International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 06 Issue: 12 | December - 2022 Impact Factor: 7.185 ISSN: 2582-3930

Enhanced Production of *Bacillus Thuringiensis Israelensis* using Solid State Fermentation Technique

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Keywords: *Bacillus thuringiensis israelensis,* solid state fermentation, *Aedes aegypti*, Bioassay, yield efficiencies, Nitrogenous supplements, biopesticide.

Abstract

Vectors play an important role in disease transmission, globally. Despite various advancements in vector management, mosquitoes are still prime vectors of dreadful diseases which influences human health and economy as well. *Bacillus thuringiensis israelensis (Bti)* is a major bacterium that produces δ-endotoxin, which can kill the certain strains of mosquito larvae. *Bti based* biopesticides can be used as an alternative technique to control insects. Biopesticides derived from *Bti* are the most prominent biological agents for selective control of insect pests. In this study we aim to produce *Bti* using different cost-effective medias based on the fruit, pulses and sea food waste and other such materials using solid state fermentation techniques (SSF). SSF has been described as the process that takes place in solid matrix in the absence or near absence of water, and the substrate requires less moisture to support the growth and metabolic activity of organisms. Here, in the present study we use different fruit wastes as carbon sources and other wastes as nitrogen source to optimize cost effective and efficient media for the growth and sporulation of *Bti*. Fruit juices of rotten fruits like pineapple, grapes, watermelon etc were used as a substrate for the production of *Bti*. Nitrogenous supplements like prawn peel powder, peanut meal extract, poultry manure was also used for the overproduction of *Bti*. Finally, the larvicidal efficiency of the novel media was also determined. This method was found to be cost effective and is functional also, and it can solve the problems caused by fruit waste accumulation. It can also reduce the problems inflicted by disease carrying vectors like mosquitoes.

Introduction

Mosquitoes are major vectors for many deadly diseases and have caused millions of people to suffer and die due to illness from time immemorial. Mosquitoes have caused serious effect on human health from ancient times and still is a threat to humans. There are more than three thousand species of mosquito, but vast majority feed mostly on rotting fruits and other sources of sugar. Only a few hundred species, including *Aedes aegypti,* need blood to survive. The World Health Organization, reports that more than 50 percent of the world's population is presently at risk from mosquito-borne diseases. Hence mosquito control and management become the most important topic with respect to maintenance of our health and disease control and management practices[1,2].

Bacillus thuringiensis subsp. *israelensis (Bti*) is the most effective bio- larvicide against mosquitoes that is available to the date. It is a gram-positive spore forming entomopathogenic bacterium first isolated in 1976. It is rod shaped, facultative anaerobic with genome size of 2.4-5.7 million base pairs[3]. It is not restricted to soil but has been isolated worldwide from different types of habitats. *Bti* kills the larvae of certain flies and mosquitoes. The main targets of this *Bti* are the larval stages of mosquitoes, black flies, and fungus gnats. It does not kill larval stages of higher flies such as house flies, stable flies etc. Aedes, Culex and Anopheles are the major susceptible mosquito genera[4].

Bti products contain the spores and parasporal crystals of Bti H-14 serotype which must be ingested by the mosquito larvae to cause mortality. Upon ingestion, the parasporal body protein crystals are solubilized in the alkaline larval midgut followed by the proteolytic activation of the soluble insecticidal crystal proteins. The toxin binds to a receptor on the midgut cell wall which results in pore formation in the cell, which eventually leads to the death of the larvae. The insecticidal effect is caused by δ-endotoxin, parasporal crystal, which contains four major proteins of 27, 65, 128 and 135 kDa. The crystal toxins are designated as Cry4a, Cry4b, Cry11Aa and Cyt1Aa. The crystal is formed at the end of sporulation. All proteins are toxic to mosquitoes, however there appears to be a synergistic interaction between the Cyt 1 Aa protein and the Cry4a and Cry11 proteins, resulting in high toxicity to mosquito larvae[5].

Our objective is to produce δ-endotoxin producing *Bti* by channelizing the wastes from various sectors as the source of energy for the production of *Bti*. Through this approach we can effectively tackle two major problems that we face now, one is accumulatiion of wastes that are mostly discarded without proper processing leading to pollution and related issues, and another to kill mosquito larvae thereby reducing the risk of mosquito borne diseases. India being geographically located in the tropical region and having tropical and subtropical climate is highly prone to mosquito borne diseases. Also, having the second largest population of the world, India produces a huge amount of waste. In India, the organic waste fraction varies between 40 and 60% of the total solid waste streams. These waste fractions can be utilized through various treatment options, such as composting as organic fertilizer and soil enhancement as well as for biogas production. Another way of utilizing these wastes is using the waste from one source to energy for another source, that is, waste to energy transformation. SSF has been described as the process that takes place in a solid matrix (inert support or support/substrate) in the absence or near absence of free water[6], but the substrate requires less moisture to support the growth and metabolic activity of microorganisms[7].

Methodology

Preparation of inoculum

Bacillus thurengiensis israelensis (MTCC 869) was purchased from IMTECH, Chandigarh, India. All the experiments were done using a pre culture prepared by transferring a loopful of frozen Bti slant culture to 20ml sterilised medium and using 0.1ml of the overnight grown culture as the inoculum. The medium composition is shown in Table 1.

CHEMICAL CONSTITUENTS	PERCENTAGE
Glucose	1 %
Peptone	0.5 %
Yeast extract	0.1 %
Calcium chloride	0.1 %
Agar	5 %

Table 1. Glucose based media composition

Preperation of fruit extracts

Three different rotten fruits were taken. Fruit used for the present study were watermelon, grapes and pineapples. 1000 g of fruit were taken and was crushed and was filtered to obtain the corresponding fruit extracts.

Solid state fermentation

Solid state fermentation of *Bti* using different carrier materials were analyzed[8]. Roller bottle method, Pebbles, wood charcoal, baby jelly and sorghum grains were used as solid substrate for successful production of *Bti*. For Roller bottle method, the glass bottles were sterilized and to this glucose media was added and a coating was made by rotating the bottle. Excess media was discarded and to the solidified media, a layer of *Bti* culture was introduced by the same method as that of medium[9]. It was then incubated at 30^0C for 48 hrs.

For solid state fermentation using pebbles, sterilized pebbles were taken and to this a coating of glucose media were given by dipping it in glucose media to form a thin layer over pebbles. After solidifying, it was then dipped in *Bti* pure culture to form a layer over the coated medium. It was then transferred to pre-sterilized glass bottles and was then incubated at 30^0C for 48 hrs.

For solid state fermentation of *Bti* using wood charcoal, to the sterilized wood charcoal, glucose media was coated by dipping it in the media and then it was further dipped it in *Bti* culture to form a layer upon medium.

Finally, solid state fermentation of *Bti* was done using baby jelly also. For this baby jelly was thoroughly washed with running tap water, 1 N H_2SO_4 and finally with distilled water. It was then sterilized and then a coating of glucose media was given by dipping it in the media. After solidification, a coating of *Bti* pure culture was done by dipping it in the culture. It was then incubated at 30^0C for 48 hrs. By measuring volume of media and inoculum before coating and after coating from media and inoculum respectively, the volume of glucose medium and culture inoculum used for coating was recorded during the procedure[10].

Elution of *Bti* biomass

After examination under light microscopy, Bti biomass was then eluted out after sporulation of culture by using 10 ml of sterilised water as eluant. Water was added to the culture flasks and mixed well with carrier materials and was kept for 1 minute before elution.

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. Larvicidal bioassay of *Bti* cultured on different carrier materials were tested against larvae of *Aedes aegypti*. Three replicates and a control were tested during each trial. The control was set up with dechlorinated tap water. 20 larvae were obtained and were released in each beaker with 100 ml of water and 10 ml of test elutes. 0.1 ml of Bti culture was used for the assay. Time dependant biolarvicidal assay was done[11]. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. The experiments were conducted under laboratory conditions at 25-30^{0}C and 80-90 % relative humidity. A total of three trials were carried out for each of the sample[12].

Optimization of fruit juice concentration for maximizing growth of *Bti*

Microbial medium was prepared using rotten grape extract, pineapple extract and watermelon extract. The concentration of extracts used were 20 %, 40 %, 60 %, 80 % and 100 % for all rotten fruit extracts. pH of the media was adjusted to 7.2 using 1 N NaOH and 2.5 % agar was added. It was then sterilized and poured to petriplates. After solidification, *Bti* culture was spread on plates using spread plate technique and was kept for incubation for 48 hrs at 30^{0}C. 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[13].

Solid state fermentation of *Bti* using different nitrogenous supplement

Three different nitrogenous supplements were used for this study. Firstly, we had prepared peanut meal extract by boiling 100 g of soaked peanut meal in 1000 ml distilled water for 30 minutes. It was then filtered with whatsman filter paper and was stored at 4^{0}C for further use. The peanut extract media was prepared by adding 0.5 % (w/v) peanut meal extract to 100 ml glucose media. Secondly, we had used prawn peel powder as nitrogenous supplement. Prawn exoskeleton and head was collected from local fish market and was washed thoroughly using tap water and then with distilled water. It was then dried in hot air oven at 60^{0}C for 24 hrs and was powdered using a grinder. The prawn peel powder supplemented media was prepared by adding 0.5 % (w/v) prawn peel powder to 100 ml glucose media. Finally, we had also tried poultry manure and NPK fertilizer as nitrogenous supplement. 0.5 % (w/v) of poultry manure and NPK fertilizer (w/v) were added to 100 ml glucose media. pH was of these supplemented media were adjusted to 7.2 using 1 N NaOH and was then sterilized. Sterilized media was then transferred to petriplates and was inoculated with Bti using spread plate technique and was incubated at 30^{0}C 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.

Results and Discussion

SSF is a fermentation mode carried out in the absence of free-flowing liquid (although containing sufficient water to allow microorganism growth), using an insoluble material that acts as both a solid support and a source of nutrients. Preliminary studies were conducted using glucose media for determining the coverage provided by different carrier materials, the usage of media and inoculum etc[14].

Inoculum and media usage comparisons of different carrier materials:

SSF of *Bti* was conducted using different carrier materials like charcoal, roller bottle, baby jelly, and pebbles. Since, the particle size shape and physical structure of each of the carrier materials differ widely, each carrier materials surface area, per unit volume was estimated. The volume of media used to give a precoating on the carrier materials used was also estimated, since SSF technology is based on the extend of surface area available. As per the table 2, it was found that minimum media usage was with roller bottle, followed by pebble, charcoal, baby jelly, sorghum. The roller bottle method shows relatively good performance by conducting larvicidal bioassay. So, this can be used as a cost-effective means for the production of *Bti* compared to existing submerged fermentation process[15,16] (Figure 1).

Carrier	Quantity (in number)	Total surface area (Cm^2)	Media used (ml)	Media coverage (Cm^2/ml)	Inoculum used (ml)
Control (petriplates)	3	75/ plate	25 / plate	3 / plate	0.1/ plate
Rolling bottle	3	184.57 / bottle	3 / bottle	61.25 / bottle	1/bottle
Pebbles	98	1230.88	34	36.202	12
Sorghum grains	3946	12047.14	76	354.3	34
Wood charcoal	68	1292	36	35.89	14
Baby jelly	210	2310	62	37.26	28

Table 2. Estimation of total surface area and usage of medium as well as inoculum when different carrier materials. Comparison of different carrier materials used for Solid State Fermentation. Here, we have given the total number of carriers used, total surface area of the carrier, media used by each carriers, media coverage i.e., surface area covered per ml and the quantity of inoculum used.

Figure 1. Solid State Fermentation of *Bti* using different carriers

Larvicidal activity of *Bti* cultured on different carrier materials

Time taken for 100 % mortality of larvae from culture grown over different carriers was checked[17]. Here, wood charcoal shows greater larvicidal efficiencies compared to other carriers. No larvae death was found in control[18]. In terms of biolarvicidal assay performance, charcoal was rated as first, roller bottle as second, pebble as third, fourth position being baby jelly and final fifth position by sorghum[19] (Figure 2).

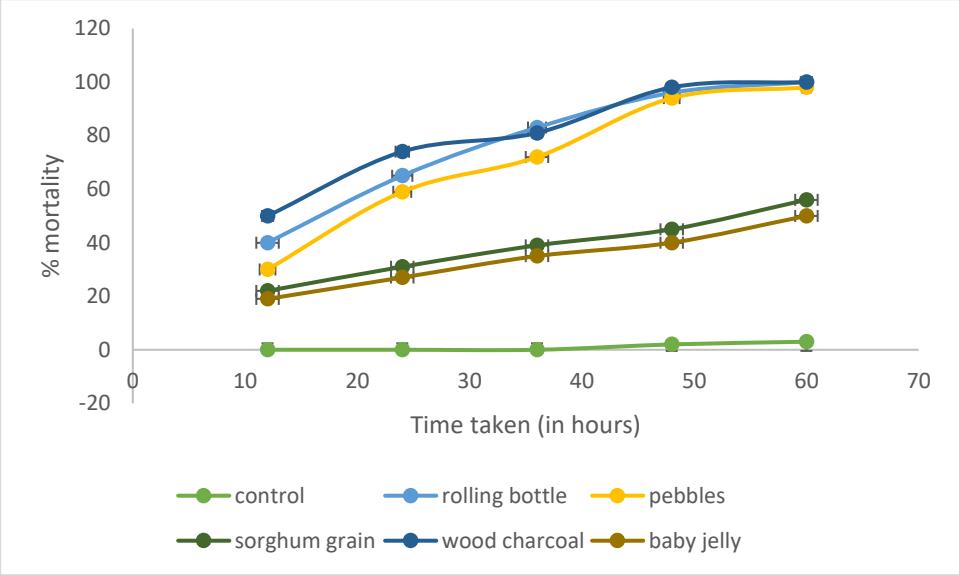

Figure 2. Time taken for larvicidal activity of eluted biomass taken from culture grown over different carriers. Here, wood charcoal carrier and rolling bottle method shows greater larvicidal efficiency than others.

Solid state fermentation of *Bti* using different fruit juice as carbon source

A simulation was performed to test the possibility of using fruit juices as a substrate for the growth of *Bti*. Biomass of *Bti* cultured on various concentrations of different fruit juices are measured[20]. The fruit juices enhanced media shows more growth of *Bti* when compared to the control. A decrease in the biomass is shown after 25 % concentration of grape juice media.

The grape juice-based media showed positive results having a biomass of 410 mg when compared to the control which produced only 380 mg. Growth enhancement was observed. Hence, the grape juice was selected to prepare a suitable media for the enhanced growth of Bti using novel technique. It was found that 20% grape juice-based media was found to be high yielding (490mg) compared to others. It also gives better biomass compared to control (glucose - based media). *Bti* can be successfully grown using pineapple juice media on solid state agar media. We have tried different concentrations of pineapple juice-based media in order to quantitate the biomass production in petriplates (figure 4). The biomass from different concentration ranging from 20 to 100% is showcased as part of the result given in table 4.3.1. It was found that biomass production, ranging from 80mg to 290 mg (from juice concentration of 20-100%) was produced with maximum growth of 360mg with 80% pineapple juice concentration, with respect to the control with biomass of 360mg. From figure 3, it was also observed that

watermelon juice can be used for the preparation of media for *Bti* cultivation. It was found that biomass production was 310 mg at 80 % watermelon juice concentration. However due to its comparatively poor biomass production rate, it was eliminated from further studies.

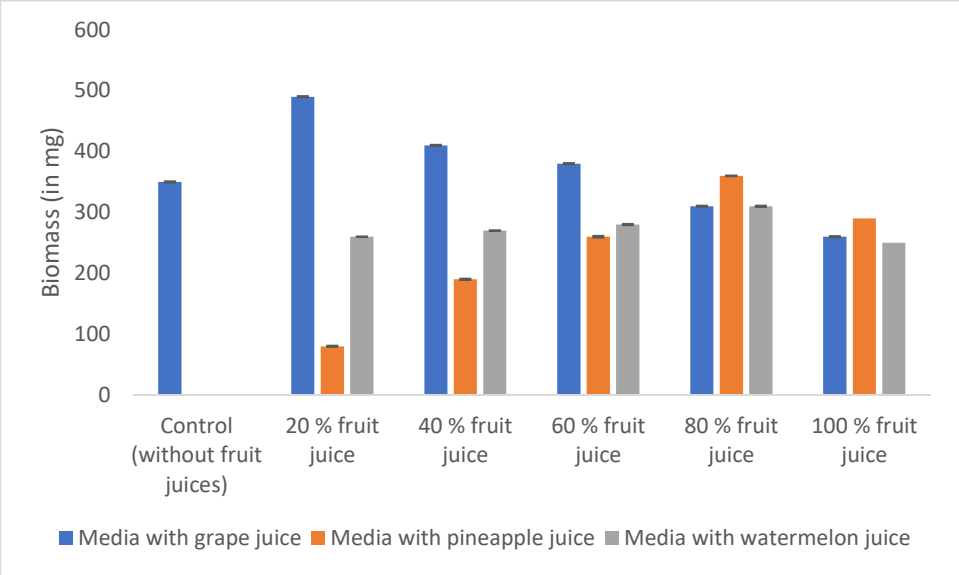

Figure 3. Biomass of *Bti* cultured on different concentrations of various fruit juice supplemented media. 20 % pineapple juice media shows minimum biomass when compared with others.

(a) (b) (c)

Figure 4. Growth of *Bti* on different fruit juice based media (a) shows *Bti* growth on 20 % grape juice supplemented media (b) shows *Bti* growth on 80 % pineapple juice supplemented media (c) shows *Bti* on 80 % watermelon juice supplemented media.

Solid state fermentation of Bti using different nitrogenous supplements

SSF of *Bti* was performed with different nitrogen supplemented media grown in petriplates (figure 6). It was found that compared to control (360 mg), there is excellent growth and biomass production of *Bti* resulting in good sporulation when peanut meal extract was supplemented (880 mg) (Figure 5). It proves that peanut meal can also be used as an excellent substitute to the very expensive Peptone, which is used in conventional microbial media.

Prawn peel supplemented media showed a greater biomass production of about 740 mg. It produced more than twice the biomass of control. Hence it can be successfully used as a protein supplement in the production of *Bti*. Due to its large-scale availability, easiness of transport, and storage, it can be scaled up to meet industrial requirements. The rich nitrogen contents, abundance and low cost makes it a very suitable raw material for the production of *Bti*. Figure 5, shows the biomass produced, when poultry manure was added as a supplement to the media. A biomass of 510 mg was observed, which proves that poultry manure can be successfully used for the production of *Bti*. The biomass data using NPK fertilizer as supplement shows that the growth and biomass production is not very promising to be employed for industrial purpose since there is only 80 mg biomass. The reason for low biomass production maybe due to ammonia production as part of microbial metabolism. Several by-products may be produced as a part of metabolism of NPK, and compounds like ammonia may cause inhibitory action on the growth of *Bti*[21]. However, based on recent studies and data obtained, the use of NPK as a supplement for *Bti* is not advised.

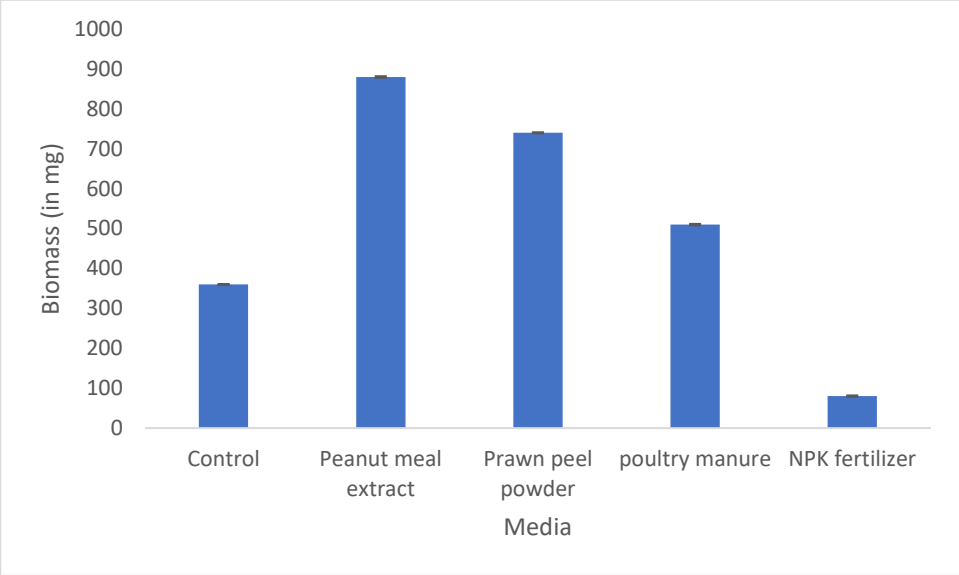

Figure 5. Biomass production by *Bti* when cultured on Control media and on NPK supplemented media. Glucose media supplemented with peanut meal extract as nitrogenous source enhance the *Bti* growth while media supplemented with NPK fertilizer does not enhance the biomass production compared with control.

(a) (b) (c)

(d) (e)

Figure 6. *Bti* grown on 0.5 % of different nitrogenous supplements a) Control b) Peanut meal extract c) Prawn peel powder d) Poultry manure e) NPK fertilizer. Here, NPK fertilizer shows less growth while peanut meal extract media shows maximum growth.

Solid state fermentation of *Bti* using fruit juice enhanced media with different nitrogenous supplements

Among the fruits used as substrate, grapes have shown to be the most promising and enhanced biomass production. It shows the maximum biomass production at the concentration of 20% as showing figure 3. Hence, it was selected for further investigations. Figure 7 shows the biomass production using rotten grape juice and various nitrogenous supplements. From the data it is evident that 0.5%(w/v) Prawn peel powder gives maximum biomass of 1100 mg compared to 400 mg produced using control. It shows that grape juice supplemented with prawn peel powder can be scaled up for industrial use, which can be a very cost-effective substitute to the costly media that are currently used . Both rotten grapes and prawn peel powder are wastes that are very cheap and abundantly available in India. Hence, these can be channelized to produce a cost-effective media for *Bti* and thereby contribute to the production of biopesticide that can control the mosquito populations which cause many deadly vector-borne diseases[22]. Among the fruit-based media prepared rotten pineapple juice-based media have shown enhanced biomass production. It shows the maximum biomass production at the concentration of 80% V/V as shown in the figure 7. So, it was selected for further investigations. 80% V/V Pine apple juice was supplemented with different nitrogen sources and its effect on the biomass was observed. Among them, pineapple juice media supplemented with 0.5%(w/v) prawn peel powder showed most enhanced biomass (2360 mg). watermelon juice media with poultry manure also showed enhanced growth (figure 8). However, more biomass was found on media supplemented with 80 % pineapple juice and 0.5 % prawn peel powder.

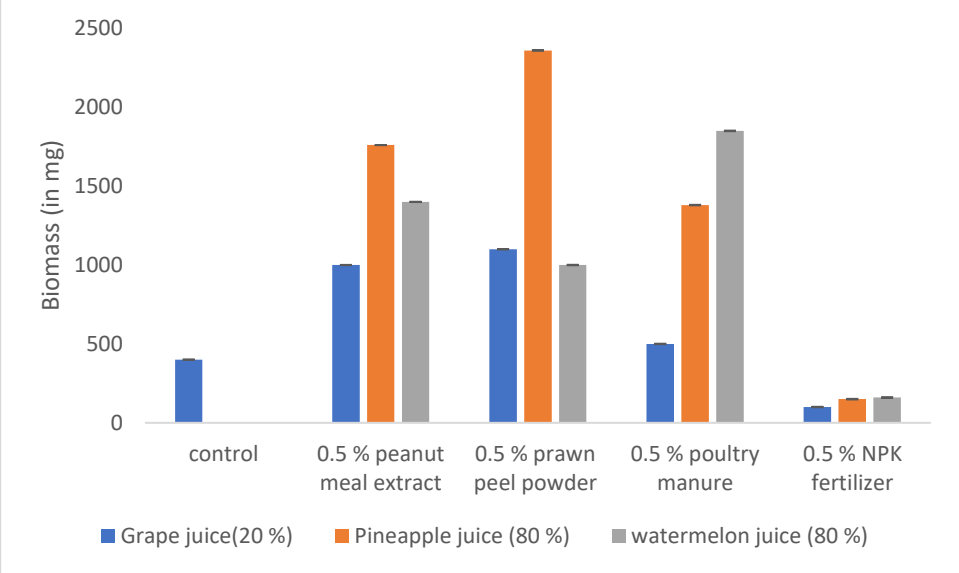

Figure 7**.** Biomass produced by *Bti* when grown on media with rotten pineapple juice as substrate incorporated with different supplements compared to that of control.

Figure 8. *Bti* grown on media with both fruit juice and nitrogenous supplements a) Control b) 20 % grape juice + 0.5 % prawn peel powder c) 80 % pineapple juice +0.5 % prawn peel powder d) 80 % watermelon juice + 0.5 % peanut meal extract

Biolarvicidal activity

Figure. 2 and figure. 7 shows that the SSF of *Bti* using rolling bottle method and Wood charcoal method with media based on 80% rotten pineapple juice supplemented with 0.5% prawn peel powder, is more effective in producing active cultures of *Bti*. The microscopic examination of the eluant also showed the presence of spores. The functionality of the spores produced was examined by the biolarvicidal assay[23] (figure 9). From the figure 2, we can observe that the biolarvicidal effects of eluate from roller bottle and wood charcoal were more effective when compared to the control. The eluate from roller bottle shows more killing efficiency than that of wood charcoal[24].

Killing efficiency of the roller bottle may be attributed to the minimal interference from external factors and good oxygen transfer rates. In the case of charcoal, its porous character can help the growth of *Bti* by trapping oxygen and nutrients, but in contrary it may also trap the toxic by-products within them, that may affect the growth of the microbe[25]. From above studies, 0.5 % prawn peel powder supplemented media with 80 % pineapple was found to be more efficient (Figure 7). So a novel media was formulated based on the results and this media was used for further assays[26] (figure 10).

(a) (b)

Figure 9.SSF of *Bti* using (a)Roller bottle method and (b)Wood charcoal coated with media made up of 80% pineapple juice supplemented with 0.5% prawn peel powder.

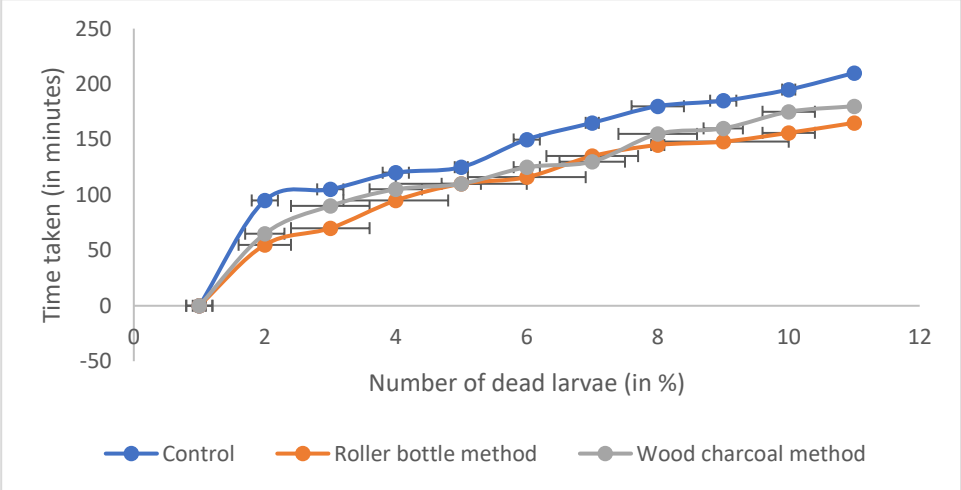

Figure 10. Biolarvicidal assay of *Bti* eluate from SSF using wood charcoal and roller bottle method with 80% pine apple juice based media supplemented with prawn peel powder.

Conclusion

Different carriers materials were used with a thin layer of medium over it, so that it can serve as a means for the growth of *Bti*[27]. Although less employed at an industrial level, SSF offers a series of advantages over submerged fermentation (SmF), such as higher yields and productivities, extended stability of products, lower production cost, lower protein breakdown (which is especially important if an enzyme is the target product), lower contamination risk, lower energy requirement, lower energy costs for sterilization, lower fermenter volume, and lower catabolite repression[28]. SSF also offers a broader possibility to use in natural agro-industrial wastes and byproducts as raw materials, which represents a key point in terms of economic feasibility, since raw material is commonly reported as one of the major expenditure incurred in enzyme production processes.Rotten Pineapple juice-based media supplemented with Prawn peel powder provides all these advantages. Rotten Grape juice supplemented with Prawn peel powder also improved the yield of *Bti* using SSF technique[29]. Among the carriers which were used for the SSF of *Bti*, wood charcoal has proved to be the best carrier material. With pineapple juice-based media supplemented with prawn peel powder and wood charcoal based and roller bottle methods, SSF of *Bti* produces active cultures of *Bti* that exhibit more potent biolarvicidal activities than the glucose medium[30]. Hence, this work can be applied for the effective production of the *Bti* based biopesticide by the use of massive amount of fruit juices and by providing huge surface area for *Bti* to grow and sporulate by the packed bed reactor (SSF technique), when compared to the traditional Submerged fermentation techniques[31,32]. This method is both cost effective and functional, as well as it can counter the social problems caused by fruit waste accumulation as well as the product can alleviate the problems inflicted by disease carrying vectors like mosquito.

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