Effect of Different Fermentation Strategies on Growth of *Bacillus Thuringensis Israelensis* and its Toxicity Towards *Aedes Aegypti*

Dr. C. Gopinathan[1], Anagha Anirudhan[2], Hana Mol K E[3]
[1]*Associate professor, Department of biotechnology, University of Calicut, Thenhipalam, Kerala, India*
[2]*Student, Department of biotechnology, University of Calicut, Kerala, India.* [3]*Research scholar, Department of biotechnology, University of Calicut, Kerala, India*

Abstract-The harmful effects of chemical pesticides on the environment and life had led to the search for biopesticides. *Bacillus thurengiensis israelensis (Bti)* is a biopesticide used to control mosquitoes worldwide. The main advantage of *Bti* over chemical insecticide is its specific activity towards diphteran insects. The growing importance of these biopesticide in insect control activities has encouraged in aiming to develop new methods to produce it in low cost. In this study we aim to to study the effect of batch, fed-batch, submerged and solid state fermentation on the growth of *Bti* using rotten grape juice as substrate. Significantly, higher biomass was obtained in fed batch compared with other fermentation techniques. Glucose medium showed less activity compared with others. Fed batch cultivation with intermittent feeding at 12 hrs of cultivation recorded highest cell density of 1.16 at 600 nm. These results support *Bti* growth using rotten grape juice as substrate can be used for cost effective production of Bti against mosquito larvae.

Keywords: *Bacillus thurengiensis israelensis*, batch fermentation, fed batch fermentation, solid state fermentation, cell growth.

INTRODUCTION

Biopesticides include products derived from naturally occurring bacteria and Insect Growth Regulators (IGRs). *Bti* have been used worldwide as a biological pesticide since 1980 for mosquito control. *Bti* was registered by EPA in 1983 and has been used as a Best Management Practice option to manage mosquito larvae. It has been proved to be a potent control for mosquito larvae (Goldberg and Margalit,1977). Since 1980 there is a continuous increment in *Bti* based products. A variety of *Bt* products are manufactured all over the world are accessible mainly to the upper class due to its cost which the poor farmers of developing countries cannot afford. Efforts to reduce the general production cost are taken by way of R&D department of biopesticide industries. *Bti* is inherently less toxic than conventional pesticides and has relatively minimal impact upon most non-target organisms. According to EPA, the toxicology of *Bti* is well-established and exhibits minimal risk to humans, pets, birds, aquatic organisms (e.g. fish and invertebrates), non-target plants and honey bees.

Presently, *Bacillus thuringiensis subsp. israelensis (Bti*) is the most effective bio larvicide against mosquitoes. 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 (Goldberg & Margalit 1977). 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, Psorophora, Culex and Anopheles are the susceptible mosquito genera. *Bti* has advantages compared to chemical insecticides in being very selective, narrow spectrum, so it is safe to other species, including humans.

Bti products contain the spores and parasporal crystals of *Bti* H-14 serotype, which is ingested by the mosquito larvae to cause mortality. Upon ingestion, the parasporal bodies 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, a 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 is a synergistic interaction between the Cyt 1 Aa protein and the Cry4a and Cry11 proteins, resulting in higher toxicity to mosquito larvae.

Nowadays, cost of production is one of the major challenges faced by the biopesticide industry. Hence, new methods have to be developed to reduce the cost of production of biopesticides. This study is to enhance the growth of *Bti* by solid state fermentation methods using rotten fruits and cheap nitrogenous supplements that could replace the standard high-cost raw materials. The raw material costs about 70% of total production cost. By cutting down the cost for the raw materials we can develop a cost-effective method to produce *Bti* based biopesticides. Commonly used nutrient sources for the production of media includes a wide range of peptones, hydrolysates, supplements majority of which are expensive for use in industrial scale. For this study we had used crude materials like prawn peel containing sufficient amount of Nitrogen rather than the pure form of peptone can reduce the cost of media considerably.

The feasibility of producing a microbial product depends on cost at which the product can be manufactured. Various different agro-industrial wastes like bagasse, whey, fish wastes and soyabean powder have been used before for producing *Bti*, which indicates that more and more agrowaste and food waste can be utilized for the production. In this study, variable volume fed-batch technique became used to triumph over catabolite repression and to enhance the production of *Bti*. Use of agro waste as substrate for the production of microbial based product is an effective method to cut down the total cost of production. This study utilizes rotten grapes as substrate and cheap nitrogenous additives such as urea, fish slurry, soyabean powder and chicken manure for the enhanced production of *Bti*.

MATERIALS AND METHODS

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

2.2. Preparation of inoculum: *Bacillus thurengiensis israelensis* (MTCC 869) was purchased from IMTECH, Chandigarh, India and was preserved in deep freezer. For inoculum preparation, $1/10^{th}$ dilution were made from 0.5 macferland standerd (ester et al., 2022 & Demarjac& larget. 1984). Composition of mGYS media is as shown in table 1. The initial pH of the media was adjusted at 7.2. The culture was then used as standard inoculum for all experiments (Dulmage. 1970).

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

Table 1. mGYS media composition

2.3. 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 in batch fermentation using a carbon source, glucose. The concentrations of glucose ranged from 1-10%. In each of these test tubes peptone (0.5%) and yeast extract (0.25%) were added as additive. pH of the media was adjusted to 7.2 and the media was then autoclaved. After cooling, medium was inoculated with 0.1 ml of *Bti* culture[9]. The test tubes were incubated for 24 hours and OD was measured at 600nm using UV spectrophotometer

(Perkin Elmer – Lambda 25) using a small aliquot of sample. This was taken as control.

2.4. Effect of different fermentation strategies using GEP as substrate:

2.4.1.Submerged fermentation: The dilutions were made from 1% to 10 % GEP (W/V). pH of the media was adjusted to 7.2 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[10].

2.4.2.Solid state fermentation: The concentration of extracts used were 20%, 40%, 60%, 80% and 100% GEP (W/V). pH was adjusted to 7.2 and 2.5% agar was then added to each conical flask. Media was autoclaved and transferred to petri dishes. After solidification of media, spread plate technique was done with *Bti* inoculum. 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. Control was also kept using 1% glucose.

2.4.3.Batch fermentation: Batch fermentation was done using 6 % GEP(W/V) as it showed maximum growth. pH was adjusted to 7.2 and the media was then autoclaved. After cooling, the media was inoculated with 1ml of inoculum. After inoculation, conical flasks were kept in a shaker for 24 hours at 30^0C.

2.4.3.1.Variable volume fed-batch fermentation: Fed-batch culture was initiated with 2 % GEP in 10ml. pH was adjusted to 7.2 and the media was then autoclaved. After cooling, the media was inoculated with 1ml *Bti* inoculum. After inoculation, conical flasks were incubated for 12 hours at 30^0C. The addition of 10 ml of 2 % GEP were done subsequently after every 12 hours under laminar air flow cabinet for remaining 108 hours till the final volume reaches 100 ml. After 108 hours, aeration was stopped and its OD was measured at 600nm. OD was compared with batch fermentation[11].

Mode of fermentation	Working volume(in ml)	Harvesting time (in hrs)	Volume of fresh media added (in ml)
Submerged fermentation	100	24	Nil
Solid state fermentation	100	48	Nil
Batch fermentation	100	48	Nil
Fed-batch fermentation	100	108	10

Table 2. Different modes of fermentation methodology used for the present study

2.5. Solid state fermentation using different nitrogenous supplement:

Four different nitrogenous supplements were used as additive along with 80% GEP(W/V) for this study. We had prepared 1 % urea, 1 % fish amino acid, 1 % of soy bean powder and 1 % poultry manure. 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 without any smell and 1 % of this material is used with 100 ml mGYS media. pH of the supplemented media were adjusted to 7.2 and was then sterilized. Sterilized media were then transferred to petriplates and was inoculated with 0.1 ml *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.

2.5. 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 was 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. Dead

larvae were identified when they failed to move after probing with a needle in the siphon or cervical region[12]. 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. The percent mortality was recorded for the average of three replicates[13,14].

RESULT

Figure 1 shows substrate repression studies using glucose and GEP as substrate. GEP media enhanced bacterial growth. GEP media also showed considerable increase in growth of *Bti*.

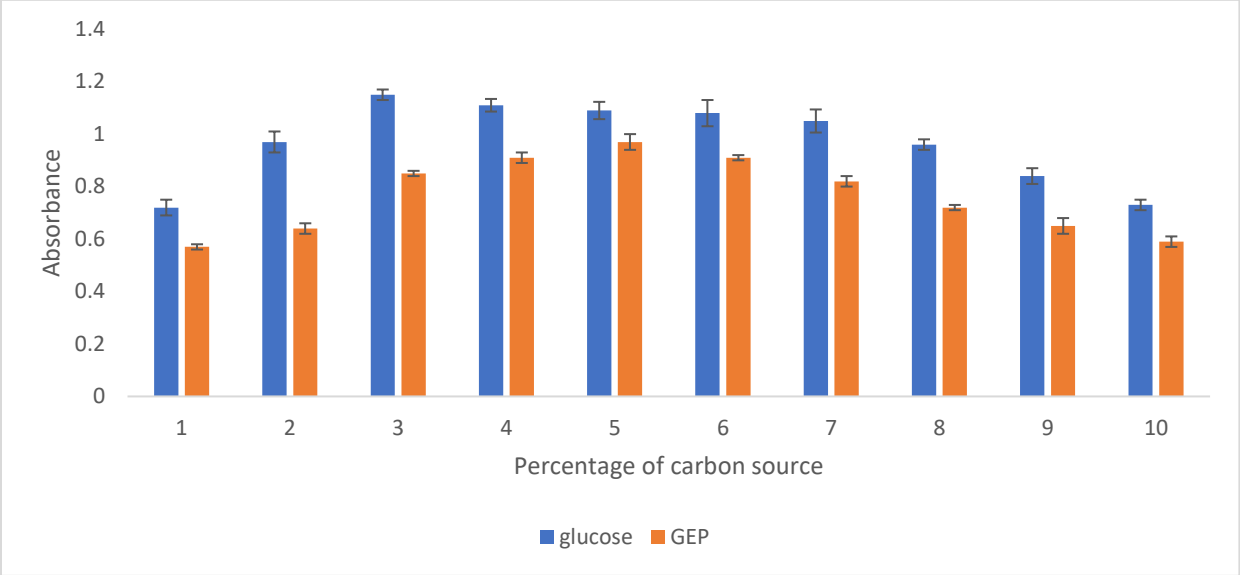


Figure 1. Submerged fermentation of *Bti* using glucose and GEP as substrates. Above graph depicts percentage of carbon source vs absorbance (mean±SE).
The bars correspond to standard error calculated from three replicates of same sample.

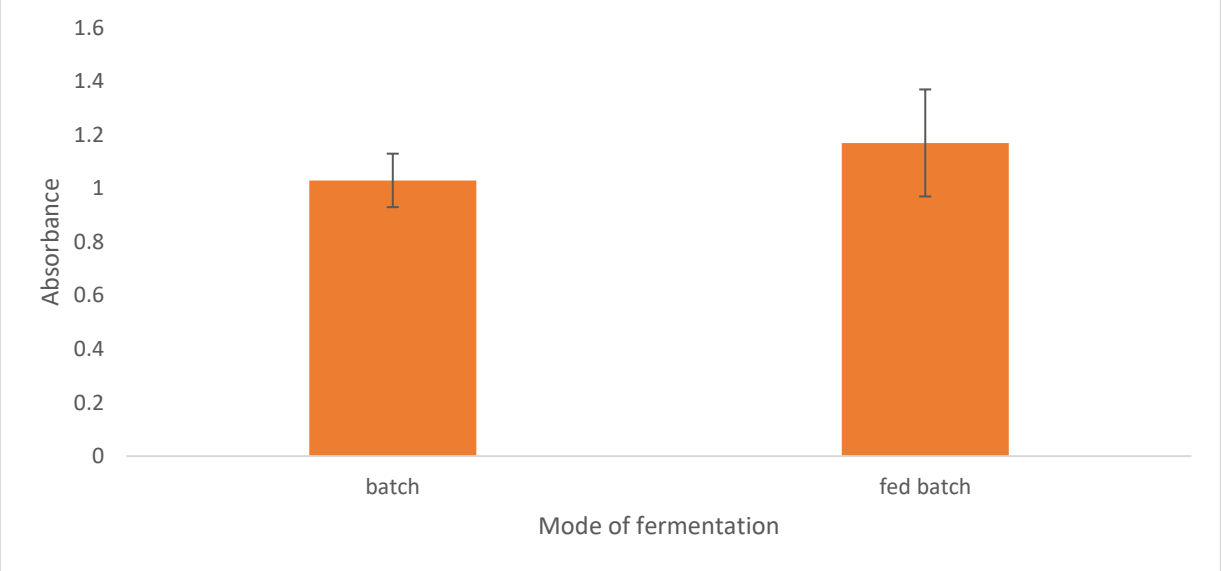


Figure 2. Fed batch fermentation was compared with batch fermentation. Batch fermentation showed maximum absorbance at 6 % GEP (W/V) while variable volume fed batch fermentation showed growth of *Bti* at 10 % *Bti*.
The bars correspond to standard error calculated from three replicates of same sample

Figure 2 compares batch fermentation with fed batch fermentation of GEP media. Fed batch showed higher activity at higher GEP concentration compared with batch fermentation. The final concentration was increased in fed batch fermentation while it was 6 % in batch fermentation.

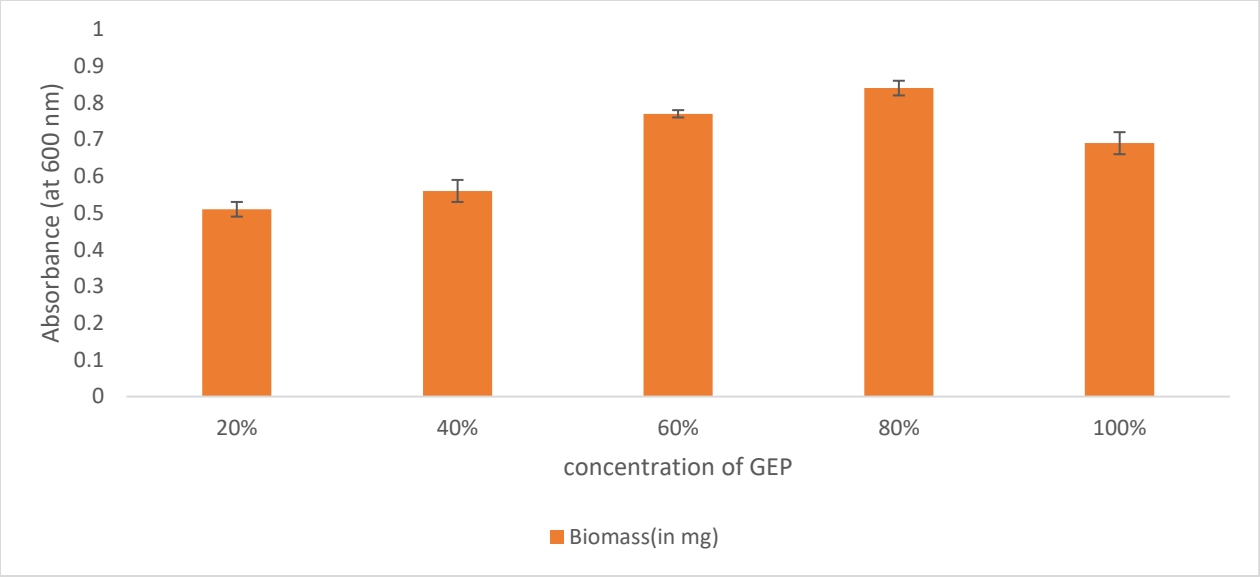


Figure 3. Solid state fermentation of *Bti* using GEP as substrate. 80% GEP (W/V) showed maximum biomass of *Bti*. The graph was plotted for concentration against biomass weight in mg (mean±SD).
The bars correspond to standard error calculated from three replicates of same sample

Figure 3 shows solid state fermentation of *Bti* in GEP media. Figure 3 shows solid state fermentation of GEP media with different nitrogenous substrates. Solid state fermentation using 80 % GEP media showed more activity. Nitrogenous supplements were added to 80 % GEP media to check their efficiency.

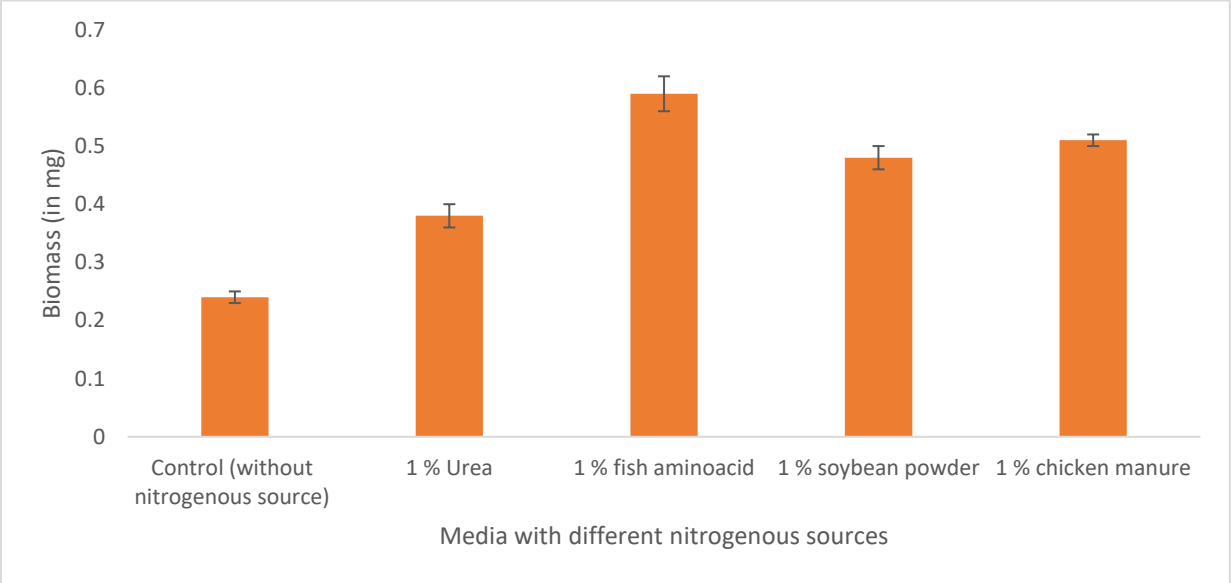
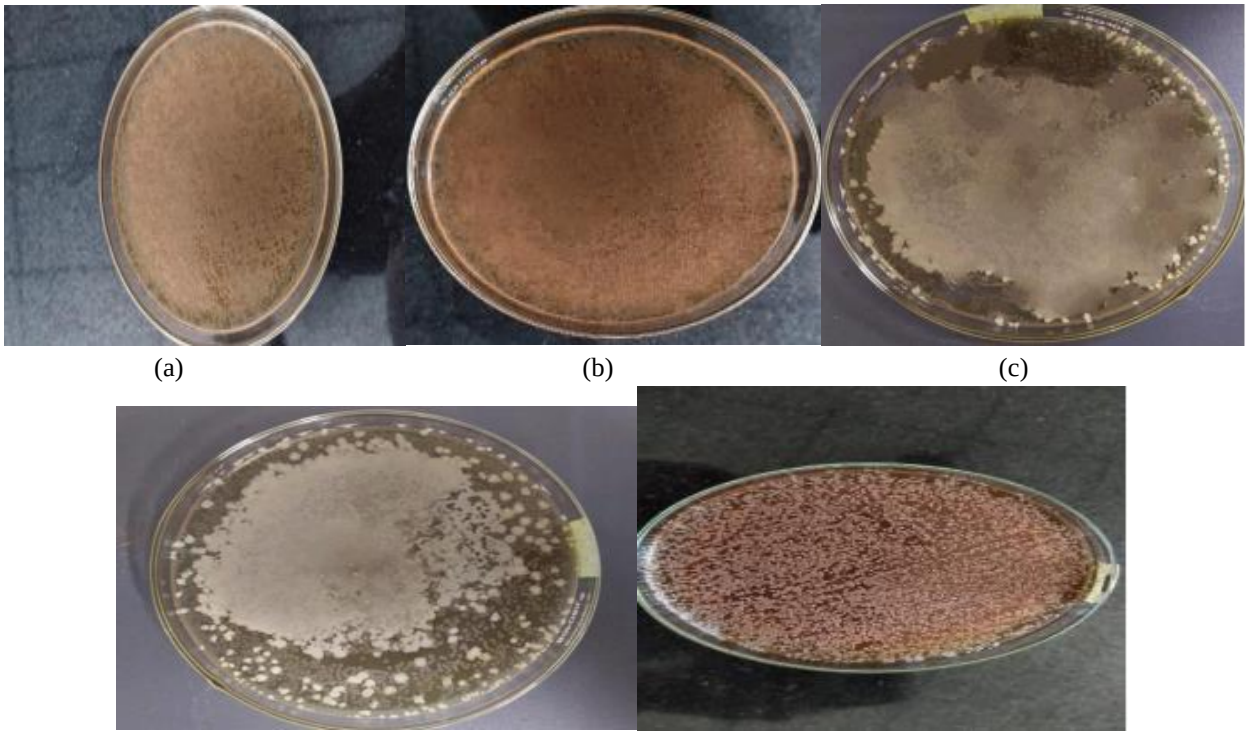


Figure 4. Biomass of *Bti* produced using different nitrogenous sources with 80% GEP supplemented media. Urea incorporated media shows minimum biomass when compared with others. 1 % aminoacid showed more activity (mean±SD).
The bars correspond to standard error calculated from three replicates of same sample

Figure 4 shows biomass formation of *Bti* grown on different nitrogenous media. From figure, we can observe that 1 % fish aminoacid supplemented media shows more activity.



(a) (b) (c)

(d) (e)

Figure 5. *Bti* grown on GEP enhanced media with different nitrogenous supplements a) Control b) Urea c) fish aminoacid d) soybean powder e) chicken manure. Culture plates showing growth of *Bti* on 80 % GEP media.

Figure 5 shows the biomass formed on different nitrogenous supplements. From this culture plate biomass were scrapped and was weighed and from that we concluded the growth of *Bti* on different nitrogenous supplements.

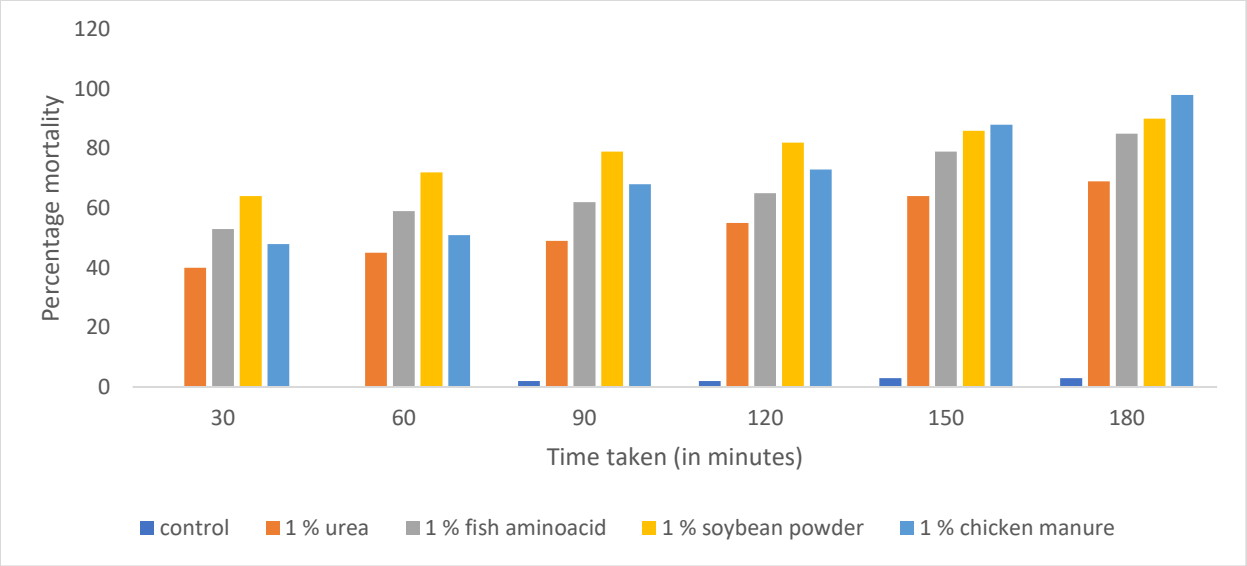


Figure 6. Biolarvicidal assay of *Bti* biomass from solid state fermentation using GEP media supplemented with different nitrogenous wastes (mean ±SD). All the nitrogenous supplements showed activity but 1 % chicken manure based biomass more mortality in larvae after 180 minutes.

The bars correspond to standard error calculated from three replicates of samples.

Figure 6 shows time dependent biolarvicidal assay of the larvae of *Aedes aegypti*. Biomass grown on four different nitrogenous supplements and 80 % GEP showed larvicidal activity but more activity was seen in 1 % fish aminoacid.

DISCUSSION

As evident from figure 1, there is substrate repression when the fermentation media was made with 10% GEP. Since it was showing repression, fed batch fermentation will be an alternative method to improve cell density for the production of *Bti* using GEP. Submerged fermentation involves inoculation of the microbial culture into the liquid medium for production of the desired product. Most of the commercial products are produced through the submerged fermentation. Repression was observed after 5 % GEP media.

Fed-batch fermentation is an operational technique in biotechnological processes where one or more nutrients (substrates) are fed to the bioreactor during cultivation and in which the product(s) remain in the bioreactor until the end of the run. Variable volume and fixed volume fed batch fermentations are the major types of fed batch culture. As the name implies, a variable volume fed-batch is one in which the volume changes with the fermentation time due to the substrate feed. The way this volume changes is dependent on the requirements, limitations and objectives of the operator. In fixed volume fedbatch, the limiting substrate is fed without diluting the culture. The culture volume can also be maintained practically constant by feeding the growth limiting substrate in undiluted form. Variable volume fed batch was more efficient in producing *Bti*. Figure 2 compares the biomass production of *Bti* using batch and variable volume fed batch fermentations. From this data it is clear that variable volume fed batch gives much more biomass compared to batch fermentations. Figure 3 shows the solid state fermentation of *Bti* using rotten grape juice as substrate. From the table it is evident that different concentrations of GEP was 20%, 40%, 60 %, 80% and 100 % showed gradual increase in biomass concentrations until 80% GEP. So for better productivity, solid state fermentation can be tried on an industrial scale. Solid state fermentation is a microbial process in which a solid material is used as the substrate or the inert support for the microorganisms growing on it. In solid state fermentation, microorganisms can sometimes grow well and produce large amounts of extracellular enzymes and other metabolites than they do in submerged fermentation (liquid).

From figure 4 &5, 80 % GEP media supplemented with 1% soyabean powder gives enhanced biomass production (0.48g) compared to control (0.25g). It was showing 63.01% increase in biomass. with 1% urea gives enhanced biomass production (0.31g) compared to control (0.25g). It is showing 21.42% increase in biomass. Since urea is a cheap nitrogen source, it can be cost effectively used for the large scale production of Bti. From figure 4 it is evident that 80 % GEP media supplemented with 1% chicken manure gives enhanced biomass production (0.51g) compared to control (0.25g). It is showing 68.42% increase in biomass. 80 % media supplemented with 1% fish slurry gives enhanced biomass production (0.59g) compared to control (0.25g). It shows 80.95% increase in biomass.

Figure 6 shows biolarvicidal assay of *Bti* produced from different nitrogenous supplements. 1 % urea after 180 minutes showed 69 % death while 1 % chicken manure supplemented GEP media showed more death i.e., 98% after 180 minutes.

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