Process development for the improved yield and cost effective production of *Bacillus thuringiensis subspecies israelensis* endotoxin based biopesticide

Thesis submitted to
University of Calicut in partial fulfillment of
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by

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CERTIFICATE

This is to certify that the thesis entitled **"Process development for the improved yield and cost effective production of *Bacillus thuringiensis subspecies israelensis* endotoxin based biopesticide"** submitted to the University of Calicut, as a partial fulfillment of Ph. D. programme for the award of the degree of **Doctor of Philosophy in Biotechnology** by **Gopinathan C**., embodies the results of bonafide research work carried out by him under my supervision and guidance in the Department of Biotechnology, and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title or recognition.

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Prof. (Dr.) K. K. Elyas
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DECLARATION

I hereby declare that the work presented in the thesis entitled **"Process development for the improved yield and cost effective production of *Bacillus thuringiensis subspecies israelensis* endotoxin based biopesticide"** submitted to the University of Calicut, as partial fulfillment of Ph. D. programme for the award of the degree of Doctor of Philosophy in Biotechnology, is original and carried out by me under the supervision of **Prof. (Dr) K. K Elyas**, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any university.

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Gopinathan C.

Dedicated to

My Beloved **Parents** and
to my amazing Teacher, Guru and exceptionally good Fermentation technologist
Prof. Ignatius Davis Konikara.

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ABBREVIATIONS

Bt	-	*Bacillus thuringiensis*
Bti	-	*Bacillus thuringiensis* israelensis
Bs	-	*Bacillus sphericus*
Cry	-	Crystal
Cyt	-	Cytotoxic
ICP	-	Insecticidal crystal proteins
%	-	Percentage
$	-	US dollar
R &D	-	Research and development
UV-B	-	Ultravoilet -B
CSTR	-	Continously stirred tank reactor
INR	-	Indian national rupee
Mm	-	Milli meter
WHO	-	World health organisation
SSF	-	Solid state fermentation
Kg	-	Kilo gram
>	-	More than
Kla	-	Volumetric mass transfer coefficient lamda
max	-	Lamda maximum
nm	-	Nanometer
cm	-	Centimeter
cm^2	-	Centimeter square
mg	-	Milligram

gm	-	Gram
psi	-	Pounds per square inch
rpm	-	Revelutions per minute
w/w	-	Weight per weight
N	-	Normal
NaOH	-	Sodium hydroxide
CaOH	-	Calcium hydroxide
HCL	-	Hydrochloric acid

CHAPER 1
INTRODUCTION

1.1 Vector born diseases

Vector-borne diseases are human illnesses caused by parasites, viruses and bacteria that are transmitted by mosquitoes, sandflies, triatomine bugs, blackflies, ticks, tsetse flies, mites, snails and lice. Many of these vectors are blood sucking insects that ingest disease-producing micro-organisms when they feed on infected individual and then transmit it to healthy persons in next bite.

1.2 Incidence and estimated report

Mosquitoes cause more human suffering than any other organism. Over one million people worldwide die from mosquito-borne diseases every year. (https: //www. mosquito. org/page/diseases)

Every year there are more than 700, 000 deaths from diseases such as malaria, dengue, schistosomiasis, human African trypanosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis and onchocerciasis, occur globally (WHO-.2020:https: //www. who. int/news-room/fact-sheets/detail/vector-borne-diseases)

In 20018, globally there were an estimated 243 million malaria cases with 8, 63, 000 deaths.89% of the reported deaths were in Africa. Most of the deaths were among children under 5 years' old and pregnant women. The annual economic costs of malaria in Africa in terms of foregone production have been estimated to be about US $12billion.

1.3 Social impact

As vectors thrive under conditions where people are poor, water is unsafe, and environments are contaminated with filth, these diseases extract their heaviest toll on the poor – the people left behind by development. Measures that control the vectors, the agents of disease, provide an excellent, but underutilized opportunity to help these people come up (https: //www. ncbi. nlm. nih. gov/pubmed/1568273) .

Usually children are more prone to being infected due to various diseases transmitted by flies, mosquitoes and other insects which act as vectors.

About 275 million people are reported to be infected with malaria every year, making it one of the most dreaded vector -borne diseses in the world and approximately 2.4 billlion people prone to new infections. Over one million children die of malaria each year.

Dengue fever is another serious vector borne tropical disease approximately 50, 000 cases of dengue hemorrahgic fever and about 100 million cases of dengue fever every year. This is especially more fatal to children.

Other regionally important vector borne diseases especially dangerous to children are Chagas disease, Japanese encephalitis, Onchocerciasis (river blindness) , Lymphatic filariasis, Yellow fever and African trypanosmiasis (sleeping sickness) .

Aquatic snail species act as the intermediate host for the water based disease schistosomiasis which mainly affects children and adolescent people.

1.4 Diseases spreading vectors

1.4.1 Mosquitoes

About 3000 species of mosquitoes have beeen identified. The female mosquito spread the disease with proboscis and extract blood from their victims, which is essential for egg laying. Diseases that are spread are Malaria, Filariasis, Yellow Fever Encephalitis, Venezuelan Equine Encephalitis, MurrayValley Encephalitis, Dengue Fever, West Nile virus, Rift Valley Fever, Chikungunya, Japanese. (https: //www. nationalgeographic. com/animals/ invertebrates/ group/ mosquitoes)

Vectors other than mosquitoes which play an important role in transmission of various diseases

1.4. 2 Sand Flies

Sand flies are blood feeders and usuallly multiplies in dark caves and burrows made by rodents like rats, which have organic matter and high humidity. They move by short flights since they are not strong fliers. Due to thier small size, its difficult to detect them, until they bagin biting. Discomfort caused due to biting continues for several days. Different types of sand flies are Black gnats, Biting Midges, Chitras, Punkies. They transmit two major diseases- Sand fly fever and Leishmaniasis.

1.4.3 Black Flies

Black flies are yet another relative of mosquitoes that are specialized for breeding in running water from small trickles to large rivers. Unlike mosquitoes, black flies feed by slashing through the skin, and they never feed indoors. They can attack in such large numbers that their salivary fluids alone can cause a person to become ill, causing a condition called“Black fly fever. ” They also vector a nematode that can live in the human body for upto

fifteenyears destroying tissues in internal organs, most notably in the eye thereby causing blindness. Different types of black flies are Turkey Gnat and White Socks. Diseases vectored are River Blindness/Onchocerciasis and Black Fly Fever.

1.4.4. Kissing Bugs

Triatomines are large insects with nocturnal habits. Usually found in structures with thatched roofs that offer hiding places during the day time. Their bites are generally painless. The insect has a habit of defecating after biting the victim, which later enters the body due to scratching the site by the victim. Different types of kissing bugs are Benchuca, Vinchuca, Chipo, Pito, Chupanca, Barbeiro, Conenose bugs, Assassin Bugs. Chagas disease is spread by this bug.

1.4.5 Ticks

Ticks in general have a much longer life cycle than a mosquito. Hard ticks feed only a few times during their lifespan, which tends to limit their odds of acquiring an infection. Nevertheless, the longevity and host selectivity of hard ticks allows them to be relatively efficient vectors. Soft ticks are long-lived nest and burrow dwellers. Like mosquitoes they can feed many times during their life span.

Ticks have a much longer life span than mosquitoes and feed few times compared to mosquitoes, which reduces the chance of getting an infection. They are efficient vectors due to the longevity and host selectivity. At the same time soft ticks are burrow and nest living creatures and can feed many times comapred to hard ticks. Diseases spread by ticks are Tick-borne Relapsing Fever, Tick-borne Encephalitis, Lyme Disease.

1.5 Importance of Vector control and success stories

For diseases where there is no effective cure, such as West nile virus, Zika virus, and Dengue fever and west nile virus, vector control remains the only way to protect human populations.

Mosquitoes being members of the culicidae family, are ectoparasites, with proboscis using which it pierces the skin and sucks the blood of the victim. Malaria, filarial diseases such as dog heartworm, and viruses such as dengue, encephalitis and yellow fever are some of the diseases vectored by mosquitoes.

The cost of treatments remains as the major barrier for many people, for diseases even for diseases which are curable. According to World health organisation (WHO,2019) estimates ecnomic losses upto 1.3 % occurs due to malaria and a child dies in every minute in africa due to malaria. So in this context both prevention and treatment of the disease assumes more importance,WHO,2019 (https: //www. who. int/news-room/fact-sheets/detail/ malaria) .

1.6 Measures taken for vector control

Massive spraying of chemical pesticides in 1940-1960s has brought a drastic reduction in many vector borne diseases. The impact of such uncensured usage was disastrous, with impacts like environmental problems and health hazards like contamination of water and food sources, poisoning of non-target flora and fauna, accumulations in food chain, which ultimately leading to biomagnifications. (Enny S. Paixão, et. al, 2018)

1.7 Chemical control and the disadvantages

Although the discovery of synthetic insecticides was a major breakthrough, rampant usage of chemical pesticides many vector borne diseases have re-emerged or spread to new geographical areas, mainly due to

insecticide resistance. Inspite of the tremendous benefits chemical insecticides provide, especially for enhancing food production and improving human health condition, they pose serious threats to non target fauna and flora, water and food sources, and also will eventually select some insect populations which are insensitive to pesticides. It also seriously affect biological control and may help secondary pest populations that are previously well controllled by its natural enemies.

1.8 Toxicity, bio-accumulation and loss of bio-diversity

A number of chemical pesticides are proven carcinogens, causing different types of lymphomas, leukemia, breast cancer, asthmatic problems, and other immunity related disorders. Its use also has very serious, socio-ecnomic and environmental ramifications. Since the cost of crude oil is increasing due to its reduced availabilty, it adds to the manufacturing cost of chemical pesticides. Moreover the efficacy of pesticides is generally decreasing due to resistance development. This has forced the industry to come up with novel more expensive pesticides, most of them not affordable by developing countries. From a field application point of view, its a fact that only a minute fraction of the sprayed pesticide is required for killing insects, and ramaining 99 % spills to the environment through water, soil and food cycles.

1.9 Biological control an its importance

In order to reduce the environmental problems due to chemical pesticides alternate methods of pest control has to be used. One of the effective method is to use microbial insecticides, which contain microragnisms, or their products. These types of products are especially valuable since they are very specific and does not affect other non-target animals including human beings. In agriculture they are safe, both for pesticide user and consumers.

1.10 Biological control agents

Formulations containing Bacteria, Fungi, virus, pheromones, and plant extracts have been in use mainly for the control of various insects which cause damage to forest and agricultural crops.

1.11 *Bacillus thuringeinsis* - Discovery and strains

Bacillus thuringiensis preprations are the most widely used in the world. Proteinaceous parasporal inclusion bodies are mainly responsible for the insecticidal activity.

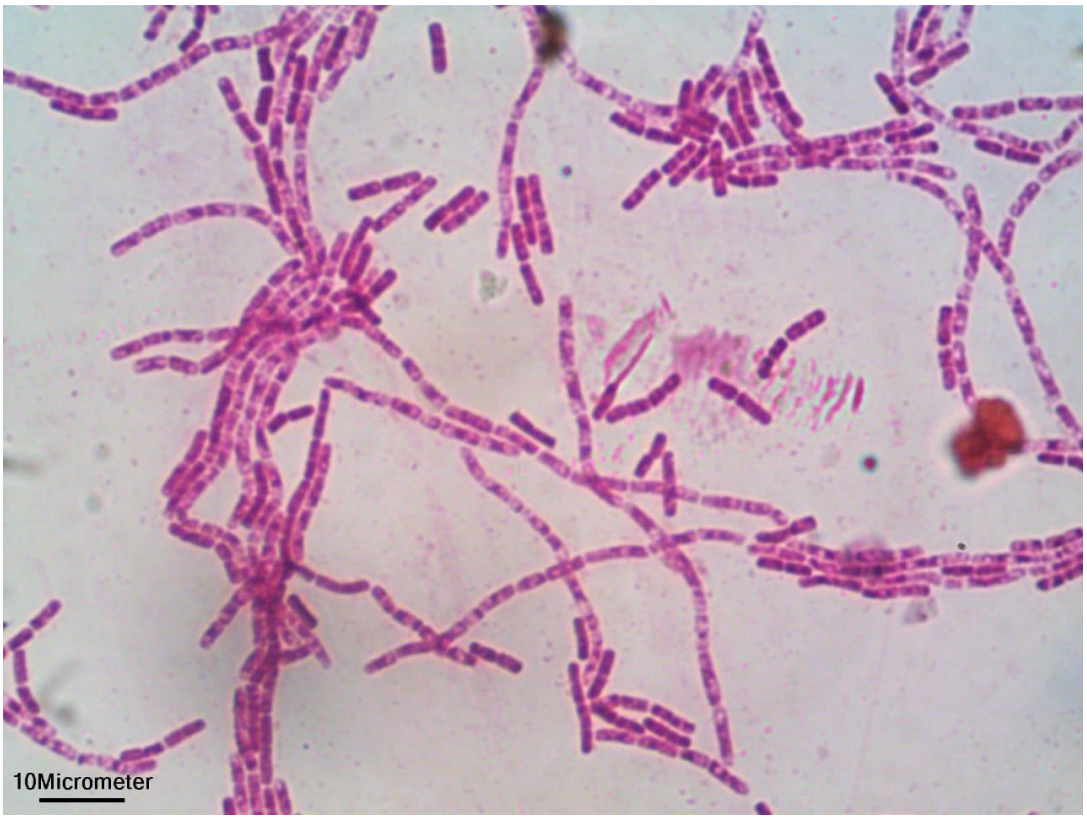

Fig: 1. *Bacillus thuringiensis israelensis sporulated cells seen under microscope by simple staining with crystal voilet .*

Bt strains have been isolated from varied habitats, including stored-product dust, and deciduous and coniferous leaves, soil and insects which

spans a wide range of insect orders which include: Diptera, Hymenoptera, Homoptera, Phthiraptera, Coleoptera, Orthoptera, Acari, and Mallophaga and other organisms such as nematodes, mites, and protozoa.

Bacillus thuringiensis subsp. israelensis (Bti) serotype H-14 is very specific in its action against dipteran insects such as mosquitoe larvae and Black flies and is the most popular microbial insecticide used world wide.

Onchocerciasis control program in west africa was great success with the use of *Bti* toxins, implemented by WHO and stands out as a good example of international co-operation for the control of infectious diseases program.

1.12. Mode of action of *Bti*

The mosquitoe larvae should feed the sporulated *Bti* cells or ICP or spore -icp complex. Depending on solubilisation the efficacy of ICP will vary, which involves protoxin to toxin conversion by proteolytic enzymes, membrane receptor binding by the C-terminal domain of the active toxin, and formation of pores by the N-terminal domain followed by lysis of epithelial cells. Multiplication of vegetative cells in the haemocoel may result in septicaemia, which results in the death of the larvae. Specificity of different *Bt* ICPs are dependent on binding to specific epithelial receptors.

Goldberg and Margalit isolated *Bti* from Israeli dessert in 1970, which has proven very effective agaist dipteran larvae, including black flies and mosquitoes. In contrast to most of the *Bt* based pesticides which are intended for topical use in agriculture, *Bacillus thuringiensis israelensis* is specifically targetted against mosquitoe larvae and therefore sparyed in water bodies and staganant pools to prevent spread of vector borne diseases.

Cry proteins (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa) and two Cyt proteins (Cyt1Aa and Cyt2Ba) are encoded by a mega plasmid which carries

the genes for all cry proteins. Since smaller particles spend more time floating, such formulations are more in demand, and easily caught by the filter feeding mosquitoe larvae. Similarly slow release formulations has been found to be very effective in field conditions.

1.13 Progress in Bt research- transgenic crops and plants

So far *Bt* products have an impeccable record in terms of safety, efficacy and environmental benefits and was found to be very much helpful in boosting ecnomic growth both in developing and industrialised countries. R and D efforts continue to create novel strains with more specific and enhanced effects and to develop transgenic plants which resists pests and curtail the scope of development of pesticide resistant strains. All these new traits will substantially help both health professionals and farmers the improved products to fight insect borne diseases and fight pests, by maintaining high crop yields and protecting the health condition of billions of people in the world.

1.14 Advantages of *Bt* over other methods

The main highlight of *Bti* products over chemical pesticides is its exeptionally high specificity against dipteran insects, predominantly due to the presence of membrane recptors in the intestine of the larvae which act as the targets for various bacterial toxins. In vertebrates these types of receptors are absent, which make it very safe for humans.

1.15 Limitation of vector control by *Bt*

Inspite of its high specificity and sefty, *Bti* products also has some draw backs. Since *Bti* products are usually sprayed in open water bodies exposed to sunlight, it is amenable to degradation by the inherent UV-B wave length radiations. Some may become totally inactive within 24 hours also. So

many plants have multiple pests, and so many *Bti* strains are required to cover the whole insect spectrum for effective crop protection. Moreover *Bti* products have to be eaten by the target insect for expected outcomes, while chemical pesticides can kill without being eaten.

1.16 Methods of *Bti* production

Conventional production is by submerged aerobic Fermentation proceess using Continously stirred tank reactor (CSTR) . Usually batch fermentation process is employed. Most of them are given as part of patent documents, so details are not avilable. Some details regarding patent infromation is given below.

A bioinsecticide formulation consisting of *Bacillus thuringiensis var israelensis***,** and its concerning manufacture- proceedings **2001-11-29** Publication of WO2001089297A2 (Elizabeth Gomes., 2000) .

Current patents related to *Bacillus thuringiensis* insecticidal crystal proteins. Shu C1, Zhang J. State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, 100193, P. R. China (Shu. C. et al., 2009) .

Abstract: This work categorizes a number of patents related to *Bacillus thuringiensis* insecticidal crystal proteins. The patents are classified into groups according to the type of toxins appearing in the claims. The purpose of the summary is to promote the application of *B. thuringiensis* insecticidal crystal proteins and the development of patentable technologies.

The earliest report on possible application of SSF in production of *Bt* appeared in a form of U. S. patent by Mechalas (1963) followed by reports by Dulmage and Rhodes (1971) and Sittig, et. al (1977) .

1.17 Bioprocess involved in *Bti* production process

High production cost still remains as the major challenge for the industrial production of *Bt* based biopesticides. Raw material cost mainly decides the ecnomics of the fermenation. Materials like waste water sludge has been tried to explore its potential as a feed stock material for *Bti* production. These types of low cost approach will be helpful for further reduction of product cost, since *Bti* products are already in the market.

The metabolic patterns of *Bacilus thuringiensis* are triphasic-vegetative growth, transition and sporulation. Most varieties are able to use glucose, fructose, starch, maltose, ribose, glycerol, organic acids, glutamate and other amino acids. Optimal endotoxin production occurs in 28 to 32 ←C pH range is 5.5 to 8.5. the usual initial pH of 6.8 to 7.2. Oxygen is essentially required for growth during vegetative growth. In addition to the manipulation of pH and temperature, optimising the composition of media and ensuring appropraite conditions with respect to dissolved oxygen is very much essential for the commercial success of the process.

1.18 Limitation of bioprocess

The cost of raw material has become one of the major limiting factor in reducing the production cost. Further since its a highly aerobic process, the supply of oxygen becomes limiting especially at high cell densities. In addition there is catabolite repression above 3% when simple sugars are used for its production.

1.19 Stability problems

Many *Bt* insecticides have a shorter shelf life than other synthetic insecticides. Generally after two or three years their effectiveness is reduced greatly. The optimal storage conditions for *Bt* products are cool, dry air and

out of direct sunlight. Moreover its production cost is high. It is also affected by solar radiations like UV radiations when it is sprayed in the seweage.

1.20 Economics of *Bti* production and Importance of cheap media

Biotechnology is the use of living systems and organisms to develop or make products, or "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" (UN Convention on Biological Diversity) .

Biotechnology mainly involves manufacturing value added compunds by using microbes. The success of this technology depends on cost effective production of microbial products at an industrial scale.

In this context the expendture incurred under various heads becomes the major deciding factor, for the ecnomic feasibility and commercial success of the enterprise. Out of the various expenditure incurred, the money spent on procuring raw materials usually becomes the major head. Naturally, ways and means have to be found out to reduce expenditure incurred due to substrates. Traditional bacterial growth media substrates like, Glucose, peptone, yeast extract, beef extract are all expensive and cannot be practised on a large scale, due to its high cost.

Alternative cheap raw materials, which can easily be assimilated by the industrial organism should be found out and a cost effective process should be developed to make it ecnomically feasible.

If a fermentation processs has to yield a product at a competitive price, the chosen micro-organism or animal cell culture should give the desired end product in predictable, ecnomically adequate, quantities. A number of basic objectives are commonly used in developing a sucessful process which will be ecnomically viable.

1. The capital investment in the fermenter and ancilliary equipment should be confined to a minimum, provided that the equipment is reliable and may be used for a range of fermentation products.

2. Raw materials should be as cheap as possible and utilized efficiently.

3. The highest yielding micro-organism should be used.

4. There should be saving in labour whenever possible and automation should be used where ever it is feasible.

5. When a batch process is operated, the growth cycle should be short as possible, to obtain the highest yield of product and allow for maximum utilization of equipment , may be possible by use of fed - batch cuture.

6. Recovery and purification procedure should be as simple and rapid as possible.

7. The effluent discharge should be kept to a minimum

8. Heat and power should be used efficiently.

9. Space requirements should be kept to a minimum, but there should be some allowance for potential expansion in production capacity.

10. All the above points must comply with safety guidelines and regulations.

In any process it is important to know the cost breakdown. Four basic components contribute to the processs cost -Raw materials, fixed costs, utilities and labour. In microbial biomasss production upto 60% is spent on raw material cost.

The cost of the various components of a production medium can have a profound effect on the overall cost of a fermentation process, since these account for 38 to 73% of the total prduction cost. Moreover the Carbon: Nitrogen ratio of the medium also has a profound effect on the growth and secretion of various microbial products (Stanbury P. F., Whitaker A., Hall S.2nd Edition, 1995)

India being an agricultural country is one of the leading producer of fruits, vegetables, tubers and cereals and pulses, which are available in huge quantities. Out the total production, almost 30% of the produce goes as wastes, due to lack of infrastructural facilities, microbial spoilage, rough handling in the field and markets.

In addition there is no systematic and centralised procurement startegies to bring the produce from the field to main processing centres.

India, the world's second largest producer of fruits and vegetables, is throwing away fresh produce worth INR 133 billion every year because of the country's lack of adequate cold storage facilities and refrigerated transport, according to the data compiled in a new report by Emerson Climate Technologies India, a business of the US-based manufacturing and technology company Emerson (Economic Times ,2013;https: //economictimes. indiatimes. com/news/economy/agriculture/india-wastes-fruits-and-vegetables -worth-rs- 13300-crore-every-year-emerson-study/articleshow/26523928)

The Emerson food wastage and cold storage report cites that have pegged the value of fruits, vegetables and grains wastage in India at INR 440 billion annually. Fruits and vegetables account for the largest portion of that wastage.

Eighteen per cent of India's fruit and vegetable production – valued at

INR 133 billion – is wasted annually, according to data from the Central Institute of Post-Harvest Engineering and Technology (CIPHET) . Two of the biggest contributors to food losses are the lack of refrigerated transport and the lack of high quality cold storage facilities for food manufacturers and food sellers (Daily Bhaskar-2013,https: //daily. bhaskar com/news/NAT-TOP-india-wastes-fruits- vegetables-worth-rs-13300-crore-every-year-emerson-report-4448764-NOR. html)

1.21. Problems to be adressed

* Lack of cost effective manufacturing procesess
* Selection of right mode of fermentation process
* Developing Simplified and cost effective down stream processing protocols

Bti being a microbial biomass based product can be cost effectively manufactured by channelising agrowastes like coconut water, Taro, Sweet potato, Purple yam, Cassava, Bagasse, Rice straw etc through proper bioprocessing (fermentation technology) , in order to covert it into *Bti* based biopesticide which consists of *Bti* cells, lysed cells, spores and delta endotoxin. This strategy will substantially reduce the production cost and will help to compete in the market with other chemical insecticides, which are generally cheaper than biopesticides. In addition the mode of fermentation carried out to get the desired microbial product also decide the cost (submerged/ batch/fed-batch/solid state, etc) . Since down stream processing also consumes huge amounts of money, cost effective simple methods were attempted including methods to extend shelf life of the products.

1.22 Objective of the research work

The main objective of this research work is to develop a high yielding cost effective process for the production of *Bacillus thuringiensis subspecies isarelensisH-14* based biopesticide.

In summary attempts were done to ;

* Produce *Bti* using a range of carbon sources like vegetable wastes, fruit wastes, coconut water, Tapioca peels/ pith, locally available tubers like Taro, Sweet potato, Purple yam and lignocellulosic wastes like bagasse and Rice straw.

* Optimize the C: N ratio of the respective media fish/chicken feather wastes.

* Optimize different growth conditions for submerged /solid state and fed batch fermentations of *Bti* for maximising the yield.

* Study the impact of dominanat amino acids in boosting amylase and delta endotoxin production of *Bti* when fed as fermentation medium additives.

* Study the factors which initiates early sporulation.

* Develop cheaper methods of down stream processing.

* Improve Shelf life and field performance of *Bti* produced using the novel process.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Vectors and its role in disease transmission

Vectors are living organisms that can transmit infectious diseases between humans or from animals to humans. Most of the vectors feed on human blood and thus ingest disease causing microbes along with it transmitting infections from animal to humans or from humans to another human being. Mosquitoes being the predominant one, and others also include different types of ticks, sandflies, bugs and fresh water snails.

Vectors are responsible for almost 17% of all infectious diseases. These types of vectors are mostly seen in tropical and subtropical areas, and usually affect the poorest populations. There were major outbreaks of chikungunya, yellow fever , Zika, Dengue and Malaria which have affected millions of people across the world, and killed so many people, and also seriously affected the health systems in many countries.

The control of vector-borne diseases can make a major contribution to poverty reduction, as it precisely targets the poor. A global health agenda that gives higher priority to vector control could save many lives and avert much suffering. Doing so is especially important for diseases like dengue and chikungunya, which have neither a vaccine nor an effective treatment. (WHO,Newsroom-Vectorbornediseases-WHO, https: //www. who. int/en/news-room/fact- sheets/detail/vector-borne-diseases)

Each year, around half a million patients with severe dengue require hospitalization. Onchocerciasis causes blindness, severe joint pain that can last for weeks, and Chagas disease in its late stage can cause heart failure and

early death in young adults. Japanese encephalitis can permanently damage the central nervous system. Schistosomiasis – the most widespread of all these diseases – contributes to poor nutritional status and poor school performance. Some forms of leishmaniasis are rapidly fatal; others cause severe facial disfigurement. Around 120 million people are currently infected with lymphatic filariasis, and about 40 million of them are disfigured and incapacitated by the disease. Lost productivity is one consequence. Stigma and social exclusion are additional sources of misery, especially for women. Many of these diseases have been historically confined to distinct geographical areas, but this situation has become more fluid due to a host of ills, including climate change, intensive farming, dams, irrigation, deforestation, population movements, rapid unplanned urbanization, and phenomenal increases in international travel and trade. These changes create opportunities for vectors and the diseases they spread to take up residence in new areas. A global brief on vector-borne diseases– WHO 2014 (www. searo. who. int/thailand/2013/ WHO_ DCO_ WHD_2014.1_eng. pdf*)*

Of all the arthropods, the Order Diptera contains the most species that transmit pathogens or are harmful to humans. Yet, of the 106 Families included in this Order, only 10 are of medical importance and, within these, relatively few species are implicated in disease transmission. For example, in the afrotropical Region, the Culicidae (mosquitoes) , widely regarded as the most important of the Diptera because of their involvement in malaria and filariasis transmission, is represented by over 780 species but only around 20 of these are important vectors of disease, representing around 2.5% of this diverse Family (Trans R Soc Trop Med Hyg 2014; 108: 179–180) doi: 10.1093/trstmh/tru020)

Mosquitoes have four distinct stages in their life cycle : Egg, larva, pupa and adult. Depending on the species a female lays between 30 and 300

eggs at a time on the surface of water. Once hatched, the larvae grow in four different stages (instars) . The first instar measures 1.5 mm in length, the fourth instar about 8-10mm. The fully grown larvae then changes into a comma shaped pupa. When mature, the pupal skin splits at one end and a fully developed adult emerges. The entire period from egg to adult takes about 7-13 days under good conditions (Wada, 1989) .

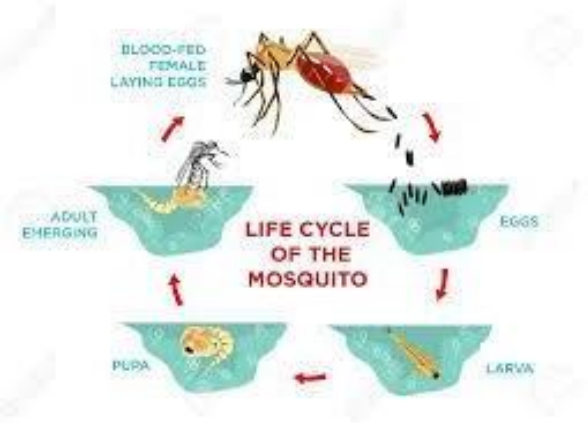

Fig. 2 Mosquito life cycle -*Bti* controls the breeding of mosquitoes by killing specifically the mosquito larvae.

Mosquito-bites not only cause nuisance and discomfort but also transmit some very dreadful diseases such as Dengue fever and Japanese encephalitis. Mosquitoes cause more human suffering than any other organism – over one million people worldwide die from mosquito-borne diseases every year. Mosquitos and other biting DipteraWHO, 2008-(https://www. who. int/water_ sanitation_health/vector007to28. pdf*)*

These diseases also exacerbate poverty. Illness and disability prevent people from working and supporting themselves and their family, causing further hardship and impeding economic development. Dengue, for example, imposes a substantial economic burden on families and governments, both in medical costs and in working days lost due to illness. According to studies conducted from eight countries, an average dengue episode represents 14.8

lost days for ambulatory patients at an average cost of US$ 514 and 18.9 days for non-fatal hospitalized patients at an average cost of US$ 1491 (A global brief on vector-borne diseases – WHO, 2014apps. who. int/iris/bitstream/10665/. . . /WHO_DCO_WHD_2014.1_eng. pdf*)*

The number of cases reported increased from 2.2million in 2010 to 3.2 million in 2015. The worst outbreak reported in Delhi, India in 2015 with over 15000 cases. More than one million deaths and 300 - 500 million cases of Malaria are still reported annually in the world. Around 120 million people are currently infected with lymphatic filariasis and about 40 million of them are disfigured and incapacitated by the disease. Vector-borne diseases account for more than 17% of all infectious diseases, causing more than 700, 000 deaths annually. More than 3.9 billion people in over 128 countries are at risk of contracting dengue, with 96 million cases estimated per year. Malaria itself causes more than 400, 000 deaths every year globally, Other diseases such as Chagas disease, leishmaniasis and schistosomiasis affect hundreds of millions of people worldwide.

Some of the vectors and diseases caused by them is given below

2.2 Mosquitoes:

Aedes -Chikungunya (Chikungunya virus) , Dengue fever (dengue virus) , Rift Valley fever (RVF virus) , Yellow fever (Yellow Fever virus) , Zika (Zika virus) .

Anopheles- Malaria (Plasmodium genus. Four species of Plasmodium can infect humans: *P. vivax, P. ovale, P. malariae and P. Falciparum.*)

Culex-Lymphatic filariasis (*Wuchereria bancrofti,* which is responsible for 90% of the cases. Brugia malayi, remaining cases) .

Sand flies - Leishmaniasis (*Leishmania major, L. tropica and L. infantum.*) , Sandfly fever (Phelebotomus fever-Phlebovirus.)

Ticks (Crimean-Congo haemorrhagic fever-Nairovirus) , Lyme disease (bacterium, *Borrelia burgdorferi*) , Relapsing fever (*Borrelia recurrentis*-borreliosis) , Rickettsial diseases (spotted fever and Q fever-*Rickettsia, Orientia, Ehrlichia, Neorickettsia, Neoehrlichia, and Anaplasma*) , Tick-borne encephalitis (Tick-borne encephalitis virus) , Tularaemia (*Francisella tularensis)* .

Triatomine bugs - Chagas disease (Protist *Trypanosoma cruzi*- American trypanosomiasis)

Tsetse flies-Sleeping sickness (*Trypanosoma brucei*-African trypanosomiasis)

Fleas – Plague (*Yersinia pestis*) , Rickettsiosis (*Rickettsia, Orientia, Ehrlichia, Neorickettsia, Neoehrlichia, and Anaplasma*)

Black flies-Onchocerciasis (Onchocerca volvulus- (River blindness)

Aquatic snails-Schistosomiasis (*Schistosoma haematobium,* S. *japonicum,* and *S. mansoni*- Bilharziasis)

Lice-Typhus and louse-borne relapsing fever-*Borrelia recurrentis*.

Table. 2.1 : Types of Mosquitoes and the diseases they transmit

Mosquitoes	**Table**
Aedes aegypti	Dengue, Yellow fever, Chikungunya, Zika virus disease
Aedes albopictus	Chikungunya, Dengue, West Nile virus Disease
Culex quinquefascicatus	Lymphatic filariasis, Saint Louis Encephalitis
Culex tarsalis	Saint Louis encephalitis, Western equine Encephalitis
Anopheles (more than 60known species can transmit disease)	Malaria, Lymphatic filariasis, (in Africa)
Haemogogus	Yellow fever

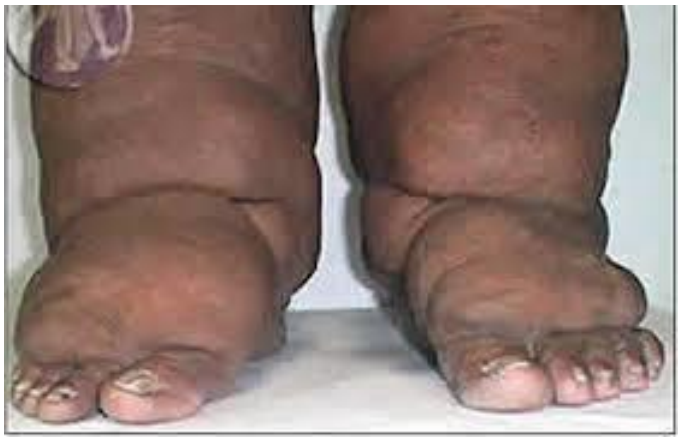

Fig. 3 Lymphatic filariasis caused by C*ulex* and A*nopheles* species.

(WHO,2014,**Vector-borne diseases - World Health Organizationhttps: //www. who. int)**

2.3 Malaria : is a parasitic disease that triggers fever, chills and a flu-like illness. Symptoms usually appear after a period of seven days or more after infection from a mosquito bite. Early diagnosis and treatment of malaria is key. If left untreated, the disease can lead to severe illness and death. The best available treatment, particularly for *Plasmodium falciparum* malaria, is artemisinin-based combination therapy (ACT) .

There is no commercially available vaccine against malaria although a promising vaccine against *P. falciparum* is currently being evaluated in a large clinical trials in seven African countries.

In 2012, there were about 207 million cases of malaria and an estimated 6, 27, 000 deaths. Increased prevention and control measures are dramatically reducing the malaria burden in many places. Since 2000, malaria mortality rates have fallen by 42% globally and by 49% in the WHO African Region. Malaria is caused by Plasmodium parasites transmitted through the bites of female Anopheles mosquitoes, which are active mainly between dusk and dawn. Around the world, malaria transmission occurs in 97 countries, putting about 3.4 billion people at risk. The disease burden is heavily concentrated in sub-Saharan Africa, where an estimated 90% of malaria deaths occur. Four out of ten malaria- related deaths occur in the two highest burden countries: The Democratic Republic of the Congo and Nigeria.

Malaria is caused by Plasmodium parasites transmitted through the bites of female Anopheles mosquitoes, which are active mainly between dusk and dawn.

Five parasite species cause malaria in humans and *Plasmodium falciparum and P. vivax* are the most common. *P. falciparum* is the most dangerous, with the highest rates of complications and mortality. This deadly form of malaria is a serious public health concern in most countries in sub-Saharan Africa. Anopheline mosquitoes are the only vectors of the Plasmodium parasites. Each of the 60 known species of Anopheles that can transmit malaria has its own biological and ecological peculiarities. Disease transmission is more intense where the mosquito species has a long lifespan and a habit of biting only humans, for example *An. Gambiae and An. funestus*, which are responsible for transmitting many of the deadly cases in Africa. (Malaria – WHO, 2019-https: //www. who. int/ith/diseases/

malaria/en/*)*

2.4 Lymphatic filariasis, commonly known as elephantiasis, is a painful and profoundly disfiguring disease. It is caused by infection with parasites classified as nematodes (roundworms) of the family Filariodidea that are transmitted through the bites of infected mosquitos. Mosquito-transmitted larvae are deposited on the skin from where they can enter the body. The larvae then migrate to the lymphatic vessels where they develop into adult worms, thus continuing a cycle of transmission.

In communities where filariasis is transmitted, all ages are affected. While the infection may be acquired during childhood its visible manifestations such as limbs oedema may occur later in life, causing temporary or permanent disability. In endemic countries, lymphatic filariasis has a major social and economic impact.

Lymphatic filariasis affects over 120 million people in 72 countries throughout the tropics and sub-tropics of Asia, Africa, the Western Pacific, and parts of the Caribbean and South America WHO, 2020 (https: //www. who. int/health-topics/lymphatic-filariasis)

2.5 Dengue fever is a severe, flu-like illness. Symptoms include high fever, severe headaches, muscle and joint pains, nausea, vomiting, swollen glands or rash. Dengue itself is rarely fatal, but severe dengue is a potentially fatal complication, with symptoms including low temperature, severe abdominal pains, rapid breathing, bleeding gums and blood in vomit. There are four known serotypes of dengue virus (DEN 1 to 4) . Recovery from infection by one provides lifelong immunity against that particular serotype. However, subsequent infections by other sero types increase the risk of developing severe dengue.

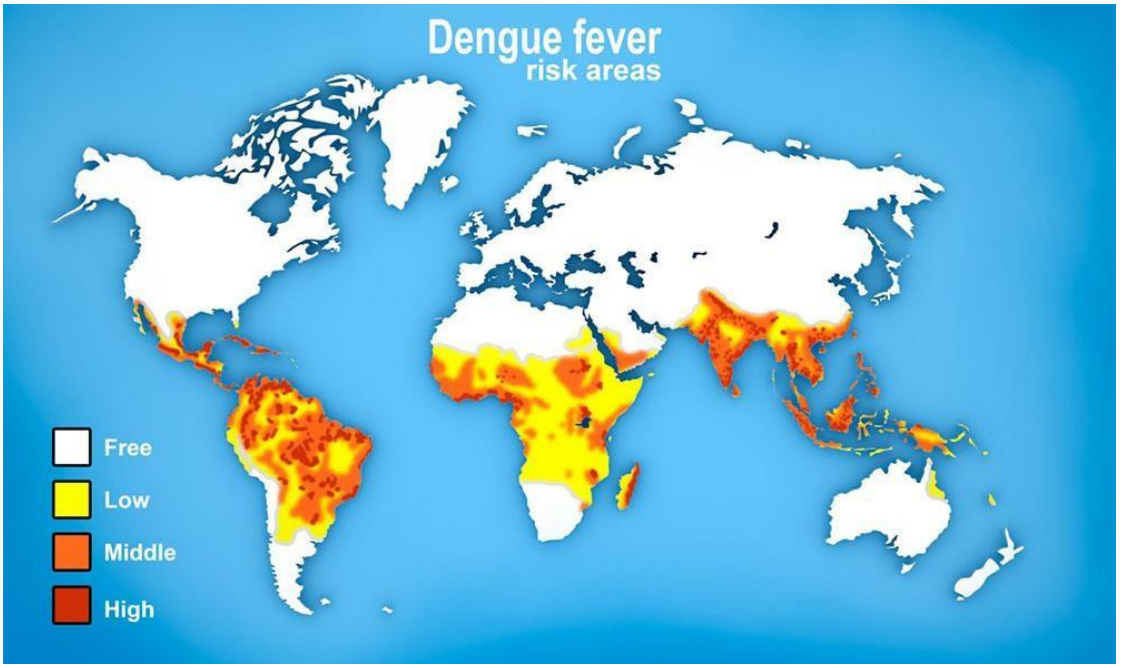

Fig.4 Prevalence of Dengue fever- caused by *Aedes albopictus*

More than 2.5 billion people – over 40% of the world's population – are now at risk of dengue. WHO estimates there may be more than 100million dengue infections worldwide every year. An estimated 500, 000 people with severe dengue require hospitalization each year, a large proportion of whom are children. About 2.5% of those affected die. The *Aedes aegypt*i mosquito is the primary vector of dengue. The virus is transmitted to humans through the bites of infected female mosquitoes. Once an infected mosquito has incubated the virus for 4–10 days, it can transmit the virus for the rest of its life. Dengue is the most rapidly spreading mosquito- borne viral disease in the world. In the past 50 years, incidence has increased 30-fold with geographical expansion to new countries and, in the present decade, from urban to rural settings.

Infected humans are the main carriers and multipliers of the virus, serving as a source of the virus for uninfected mosquitoes. Patients who are already infected with the dengue virus can transmit the infection (for 4–5

days; maximum 12) via Aedes mosquitoes once their first symptoms appear. The *Ae. aegypti* mosquito lives in urban habitats and breeds mostly in man-made containers. This species is a day time feeder; its peak biting periods are early in the morning and in the evening before dusk. Female Ae. aegypti bite multiple people during each feeding period (Dengue virus disease -Better Health Channel-2014, https: //www. betterhealth. vic. gov. au/health/Dengue-virus-disease)

2.6 C**hikun gunya:** Symptoms are fever and severe joint pain that can last for weeks. Other symptoms include muscle pain, headache, nausea, fatigue and rash.

Most patients recover fully, but in some cases joint pain may persist for several months, or even years. Occasional cases of eye, neurological and heart complications have been reported, as well as gastrointestinal complaints. Serious complications are not common but, in older people, the disease can contribute to the cause of death.

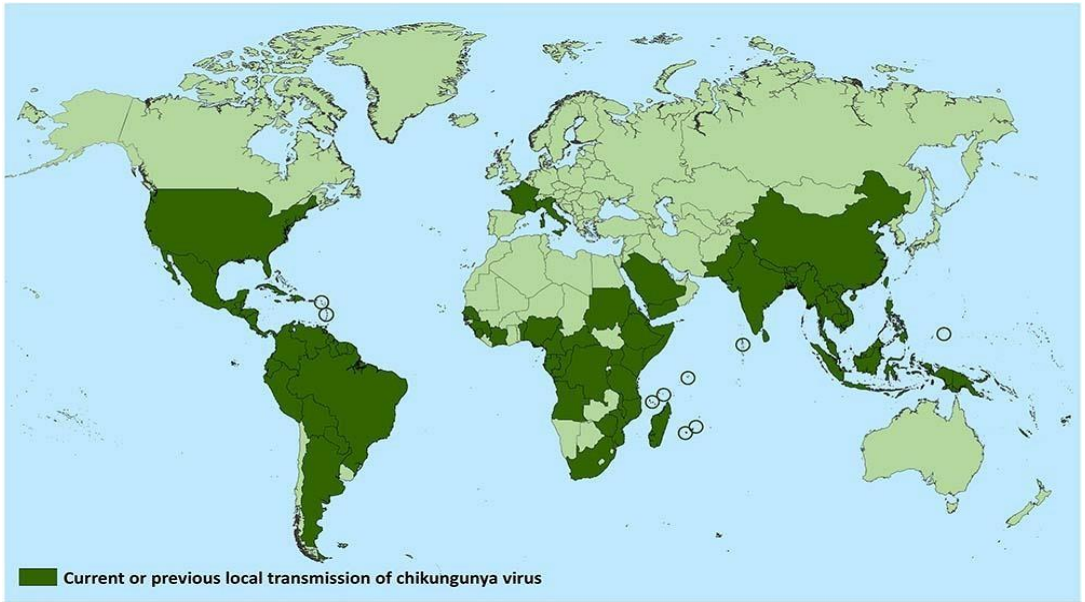

Fig. 5 Prevalence of Chickun gunya- caused by *Aedes aegyptii*

Often symptoms are mild and the infection may go unrecognized, or be misdiagnosed in areas where dengue occurs The disease typically consists of an acute illness with fever, skin rash and incapacitating joint pains that could last for several months. Chikungunya shares the same vectors, symptoms and geographical distribution as dengue, except for the presence of joint pains.

There is no specific treatment for chikungunya. Symptomatic or supportive treatment basically comprises rest and use of acetaminophen or paracetamol to relieve fever and a non-steroidal anti-inflammatory agent to relieve joint pain. Chikungunya occurs in Africa, Asia and the Indian subcontinent. In recent decades, there have been outbreaks of the disease in countries that have never recorded cases before.

In Asia and the Indian Ocean region, the main vectors of chikungunya are *Aedes albopictus and Ae. aegypti.* In Africa, a larger range of Aedes species transmit the virus as well as *Culex annulirostris, Mansonia uniformis and Anopheles* species. WHO, 2020 (https: //www. who. int/news-room/fact-sheets/detail/chikungunya }

2.7 Yellow fever, the original viral haemorrhagic fever, was one of the most feared lethal diseases before the development of an effective vaccine. Every year there are around 200, 000 cases of illness and 30, 000 deaths from yellow fever. The number of yellow fever cases has increased over the past two decades due to declining population immunity to infection, deforestation, urbanization, population movements and climate change. The first symptoms of the disease usually appear 3–6 days after infection. The first, or ‘acute’, phase is characterized by fever, muscle pain, headache, shivers, loss of appetite, nausea and vomiting. After 3–4 days, most patients improve and symptoms disappear. However, 15% of patients enter a ‘toxic’ phase: fever returns and the patient develops jaundice and sometimes bleeding, with blood appearing in the vomit. There is no specific treatment for yellow fever.

Supportive care needed to treat dehydration, respiratory failure, fever and associated infections is not easily available to many yellow fever patients. About 50% of patients who enter the toxic phase die within 10–14 days. Vaccination is the most important preventive measure against yellow fever. A single dose of the vaccine provides life-long immunity.

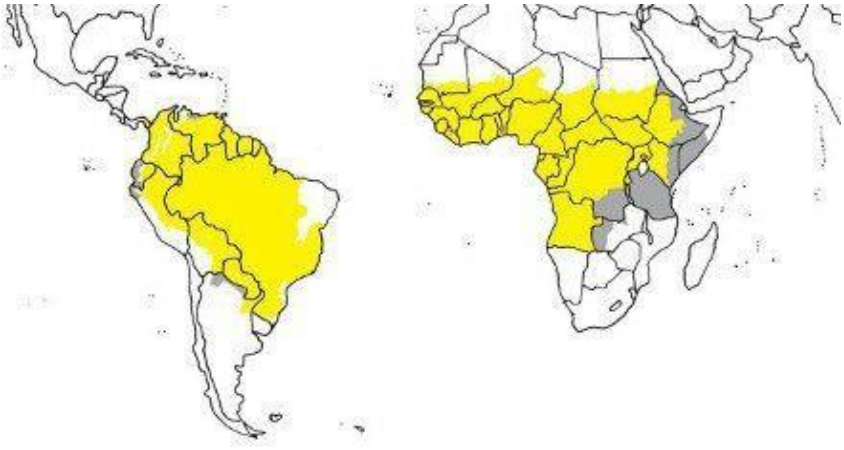

Fig. 6 Prevalence of yellow fever- caused by *Haemogogus species*

Yellow fever is transmitted by the *Haemagogus and Aedes* species of mosquitoes between monkeys and humans. Outbreaks have occurred in recent years in seasonal workers, nomadic and displaced people in several African countries.

In the past 30 years, dramatic increases in the distribution of the urban mosquito vector, Ae. aegypti, as well as the rise in air travel, have increased the risk of introduction and spread of yellow fever to North and Central America, the Caribbean and Asia (Monath. P. Thomas, 2001*)*

2.8 Japanese encephalitis virus is transmitted to humans through infected *Culex* mosquitoes. Most human infections are asymptomatic or result in only mild symptoms; however, a small percentage of infected people develop inflammation of the brain (Encephalitis) , with symptoms including sudden onset of headache, high fever, disorientation, coma, tremors and convulsions.

One quarter of severe cases can be fatal, and 30% of those who survive severe infection have lasting central nervous system damage. There is no specific treatment for the disease; supportive care and management of complications can provide some relief. The vaccine against Japanese encephalitis is the single most effective preventive measure against this disease.

Japanese encephalitis causes an estimated 50, 000 cases and 10, 000 deaths every year, mostly of children aged less than five. It occurs across Asia, from the islands of the Western Pacific in the east to the Pakistani border in the west, and from the Republic of Korea in the north to Papua New Guinea in the south. While cases have been reduced considerably in Japan, the Republic of Korea, Taiwan and China, the disease has expanded to India, Nepal and Sri Lanka where it has become a substantial public- health problem.

Transmission of the Japanese encephalitis virus occurs primarily in rural agricultural areas, particularly around flooded rice fields, but can also occur near urban centres. In temperate areas of Asia, transmission of the virus is seasonal (CDC,2019-Transmission of Japanese Encephalitis Virus - CDC, 2019https://www. cdc. gov/japaneseencephalitis/transmission/index. html)

The impact of yellow fever virus is very much devastating. The priority is for vector control. Vector control in many countries have considerably reduced mortality rates, especially among children. Large scale movement of people increases the risk. Since vector control can used as an effective tool aginst multiple diseases, different methods can be integrated to combat the same. WHO therefore recommends integrated vector management for implementing startegies for vector control Vector control-Wikipedia, 2020 (https: //en. wikipedia. org/wiki/Vector_control)

2.9 Strategies to counter vector borne diseases and success stories

Thailand is one of the few countries which is successful in reducing dengue from 14% in 1958 to 0.09% in 2013. The credit of this great success goes to the national polcy and commitment of administrators (WHO, 2014 Vector-borne diseases, Report of an informal expert consultation, SEARO, New Delhi, 7–8 April page 7)

Similarly singapore is one of the south east asian countries that had implemented a very successful dengue conrol programme, by making use of source reduction of *Aedes aegyptii*, public education, and law enforcement. It invoked the disease bearing insects act (1968) , which resulted in containment of the disease Eng-Eong-Ooi, 2006.

With the wide usage of insecticide-treated nets (ITNs) , particularly the long-lasting insecticidal nets (LLINs) , the incidence of mosquitoe borne diseases has come down significantlly, especially malaria. Ethiopia has adopted the global malaria elimination campaign, as clearly stipulated in its current national strategic plan for malaria prevention and control (Deressa, et. al.
2014) .

The effectiveness of ITNs/LLINs against malaria, however depends on species, acceptance and proper utilization. One of the draw back is usage during only bed time. The feeding behaviour of various species of mosquitoes has a profound impact on the effectiveness of treated nets. Ususally mosquitoes can bite during anytime of the day, when people are involved in different activities. A study coducted revealed that the most intense biting hours between 18-20 PM (indoors) and 22-24 PM (outdoors) (Buame CBA. et. al, 2009) .

Repellents provide ususally good protection from mosquitoe bites. The

use of di-ethyl 3-methylbenzamid (DEET) mosquitoe repellant was associated with a 56% reduction in malaria cases in pakistan. Repellant with treated nets has found be very effective. The use of repellants during evening time, can improve the effectiveness of LLINs. A trial conducted in bolivian amazon region showed that some plant derived insect repellants combined with nets has found to be very effective in reducing malaria infection, especially due to P. Vivax (Moore. et. al, 2007) .

2.10 Side effects of pesticide usage: The process of accumulation of heavy metals and persisitant chemical pesticides in tissues and internal organs as they move up the food chain is refferred to as biomagnification. Since mosquitoes aquired resistance, many diseases came back with more health impact. (Biomagnification-Wikipedia (https: //en. wikipedia. org/wiki/ Biomagnification*)*

The use of chemical/synthetic pesticides has caused severe health problems in humans and animals. Dursban, a common pesticide used in hospitals, schools, and in agricilture is banned in 2000 by USEPA due to its high risk to children.

DDT which was widely used to control malaria was found to have caused a sharp decline in the population of amphibians, frogs, raptorial birds, and other predators. Thinning of egg shell was caused for birds like, kite, hawks, eagles, vultures, etc. (Rattner B A, 2009) .

Pesticides are of generally two types: Larvicides and adulticides. When it is not possible to eliminate stagnant water that may become breeding grounds of mosquitoes, larvicides can be applied to the water bodies. Since mosquitoe larvae are found in stagnant water sources, they can be easily treated with pesticides. [Pesticides used for Outdoor Mosquito Control, 2010].

Synthetic pesticides used fall mainly to four classess namely, organochlorines, organophosphates, pyrethroids and carbamates. These can cause either acute or chronic effects both in humans and animals, especially in

the nervous, endocrine, and reproductive systems.

The use of synthetic organic pesticides has had serious economic, social and environmental ramifications. Economically, the rapidy increasing cost for development and production of petrochemically derived insecticides, together with the declining effectiveness due to widespread insect resistance. As a result, the chemical pesticide industry continues to develop new more expensive compounds and increasing pesticide price. Socially and ecologically they have caused death and disease in humans and damaged the environment. It is estimated that only a minute fraction of the insecticides applied is required for suppression of the target pest. The remainder, more than 99.9%, enters the environment through soil, water and food cycles (Metcalf, 1986) .

DDT was the was the very first used pesticide to control malaria, dengue and typhus. With its rampant usage in agricultural fields and related side effects, led most of the countries to ban its production and use. The production and use of DDT is strictly restricted by an international agreement known as the Stockholm Convention on Persistent Organic Pollutants[World Health Organization manual, 2014].

Resistance to such insecticides among mosquito-species is frequent due to loss of sensitivity of the insect's acetylcholinesterase to organophosphates and carbamates (Weill et al., 2003) . Genes conferring insecticide resistance have been spreading in vector populations, particularly in vectors of pathogens causing malaria and dengue (Ranson et al., 2002, 2001) .

2.11 Integrated Pest Management (IPM) is highly recommmended by The Agriculture, Fisheries and Conservation Department, which includes surveillance, species identification, source reduction, mosquito habitat modification, biological control inspection and surveilance.

2.12 Microbial insecticides: Chemical insecticides are more widely used than microbial based. Microbial insecticides also called as biological control agents /biological pathogens have at least in part replaced some very toxic chemical pesticides. A number of bioagents formulated with bacteria, viruses, fungi, pheromones, and plant extracts are used to control insect pests. (Biological Pest Control — A Review - Bacterial Toxin Application and Effect of environmental factors, 2013)

Viruses survive within host organisms and multiply to cause a disease. Viruses can act as one of the most effective and safe biocontrol agent to control a variety of diseases. For some fruit pests, commercial virus preprations are available. Some viruses can cause outbreaks so additional inputs are not required. Baculoviruses can infect 600 insect species worldwide.

Most baculoviruses infect caterpillars, which are the immature form of moths and butterflies. Insect viruses are potent population regulators of many caterpillar pests. All are highly specific in their host range, usually limited to a single type of insect. These include Gemstar LC (Certis USA) [(nuclear polyhedrosis virus of Heliothis/Helicoverpa spp. (e. g., corn earworm, tobacco budworm)] Spod-X LC (Certis USA) [(nuclear polyhedrosis virus of *Spodoptera* spp. (e. g., beet armyworm)] CYD-X (Certis USA) and Virosoft CP4 (BioTEPP, Inc.) (granulosis virus of *Cydia pomonella*, the codling moth); and CLV LC (Certis USA) [nuclear polyhedrosis virus of *Anagrapha falcipera*, the celery looper].

2.13 History of *Bacillus thuringiensis based biopesticides:* *Bacillus thuringiensis*sero*var israelensis* (*Bti*) is the most effective bio-larvicide against mosquitoes that is available to date (Goldberg and Margalit, 1977; de Barjac, 1978; Tyrell et al., 1979; deBarjac 1984; Federici et al., 1990; Mahammod, 1998; Su and Mulla, 1999) . *Bti* is a gram positive, rod shaped,

facultative aerobic, spore forming bacterium having genome size of 2.4 to\ 5.7 million base pairs. The prevalence of this strain is not restricted to soil but has been isolated world wide from many types of habitats (Schnepf et. al., 1998) .

Bt has advantages compared to chemical insecticides in being very selective, narrow spectrum, so very safe to other species. (Travis R. Glare and Maureen O'Callaghan, 1998) . **C**hemical insecticides are also major pollutants of the environment. Since *Bti* has multi protein /toxin complex, the possibility of insect developing ressistance is very low (Wirth et al., 1998) .

Bt is a spore former, , the toxicity is attributed to the production of crystal toxic proteins. Related species is *Bacillus cereus*, causing food poisoning. *Bt* carries plasmid encoded crystal proteins

Bacillus thuringiensis Toxins: An Overview of their Biocidal Activity). *Bt* is almost ubiquitous in its occurrence, including tundra, beaches, dessert etc. mostly used in agricultuture and in urban aerial spraying programs.200 different proteins are produced by over 10000 different species of *Bt*.

Bt is the leading biopesticide used n the world, with more than 90% of all biological products used for plant protection are *Bt* based. Spores and crystal toxins are the major part of active preprations. With the discovery of new srains, the action spectrum has broadened, which has resulted in using *Bt* against mosquitoes and defoliating pests. Genetic engineering have produced *Bt* which target mites, nematodes and protozoa, further expanding their potential as biocontrol agents.

Feld trials were carried out in cuba against tobacco budworm *Heliothis virescens (F.) and grass looper Mocislatipes Guenée (Lep., Noctuidae) with* commercial products manufactured in USA, France and the former Soviet Union. Later cuba started *Bt* production using simple cost effective methods which includes using static liquid culture based on waste products from the sugar industry or other crop production.

2.13.1 *Bacillus thuringiensis*

Bacillus thuringiensis was first discovered in 1901 by a Japanese biologist named Shigetane Ishiwatari. He discovered this when he was researching Sudden Collapse Disease, that afflicted silkworms. It was later made known that *B. thuringiensis* was responsible for this. This discovery was forgotten until Ernst Berliner rediscovered *B. thuringiensis* in 1911. He was researching the cause for the death of moths in the German town of Thuringia. This is where the name *thuringiensis* came from. Originally the bacteria had been called *Bacillus sotto* by Ishiwatari, but this was over ruled after Berliner's discovery. Berliner discovered the crystal inside the bacteria but it was unknown what the crystal did until later. *Bacillus thuringiensis* was unused in the main stream market until 1920 when farmers were reported to spray it on their fields. Finally in 1938, France started to market these bacteria to farmers as Sporine. This was marketed to kill flour moths**.**

Many methods have been used to identify and classify the thousands of strains of *Bti,* which were isolated from various natural habitats in decades of *Bti* research. These methods began with bioassays and traditional biochemical methods, then serotyping and polymerase chain reaction (PCR) , etc. The most useful early system proved to be serotyping based on H (or flagellar) antigens. The H serotypes led to the present growing list of subspecies, now numbering over 80, listed by Glare and I'M Callaghan (2000) . Among these subspecies, *Bacillus thuringiensis israelensis (Bti)* or serotype H-14, exhibited acute toxicity towards diptera insects such as mosquitoes and blackflies (de Bajrac, 1978) .

Bt can be found almost every where in the world. Surveys have indicated that *Bt* is distributed in the soil sparsely but frequently worldwide. *Bt* has been found in all types of terrain, including beaches, desert, and tundra habitats. The insecticidal properties of these bacteria are primarily due to the

production of toxic proteins during sporulation. The key proteins are cry1Aa (27.3kDa) , cry4A (128kDa) , cry4B (134kDa) and cry11A (72kDa) . All proteins are toxic to mosquitoes; however there appears to be synergistic activities of these protein complexes. *Bti* treated mosquito larvae generally cease feeding within 1 hour, show reduced activity by 2 hours, extreme sluggishness by 4 hours and general paralysis by 6 hours after ingestion.

2.13.2 ***Bacillus thuringiensis strains***: A large number of strain of *Bt* have been isolated from which to date. *Bt* as currently recognized is actually a complex of subspecies. They have grouped in 79 serotypes (Zeigler, 1999) . The first *Bt* strain was isolated from diseased larvae of the silkworm, *Bombix mori*, in Japan by Ishiwata (1901) . Iwabushi (1908) described the Bacillus as *Bacillus sotto*. Mitani & Wataral (1916) purified a highly toxic substance from sporulated *Bt* cultures. It was not officially described, however, until it was reisolated by Berliner in 1915 from diseased larvae of the Mediterranean flour moth, *Anagasta kuehniella*, in Thuringia, Germany, hence the derivation of species name *thuringiensis* (Federici, 1999) .

A *Bt* strain was isolated again by Mattes (1927) and described briefly the inclusion rhomboidal body. The activity of the *Bt* strains against lepidopteran larvae was described by Metalnikov (1930) and by Husz (1931) . The association of the inclusion bodies of *B. thuringiensis* with toxicity against insects was established by Steinhaus. In 1951 published a paper which described the morphology of *Bt* and its possible use in the biological control against the alfalfa caterpillar. In France, a product named “Sporeine” was developed and used against *Ephestia kuhniella* (Lepidoptera) (Jacobs, 1950) . Hannay, (1953) described a parasporal body in bipyramidal shape produced by the bacterium during sporulation and suggested that the crystal was involved in the toxic activity. The protein nature of the crystals was determined by Hanna and Fitz-James (1955) .

2.13.3 Features of *Bacillus thuringiensis* strains : *Bt* is a facultative aerobic, gram-positive bacterium that forms characteristic protein inclusions adjacent to the endospore . The crystalline inclusion are composed of proteins known as ICPs crystal proteins. Cry proteins, or delta--endotoxins is the basis for commercial insecticidal formulations of *Bt*. Insecticides containing *Bt* in pest control programs is now considered as a viable strategy, which has proven to be both safe and reliable over the last 45 years (Chungjatupornchai et al.1988)

2.13.4 Biological Control of Mosquito Larvae by *Bacillus thuringiensis subsp. Israelensis*

According to Federici (1999) the main reasons for the success of *Bt* include (i) the high efficacy of its insecticidal proteins (ii) the existence of a diversity of proteins that are effective against a range of important pests (iii) its relativey safety to nontarget insect predators and parasites (iv) its easy to mass produce at a relatively low cost, and (v) its adaptability to conventional formulations and application technology. Advantages and disadvantages of Bt with chemical insecticides were summarized by Rowe and Margaritis (1987)

Bt is a crystalliferous spore-forming bacterium having close genetic relationship with *B. cereus, B. anthracis and B. mycoides* (Höffe & Whiteley, 1989) . The classification of *Bt* is difficult because DNA sequencing studies of conserved gene regions of theses species have suggested that they belong to a single species. *Bt* strains are distinguished from *B. cereus, B. anthracis and B. mycoides* by the ability to produce parasporal crystalline inclusions during sporulation (de Barjac, 1978) . Crystal formation is the criterion for distinguishing between *B. cereus and Bt,* otherwise they could be considered as the same species. Research based on a comparative study of 16s rRNA sequences, *Bt and B. cereus var. mycoides* differed from each other and from *B. anthracis* and cereus by less than nine nucleotides (Ash and Collins 1992) . Chen & Tsen (2002) amplified 16S rDNA and gyrB gene by PCR and they found that the discrimination between *B. cereus and Bt* strains, when a large number of Bacillus strains were tested was difficult. They proposed, to

distinguish *Bt from B. cereus,* a single feature, such as the presence of a parasporal crystal protein or cry gene is reliable. Several attempts were made to classify Bt strains. de Barjac and Frachon (1990) didn´t find correlation between biochemical reactions and 27 H serotypes using 1, 600 *Bacillus. thuringiensis* isolates. They demonstrated that the current biochemical tests have no value as the sole criteria for differentiating *Bt* strains. Another approach of these authors for classification was the use of susceptibility to certain bacterial viruses called phages. There are 14 bacteriophages that have been used for *Bt* but phage typing is inconsistent with serotyping and does not permit classification. They found frequent cross-reactions. One widely clasification system for *Bt* strains is based in the determination of the H-flagellar antigen technique described by Barjac & Bonnefoi (1962) **.** This technique needs very motile bacterial cultures to prepare flagellar suspensions. These suspensions are titrated against antisera directed against *B. thuringiensis* strains of each serotype. Presently *B. thuringiensis* strains are classified within 79 serotypes.

Bt strains that had discovered previously to 1977 were pathogens towards lepidopteran larvae. However, by 1975 *B. thuringiensis* sero*var israelensis* was discovered toxic to mosquito larvae (Goldberg & Margarit 1977) and in 1983 a strain from *Bt serovar morrisoni* was found to be pathogenic to Coleoptera larvae (Krieg et al.1983) . According to these findings, serological classification, although still in use as a basic method to classify *Bt* strains could not be related with pathogenicity. Subsequently studies showed that, within a serotype, different activity spectra can be found. For example, some strains of *B. thuringiensis serotype morrisoni* in their parasporal inclusión bodies contain different proteins and have activity against Diptera, Coleoptera or Lepidoptera. With the knowledge of sequence of the genes that encode the proteins cry it was proposed a classification based on the cry toxin genes. Höfte and Whiteley (1989) proposed a nomenclature clasification scheme for *Bt* crystal proteins based in their structural aminoacid sequence, deduced from the DNA and host range. They

named cry (crystal protein) genes and their related proteins, “Cry proteins”. They clasified 42 Bt crystal protein genes into 14 distinct genes grouped into 4 major classes. The classes were CryI (Lepidoptera-specific) , CryII (Lepidoptera-and Diptera-specific) , CryIII (Coleoptera-specific) , and CryIV (Dipter-specific) .

2.14 Discovery of *Bti*

In 1975-76 under a World health organisation sponsored project, a new *Bti* strain was discovered in Negev desert in Israel by Goldberg and Margalit (1977) . The strain was isolated from dead mosquito larvae of *culex* species. Later was identified as *Bacillus thuringiensis israelensis*, serotype H-14 according to its flagellar antigenicity

2.14.1 Properties of *Bti*

Bacillus thuringiensis israelensis is serving as an ideal candidate for mosquito control because of its high mosquito larvicidal activity, environmental friendliness, safety to non-target organisms and mammals, ease of production and amenability to a variety of formulations like dust, pellets, sprays, briquettes, capsules, granules, etc. Moreover the chance of development of resistance by mosquitoes to its products is very low due to its multi-toxin complex.

Fig.7 *Bacillus thuringiensis israelensis* based commercial formulations which is used to control *Aedes, Culex and Anopheles* species of mosquitoes.

2.14.2 Mode of action of *Bti*

Bti products contain spores and parasporal crystals of *Bti* H-14 serotype which must be ingested by the larval stage of the mosquito to cause mortality following ingestion, the parasporal crystals are solubilized in the alkaline larval midgut, followed by a proteolytic activation of the protoxin into active toxin. The toxin binds to a receptor on the midgut epithelium resulting in pore formation in the cell, which leads to the death of the Lava.

The sporulated *Bt* with ICP or spore–ICP complexes must be ingested by a susceptible insect larva. The efficacy of the ICP depends on the solubilization in the midgut, the conversion of the protoxin to the biologically active toxin by proteolytic enzymes, specific membrane receptor binding by the C-terminal domain of the active toxin, and pore formation by the N-terminal domain with subsequent lysis of the epithelial cells. Spore germination and proliferation of the vegetative cells into the haemocoel may result in a septicaemia, contributing to the cause of death. Receptor binding by the ICP is the major determinant of host specificity by the different *Bt* ICPs.

The insecticidal properties of these bacteria are primarily due to the production of toxic proteins during sporulation. The key proteins are cry1Aa (27.3kDa) , cry4A (128kDa) , crt4B (134kDa) and cry11A (72kDa) . All proteins are toxic to mosquitoes; however there appears to be synergistic activities of these protein complexes. *Bti* treated mosquito have larvae generally cease feeding within 1 hour, show reduced activity by 2 hours, extremely sluggishness by 4 hours and general paralysis by 6 hours after injection.

2.14.3 δ-Endotoxins of *Bti*

The original isolate of *Bti* harbours eight circular plasmids ranging in

size between 5 and 210 kb and a linear replicon of approximately 16 kb . The larvicidal activity of *Bti* resides in at least four major crystal protoxins, of 134, 128, 72 and 27 kDa, encoded by *cry4Aa*, *cry4Ba*, *cry11Aa* and *cyt1Aa* respectively, all mapped on the 128 kb plasmid known as pBtoxis . In addition, pBtoxis contains *cry10Aa*, *cyt2Ba* and *cyt1Ca*: Cry10Aa and Cyt2Ba accumulate in small amounts in the parasporal body and seem to contribute to the overall toxicity of *Bti*. The large protoxins (Cry4Aa and Cry4Ba) have conserved C-terminal halves participating in spontaneous crystal formation via inter- and intra-molecular disulphide bonds, whereas the smaller (Cry11Aa and Cyt1Aa) do not possess this domain and hence require assistance in crystallization . The *cry11Aa* is organized in an operon together with *p19* and *p20*. The P20 accessory protein stabilizes both Cyt1Aa and Cry11Aa in recombinant *Escherichia coli*, *Pseudomonas putida* and *Bt* by interactions with the nascent polypeptides thus protecting these protoxins from proteolysis .

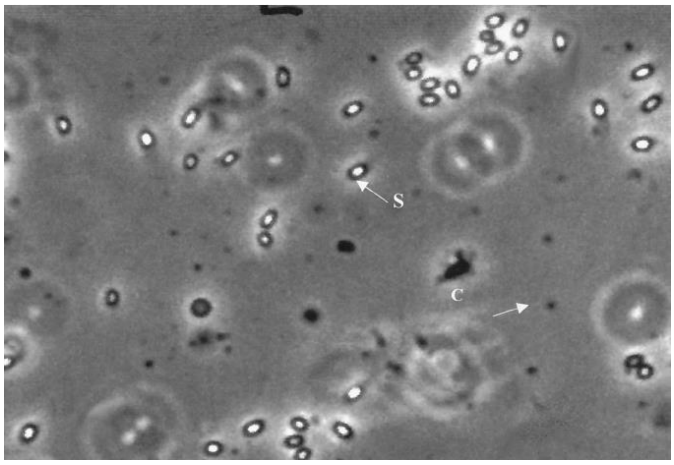

Fig.8 *Bacillus thuringiensis israelensis* spores and crystal toxins

The Cry and Cyt toxins are membrane-perforating proteins although unrelated structurally and differ in their requirement of essential membrane components; the Cry's bind to membrane receptors whereas Cyt1Aa binds with high affinities to unsaturated phospholipids.

Cry4Aa, encoded by a sequence of 3543 bp (1180 amino acids) , is highly toxic to larvae of *Culex* and less to *Anopheles* and *Aedes* and Cry4Ba, encoded by a sequence of 3408 bp (1136 amino acids) , has high larvicidal activities against *Anopheles* and *Aedes* but very low against *Culex* Consistent with the differential specificities, the identity between the amino acid sequences of their N-termini toxic portions is only about 30% (55% similarity) . Cry11Aa is encoded by a sequence of 1929 bp (643 amino acids) and displays high larvicidal against the larvae of *Aedes* and *Culex* but lower against *Anopheles*.

The larvicidal activity of Cyt1Aa, encoded by a sequence of 744 bp (248 amino acids) , is low against all three mosquito genera . It is cytolytic *in vitro* to cells of certain vertebrates and invertebrates and highly mosquito species-specific *in vivo*, implying a specific mode of action . The cytotoxicity seems to derive from an interaction between its hydrophobic segment and membrane phospholipids. The sequence of Cyt1Aa has no homology to Cry polypeptides but the toxins plays a critical role in delaying selection for resistance to *Bti*'s Cry proteins (*WHO*, 2009).

2.15 *Bt* based transgenic crops

Due to harsh climatic conditions which is prevailing in the field, *Bt* pesticides usually have short life. So it needs to be sprayed several times to control the target species, increasing the amount and the expenditure and labour needed for spraying. Sap sucking insects usually escape the spraying (Georgina , et. al.2001).

As an alternative transgenic crops with *Bt* cry genes were made. Some companies like plant genetic systems poineered such research. (Fischhoff *et al.* , 1987; Vaeck *et al.* , 1987; Perlak *et al.* , 1991)

The main advantage of *Bti* over chemical insecticides is its highly

specific activity towards dipteran insects, due to the presence of membrane receptors in the insect gut serving as targets for the bacterial toxins. Due to the absence of such receptors in vertebrates, the *Bti* is considered safe for human health. It was claimed that the toxins and spores were not persistent in the environment with virtually no residual effects, even in environments prone to seasonal applications. Furthermore, almost no dispersion of the spores was observed in the soil, and contamination of ground water seems very unlikely. Finally, due to the complex structure of *Bti* toxins, many authors emphasized that the acquisition of resistance in exposed insects would require multiple mutations at different loci, and is therefore largely delayed under natural conditions. (Schnepf E, et. al.1998, Gill. et. al, 1987, Thomas, e. al.1983, De Respinis. et. al.2006, Akiba. et. al, 1991, Becker, et. al, 1993, Georghiou. et. al.1997) , WHO report, 1999) .

B. thuringiensis also has its disadvantages. Many strains are susceptible to the sunlight and their potency will degrade within a week. Some newer strains used on leaf beetles can become ineffective within 24 hours. Also, because *Bt* insecticides are highly specific, many varieties must be used if full protection of the crops is required. They will not be that effective on crops that have multiple pests. Because *Bt* insecticides are only useful when their are eaten, sometimes they are ineffective because some insects such as codling moth don't get the chance to eat it.

Applying *Bti* for mosquito control is limited by short residual activity of current preparations under field conditions due to: (i) sinking to the bottom of the water body; (ii) adsorption onto silt particles and organic matter; (iii) consumption by other organisms to which it is nontoxic; and (iv) inactivation by sunlight. In order to overcome these shortcomings, the δ-endotoxin genes have already been expressed individually or in combinations in various Gram-positive and -negative species . Best results were achieved by expressing the genes encoding binary toxin of *B. sphaericus in Bti*. The recombinant bacteria

were highly potent against fourth instar larvae of *Cx. quinquefasciatus* and *Cx. tarsalis*, even to lines selected for resistance to the binary toxin. Higher toxicity against fourth-instar *Cx. quinquefasciatus* was achieved in recombinant acrystalliferous *Bti* strain that produces the combination of *B. sphaericus* binary toxin together with Cyt1Aa of *Bti* and Cry11Ba from *Bt* subsp. *Jegathesan*. (Leopoldo Palma, 2014, Federici. et. al, 2010, Margalith. et. al.2000, Park. et. al.2005).

2.16 Methods of *Bti* production

Conventional production is by submerged aerobic Fermentation proceess using CSTR reactor. More recent updates about current manufacturing strategies is given in papers published by Vimala Devi, P.S.et al (2020),Vimala Devi, P.S.et al(2018). Amnah M. Hasanain et al.(2017)

Although there are reports regarding batch production of Bt and fed batch production, so far no reports regarding semicontinous prodction. The production Bt was assesed during an Semi continous process for vegetative growth and sporulation. The potency of the pesticide produced during aeration and without aeration was also studied. (Millena da Silva et al. 2011)

2.16.1 Mass production of *Bt* and *Bs*

In commercial production of *Bt* and *Bs* priority was given for optimisation of bioprocess parameters to have maximum yield with minimum inputs of raw materials and energy, since both have very high ecnomic importance in the field as excellent biocontrol agents. (Salama et al.1983) and Sachdeva et al. (1999) reported that the ecnomic feasibilty of *Bt* and *Bs* production is highly dependant on process optimization, raw material costs, efficiency of the strain, batch time, down stream process, and final formulation of the product. In *Bt* commercial production the raw material costs usually fluctuate betwen 30-40% of the production cost. (Ejiofor 1991 and Lisansky et al.1993) .

Cost efective production of *Bt* thus should focus on using production media made of cheap and locally available raw materials including agro-industrial products (AMPOFO, 1995) **.**

Various strategies were used to design media that can support good sporulation and crystal toxin production on a large scale, in a cost effective way. Materials like citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupal skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal, residues from chicken slaughter house, fodder yeast, cheese whey, and corn steep liquor. etc were used as raw materials.

2.16.2 Bioprocess involved in the production process

The search for suitable media for industrial production of *Bt* has been the objective of several studies reported in the literatures. An early submerged fermentation medium for *Bt* production was reported by Megna (1963) . He used cotton seed medium containing beet molasses (1%) , corn steep solids (0.85%) and calcium carbonate (0.1%) . While the production medium contained beet molasses (1.86 %) , corn steep solids (1.7%) , cotton seed flour, (1.4%) and calcium carbonate (0.1%) . The yield was 2.5×10^9 colony forming units (CFU) /ml.

Dulmage (1970) devised a fermentation medium based on defatted cotton seed flour, which supported the production of large yield of δ-endotoxin by the tested *Bt* strains. The same author (Dulmage, 1971) constructed three fermentation media including a novel medium with defatted soybean meal flour as the major component for production of *Bt* sero-type 3. In later study, Dulmage and DE Barjac (1973) reported fermentation media for *Bt* δ-endotoxin production based upon cotton seed flour and corn steep liquor. (Mechalas, et. al, 1963, Megna, et. al, 1963, Dulmage, et. al, 1970, Dulmage, et. al, 1971. Dulmage, et. al.1973) .

2.17 Growth aspects of *Bti*: The metabolic patterns of *Bacilus thuringiensis* are triphasic- vegetative, growth, transition and sporulation. Most varieties are able to use glucose, fructose, starch, maltose, ribose, glycerol, organic acids, glutamate and other amino acids. Optimal endotoxin production occurs in 28 to 32 ºC. pH range is 5.5 to 8.5. The ussual initial pH is 6.8 to 7.2. Oxygen is essentially required for growth and during vegetative growth. In addition to the manipulation of pH and temperature, optimising the composition of media and ensuring appropriaite conditions with respect to dissolved oxygen (Vinod bihari, 1998) .

2.18 Fermentation media for production of both *Bti* and Bs

In 1985, Dharmsthiti et al. constructed two media using HDL, a by-product from a mono-sodium glutamate factory. These media were found to be highly suitable for culturing *Bti* and *Bs*. These media contained 0. 05% K2HPO4 and 4% HDL (H4 medium) or 0. 05% K2HPO4 and 7% HDL (H7 medium) . The sporulation and LC50 values of *Bti* grown in H4 medium at 48 hr were 2.5 × 108 CFU/ml and 10–7 (dilution) , respectively. While those of Bs grown in H7 medium were 1.4 × 109 CFU/ml and 10–8, respectively (J. Basic Microbiol.2006, Magda A.1985) .

Kuppusamy and Balaraman (1991) studied the effect of corn steep liquor as a growth medium for production of three strains of *Bti* and two strains of Bs as compared with a medium based on peptone and yeast extract using a laboratory fermentor. They concluded that corn steep liquor can effectively replace both peptone and yeast extract in the media for large scale production of the two larvicidal bacilli.

Desai and Shethna (1991) formulated three fermentation media for growth of *Bti* and *Bs* 1593 using defatted ground nut cake as the first nitrogen source and gram flour, soy bean and defatted milk powder as the second

nitrogen source. They reported that medium containing gram flour showed the highest toxicity in case of *Bti*, whereas medium containing defatted milk powder enhanced toxicity of *Bs* 1593.

Gangurde and Shethna (1995) studied the growth of *Bti* and *Bs* in media based on defatted mustard seed meal. They obtained appreciable growth and larvicidal activity of the tested *Bti* and *Bs* strains. Moreover, the potencies of *Bs* in medium with 4% defatted mustard seed meal were comparable with those of international reference standards.

2.19 Solid state fermentation (SSF)

Under the circumstances of the developing countries, the use of submerged fermentation for *Bt* and *Bs* production may not be economically feasible due to the high cost of submerged fermentation equipments such as the cost of the well-equipped deep-tank fermentor, high-speed cooling centrifuge as well as drying facilities e. g. spray dryer. Accordingly, the SSF methodology offers an alternative approach. Advantages of solid state fermentations are: 1.low cost methodology (2) low waste water output, (3) low capital investments, (4) some spore-forming microorganisms only sporulate when grown on a solid substrate (Mudgett 1984, Walter and Paau 1992 and Capalbo, 1995) .

Till date so many biotech products have been manufactured using SSF technology, but very little information is available for SSF technology for the production of *Bt* and other biopesticides.

The earliest report on possible application of SSF in production of *Bt* appeared in a form of U. S. patent by Mechalas (1963) followed by reports by Dulmage and Rhodes (1971) and Sittig (1977) .

In China novel cost effective methods were developed using simple

and ecnomic SSF methods. The medium contained wheat bran, rice husk and calcium powder which has proved to be power saving in different provinces of china **(**Wang. et. al.1988) .

Capalbo and Moraes (1988) carried out a study on the production of *Bt* by SSF methodology. They used a group of available agro-industrial by-products as growth media including wastes from pulp and paper industry, residual fermented malt from beer industry, meal from residual cookies and biscuits from bakery industry as well as meal from chicken slaughter house residues. They reported that the successful production of *Bt* formulations with high sporulation titers occurred by using paper pulp and fermented malt. However, no detailed information on the experimental design and fermentation conditions used were given. (Yang. et. al .1994)

Several agroindustrial waste materials were used as the solid media for the culture and production of delta -endotoxin by *Bt*. Various bioprocess parameters were tested including inoculum size, pH, seed age, initial moisture content, amount of plant ash used, and fermentation temperature. Biolarvicidal assays against *Seaio-thisa cinereasra* and *Pieris rapes* proved the high potency of the biopesticides. (Capalbo, et. al, 1995.)

2.20 Limitation of bioprocess and important parameters

The cost of raw material has become one of the major limiting factor in reducing the production cost. Further since its a highly aerobic process, the supply of oxygen becomes limiting especially at high cell densities. (Donald. F. et al., 1988) .

2.21 Product stability problems

Many *Bt* insecticides have a shorter shelf life than other synthetic insecticides. Generally after two or three years their efectiveness is reduced

greatly. The optimal storage conditions for *Bt* products are cool, dry air and out of direct sunlight. Moreover its production cost is high. Its also affected by solar radaiations like UV radiations when its sprayed in the seweage.

2.22 Ecnomics of *Bti* fermentation

Biotechnology mainly involves manufacturing value added compunds by using microbes. The success of this technology depends on cost effective production of microbial products at an industrial scale.

In this context the expendture incurred under various heads becomes the major deciding factor, about the ecnomic feasibility and commercial success of the enterprise. Out of the various expenditure incurred, the money spent on procuring raw materials usually becomes the major head. Natuarally, way and means have to be found out to reduce expenditure incurred due to substrates. Traditional bacterial growth medium substrates like, Glucose, peptone, yeast extract, beef extract are all expensive and cannot be practised in large scale, due to high cost.

Alternative cheap raw materials, which can easily be assimilated by the industrial organism should be found out and a cost effective process should be developed to make it ecnomically feasible.

If a fermentation processs has to yield a product at a competitive price, the chosen micro-organism or animal cell culture should give the desired end product in predictable, ecnomically adequate, quantities. A number of basic objectives are commonly used in developing a sucessful process which will be ecnomically viable.

1. The capital investment in the fermenter and ancilliary equipment should be confined to a minimum, provided that the equipment is reliable and may be used for a range of fermentation products.

2. Raw materials should be as cheap as possible and utilized efficiently.

3. The highest yielding micro-organism should be used.

4. There should be saving in labour whenever possible and automation should be used where ever it is feasible.

5. When a batch process is operated, the growth cycle should be short as possible, to obtain the highest yield of product and allow for maximum utilization of equipment. To achieve this it may be possible to use fed - batch cuture.

6. Recovery and purification procedure should be as simple and rapid as possible.

7. The effluent discharge should be kept to a minimum

8. Heat and power should be used efficiently.

9. Space requirements should be kept to a minimum, but there should be some allowance for potential expansion in production capacity.

10. All the above points must comply with safety guidelines and regulations.

To make the industrial production enomically viable, it is important to know the cost breakdown. The main components which contribute to production cost are raw materials, fixed costs, utilities and labour.

The cost of the various components of a production medium can have a profound effect on the overall cost of a fermentation process, since these account for 38 to 73% of the total prduction cost. Moreover the carbon: nitrogen ratio of the medium also has a profound effect on the growth and secretion of varoius microbial products.

In the manufacturing of biomass based biotech products, upto 60% is spent on procuring raw materials (Stanbury, whittaker, 1995).

2.23 Technical problems adressed by this research work

Bti being a biomass based product can be cost effectively manufactured by channelising agrowastes like coconut water, Taro, sweet potato, purple yam, cassava, bagasse, rice straw etc through proper bioprocessing (Fermentation technology) , in order to convert it into *Bti* based biopesticide which consists of *Bti* cells, lysed cells, spores and delta endotoxin. This strategy will substantially reduce the production cost and will help to compete in the market with other chemical insecticides, which are generally cheaper than biopesticides. In addition the mode of fermentation carried out to get the desired microbial product also decide the cost (submerged/ batch/fed- batch/solid state, etc) . Since down stream processing also consumes huge amounts of money, cost effective simple methods were attempted including methods to extend shelf life of the products.

2.24 Potential agro wastes for the cost effective production of *Bti*

A brief description of various agro wastes suitable for *Bti* production is listed.

2.24.1 Coconut water

Glucose and fructose are predominant in the coconut water. Its concentration gradually increases from 1.5 % to 5.5 % in the early months and falls to 2% by full maturation. Sucrose appears on maturation, instead of glucose and fructose. It also contains minerals like potassium, sodium, calcium, phosphorous, iron, copper, sulphur and chlorides. Majority being pottassium the concentration varies depending on maturing.

Fig.9 Large scale coconut de-husking machine which is required for high volume production of *Bti* biopesticide using coconut water as the raw material

2.24.2 Elephant yam is cultivated throughout the asia. Tubers are in demand after rice and maize. Mostly consumed in india and srilanka. Elsewhere they are seen as a famine crop, to be used when more popular staples, such as rice, are in short supply.

2.24.3 Cassava or tapioca is a native of south america and now widely cultivated in different countries including Srilanka, Philippines, Myanmar, China, Indonesia, Malaysia, India, Carribean islands, Central america, and Mexico. The adventitious roots store carbohydrates/starch. It is predominanatly cultivated in the southern states in india. In addition it is also cultivated in Madhya Pradesh, Pondicherry, Nagaland, Tripura, Mizoram and the Andaman & Nicobar group of Islands, Andhra, and karnataka. Used as raw material for starch& sago industry and as supplement for animal, fish and poultry feeds. Thailand and India are major exporters of cassava starch to international market. Mostly used in paints, medicines and health drinks as filler and processed to make vermicilli, chips, papppads.

Fig.10 Large scale tapioca peeling machine which is required for high volume production of *Bti* biopesticide using tapioca wates as the raw material.

2.24.4 Sweet potato: Roots are ecnomically important. Its grown in tropical and subtropical world and warmer areas. Its rich in carbohydrates and vitamins.

Fig.11 Sweet potato- cheap tuber which is rich in starch and is usually not much in demand . Huge quantities accumulate as wastes.

2.24.5 TARO: Rich in carbohydrates and is cultivated mainly in asia /pacific regions.

Fig.12 Taro- huge amounts of this tuber gets accumulated as agro wastes.

2.24.6 YAM: Mostly cultivated in Latin America, the Caribbean and Oceania, asia and africa. It is usually used as grilled, boiled, baked, smoked, barbecued, roasted or fried.

Fig.13 Purple yam- cheaply available tuber which is rich in starch and is usually not much in demand . Huge quantities accumulate as wastes.

2.24.7 Bagasse*:* Bagasse is a heterogeneous material containing around 30-40 percent of "pith" fibre, which is derived from the core of the plant and is mainly parenchyma material, and "bast", "rind", or "stem" fibre, which makes up the balance and is largely derived from sclerenchyma material.

Fig.14 Bagasse raw and delignified- enormous quantities of this material is burnt in the field. This material being lignocellulosic can be used for the production of microbial metabolites , including *Bti*

A typical chemical analysis of washed and dried bagasse contains :

Cellulose	45–55 %
Hemicellulose	20–25 %
Lignin	18–24 %
Ash	1–4 %
waxes	<1 %

Bagasse can be used as a cost effective raw material to generate electricity. Steam is generated by buring it and used to turn turbines to produce power.30 % of crushed sugar cane comes as bagasse. Bagasse production corelates with sugar cane production.

2.24.8 Rice straw: Each kg of milled rice produces 0.7-1.4 kg of rice straw depending on varieties, Rice straw is separated from the grains after the plants are threshed either manually, using stationary threshers or, more recently, by using combine harvesters (Rice straw - IRRI Rice Knowlede Bank) .

Fig.15 Rice straw- 800 million tons is produced annually , most of which is wasted or burned. Being lignocellulosic can be used as carbon source for the production of various biotech products.

2.25 *Bti* medium formulations: Though the high efficacy and specificity of *Bti* are useful in controlling mosquitoes, the cost to grow and produce *Bti*, through highly refined laboratory bacterial culture medium, is high. The cost of *Bti* production depends on many factors; however, the raw material cost is one of the most important criteria, which may comprise >70% of the overall production cost (Ejiofor, 1991) . Therefore, the selection of growth medium or raw material is critical for commercial production of these biopesticides. In order to encourage the commercial production of biopesticides, utilization of less expensive raw material is advisable (Mummigatti & Raghunathan, 1990)

Several raw materials (industrial and agricultural by-products) have been tested successfully in mosquito-control programme, as an alternative culture media, for the production of *Bacillus sphaericu*s and *Bti* (Saalma et al., 1983; Obeta & Okafor, 1984; Kumar et al., 2000; Poopathi et al., 2002) .

The feasibility of producing a given microbial product depends, to a large extent on the cost at which the product can be produced. This, in turn, depends on the efficiency with which the organism produces this product, the fermentation parameters (Temperature, pH, aeration, etc.) , the means of recovery and formulation and finally, the cost of the medium used in the fermentation (Ejiofor and Okafor, 1988) . Although the introduction of cheap agro- industrial by-products in fermentation media of *B. thuringiensis* H-14 has not elicited enough interest many workers have produced very potent formulations using various raw materials as media components **(**Dulmage, 1981; Hertlein et al., 1981, Salama and Okafor, 1984; Okafor, 1987; WHO, 1983) .

Chicken/poultry feathers available globally as a cheap bio-organic wastes are a very useful substrate for the industrial production of mosquito pathogenic bacteraia *Bti,* especially in devloping countries, towards mosquito

control programs (Subbiah poopathi, 2008) .

Study was conducted to explore the possibility of degrading chicken feathers, discarded as environmental waste, from poultry processing industries, for bacterial culture media preparation to produce mosquitocidal toxins. Chicken feather powder (0.5%) was used, for the preparation of bacterial culture media. It enabled the complete degradation of feather waste by the entomopathogenic bacteria (*Bacillus sphaericus* and *Bacillus thuringiensis serovar israelensis*) , thus helping to overcome the problem of dumping of poultry feather waste into the environment. The new fermentation technique also facilitated the effective storage of raw material (feathers) , enabling easy transportation and convenience in handling and application. The bioremediation method thus envisaged proved to be cost effective for the preparation of culture media with high efficacy of bacterial toxin production and mosquito vector control. This technique possesses the dual benefits of efficient biodegradation of environmental bio-organic waste (Chicken feathers) and effective production of mosquitocidal biopesticides.

Agro industrial waste sugar cane bagasse was used as a suitable substrate for the production of *Bti* in combination with soya bean powder. Water extract of bagasse was used, without delignification (Poopathi. S, 2013)

Sugar cane bagasse is a renewable resource that can be used to produce biopesticide for the control of mosquito vectors. It was demonstarted 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.

A mediun for the growth of *Bti* with soybean as substrate was described. (K, Balaraman, 2006) *Bacillus thuringiensis var israelensis* (*Bti*) has been widely used in mosquito control programs, but the large scale production of this Bacillus is expensive because of the high cost of the medium. Attempt was made to develop a cost-effective medium, based on inexpensive, locally available raw materials including soybean flour (*Glycinemax*) , Groundnut cake powder (*Arachis hypogea*) , and wheat bran extractG (*Triticum aestivum*) by using 100-L fermentor. Sporulation, toxicity, and biomass were satisfactory after *Bti* was produced on all the three media. Use of the soy bean culture medium resulted in maximum toxicity (LC50

8.89 ng/ml against *Culex quinquefasciatus* IIIrd instar larvae) , highest spore count (0.48×10^{11} c. f. u. /ml) , and maximum biomass (7.8 g/L) within a short fermentation time of 24 h. Hence, this soy bean-based culture medium was considered most economical for the large scale industrial production of *Bti.*

Barley based medium was used for the production of *Bti* (P. S. vimaladevi, 2015) . Studies conducted for the multiplication of *Bacillus thuringiensis* (*Btk*) using barley *Hordeumvulgare* as the carbon source led to the development of a protocol for the cost-effective, mass production of *Bt*. The production employs the simple shake flask method and can be easily adopted with a production potential of 1.5 kg *Bt* per day approximately at an overall production cost of Rs.360 per kg (8 US dollars) . The protocol is suitable for promoting localized production of *Bt* at the village/district level. The product when tested as 0.1% (w/v) spray against the castor semilooper, *Achoea janata* proved highly effective, causing immediate feeding cessation of the larvae followed by 85% and 100% mortality by 48 and 72 h after

treatment, respectively.

Potato extract based medium was used by Poopathi et, al for the successful production of *Bti* (Poopathi. S, 2002) . Mosquito larvicides like *Bacillus sphaericus* and *Bacillus thuringiensis* serovar. *israelensis*have been widely and effectively used in mosquito control programs, but the industrial production of these Bacilli is expensive. Attempt was done to develop three cost-effective media, based on cheap sources, potato, common sugar and bengal gram. Growth and production of the insecticidal proteins from these bacteria were satisfactory. Bioassay studies with different mosquito larvae showed considerable toxicity. Therefore the investigation suggests that potato- based culture media are more economical for the industrial production of *B. sphaericus* and *B. thuringiensis* serovar. *israelensis*.

A novel cost effective medium for the production of *Bacillus thuringiensis* subspecies, israelensis for mosquito control was developed by Subbiah poopaathi (Subbiah poopaathi. et. al, 2012) .

Attempts were done to widen the scope in developing cost-effective culture medium for *Bti* production, based on the raw materials available on the biosphere, including coconut cake powder, CCP (*Cocos nucifera*) , Neem cake powder, NCP (*Azadirachta indica*) and Groundnut cake powder, GCP (*Arachis hypogea*) . Among these raw materials, the biomass production of *Bti*, sporulation and toxin synthesizing from 'CCP' in combination with mineral salt ($MnCl_2$) was comfortably satisfactory. Bioassays with mosquito species (*Culex quinquefasciatus, Anopheles stephensi and Aedes aegypti*) and field trials were also satisfactory. The present investigation suggests that coconut cake-based culture medium can be used as an alternative for industrial production of *Bti* in mosquito-control programme. Therefore, the study is very important from the point of effective production of *Bti* from cost-effective culture medium for the control of mosquito vectors.

A bioinsecticide with high specificity, easy environmental degradation and low production costs could eliminate the ecological, economic and health impacts of chemical compounds used for control (Brar et al., 2006) . However, bioinsecticide production has not yet resulted in high sales levels throughout the world. New products and formulations are being developed for commercialization (Hynes and Boyetchko, 2006) . Parameters of fermentation and toxin production are particularly important for optimum product yield. The evaluation of various substrates is also an important factor, including the potential exploitation of agricultural products and agri-business waste (Prabakaran and Balaraman, 2006) . Various researchers have explored alternatives to the preparation of several less expensive culture media for *Bti* biopesticide production. Often, locally available, cost-effective substrates have been used and have been shown to achieve comparable or better results than those obtained using conventional medium. Prabakaran and Balaraman (2006) attempted to develop a medium based on raw materials including soybean flour (*Glycine max*) , groundnut cake powder (*Arachis hypogea*) , and wheat bran extract (*Triticum aestivum*) in a 100-L fermentor. Prabakaran et al. (2008) made a cost-effective medium with coconut water, which is a raw material that is abundantly available as a waste product from the coconut oil industry. Yezza et al. (2006) conducted the bioconversion of industrial waste water and waste water sludge into a biopesticide in a pilot fermentor.

The final fermented *Bt* broth comprises of spores, cell debris, inclusion bodies, enzymes and other residual solids, which needs to be recovered efficiently to be utilized in subsequent formulation step. Generally, depending on the desired entomotoxicity of final product and scale of production, the processing required varies significantly. Key factors governing the choice of harvesting strategy include process throughput, physical characteristics of product and impurities and desired end-product concentration.

2.26 Hosts susceptible to *Bti* (Glare. Travis and Maureen O'Callaghan, 1998)

Aedes aegypti,

Ae. Albopictus.,

Ae. Atlanticus,

Ae. Atropalpus,

Ae. ampestris,

Ae. canadensis,

Ae. Cantans,

Ae. Cantator,

Ae. Caspius,

Ae. Cataphylla,

Ae. Cinereus,

Ae. Communis,

Ae. Detritus,

Ae. Dorsalis,

Ae. Dupreei,

Ae. Fitchii,

Ae. Flavopictus,

Ae. Hexodontus,

Ae. implicatus.

Ae. melanimon.

Ae. elanion.

Ae. mercurator.

Ae. nigromaculis.

Ae. polynesiensis.

Ae. pseudoscutellaris.

Ae. pullatus.

Ae. punctor.

Ae. sierrensis

Ae. sollicitan.

Ae. spencerii (& varieties) .

Ae. squamiger.

Ae. sticticus.

Ae. stimulans.

Ae. taeniorhynchus.

Ae. togoi.

Ae. tomentor

Ae. triseriatus.

Ae. rusticus.

Ae. vexans.

Ae. ventrovittis.

Ae. vigilax.

Anopheles albimanus

An. annulipes.

An. anthropophagus

An. arabiensis.

An. atroparvus.

An. balabacensis.

An. culicifacies.

An. crucians.

An. dthali.

An. gambiae complex.

An. hyrcanus.

An. karwari.

An. maculatus.

An. maculipennis.

An. multicolor

An. nigerrimus.

An. pharoensis.

An. pulcherrimus.

An. quadrimaculatus.

An. sacharovi.

An. sinensis.

An. stephensi.

An. subpictus.

An. sundaicus.

An. superpictus.

An. vagus.

Armigeres durhami.

Ar. kesseli.

Ar. subalbatus.

Culex annulirostris

Cx. antennatus.

Cx. declarator.

Cx. erraticus

Cx. fuscanus.

Cx. laticinctus.

Cx. nigripalpu

Cx. orientalis.

Cx. peus.

Cx. pipiens complex.

Cx. pseudovishnui.

Cx. quinquefasciatus.

Cx. Restuans

Cx. salinarius.

Cx. sitiens.

Cx. tarsalis.

Cx. theileri.

Cx. tritaeniorhynchus.

Cx. vishnui.

Culiseta alaskaensis

Cs. incidens Cs. inornata. Cs. longiareolata. Cs. Melanura Cs. nigripalpus.

Mansonia spp. *Ma. bonneae. Ma. dyari. Ma. indiana.*

2.27 Factors affecting δ-dotoxin production

There are several factors that influence the production of crystals:

(1) Carbon source:

When the Carbon source gets exhausted in the fermentation, its absence triggers sporulation. The use of one or other carbon source affects the biological activity and the morphology of the crystals (Dulmage, 1970) .

(2) Nitrogen source:

An appropriate source of amino acids provides high growth rates and high sporulation. Its absence delays sporulation and results in low yield of Cry proteins (Goldberg et al.1980)

(3) Carbon: Nitrogen (C/N) ratio:

Several authors have recommended a carbon nitrogen ratio of 7.5: 1. (Salama et al.1983; Foda et al.1985) on an average 10: 1 ratio is used.

(4) Oxygen. High aeration rates are important for high sporulation and Toxin formation. As kLa increases, biomass and Cry protein formation also increases (Rowe & Margaritis, 1987) .

(5) **pH**. Optimum pH for thegrowth of *Bti* is 6.8-7.2. If pH rises to 9. 0 Cry proteins can be dissolved.

(6) **Temperature**. Optimum temperature for *Bti* is 28-32^{0}C. Higher temperatures favor plasmid loss or *Bt* mutants (Rowe & Margaritis, 1987) .

2.28 Susceptibility of mosquito species to *B. thuringiensis* serotype *israelensis:* *Bti* is highly pathogenic against *Culicidae* (mosquitoes) and *Simuliidae* (blackflies) , and has some virulence against certain others *Diptera,* especially *Chironomidae* (midges) . First instar of larvae is more susceptible to *Bti* than the fourth instar because larvae are actively feeding in their early stages (Mulla *et al.1*990) . Pupa does not feed and therefore is not affected by *Bti*. For almost all species tested, increasing age of the larvae resulted in reduced susceptibility in mosquito (Chen et al.1984; Mulla *et al.1*985) . *Bti* was found to be specific toxic to larvae of 109 mosquito species.

Table.2.2: Larvicidal activity of *Bti* on different mosquito species (Glare & O´Callaghan 1998) .

Mosquito genus	**Species**
Aedes	40
Anopheles	27
Culex	19
Culiseta	5
Mansonia	5
Psorophora	3
Armigeres	3
Toxorhynchites	2
Limatus	2
Trichophospon	1
Uranotaenia	1
Tripteroides	1
	Total 109

2.29 Production cost of *Bti*- A challenge for biopesticide comercialization

Since mosquitoe related diseases cause serious ecnomic and social problems, there is a need to manufacture huge volumes of this ecofriendly biopesticide. Many countries need cost effective solutions for mosquitoe control, so now more attention is drawn towards low cost production of *Bti* which can be achieved through optimization of culture conditions using appropriate media. The cost to grow and produce *Bti* using highly refined media is very high. Although the cost of production depends on many factors, raw material cost dominates, which may comprise more than 70% of the overall production cost. (Ejiofor, 1991) .

Very less published data is available for the use of low cost medium ingedients for mass manufacturing of *Bti*. Its found that a medium with starch

or molassess as carbohydrate source in combination with mung beans as protein supplement were used to over produce delta endotoxin of *Bti* (Mummigatti and Raghunathan, 1990) .

Media formulation and optimization are key considerations in development of bioprocesses that can produce affordable biological agents, yet limited progress has been made in this area to satisfy market opportunities for affordable commercial biological insecticide products.

Its proved many times that carbon, nitrogen and macronutrients dramatically influence the spore production and toxicity of *Bti* (S. Yan, et al., 2007; Gouda et al., 2001) . Commonly used nutrient sources include, peptones, extracts and hydrolysates, many are expensive from an industrial manufacturing point of view since it involves huge volumes (Nohata and Kurane, 1997; Vuolanto et al., 2001) . Increase in biomass, toxin production, and spore formation are all very much affected by growth medium components and its imperative to have detailed study about it to make advancement in the design of low-cost culture medium for the overall efficient manufacturing of this highly sought after biopesticide.

For vigorous growth and toxin production, ample quantities of amino nitrogen must be avilable in the fermentation media. Depending on the concentration there will be fluctuations in biomass and toxin production. Increase in initial amino nitrogen in the culture medium increases the biomass, spore count and toxicity (Prabakaran and Hoti, 2008) . Thus it of paramount importance to look for cheaper nitrogen sourcess, since conventional sources like peptones, casein hydrolysate, yeast extract have proved to be very expensive. At the same time rotten fish and poultry feathers has been found to be promising.

2.30 Down stream processing strategies: Since *Bti* being a biomass based product, cell separtion is an important down stream operation which can be achieved by either filtration or centrifugation.

Certain materials act as filter aids which improves the rate of filtration by improving voidage, when such down stream methods are used for the separation of bacterial biomass based biotech product.

2.30.1 Guar gum: Gaur gum also called guaran, is a galactomanan polysaccharide extracted from guar beans. Due to its thickening and stabilizing properties its widely used in food, feed and industrial applications. usually marketed as a free flowing off -white powder.

Its mainly grown in India, Pakistan, U. S. , Australia and Africa, with india leading in production with 2.5-3 million tonns per annum. US has produced 5000-140000 tonns of gaur over the last 5 years. Gaur gum is an exo-polysaccharide made up of galactose and mannose. It has the ability to with stand temperatures of even 80 degrees celsius for 5 minutes.

Some of the Industrial applications of Gaur gum are in:

- Textile industry – sizing, finishing and printing
- Paper industry – improved sheet formation, folding and denser surface for printing
- Explosives industry – as water proofing agent mixed with ammonium nitrate, nitroglycerin, etc.
- Pharmaceutical industry – as binder or as disintegrator in tablets; main ingredient in some bulk-forming laxatives
- Cosmetics and toiletries industries – thickener in toothpastes, conditioner in shampoos.

The largest market for guar gum is in the food industry. Applications include:

- In baked goods, it increases dough yield, gives greater resiliency, and improves texture and shelf life; in pastry fillings, it prevents "weeping" (syneresis) of the water in the filling, keeping the pastry crust crisp. It is primarily used in hypoallergenic recipes that use different types of whole-grain flours. Because the consistency of these flours allows the escape of gas released by leavening, guar gum is needed to improve the thickness of these flours, allowing them to rise as a normal flour.
- In dairy products, it thickens milk, yogurt, kefir, and liquid cheese products, and helps maintain homogeneity and texture of ice creams and sherbets. It is used for similar purposes in plant milks.
- For meat, it functions as a binder.

In *Bti* fermentations, the product being biomass based, is seperated from the fermentation broth by doing centrifugation. Filtration of fermented broth is another technology option. In this work gaur is used as a filter aid.

2.30.2 Centrifugation is a technique which involves the application of centrifugal force to separate particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed. There is a correlation between the size and density of a particle and the rate that the particle separates from a heterogeneous mixture, when the only force applied is that of gravity.

$$v_c = \frac{d^2 (\rho_p - \rho_l)}{18\eta} r\omega^2$$

Fig.16 Equation for rate of settling of a particle under centrifugal force

The rate of centrifugation is specified by the angular velocity usually expressed as revolutions per minute (RPM) , or acceleration expressed as X g. The conversion factor between RPM and g depends on the radius of the centrifuge rotor. The particles'settling velocity in centrifugation is a function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity. The most common application is the separation of solid from highly concentrated suspensions, which is used in the treatment of sewage sludges for dewatering where less consistent sediment is produced.

2.30.3 Filtration is any of various mechanical, physical or biological operations that separate solids from fluids (liquids or gases) by adding a medium through which only the fluid can pass. The fluid that passes through is called the filtrate. In physical filters over sized solids in the fluid are retained and in biological filters particulates are trapped and ingested and metabolites are retained and removed. For example, in animals (including humans) , renal filtration removes wastes from the blood, and in water treatment and sewage treatment, undesirable constituents are removed by absorption into a biological film grown on or in the filter medium, as in slow sand filtration.

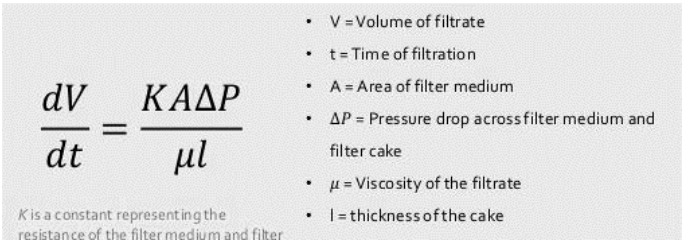

Fig.17 Equation for the rate of flow through a conventional filter

Filtration is used to separate particles and fluid in a suspension, where the fluid can be a liquid, a gas or a supercritical fluid. Depending on the application, either one or both of the components may be isolated.

CHAPTER 3
MATERIALS AND METHODS

The strain: The strain used in all these experiments was *Bacillus thuringiensis subspecies israelensis H-14.* The bacteria was grown in a 500ml conical flask using submerged fermentation technique. The parent strain was maintained as sporulated cultures on modified glucode yeast extract salts (mGYS) agar slants containing 0.3% glucose and it was stored at 4°C and subcultured every 3months.

3.1.1 Composition of mGYS medium

Glucose	-	0.3%
Ammonium sulphate	-	0.2%
di-pottassium hydrogen phosphate	-	0.5%
yeast extract	-	0.2%
Magnesium sulphate	-	0. 02%
Calcium chloride	-	0. 008%
Manganese sulphate	-	0. 005%

pH-7.2

In all the cases the cultivation of bacteria began with a pre-culture stage. A loopfull of the refrigerated preserved culture which was routinely used for storage was transferred to 20ml. of mGYS broth in 100 ml conical flask and incubated for 12 hours keeping the flask stagnanant. For all the experiments conducted, the following culture media were used. Media developed in the bioprocess lab with components, Glucose 1%, 0.5% peptone and 0.1% yeast extract was used for the routine culture of the bacteria , required for microscopy, sporulation status etc **(Gopinathan, C. et. al.2019)**

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3.2 Media formulations

A preliminary investigation regarding the growth and sporulation status of *Bacillus thuringiensis subspecies israelensis* on a range of media formulations were done.

3.2.1 The production of *Bti* based biopesticide using coconut water based media (submerged fermentation) .

Materials used: Coconut water, Peptone, yeast extract, and calcium chloride. Methods: Different liquid media were prepared with 100. ml coconut water alone, coconut water +0.1% peptone, coconut water + 0.1% peptone+0.1% yeast extract, coconut water + 0.1% peptone+0.1% yeast extract+ 0.1% $CaCl_2$.

A pre-culture was prepared by inoculating a loopful of *Bti* into 20ml. of sterilsed mGYS medium and kept stagnant overnight. 0.1ml of the preculture was inoculated into the respective four flasks containing four different media described above and all the plates were incubated for 48 hours . The biomass production and sporulation time was noted.

3.2.2 The production of *Bti* based biopesticide using coconut water with agar based media

100. ml coconut water alone, Coconut water +0.1% peptone+2% agar, coconut water + 0.1% peptone+0.1% yeast extract+2% agar, coconut water + 0.1% peptone+0.1% yeast extract+0.1% $CaCl_2$+2% agar.

A preculture was prepared by inoculating a loopful of *Bti* into 20ml. of sterilsed mGYS medium and kept stagnant overnight. 0.1ml of the preculture was inculated into the respective petri plates containing four different media described above and all the plates plates were incubated for 48 hours . The

biomass production and sporulation time was noted.

3.2.3.1 The production of *Bti* based biopesticide using Rice straw with agar based media

Materials used: **Rice straw (RS)** powder, peptone, yeast extract Methods: Different solid media were prepared with 1 % Rice straw alone, 1 % RS + 0.1% peptone +2% agar , 1 % RS + 0.1% peptone + 0.1 % yeast extract+2% agar. 0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the three different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.3.2 The production of *Bti* based biopesticide using chemically delignified (NaOH) straw based medium with agar.

Materials used: Rice straw, peptone, yeast extract, sodium hydroxide

Methods: Different Solid media were prepared with

1% Delignified rice straw powder alone+2% agar , 1% Delignified rice straw powder + 0.1% peptone +2% agar, 1% Delignified rice straw powder + 0.1% peptone + 0.1% yeast extract+2% agar.

1N NaOH was used to delignify it and the resulting delignified straw was used for further experiments.10 gm. straw was dipped in 100. ml sodium hydroxide solution for 1 hour and the supernatent was discarded. The residue was washed with distilled water. The residue was resuspended in 100ml distilled water, supplemented with 100. mg yeast extract and pH was adjusted to 7.2, then 2gm. agar was also added, then it was autoclaved for 15 minutes at 15 psi at 121ºC. After cooling to room temepreature, poured into plates and it was inoculated with 0.1ml of *Bti* preculture and allowed to grow.

0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing three different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.3.3: The production of *Bti* based biopesticide using chemically delignified (CaOH) straw based medium with agar

Materials used: Rice straw, peptone, yeast extract, calcium hydroxide

Methods: Different Solid media were prepared with

1% Delignified rice straw powder +2% agar, 1% Delignified rice straw powder + 0.1% peptone +2% agar, 1% Delignified rice straw powder + 0.1% peptone + 0.1% yeast extract +2% agar.

1N CaOH was used to delignify it and the resulting delignified straw was used for further experiments.10gm straw was dipped in 100. ml calcium hydroxide solution for 1 hour and the supernatent was discarded. The residue was washed with distilled water. The residue was resuspended in 100ml distilled water, supplemented with 100. mg yeast extract and pH was adjusted to 7.2, then 2gm agar was also added, then it was autoclaved for 15 minutes at 15 psi at121°C. After cooling to room temepreature, 0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the three different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.3.4: The production of *Bti* based biopesticide using Biologically delignified (*Trichoderma reesei*) straw based medium with agar

Materials used: Rice straw, peptone, yeast extract, *Trichoderma reesei* fungus, polypropylene covers.

Methods: Different Solid media were prepared with

1% Delignified rice straw powder alone +2% agar, 1% Delignified rice straw powder + 0.1% peptone +2% agar, 1% Delignified rice straw powder + 0.1% peptone + 0.1% yeast extract +2% agar.

Delignifcation of rice straw was done by growing *Trichoderma reesei* on the rice straw, which is an excellent lignase enzyme producer. For this solid state fermentation method was used.500. gm rice straw is submerged in 5 litres water for a period of 24 hours. The excess water is drained off and the rice straw is packed in polyproplene bags of dimension 10x20cm as layers.25 gm (5% w/w) of *Trichoderma reesie* commercial formulation was laid and polythene bags were closed and incubated for 15 days in dark. The bags were punctured with a sterile pin, to allow gas exchange. After 15 days the fully grown rice starw substratum was used for growing *Bti*.1. gm of this sample was suspended in 100. ml distilled water supplemented with 100. mg of yeast extract and the pH was adjusted to 7.2, 2 gm agar was was added and then autoclaved, 121ºC, for 15 min, at 15 psi. After cooling, poured into petri plates and sporulation time was noted.

3.2.3.5: The production of *Bti* based biopesticide using Biologically delignified (*Pleurotus florida*) straw based medium with agar.

Materials used: Rice straw, peptone, yeast extract, *Pleurotus* fungus, polypropylene covers

Methods: Different Solid media were prepared with

1% Delignified rice straw powder alone +2% agar, 1% Delignified rice straw powder + 0.1% peptone +2% agar, 1% Delignified rice straw powder + 0.1% peptone + 0.1% yeast extract+2% agar.

Delignifcation of rice straw was done by growing *Pleurotus florida* on

the rice straw, which is an excellent lignase enzyme producer. For this solid state fermentation method was used.500. gm rice straw is submerged in 5 litres water for aperiod of 24 hours. the excess water is drained off and the rice straw is packed in polyproplene bags of dimension 10x20cm as layers.25 gm (5% w/w) of *Pleurotus florida* commercial formulation was laid and polythene bags were closed and incubated for 15 days in dark. The bags were punctured with a sterile pin, to allow gas exchange. After 15 days the fully grown rice starw substratum was used for growing *Bti.1*. gm of this sample was suspended in 100. ml distilled water supplemented with 100. mg of yaest extract and the pH was adjusted to 7.2, 2gm agar was added and then autoclaved, 121ºC, for 15min, at 15psi. After cooling, poured into petri plates and sporulation time was noted.

3.2.3.6 The production of *Bti* based biopesticide using delignified rice straw (NaOH) -agar based medium supplemented with fish slurry

Delignification of rice straw was done as described earlier mentioned in section: 3.2.3.2.1. gm residue was resuspended in 100 ml. distilled water and supplemented with 0.1, 0.2, 0.3, 0.4, and 0.5ml of fish slurry and yeast extract. pH was adjusted to 7.2, then 2gm agar was also added, it was autoclaved for 15 minutes at 15 psi and 121°C. After cooling to room temepreature, poured into plates and it was inoculated with 0.1ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.3.7 The production of *Bti* based biopesticide using delignified rice straw (CaOH) -agar based medium supplemented with fish slurry

Delignification of rice straw was done as described earlier mentioned in section: 3.2.3.3.1. gm residue was resuspended in 100ml distilled water supplemented with 0.1, 0.2, 0.3, 0.4, and 0.5ml of fish slurry and yeast

extract. pH was adjusted to 7.2, then 2gm agar was also added, then it was autoclaved for 15 minutes at 15 psi and 121°C**.** After cooling to room temepreature, poured into plates and it was inoculated with 0.1ml of *Bti* preculture and allowed to grow for 48 hours.

3.2.3.8 The production of *Bti* based biopesticide using delignified rice straw (*Trichoderma reesie*) -agar based medium supplemented with fish slurry

Delignification of rice straw was done as described earlier mentioned in section: 3.2.3.4. 1. gm of this sample was suspended in 100. ml distilled water supplemented with 100. mg of yeast extract + 0.1, 0.2, 0.3, 0.4, and 0.5ml of fish slurry and the pH was adjusted to 7.2, agar 2gm. was added and then autoclaved at 121ºC, for 15min, at15psi. After cooling, poured into petri plates. 0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the five different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.3.9 The production of *Bti* based biopesticide using delignified rice straw (P*leurotus florida*) -agar based medium supplemented with fish slurry

Delignification of rice straw was done as described earlier mentioned in section: 3.2.3.5.500. gm rice straw is submerged in 5 litres water for a period of 24 hours. The excess water is drained off and the rice straw is packed in polyproplene bags of dimension 10x20cm as layers.25 gm (5% w/w) of *Pleurotus florida* commercial formulation was laid and polythene bags were closed and incubated for 15 days in dark. The bags were punctured with a sterile pin, to allow gas exchange. after 15 days the fully grown rice starw substartum was used for growing *Bti*.1. gm of this sample was

suspended in 100. ml distilled water supplemented with 100. mg of yeast extract + 0.1, 0.2, 0.3, 0.4, and 0.5ml of fish slurry and the pH was adjusted to 7.2, Agar 2gm. was added and then autoclaved at 121ºC, for 15min, at 15psi. After cooling, poured into petri plates. 0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the five different media, described above and all the flasks were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.3.10 The production of *Bti* based biopesticide using *Pleurotus florida* fungi pretreated rice straw hydrolysed with cellulase enzyme from *Aspergillus niger.*

Deglinification was done as described earlier mentioned in 3.2.3.5. The resultant cellulose hydrolysis was done with cellulase enzyme from *Aspergillus niger* (20.3 units/ mg) 10. gm of rice straw was dipped in 100. ml of cellulase enzyme solution (10 mg/100ml) for 1 hour and the residue was discarded. To 100ml supernatent supplemented with 100 mg yeast extract and 0.1% peptone. Then the pH was adjusted to 7.2. Then it was autoclaved for 15psi at 121°C. After cooling to room temperature, it was inoculated with 0.1ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

Supplementation with chicken feather hydrolysate

Prepration of chicken feather hydrolysate: The chicken feathers were collected from poultry farms and washed 3 times to remove dirt and other debris like blood and then it was subjected to treatment with chloroform : Methanol (1: 1 v/v) for 12 hours. The resulting defatted feathers were filtered and then dried in sun light. Then it was powdered and the subjected to 0.1N NaOH treatment at 90°C for 30 minutes. 1 gm was used for hydrolysis.After hydrolysis the resulting material is filtered and the filtrate was used for further studies.

3.2.3.11 The production of *Bti* based biopesticide using Chemically delignified rice straw (NaOH) supplemented with chicken feather hydrolysate.

Delignification was done with 1. N sodium hydroxide as in 3.2.3.2.10 gm. of rice straw was dipped in 100 ml of sodium hydroxide for 1 hour, and the supernatent was discarded. The residue was washed with distilled water.1 gm. residue was resuspended in 100. ml distlled water supplemented with 0.1ml, 0.2ml, 0.3ml, 0.4. ml and 0.5ml of chicken feather hydrolysate. Then the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at 121 °C**.** After cooling to room temperature, it was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.3.12 The production of *Bti* based biopesticide using Chemically delignified rice straw (CaOH) supplemented with chicken feather hydrolysate. The experiment was done as desribed earlier in section3.2.3.7**.** The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.3.13 The production of *Bti* based biopesticide using *Trichoderma reesei* delignified rice straw supplemented with chicken feather hydrolysate : Delignification of rice straw was done as described in section 3.2.3.4.1gm. of this sample was suspended in 100. ml distilled water supplemented with 0.1, 0.2, 0.3, 0.4, and0.5 ml chicken feather hydrolysate

100. mg of yeast extract and the pH was adjusted to 7.2. then it was autoclaved for 15 minutes at 15 psi at 121ºCC**.** After cooling to room temperature, it was inoculated with 0.1. ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.3.14 The production of *Bti* based biopesticide using *Pleurotus florida* delignified rice straw supplemented with chicken feather hydrolysate: The experiment was done as described earlier as desribed in section 3.2.3.5 1. gm. of this sample was suspended in 100. ml distilled water supplemented with 100. mg of yeast extract+ +0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml chicken feather hydrolysate and the pH was adjusted to 7.2. then it was autoclaved for 15 minutes at 15 psi at 121°C. After cooling to room temperature, it was inoculated with 0.1. ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.4.1 The production of *Bti* based biopesticide using bagasse based media

Materials used: Bagasse (BG) , peptone, yeast extract Methods: Different solid media were prepared with 1% Bagasse powder alone+2%agar , 1% BG + 0.1% peptone+ 2%agar , 1% BG + 0.1% peptone + 0.1% yeast extract+ 2%agar A preculture was prepared by inoculating a loopful of *Bti* into 20ml. of sterilsed mGYS medium and kept stagnant overnight. 0.1ml of the preculture was inculated into the respective petri plates containing three different media described above and all the plates plates were incubated for 48 hours . The biomass production and sporulation time was noted.

3.2.4.2 The production of *Bti* based biopesticide using chemicaly delignified (NaOH) bagasse .

Delignification was done as described in section: 3.2.3.2.1gm. The residue was resuspended in 100. ml distlled water supplemented with 100. mg of yeast extract and the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at 121°C. After cooling to room temperature, It was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.4.3 The production of *Bti* based biopesticide using Chemically delignified (CaOH) bagasse : Delignification was done as described in section: 3.2.3.3.10 gm. of bagasse was dipped in 100 ml of calcium hydroxide for 2 hours, and the supernatent was discarded. The residue was washed with distlled water. The residue was resuspended in 100. ml distlled water supplemented with 100. mg of yeast extract and the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at 121°C**.** After cooling to room temperature, It was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.4.4 The production of *Bti* based biopesticide using NaOH delignified bagasse based medium supplemented with fish slurry

Delignification was done as described in section: 3.2.3.2. The residue was resuspended in 100. ml distlled water supplemented with 100. mg of yeast extract+ 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry and the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at 121°C**.** After cooling to room temperature, it was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.4.5 The production of *Bti* based biopesticide using CaOH delignified bagasse based medium supplemented with fish slurry.

Delignification was done as described in section 3.2.3.3. The residue was resuspended in 100. ml distlled water supplemented with 100. mg of yeast extract + 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry and the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at 121 °C. After cooling to room temperature, it was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration,

sporulation status and larvicidal activity were determined**.**

3.2.4.6 The production of *Bti* based biopesticide using *Trichoderma reesei* delignified Bagasse based medium supplemented with fish slurry

1 gm. of this sample was suspended in 100. ml distilled water supplemented with 100. mg of yeast extract+ 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry and the pH was adjusted to 7.2. then it was autoclaved for 15 minutes at 15 psi at 121C**.** After cooling to room temperature, it was inoculated with 0.1. ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.4.7. The production of *Bti* based biopesticide using *Pleurotus florida* delignified Bagasse based medium supplemented with fish slurry The experiment was done as described earlier as mentioned in section 3.2.3.5

1 gm. of this sample was suspended in 100. ml distilled water supplemented with 100. mg of yeast extract+ 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry and the pH was adjusted to 7.2. then it was autoclaved for 15 minutes at 15 psi at 121°C**.** After cooling to room temperature, it wasinoculated with 0.1. ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.4.8 The production of *Bti* based biopesticide using *Pleurotus florida* delignified Bagasse (and hydrolysed with cellulase from *Aspergillus niger)* based medium supplemented with fish slurry: The delignification was done as descibed earlier described section 3.2.3.5.1. gm residue was resuspended in 100. ml distilled water supplemented with 100 mg of yeast extract and 100mg peptone. Then the pH was adjusted to 7.2. Then it was autoclaved for 15 minutes at 15 psi at **121 °**C**.** After cooling to room

temperature, it was inoculated with 0.1 ml of *Bti* preculture and allowed to grow for 48 hours. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.5.1 The production of *Bti* based biopesticide using taro agar based medium

Fig.18. Taro powder- millling or powdering increases surface area which in turn increases the assimilabilty of the raw material by the microbial enzymes.

Materials used: Taro, peptone, yeast extract, calcium chloride. Methods: Different solid media were prepared with1% Taro powder alone +2% agar, 1% Taro powder + 0.1% peptone +2% agar, 1% Taro powder + 0.1% peptone + 0.1% yeast extract+2% agar, 1% Taro powder + 0.1% peptone + 0.1% yeast extract+ 0.1% calcium chloride+2% agar.0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.5.2 The production of *Bti* based biopesticide using hydrolysed taro (HCl) agar based media

Materials used: Taro, peptone, yeast extract, calcium chloride, 1N HCl

Methods: Different solid media were prepared with 1% Taro powder alone +2% agar, 1% Taro powder + 0.1% peptone +2% agar, 1% Taro powder + 0.1% peptone + 0.1% yeast extract +2% agar, +2% agar, 1% Taro powder + 0.1% peptone + 0.1% yeast extract+ 0.1% calcium chloride

1N HCl was used to pre- treat the Taro and neutralised with 1N NaOH and the resulting hydrolysed taro was used for further experiments. 0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.5.3 Solid state fermentations of *Bti* using taro powder and bengal gram agar based media.

Materials used: Taro, peptone, yeast extract, bengal gram powder and coconut water.

Methods: Different solid media were prepared with 1gm. Taro powder +0.5gm bengal gram powder+0.25% yeast extract+2% agar in 100ml coconut water, 0.5 gm peptone in 100ml cocounut water, and 0.5gm peptone and 0.25gm yeast extract in 100ml coconut water.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.6.1 The production of *Bti* based biopesticide using Sweet potato agar based medium

Materials used: Sweet potato (SP) , peptone, yeast extract, calcium chloride
Methods: Different solid media were prepared with1% SP powder alone +2% agar, 1% SP powder + 0.1% peptone +2% agar, 1% SP powder + 0.1%

peptone + 0.1% yeast extract +2% agar, 1% SP powder + 0.1% peptone + 0.1% yeast extract+ 0.1% Calcium chloride+2% agar.

0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.6.2 Solid state fermentations of *Bti* using Sweet potato powder and bengal gram agar based media.

Materials used: Sweet potato (SP) , peptone, yeast extract, bengal gram powder and coconut water

Methods: Different solid media were prepared with

1gm. SP powder +0.5gm bengal gram powder+0.25% yeast extract+2% agar in 100ml coconut water, 0.5 gm peptone in 100ml cocounut water, and 0.5gm peptone and 0.25gm yeast extract in 100ml coconut water.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.7.1 The production of *Bti*based biopesticide using Purple yam (PY) agar based media

Materials used: Purple yam (PY) , peptone, yeast extract, calcium chloride
Methods: Different solid media were prepared with 1% PY powder alone +2% agar, 1% PY powder + 0.1% peptone +2% agar, 1% PY powder + 0.1% peptone + 0.1% yeast extract +2% agar, 1% PY powder + 0.1% peptone + 0.1% yeast extract + 0.1% Calcium chloride+2% agar.

0.1ml *Bti* preculture was prepared and inoculated into the respective

petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.7.2 The production of *Bti* based biopesticide using Purple yam agar based medium with fish slurry supplementation: To 1. gm purple yam powder 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry was added. 0.1. ml *Bti* pre culture is inoculated into each of the media using laminar air flow cabinet. The plates were incubated for 48 hours at room temperature. The biomass was estimated by scraping and weighing the wet biomass. Sporulation status and larvicidal activity were also determined.

3.2.7.3. **Solid state fermentations of *Bti* using purple yam powder and bengal gram based medium .**

Materials used: purple yam (PY) , peptone, yeast extract, bengal gram powder and coconut water

Methods: Different solid media were prepared with 1gm. PY powder +0.5gm bengal gram powder+0.25% yeast extract+2% agar in 100ml coconut water, 0.5 gm peptone in 100ml cocounut water, and 0.5gm peptone and 0.25gm yeast extract in 100ml coconut water.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.8.1 The production of *Bti* based biopesticide pineapple peel (PP) agar based medium :

Methods: Different solid media were prepared with 1% PP powder alone +2% agar, 1% PP powder + 0.1% peptone +2% agar, 1% PP powder + 0.1% peptone + 0.1% yeast extract +2% agar. And all the media was sterilised

at 15 psi for 15 minutes at 121°C**.** It was then poured into sterile petridishes . 0.1. ml *Bti* pre culture is spread uniformly over the agar medium using laminar air flow cabinet. The plates were incubated for 48 hours at room temperature. The biomass production and sporulation time was noted.

3.2.8.2. The production of *Bti* based biopesticide using Pineapple peel agar based medium with fish slurry supplementation: To 1. gm Pine apple peel powder 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry was added. 0.1. ml *Bti* pre culture is inoculated into each of the media using laminar air flow cabinet. The petridishes were incubated for 48 hours at room temperature. The biomass was estimated by scraping and weighing the wet biomass.

3.2.8.3 The production of *Bti* based biopesticide using pineapple peel agar based medium with Chicken feather hydrolysate (CHF) : To 1. gm pine apple peel powder 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of CHF was added. 0.1. ml *Bti* pre culture is inoculated into each of the media using laminar air flow cabinet. The petridishes were incubated for 48 hours at room temperature. The biomass was estimated by, scraping and weighing the wet biomass. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.9.1 The production of *Bti* based biopesticide using Tapioca peel agar based medium: Materials used: tapioca peel (TP) , peptone, yeast extract.

Methods: Different solid media were prepared with
1% TP powder alone +2% agar, 1% TP powder + 0.1% peptone +2% agar, 1% TP powder + 0.1% peptone + 0.1% yeast extract +2% agar. 0.1ml *Bti* preculture was prepared and inoculated into the respective petriplates containing the four different media, described above and all the plates were incubated for 48 hours. The biomass production and sporulation time was noted.

3.2.9.2 The production of *Bti* based biopesticide using Tapioca peel agar based medium with fish slurry supplementation: To 1. gm Tapioca peel powder 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry was added. 0.1. ml *Bti* pre culture is inoculated into each of the media using laminar air flow cabinet. The petridishes were incubated for 48 hours at room temperature. The biomass was estimated by , scraping and weighing the wet biomass. The biomass concentration, sporulation status and larvicidal activity were determined.

3.2.10.1 The production of *Bti* based biopesticide using vegetable waste (VW) agar based medium:

Methods: Different solid media were prepared with 1% VW alone +2% agar, 1% VW + 0.1% peptone +2% agar, 1% VW + 0.1% peptone + 0.1% yeast extract +2% agar.

And all the media was sterilised at 15 psi for 15 minutes at 121°C**.** It was then poured into sterile petridishes . 0.1. ml *Bti* pre culture is spread uniformly over the agar medium using laminar air flow cabinet. The plates were incubated for 48 hours at room temperature. The biomass production and sporulation time was noted.

3.2.10.2 The production of *Bti* based biopesticide using vegetable waste agar based medium with fish slurry supplementation: To 1. gm mixed vegetables paste 0.1ml, 0.2ml, 0.3ml. 04 ml. and 0.5 ml of fish slurry was added. 0.1. ml *Bti* pre culture is inoculated into each of the media using laminar air flow cabinet. The petridishes were incubated for 48 hours at room temperature. The biomass was estimated by , scraping and weighing the wet biomass. The biomass concentration, sporulation status and larvicidal activity were determined.

3.3 Process development for the cost effective production of *Bacillus thuringiensis subspecies israelensis* using Fed-batch fermentation.

3.3.1 Batch fermentations with different carbon sources: Substrate repression studies

In order to assess the maximum level of initial glucose concentration, which *Bacillus thuringiensis subspecies israelensis* can tolerate in liquid culture, a number of media containing increased concentrations of particular carbon sources were prepared. The concentration of each carbon source, giving rise to the crab-tree effect was thus determined.

Preparation of the different media and inoculation

The quantities of the media additives are expressed in percentage except otherwise mentioned. The starting pH for each of the medium was set to 7.3, except otherwise mentioned. All the media were autoclaved in 500ml conical flasks of equal diametrical dimensions at 15 lbs. [sq. inch]$^{-1}$ for 15 minutes at 121°C. After cooling, each medium was inoculated with 1ml of the preculture. The flasks were aerated using rotary shaker at an RPM of 180. The cells were allowed to grow for 48 hours before being harvested and further experimented on.

3.3.1.1. Composition of medium for batch mode fermentation of *Bti* using glucose as the carbon source for substrate repression studies

The quantities are expressed in percentage unless otherwise mentioned:

Glucose	ranged from 1-4
Peptone	0.5
Yeast extract	0.1
Distilled water	150ml

3.3.1.2 Composition of medium for batch mode fermentation of *Bti* using sucrose as the carbon source for substrate repression studies

Sucrose	ranged from 1-6
Peptone	0.5
Yeast extract	0.1
Distilled water	150ml

3.3.1.3 Composition of medium for batch mode fermentation of *Bti* using cane sugar juice as the carbon source for substrate repression studies

The quantities are expressed in percentage unless otherwise mentioned: Cane sugar ranged from 1-4

Peptone	0.5
Yeast extract	0.1
Distilled water	150ml

3.3.1.4 Composition of medium for batch mode fermentation *Bti* using jaggery as the carbon source for substrate repression studies

The quantities are expressed in percentage unless otherwise mentioned:

Jaggery	ranged from 1-4
Peptone	0.5
Yeast extract	0.1
Distilled water	150ml

3.3.1.6 Composition of medium for batch mode fermentation of *Bti* using tapioca peel powder as the carbon source for substrate repression studies

Tapioca powder	ranged from 1-6
Peptone	0.5
Yeast extract	0.1
Distilled water	150ml

3.3.2 Fed-batch mode of experiments

The process

To get rid of the substrate repression and to achieve increased cell density, and in turn higher toxin production, fed-batch mode of operation was designed. In order to address the problem of substrate repression, the operations were started with an initial carbon concentration of 3%. The final concentrations were achieved by additional increments of the carbon source at the rate of 1% at each 12 hours interval. Thus, the fed-batch mode was carried in the variable volume fed batch mode and not the fixed volume mode. After inoculation the cultures were allowed to grow for a period of twelve hours. Then autoclaved 1% solution of the respective carbon source, dissolved in minimum volume of distilled water, was added aseptically in the laminar airflow chamber to the respective flasks, and again allowed to grow for twelve hours before the next addition. After the final addition, the culture was allowed to grow for 24 hours with intermittent light microscopy being done to check the sporulation status of the culture. If the culture sporulated by that time, the cells were harvested, and if the culture was still growing then the next microscopy was done again after a gap of twelve hours.

The quantities of the media additives are expressed in percentage

except otherwise mentioned. The starting pH for each of the medium was set to 7.2, except otherwise mentioned.

3.3.2.1 Composition of medium for fed-batch mode fermentation of *Bti* using glucose as the carbon source

Glucose (initial)	3%
Peptone	0.5%
Yeast extract	0.1%
Distilled water	150ml

3.3.2.2 Composition of medium for fed-batch mode fermentation of *Bti* using sucrose as carbon source

Sucrose (initial)	3%
Peptone	0.5%
Yeast extract	0.1%
Distilled water	150ml

3.3.2.3 Composition of medium for fed-batch mode fermentation *Bti* using cane sugar juice as the carbon source

Cane sugar (initial)	3%
Peptone	0.5%
Yeast extract	0.1%
Distilled water	150ml

3.3.2.4 Composition of medium for fed-batch mode fermentation of *Bti* using jaggery as the carbon source

Jaggery (initial)	3%
Peptone	0.5%
Yeast extract	0.1%
Distilled water	150ml

3.3.2.5 Fed-batch fermentation *Bti* using glucose as the carbon source and soya bean powder as the nitrogen source

Glucose (initial)	3%
Soya bean	1%
Yeast extract	0.1%
Distilled water	150ml

3.3.2.6 Fed Batch fermentation of *Bti* using cane sugar juice as the carbon source and without yeast extract supplement

Cane sugar	initial -3%
Peptone	0.5
Distilled water	150ml

3.4. The production of *Bti* based biopesticide: Batch fermentation *Bti* using jaggery as the carbon source and without yeast extract supplement

Jaggery	3%
Peptone	0.5%
Distilled water	150ml

3.5 Batch fermentation of using jaggery as the carbon source and soya bean powder as supplement.

Jaggery	3%
soya bean powder	1%
Yeast extract	0.1%
Calcium carbonate	0.1%
Distilled water	150 ml

3.6. Batch fermentation of *Bti* to study the effect of Calcium carbonate on sporulation with jagerry as carbon source and peptone as the nitrogen source.

Jaggery	3%
Peptone	0.5%
Yeast extract	0.1%
Calcium carbonate	0.1%
Distilled water	150 ml

3.7 The production of *Bti* based biopesticide : Solid State fermentation of *Bacillus thuringensis subspecies israelensis*

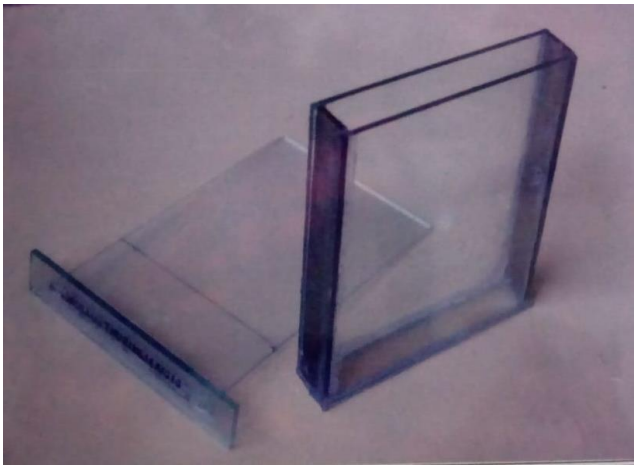

Fig.18 Glass plate and chamber used for Solid state fermentation *Bacillus thuringensis*-molten agar medium is taken in the glass chamber and the glass plate is immersed in it to give a thin coating of the agar media on both sides. After cooling of the media, the glass plate is immersed in another glass chamber containing pure culture of *Bti* to give a uniform inoculum coating on both sides and incubated. This method is very cost effctive and substantially increases sporulation and delta endotoxin production of *Bti.*

Since this *Bacillus thuringensis* sporulates profusely on solid surface compared to submerged culture attempts have been made to optimise suitable growth conditions for maximum sporulation as well as toxic crystal production. The materials used for this study is as follows

1. Glass petriplate of total surface area 78.5 cm^2
2. Glass chamber containing submerged glass plate with a of a total surface area of 297 cm^2.

3.7.1.1 Preparation of agar plates for surface culture studies

A petriplate of total surface area 78.5 cm^2 was taken and autoclaved at 15 Ibs. /sq. inch for 15 minutes at 121°C.10 ml of molten agar media

waspoured into the petriplate so that it forms a thin layer of agar. The agar media was allowed to solidify and 0.1 ml of inoculum was spread over the thin agar layer using a glass spreader. The plates were then incubated until sporulation at room temperature and culture was harvested.

3.7.1.2 Harvesting of the sporulated culture from petriplate

10 ml of tap water was poured to the sporulated agar plate. The culture was gently swirled and poured into a 25 ml beaker.10ml of this concentrate suspension is then made up to 100 ml using tap water. This is used for further assay.

3.7.2.1 Preparation of glass plates for surface culture studies

Glass chamber of dimension 16 x 13 x 25 cm containing 70 percent of molten agar medium were taken and glass plates were dipped in it for about 1 minute.

The plates were then taken out, so that a uniform thin layer of solidified agar is made on the glass plate.

Another glass chamber of the same dimension which contained 70 percent of *Bacillus thuringensissubsp. isralensis* pure culture was taken and the already prepared agar glass plate was dipped in it for 1 minute. So that the surface of the agar was evenly coated with a thin film of inoculum.

This plate was placed in glass chamber and incubated at room temperature till sporulation.

3.7.2.2 Harvesting of the sporulated culture from glass plate

The glass plate was washed with 40 ml of tap water to a glass trough. 10 ml of this concentrate suspension is then made up to 100 ml using tap water. This is used for further assay.

3.8 Estimation of the cell density

The cell density measurements were done using spectrophotometer using the 600-900 nm filter for the readings. The absorbances were noted at lamda**max**= 600nm. The zero correction in each case was achieved using the initial fermentation broth without the inoculum.1: 10 dilution of the fermented broth was taken for each of the cases to note the absorbance value. The absorbance thus observerd were multiplied by the dilution factor to arrive at the appropriate cell density values.

3.9 Harvesting of the cells from the fermented broths and determination of the wet and dry weights of *Bti* cells

A round-bottom centrifuge tube of 50ml capacity was taken in each case, washed with extran, rinsed with distilled water and autoclavd. The weight of the empty tube was taken using a digital weighing balance.40ml of the fermented broth was then transferred to the tube aseptically in the laminar airflow chamber. It was then centrifuged. Centrifugation was carried at 5000 rpm (1900g) at a temperature of 15ºC for 30 minutes. After centrifugation, the supernatant was discarded and the weight of the centrifuge tube was again taken using wighing balance. The difference between the final and the initial weights gave the wet weight. The same was used for calculating the wet weight corresponding to 100ml of the fermented broth. The dry weight for 100 ml of the fermented broth was calculated by multiplying the weight for the same volume by a factor of 0.4.

3.10 Biossay

3.10.1 Collecting and rearing the larvae

A mixed breed of larvae was collected from wastewater. First and second stage of larvae of the tiger mosquito, *Aedes aegypti*, was manually isolated from the collected mixed breed of larval population.

3.10.2 Conducting the bioassay

3.10.2 conducting bioassay Bioassay was done according to WHO guidelines.Although both lethal concentration 50(LC_{50}) and time dependant assay are done to assess the potency of Bti formulations,considering the number of samples and infrastructure limitations,time dependant assay was done for all the samples. A few hours before setting up of individual experiments 3-4 mm long mosquito larvae were removed using 10.ml glass pipettes with the back side/ blunted orifice to avoid injury to the larvae.

Fig.20 Mosquito larvae collected for bioassay-

They were collected in large numbers to facilitate quick transfer of the larvae into test beakers without any substantial transfer time difference between the first and last beakers. The wet Bti biomass obtained after centrifugation (or from solid agar plates)was suspended in 100.ml tap water and mixed well .Ten larvae were taken in 100 ml beaker containing 90 ml tap water and to it 10 ml of fermented broth/culture was added and mixed well and time was noted as $t=t_0$ In addition to test beakers a similar set up was prepared ,but without any culture added to it(Ten larvae suspended in 100 ml water).This was used as the control.The mortality of each of the larvae was noted against the respective time points from the start up time point(t_0) ,so as to get ten different different time points for 10 larvae.The last time point

corresponding to the death of 10 th larvae is taken for comparison with other formulations.similar time points generated with respect to other formulations/ fermentation broths and compared for assessment of toxicity and shown in the respective tables as Bioassay killing time in minutes.

3.11 Down stream processing of *Bti* .

3.11.1 Study of settling of delta endotoxin of *Bacillus thuringiensis var israelensis* at different alum concentrations

Fig.21 Alum (commercial grade) which is cheap and used as a flocculant. Flocculation of *Bti* cells will substantially reduce filtration/centrifuagation time and costs.

In this context we have used alum to enhance settling of *Bti*cells, cell debris, delta endotoxin etc in the fermentation residues. Settling under gravity is the most ecnomical method of settling the cells and other fermentation residues. The main draw back of this technique is that it takes lot of time to give a clear supernatent, which cant be spent considering the value of time in industry. So we have used primarily alum, which was mixed with a fixed volume of fermentaion broth and the the settling time was compared with control, without alum.

800ml of the sporulated *Bti* culture which was taken in 8 different 100. ml beakers. To the respective beakers various concentrations of alum

(commercial grade) 0.05, 0.1, 0.15, 0.20, 0.25, 0.5, 1 and 2% were added, mixed well and poured the contents into the respective 100. ml measuring cylinders. The time taken for settling was observed and noted in each case.

3.11.2.1 Effect of alum on filtration of the fermented broth of *Bacillus thuringiensis var israelensis.*

In Biotech industries One of the main down stream processing technique used to separate cells and other solid debris is filtration. The process is restricted by low voidage, since the particles present in fermentation broth are ususally too small (bacteria) . By addition of alum and resulting flocculation, floccules which are bigger in diameter are formed which effectively increases voidage, thus improving flux through the filter.

200 ml. of sporulated *Bacillus thuringiensis var. israelensis* culture was prepared and transferred to two glass beakers 100ml each. (500. ml) . In one glass beaker, 0.5 gm alum was added and mixed well. Both the cultures were allowed to settle down at the bottom of the beaker.10. ml of each of the sample was filtered using a glass funnel with whatman Number.1 filter paper as filtering medium, into a conical flask. The time taken by each sample for complete filteration was noted. The surface area of filter paper used was 63.585 cm. sq.

3.11.2.2 Optimisation of alum concentration for improved filtration rate using conventional filter.

In order to know the optimum concentration of alum which will give improved flow rates through filter, the following experiment was conducted. 600ml of sporulated *Bacillus thuringiensis var. israelensis* culture was prepared and transferred to 6 glass beakers (500. ml) . In five glass beakers, 0.1, 0.3, 0.5, 0.7, 0.9 gm alum were added and mixed well. The other 6^{th} flask was kept as control withou adding any alum. Both the cultures were allowed

to settle down at the bottom of the beaker.10. ml of each of the sample was filtered using a glass funnel with Whatman Number.1 filter paper as filtering medium, into a conical flask. The time taken by each sample for complete filteration was noted.

3.11.3 Effect of Ferric chloride on filtration of the fermented broth of *Bacillus thuringiensis* var *israelensis*

200ml of sporulated *Bacillus thuringiensis var. israelensis culture* was prepared and transferred to two glass beakers 100ml each (500. ml) . In one glass beaker, 0.5 gm Ferric chloride was added and mixed well. Both the cultures were allowed to settle down at the bottom of the beaker.10. ml of each of the sample was filtered using a glass funnel with whatman Number.1 filter paper as filtering medium, into a conical flask. The time taken by each sample for complete filteration was noted.

Fig.22 Ferric chloride (commercial grade) which is cheap and readily available used as a flocculant . Flocculation of *Bti* cells will substantially reduce filtration/centrifuagation time and costs both of which are power intensive operations in the down stream operations in biotech industry.
Effect of Aluminium sulphate on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*

200ml of sporulated *Bacillus thuringiensis var. israelensis* culture was prepared and transfeered to two glass beakers, 100ml each (500. ml) . In one glass beaker, 0.5 gm Aluminium sulphate was added and mixed well. Both

the cultures were allowed to settle down at the bottom of the beaker.10. ml of each of the sample was filtered using a glass funnel with what man Number.1 filter paper as filtering medium, into a conical flask. The time taken by each sample for complete filteration was noted.

3.11.4 Effect of Moringa seeds on filtration of the fermented broth of *Bacillus thuringiensis* var *israelensis*.

200ml of sporulated *Bacillus thuringiensis var. israelensis* culture was prepared and transferred to two glass beakers , 100ml each (500. ml) . In one glass beaker, 0.5 gm powdered dy moringa seeds was added and mixed well. Both the cultures were allowed to settle down at the bottom of the beaker.10. ml of each of the sample was filtered using a glass funnel with whatman Number.1 filter paper as filtering medium, into a conical flask. The time taken by each sample for complete filteration was noted.

Fig.23 Moringha seeds- used as natuaral flocculant to agglomerate *Bti* cells in the fermentation broth making further down stream operations cost effective.

3.11.5 Effect of Gaur gum on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*

200ml of sporulated *Bacillus thuringiensis var. israelensis* culture was prepared and transfeered to two glass beakers , 100ml each (500. ml) . In one

glass beaker, 0.5 gm Gaur gum powder was added and mixed well. Both the cultures were allowed to settle down at the bottom of the beaker.10. ml of each of the sample was filtered using a glass funnel with whatman Number.1 filter paper as filtering medium, into a conical flask. The time taken by each sample for complete filteration was noted. The surface area of filter paper used was 63.585 cm. sq

3.12 Efficacy of *Bti* in Field conditions

3.12.1 Effect of temperature on the stability of delta endotoxin of *Bti*: In order to study the impact of temperature on *Bti* formulations, 99. ml tap water was taken in each of the glass beakers. To it 1.0 ml sporulated culture of *Bti var. Israelensis* was added. The contents were mixed well. The respective beakers were heated to 30, 40, 50, 60, 70 80 and 90 °C**.** Once the beakers attained the respective temperatures, they were allowed to cool to room temoreature. To each of these beakers 10 larvae were added and the time taken for the larvicidal effect of delta endotoxin was noted.

3.12.2 Effect of temperature on the stability of delta endotoxin of *Bti-w*ith one hour holding-100. ml of tap water was taken in each of the glass beakers. To it, 1ml sporulated culture of *Bacillus thuringiensis varety israelensis* was added. The contents were mixed well. The respective beakers were heated to 40 °C and 50°C. The respective temperatures were maintained for one hour, cooled to room temperature and added 10 larvae in each beaker. The time taken for larvicidal effect by the delta endotoxin was noted.

3.12.3 Effect of Sunlight on the stability of delta endotoxin of *Bacillus thuringiensis variety israelensis*

50. ml of sporulated culture of *Bti* was taken in a 500ml beaker and it was exposed to direct sunlight. Aafter every 24 hours the total volume was made upto the appropriate level using tap water in order to compensate for the

evapouration loss.1ml. of the sunlight exposed sporulated culture was removed every day and bioassay was performed. For this 99. ml tap water was taken in a glass beaker.1. ml sunlight exposed culture was added, mixed well. To it 10 larvae were added and time taken for the larvicidal effect was noted. The experiment was done for a period of 30 days.

3.12.4.1 Effect of UV on the stability of delta endptoxin of *Bti*

1.0 ml of the sporulated culture of *Bti* was taken in four different glass petriplates. They were placed with their lids open and allowed to be exposed to UV light for a period of 30 minutes, 60 minutes, 90 minutes and 120 minutes repectively.90. ml of tap water was taken in four different glass beakers.10ml of the UV exposed sporulated culture was added to the respective beakers. Mixed well, 10 larvae were added to each beaker and time taken for the larvicidal effect of delta endotoxin was noted.

3.12.4.2 Effect of Aloe vera as a UV protectant in *Bti* formulation

100 mg. of Aloe vera gel dry powder was mixed with1. 0 ml of the sporulated culture of *Bti* and kept four 24 hours. Then it was taken in four different glass petriplates 20 ml each. They were placed with thier lids open and allowed to be exposed to UV light of wave length 253.7nm for a period of 30 minutes, 60 minutes, 90 minutes and 120 minutes repectively.99. ml of tap water was taken in four different glass beakers.1. 0ml of the UV exposed sporulated culture was added to the respective beakers. mixed well 10 larvae were added to each beaker and time taken for the larvicidal effect of delta endotoxin was noted.

Fig.24 Aloe vera- used as a natuaral UV protectant in *Bti* formulation.

3.12.5 Effect of pH on the stability of delta endptoxin of *Bti*

90. ml of tap water was taken in each of the eight glass beakers. To it 10 ml of the sporulated culture of *Bti* was added. Mixed well. The pH of the beakers were adjusted using a pH meter to pH 3, 4, 5, 6, 7, 8, 9 and 10 respectively. After 12 hours, bioassay was done.10 larvae were added to each of the beakers and time taken for larvicidal effect of the delta endotoxin was noted.

3.12.6 Effect of various protein preservatives on the action of delta endotoxin of *Bti*.

200. ml of sporulated culture of *Bti* was prepared using the media. The culture was distributed into four beakers of 50ml each. To the respective beakers, 0.5 gm ammonium sulphate, 0.5 gm ammonium acetate, 0.5 gm sodium metabisulphate were added and mixed well. The beakers were maintained for 30 days. Every 5^{th} day, 1ml of the sporulated culture was taken and bioassay was performed. For this 90. ml of tap water was taken in four glass beakers and to it 10ml sporulated culture of *Bti* was added from the respective four test beakers and mixed well to it 10 mosquitoe larvae were added and time taken for the larvicidal effect by delta endotoxin was noted.

CHAPTER 4

RESULTS

4.1 Production of *Bti* based biopesticide : Media formulations

Batch fermentations of *Bti* using Coconut water as the growth media

Table 4.1.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in submerged fementaion (SmF) and Solid state fermentation (SSF) using coconut water as the main raw material.

Table.4.1.1: Batch fermentations of *Bti* using Coconut water as the growth media

Submerged culture				**Solid state fermentation with 2% agar**		
Media composition	**Wet weight (in gm) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: : ± 0.25 hr)**	**Bioassay-killing time SmF -in min- 1/10 dilution (SD: : ± 15 min)**	**Wet weight (in gm) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in SSF culture (in hr) (SD: : ± 0.25 hr)**	**Bioassay-killing time SSF in min- 1/10 dilution. (SD: : ± 15 min)**
Coconut water alone (CW)	0.91	51	174	1. 0465	47	59
CW + 0.1%peptone	0.98	48	168	1.2558	42	148
CW +0.1% peptone+0.1%yeast extract	1.21	45	147	1.31859	38	130
CW +0.1% peptone+0.1%yeast extract+0.1%Calcium chloride	1.54	40	137	1.59	26	118

Solid state fermentations of *Bti* usingCoconut water as the growth media

Table 4.1.2 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown inSSF using coconut water as the main raw material and bengal gram as nitrogen supplement instead of costly peptone.

Table 4.1.2: Solid state fermentations of *Bti* usingCoconut water as the growth media

Media composition	**Wet weight (in gm) per 100 ml of media (SD: : ± 10%)**	**Sporulation time in SSF culture- in min. (SD: : ± 15 min)**	**Bioassay-killing time SSF (in min) 1/10 dilution (SD: : ± 15 min)**
Coconut water 100 ml (CW) + Bengal gram powder 0.5 % + yeast extract 0.1%+ 2% agar	1.297	47	150
CW + 0.5%peptone 2%+ Agar	1.39	40	131
CW+0.5%peptone+0.1%yeast extract +2% agar	1.43	35	125

Table 4.2.1: Batch fermentations of *Bti* using Rice straw as the growth media

Table 4.2.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using rice straw as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in gm) per 100 ml of broth (SD: : ± 10%)**	**Sporulation time-in hr. (SD: : ± 0.25 hr)**	**Bioassay-killing time SSF -in min (SD: : ± 15 min)**
Rice straw powder (RS) 1%+2% agar	0. 03	67	480
1% RS+ 0.1% pep+2% agar	0. 0315	55	378
1% RS+ 0.1% pep + 0.1% Y. E+2% agar	0. 0311	47	320

Table.4.2.2 Batch fermentations of *Bti* using delignified rice straw (NaOH) as the growth media

Table 4. .2.2 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using sodium hydroxide) rice straw as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in gm) per 100 ml of broth (SD: : ± 10%)**	**Sporulation time-in hr. (SD: : ± 0.25 hr)**	**Bioassay- killing time SSF -in min (SD: : ± 15 min)**
DegnifiedRice straw (DRS) powder 1%+2% agar	0.19	55	360
1% DRS+ 0.1% pep+2% agar	0.21	43	290
1% DRS+ 0.1% pep + 0.1% Y. E+2% agar	0.225	38	230

Table.4.2.3: Batch fermentations of *Bti*usingdelignified Rice straw (CaOH) as the growth media

Table 4. .2.3 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using calcium hydroxide) rice straw as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in gm) per 100 ml of broth (SD: : ± 10%)**	**Sporulation time -in hr (SD: : ± 0.25 hr)**	**Bioassay-killing time SSF-in min. (SD: : ± 15 min)**
Delignified Rice straw powder 1%+2% agar	0.179	56	370
1% DRS+ 0.1% pep+2% agar	0.19	45	321
1% DRS+ 0.1% pep + 0.1% Y. E+2% agar	0.223	36	245

Table.4.2.4: Batch fermentations of *Bti* using delignified rice straw (*Trichoderma reesei)* as the growth media

Table 4.2.4 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (with *Trichoderma reesei*) rice straw as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in gm) per 100 ml of broth (SD: ±10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
Rice straw powder 1%+2% agar	0.122	48	350
1% DRS+ 0.1% pep+2% agar	0.131	39	311
1% DRS+ 0.1% pep + 0.1% Y. E+2% agar	0.139	34	280

Table.4.2.5: Batch fermentations of *Bti* using delignified rice straw *(Pleurotus florida)* as the growth media

Table 4. .2.4 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (with *Pleurotus florida*) rice straw as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in gm) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
Rice straw powder 1%+2% agar	0.119	40	375
1% DRS+ 0.1% pep+2% agar	0.123	37	311
1% DRS+ 0.1% pep + 0.1% Y. E+2% agar	0.194	32	265

Table.4.2.6 : Batch fermentations of *Bti* using delignified rice straw (NaOH) supplemented with fish slurry

Table 4. .2.6 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using sodium hydroxide) rice straw as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time -in hr) (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
Delignified Rice straw powder 1% + 0.1ml fish slurry (FS) + 0.1gm yeast extract+2% agar	0.226	49	222
1% DRS+ 0.2ml FS +0.1gm YE+2% agar	0.267	44	210
1% DRS+ 0.3ml FS +0.1gm YE+2% agar	0.285	42	200
1% DRS+ 0.4ml FS +0.1gm YE+2% agar	0.292	36 -38	213
1% DRS+ 0.5ml FS +0.1gm YE+2% agar	0.273	41	219

Table.4.2.7: Batch fermentations of *Bti* using delignified rice straw (CaOH) supplemented with fish slurry

Table 4. .2.7 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using calcium hydroxide) rice straw as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time-in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF -in min (SD: ± 15 min)**
Delignified Rice straw powder 1% + 0.1ml fish slurry+ 0.1gm yeast extract+2% agar	0.189	44	240
1% DRS+ 0.2ml FS +0.1gm YE+2% agar	0.194	40	235
1% DRS+ 0.3ml FS +0.1gm YE+2% agar	0.226	37	231
1% DRS+ 0.4ml FS +0.1gm YE+2% agar	0.224	39	236
1% DRS+ 0.5ml FS +0.1gm YE+2% agar	0.223	38	243

Table.4.2.8: Batch fermentations of *Bti* using delignified rice straw (*Trichoderma reesei*) supplemented with fish slurry

Table 4. .2.8 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Trichoderma reesei*) rice straw as the main

raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSFin min (SD: ± 15 min)**
Delignified Rice straw powder 1% + 0.1ml fish slurry+ 0.1gm yeast extract+2% agar	0.125	40	285
1% DRS+ 0.2ml FS +0.1gm YE+2% agar	0.133	37	273
1% DRS+ 0.3ml FS +0.1gm YE+2% agar	0.143	38	267
1% DRS+ 0.4ml FS +0.1gm YE+2% agar	0.148	36	260
1% DRS+ 0.5ml FS +0.1gm YE+2% agar	0.153	34	264

Table.4.2.9: Batch fermentations of *Bti* using delignified rice straw *Pleurotus florida* fungi supplemented with fish slurry

Table 4. .2.9 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Pleurotus florida*) rice straw as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
Delignified Rice straw powder 1% + 0.1ml fish slurry+ 0.1gm yeast extract+2% agar	0.122	42	295
1% DRS+ 0.2ml FS +0.1gm YE+2% agar	0.138	38	283
1% DRS+ 0.3ml FS +0.1gm YE+2% agar	0.2143	35	271
1% DRS+ 0.4ml FS +0.1gm YE+2% agar	0.217	32	260
1% DRS+ 0.5ml FS +0.1gm YE+2% agar	0.216	32	269

Table.4.2.10: Batch fermentations of *Bti* using delignified rice straw (*Pleurotus* fungi) and pretreatment with cellulase enzyme from *Aspergillus niger.*

Table 4.2.10 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Pleurotus florida*) rice straw as the main raw material (carbon source) and further hydrolysed using cellulase enzyme secreted by *Aspergillus niger* fungi. To the resulting hydrolysate peptone and yeast extract were added as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSFin min (SD: ± 15 min)**
Delignified Rice straw powder 1%+2% agar	0.113	38	290
1% DRS+ 0.1% peptone+2% agar	0.139	32	267
1% DRS + 0.1% pep+0.1%YE+2% agar	0.143	38	232 -248

Chicken feather hydrolysate as protein supplement

Figure 25: Poultry feathers after Methanol: Chloroform treatment

Table.4.2.11: Batch fermentations of *Bti* using delignified rice straw (NaOH) supplemented with chicken feather hydrolysate

Table 4. .2.11 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using sodium hydroxide) rice straw as the main raw material (carbon source) and chicken feather hydrolysate yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
1. gm DRS+ 0.1ml CFH + 0.1gm yeast extract+2% agar	0.179	47	232
1. gm DRS+ 0.2ml CFH + 0.1gm yeast extract+2% agar	0.189	44	220
1. gm DRS+ 0.3ml CFH + 0.1gm yeast extract+2% agar	0.193	39	214
1. gm DRS+ 0.4ml CFH + 0.1gm yeast extract+2% agar	0.187	47	221
1. gm DRS+ 0.5ml CFH + 0.1gm yeast extract+2% agar	0.177	46	217

Table.4.2.12: Batch fermentations of *Bti* using delignified rice straw (CaOH) supplemented with chicken feather hydrolysate

4.2.12 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using calcium hydroxide) rice straw as the main raw material (carbon source) and chicken feather hydrolysate and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm DRS+ 0.1ml CFH + 0.1gm yeast extract+2% agar	0.176	43	247
1. gm DRS+ 0.2ml CFH + 0.1gm yeast extract+2% agar	0.183	39	238
1. gm DRS+ 0.3ml CFH + 0.1gm yeast extract+2% agar	0.213	39	234
1. gm DRS+ 0.4ml CFH + 0.1gm yeast extract+2% agar	0.211	37	239
1. gm DRS+ 0.5ml CFH + 0.1gm yeast extract+2% agar	0.191	39	243

Table.4.2.13: Batch fermentations of *Bti* using delignified rice straw (*Trichoderma reesei*) supplemented with chicken feather hydrolysate

Table 4. .2.13 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Trichoderma reesei*) rice straw as the main raw material (carbon source) and chicken feather hydrolysate and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm DRS+ 0.1ml CFH + 0.1gm yeast extract+2% agar	0.128	49	315
1. gm DRS+ 0.2ml CFH + 0.1gm yeast extract+2% agar	0.134	45	302
1. gm DRS+ 0.3ml CFH + 0.1gm yeast extract+2% agar	0.147	41	298
1. gm DRS+ 0.4ml CFH + 0.1gm yeast extract+2% agar	0.115	38	285
1. gm DRS+ 0.5ml CFH + 0.1gm yeast extract+2% agar	0.151	37	294

Table.4.2.14: Batch fermentations of *Bti* using delignified rice straw (*Pleurotus florida*) supplemented with chicken feather hydrolysate

Table 4. .2.14 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Pleurotus florida*) rice straw as the main raw material (carbon source) and chicken feather hydrolysate and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm DRS+ 0.1ml CFH + 0.1gm yeast extract+2% agar	0.112	45	335
1. gm DRS+ 0.2ml CFH + 0.1gm yeast extract+2% agar	0.118	43	328
1. gm DRS+ 0.3ml CFH + 0.1gm yeast extract+2% agar	0.210	33	219
1. gm DRS+ 0.4ml CFH + 0.1gm yeast extract+2% agar	0.214	37	302
1. gm DRS+ 0.5ml CFH + 0.1gm yeast extract+2% agar	0.119	39	295

Table 4.3.1: Batch fermentations of *Bti* using Bagasse as the growth media

Table 4. .3.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using bagasse as the main raw material (carbon source) and peptone

and yeast extract as medium supplements

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
Bagasse (BG) 1%+2% agar	0. 027	74	520
1% BG+ 0.1% pep+2% agar	0. 03	63	432
1% BG+ 0.1% pep - 0.1% Y. E+2% agar	0. 031	59	350

Table 4.3.2 : Batch fermentations of *Bti* using delignified bagasse (NaOH) as the growth media

Table 4. .3.2 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified bagasse (using NaOH) as the main raw material (carbon source) and peptone and yeast extract as medium supplements

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
DelignifiedBagasse powder 1%+2% agar	0.183	44	335
1% DBG + 0.1% pep+2% agar	0.191	39	315
1% DBG+ 0.1% pep + 0.1% Y. E+2% agar	0.217	35	222

Table.4.3.3: Batch fermentations of *Bti* using delignified bagasse (CaOH) as the growth media

Table 4. .3.3 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified bagasse (using CaOH) as the main raw material (carbon source) and peptone and yeast extract as medium supplements

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
Bagasse powder 1%+2% agar	0.167	47	315
1% DBG + 0.1% pep+2% agar	0.175	44	237
1% DBG+ 0.1% pep + 0.1% Y. E+2% agar	0.1827	34	220

Table.4.3.4: Batch fermentations of *Bti* using delignified bagasse (NaOH) supplemented with fish slurry

Table 4. .3.4 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using Sodium hydroxide) bagasse as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ±15 min or 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm Delignified BG+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.189	48	245
1. gm Delignified BG+ 0.2ml fish slurry + 0.1gm yeast extract+2% agar	0.196	48	243
1. gm Delignified BG+ 0.3ml fish slurry + 0.1gm yeast extract+2% agar	0.2149	45	239
1. gm Delignified BG+ 0.4ml fish slurry + 0.1gm yeast extract+2% agar	0.203	42	238
1. gm Delignified BG+ 0.5ml fish slurry + 0.1gm yeast extract+2% agar	0.203	38	235

Table.4.3.5: Batch fermentations of *Bti* using delignified bagasse (CaOH) supplemented with fish slurry

Table 4.3.5 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using Calcium hydroxide) bagasse as the main raw

material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ±0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm Delignified BG+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.173	44	255
1. gm Delignified BG+ 0.2ml fish slurry + 0.1gm yeast extract+2% agar	0.177	41	251
1. gm Delignified BG+ 0.3ml fish slurry + 0.1gm yeast extract+2% agar	0.186	39	251
1. gm Delignified BG+ 0.4ml fish slurry + 0.1gm yeast extract+2% agar	0.183	40	238
1. gm Delignified BG+ 0.5ml fish slurry + 0.1gm yeast extract+2% agar	0.179	42	239

Table.4.3.6: Batch fermentations of *Bti* using delignified bagasse (*Trichoderma reesei*) supplemented with fish slurry

Table 4. .3.6 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Trichoderma reesei*) bagasse as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm Delignified BG+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.11	47	325
1. gm Delignified BG+ 0.2ml fish slurry + 0.1gm yeast extract+2% agar	0.15	45	319
1. gm Delignified BG+ 0.3ml fish slurry + 0.1gm yeast extract+2% agar	0.127	43	301
1. gm Delignified BG+ 0.4ml fish slurry + 0.1gm yeast extract+2% agar	0.132	39	299
1. gm Delignified BG+ 0.5ml fish slurry + 0.1gm yeast extract+2% agar	0.141	39	295

Table.4.3.7: Batch fermentations of *Bti* using delignified bagasse (*Pleurotus florida fungi*) supplemented with fish slurry

Table 4. .3.7 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Pleurotus florida* fungi) bagasse as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
1. gm Delignified BG+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.117	49	295
1. gm Delignified BG+ 0.2ml fish slurry + 0.1gm yeast extract+2% agar	0.124	45	291
1. gm Delignified BG+ 0.3ml fish slurry + 0.1gm yeast extract+2% agar	0.119	40	286
1. gm Delignified BG+ 0.4ml fish slurry + 0.1gm yeast extract+2% agar	0.204	35	285
1. gm Delignified BG+ 0.5ml fish slurry + 0.1gm yeast extract+2% agar	0.200	42	280

Table.4.3.8: Batch fermentations of *Bti* - Biological delignification of Bagasse with *Pleurotus florida* and pretreatment with cellulase enzyme from *Aspergillus niger*

Table 4.3.8 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using delignified (using *Pleurotus florida*) bagasse as the main raw material (carbon source) and further hydrolysed using cellulase enzyme secreted by *Aspergillus niger* fungi. To the resulting hydrolysate peptone and yeast extract were added as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
1. gm Delignified BG+ 0.1gm yeast extract+2% agar	0.189	49	255
1. gm Delignified BG + 0.1gm peptone+2% agar	0.192	45	251
With 1. gm Delignified BG+0.1gm peptone+0.1gm yeast extract+2% agar	0.113	43	249

Table.4.4.1: Batch fermentations of *Bti* using Taro as the growth media

Table 4. .4.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Taro as the main raw material (carbon source) and peptone and yeast extract and clacium chloride as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
Taro 1%+2% agar	0.22	54	67
1% Taro + 0.1% pep+2% agar	0.2464	47	59
1% Taro + 0.1% pep + 0.1% Y. E+2% agar	0.2648	40	55
1% Taro + 0.1% pep + 0.1% Y. E + 0.1% $CaCl_2$+2% agar	0.31	23	52

Table.4.4.2: Batch fermentations of *Bti* using hydrolyzed Taro as the growth media

Table 4. .4.2 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using hydrolysed Taro (with HCl) as the main raw material (carbon source) and peptone and yeast extract and clacium chloride as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
Taro 1%+2% agar	0.29	38	77
1% Taro + 0.1% pep+2% agar	0.32	35	68
1% Taro + 0.1% pep + 0.1% Y. E+2% agar	0.357	34	61
1% Taro + 0.1% pep + 0.1% Y. E + 0.1% $CaCl_2$+2% agar	0.41	19	48

Table 4.4.3: Solid state fermentations of *Bti* using Taro powder as the growth media

Table 4. .4.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Taro as the main raw material (carbon source) and bengal gram, and yeast extract as medium supplements. Instead of water coconut water was used .

Media composition	**Wet weight (in grams) per 100 ml of media (SD: ± 10%)**	**Sporulation time in SSF culture in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF (1/10 dilution) in min (SD: ± 15 min)**
Taro power 1%+ Bengal gram powder 0.5 % + 0.1% yeast extract : 0.25%+100ml CW++2% agar	1.7	34	49
CW+0.5%pep+2% agar	1.40	42	135
CW+0.5%pep+0.1%YE+2% agar	1.54	38	125

Table.4.5.1: Batch fermentations of *Bti* using Sweet potato as the growth media

Table 4. .5.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Sweet poato as the main raw material (carbon source) and peptone and yeast extract and clacium chloride as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min)**
Sweet potato (SP) 1%+2% agar	0.272	57	65
1% SP + 0.1% pep+2% agar	0.310	47	59
1% SP + 0.1% pep - 0.1% Y. E+2% agar	0.36	44	54
1% SP + 0.1% pep + 0.1% Y. E + 0.1% $CaCl_2$+2% agar	0.39	26	46

Table 4.5.2: Solid state fermentations of *Bti* using Sweet potato powder and bengal gram powder as the growth media

Table 4. .5.2 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Sweet poato as the main raw material (carbon source) and bengal gram , yeast extract as medium supplements

Media composition	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Sporulation time in SSF culture in hr (SD: ± 0.25 hr)	Bioassay-killing time SSF in min (SD: ± 15 min) 1/10 dilution
Sweet potato powder -1% (CW) + Bengal gram powder 0.5 % + 0.1% yeast extract : 0.25%+2% agar	1.57	47	44
CW+0.5%pep+2% agar	1.39	39	123
CW+0.5%pep+0.1%Y. E+2% agar	1.44	32	121

Table 4.6.1: Batch fermentations of *Bti* using Purple yam powder as the growth media

Table 4.6.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using purple yam as the main raw material (carbon source) and peptone and yeast extract and clacium chloride as medium supplements

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr) 1/10 dilution**	**Bioassay-killing time SSF in min (SD: ± 15 min)**
Purple yam powder (PY) 1%+2% agar	0.258	58	69
1% PY + 0.1% pep+2% agar	0.297	53	65
1% PY + 0.1% pep+-0.1% Y. E+2% agar	0.322	49	61
1% PY + 0.1% pep+-0.1% Y. E + 0.1% $CaCl_2$ +2% agar	0.35	29	49

Table 4.6.2 Batch fermentations of *Bti* Growing on Purple yam based medium with fish slurry supplementation

Table 4. .3.7 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using purple yam as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr) 1/10 dilution**	**Bioassay-killing time SSF in min (SD: ± 15 min) 1/10 dilution**
1. gm Purple Yam + 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.2 03	41	135
1. gm PY +0.2ml FS +0.1gm YE+2% agar	0.2 07	39	127
1. gm PY +0.3ml FS +0.1gm YE+2% agar	0.2 17	36	115
1. gm PY +0.4ml FS +0.1gm YE+2% agar	0.2 14	37	124
1. gm PY +0.5ml FS +0.1gm YE+2% agar	0.2 07	39	127

Table 4.6.3: Solid state fermentations of *Bti* using Purple yam powder as the growth media

Table 4.6.3 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Purple yam as the main raw material (carbon source) and bengal gram, and yeast extract and calcium chloride as medium supplements. Instead of water, coconut water was used.

Media composition	Wet weight (in grams) per 100 ml of media (SD: ± 10%)	Sporulation time in SSF culture in hr (SD: ± 0.25 hr)	Bioassay-killing time SSF in min (SD: ± 15 min) 1/10 dilution
Purple yam powder -1% + Bengal gram powder 0.5% + yeast extract : 0.25%+2% agar	1.39	47	55
CW + 0.5%peptone+2% agar	1.37	45	137
CW + 0.5%peptone+0.25%yeast extract+2% agar	1.5	35	120
CW + 0.5%peptone+0.25% yeast extract+Calcium chloride+2% agar	1.57	28	113

Table.4.7.1 Batch fermentations of *Bti* using Pineapple peel as the growth media

Table 4.7.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Pine apple peel as the main raw material (carbon source) and peptone and yeast extract as medium supplements

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ±10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min or) 1/10 dilution**
Pineapple Peel 1%+2% agar	1.87	67	415
1% PP + 0.1% pep+2% agar	2.15	59	390
1% PP + 0.1% pep + 0.1% Y. E+2% agar	2.21	46	330

Table.4.7.2: Batch fermentation of *Bti :* Growth on Pineapple peel based medium with fish slurry supplementation

Table 4.7.2 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Pine apple peel as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ±15 min) 1/10 dilution**
1. gm Pine apple peel waste+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	1.93	63	310
1. gm PP waste+0.2ml FS +0.1gm YE+2% agar	2.21	55	307
1. gm PP waste+0.3ml FS +0.1gm YE+2% agar	2.29	51	300
1. gm PP waste+0.4ml FS +0.1gm YE+2% agar	2.34	44	293
1. gm PP waste+0.5ml FS +0.1gm YE+2% agar	0.22	47	290

Table.4.7.3: Batch fermentations of *Bti* using Pineapple peel supplemented with chicken feather hydrolysate

Table 4.7.3 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Pine apple peel as the main raw material (carbon source) and chicken feather hydrolysate and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min) 1/10 dilution**
1. gm Pineapple peal + 0.1ml CFH + 0.1gm yeast extract+2% agar	1.78	59	340
1. gm PP + 0.2ml CFH + 0.1gm yeast extract+2% agar	2.11	51	331
1. gm PP + 0.3ml CFH + 0.1gm yeast extract+2% agar	2.20	49	327
1. gm PP + 0.4ml CFH + 0.1gm yeast extract+2% agar	2.27	41	321
1. gm PP + 0.5ml CFH + 0.1gm yeast extract+2% agar	2.16	40	315

Table.4.8.1: Batch fermentations of *Bti* using Tapioca peel as the growth media

Table 4.8.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Tapioca peel as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay- killing time SSF in min (SD: ± 15 min) 1/10 dilution**
Tapioca Peel 1%+2% agar	0.217	38	411
1% TP + 0.1% pep+2% agar	0.236	33	370
1% TP + 0.1% pep + 0.1% Y. E+2% agar	0.264	29	355

Table 4.8.2: Batch fermentation of *Bti* Growth on Tapioca peel based medium and with fish slurry supplementation

Table 4.8.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Tapioca peel as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSFin min (SD: ± 15 min) 1/10 dilution**
1. gm Tapioca peel waste+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.189	57	150
1. gm TP waste+0.2ml FS +0.1gm YE+2% agar	0.213	51	143
1. gm TP waste+0.3ml FS +0.1gm YE+2% agar	0.224	39	139
1. gm TP waste+0.4ml FS +0.1gm YE+2% agar	0.217	39	137
1. gm TP waste+0.5ml FS +0.1gm YE+2% agar	0.2 04	44	135

Table.4.9.1: Batch fermentations of *Bti* using Vegetable waste as the growth media

Table 4.9.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Vegetable as the main raw material (carbon source) and peptone and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 0.25 hr)**	**Bioassay-killing time SSF in min (SD: ± 15 min) 1/10 dilution**
Vegetable waste 1%+2% agar	0.273	39	415
1% Veg. + 0.1% pep+2% agar	0.286	28	392
1% Veg. + 0.1% pep + 0.1% Y. E+2% agar	0.361	32	380

Table.4.9.2: Batch fermentation of *Bti* using vegetable wastes based medium with fish slurry supplementation

Table 4.9.1 shows the data of *Bacillus thuringiensis subspecies israelensis* biomass yield, sporulation timings and bioassay data when grown in SSF using Tapioca peel as the main raw material (carbon source) and fish slurry and yeast extract as medium supplements.

Solid state fermentation			
Media composition	**Wet weight (in grams) per 100 ml of broth** (SD: ±10%)	**Sporulation time** in hr **(SD: ± 0.25 hr)**	**Bioassay- killing time SSF** in min **(SD: ± 15 min) 1/10 dilution**
1. gm Vegetable waste+ 0.1ml fish slurry + 0.1gm yeast extract+2% agar	0.193	57	135
1. gm VG waste+0.2ml FS +0.1gm YE+2% agar	0.225	45	127
1. gm VG waste+0.3ml FS +0.1gm YE+2% agar	0.229	40	115
1. gm VG waste+0.4ml FS +0.1gm YE+2% agar	0.227	34	111
1. gm VG waste+0.5ml FS +0.1gm YE+2% agar	0.221	36	123

Batch fermentations of *Bti*: Substrate repression studies

Table 4.10.1: Batch fermentations of *Bti* using glucose as the carbon source for substrate repression studies

Table 4.10.1 shows the batch (submerged) fermentation of *Bti* using glucose as the main carbon source ranging from 1-4% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Glucose) + 0.1% YE + 0.5% Peptone	**Absorbance at 600 nm** (SD: ± 0.5 OD)	**Wet weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Dry weight (in grams) per 100 ml of broth** (SD: ± 10%)	**Bioassay- killing time SmF** in min **(SD: □ 15 min) 1/10 dilution**
1	5.56	1. 02	0.408	265
2	6.32	1.92	0.768	163
3	9.60	3.64	1.456	104
4	8.71	2.72	1. 088	116

Table 4.10.2 Batch fermentation of *Bti* using sucrose as the carbon source for substraterepression studies

Table 4.10.2 shows the batch (submerged) fermentation of *Bti* using sucrose as the main carbon source ranging from 1-6% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Sucrose) + 0.1% YE + 0.5% Peptone	Absorbance at600nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay-killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	3.8	0.923	0.2714	332
2	4.20	0.976	0.2981	276
3	5. 000	1. 0940	0.43760	239
4	7. 000	1.7214	0.68856	188
5	7.564	2. 0300	0.81200	142
6	6.630	1.6200	0.64800	199

Table 4.10.3 Batch fermentation of *Bti* using Jaggery as the carbon source for substrate repression studies

Table 4.10.3 shows the batch (submerged) fermentation of *Bti* using Jaggery as the main carbon source ranging from 1-4% and peptone and yeast extract as the medium supplements. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Jaggery) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	5.6	2.9	0.87	123
2	7.8	3.8	1.1	115
3	10.600	4.895	2.3580	73
4	10.250	3.564	1.4156	109

Table 4.10.4 Batch (submerged) fermentation of *Bti* using cane sugar juice as the carbon source for substrate repression studies

Table 4.10.4 Shows the batch (submerged) fermentation of *Bti* using cane sugar juice powder as the main carbon source ranging from 1-4% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet

weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Cane Sugar Juice) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	4.1	2.3	0.575	29
2	5.7	3.9	0.976	37
3	8.30	4.25	1.700	44
4	4.19	2.44	0.976	115

Table.4.10.5 The production of *Bti* based biopesticide: Batch fermentation *Bti*using jaggery as the carbon source and soya bean powder as the protein supplement.

Table : 4.10.5 shows the biomas data using jagerry as carbon source and soyabean powder as nitorgen source. Since jaggery is mostly having carbohydrates like sucrose with less nitrogen, soybean powder which is a rich source of proteins was used as the supplement instead of costly peptone and yeast extract as additive. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage Of Carbon Jaggery 1% + 0.1% YE + 0.5% soya bean powder	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	5.6	5.1	2.55	105
2	7.97	3.9	1.71	167
3	10.89	4.98	1.32	163
4	10.65	4.87	1.27	165

Table.4.10.6 The production of *Bti* based biopesticide: Batch fermentation *Bti* using jaggery as the carbon source and without yeast extract supplement.

Table : 4.10.6 shows the biomas data using jagerry (1-4%) as the sole carbon source . Although yeast extract contains various vitamins and minerals it is costly and since jaggery already contains many vitamins and minerals, the growth aspects of *Bti* in jaggerry based medium was done, which will substantially reduce production cost. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon Jaggery 1%	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	4.8	2.12	0.53	127
2	6.7	3.21	0.817	93
3	9.1	3.75	0.98	79
4	10.0	4.3	1.098	80

Table 4.10.7 Batch fermentation of *Bti* using tapioca peel powder as the carbon source for substrate repression studies

Table 4.10.7 shows the batch (submerged) fermentation of *Bti* using tapioca peel powder as the main carbon source ranging from 1-6% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (tapioca peel powder) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay-killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	3.9	0.945	0.227	173
2	4.7	2.56	0.68	122
3	6.780	5.680	2.272	83
4	6.180	6.240	2.496	63
5	7.710	6.690	2.784	48
6	8.230	12.15	4.860	39

Table 4.10.8 Batch fermentation of *Bti* using Taro powder as the carbon source for substrate repression studies

Table 4.10.8 shows the batch (submerged) fermentation of *Bti* using taro powder as the main carbon source ranging from 1-6% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Taro powder) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	4. 0	2.11	0.54	167
2	4.9	2.79	0.61	122
3	7.4	6.39	2.3	80
4	7.8	6.58	2.5	87
5	8.8	7.3	2.9	93
6	8.3	6.9	2.7	89

Table 4.10.9 Batch fermentation of *Bti* using Sweet potato as the carbon source for substrate repression studies

Table 4.10.9 shows the batch (submerged) fermentation of *Bti* using sweet potato powder as the main carbon source ranging from 1-7% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Sweet potato) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	3.7	1.79	0.44	177
2	4.6	2.55	0.69	127
3	6.9	5.7	2.1	84
4	7.3	6.5	2.3	79
5	7.9	6.7	2.7	85
6	8.5	7.1	3. 0	91
7	7.9	6.6	2.6	87

Table 4.10.10 Batch fermentation of *Bti* using Purple yam as the carbon source for substrate repression studies

Table 4.10.10 shows the batch (submerged) fermentation of *Bti* using purple yam powder as the main carbon source ranging from 1-7% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Purple yam) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
1	3.5	4.3	1.35	1.3
2	4.3	4.6	1.49	98
3	6.3	5.1	1.9	91
4	6.8	5.8	2.3	83
5	7.5	6.7	2.6	81
6	8.1	6.9	2.8	77
7	7.7	6.6	2.5	79

Fed-Batch fermentations of *Bti*

Table.4.11.1 Fed-batch fermentation of *Bti* using glucose as the carbon source

Table 4.11.1 shows the Fed-batch (submerged) fermentation of *Bti* using glucose as the main carbon source ranging from 4-8% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Glucose) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
4	5. 010	1.4530	0.68120	125
5	8.913	3.8460	1.53840	125
6	11.29	8. 0600	3.22400	99
7	9. 050	5. 032	2.48128	146
8	6.900	4.5458	1.61832	67

Table 4.11.2 The production of *Bti* based biopesticide: Fed-batch fermentation *Bti* using glucose as the carbon source and soya bean powder as the nitrogen sourced biopesticdie

Percentage of Carbon (Glucose) + 0.1% YE + 0.5% Soyabean powder	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
4	1.55	5.2	0.75	135
5	3.9	8.87	1.54	140
6	8.5	11.4	3.8	103
7	9.56	9.8	0.56	157
8	0.75	6.7	0.75	77

Table.4.11.3 Fed-batch fermentation of *Bti* using sucrose as the carbon source

Table 4.11.3 shows the Fed-batch (submerged) fermentation of *Bti* using sucrose as the main carbon source ranging from 4-8% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bio-assay data are shown in this table.

Percentage of Carbon (Sucrose) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
4	2.500	0.533	0.2134	148
5	4.450	0.706	0.2824	147
6	7.540	0.806	0.3224	134
7	8.341	2. 026	0.8104	101
8	2.341	0.453	0.1812	200

Table.4.11.4 Fed-Batch fermentation of *Bti* using cane jaggery as the carbon source

Table 4.11.4 shows the Fed-batch (submerged) fermentation of *Bti* using Jagerry as the main carbon source ranging from 4-12% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Jaggery) + 0.1% YE + 0.5% Peptone	**Absorbance at 600 nm (SD: ± 0.5 OD)**	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Dry weight (in grams) per 100 ml of broth (SD: ± 10%**	**Bioassay-killing time SmF in min (SD: ± 15 min) 1/10 dilution**
4	8.5000	2.05	0.820	99
5	9. 0300	2.52	1. 008	86
6	9.3500	3.38	1.352	71
7	9.7000	3.64	1.456	63
8	10.700	4.51	1.804	47
9	15. 000	4.61	1.844	43
10	15.323	4.92	1.968	38
11	16. 000	5.30	2.120	31
12	9.2000	2.91	1.164	83

Table.4.11.5 Fed-batch fermentation *Bti* using cane juice powder as the carbon source

Table 4.11.5 shows the Fed-batch (submerged) fermentation of *Bti* using sugar cane juice powder as the main carbon source ranging from 4-12% and peptone and yeast extract as the medium supplement. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Cane sugar juice) + 0.1% YE + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
4	8.250	2.61	1. 044	80
5	9. 025	2.83	1.132	78
6	10.90	3.35	1.340	64
7	11.97	3.65	1.460	61
8	14. 02	5.27	2.108	46
9	15.19	5.60	2.240	39
10	15.98	6.31	2.524	32
11	16.67	6.99	2.796	29
12	9.36	3.16	1.264	71

4.11.6 Fed Batch fementation of *Bti* using cane sugar juice as the carbon source and without yeast extract supplement

Table.4.11.6 Fed Batch fermentation of *Bti* using cane sugar juice as the carbon source and without yeast extract supplement. yeast extract although being rich in nutrients is a very costly substrate /additive. so the growth aspects of *Bti* without supplmentation was studied. Absorbance at 600nm, wet weight, dry weight and bioassay data are shown in this table.

Percentage of Carbon (Cane sugar juice) + 0.5% Peptone	Absorbance at 600 nm (SD: ± 0.5 OD)	Wet weight (in grams) per 100 ml of broth (SD: ± 10%)	Dry weight (in grams) per 100 ml of broth (SD: ± 10%)	Bioassay- killing time SmF in min (SD: ± 15 min) 1/10 dilution
4	7.6	2.23	0.87	103
5	8.45	2.45	0.95	98
6	8.9	2.89	1.11	87
7	10.3	3.1	1.27	83
8	12.87	4.76	1.73	75
9	13.22	4.95	1.84	67
10	13.55	5.32	1.89	57
11	14.2	5.73	2.21	54
12	7.89	2.16	0.97	47

Solid state fermentations of *Bti*

Preparation of glass plates for surface culture studies: (Glass chamber containing submerged glass plate of a total surface area of 297 cm^2.)

Glass chamber of dimension 16 x 13 x 25 cm (297 cm^2**)** containing 70 percent of molten agar medium were taken and glass plates were dipped in it for about 1 minute. The respective media were prepared using coconut water, Taro powder, purple yam powder, sweet potato powder, Jaggery, sugar cane juice powder were used at 1% concentration supplemented with bengal gram powder 0.5% and yeast extract 0.25% mixed with 2% agar.

In coconut water based medium, coconut water replaced tap water and same additives were used. The plates were then taken out, so that a uniform thin layer of solidified agar is made on the glass plate. Another glass chamber of the same dimension which contained 70 percent of *Bacillus thuringensis subsp. isralensis culture* was taken and the already prepared agar glass plate was dipped in it for 1 minute. So that the surface of the agar was evenly coated with a thin film of inoculum. This plate was placed in another glass chamber and incubated at room temperature till sporulation.

Table 4.12.1: Solid state fermentations of *Bti* using Jaggery powder as the growth media

Shows the solid state fermentation dta of *Bti* grown in SSF mode using glass plates with jaggerry powder as the main carbon source with bengal gram and yeast extract as medium supplements. Instead of water, coconut water was used. Wet weight, sporulation data and bioassay data are shown in this table.

Media composition	**Wet weight (in grams) per 100 ml of medium** (SD: ± 10%)	**Sporulation time** in hr **(SD: ± 15 min or 0.25 hr)**	Bioassay-killing time SmF in min **(SD: ± 15 min) 1/10 dilution**
Jaggery - 1gm (CW) + Bengal gram powder 0.5 gm + yeast extract : 0.25gm	1.56	37	35
CW+0.5g peptone	1.36	41	127
CW+0.5g pep++ 0.1g Y. E	1.43	37	124

Table 4.12.2: Solid state fermentations of *Bti* using Sugar cane juice powder as the growth media

Shows the solid state fermentation data of *Bti* grown in SSF mode using glass plates with sugar cane juice powder as the main carbon source with bengal gram and yeast extract as medium supplements. Instead of water, coconut water was used. Wet weight, sporulation data and bioassay data are shown in this table.

Media composition	**Wet weight (in grams) per 100 ml of broth (SD: ± 10%)**	**Sporulation time in hr (SD: ± 15 min or 0.25 hr)**	**Bioassay-killing time SmF in min (SD: ± 15 min) 1/10 dilution**
Sugar cane juice powder - 1gm (CW) + Bengal gram powder 0.5 gm + yeast extract : 0.25gm	1.84	35	33
CW+0.5g peptone	1.37	41	127
CW+0.5g pep++ 0.1g Y. E	1.46	33	122

4 .13 Down stream processing of *Bti*

Table 4.13.1 shows the show sthe settling time of of *Bacillus thuringiensis var israelensis* at different alum concentrations. In this context we have used alum to enhance settling of *Bti* cells, cell debris, delta endotoxin etc in the fermentation residues. Settling under gravity is the most ecnomical method of settling the cells and other fermentation residues. The main draw back of this technique is that it takes lot of time to give a clear supernatent, which can't be spent considering the value of time in industry. So we have used primarily alum with varying concentrations, which was mixed with a fixed volume of fermentaion broth and the the settling time was compared with control, without alum.

Table 4.13.1 Study of settling of delta endotoxin of *Bacillus thuringiensis subspecies isreaelensis at* at different alum concentrations.

% Alum used	**Time of settling -** in min. **(SD: ± 2 min)**
0.05	70
0.1	60
0.15	28
0.2	20
0.25	18
0.5	17
1	19
2	20

4.14.1 Effect of alum on filtration of the fermented broth of *Bacillus thuringiensis var israelensis.*

Table 4.14.1 shows the Effect of alum on filtration of the fermented broth of *Bacillus thuringiensis var israelensis.* In Biotech industries. One of the main down stream processing technique used to separate cells and other solid debris is filtration. The process is restricted by low voidage, since the particles present in fermentation broth are ususally too small (Bacteria) . By addition of

alum , which results in flocculation, floccules which are bigger in diameter are formed which effectively increasess voidage, thus improving flux through the filter. Table 4.14.1shows the filtration rates with and without alum.

Filtration done	**Time (in minutes) (SD ± 30 sec)**
With Alum	4
Without Alum	7

Table 4.14.2 Optimisation of alum concentration for improved filtration rate using conventional filter.

In order to know the optimum concentration of alum which will give improved flow rates through filter, the following experiment was conducted.

% of Alum used (%)	**Filtration time in minutes (SD ± 30 sec)**
0	7
0.1	5
0.3	4 .5
0.5	4
0.7	3 .5
0.9	3.5

Table 4.14.3 Effect of Ferric chloride on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*. By addition of ferric chloride , which results in flocculation, floccules which are bigger in diameter are formed which effectively increasess voidage, thus improving flux through the filter. Table 4.14.3shows the filtration rates with and without ferric chloride.

Filtration done	**Time (in minutes) (SD ± 30 sec)**
With Ferric chloride	5.5
Without Ferric chloride	7

Table 4.14.4 Effect of Aluminium sulphate on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*.

By addition of aluminium sulphate which a industrial flocculant , results in flocculation, floccules which are bigger in diameter than individual bacteria are formed which effectively increases voidage, thus improving flow rate through the filter. Table 4.14.4shows the filtration rates with and without aluminium sulphate.

Filtration done	**Time (in minutes) (SD ± 30 sec)**
With Aluminium sulphate	4 minutes 10 seconds
Without Aluminium sulphate	7

Table 4.14.5 Effect of Moringa seeds on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*. By addition of moringha seeds which is a natuaral flocculant , which results in flocculation, floccules which are bigger in diameter are formed which effectively increases voidage, thus improving flow rate through the filter. Table 4.14.5shows the filtration rates with and without moringha seed powder.

Filtration done	**Time (in minutes) (SD ± 30 sec)**
With Moringa seed powder	5
Without Moringa seed powder	7

Table 4.14.6 Effect of Gaur gum on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*. By addition of gaur gum powder which is also a natuaral flocculant , results in flocculation, floccules which are bigger in diameter than individual bacteria are formed which effectively increases voidage, thus improving flow rate through the filter. Table 4.14.6

4.14.6 shows the filtration rates with and without gaur gum.

Filtration done	Time (in minutes) (SD ± 30 sec)
With Gaur gum	6 minutes 10 seconds
Without Gaur gum	9

4.15 Stability of *Bti* under Field conditions

Table 4.15.1.1 Effect of temperature on the stability of delta endotoxin of *Bti*

Bacillus thuringiensis formulations are usually sprayed in stagnant water bodies, where mosquitoes breed. So they are amenable to direct exposure to sunlight and heat. The mosquito larvae may not ingest the delta endotoxins in the first day. So the stability of these toxins over a period of time assumes importance from a commercial point of view. The stability of these biopesticide preprations were studied under different temperatures and plotted in Table.4.15.1

Temperature (←C)	Bioassay- killing time in min **(SD: ± 15 min) 1/10 dilution**
30	60
40	65
50	73
60	85
70	93
80	111
90	123

Table 4.15.1.2 Heat stabilty of *Bti* with one hour holding

Shows the time to kill 10 larvae after holding for 40 and 50 degrees. Water retains heat compared to land, and cooling is a slow process.

Temperature (°C)	Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution
40	79
50	85

Table 4.15.2.1 Effect of sunlight on the stability of delta endotoxin of *Bacillus thuringiensis variety israelensis.* The stability of *Bti* over a period of 30 days was studied, after exposing to sunlight .

No. of days of exposure	Bioassay- killing time in min **(SD: ± 15 min or) 1/10 dilution**
0	95
1	97
2	102
3	109
4	114
5	118
6	124
7	128
8	136
9	145
10	151
11	156
12	164
13	169
14	177
15	182
16	188
17	195
18	201
19	217
20	215
21	219
22	225
23	233
24	239
25	244
26	248
27	254
28	258
29	263
30	267

Table 4.15.2.2 Effect of UV light on the stability of delta endotoxin

***Bacillus thuringiensis* subspecies israelensis**

Sun light contains UV -A and B , which are detrimental to the stabilty of insecticidal proteins. So UV exposure was done for a period upto 120 minutes.

Time of exposure to UV (in minutes)	**Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution**
Control	67
30	75
60	82
90	93
120	105

Table 4.15.2.3 Effect of Aloe vera as a UV protectant in *Bti* formulation

Time of exposure to UV (in minutes)	**Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution**
Control	67
30	71
60	79
90	86
120	94

Table 4.15.3 Effect of pH on the stability of delta endptoxin of *Bti*

Table.4.17.1 shows the the effect of varying pH on the stability of *Bti* delta endotoxin. The sewage water ususaly fluctuates in pH and being proteins its functionality is affected. The stabilty under a range of pH was studied and biolarvicidal time noted.

pH of the culture	Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution
3	71
4	68
5	67
6	65
7	63
8	69
9	84
10	91

Table 4.15.4 Effect of various protein preservatives on the action of delta endotoxin of *Bti*. The active ingredient being protein in *Bti* formulations , preservation of its functionality over a period of time in contaminated field sites is of paramount importance for the effctive control of mosquito larvae. Protein preservatives which are cheap and readily available are used and its preservative action was noted from 0 to 30 days.

Protein preservatives used	No. of days of exposure	Control Without Ammonium Acetate	Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution
		Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution	
Ammonium Acetate	0	79	65
	5	81	69
	10	93	75
	15	90	78
	20	98	83
	25	101	87
	30	103	91

Protein preservatives used	No. of days of exposure	Control Without Ammonium Sulphate	Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution
		Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution	
Ammonium Sulphate	0	77	68
	5	84	75
	10	90	81
	15	95	89
	20	902	94
	25	102	91
	30	110	107

Protein preservatives used	No. of days of exposure	Control Without Sodium meta bisulphate	Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution
		Bioassay- killing time in min (SD: ± 15 min) 1/10 dilution	
Sodium meta bisulphate	0	78	66
	5	83	68
	10	85	71
	15	82	75
	20	89	77
	25	87	80
	30	93	84

CHAPTER 5
DISCUSSION

Bacillus thuringiensis suscpecies israelenis (Bti) is the only microbe which has been successfully exploited commercially for the management of insect pests specifically mosquitoes. *Bt* accounts for 95% of the world market for microbial pest control agents due to its twin advantages of safety to natuaral enemies, honey bees , etc and its rapid and specific action against target insect pests.

Presently *Bti* based products are the most popular biopesticide against mosquitoes, available in the market, still its use is mainly restricted to mostly developed countries due to its high cost of production. In order to make it affordable for use in developing countries especially tropical and equitorial countries , where the climate is conducive for mosquito breeding, its production cost has to be further reduced. The work is aimed at developing a cost effective processs for the improved yield of *Bacillus thuringiensis subspecies israelensis* endotoxin based biopesticide.

5.1 Batch fermentations of *Bti* using Coconut water as the growth media

Table.4.1.1 reveals submerged fermentation and solid state fermentation based data of of *Bti* using Coconut water as the growth media. It was shown that under submerged fermentaions the biomass output was generally lower than solid state fermentation mode. This may be due to repressive nature of carbon/ sugars present in coconut water which is around 5-5.5%. Supplemenation of peptone and yeast extract have shown it has got a positive effect on improving biomass production in both submerged and SSF, since natural coconut water is having very less protein and other essential aminoacids. In addition, supplementation of calcium chloride in both modes

has found to be beneficial in increasing biomass output, reducing sporulation time and correspondingly larvicidal assay killing time. This proves that supplementary calcium in the medium augments better synthesis of calcium dipicolinic acid, which is an integral part of bacterial endospore. Better sporulation also encourages delta endotoxin syntheisis, which drastically reduces the time required to kill mosquitoe larvae, indicating high potency of the formulation.

100 grams of coconut water contains 19Calories, Fat 0.2 g, Saturated fat 0.2g, Sodium 105 mg, Potassium 250 mg, Total Carbohydrate 3.7 g, Dietary fiber 1.1 g, Sugar 2.5 g, Protein 0.7 g, Vitamin C 4mg, Calcium 2mg, Iron 1mg, Magnesium 6 mg.

5.1.1 Solid state fermentations of *Bti* using Coconut water as the growth media

Table 4.1.2 shows the Solid states fermentation data of *Bti* produced using Coconut water (CW) as the growth media. Usually *Bti* sporulates better in solid media than liquid media. it was found that a medium with CW, bengal gram powder with yeast extract the wet weight of biomass produced is 1.297 gm, where is CW and peptone gives 1.39 and additionally with yeast extract 1.43gm. Sporulation time was 47, 40 and 35 respectively and biolarvicidal killing time was 150, 131, 125 minutes. It was found that the biomass was found to be higher compared to submerged fermentation and the sporulation time was lower and related bioasay killing time also lower, proving that SSF with CW is a better method of production compared to conventional submerged method.

5.2.1: Batch fermentations of *Bti* using Rice straw as the growth media

Table.4.2.1 shows the growth of *Bti* using rice straw as the main carbon source/ media. It was found that raw untreated rice straw gives

negligible biomass output. In addition even supplementation with peptone, and yeast extract etc also didnt improve the biomass output to be viable in a ecnomical way. The reason for this can be attributed to the unique biochemical structure of the rice straw and the inability of *Bti* to secrete sufficient quantities of extracellular hydrolytic enzymes necessary for assimilating the biopolymers inherent in the rice straw. With raw rice straw only the biomass output was 0. 03gm, with suppplementation of peptone 0. 0315gm and by additional supplementation of yeast extract it was 0. 0311gm.

Rice straw (RS) is unique in its structure due to the presence and peculiar arrangement of cellulose (40%) hemicellulose (26%) and lignin and ash 18%.139. (Yoswathana and Phuriphipat, 2010) .

India being the second largest producer of rice straw after china. india has 30, 000 varieties of paddy (Goh, et al., 2010) . The RS cell walls constitute three biopolymers, namely, cellulose, hemicellulose, and lignin. Cellulose is a homopolymer of cellobiose (two units of glucose) , which are linked by -1, 4-glycosidic bonds. The complete hydrolysis of cellulose yields glucose, which is a preferable carbon source for commonly used fermenting microorganisms in industry. Hemicellulose contains a mixture of polysaccharides by which xylan represents a major portion. Xylan consists of xylose units, which are linked by -1, 4-glycosidic bonds to form xylan main chain. Typically, the main chain is substituted with some sugars (i. e., arabinose, galactose, and glucose) , sugar acids (i. e., glucuronic acid) , and ferulic and ρ-coumaric acids, depending on the source of xylans. Lignin is a large complex polymer of unrepeated phenolic monomers. It significantly contributes to the water conduction and defense systems in plants. However, the hydrophobicity and complex structure (also heterogeneity) of lignin pose challenges for biomass processing and utilization. Since *Bti* is a very poor producer of cellulase enzyme, the results generated tallies. (Ling., et. al .2012).

5.2.2 Batch fermentations of *Bti* using Delignified rice straw (NaOH) as the growth media.

Compared to other pretreatment methods, alkali pretreatments are the most effective ones because of its ability to increase cellulose accessibility and lignin removal capability (Ravindran and Jaiswal 2016;Zhang et al. 2012)

. Alkali pretreatments work on the principle of saponification (de-esterification) of xylan-hemicellulose and lignin intermolecular crosslinking ester bonds. As much as the crosslinks (bonds) removed, lignocellulose porosity increased. When NaOH is used as a pretreatment process, the internal surface area increases and crystallinity and degree of crystallinity are increased due to the swelling of lignocellulose material caused by NaOH that eventually leads to the removal of amorphous parts (Zhao et al. 2017) .

Sodium hydroxide, also known as lye and caustic soda, is an inorganic compound with the formula NaOH. It is a white solid ionic compound consisting of sodiumcations $Na^+$and hydroxideanions OH^-.

Sodium hydroxide is a strong alkali that hydrolyses proteins and carbohydrates at ordinary ambient temperatures and may cause severe chemical burns. It is highly soluble in water, and readily absorbs moisture and carbon dioxide from the air.

Sodium hydroxide is used in many industries: In the manufacture of pulp and paper, textiles, drinking water, soaps and detergents, and as a drain cleaner. Worldwide production in 2018 was approximately 60 million tonnes, while demand was 51 million tonnes. Since it is a cheap compound it can be used very cost effctively for the large scale production of *Bti* after proper hydrolysing the rice straw derived cellulose and using the resulting glucose as carbon source.

Since *Bti* is a poor producer of cellulase enzyme and due to the highly complicated structure of lignocellulosic structure of rice straw, Chemical hydrolysis with alkali, (NaOH) was attempted. Alkaine hydrolysis will break the lignin outer coat resulting in delignification and more over the exposed cellulose microfibrils will be digested /cleaved to release glucose monomers. The growth and biomass production clearly shows the enhanced biomass output using delignified rice straw.

Biomass output was 0.19 gm with 1% delignified rice straw, compared to raw rice straw 0. 03 gm and with supplementations (1%DRS+0.1% peptone, 1% yeast extract) biomass out put was 0.225 gm compared to 0. 0311gm. The biolarvicidal asssay time achieved was 230 minutes. This clearly proves that chemical delignification of rice straw is a pre-requiste for enhanced *Bti* production from rice straw as the substrate.

5.2.3: Batch fermentations of *Bti* using delignified rice straw (CaOH) as the growth media

Calcium hydroxide (traditionally called slaked lime) is an inorganic compound with the chemical formula CaOH. It is a colourless crystal or white powder and is produced when *quicklime* (calcium oxide) is mixed, or slaked with water. It has many names including hydrated lime, caustic lime, builders' lime, slack lime, cal, or pickling lime. Calcium hydroxide is used in many applications, including food preparation. Lime water is the common name for a saturated solution of calcium hydroxide. Since it is cheaper than sodium hydroxide and easily available in bulk quantities there is possibility of using this chemical for hydrolysis of straw and production of *Bti* in a cost effctive manner, although there is a slight drop in biomass production.

Delignification of rice straw was done using mild alkaline calcium hydroxide. Sodium hydroxide being a strong alkali will create environmental

pollution when the process is scaled up. As a solution to this calcium hydroxide which is less polluting can be used. Table 4.2.3 gives the biomass output data with delignified rice straw powder (1%) and with supplentations of peptone and yeast extract which are 0.179 and 0.223 gm respectively. The culture has been found to sporulate in 46 hours which is much lesser compared to control (48 hurs) . Bioasssay related killing time is 245 minutes which proves high potency.

5.2.4 : Batch fermentations of *Bti* using delignified rice straw (*Trichoderma reesei*) as the growth media

Trichoderma reesei is a mesophilic and filamentousfungus. *T. reesei* can secrete large amounts of cellulolytic enzymes (cellulases and hemicellulases) . Microbial cellulases have industrial application in the conversion of cellulose, a major component of plant biomass, into glucose. *Trichoderma reesei* produces a number of extracellar enzymes . It produces at least four endo-1, 4-β-xylanases (XYN I, II, III and IV, EC 3.2.1.8, , two β-xylosidases (EC 3.2.1.37) , two endo-1, 4-β-D-glucan cellobiohydrolases (CBH I and II, EC3.2.1.91) , five endo-1, 4-β-D-glucan-4-glucanohydrolases (EG I, II, III, IV and V, EC 3.2.1.4) and two β-D-glucosidases (BGL I and II, EC 3.2.1.21) (Bailey et al., 1993; Karlsson et al., 2001;Nogawa et al., 2001; Xu et al., 1998; Zeilinger et al., 1996) . Several other enzymes are also produced by T. reesei: β-mannanase (EC 3.2.1.78) , β-mannosidase (EC 3.2.1.25) , α-L-arabinofuranosidase (EC 3.2.1.55) , α-galactosidase (EC 3.2.1.22) , acetylxylan esterases (EC 3.1.1.72) and laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) (Karlsson et al., 2001; Roche et al., 1995; Shabalin et al., 2002) .

Due to the ability of *Trichoderma reesei* to produce cellulases and laccases. It was used to delignify and to produce *Bti* biomass from rice straw. From Table 4.10 it was shown that with delignified rice straw (1%) produced

a biomass of 0.122gm, while with supplementations the biomass has found to increase 0139. the sporulation time was 34 hours while bioassay based kiilling time was 280 minutes.

5.2.5: Batch fermentations of *Bti* using delignified rice straw (*Pleurotus florida*) as the growth media. *Pleurotus* is a genus of gilled mushrooms which includes one of the most widely eaten mushrooms, *P. ostreatus*. Species of *Pleurotus* may be called oyster, abalone, or tree mushrooms, and are some of the most commonly cultivatededible mushrooms in the world *Pleurotus* species, commonly known as oyster mushroom, are edible cultivated world wide . It is characterized by its high protein content and is the easiest, fastest and cheapest to grow, require less preparation time and production technology compared with other mushrooms. Bioconversion of lignocellulosic residues through cultivation of *Pleurotus* species offers the opportunity to utilize renewable resources in the production of edible protein rich food (Sanchez *et al*., 2002) . Various agricultural byproducts are being used as substrates for oyster mushroom cultivation. Some of these wastes include rice straw, wheat straw, banana leaves, peanut hull, corn leaves, mango fruits and seeds and sugarcane leaves (Cangy and Peerally, 1995;Mandeel *et al*., 2005) . However, widely used substrates for cultivation of oyster mushroom are rice straw and sawdust (Thomas *et al*., 1998) . Bioconversions of hard lignocellulosic components by oyster mushrooms are due to their ability to produce high levels of cellulases, hemicellulases (Vishal *et al*., 2006) and liginases (Tellez-Tellez *et al*., 2005) . In parallel bioconversion technology can increase the nutritional values of wastes to use as animal fodder and increasing high values of reducing sugars able to ferment producing other sources of energy.

From **Table 4.2.5** It can be seen that the biomass output of *Bti* considerably increased after delignification of rice straw using *Pleurotus*

florida mushroom. Further increase was achived by supplentation of peptone and yeast extract. The biomass out put with delignified rice straw only was 0.119, with peptone supplementation 0.13 and with additional supplementation with yeast extract was 0.193. sporulation timings were 40, 37, and 32 hours while bioasssay killing time was (SSF) 375, 311, 265 minutes.

5.2.6: Batch fermentations of *Bti* using delignified rice straw (NaOH) supplemented with fish slurry

C: N ratio of rice straw is 51. So there is need for incorporate nitrogen in to the medium in order to satisfy the microbial requirement. In this context fish is found to be a cheap medium supplement, considering the total quantity of fish wastes avaiable in the country.

Fishing in India is a major industry in its coastal states, employing over 14 million people. In 2016-17, the country exported 11, 34, 948 metric tonnes of sea food worth US$5.78 billion (₹ 37, 870.90 crore) , frozen shrimp being the top item of export. According to the Food and Agriculture Organization (FAO) of the United Nations, fish production has increased more than tenfold since 1947 and doubled between 1990 and 2010.

India has 8, 129 kilometres (5, 051 mi) of marine coastline, 3, 827 fishing villages and 1, 914 traditional fish landing centers. India's fresh water resources consist of 195, 210 kilometres (121, 300 mi) of rivers and canals,

2.9 million hectares of minor and major reservoirs, 2.4 million hectares of ponds and lakes, and about 0.8 million hectares of flood plain wetlands and water bodies. As of 2010, the marine and freshwater resources offered a combined sustainable catch fishing potential of over 4 million metric tonnes of fish. In addition, India's water and natural resources offer a ten fold growth potential in aquaculture (farm fishing) from 2010 harvest levels of 3.9 million metric tonnes of fish, if India were to adopt fishing knowledge, regulatory

reforms and sustainability policies.

As per **Table.4.2.6** chemically delignified rice sraw (NaOH) with various concentrations of fish slurry and with yeast extract supplementation, the biomass out put was 0.226, 0.267, 0.285, 0.292, 0.292 (with 0.1ml, 0.2, 0.3, 0.4and 0.5 fish sluury) 0.4 ml was found to be optimum which gives maximum output, the control was having 0.225gm with peptone. Sporulation timing was in the range of 36-38 hours. and killing time was 213 minutes. This has conclusively proved that supplementation of fish slurry will greatly enhance yield and profitability of this biopesticide.

5.2.7: Batch fermentations of *Bti* using delignified rice straw (CaOH) supplemented with fish slurry

As per **Table.4.2.7** chemically delignified rice straw (CaOH) with various concentrations of fish slurry and with yeast extract supplementation, the biomass out put was 0.189, 0.194, 0.226, 0.224, 0.223 (with 0.1ml, 0.2, 0.3, 0.4and 0.5 fish sluury) 0.4 ml was found to be optimum which gives maximum output, the control was having 0.223. gm with peptone. This is really cost effective when a comparison of the price of peptone and fish slurrry cost is done. Sporulation timing was 39 hours. and killing time was 236 minutes. This has conclusively proved that supplementation of fish slurry will greatly enhance yield and profitability of this biopesticide.

5.2.8: Batch fermentations of *Bti* using delignified rice straw (*Trichoderma reesei*) supplemented with fish slurry

From. **Table.4.2.8** it is eveident that the *Bti* biomass production can be considerably enhanced by delignifying the rice straw with *Trichoderma reesei* and supplementing with the right concentration of fish slurry. Biologicaly delignified rice sraw (*Trichoderma reesei*) with various concentrations of fish slurry and with yeast extract supplementation, the biomass out put was 0.125,

0.133, 0.143, 0.148, 0.153 (with 0.1ml, 0.2, 0.3, 0.4 and 0.5 fish sluury) 0.5 ml was found to be optimum which gives maximum output, the control was having 0.139. gm with peptone. This method of production has the potential for scale up since *Trichoderma reesei* can cultured and grown easily using agrowastes. Sporulation timing was 34 hours. and killing time was 264 minutes. This has again proved that supplementation of fish slurry will greatly enhance yield and profitability of this biopesticide.

5.2.9: Batch fermentations of *Bti* using delignified rice straw *Pleurotus florida* fungi supplemented with fish slurry.

From. **Table.4.2.9** it is very clear that the *Bti* biomass production can be considerably improved by delignifying the rice straw with *Pleurotus florida* and supplementing with the right concentration of fish slurry. Biologicaly delignified rice sraw (*Pleurotus florida*) with various concentrations of fish slurry and with yeast extract supplementation, the biomass out put was 0.122, 0.138, 0.2143, 0.217, 0.216 (with 0.1ml, 0.2, 0.3, 0.4and 0.5 fish sluury) 0.4 and 0.5 ml was found to be optimum which gives maximum output, the control was having 0.194. gm with peptone. This method of production has the potential for scale up since *Pleurotus florida* can cultured and grown easily using agrowastes. Sporulation timing was 34 hours. and killing time was 264 minutes. This has again proved that supplementation of fish slurry will greatly enhance yield and profitability of this biopesticide.

5.2.10: Batch fermentations of *Bti* using delignified rice straw (*Pleurotus fungi*) and pretreatment with cellulase enzyme

Table.4.2.10 data shows the biomass, sporulation time and biolarvicidal -killing time of *Bti* grown using *Pleurotus* fungi delignified rice staw further treated with cellulase enzyme. The biomass production using 1%

DRS, 0.1% pepetone and 0.1% yeast extract is 0.143 gm which is higher than the control (*Pleurotus* treated only-0.194) . Since this method releases free glucose, exess concentration of which supresess *Bti* growth due to substrate repression, the biomass concentration is slightly lower than the control.

5.2.11: Batch fermentations of *Bti* using delignified rice straw (NaOH) supplemented with chicken feather hydrolysate

The poultry feather is the most important waste by-product resulting from poultry processing plants, reaching billions of tons annually worldwide. Specifically, the Tunisian contribution is about 20, 000 tons. This by-product presents 10% of the total weight of poultry. The feather is a rich protein source because it is the most abundant keratinous protein in nature, representing 80–90% of the total composition of the feather. Keratin has a high stable mechanical structure attributed to the high degree of cross-linking by disulfide bonds, hydrogen bonds and hydrophobic interactions.

Table.4.2.11 shows the batch fermentations of *Bti* using delignified rice straw (NaOH) supplemented with chicken feather hydrolysate. Like fish hydrolysate chicken or poultry feather hydrolysate is also a rich source of various amino acids. Since the cost of peptone is exhorbitant, *Bti* biomass production using chicken feather hydrolysate is a cost effective method to manuafcture this highly sought after biopesticide.

Table 4.2.11 shows the biomass, sporulation time and larvicidal assay killing time of *Bti* produced with various quantities of chicken feather hydrolysate mixed with delignified rice straw (NaOH) with additional yeast supplementation. Biomass production with various concentrations of CFH (0.1, 0.2. 0.3, 0.4, 0.5) were 0.179, 0.189, 0.193, 0.187, 0.177 gms. 0.3ml was found be optimum, with a sporulation time of 39-45 hours and killing time of 214 minutes. The biomass produced is lower compared to similar condition

with fish hydrolysate, since fish proteins (Globular proteins) are more digestible or easily hydrolysable than poultry feather, which is beta pleated sheet structure.

5.2.12: Batch fermentations of *Bti* using delignified rice straw (CaOH) supplemented with chicken feather hydrolysate

Table 4.2.12 shows the biomass, sporulation time and larvicidal assay killing time with various quantities of chicken feather hydrolysate mixed with delignified rice straw (CaOH) with additional yeast supplementation. Biomass production with various concentartions of CFH (0.1, 0.20.3, 0.4, 0.5) were

0.176, 0.183, 0.213, 0.211, 0.1191 gms. 0.3ml was found be optimum, with a sporulation time of 39-43 hours and kiling time of 234 minutes. The biomass produced is lower (just like in previous case with NaOH) compared to similar condition with fish hydrolysate, since fish proteins (Globular proteins) are more digestible or easily hydrolysable than poultry feather, which is beta pleated sheet structure.

5.2.13 Batch fermentations of *Bti* using delignified rice straw (Trichoderma reesei) supplemented with chicken feather hydrolysate

Table 4.2.13 shows the biomass, sporulation time and larvicidal assay killing time with various quantities of chicken feather hydrolysate mixed with delignified rice straw (*Trichoderma reesei*) with additional yeast supplementation. Biomass production with various concentrations of CFH (0.1, 0.20.3, 0.4, 0.5) were 0.128, 0.134, 0.147, 0.115, 0.151 gms. 0.5ml was found be optimum, with a sporulation time of 37 hours and killing time of 294 minutes. The biomass produced is slightly lower compared to similar condition with fish hydrolysate, may be due to the easy assimilabilty of fish proteins (Globular proteins) compared to poultry feather, which is relatively resistant to biodigestion.

5.2.14 Batch fermentations of *Bti* using delignified rice straw (Pleurotus florida) supplemented with chicken feather hydrolysate

Table 4.2.14 shows the biomass, sporulation time and larvicidal assay killing time with various quantities of chicken feather hydrolysate mixed with delignified rice straw (*Pleurotus florida*) with additional yeast supplementation. Biomass production with various concentartions of CFH (0.1, 0.20.3, 0.4, 0.5) were 0.112, 0.118, 0.210, 0.214, 0.119 gms. 0.4ml was found be optimum, with a sporulation time of 37 hours and kiling time of 302 minutes. The biomass produced is lower compared to similar condition with fish hydrolysate, may be due to the easy assimilabilty of fish proteins (Globular proteins) compared to poultry feather, which is relatively resistant to biodigestion.

5.3.1 Batch fermentations of *Bti* using Bagasse as the growth media

Table 4.3.1 The growth of *Bti* in untreated raw bagasse medium also did not give good biomass output, due to reasons mentioned above, since it is also having similar biochemical structure and *Bacillus thuringiensis* lacks the ability to produce corresponding enzymes to cleave and utilize the resulting monomers for energy production and product secretion.

Table 4.3.1 The growth of *Bti* in untreated raw bagasse medium also did not give good biomass output, due to reasons mentioned above, since it is also having similar biochemical structure and *Bacillus thuringiensis lack*s the ability to produce corresponding enzymes to cleave and utilize the resulting monomers for energy production and product secretion. The biomass production with 1% bagasse, with supplementation of 0.1% peptone and with supplementation of 0.1% peptone and 0.1% yeast extract were 0. 027, 0. 03 and 0.031 gm respectively. This biomass out put is not ecnomical, commercially useful.

5.3.2 : Batch fermentations of *Bti* using delignified bagasse (NaOH) as the growth media

Table.4.3.2 Since *Bti* is not a good producer of Cellulase enzyme and due to the highly complicated structure of lignocellulosic structure of bagasse, Chemical hydrolysis with alkali, (NaOH) was attempted. Alkaine hydrolysis will break the lignin outer coat resulting in delignification and more over the exposed cellulose microfibrils will be digested /cleaved to release glucose monomers. The growth and biomass production shows the enhanced biomass output using delignified bagasse.

Biomass output was 0.183 gm with 1% delignified bagasse , compared to raw bagasse 0. 027 gm and with supplementations (1%BG+0.1% peptone, 1% yeast extract) biomass out put was 0.217 gm compared to 0. 031gm. The biolarvicidal assay time achieved was 227 minutes. This clearly proves that there is a technical requirement for chemical delignification of bagasse which is a pre requiste for enhanced *Bti* production from bagasse as the substrate.

5.3.3 Batch fermentations of *Bti* using delignified bagasse (CaOH) as the growth media

Table.4.3.3 shows the data regarding Chemical hydrolysis with alkali, (CaOH) . The growth and biomass production shows the enhanced biomass output using delignified bagasse.

Biomass output was 0.167 gm with 1% delignified bagasse , compared to raw bagasse 0.027 gm and with supplementations (1%BG+0.1% peptone

+0.1% yeast extract) biomass out put was 0.187 gm compared to 0. 031gm. The biolarvicidal assay time achieved was 220 minutes. This clearly proves that there is tremendous impact on chemical delignification of bagase

which is a pre-requiste for enhanced *Bti* production from bagasse as the substrate.

5.3.4 Batch fermentations of *Bti* using delignified bagasse (NaOH) supplemented with fish slurry

Table.4.3.4 shows the data regarding Chemical hydrolysis with alkali, (NaOH) with supplementations of various concentrations of fish sluury. The growth and biomass production shows the enhanced biomass output using fish slurry supplementation.

Biomass output was with various concentrations of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.189, 0.196, 0.2149, 0.203, 0.203 gm. The optimum was found to be 0.3ml with sporulation time of 45 minutes The biolarvicidal assay time achieved was 239 minutes. This clearly proves hat there is good impact on bagasse by nitrogen supplementation which enhances *Bti* production from bagasse as the substrate.

5.3.5 Batch fermentations of *Bti* using delignified bagasse (CaOH) supplemented with fish slurry

Table.4.3.5 shows the data regarding Chemical hydrolysis with alkali, (CaOH) with supplementations of various concentrations of fish sluury. The growth and biomass production shows the enhanced biomass output using fish slurry supplementation although the total biomass out put with this method is generally lesser than with NaOH.

Biomass output was with various concentrations of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.173, 0.177, 0.186, 0.183, 0.179 gm. The optimum was found to be 0.3ml with sporulation time of 39 minutes The biolarvicidal assay time achieved was 251 minutes. This clearly proves that there is good impact on bagasse by nitrogen supplementation which

enhances *Bti* production from bagasse as the substrate.

5.3.6 Batch fermentations of *Bti* using delignified bagasse (Trichoderma reesei) supplemented with fish slurry

Table.4.3.6 shows the data regarding biological pretreatment of bagasse with *Trichoderma reesei* fungi, with supplementations of various concentrations of fish sluury. The growth and biomass production shows the enhanced biomass output using fish slurry supplementation.

Biomass output was with various concentrations of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.111, 0.15, 0.127, 0.132, 0.141 gm. The optimum was found to be 0.5ml with sporulation time of 39 minutes The biolarvicidal assay time achieved was 295 minutes. Thus conclusively proving that there is good impact on bagasse by nitrogen supplementation with fish slurry which enhances *Bti* production from bagasse as the substrate. Biotechnological method of pretreatment has the potential to reduce pollution caused by chemicals, when it is scaled up for industrial production.

5.3.7 Batch fermentations of *Bti* using delignified bagasse (Pleurotus florida fungi) supplemented with fish slurry

Table.4.3.7 shows the data regarding biological pretreatment of bagasse with *Pleurotus florida* fungi, with supplementations of various concentrations of fish slurry. The growth and biomass production as expected shows the enhanced biomass output using fish slurry supplementation.

Biomass output was with various concentrations of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.117, 0.124, 0.119, 0.204, 0.200 gm. The optimum was found to be 0.4ml with sporulation time of 35 minutes The biolarvicidal assay time achieved was 285 minutes. The overall biomass output being lower than with biological pretreatment with *Trichoderma*, still

found to be good for industrial scale of this process. There is additional advantage that the additional income can be generated by producing oyster mushroms (First flush) .

5.3.8 : Batch fermentations of *Bti* using delignified bagasse (Pleurotus fungi) and pretreatment with cellulase enzyme

Table.4.3.8 data shows the biomass, sporulation time and biolarvicidal -killing time of *Bti* grown using *Pleurotu*s fungi delignified bagasse which was further exposed to treatment with cellulase enzyme. The biomass production using 1% BG, 0.1% pepetone and 0.1% yeast extract is 0.113 gm. the sporulation time in this case is 43 minutes and bioassay larvicidal time is 249 miuntes.

5.4.1 Batch fermentations of *Bti* using Taro as the growth media

Table.4.4.1 shows the biomass and sporulation time dependant data of *Bti*grown using Taro waste as the main carbon source. Biochemical content of taro is as follows:

Moisture 63-85%, Carbohydrate (mostly starch) 13-29%, Protein -1.4-3. 0%, Fat-0.16-0.36%, Crude Fibre 0.60-1.18%, Ash-0.60-1.3%, Vitamin C 7-9 mg/100 g, Thiamine-0.18 mg/ g, Riboflavin-0.11 mg/100 g, Niacin-0.9 mg/100 g.

From the biochemical composition itself it looks like a good medium for the growth of Bacilli with 1% taro the biomass ouput was 0.22 gm, while with additives 0.1% peptone, 0.1% yeast extract and 0.1% calcium chloride, there was substantial improvement in biomass output (0.2648) and there is corresponding reduction in kiling time (40 minutes) .

5.4.2 Batch fermentations of *Bti* using hydrolyzed Taro as the growth media

Table 4.4.2 shows the growth and sporuation of *Bti* in pretreated taro

based medium. The pretreatment was done to hydrolyze the inherent starch in taro to its corresponding monomers, so the viscocity of the medium will decrease and the overall growth of *Bti* will increase. With hydrolysed taro the biomass output was 0.29 compared to unhydrolysed taro (0.22) , which proves that pretretment and hydrolysis of Taro with HCl is very much beneficial for enhancing *Bti* biomass from this other wise wasted agro resource. Supplementations with peptone, yeast extract and calcium chloride have all proved to increase biomass output and also delta endotoxin synthesis as is evident from the reduced Kiling time (23-24 and 19-20 hrs) .

5.4.3 Solid state fermentations of *Bti* using Taro powder as the growth media

Table.4.4.3 data shows the biomass, sporulation time and biolarvicidal killing time of *Bti* grown using taro powder through solid state fermentation technology. The biomass production using 1% Taro, 0.5% bengal gram powder and 0.25% yeast extract dissolved in cocounut water is 1.7 gm. The sporulation time in this case is 34 minutes and bioassay larvicidal time is 49-50 miuntes. With coconut water and peptone biomass is 1.40, sporulation time is 42minutes and biolarvicidal assay time is 135 minutes. The combination of coconut water and yeast extract gave an biomass out put of 1.54 gm, sporulation time of 38 minutes and larvicidal assay time of 125 minutes. since there is huge availability of coconut water which is now under utilised there is immense scope for making use of this highly valuable natural resource for the cost effective production of *Bti*. In addition incorporation of taro with supplementation has found to be very promising as cost effective subtrates and alternative to costly substrates like glucose and peptone. Solid state fermentation usually gives better out put of *Bti* biomass and spore/delta endotoxin compared to submerged fermentation, and the effluent discharge from it is also minimal which further reduces process cost.

5.5.1 Batch fermentations of *Bti* using Sweet potato as the growth media

Besides simple starches, raw sweet potatoes are rich in complex carbohydrates, dietary fiber and beta-carotene (provitamin A carotenoid) , with moderate contents of other micronutrients, including vitamin B5, vitamin B6 and manganese .

Table 5.1 Biochemical composition of sweet potato

Energy	378 kJ (90 kcal)
Carbohydrates	20.7 g
Starch	7. 05 g
Sugars	6.5 g
Dietary fiber	3.3 g
Fat	0.15 g
Protein	2. 0 g

Sweeet potato also has high content of starch and other nutrients which makes it one of the favourite substrates for *Bti* fermentations. from table 4.5.1 it is evident that with 1% sweet potato the biomass output was 0.272 and with supplementations with peptone, yeast extract and calcium chloride it has considerably increased to 0.39 gm. correspondingly there was reduction in larval killing time (26 minutes) , which is an index of good sporulation and delta endotoxin synthesis.

5.5.2 Solid state fermentations of *Bti* using Sweet potato powder as the growth media

Solid state fermentation is advantageous for the production of *Bti* in so many ways. It is not only cost effctive with less power consuming steps but also discharge very less effluent. From table 4.5.2 it is evident that with 1%

sweet potato, 0.5% bengal gram powder and 0.25% yeast extract powder the biomass output was 1.57 gm compared to CW, peptone and yeast extract. (1.44gm) . The biolarvicidal assay killing time data was 44 minutes, which is an index of good sporulation and delta endotoxin synthesis.

5.6.1 Batch fermentations of *Bti* using Purple yarm powder as the growth media

Table 5.2 The biochemical composition of purple yam

Water/100gm	69.6 g
Energy	118 Kcal
Protein	1.53 g
Total Fat (lipid)	0.17 g
Carbohydrate	27.89 g
Total dietary Fiber	4.1 g

It is a rich a source of carbohydrates approximately 27% and with almost 1.53 % protein. Table 4.6.1 shows the biomass output data of *Bti* with purple yam (1%) which is 0.258, and with supplementations with peptone, yeast extract and calcium chloride (0.1% each) the biomass output was 0.35. the sporulation time showed a marked decrease with incorporation of calcium chloride 29 minutes compared to without calcium chloride 58 minutes. This proves that purple yam wastes can be effectively channelised for the growth and delta endotoxin production of *Bti* in a cost effective way.

5.6.2 : Batch fermentation of *Bti* Growth on Purple yam based medium with fish slurry supplementation

Table.4.6.2 shows the data regarding growth of *Bacillus thuringiensis israelensis* using purple yam with supplementations of various concentrations of fish slurry. The growth and biomass production shows the enhanced biomass output using fish sluury supplementation.

Biomass output was with various concentartions of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.203, 0.207, 0.217, 0.214 and 2. 07 gm. The optimum was found to be 0.3ml with sporulation time of 36 minutes. The biolarvicidal assay time achieved was 115 minutes. This clearly proves that mixing the right nitrogen source like fish wastes can greatly enhance the production of *Bti* using this much abundant and cheap source of carbohydrate.

5.6.3 Solid state fermentations of *Bti* using Purple yam powder as the growth media

Table.4.6.3 shows the data regarding growth of *Bacillus thuringiensis israelensis* using purple yam using solid state fermentation technique. Solid state fermentation has multiple benefits for the production of *Bti* in so many ways. It is not only cost effective with less power consuming steps but also discharge very less effluent. From table 4.6.3 it is evident that with 1% purple yam, 0.5% bengal gram powder and 0.25%yeast extract powder the biomass output was 1.39 gm compared to CW, peptone and yeast extract. (1.5gm) . The biolarvicidal assay killing time data was 55 minutes, which is an index of good sporulation and delta endotoxin synthesis. Comparing the cost of peptone and bengal gram and also keeping purple yam as the principal carbon source this medium can really create good impact in the large scale manufacturing of *Bti*.

5.7.1 Batch fermentations of *Bti* using Pineapple peel as the growth media

Table 5.3 Physical and chemical constituents of Pineapple pulp and wastes

1	Moisture (%)	87.3
2	Ash content (mg/100g)	1.8
3	Titratable acidity (%)	2. 03
4	Ascorbic acid (mg/100g)	21.5
5	Reducing sugars (%)	10.5
6	Non reducing sugars (%)	7.4
7	Total soluable solids (%)	13.3
8	Total sugars (%)	8.66
9	Crudefibre (g/100g-fw)	0.41
10	Protein (mg/100g)	7.2

From the data its evident that the sugar concentration is 8.66 % and protein content is 7.2% making it a cheaper and suitable substrate for *Bti* manufacture. From table 4.7.1 it is evident that good biomass production and toxin synthesis is possible. Pine apple peel 1%, with 0.1% peptone, and with 0.1% peptone and 0.1% yeast extract the biomass out put were 1.87, 2.15, 2.21 gm. sporulation timing and larvicidal assay timing were 46 imutes and 330 minutes. Sporulation is delayed may be due to availability of free assimilable sugars.

5.7.2 Batch fermentation of *Bti* : Growth on pineapple peel based medium with fish slurry supplementation

Table.4.7.2 shows the data regarding growth of *Bacillus thuringiensis israelensis* using Pine appple peel with supplementations of various concentrations of fish sluury. The growth and biomass production shows the enhanced biomass output using fish sluury supplementation.

Biomass output was with various concentartions of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 2. 03, 2. 07.2.17, 2.14 and 2. 07 gm. The optimum was found to be 0.3ml with sporulation time of 36 minutes. The biolarvicidal assay time achieved was 115 minutes. This clearly proves that mixing the right nitrogen source like fish wastes can greatly enhance the production of *Bti* using this much abundant and cheap source of carbohydrate.

5.7.3 Batch fermentations of *Bti* using Pineapple peel supplemented with chicken feather hydrolysate

Table.4.7.3 shows the data regarding growth of *Bacillus thuringiensis israelensis* using Pine apple peel with supplementations of various concentrations of chicken feather hydrolysate. The growth and biomass production shows the enhanced biomass output using CHF supplementation. Biomass output was with various concentrations of CHF (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 1.78, 2.11, 2.20, 2.27, 2.16 gm. The optimum was found to be 0.4ml with sporulation time of 41 minutes The biolarvicidal assay time achieved was 321 minutes. The data shows that CFH can be effcetively used to enhance biomass production of *Bti* although its not as effctive as fish hydrolysate, due to the unique structure of feathers and difficulty involved in hydrolysis of protien to amino acids.

5.8.1 Batch fermentations of *Bti* using Tapioca peel as the growth media

From table 4.8.1 shows the growth and sporulation data of *Bti* grown using tapioca peel using SSF. It is evident that good biomass production and toxin synthesis/production is possible. Tapioca peel 1%, with 0.1% peptone, and with 0.1% peptone and 0.1% yeast extract the biomass out put were 0.217, 0.236. 0.264. Sporulation timing and larvicidal assay timing were 29 minutes and 335 minutes.

5.8.2 Batch fermentation of *Bti* Growth on Tapioca peel based medium with fish slurry supplementation

Table.4.8.2 shows the data regarding growth of *Bacillus thuringiensis israelensis* using tapioca peels with supplementations of various concentrations of fish slurry. The growth and biomass production shows the enhanced biomass output using fish sluury supplementation.

Biomass output was with various concentrations of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.189, 0.213, 0.224, 0.217, and 0.204 gm. The optimum was found to be 0.3ml with sporulation time of 39-40 minutes The biolarvicidal assay time achieved was 139 minutes. As expected mixing the right nitrogen source like fish wastes which is available in huge quantities has helped to increase the production of *Bti* using this much abundant and cheap source of carbohydrates.

5.9.1 Batch fermentations of *Bti* using vegetable wastes as the growth media

Table 4.9.1 show the data regarding usage of vegetable wastes gives excellent biomass growth and toxin production. Considering the huge availabilty of this material, process scale up has to be attempted. Vegetable wastes alone produces bimass output of 0.273 and with supplementations 0.361 gm. Sporulation time was found to be shorter and delta endotoxin production was found to be good in corelation with bioasssay killing time. Pre-cooking of veg wastes will help to extract more nutrients from vegetable wastes.

5.9.2 Batch fermentations of *Bti* using vegetable waste based medium with fish slurry supplementation

Table.4.9.2 When supplemented with different concentrations of fish slurry the overall yield of biomassss and toxin production increases.

Biomass output was with various concentrations of fish slurry (0.1, 0.2, 0.3, 0.4 and 0.5ml) biomass out put was 0.193, 0.225, 0.229, 0.227, 0.231 gm. The optimum was found to be 0.3ml with sporulation time of 39-40 minutes The biolarvicidal assay time achieved was 115 minutes. The data reveals the immense posssibilities of large scale *Bti* manufacturing, since both these raw materials are abundant in excess throughout the world.

5.10.1 Batch fermentations of *Bti* using glucose as the carbon source for substrate repression studies

Table 4.10.1 The biomass yield of *Bti* at different concentrations of glucose which increases to 3%, but decreases after 3%. so the tolerance limit of *Bti* is 3% above which actually it repressess further growth which is a limitation when running batch reactors.

5.10.2 Batch fermentation of *Bti* using sucrose as the carbon source for substrate repression studies

Table.4.10.2 Shows the biomass yield of *Bti* using sucrose as the carbon source at different concentrations. Sucrose concentrations ranging from 1 to 6 % were used. Repression was shown after 5% due to the structure of sucrose, which is disaccharide, and since no free floating glucose is available in the medium. In this context feeding sucrose in batch fermentation of *Bti* will be more profitable, which can further increase biomass due to extended growth. Sucrose is also cheaper than glucose.

5.10.3 Batch fermentation of *Bti* using jaggery as the carbon source for substrate repression studies

Table.4.10.3 Shows the biomass yield of *Bti* using jaggerry as the carbon source at different concentrations. Sucrose concentrations ranging from 1 to 4% were used. Repression was shown after 3% due to the biochemical

composition of jaggerry, and since no free floating glucose is available in the medium. the biomass ouput is more than with sucrose. In this context feeding jaggerry in batch fermentation of *Bti* will be more profitable, which can further increase biomass due to the presence of multicomponents and richness of the medium. Jaggerry is is also cheaper than glucose.

5.10.4 Batch fermentation of *Bti* using cane sugar juice as the carbon source for substrate repression studies

Table 4.10.4 shows the biomass data using jaggerry as carbon source and soyabean as nitrogen source. Since jaggery is mostly having carbohydrates like sucrose with less nitrogen, soybean powder which is a rich source of proteins was done. It was found that compared to control (jaggerry alone) it was increasing 5.1 gm compared to 4.89 with respect to control, proving that the supplementation with soybeans is ecnomical for the cost effective production of *Bti*.

5.10.5 The production of *Bti* based biopesticide: Batch fermentation *Bti* using jaggery as the carbon source and without yeast extract supplement

The production of *Bti* based biopesticide: Using jaggery as the carbon source and soya bean powder as protein source

Table 4.10.5 shows the biomass yield differnce without yeast extract which is a costly ingredient in fermentations. The wet weight was 4.12 at 3%, compared to 4.89 with yeast extract. Here only peptone was used as the supplement. yeast extract being highly expensive its addition will greatly enhance the cost of manufacturing.

5.10.6 The production of *Bti* based biopesticide: Batch fermentation *Bti* using jaggery as the carbon source and without yeast extract supplement

Table.4.10.6 Reveals the *Bti* biomass data when the fermentation is run in batch mode with cane sugar juice as the substrate. The highest yield was with 3 % further reducing. The biomass yield is 4.25 gm compared to 3.64 with glucose, which proves fresh sugar cane juice has a profound effect on the yield of *Bti* production and sporulation.

5.10.7 Batch fermentation of *Bti* using tapioca peel powder as the carbon source for substrate repression studies

Table.4.10.7 Gives the batch fermentation data with tapioca peel powder. No repression of growth was found till 6%. Tapioca starch being polymer donot show substrate repression, and further decrease after 6% is due to difficulties in oxygen transfer due to increased viscocity. The problems arising due to viscocity can be tackled after appropriate pretreatment of susbtrate.

5.10.8 Batch fermentation of *Bti* using Taro powder as the carbon source for substrate repression studies

Table.4.10.8 Gives the batch fermentation data with Taro powder. No repression of growth was found till 5%. Taro also do not show substrate repression, and further decrease after 5% is due to difficulties in oxygen transfer due to increased viscocity. The problems arising due to viscocity can be tackled after appropriate pretreatment of taro with amylases.

5.10.9 Batch fermentation of *Bti* using Sweet potato as the carbon source for substrate repression studies

Table.4.10.9 Gives the batch fermentation data with Sweet potato. No repression of growth was found till 6%. Sweet potato didnt show substrate

repression, and further decrease after 6% is due to difficulties in mass transfer especially oxygen transfer due to increased viscocity. The problems arising due to viscocity can be tackled after appropriate pretreatment techniques by mass producing amylases using SSF technology.

5.10.10 Batch fermentation of *Bti* using Purple yam as the carbon source for substrate repression studies

Table.4.10.10 Gives the batch fermentation data with Purple yam powder. No repression of growth was found till 6%. Purple yam powder didnt show substrate repression, and further decrease after 6% is due to difficulties in mass transfer especially of oxygen transfer due to increased viscocity.

5.11.1 Fed-batch fermentation of *Bti* using glucose as the carbon source

Table.4.11.1 shows the biomass build up data of *Bti* grown using glucose as the carbon source in fed batch mode. It was found that biomass keep on increasing beyond 3% without repression till 6%. This has a profound impact on the overall biomass accumulated at the end of fermentation, sporulation and biolarvicidal killing time. wet weight accumulated at 6% is 8.060 gm compared to 3.21 with batch mode.

5.11.2 The production of *Bti* based biopesticide: Fed-batch fermentation *Bti* using glucose as the carbon source and soya bean powder as the nitrogen sourced biopesticdie

Table.4.11.2 Gives the biomass related data of *Bti* grown using glucose and soya bean powder. Soy bean being rich in proteins (54%) can be used as a substitute for the costly peptone additive. Compared with peptone based data it gives good output and the biomass keep on increasing upto a glucose concentration of 4%. Still compared to cost of peptone the fed batch fermentation using soybean will be ecnomical.

5.11.3 Fed-Batch fermentation of *Bti* using sucrose as the carbon source

Table.4.11.3 Gives the biomass related data of *Bti* grown using sucrose . The biomass keep on increasing upto a glucose concentration of 7%. This is very promising resullt considering the cost of sucrose compared to glucose. Above 8% there is substantial reduction in biomass yield may be due to combined effect of catabolite represssion and viscocity of fermentation broth.

5.11.4 Fed-Batch fermentation of *Bti* using jaggery as the carbon source

Table.4.11.4 Gives the biomass related data of *Bti* grown using jaggerry. Jaggerry being rich in varoius nutrients gives excellent biomass output. The biomass keep on increasing upto a glucose concentration of 11%. This will have a tremendous impact on the overall ecnomics of *Bti* manufacturing. Above 11 % there is substantial reduction in biomass yield may be due to combined effect of catabolite represssion/product repression and viscocity of fermentation broth.

5.11.5 Fed-Batch fermentation of *Bti* using cane sugar juice as the carbon source

As per the **Table.4.11.5** biomass yield of *Bti* produced using fed batch fermentation using sugar cane juice as the carbon source. Sugar cane juice supplemented with peptone and yeast extract is very rich in minerals, vitamins, amino acids etc. The biomass yield increased upto 11 % with wet weight of 6.99 gm. Correspondingly the killing time for larvae was 29 minutes in 1/10 dilution, which is a reflection of good delta endotoxin synthesis. Use of sugar cane juice is ecnomical since all other forms of sugar has to go through various stages of processsing, which natuarally contributes to cost increase.

5.11.6 Fed Batch fermentation of *Bti* using cane sugar juice as the carbon source and without yeast extract supplement

Table.4.11.6 Fed Batch fermentation of *Bti* using cane sugar juice as the carbon source and without yeast extract supplement. Yeast extract although being rich in nutrients is a very costly substrate /additive. So the growth aspects of *Bti* wihout supplmentation was studied. The biomass concentration increased upto 8% which gives almost similar pattern of growth compared to sugar cane juice with yeast extract, but the maximum achieved was slightly lower compared to the control. Proving that yeast extract is an indespensable component which is essential for improved biomasss ouput.

5.12.1 Solid state fermentations of *Bti* using Jaggery powder as the growth media

Table.4.12.1 Shows the biomass data of *Bti* grown using jaggerry as the carbon source through solid state fermentation technology. Usually the *Bti* shows intense sporulation when grown under SSF. Moreover since coconut water has replaced water in this particular media, it contributes further to the enhanced biomas, sporulation and high delta endotoxin synthess. Jaggery with bengal gram powder yielded biomass of 1.56, compared to coconut water with peptone additive, which is 1.36. This is an increase of around 20% more compared to control, which is significant in terms of ecnomics. There is good scope of utilizing massive amounts of coconut water, jaggerry and bengal gram powder through solid state fermentaion for the cost effective manufacturing of this highly sought after biopesticide.

5.12.2: Solid state fermentations of *Bti* using Sugar cane juice powder as the growth media

Table.4.12.2 Shows the biomass data of *Bti* grown using sugar cane juice powder as the carbon source through solid state fermentation technology.

Here also *Bti* shows intense sporulation when grown under SSF. Moreover since cocconut water has replaced water in this particular media, it contributes further to the enhanced biomas, sporulation and high delta endotoxin synthess. Sugar cane juice powder with bengal gram powder yielded biomass of 1.84, compared to coconut water with peptone additive, which is 1.37, which is very much significant in terms of ecnomics. There is good scope of utilizing massive amounts of coconut water, sugar cane juice powder and bengal gram powder through solid state fermentaion for the cost effective manufacturing of this highly useful biopesticide.

5.13.1 Study of settling of delta endotoxin of *Bacillus thuringiensis* subspecies israelensis at different alum concentrations.

From **Table 4.13.1** it is evident that from the range of Alum concentrations used, 0.25% was the optimum. Alum being a cost effctive flocculant will not contribute much to the overall cost of production/down stream processing. So can be effectively used to reduce the settling of biomasss, since *Bti* is basically being a biotech product.

5.14.1 Effect of alum on filtration of the fermented broth of *Bacillus thuringiensis* subspecies israelensis.

Table.4.14.1 shows the filtration of fixed quantity *Bti* broth with alum and without alum. There was 3 minutes difference in timing, which is significant in scale up. since the cost of additive/alum is negligible it can be used in scale up to have substantial reduction in filtration time, and to have an all together less time consuming down stream processsing, in order to get finished product. Alum affects the voidage of filter cake which gets deposited during conventional filtration.

5.14.2 Optimisation of alum concentration for improved filtration rate of *Bti* using conventional filter.

Table.4.14.2 gives the filtration rate data regarding the filtration rates given by range of alum concentraion from 0.1%, 0.3%, 0.5%, 0.7%and 0.9%. it was found that 0.7 % give sthe shortest filtration timings of 3 minutes and 50 seconds. This data will help to ecnomise the usage of alum in a very cost effctive way.

5.14.3. Effect of Ferric chloride on filtration of the fermented broth of *Bacillus thuringiensis* subspecies israelensis.

Table 4.14.3 shows the effect of Ferric chloride as a floccullant in enhancing the filtration rates of *Bti* fermentation broths. Compared to control the time taken for a fixed volume was 1 minute and 30 seconds lesser, which is significant in scale up. In addition it is suitable to be used in scale up since it is very cheap, and already used on a technical scale in water purification.

5.14.4 Effect of Aluminium sulphate on filtration of the fermented broth of *Bacillus thuringiensis* subspecies israelensis.

Table 4.14.4 shows the Effect of Aluminium sulphate on filtration of the fermented broth on *Bacillus thuringiensis var israelensis.* Aluminium sulphate is very cheap and is an effective flocculant. Since due to particle size increase there will be more voidage it improves filtration rates with *Bti* fermented broth. The time taken to filter a fixed volume was 4 minutes and 10 seconds compared to control which gives 7 minutes.

5.14.5 Effect of Moringha seeds on filtration of the fermented broth of *Bacillus thuringiensis* subspecies israelensis.

Table.4.14.5 Shows the effect of Moringha seeds on filtration of the fermented broth of *Bacillus thuringiensis var israelensis*. Compared to control

2 minutes reduction in filtration time was noted. This assumes lot of importance its easy and cost effctive method. its organic and non toxic and work like a flocculant.

5.14.6 Effect of Gaur gum on filtration of the fermented broth of *Bacillus thuringiensis* subspecies israelensis.

Table.4.14.6 shows Effect of gaur gum on filtration of the fermented broth on *Bacillus thuringiensis var israelensis*. Gaur gum extracted from gaur, is very cheap and is an effective flocculant. Since due to particle size increase there will be more voidage it improves filtration rates with *Bti* fermented broth. The time taken to filter a fixed volume was 6minutes and 10 seconds compared to control which gives 9 minutes.

5.15.1.1 Stability of *Bti* formulations under field conditions Effect of temperature on the stability of dleta endotoxin of *Bti*

Table 4.15.1.1 shows the effect of temperature on the stability of delta endotoxin. Respective beakers were heated to the desired temperature starting from 30 to 90 degrees. It was found that with increase in temperature the larvicidal killing time increases with 123 minutes at 90 degrees. Maximum activity was seen at 30 degrees.

5.15.1.2 Heat stability of *Bti* with one hour holding

Table 4.15.1.2 shows the effect of temperature on the stability of delta endotoxin with one hour holding. Respective beakers heated to the desired temperature starting from 40 to 50 degrees and held for one hour. It was found that the larvicidal killing time increases with 50 minutes (77 minutes) compared to 40degrees (69 minutes) . These temperature variations are normal in the field.

5.15.2.1 Effect of Sunlight on the stability of delta endotoxin *Bacillus thuringiensis* subbspecies israelensis

Table 4.15.2.1 Shows the Effect of sunlight on the stability of delta endotoxin of *Bacillus thuringiensis variety israelensis*. Since it is a biopesticide sprayed in stagnant water to control mosquitoe larvae, it is exposed to sunlight and related damage. How a particular *Bti* formulation will work after exposure can be studied only after exposing the product to sunlight for a fixed period of time and doing biolarvicidal assay. With respect to control which took 95 minutes without exposure. It was found that there is gradual increase of biolarvicidal killing time with respect to number of days exposure time starting from 197 minutes (1^{st} day) to 267 minutes for 30^{th} day. So its important that the biopesticide should be preferably ingested early by the larvae or it should be formulated in such a way to reduce the damage by sunlight exposure.

5.15.2.2 Effect of UV light on the stability of delta endotoxin *Bacillus thuringiensis* subbspecies israelensis

Table.4.15.2.2 shows the effect of UV light on the bioactivity of delta endotoxin. Sunlight contains visible, ultra voilet and infra red wave lenghths. out of these UB rays generally affect the biological activity of delta endotoxin. Cultures of *Bti* exposed to UV light for varying periods of time (30.60.90.120) took 75, 82, 93, 105 minutes respectively to kill all 10 larvae in the beaker. so ultra voilet radiations over a period of time deleteriously affects the activity of *Bti* formulations.

5.15.2.3 Effect of Aloe vera as aUV protectant in *Bti* formulations

Table 4.15.2.3 shows the efect of Aloe vera gel in protecting the *Bti* delpta endotoxin biological activity from harmful UV rays. It was found that *Bti* formulations mixed with Aloe vera gel and exposed for time periods ranging

from 30 minutes to 120 minutes were having reduced killing times compared to control. When control showed bioassay larvicidal killing time of 105 minutes for 120 minutes exposure, with Aloe vera within 95 minutes all larvae died , proving the effectiveness of Aloe vera as natural UV protectant which can be tried in commercial formulations.

5.15.3 Effect of pH on the stability of delta endotoxin of *Bti*

Table 4.15.3 shows effect of pH on the stability of delta endptoxin of *Bti*. Being protenacious in nature delta endo toxin of *Bti* is amenable to changes and denaturations with variations of pH which occurs in environment. It was found that with respect to control, the time taken to kill all the 10 larvae were 71, 68, 67, 65, 63, 69, 84 minutes for pH of 3. 0, 4. 0, 5.

0, 6. 0, 7. 0, 8. 0, 9. 0 respectively. This has proved that the delta endotoxin bio activity is very much affected by variations in pH and the optimum was found to be pH 7. 0 with a biolarvicidal killing time of 63 minutes.

5.15.4 Effect of various protein preservatives on the action of delta endotoxin of *Bti*.

Table.4.15.4 shows the Effect of various protein preservatives on the action of delta endotoxin of *Bti*. Mixing different protein preservatives as part of final *Bti* formulation will greatly enhance the shelf life and biological activity in field conditions. Various protein preservatives like Ammonium sulphate, Ammonium acetate, Sodium metabisulphate were used. It was found that with respect to control, with Ammonium acetate the killing timeswere 65, 69, 75, 78, 83, 87, 91 minutes and with Ammonium sulphate 68, 75, 81, 89, 94, 99, 107, with sodium metabisulphite 66, 68, 71, 75, 77, 80, 84, minutes respectively for 0, 5, 10, 15, 25 and 30^{th} days. Out of that sodium meta bisulphite was found tobe very useful in terms of low killing time of larvae and amonium actate was found to be performing as the second best material. Cost wise Ammonium acetae and ammonium sulphate will be cheaper.

CHAPTER 6
CONCLUSION

- Considering the quantity and easiness of usage coconut water is an excellent raw material for the cost effective production of *Bti*.
- Spoiled/rotten tuber crops like Tapioca, Taro and Purple yam have found to be very promising substrates for the cost effctive production of *Bti* , espcially in solid state fermentation mode.
- Agro wastes like rice straw and bagasse can be effctively channelised for the production of *Bti* after apprpriate chemical/biological pretreatment menthods. Fish hydrolysate has found to be more effective than chicken feather hydrolysate in enhancing yield.
- Fed batch fermentation is an effective technique to enhance the yield of *Bti* overcoming substrate repression , especially with monomeric and dimeric substrates like glucose , sucrose and jaggerry.
- Solid state fermentation of *Bti* using glass plates gives maximum yield of delta endotoxin at minimum cost. Disposal of spent agar medium during scale -up may be an issue and technical process has to be developed.
- Alum , Ferric chloride and Aluminium sulphate have all proved to be very good rate enhancing materials when used in down stream operations like filtration/centrifugation.
- Gaur gum and Moringha seeds powder have proved to be good natural filter aids for the cost effective filtration of *Bti* fermentation broth.

- Aloe vera can be used in *Bti* formulations to reduce the effect of ultravoilet rays in the sunlight and to maintain its insecticidal activity in the field.

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