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Cost Effective Production of *Bacillus thuringiensis israelensis* using Rotten Pineapples

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Abstract: ***Vector-borne ailments are illnesses that are transmitted by way of vectors. These vectors can elevate infective pathogens such as viruses, bacteria and protozoa from one host to another. For the prevention of these diseases, vector management could be a powerful preventive tool. Use of biopesticide is safer than use of chemicals or chemical derived pesticides posing low risk to environment and humans. Microbial insecticide is a most extensively used biopesticide. Bacillus thuringiensis israelensis (Bti) serves as an ideal candidate for mosquito control due to the fact of its excessive mosquito larvicidal activity, environmental friendliness, safety to non-target organisms and mammals, ease of production and ability to withstand various formulations like a dust, pellets, sprays, briquettes, capsules, granules, etc. In the present study, using pineapple juice as substrate, effect of submerged fermentation, solid state fermentation, batch fermentation and fed batch fermentation on the cultivation of Bti was investigated. Compared with batch fermentation, fixed volume fed-batch fermentation showed increase in biomass by 23.8%. 80 % solid state fermentation showed maximum biomass among all the cultivation. Studies have also been conducted using cheap industrial substrate together with chicken manure, fish waste, urea and soybean powder as nitrogenous sources which will greatly help in cutting down production cost of Bti. Finally, biolarvicidal activity of the Btiwere also evaluated using Aedes aegypti. 70-98 % mortality was also observed in pineapple extract media supplemented with different nitrogenous wastes.***

Keywords: *Bacillus thuringiensis israelensis*, Fed batch fermentation, Batch fermentation, Fixed volume fed-batch fermentation, Solid-state fermentation, Biopesticide

1. Introduction

Mosquito bites no longer remains as a nuisance and pain it additionally transmits some dreadful illnesses such as Dengue fever, chickungunya, malaria, etc. Mosquitoes cause enormous human suffering than any other organisms - over 700 million human beings die from mosquito borne ailments each year (WHO 2021). The control of vector borne diseases can make a fundamental contribution to poverty reduction, as it mostly affects the poor. Doing so is especially vital for illnesses like a dengue and Chikungunya, which have neither a vaccine nor an effective treatment. Mosquito borne diseases exert a huge burden of mortality and morbidity. Main methods for mosquito control include elimination or management of larval habitats using biological or chemical agents. Biological control of mosquito vectors have advantage of safety to environment and other non-target organisms, and there is less risk of mosquito resistance developing.

Batch fermentation is a 'closed system' where the substrate and producing microorganism are added to the system and are not removed until the fermentation is complete. Another way of keeping nutrients from becoming a limiting factor is to have intermittent additions of substrate during cultivation. This is called fed-batch fermentation, which is a partly open system. Solid state fermentation (SSF) have been described as the process that takes place in a solid matrix (inert support) in the absence or near absence of water.

Bti production can be done by the optimization of a suitable medium. The cost to develop and produce *Bti*, through refined laboratory bacterial culture medium is high. The cost of *Bti* production relies upon many factors; however, the raw material cost is one of the most essential criteria, which may compromise greater than 70% of the whole manufacturing cost (Bravo et al. 2007). Another component responsible for the greater cost of *Bti* manufacturing is the limitation caused by catabolite repression which restricts biomass productivity. Catabolite repression can be overcome the usage of fed-batch fermentation (Butko P. 2003). Fed-batch fermentation is a manufacturing approach in between batch and continuous fermentation. In view of increase in productivity, it is utilized for the manufacturing of penicillin and other industrial products (C. gopinathan et al. 2016). In this work, experiments have been executed by use of fixed-volume fed-batch fermentation. The main advantages of fed batch fermentation over conventional batch mode of fermentation are production of high cell densities, control over the production of by-products or catabolite repression effects, the mode of operation can overcome and control deviations in the organism's growth pattern as found in batch fermentation, allows the replacement of water loss by evaporation and no special equipment is required as compared with the batch fermentation mode of operation. In fixed volume fed-batch, volume of the culture medium is kept constant, used media is replaced through equal volume of fresh media at certain time interval (Chen et al. 2013). This helps in diluting the inhibitory

by-products or metabolites and will enhance the biomass production. Raw materials for the fermentation process needs to be less expensive, feasible and has to be utilized efficiently (Chilcott et al. 1990).

2. Materials and methods

Preparation of pineapple extract powder (PEP): PEP was prepared by adding 100 g of rotten pineapple into 100 ml of distilled water. It was then boiled for 10 minutes at 100^0C. Then, the solution was filtered using whatsman filter paper and the filtrate was then kept in a hot air oven at 80^0C for 24 hours.

Preparation of inoculum: *Bacillus thurengiensisisraelensis* (MTCC 869) was purchased from IMTECH, Chandigarh, India and was preserved in deep freezer. For inoculum preparation, the frozen culture was thawed by keeping it in water bath at 30ºC. Aloopfull of culture was then transferred to 100 ml mGYS media and was incubated at 30ºC for 20 hour when optical density reaches 2.5-3.0. The culture was then centrifuged for 10 minutes at 3500 rpm at 10ºC. the pellet was then diluted with mGYS media to reach approximately a concentration of 10^8 CFU/ml (ester et al., 2022 & Demarjac & larget. 1984). Composition of mGYS media is as shown in table 1. The culture was then used as standard inoculum for all experiments (Dulmage. 1970).

Table 1: mGYS media composition

Chemical Constituents	Percentage Composition
Glucose	0.3
Ammonium sulphate	0.2
Di-potassium hydrogen phosphate	0.5
Yeast extract	0.2
Magnesium sulphate	0.02
Calcium chloride	0.008
Manganese sulphate	0.005
pH	7.2

Substrate repression studies using glucose as substrate: In order to assess the maximum level of carbon source which can be used in batch fermentation without substrate repression, this experiment was done using glucoseas carbon source. The concentrations of glucose ranged from 1-10%. Peptone (0.5%) and yeast extract (0.25%) were also added to each test tubes. pH of the media was adjusted to 7.2 using 1 N NaOH and the media was then autoclaved. After cooling, medium was inoculated with 0.1 ml of *Bti* culture (Federici et al. 1990). The test tubes were incubated for 24 hours at 30ºC and OD was measured at 600nm using UV spectrophotometer (Perkin Elmer - Lambda 25) using a small aliquot of sample.

Substrate repression studies using PEP as substrate:

Submerged fermentation: Microbial medium was prepared using PEP powder. The dilutions are made from 1% to 10 % PEP (W/V) in each test tubes. pH of the media was adjusted to 7.2 using 1 N NaOH and the media was then autoclaved. After cooling, medium was inoculated with 1 ml of *Bti* culture. The test tubes were incubated for 24 hours at 30^0C and OD was measured at 600nm using UV spectrophotometer (Perkin Elmer - Lambda 25) using a small aliquot of sample (Foda et al. 1985).

Solid state fermentation: The concentration of extracts used were 20%, 40%, 60%, 80% and 100% PEP (W/V). pH was adjusted to 7.2 using 1N NaOH and 2.5% agar was added to each conical flask. Media was then autoclaved and transferred to petri dishes. After solidification of media 0.1 ml *Bti* inoculum was added and was then spreaded uniformly all over the surface of petri plates using a cotton swab. It was then incubated for 48 hours at 30^0C. After incubation, colonies were scraped out from the petriplates using a scraper onto a butter paper and the biomass was weighed using a weighing balance.

Batch fermentation: The microbial medium was prepared with 5% PEP (W/V) in batch culture. pH of the media was adjusted to 7.2 using 1 N NaOH and the media was then autoclaved. After cooling, the media was inoculated with 1ml of inoculum. After inoculation, both of the conical flasks were kept in a shaker for24 hours at 30^0C.

Fed-batch fermentation: Fed-batch culture was initiated with 2 % PEP media in 100ml distilled water. pH was adjusted to 7.2 using 1 N NaOH and the media was then autoclaved. After cooling, the media was inoculated with 1ml of *Bti* inoculum. After inoculation, both of the conical flasks were incubated in a shaker for 12 hours. After 12 hours, 20 ml of extract was discarded and 20ml of freshly prepared 2 % PEP is added. Three more additions were done, after every 12 hours. After final addition of 2 % PEP, final concentration was made to 10 % and the total volume remains same i.e., 100ml. After 66 hours aeration was stopped and its OD was measured at 600nm. OD was compared with the control (G P longobordi. 1994).

Table 2: Different mode of fermentation methodology used for the present study

Mode of fermentation	Working Volume (in ml)	Harvesting time (in hrs)	Volume of fermented broth removed (in ml)	Volume of fresh media added (in ml)
Submerged fermentation	100	24	Nil	Nil
Solid state fermentation	100	48	Nil	Nil
Batch fermentation	100	24	Nil	Nil
Fed-batch fermentation	100	66	20	20

Solid state fermentation using different nitrogenous supplement:

Solid state fermentation technique was used for this study. Four different nitrogenous supplements were used as additive and 80% PEP (W/V) were used as substrate for this study. Initially, we had prepared 1 % urea. Secondly, we had used fish amino acid as nitrogenous supplement. Fish aminoacid was prepared by placing jaggery and rotten fish in alternate layers (sardine) in a tight container and was kept buried in soil for one month. The resultant liquid will be black in colour and was filtered. The filtrate was then used for our study. 1 % of the filtrate was used with 80% PEP media. 1 % of soy bean powder and 1 % poultry powder was also used as nitrogenous supplements. pH of these supplemented media were adjusted to 7.2 using 1 N NaOH and was then sterilized. Sterilized media were then transferred to petriplates and was inoculated with *Bti* using spread plate technique and was incubated at 30^0C for 48 hours. After incubation, colonies were scraped out from the petriplates using a scraper onto a butter paper and the biomass was weighed using a weighing balance.

Larvicidal assay

The larvae were collected from Departmental Garden, Department of biotechnology, University of Calicut. The larvae were identified by Dr. Raghu, Assistant Director, Centre for Disease Control, Kallayi. The larvae were identified as *Aedes aegypti*. Larvae were kept in plastic containers with tap water. The tests were conducted in petri plates. Standard WHO protocol for time dependent assay with slight modifications were adopted for the study. Three replicates and a control were tested during each trial. The control was set up with dechlorinated tap water. 10 third instar larvae were obtained and were released in each petri plates with 90 ml of water and 10 ml of test sample. Concentration of test sample was 50 mg/L of *Bti*. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region (Goldberg & Margalit. 1977). The experiments were conducted under laboratory conditions at 25-30^0C and 80-90 % relative humidity. A total of three trials were carried out. The percent mortality were recorded for average of three replicates (Mahmmod. 1998; Metcalf. 1986).

3. Results

An experiment conducted to study the effect of different concentrations of glucose on growth of *Bti*in batch culture revealed that the growth increases as the sugar concentration is increased till it reaches a threshold limit of 3% and reduces with further increase in the concentration of glucose (Nduka. 2018). As shown in figure 1. glucose has repressive effect on growth and biomass production of *Bti*. While PEP media reaches a threshold limit of 5 % and further increase in concentration of PEP, *Bti*growth reduces.

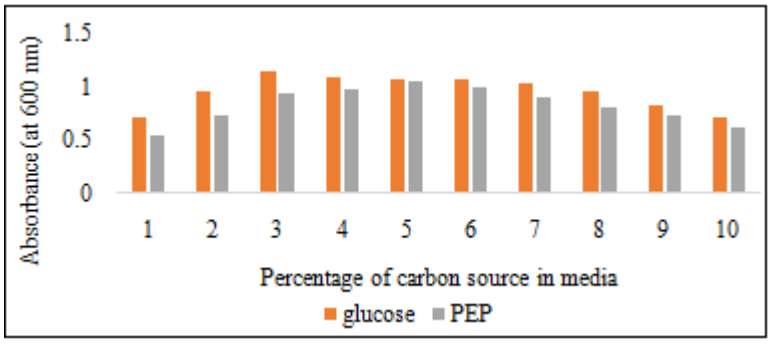


Figure 1: Substrate repression studies of *Bti* using glucose and PEP as substrate (submerged fermentation)

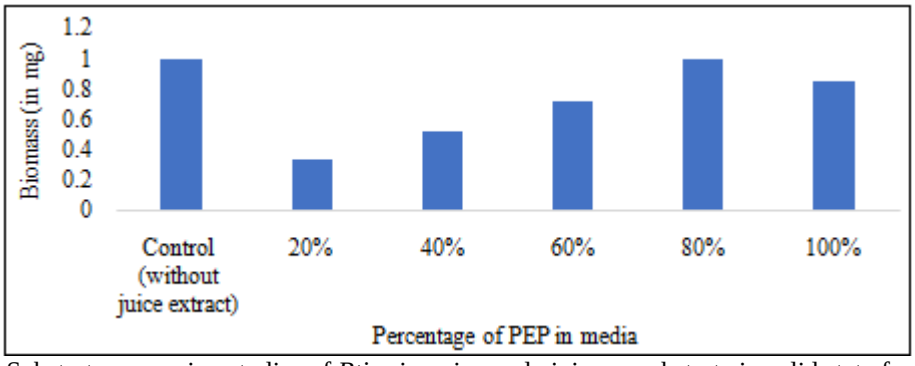


Figure 2: Substrate repression studies of *Bti* using pineapple juice as substrate in solid state fermentation

Figure 2 shows the substrate repression study of *Bti* in solid state fermentation mode. Figure 2 further indicates that *Bti* grown on solid state fermentation mode shows maximum growth as the process takes place in a solid matrix for the bacterium to grow.

From figure 1 we found that biomass was maximum when PEP concentration reaches 5% (W/V) (Raju et al. 2016). So, batch cultivation was done at 5 %. In fed-batch cultivation, nutrients were added aseptically; it is a partially closed system, and the volume of liquid culture in the bioreactor increases as the culture is systematically added. Here, fixed volume fed batch was also studied. As compared to batch, fixed volume fed-batch shows 23.8% increase in biomass as shown in the Figure 3. So, it is proved that fixed volume fed batch is an effective technology to overcome the substrate repression and to have cost effective production of *Bti*.

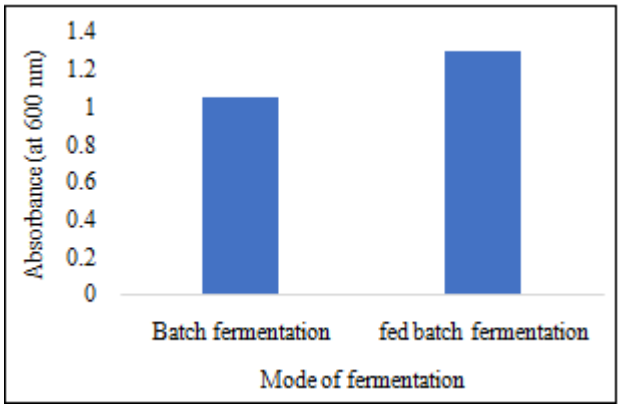


Figure 3: Substrate repression studies of *Bti* using PEP as substrate (Fed batch fermentation)

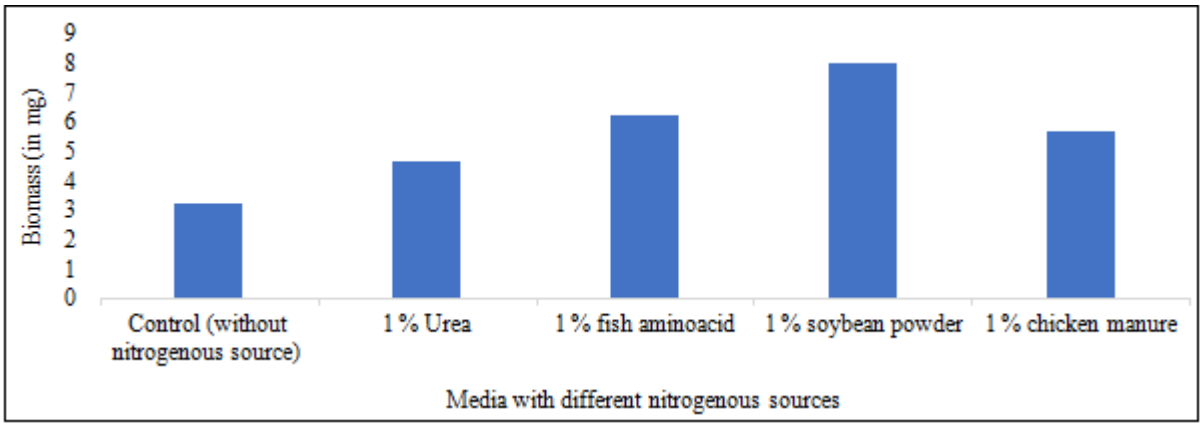
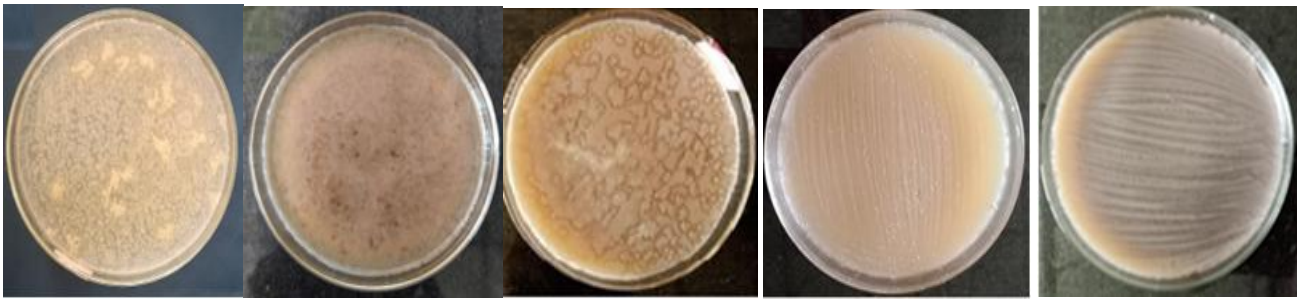


Figure 4: Biomass of *Bti* produced using different nitrogenous sources with 80% PEP supplemented media. Urea incorporated media shows minimum biomass when compared with others.



(a) (b) (c) (d) (e)

Figure 5: *Bti* grown on PEP enhanced media with different nitrogenous supplements a) Control b) Urea c) fish aminoacid d) soybean powder e) chicken manure. Here, 1 % soybean powder shows more growth.

Figure 4. shows growth of *Bti* on 80 % PEP media supplemented with different nitrogenous substrate. Urea, fish amino acid, soybean powder, chicken manure were used as different nitrogenous sources. Figure 5. shows the *Bti* grown on 80 % PEP media. From these figures, it is clear that 1 % soybean powder shows more activity than other supplements. 1 % fish amino acid also showed increase in biomass. Biomass was maximum at 1 % soybean powder and was 8.06 mg.

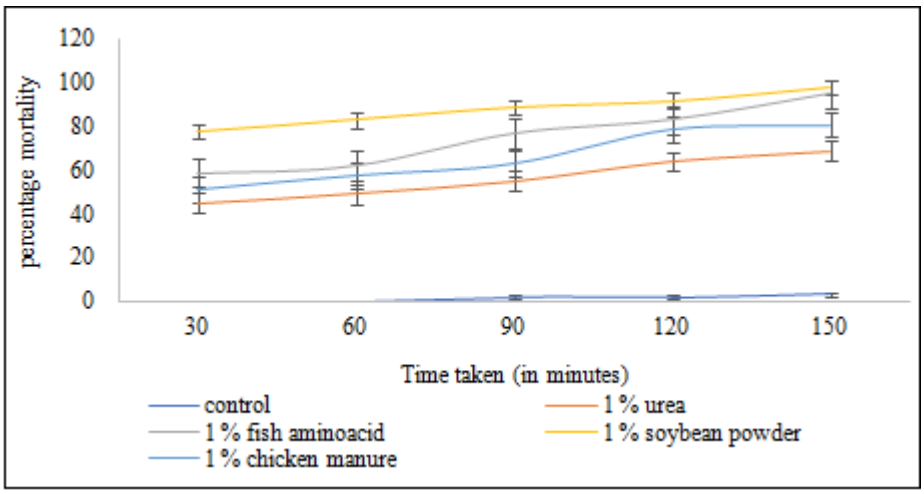


Figure 6: Biolarvicidal assay of *Bti* biomass using 80% PEP media supplemented with different nitrogenous wastes (solid state fermentation).

Figure. 6 shows biolarvicidal assay of 80 % PEP supplemented media with different nitrogenous wastes. All the supplements showed promising results with biolarvicidal assay. Fish amino acid and soybean powder showed more activity (Wada. 1989; Wirth et al., 1998). 1 % soybean powder showed 98 % mortality in 60 hrs while 1% fish aminoacid shows 95 % mortality in 60 hrs. 1 % urea showed less activity compared with other nitrogenous supplements.

4. Discussion and Conclusion

The current study mainly focuses on the development of cost-effective media for the production of *Bti*. Media was developed using rotten pineapple and effect of different fermentation strategies on growth of *Bti* on media supplemented with PEP were also studied. Figure 1 shows submerged fermentation using PEP media. It is evident from figure 1. That as the concentration of PEP increases, the biomass increases till the effective concentration of PEP reaches 5%. With further increase in PEP concentration the biomass decreases due to high concentration of sugar, there is a repressive effect on the growth of *Bti*.

The process of solid state fermentation is performed on a solid substrate with low moisture content, with the advantages of high product concentration. The required water content in solid state fermentation is absorbed by the substrate in a solid matrix and offers more advantages for the transfer of oxygen which will enhance the growth of microorganism. Thus, solid state fermentation exhibits several advantages like easy gas exchange, less water consumption, pH control, tray fermenters are required for SSF and thus less effort is needed for downstream processing. The major disadvantage of this method is the labor cost. Solid state fermentation was done with PEP media and tolerates up to 80% PEP media (Mulla et al. 1990). So, solid state fermentation mode is very much effective for the cost-effective production of *Bti* (Stanbury & whitacker. 2003).

Later, a comparison between batch and fed-batch fermentation were done. In batch process, all nutrients were provided at the beginning of the cultivation, and no additional nutrients were added. The leading advantages of batch process are short duration, less chance of contamination as no nutrients are added during the fermentation process. But it has several disadvantages like time consumption during sterilization of bioreactors, the difficulty of maintaining sterility between uses, prolonged lag phase, and less productivity. A fed-batch culture is more productive as it yields more with controlled sequential additions of nutrients, allows higher cell densities, and prolonged product synthesis. The major advantage of fed batch over batch culture is long-term synthesis of products, by increasing the amount of product by increasing number of cells, which is proportional to concentration of biomass. Greater efficiencies can be achieved in the process with controlled additions of nutrients; it allows the bioreactor to be used for extended production periods. This procedure involves feeding the bioreactor with the limiting substrate in a concentrated form so that there is no notable increase in volume (Ranson et al. 2010). From figure 3, we had proved that fixed volume fed batch is more effective than batch fermentation for *Bti* production using PEP media.

Finally, 80 % PEP media, which showed more *Bti*grow thin solid state fermentation were supplemented with different nitrogenous supplements. Firstly, we had used urea. Urea is the most important nitrogenous fertilizer because of its high nitrogen content (46%N). It showed substantial increase in biomass by 44.45%. So, this strategy can be used in industrial scale to boost the biomass production (Salam et al. 1983). Apart from the quality losses in the supply chain, in Worldwide, more than 25% of total fish is wasted per year. Fish waste, which is rich in nitrogen can be used along with PEP media for *Bti* production. Media enhanced with 1 % fish aminoacid showed increase in biomass by 93 %. India is Asia's second largest producer of soybeans, and it accounts for 3.95 percent of global production. From 2004-05 season to 2019-20 season, there has been a compound annual growth rate of 11.6 % soybean production in the country, according to the Federation of Indian Chambers of Commerce and Industry

(FICCI). Most of the soybean is produced in Rajasthan, Andhra Pradesh, Karnataka, Chhattisgarh, and Gujarat. Using soybeans there was substantial increase in biomass upto 142% (Schnepf et al. 1998). So, we can use soybean powder in the industrial scale to enhance the *Bti* production. Chicken manure contains 0.5% to 0.9% nitrogen, 0.4% to 0.5% phosphorus, and 1.2% to 1.7 % potassium. One chicken produces approximately 8-11 pounds of manure monthly (Su et al.1999). Poultry manure can create significant threats to soil and living organisms if not processed properly. By using chicken manure as nitrogenous additive, substantial increase in *Bti* biomass up to 75% was found. So, we can also use chicken manure for large scale *Bti* production and this will help in reducing the environmental pollution that is caused by chicken manure (Travis & Maureen. 1998; Tyrell et al., 1979).

Biolarvicidal assay was also done to confirm the larvicidal activity of *Bti* produced. Time dependent assay was done using third instar larvae of *Aedes aegypti*. PEP media supplemented with 1 % soybean powder showed maximum mortality than other PEP media supplemented with other nitrogenous sources.

This technology has proved that it is possible to channelize massive amounts of rotten pineapple wastes as a substrate for *Bti* production that is available in our country for cost-effective production of *Bti*which is used as abiopesticide (Woijciec & Korsten.2002). Fixed volume fed batch fermentation give increased biomass in lesser time without substrate repression compared to batch fermentation (Li et al. 2010). Solid state fermentation tolerates more sugar concentration than submerged fermentation. So, it can be concluded that SSF produce more sporulated biomass than other types of fermentation (D MF & I O. 1997). Considerable increases in *Bti* biomass using different nitrogen sources such as Urea, Fish aminoacid, soybean powder and chicken manure powder which were used as nitrogenous additives showed increase in biomass compared to control (Weiwei et al. 2013). Fish aminoacid had found to be cheapest and most promising for enhancing biomass production from pineapple waste to produce *Bti* based biopesticide (Magda et al. 2019). Hence, this work will allow low-cost production of active *Bti* based products and will thus alleviate the socio-economic problems inflicted by disease carrying vector, mosquitoes (Dana et al. 2020).

Abbreviations
SSF-solid state fermentation, *Bti-Bacillus thuringiensis israelensis*, PEP-pineapple processing waste extract, W/V - weight/ volume

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Statements and Declarations
Dr. Gopinathan C designed the experiments. Devika M shaju and Hana mol K E conducted all the experiments. Hana mol K E and Dr Gopinathan C analyzed the data. Hana mol K E wrote the manuscript. All the author's read and approved the manuscript. The authors declare that they have no competing interests.

Competing Interests
The authors have no financial or non-financial interests to disclose

Author's Contributions
Dr. Gopinathan C designed the experiments. Devika M shaju and Hana mol K E conducted all the experiments. Hana mol K E and Dr Gopinathan C analyzed the data. Hana mol K E wrote the manuscript. All the author's read and approved the manuscript

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