Hamon, S. (1992). Characterization and toxicity to mosquito larvae of four *Bacillus sphaericus* strains isolated from Brazilian soils. *Journal of Invertebrate Pathology* **60**: 10-14.

Sekar, V. & Carlton, B.C. (1985). Molecular cloning of the delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. *Gene* **33**: 151-158.

Sekar, V. (1986). Biochemical and immunological characterization of the cloned crystal toxin of *Bacillus thuringiensis* var *israelensis*. *Biochemistry Biophysics and Research Communication* **137**: 748-751.

Silva-Filha, M.H., Nielsen-LeRoux, C. & Charles, J.F. (1999). Identification of the receptor for *Bacillus sphaericus* crystal toxin in the brush border membrane of the mosquito *Culex pipiens* (Diptera: Culicidae). *Insect Biochemistry and Molecular Biology* **29**: 711-721.

Skovmand, O., Thiery, I., Benzon, G.L., Sinegre, G., Monteny, N. & Becker, N. (1998). Potency of products based on *Bacillus thuringiensis* var. *israelensis*: inter laboratory variations. *Journal of American Mosquito Control Association*. **14**: 298-304.

Suh, K.N., Kain, K.C. & Keystone, J.S. (2004). Malaria. *Canadian Medical Association Journal* **170**: 1693-1702.

Thiery, I., Ofori, J., Dumanoir, V.C., Hamon, S. & de Barjac, H. (1992). New mosquitocidal strains from Ghana belonging to serotypes H3, H6 and H48 of *Bacillus sphaericus. Applied Microbiology and Biotechnology* **37**: 718-722.

Thiery, I., Baldet, T., Barbazan, P., Becker, N., Junginger, B. & Mas, J.P. (1997). International indoor and outdoor evaluation of *Bacillus sphaericus* products: complexity of standardizing outdoor protocols. *Journal of American Mosquito Control Association* **13**: 218-226.

Tinelli, R. & Bourgouin, C. (1982). Larvicidal toxin from *Bacillus sphaericus* spores: isolation of toxic components. *FEBS Letters* **142**: 155-158.

Thomas, J.E., Parry, J.N., Schwinghamer, M.W. & Dann, E.K. (2010). A novel mastreviruses from chickpea (*Cicer arietinum*) in Australia. *Archives of Virology* **155**: 1777-1788.

Ventosilla, P. & Guerra, H. (1997). Pilot production using whole coconuts and application in the field of *Bacillus thuringiensis* var. *israelensis* for biological control of *Anopheles* in malaria endemic areas in Peru. *Revista de Medicina Exp. Del. Instituto Nacional de Salud Segunda Epoca* **15**: 61-70.

Wirth, M.C., Delecluse, A., Federici, B.A. & Walton, W.E. (1998). Variable cross-resistance to *Cry* 11B from *Bacillus thuringiensis* subsp. *jegathesan* in *Culex quinquefasciatus* (Diptera: Culicidae) resistant to single or multiple toxins of *Bacillus thuringiensis* subsp. *israelensis. Applied Environmental Microbiology* **64**: 4174-4179.

WHO. (1985). Informal consultation on the development of *Bacillus sphaericus* as a microbial larvicide. TDR/BVC/ sphaericus; 85.3:1.

WHO. (2006). Global program to eliminate lymphatic filariasis. *Weekly Epidemiological Records*, WHO, Geneva. **81**: 221-232.

Yousten, A.A., Fretz, S.B. & Jelley, S.A. (1985). Selective medium for mosquito-pathogenic strains of *Bacillus sphaericus. Applied Environmental Microbiology* **49**: 1532-1533.

Zanzi, R., Sjostrom, K. & Bjornbom, E. (1995). Rapid pyrolysis of bagasse at high temperature. Proceedings of Third Asia-Pacific International Symposium on Cumbustion Energy utilization, Hong Kong **1**: 211-215.