Insecticidal activity of indigenous *Bacillus thuringiensis* strains against Tephritids fruit fly pests affecting fruits and vegetables

Ph. D. Thesis

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Insecticidal activity of indigenous *Bacillus thuringiensis* strains against Tephritids fruit fly pests affecting fruits and vegetables

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DEDICATED
TO MY
BELOVED PARENTS &
LOVELY CHILDREN

পিএইচ.ডি./ডি.বি.এ./এম.ফিল.

(থিসিসে Plagiarism নেই মর্মে প্রত্যয়নপত্র)

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(খ) ইংরেজীতে..... MD. ABDUL BARI .....

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থিসিসের শিরোনাম..... Insecticidal activity of indigenous *Bacillus thuringiensis* strains against Tephritids fruit fly pests affecting fruits and vegetables .....

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We approve the submission of his Ph.D. Dissertation.

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ABSTRACT

The extensive and indiscriminate use of synthetic chemical insecticides in agriculture and forestry has led to several unintended and detrimental consequences, including environmental pollution, the development of pest resistance, the mortality of non-target organisms, and negative public health effects. These challenges are particularly pronounced in developing countries like Bangladesh, where pest infestations significantly reduce crop productivity and economic gains. As a sustainable and environmentally safer alternative, microbial biopesticides, particularly those derived from *Bacillus thuringiensis* (*Bt*), have emerged as a promising solution. *Bt*-based biopesticides are host-specific, biodegradable, and effective, offering significant potential to reduce chemical pesticide dependency.

This study aimed to evaluate insecticidal activity of previously isolated, characterized and preserved indigenous *Bacillus thuringiensis* (*Bt*) strains from different eco-regions of Bangladesh against four economically important Tephritid fruit fly pests, *Bactrocera dorsalis, B. zonata, Zeugodacus cucurbitae* and *Z. tau*. These pests are known to cause extensive damage to fruits and vegetables, impacting local consumption, export opportunities, and national food security. A total of 44 *Bt* strains were isolated, identified, and screened through a comprehensive suite of phenotypic, genetic, proteomic, and toxicity analyses. The overarching goal was to identify highly potent *Bt* strains and validate their effectiveness under both laboratory and field conditions.

Initial screening of the 44 native *Bt* isolates showed that 16 strains could induce greater than 50% and three potential strains JSd1, SaS6, and JDc1 exceeded 80% larval mortality in all four Tephritid species tested. *Bt* strain JSd1 consistently demonstrated the highest efficacy, inducing 97% mortality in *B. dorsalis*, 95% in *B. zonata*, 95% in *Z. cucurbita*e, and 92% in *Z. tau*, outperforming even the reference *Btk* HD-73 and *Bts* T84A1. Further toxicological analyses, including lethal concentrations (LC_{50}) and lethal time (LT_{50}) values, confirmed JSd1's superior performance. LC_{50} values ranged from 0.431 to 0.472 mg/ml and LT_{50} values ranged between 54.09 and 55.17 hours, which are significantly lower than other strains, indicating high potency at reduced concentrations, while faster action and quicker pest knockdown.

Beyond bioassays, the study also evaluated the biological quality parameters of insects exposed to *Bt* treatment infested with preferred hosts, which are critical in understanding sub-lethal effects and potential population suppression in pest communities. *Bt* JSd1, SaS6, and JDc1 significantly reduced pupal yield, pupal weight, adult emergence percentage, and flying ability

across all four Tephritid species. For instance, *Bt* JSd1-treated groups consistently demonstrated the lowest pupal yield (as low as 99 ± 2.081 in *Z. tau*), lowest pupal weight (~8 mg), and lowest adult emergence (ranging from 39–53%).

Emergence of malformed or half-emerged adults was also significantly higher in treated groups, while sex ratio distortion was minimal, suggesting that these strains primarily impacted general viability rather than sex-linked mortality. These results indicate a substantial decline in the reproductive and survival potential of these pest populations upon exposure to *Bt* biopesticides, making them highly effective components in Integrated Pest Management (IPM) strategies.

To understand the genetic basis of its high efficacy, whole genome sequencing of *Bt* JSd1 was conducted. Genomic analysis revealed the presence of multiple *Cry* and *Vip* insecticidal genes, notably *Cry*22A and *Vip*3A, which are known for their effectiveness against Dipteran insects. Additionally, several virulence factors and biosynthetic gene clusters associated with secondary metabolite production were identified, contributing to the strain's broad-spectrum insecticidal capability and environmental adaptability. This genetic richness further reinforces the potential of *Bt* JSd1 as a candidate for next-generation bioinsecticide development.

Finally, field validation was carried out using the formulated *Bt* biopesticide (compared with chemical pesticides) on *Solanum melongena* (brinjal), a crop heavily impacted by the Eggplant Fruit and Shoot Borer (EFSB. Four-time foliar applications (Each of 100 ml volume containing 25.7 mg spore crystal mixture) of the *Bt* preparation reduced EFSB infestation to just 10%, compared to significantly higher levels in untreated controls, with no significant difference with the chemical pesticide. The average yield per plant increased from 1.45 kg (control) to 3.85 kg in the treated group, effectively matching or surpassing yields from chemically treated plots. Importantly, there were no observed negative impacts on beneficial insect populations or surrounding flora, highlighting the ecological safety of the product.

Overall, this study provides compelling evidence for the viability and impact of indigenous *Bt* strains, particularly JSd1, as potent, safe, and affordable biocontrol agents, offering a sustainable solution to the challenges of chemical pesticide overuse, contributing to improved agricultural productivity, ecological health, and food security. Their local origin also enables domestic production and reduces reliance on imported formulations, supporting national bioeconomy goals.

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The Author, December 04, 2025

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ABBREVIATIONS AND SYMBOLS

%	Percentage
~	Tilde, here used to indicate a range
Aa	Amino acid
BARI	Bangladesh Agricultural Research Institute
BLAST	Basic local alignment search tool
BLASTn	Nucleotide BLAST
BLASTp	Protein BLAST
bp	Base pair
Bt	*Bacillus thuringiensis*
CDD	Conserved domain database
CDS	Coding DNA Sequence
ClustalW	Clustal alignment format without base/residue numbering
cm	Centimeter
Cry	Crystalline inclusion protein of *Bacillus thuringiensis*
Cyt	Cytolytic inclusion protein of *Bacillus thuringiensis*
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
EA1	Extractable antigen-1
EBI	European bioinformatics institute
EDTA	Ethylene diamine tetra-acetic acid
et al.	And others
Et-Br	Ethidium bromide
Etx	Epsilon toxin
× g	g-force or relative centrifugal force (rcf)
g/gm	Gram
HeLa	HeLa is a cervical cancer cell line derived from the name of the patient, Henrietta Lacks
Ig	Immunoglobulin
in silico	Silicon in computer chips / in or on a computer
kb	Kilobase
kDa	Kilodalton
Kg	Kilogram
L	Liter
LB	Luria Bertani
M	Molar
MAFFT	Multiple alignment using fast fourier transform

MEGA	Molecular evolutionary genetics analysis
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimole
Mtx	Mosquitocidal toxins
MUSCLE	Multiple sequence comparison by log- expectation
MW	Molecular weight
NA	Nutrient agar
NCBI	National centre for biotechnology information
Ng	Nanogram
NGS	Next generation sequencing
Nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PGAP	Prokaryotic genome annotation pipeline
pH	Negative logarithm of hydrogen ion concentration
PS	Parasporin
RAST	Rapid annotations using subsystems technology
RNA	Ribonucleic acid
rpm	Rotation per minute
rRNA	Ribosomal RNA
RT	Room temperature
Sap	Surface array protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Scanning electron microscope
S-layer	Surface layer
SLH	Surface layer homology
SLP	Surface layer protein
SMART	Simple modular architecture research tool
spp.	Species
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA

TE	Tris EDTA buffer
TEMED	Tetramethylethylenediamine
UniProt	Universal protein resource
UV	Ultraviolet
v/v	Volume per volume e.g. (mL/mL)
Vero	Kidney epithetical cell line derived from an African Green Monkey. Vero gets its name from a derivation of green kidney, "Verda Reno"
Vip	Vegetative insecticidal protein
vol.	Volume
w/v	Weight per volume e.g. (g/mL)
WGS	Whole genome sequencing
A	Alpha
B	Beta
Γ	Gamma
Δ	Delta
µ	Micro
µg	Microgram
µL	Microliter
µm	Micrometer
3D	Three dimensional

CHAPTER 1

INTRODUCTION

INTRODUCTION

Agriculture is still Bangladesh's largest source of earnings, although it only makes up 12% of the country's GDP due to the fact that the country is rapidly ending up being progressively industrialized and urbanized (Chowhan *et al.*, 2023; Hossain *et al.*, 2022). The sector is ending up being better and much better, however there are still challenges, particularly with insect pest that make food less safe, lower farmers' earnings, and lessen the quality of exports. Pests are specifically impacting veggies and fruits, and they can cut their typical yearly output by 15% to 25%, depending upon the crop and the climate where it grows (Ali *et al.*, 2021; Mian *et al.*, 2016).

Bangladeshi farmers generally employ synthetic chemical pesticides such organophosphates, neonicotinoids, carbamates, and pyrethroids when pests are a concern. They often use these substances without understanding how much to utilize, how safe they are, or how to cope with resistance (Dasgupta *et al.*, 2007). Long-term effects of this method consist of the expansion of insect populations that are resistant to the pesticides, the disturbance of the balance of nature, contamination of the environment, and major health risks such neurological and reproductive concerns and cancer (Aktar *et al.*, 2009). It's strange that over 73% of the vegetables from Bangladesh that were tested contained more pesticides than are authorized.

The Integrated Pest Management Collaborative Research Support Program (IPM CRSP) started in 1998 with the function of choosing safer and more sustainable by improving education, presentations, and farmer participation(Koppenhöfer *et al.*, 1999). Bio-intensive Integrated Pest Management (BIPM) utilizes ecological strategies such crop rotation, trap cropping, biological control firms, and biopesticides. *Bacillus thuringiensis* (*Bt*) is the most widely known microbial biopesticide worldwide. It is safe for the environment, only damages to specific hosts, and has actually been demonstrated to kill insects(Chandler *et al.*, 2011; Sanahuja *et al.*, 2011).

Bt is a Gram-positive germ that forms spores and produces insecticidal crystalline (Cry) and vegetative (Vip) proteins all the time. These proteins are selectively bound by the target insect larvae midgut receptors and form pores that upset cellular osmoregulation and result in larval death. *Bt* formulations have been effectively utilized against lepidopteran, dipteran, and coleopteran pests. Target specificity and biodegradability make *Bt* an useful tool for sustainable pest control, especially in pesticide resistance management and environmental conservation. While *Bt* is applied on genetically modified crops worldwide, evolution of resistance and gene

migration have rendered *Bt* sprays or foliar application a mode of choice in nations like Bangladesh where the use of GM crops is limited.

There is sufficient but unexploited microbial wealth in Bangladesh, with indigenous *Bt* strains bred from varied ecological niches like soil, insect cadavers, and grain storage environments (Shishir *et al.*, 2014). These native strains have the potential to impart more potency and environmental flexibility when used in native pest control schemes. But the majority of *Bt* research and product development in Bangladesh relies on foreign isolates with very little genomic identification and indigenous isolate field testing. Hence, there are no cost-effective region-specific biopesticides suitable to the varied agroecological conditions of the nation.

One of the most significant orders of insect pests causing enormous damage to vegetable and fruit crops in Bangladesh is family Tephritidae, or fruit flies. Tephritid fruit flies include over 5,000 species worldwide (Pape *et al.*, 2011), the majority of which are of economic significance. Of these, species within the genera *Bactrocera* and *Zeugodacus* are the most devastating in Bangladesh. There was a recent entomological publication that has boosted the nation's known Tephritid diversity from merely seven species in 2013 to over thirty-four by 2021 (Leblanc *et al.*, 2013, 2019, 2021). Two further species were noted by (Khan *et al.*, 2024), in 2024. Bangladesh has recently reported twelve new country records, including an annotated checklist of non-Dacini species (Hossain *et al.*, 2024).

These are some of the most significant pest species: *Bactrocera dorsalis* (Oriental fruit fly), *Bactrocera zonata* (Peach fruit fly), *Zeugodacus cucurbitae* (Melon fly), and *Zeugodacus tau* (Pumpkin fruit fly). These traits infect mangoes, guavas, bananas, papayas, watermelon, peach, fig, citrus, zucchini, tomato, brinjal, cucumber, bitter gourd, pumpkin, ridge gourd, sponge gourd, bottle gourd, snake gourd, cabbage, red cabbage, cauliflowers, cucurbits, and other valuable fruits and vegetables. Female flies lay eggs inside the fruit tissue with the help of an elongate modified ovipositor. The larvae, upon hatching, feed inside and destroy much of the tissue, leading to rot and secondary microbial infections and usually rendering the fruits unmerchantable. Infestation by fruit fly is as much as 85.7% during fruiting seasons, resulting in colossal loss of quantity and quality (Boopathi *et al.*, 2017). It is a vast economic loss where *Z. tau* alone causes an estimated loss in crops of over three billion dollars in China (Fang *et al.*, 2015; Jaleel *et al.*, 2018).

Bait sprays, chemical insecticides, and traps are used in the traditional management of fruit flies. The chemical control strategies are saddled with problems of resistance, residues, non-target

toxicity, and environmental degradation. Microbial biocontrol agents such as *Bt* offer cleaner and environmentally more friendly options with biological control. A number of researches carried out in India, Brazil, and other Tephritid-infested regions confirmed the larvicidal efficacy of *Bt* against Tephritids and warranted its application in integrated fruit fly management (Paranhos *et al.*, 2019; Singh & Dhkal, 2024; Sood *et al.*, 2019).

Strain dependency of the toxin is of very critical importance in developing the efficient *Bt* formulations. Local strains, which have natural resistance towards local environment and pest conditions, are the potential hope candidates to develop targeted biopesticides. In spite of this potential, limited research in Bangladesh has systematically examined the diversity, cry gene characterization, or insecticidal activity of the native *Bt* strains against local species of Tephritids. The combined effort at isolating, identifying, and characterizing such strains would help in developing environmentally friendly fruit fly management practices consistent with national IPM programs.

A strategic shift to biopesticides, particularly those produced from locally adapted *Bt* strains, would de-emphasize Bangladesh's reliance on poisonous chemicals, introduce sustainable agriculture, and enhance crop quality and safety. This is in congruence with the global movement to reduce agrochemical inputs and promote biodiversity conservation. With increasing pest pressure and climate dynamics driving, identification and utilization of indigenous microbial assets such as *Bt* are an indispensable and vital step in the country's enhanced crop resilience and food safety.

CHAPTER 2

LITERATURE REVIEWS AND OBJECTIVES

2.1 *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is a distinguished species among the *Bacillus* genus for insecticidal potential and is used as a biocontrol agent. This bacterial species produces insecticidal proteins (δ-endotoxins) during the sporulation phase as parasporal inclusions, which predominantly comprise one or more proteins called Cry and Cyt toxins. These protein toxins are highly selective to their target insect, are innocuous to humans, vertebrates, and plants, and are also completely biodegradable. Therefore, *B. thuringiensis* is a viable alternative for the control of insect pests in agriculture and disease vectors of importance in human health (Helgason *et al.*, 2000). Numerous *B. thuringiensis* strains have been isolated that show activity towards lepidopteran, dipteran, or coleopteran insects. *Bt* is a soil-dwelling, Gram-positive bacteria that is commonly employed as a biopesticide against insect pests, particularly Tephritid fruit flies (*Bactrocera*, *Zeugodacus*, *Ceratitis*, *Anastrepha*). While *Bt* is well known for suppressing lepidopteran and coleopteran pests, some strains are also toxic to dipterans, including different fruit flies.

B. thuringiensis strains are also active against Hymenoptera, Homoptera, Orthoptera, and Mallophaga insect orders and against other noninsect organisms like nematodes, mites, and protozoa have been isolated (Crickmore *et al.*, 1998). *Bt*, a member of the *Bacillaceae* family, is rod-shaped, motile, Gram-positive, and a facultative anaerobe. It is also a spore-forming bacterium, typically measuring 1.0-1.2 μm wide and 3.0-5.0 μm long. According to the List of Prokaryotic names with Standing in Nomenclature (LPSN) server (www.bacterio.net), there are 25 subspecies of *B. thuringiensis* (accessed on 25 April 2025). As a spore-forming bacterium, it can create extremely strong endospores that help it to survive in very hostile environments, including heat, radiation, and desiccation. Common habitats include soil, plant surfaces, and aquatic ecosystems, all of which feature *B. thuringiensis* (Bravo *et al.*, 2017; Nicholson *et al.*, 2000; Schnepf *et al.*, 1998). This bacterium's spores are ellipsoidal, but they are primarily cylindrical and found in the central or paracentral region of the mother cell (Figure 2.1).

Research on *B. thuringiensis* (*Bt*) began in 1901 when Japanese biologist Ishiwata Shigetane first found the bacterium separated from sick silkworm larvae (*Bombyx mori*). First calling it *Bacillus sotto*, he noted its pathogenicity for insects (Ishiwata, 1901). German scientist Ernst Berliner separately rediscovered the bacterium in Thuringia province, Germany, from sick flour moth larvae (*Ephestia kuehniella*) ten years later, in 1911.

Berliner called it *Bacillus thuringiensis* and detailed its unusual ability to generate protein crystals during sporosis, which were subsequently connected to its insecticidal effects (Berliner, 1915).

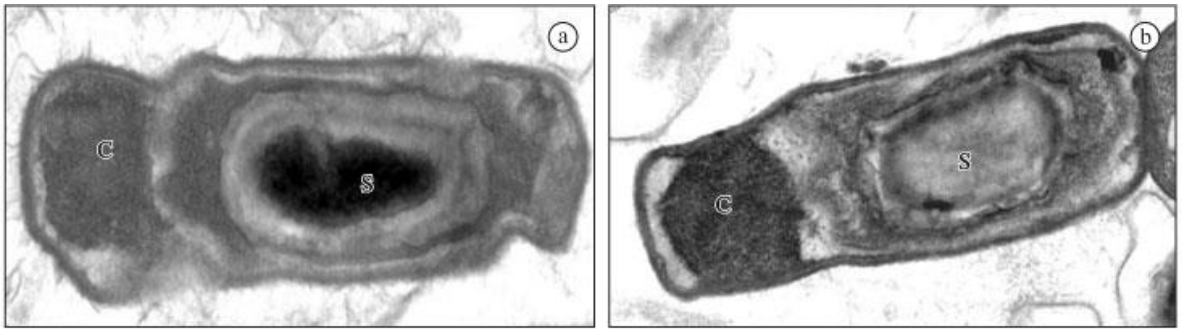


Figure 2.1: Transmission electron microscopy of *B. thuringiensis* isolates (a & b) s = spore, c = crystal (Fiuza *et al.*, 2012)

2.2 The history of *B. thuringiensis* research and its recent advances

In 1916, it was demonstrated that the toxicity arising from sporulated cultures was due to an endotoxin protein (Aoki & Chigasaki, 2016). Later, the re-isolation of the Berliner's strain led to the observation of an additional structure within the sporangia, distinct from the spore (Mattes, 1927). This structure, known as the parasporal crystal inclusion, was hypothesized to be responsible for insecticidal activity (Hannay, 1953). Subsequent studies confirmed this hypothesis, showing that ingestion of alkali-treated spores resulted in paralysis, septicemia, and ultimately death in target organisms (Angus, 1954).

Bt stayed a topic of little interest for many years, mostly under study for its function in insect disease. By the 1930s, researchers recognized its insecticidal properties, and France introduced the first commercial *Bt*-based biopesticide under the name Sporeine in 1938. This marked the beginning of its application in pest management. However, researchers started looking at its potential as a biological insecticide in the 1950s. *Bt*-based biopesticides were being commercially manufactured and applied in agriculture to control pests in crops such as cotton and corn by the 1960s (Dulmage, 1970).

During the 1980s and 1990s, improvements in molecular biology assisted in the cloning and insertion of cry genes into plant genomes, paving the method for the development of genetically modified crops. In 1996, the very first transgenic *Bt* crops were commercially released, including *Bt* cotton revealing Cry1Ac and *Bt* maize expressing Cry1Ab. These

geneticallycrafted crops provided season-long security versus major insect pests, therefore substantially reducing the dependence on artificial pesticide applications. Their adoption expanded quickly on a worldwide scale, with millions of hectares cultivated annually in countries such as the United States, India, and China (Abbas, 2018). Advances in genomics, protein engineering, and eco-friendly research studies have pressed recent *B. thuringiensis* (*Bt*) research into many innovative instructions. Broad utilizes follow from determining brand-new *Bt* strains and toxins, such as Cry proteins efficient versus nematodes and other non-insect pests (Soberón *et al.*, 2007). Protein engineering methods have actually made it possible for the modification of *Bt* toxins, consisting of the generation of hybrid Cry proteins through which efficacy has been enhanced, target ranges have been increased, and insect resistance has been challenged.

Researchers have been looking into ways to manage resistance, like gene pyramiding (stacking many *Bt* genes in crops) and refugia (keeping areas of non-*Bt* crops) to slow down the development of resistance (Tabashnik *et al.*, 2013). Beyond agriculture, *Bt* toxins are under research for non-agricultural uses, including public health, where *Bt* subsp. *israelensis* is extensively deployed to eradicate mosquito larvae, so helping to prevent vector-borne diseases, like malaria and dengue (Benelli *et al.*, 2016). Additionally, constant environmental and security research exposes that *Bt* contaminants are rather selective in targeting insects, hence providing low damage to non-target types, including people and useful insects (Mendelsohn *et al.*, 2003). These successes highlight *Bt*'s flexibility and pledge in tackling environmental sustainability, public health, and farming barriers worldwide.

2.3 Beneficial applications of *B. thuringiensis*

Over the last several years, *B. thuringiensis* (*Bt*) has actually gotten worldwide acknowledgment not simply for its insecticidal properties nevertheless similarly for its broad possible applications in agriculture, biotechnology, and public health. Its flexibility establishes from the diversity of toxins it produces and its compatibility with environment-friendly insect control practices. Among the most popular applications of *B. thuringiensis* (*Bt*) is its use as a biopesticide for the management of insect pests in farming. *Bt*-based formulations have demonstrated efficacy against several major insect orders, including Lepidoptera (e.g., *Helicoverpa armigera*, *Plutella xylostella*), Diptera (e.g., *Aedes aegypti*, *Culex quinquefasciatus), and Coleoptera (e.g.,* Leptinotarsa decemlineata). People often utilize these biopesticide products as foliar sprays, soil

drenches, or bait formulas. They are really beneficial for growing veggies, fruits, pulses, and cereal grains.

A substantial turning point in farming biotechnology was the development of genetically customized (GM) crops crafted to reveal cry genes stemmed from *B. thuringiensis* (*Bt*). Noteworthy examples consist of *Bt* cotton, exposing cry1Ac for the control of bollworms; *Bt* maize, exposing cry1Ab and cry2Ab to combat stem borers and earworms; and *Bt* brinjal (eggplant), exposing cry1Ac, which was substantially advertised in Bangladesh. These transgenic crops supply season-long security versus targeted insect pests, substantially reduce the requirement for chemical pesticide applications, and contribute to enhanced crop yields and increased farmer income. Particularly in agriculture and pest control, *B. thuringiensis* (*Bt*) is becoming ever more vital. Throughout sporosis, it produces crystalline endotoxins (Cry and Cyt proteins) with extremely particular insecticidal action against a number of insect larvae, including Lepidoptera, Diptera, and Coleoptera species (Sanahuja *et al.*, 2011). Since these toxins disrupt the midgut epithelium of susceptible insects, which results in their death, *Bt* is a green alternative to chemical pesticides (E. Schnepf *et al.*, 1998). Genetically modified *Bt* has also been developed into crops, including *Bt* cotton and *Bt* maize, to confer resistance against insect pests, so greatly reducing the demand for synthetic pesticides and improving crop yield (ISAAA, 2017).

Bacillus thuringiensis (*Bt)*, particularly the subspecies *B. thuringiensis israelensis* (*Bti*), has played a pivotal role in public health initiatives aimed at controlling disease vectors such as *Aedes* (responsible for transmitting dengue and Zika viruses), *Anopheles* (malaria), and *Culex* species (filariasis and West Nile virus). *Bti*-based formulations, which contain a combination of Cry4, Cry11, and Cyt toxins, are commonly applied to mosquito breeding habitats, including stagnant water bodies, rice paddies, and urban drainage systems. These biolarvicides are extremely valued due to their specificity in targeting mosquito larvae, minimal environmental impact on non-target organisms such as fish, amphibians, and people, and their lowered risk of resistance development owing to the existence of numerous toxic substance components. Beyond farming, *Bt*-based solutions are utilized in vector control programs to target *Aedes*, *Anopheles*, and *Culex* larvae, hence preventing mosquito-borne disease (Lacey *et al.*, 2015). Under research for applications in aquaculture, agriculture, and even medication is *Bt*, where its antimicrobial homes and possible bioinsecticide worth point to sustainable choices (Bravo *et al.*, 2011). These numerous usages highlight the environmentally friendly and financial value of *B. thuringiensis* in modern-day biotechnology. The biodegradability and advantageous security

profile of *B. thuringiensis* (*Bt*) have in fact developed it as a crucial element in organic farming. It fulfills the requirements for ecologically sustainable insect management by avoiding groundwater contamination, showing no toxicity to birds, mammals, or useful arthropods, and supporting the health of pollinators. As an outcome, *Bt* contributes considerably to climate-resilient agricultural practices and help in reducing the carbon footprint associated to crop security methods.

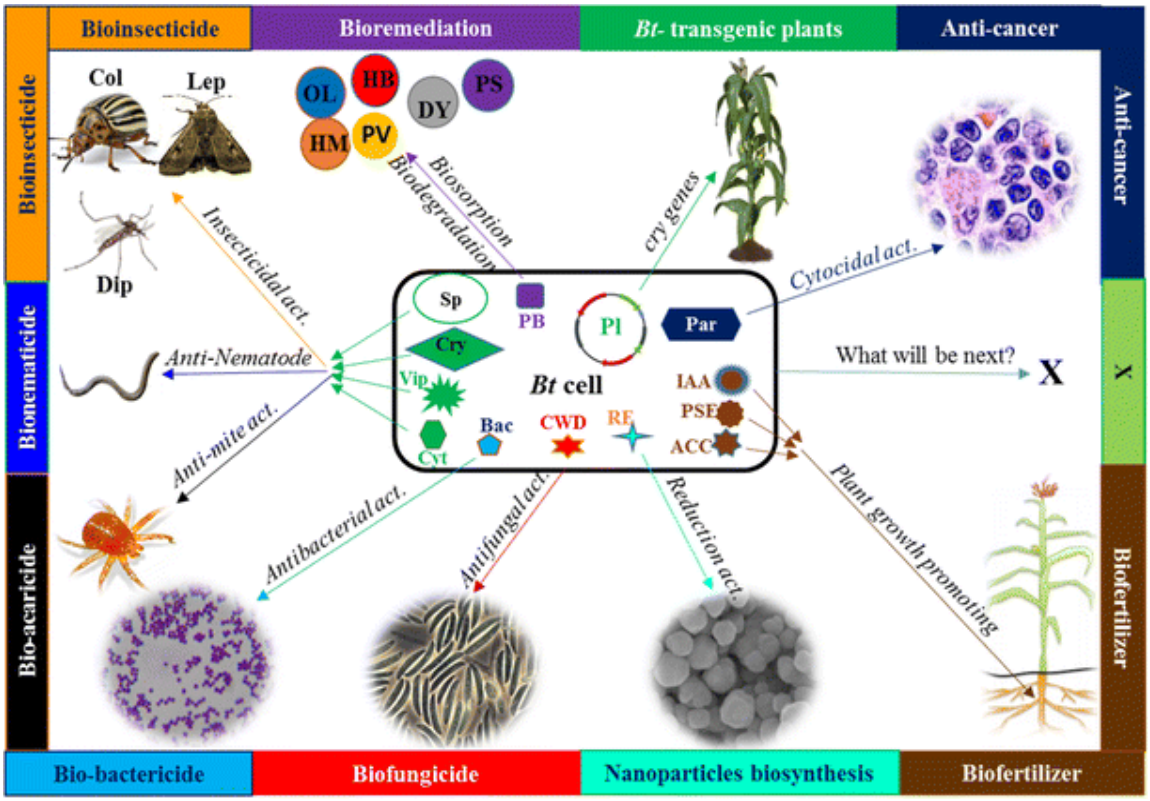


Figure 2.2: Diagrammatic overview of *Bt*'s applications, including transgenic plants, bioremediation, bioinsecticidal activity, nanoparticle synthesis, biofertilizers, and potential to anticancer properties (Jouzani *et al.*, 2017).

2.4 The ecology and diversity of *B. thuringiensis*

Bacillus thuringiensis (*Bt*) is a good biological control agent because it has a lot of different genes and lives in a lot of different places. Its capability to adjust to numerous environments, relate to varied organisms, and produce a broad spectrum of contaminants has actually made it a versatile gamer in agricultural and natural communities (Ragasruthi *et al.*, 2024).

B. thuringiensis (*Bt*) has been separated from a wide variety of ecological specific niches, including soil, the phyllosphere, infected insects, kept items, decline disposal websites, and the excreta of herbivorous animals. Significantly, studies have actually revealed that approximately

30--100% of spore-forming bacteria present in the phyllosphere are determined as *B. thuringiensis*. This extensive event highlights the ecological flexibility and hereditary diversity of *B. thuringiensis*, which varies substantially depending upon the source of seclusion (Argôlo-Filho & Loguercio, 2013). An analysis of 27,000 isolates gathered from 100 soil samples all over the world showed that *B. thuringiensis* could be separated everywhere, including tundra, desert, and beach environments (Thammasittirong & Attathom, 2008).

Researchers looking at rice field soils observed that *Bt* stress there can be quite various; their crystals have various shapes, they respond differently to prescription antibiotics, and they bring various kinds of plasmids (Das & Dangar, 2007). Some soil samples even revealed *Bt* strains that are much better matched to specific temperatures or may act like different kinds of pathogens (Swiecicka *et al.*, 2013). There is a lot of variety in *Bt* found in soil, not just in the shapes of the crystals, but also in how they react to prescription antibiotics and other biochemical traits (Chatterjee *et al.*, 2007). People are still arguing about what *Bt* really does in the wild. Some see it generally as a pathogen, while others believe it's more like a survivor that makes the most of whatever environment it ends up in. Recent research study even hints that nematode may assist spread *Bt* around, which could be why it shows up in locations where there aren't numerous insects (Ruan *et al.*, 2015). All in all, *Bt* seems incredibly good at adapting and finding a way to fit into lots of different ecosystems.

Bt has also been isolated from the gut of various insects, where it may exist as a commensal or opportunistic pathogen. In plants, *Bt* can function as a rhizobacterium, enhancing root health or suppressing soil-borne pathogens indirectly through antimicrobial compounds such as Zwittermicin A (Li *et al.*, 2020). These associations show that *Bt* is not merely an insect killer but a microbial player in complex ecological interactions.

2.5 Systematic position of *B. thuringiensis*

B. thuringiensis is classified within the domain of Bacteria and follows this hierarchy:

Domain: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: *Bacillus*
Species: *Bacillus thuringiensis*

Bt is really closely related to *B. cereus* and *B. anthracis*; they share over 99% similarity in their 16S rRNA genes. What sets *Bt* apart, though, is that it makes special parasporal crystals (Cry toxins) during sporulation, and these are carried on plasmids (Rasko *et al.*, 2005). Traditionally, scientists used serotyping, based on differences in the flagellar (H) antigens, to sort *Bt* into more than 100 different groups, or serovars. For example, subsp. *kurstaki* (H3a3b) targets moths and butterflies (Lepidoptera), while subsp. *israelensis* (H14) is great against flies and mosquitoes (Diptera) (Porcar *et al.*, 1999). Right now, scientists have better tools, like plasmid profiling and whole-genome sequencing, to dig deeper into *Bt.* What they've noticed is that most of the important toxin genes, like *cry*, *vip*, and *cyt*, aren't sitting on the main chromosome. They're hanging out on plasmids instead. Since plasmids can move around pretty easily, these toxin genes can jump from one strain to another. That's probably why it's sometimes tricky to draw clear lines between different species in the *B. cereus* group (Liu *et al.*, 2015). Instead of just looking at serovars, researchers often classify *Bt* strains by the types of toxins they produce, Cry proteins (which are divided into 75 families), Cyt toxins, and Vip proteins, because these determine which pest they target (Chakroun *et al.*, 2016). In terms of practical use, *Bt* strains are grouped by the pests they control, like those that specifically target caterpillars (subsp. *kurstaki*), or mosquitoes (subsp. *israelensis*), or even genetically modified strains that can hit multiple pests at once (Sanahuja *et al.*, 2011). *Bt* has been a huge help in pest control, but pinning down exactly how to classify it hasn't been easy. It's so closely related to *B. cereus* and *B. anthracis* that scientists still argue about where to draw the line (Helgason *et al.*, 2000). As researchers keep digging into its genome, and figuring out more about its toxins, the way we sort and name *Bt* keeps changing too.

2.6 The toxins of *B. thuringiensis*

Bacillus thuringiensis (*Bt*) produces a range of insecticidal proteins that serve as the main system of its entomopathogenic activity. These impurities, typically Cry (crystal), Cyt (cytolytic), and Vip (vegetative insecticidal proteins), are accountable for *Bt*'s capability to kill particular insect larvae. They are manufactured throughout numerous stages of bacterial development and act through different modes of action, primarily targeting the insect midgut epithelium. The genes encoding these pollutants are usually found on big plasmids, contributing to the diversity and specificity of *Bt* strains (Kumar *et al.*, 1996).

Vegetative cellular division and spore generation are the two separate stages of *Bt* cell development. Seven different stages are involved in the development of the spore and crystal. Following stage II of sporulation, the insecticidal proteins are synthesized and accumulate in the

mother cell as a crystal, which can make up as much as 25% of the dry weight of the sporulated cells (Bulla *et al.*, 1980). These crystals might be spherical, rectangular, cuboidal, flat rhomboid, bipyramidal, or pyramidal. A bipyramidal crystal is the most prevalent type. The crystals may contain one or more Cry proteins, also known as delta-endotoxins. These proteins have molecular weights ranging from 30 to 140 kDa and, when consumed by target pests, primarily insects, are transformed into poisonous peptides.

B. thuringiensis produces varieties of toxins, and crystal proteins (Cry) are the major group (Cooper, 1994). They aggregate into crystalline inclusions that are solubilized and activated in the alkaline environment of the insect midgut. Activated toxins bind to specific receptors on the epithelial cells, leading to pore formation, cell lysis, and ultimately, insect death. Cry proteins are classified into different families (Cry1, Cry2, Cry3, etc.) based on their amino acid sequence similarity and insecticidal spectrum. Cry1 and Cry2 toxins are particularly effective against lepidopteran pests, while Cry3 proteins target coleopterans. Cyt proteins (cytolytic δ-endotoxins) are also produced during sporulation but are structurally and functionally distinct from Cry proteins. These proteins exhibit broad-spectrum activity, primarily against Dipteran insects such as mosquitoes. Unlike Cry toxins, Cyt proteins do not require specific receptors for binding; instead, they insert directly into the cell membrane, disrupting its integrity through detergent-like action (Federici *et al.*, 2003).

Cry and Cyt proteins are made throughout sporulation, whereas vegetative insecticidal proteins (Vip) are produced throughout the vegetative development stage. Vip proteins supply an alternate technique of action by targeting the midgut cells of lepidopteran pests, particularly Vip1 and Vip2 (binary toxins) and Vip3 (single chain) (Estruch *et al.*, 1996). The most extensively investigated element, Vip3A, has been contributed to genetically engineered crops like *Bt* maize and cotton, offering extended defense against pests and delaying the advancement of resistance to Cry proteins (Chakroun *et al.*, 2016).

Both Cry and Cyt toxic substances are effective versus insects, nematodes, and human cancer cells. Researchers studied the structure and mode of action of *B. thuringiensis* toxic substances thoroughly and revealed three main classifications: three-domain type α-PFTs, Cyt contaminant type β-PFTs, and aerolysin type β-PFTs (Xu *et al.*, 2014). The toxins of *B. thuringiensis* subsp. *israelensis* (*Bti*), residing in 4 major and 2 minor polypeptides encoded by particular genes, have larvicidal activity, and they are particularly effective versus mosquito and black fly larvae. The mix of Cry and Cyt toxins in *Bti* develops a synergistic effect, avoiding resistance

development in target organisms (Ben-Dov, 2014). Additionally, throughout vegetative development, *B. thuringiensis* produces other insecticidal proteins, called Vips and Sip (Palma *et al.*, 2014).

2.7 Sporulation and parasporal inclusion formation of *B. thuringiensis*

B. thuringiensis produces insecticidal parasporal crystals throughout sporulation. Contrary to earlier hypotheses, these crystals form independently of the forespore septum, exosporium, or mesosomes. Crystal development begins when engulfment starts and is nearly total by the time the exosporium types. The synthesis of the crystal protein happens simultaneously with the look of crystal antigens. Sporulation is a crucial stage in the life process of *B. thuringiensis* (*Bt*), during which the bacterium undergoes a series of biochemical and morphological changes to produce resistant endospores and insecticidal parasporal crystals. These crystals, formed alongside spores, are the main representatives of insect toxicity and are accountable for the success of *Bt* as a microbial biopesticide (Bechtel & Bulla, 1976).

Sporulation is activated by ecological tension, a lot of typically the exhaustion of necessary nutrients such as nitrogen, phosphorus, or carbon. When triggered, the bacterium shifts from active vegetative development into an inactive and resistant spore state. This process includes uneven cellular division, forming a forespore and a mom cell, each undergoing specific developmental programs (Hilbert & Piggot, 2004). Throughout the late sporulation phases (especially phase V), the mom cell of *Bt* manufactures insecticidal crystal proteins (generally Cry and Cyt). These proteins aggregate into parasporal inclusions surrounding to the establishing spore. The shape, size, and protein structure of these crystals vary depending on the strain and the specific Cry/Cyt genes expressed. These additions might appear bipyramidal, cuboidal, spherical, or irregular under electron microscopy. Sporulation and crystal protein synthesis are tightly controlled by a waterfall of sigma aspects and transcriptional regulators. Secret sigma elements included include SigF, Sige, sigk, and sigg, which are sequentially set off in the forespore and mom cell compartments. In addition, *Bt*-specific regulators such as Spo0a, cry, and sigh gene promoters include to the precise timing of spore and crystal development (Park *et al.*, 2022).

Upon conclusion of spore and crystal formation, the mom cell goes through autolysis, launching both aspects into the environment. This process is necessary for *Bt*'s function as a biocontrol agent, as the released crystals can stay steady in soil and on other surfaces till consumed by a susceptible insect host. The concurrent production of crystals and spores makes sure both the

survival of the bacteria under unwanted conditions and efficient dissemination of insecticidal toxic substances. This dual method boosts the ecological physical conditioning of *Bt* in agricultural environments, where sporulation can be triggered by changing soil or plant nutrient conditions (McGaughey *et al.,* 1998).

Proteases, amino acid metabolism, and the usage of kept poly-β-hydroxybutyrate and acetoin all aid to metabolically manage crystal development. In addition, the pentose phosphate path, a changed tricarboxylic acid cycle, and overexpression of F0F1-ATPase subunits all add to energy production required for both sporulation and crystal development (Wang *et al.*, 2013). Serological studies show that crystal antigens are losing out on in vegetative cells however emerge throughout sporulation, correlating with both crystal production and advancement (Monro, 1961).

2.8 The morphological diversity of toxicidal inclusions

The trademark function of *B. thuringiensis* (*Bt*) is the production of proteinaceous parasporal inclusions throughout sporulation. These crystalline structures, composed generally of Cry and Cyt pollutants, show a big series of morphological types. The diversity in shape, size, and ultrastructure of these additions is carefully related to the kind of insecticidal protein produced, the particular *Bt* pressure, and its target insect group. The morphology of crystalline inclusions produced by *B. thuringiensis* differs depending upon the toxic substance household and serotype. Bipyramidal crystals are most commonly related to Cry1 proteins, which are generally produced by *Bt kurstaki* and *Bt aizawai* pressures and show insecticidal activity versus Lepidoptera. Cuboidal crystals are formed by stress revealing Cry2 and particular Cyt impurities, which typically target both Lepidopteran and Dipteran insects. Irregularly or spherical shaped crystals are particular of Cry11, cyt, and cry4 contaminants produced by *Bt israelensis*, and are mainly reputable against Dipteran insects such as blackflies and mosquitoes. In addition, flat rectangular or composite crystals are observed in pressures such as *Bt tenebrionis*, which are active against Coleopteran pest (Gill *et al.*, 1992).

The toxicidal inclusions made by *B. thuringiensis* have different effects on insects depending on their shape. Both cuboidal inclusions, which are harmful to dipterans, and bipyramidal inclusions, which are harmful to lepidopterans, develop in the *kurstaki* HD-1 strain. Confirming their varied toxicity, chymotrypsin-like P-III, a protease discovered in silkworm intestine, can dissolve bipyramidal inclusions but not cuboidal ones. Three kinds of inclusions were found in *B. thuringiensis israelensis*; among the type 2 inclusions was a 65 kDa protein showing limited

toxicity to mosquitoes (Ibarra *et al.*, 1986). Another entomopathogenic bacteria, *Photorhabdus luminescens*, generates two different crystalline inclusions: rectangular (type 1) and bipyramidal (type 2), each made of one single 11 kDa protein subunit. These inclusions show up in late exponential growth and account for 40% of the stationary phase's cellular protein; they are not harmful to *Galleria mellonella* larvae (Bowen & Ensign, 2001).

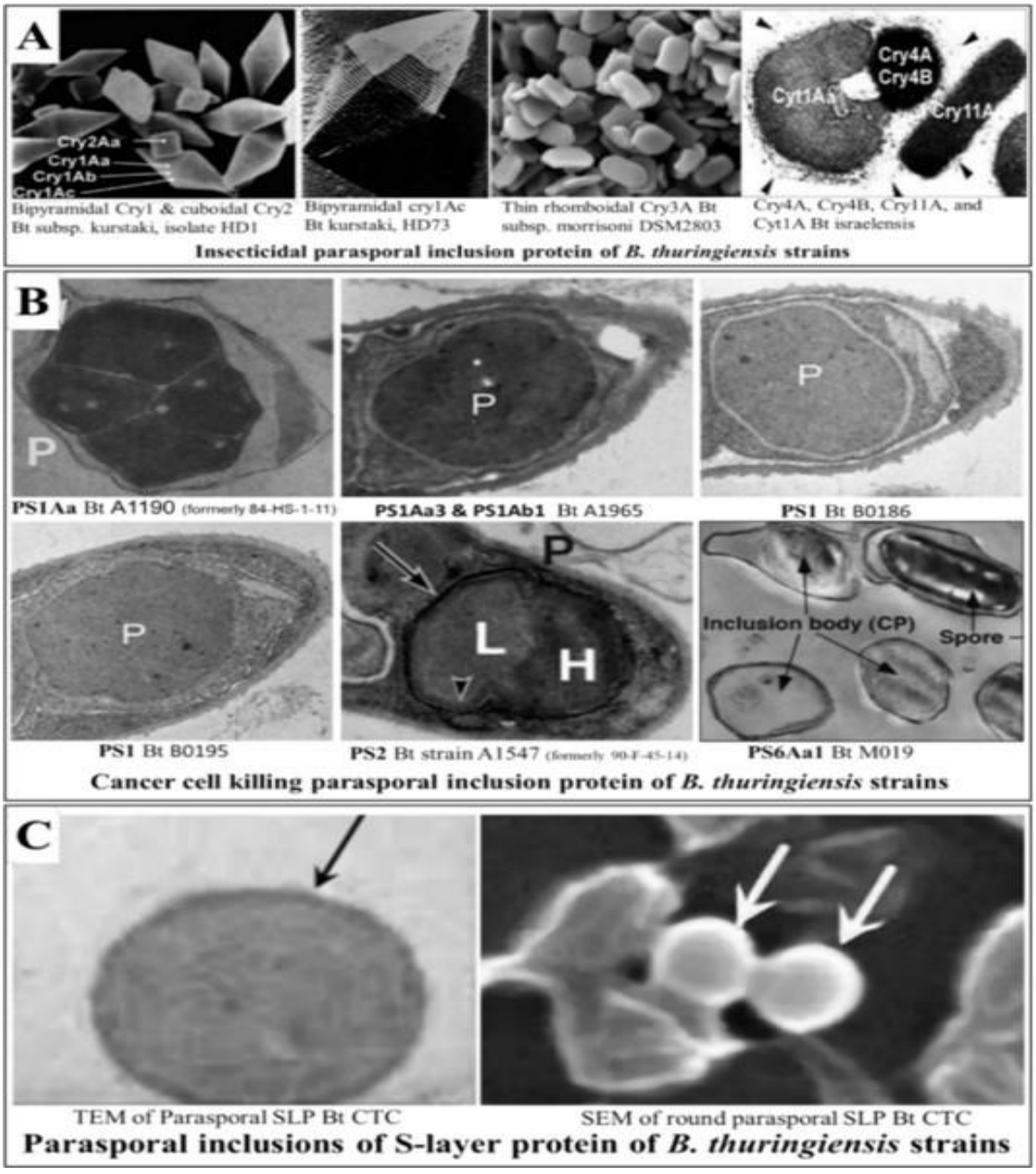


Figure 2.3: Scanning and transmission electron microscopic image displaying different crystal shapes of *Bt* strains. (A) insecticidal inclusions (Sawaya *et al.*, 2014); (B) anticancer inclusions(Nagamatsu *et al.*, 2010); (C) S-layer inclusions (Zhu *et al.*, 2008).

Morphological diversity in crystals contributes to *Bt*'s host specificity. The structural form affects how easily a toxin is solubilized and activated in the insect gut, impacting the level and

speed of toxicity. Therefore, understanding and selecting strains with optimized crystal morphology is essential for developing efficient and robust *Bt*-based biopesticides (Knowles, 1994).

2.9 Properties of toxocidal inclusions in *B. thuringiensis*

B. thuringiensis generates parasporal inclusions carrying insecticidal proteins termed δ-endotoxins or Cry proteins. These inclusions have diverse shapes, such as bipyramidal or cuboidal, and comprise some toxins with distinct molecular weights (Samasanti *et al.*, 1986). The toxicity and selectivity of these inclusions rely on their protein composition and solubility in the insect gut (Aronson *et al.*, 1991). Upon ingestion, the inclusions dissolve in the midgut, generating protoxins that are activated by proteases. The active poisons subsequently harm the midgut epithelium, culminating in insect death. Different Cry proteins target various pest orders; for example, CryIA(b) and CryIC are efficacious versus lepidopteran pests, while CryIVD (72 kDa) is destructive to mosquitoes. Understanding the molecular basis of contaminant selectivity and effectiveness is crucial for strengthening *B. thuringiensis* as a biological insecticide (Chang *et al.*, 1992).

The parasporal crystalline inclusions produced by *B. thuringiensis* (*Bt*) are the main elements accountable for its insecticidal action. These crystals are composed primarily of δ-endotoxins, especially Cry and Cyt proteins, which display high insect specificity and activity. The physicochemical and biological residential or commercial properties of these inclusions are crucial to understanding how *Bt* exerts its toxic effects and how it can be optimized as a biopesticide. The primary constituents of the crystalline inclusions are big, insoluble protoxins varying from 60 to 140 kDa. These protoxins are synthesized throughout sporulation and are packaged into extremely purchased protein crystals. Cry proteins normally comprise the significant part, although Cyt proteins might also be present, specifically in Dipteran-active stress such as *Bt israelensis* (E. Schnepf *et al.*, 1998). Some crystals may include mixes of several Cry proteins or Cry and Cyt proteins, depending upon the strain's hereditary content (Crickmore *et al.*, 1998). Among the specifying features of *Bt* crystals is their pH-dependent solubility. They stay steady under slightly acidic and neutral conditions however liquify quickly in the alkaline environment of the insect midgut (pH 9-11). This solubility sets off the activation process, where midgut proteases cleave the protoxins into active toxic substance pieces that can bind to epithelial receptors (Gill *et al.*, 1992). The purchased structure of the crystal gives resistance to environmental destruction. The compact packaging of the protein subunits makes the inclusions extremely durable, enabling *Bt* to continue soil and foliage for prolonged periods.

This stability also makes sure that *Bt* preserves insecticidal potential even under varying field conditions (Bravo *et al.*, 2011). Nevertheless, excessive UV direct exposure can denature crystal proteins, which is why UV protectants are sometimes contributed to industrial formulations. The toxic activity of *Bt* crystals depends not just on the Cry or Cyt protein composition but also on the crystal's ability to liquify and launch active toxic substances at the correct time and area. Uniqueness is provided by both the molecular structure of the contaminant and the existence of suitable receptors in the insect midgut. As an outcome, different crystals show selective toxicity towards unique insect orders, making *Bt* a safe choice for non-target organisms (Federici *et al.*, 2003; Knowles, 1994).

2.10 Solubilization and activation of Cry toxin

The insecticidal action of Cry proteins from *B. thuringiensis* (*Bt*) is highly depending on their solubilization and activation in the insect gut. These procedures are necessary actions that transform the non-active protoxins within the parasporal crystals into active toxic substances capable of binding to midgut epithelial cells and causing larval death. Comprehending the physicochemical conditions that drive these transformations is essential to enhancing *Bt*'s efficiency as a biopesticide. Cry proteins are at first produced in an insoluble crystalline type during sporulation. These protoxins remain steady in the external environment up until ingested by a vulnerable insect host. In the larval mid gut especially in Lepidoptera and Diptera the alkaline pH (typically ranging from pH 9 to 11) facilitates the dissolution of the crystal matrix. This pH-dependent solubility ensures that Cry toxins remain inactive outside the host and become activated only in the target insect, thereby limiting off-target effects (Gill *et al.*, 1992; Schnepf *et al.*, 1998).

Once solubilized, the protoxin (generally 130–140 kDa) undergoes enzymatic cleavage by midgut proteases such as trypsin-like or chymotrypsin-like enzymes. These cleavages remove non-toxic segments from the N- and C-terminal regions, generating an active core toxin of approximately 60–70 kDa. The activated Cry toxin adopts a three-domain structure crucial for receptor binding and membrane insertion (Bravo *et al.*, 2007). Proteolytic processing is highly specific and can influence the potency of the toxin. Improper activation may result in reduced binding affinity or incomplete pore formation, thereby weakening insecticidal effects. Additionally, some insects may possess gut proteases that degrade Cry toxins nonspecifically, leading to natural resistance (Oppert *et al.*, 2000). The solubilization efficiency of Cry protoxins is affected not only by pH but also by the presence of reducing agents and divalent cations such

as Ca^{2+} and Mg^{2+}. These ions stabilize certain structural regions of the toxin and influence folding and membrane interaction. For example, Ca^{2+} has been reported to enhance the binding of Cry1 toxins to brush border membrane vesicles in Lepidopteran midguts (Knowles, 1994). Any disruption in the solubilization or activation steps can contribute to reduced *Bt* efficacy. Field-evolved resistance in insects often involves changes in gut protease expression or gut pH, which prevent proper Cry toxin activation. Therefore, engineering Cry variants that activate under broader physiological conditions is a current strategy for overcoming resistance (Pardo-López *et al.*, 2013; Tabashnik *et al.*, 2013).

Cry toxins of *Bacillus thuringiensis* are produced as inactive protoxins as crystalline inclusions and need to be solubilized and activated before they are effective as insecticides. Solubilization occurs in the alkalinity of the insect midgut, where the crystalline inclusions are dissolved and the protoxins are processed by midgut proteases. Activation involves a proteolytic autocatalyzed reaction that transforms the protoxins into smaller fragments of toxin that are competent enough for binding on specific receptors on the surface of midgut epithelial cell surface such as cadherins, aminopeptidases and alkaline phosphatases that results in pore formation and cell lysis (Liu *et al.*, 2021; López-Molina *et al.*, 2021; Sakdee *et al.*, 2023). Binding of Cry toxins on these receptors plays a crucial role in insecticidal activity by permitting toxin insertion into the cell membrane and pore development that results in cell ion imbalance and cell death (Pardo-López *et al.*, 2013).

Resistance advancement in the pest involves mechanisms such as loss of affinity of the toxin for binding due to anomalies of the toxic substance binding site of the receptor, guideline of expression of the receptor and the role of detoxifying enzymes (Guo *et al.*, 2020). It is important that these mechanisms are illuminated as a basis for establishing brand-new steps for breaking down the resistance and boosting the efficacy of Cry toxic substance as an insecticide. Performance of aggregation and activation and aggregation might vary among various Cry toxic substances and insect types, and hence the overall toxicity is figured out.

2.11 Cry toxins diversity in *B. thuringiensis* strains

One of the most remarkable functions of *Bacillus thuringiensis* (*Bt*) is its huge selection of crystal (Cry) proteins, showing its evolutionary complexity and adaptability as a biocontrol agent. These insecticidal proteins are encoded by cry genes, many of which are situated on big, transmissible plasmids. *B. thuringiensis* pressures show a large variety of Cry toxins, with over 789 distinct cry genes currently determined (Nair *et al.*, 2018). Even though they are in the pro-toxin state, the majority of commercial plants express the same *B. thuringiensis* genes found in bioinsecticides. The Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, and Cry1D proteins are found in the main bioinsecticides that are sold globally. The proteins of the families Cry1A, Cry1C, and Cry1F are the primary proteins utilized in pest management, as evidenced by their presence in transformed products and plants (Baktavachalam *et al.*, 2015).

The diversity of Cry toxins contributes to the high specificity of *Bt* against various insect orders and plays a crucial role in the development of targeted and environmentally safe biopesticides. Cry1Ba, Cry2A, Cry4Aa, Cry10Aa, Cry11Aa, are the effective key toxins against Tephritids fruit flies. Cry4Aa, Cry11Aa very much effective against Tephritids fruit flies specifically to *B. dorsalis* resulting 60 to 80 percent larval mortality.

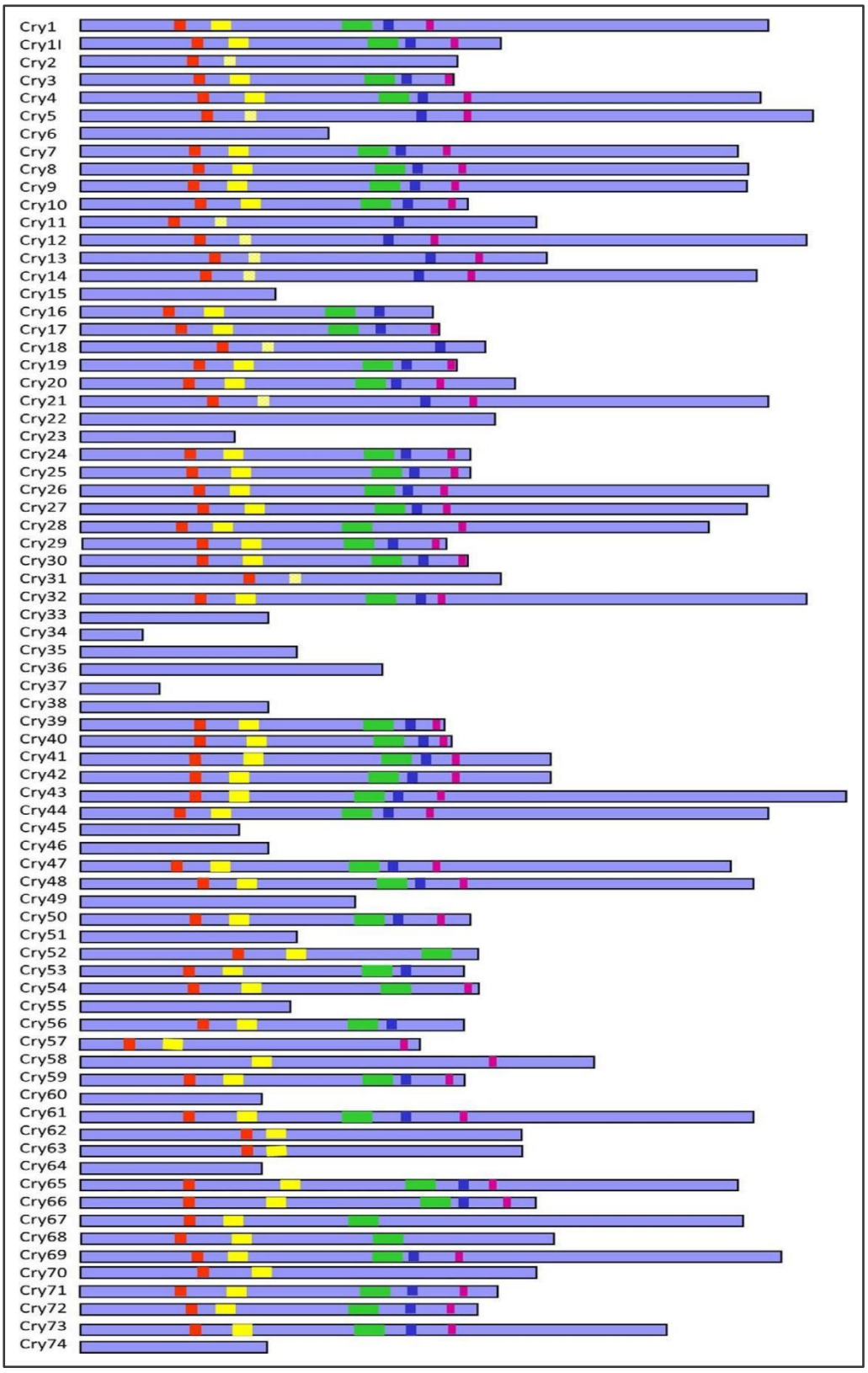


Figure 2.4: Diagrammatic representations of the diversity of *Bt* Cry toxins. Length of each toxin is clearly shown, and the five conserved blocks are remarked with colored inserts (Adang *et al.*, 2014).

This diverse pool was pictured with the discovery of some unusual strains, such as the H3 strain from Lebanese soil which produces 11 unique Cry proteins, thus exhibiting the capabilities of this bacterium with the application of further insecticidal activities (Fayad *et al.*, 2020). The broad-spectrum insecticidal activity of certain strains, like *B. thuringiensis* serovar galleriae BTG, is attributed to the presence of multiple cry genes, enabling them to target a wide range of insect orders, including Lepidoptera, Coleoptera, Diptera, and Hemiptera (Arsov *et al.*, 2023). The circulation of lepidopteran-specific contaminant genes reveals distinctions between tension, with particular profiles being more plentiful in specific sources (Sauka & Benintende, 2017). Structural and evolutionary research study studies have actually shown considerable variety within Cry proteins, which permits targeting distinct insect orders and the possibility of crossing orders in terms of toxicity (Das *et al.*, 2021). Genomic research studies even more exposed the existence of partial Cry gene series, which combine to create a new active Cry toxic substance, suggesting that partial genes are an appealing source for discovering unique Cry contaminants (Sajid *et al.*, 2018). Such genetic variability would work in creating strains with particular insecticidal homes and in handling insect resistance (Queiroz *et al.*, 2023). The development of insect resistance to Cry pollutants such as Cry1A, Cry1F, Cry3bb, cry2a, and cry1c represents a significant restriction to the continual efficiency of *B. thuringiensis* (*Bt*)-based crops and biopesticide solutions (Carrière *et al.*, 2016). The range of Cry toxins in tension of *B. thuringiensis* represents its increased capacity as a bioinsecticide while likewise providing insights towards the improvement of brand-new pest control avenues.

2.12 The 3-D crystallographic structure of the three-domain Cry toxin

The 3-D crystallographic structure of three-domain Cry hazardous substances, which are created by *B. thuringiensis*, is necessary for comprehending their insecticidal activity. The harmful compounds are globular particles and consist of three domains (Figure 2.5). The N-terminal domain, a plan of alpha-helices that is accountable for membrane insertion and pore development; the middle domain, which is largely included beta-sheets and is accountable for receptor binding; and the C-terminal domain, which helps in the stability of the overall pollutant (Thamwiriyasati *et al.*, 2022). The alpha-helical bundle of the N-terminal domain, in specific helix alpha4, performs a necessary function in the development of transmembrane pores, leading to cell lysis in target insects (Torres *et al.*, 2023). The beta-sheet conformation of the middle domain is essential to the identification and binding of specific receptors on the surface area of insect cells, determining the uniqueness of the toxin (Boonserm *et al.*, 2005).

Structural studies have actually shown that domain swaps and recombination occasions are accountable for the diversity and uniqueness of Cry contaminants, allowing them to adapt to diverse insect targets (Shikov *et al.*, 2021). Sophisticated methods such as X-ray crystallography and Alphafold-2 have yielded information on these structures, while challenges persist in the complete elucidation of the kinetics of membrane insertion and pore formation (Huang *et al.*, 2016). Elucidating these structural aspects is critical for the design of more effective biopesticides. Insects have evolved several resistance mechanisms, such as receptor gene mutations; however, modified Cry toxins that can oligomerize in the absence of receptors hold potential in being able to overcome resistance.

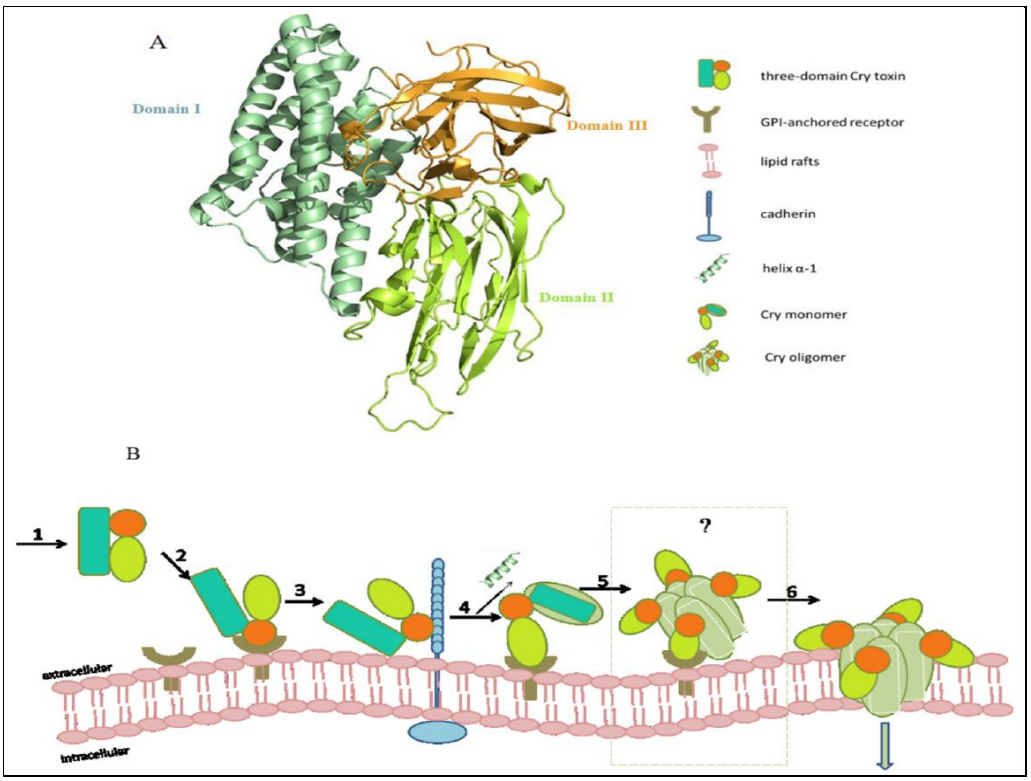


Figure 2.5: The pore formation model of three-domain Cry1A toxin in the midgut lipid rafts. **(A)** Ribbon diagram of Cry1Aa structure. Three domains are colored in pale green, lemon and bright orange, respectively; **(B)** Sequential steps of the pore formation model (Xu *et al.*, 2014)

Several three-domain Cry toxins produced by *Bacillus thuringiensis* (*Bt)* have been structurally resolved using X-ray crystallography, providing critical insights into their functional domains and insecticidal specificity. These toxins target a broad spectrum of insect orders, including Lepidoptera, Diptera, Coleoptera, and Nematoda, and are typically composed of three conserved domains: Domain I (involved in pore formation), Domain II (responsible for receptor binding and specificity), and Domain III (contributing to structural stability and additional receptor

interactions). Cry1Aa and Cry1Ac originate from *B. kurstaki* pressures and are triggered by trypsin. They are only effective versus Lepidopteran pests and have a comparable domain organization (Grochulski *et al.*, 1995).

2.13 The virulence aspects of B. thuringiensis

The entomopathogenic germs *B. thuringiensis* (*Bt*) is widely known around the world for its insecticidal homes through its Cry and Cyt proteins (Höfte & Whiteley, 1989). In addition to popular crystal (Cry) and cytolytic (Cyt) proteins, *B. thuringiensis* (*Bt*) has a different set of virulence aspects that add to its entomopathogenicity. These factors enhance the germs's capability to infect, continue, and get rid of insect hosts, regularly acting synergistically with the primary pollutants. While Cry and Cyt proteins begin the toxic response, secondary virulence aspects assist in immune suppression, tissue wear and tear, and nutrient acquisition within the host (Raymond *et al.*, 2010).

Bt brings numerous virulence aspects that include Vip proteins, enterotoxins, hemolysins, phospholipases, proteases, and degradative enzymes (Vilas-Bôas *et al.*, 2012). Such qualities enable it to colonize insect hosts effectively while preventing immune reactions and ruining the extracellular matrix. The expression of numerous virulence elements is managed by PlcR, a pleiotropic transcriptional activator found in the *B. cereus* group (Agaisse *et al.*, 1999). PlcR controls cell-surface proteins, enterotoxins, and degradative enzymes, and these genes are spread throughout the chromosome rather of being clustered in a pathogenicity island. Thus, this major variety of virulence characteristics justifies the host uniqueness and the ecological specific niche of *B. thuringiensis* (Malovichko *et al.*, 2019).

Bt can produce hemolysins such as hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and cytotoxin K (CytK), all of which are likewise found in *B. cereus*. These components can lyse pest and mammalian cells, destructive epithelial barriers, and promoting systemic infection. Though mainly studied in the context of intestinal condition in people, they may also play an encouraging function in pest pathogenesis by destructive gut or hemocoel tissues following epithelial disruption by Cry toxins (Molva *et al.*, 2009). In addition, chitinases may promote nutrient release from dead host tissue, supporting bacterial replication. *Bt* produces chitinases during infection to breach these barriers and enhance toxin penetration. These enzymes may help with Cry toxin access to midgut epithelial receptors by partially breaking down the protective peritrophic matrix (Arora *et al.*, 2003).

B. thuringiensis (*Bt*) pressures produce a series of extracellular proteases, notably metalloproteases such as InhA and serine proteases like camelysin, which play vital roles in the pathogenesis of insect hosts. These enzymes contribute to the overall insecticidal activity of *Bt* by deteriorating host immune proteins, improving the activation of Cry toxic substances, and facilitating bacterial evasion from host immune defences (Fedhila *et al.*, 2002). Particularly, the metalloprotease InhA has been shown to degrade insect antimicrobial peptides and immune-related proteins present in the hemolymph, therefore promoting bacterial survival following the initial gut epithelial interruption brought on by Cry toxins.

Some strains of *B. thuringiensis* (*Bt*) produce thermostable β-exotoxins that hinder RNA polymerase activity, therefore interfering with protein synthesis in both insect and mammalian cells. These toxins exhibit broad-spectrum and potent activity; however, their inclusion in commercial biopesticide formulations is prohibited due to their high toxicity to non-target organisms. Despite this limitation, β-exotoxins underscore the extensive arsenal of virulence mechanisms that *Bt* can utilize under natural conditions. Although Cry toxins are recognized as the primary insecticidal agents, the effectiveness of *Bt* is significantly enhanced by the synergistic action of auxiliary virulence factors. For instance, chitinases degrade the insect peritrophic matrix, facilitating Cry toxin access to epithelial cells; proteases contribute to the activation of Cry protoxins; and hemolysins, along with enterotoxins, aid in systemic invasion following the disruption of gut integrity (Bravo *et al.*, 2015). This multifactorial and coordinated strategy underlies *Bt'*s high efficacy, even against insect populations that exhibit resistance or partial tolerance to individual toxins.

2.14 The δ (Delta)-Endotoxins

Bt produces δ-endotoxins as insecticidal proteins appearing as crystalline inclusion bodies during sporulation. The toxins make up 30% of the protein of the cell and are environmentally safe biopesticides that control 90% of the world market. The δ-endotoxins have been classified as multi-domain proteins with three discrete parts: the pore-formation region of the cell membrane of the larval midgut (Domain I), the insect specificity region (Domain II), and the structural stability region as well as the binding region (Domain III) (Saraswathy & Kumar, 2004). The protoxins dissolve when ingested by susceptible insect larvae inside the alkaline midgut and are activated by proteases. The toxins then bind to the epithelial cell surface receptors and cause the development of leakage channels leading to cell rupture and finally the insect's death by starvation or septicemia (Li, 1996). Structures of δ-endotoxins have provided fundamental

insights from the computational analysis of protein sequences, while recent advances in protein engineering have improved both insecticidal activity and specificity (Hodgman & Ellar, 1990).

Bacillus thuringiensis (*Bt*)-based insecticides primarily consist of formulated blends of delta-endotoxin crystals and *Bt* spores, with the spores enhancing the toxicity of the crystalline proteins. These insecticides are ineffective against adult insects and require ingestion by feeding larvae to exert their toxic effects. During sporulation, *Bt* produces crystals composed of delta-endotoxins, which, upon ingestion, dissolve in the larval midgut, functioning as potent toxins. These toxins bind specifically to receptors on the membranes of midgut epithelial cells, initiating proteolytic processing of the solubilized protein crystals into active toxins. This binding induces the formation of pores or channels in the epithelial cell membrane, leading to cellular paralysis, disruption of digestion, cessation of feeding, and ultimately larval death (Silberhorn, 2005). The time to mortality varies from hours to weeks, contingent upon the insect species and the quantity of *Bt* ingested, highlighting the specificity and potency of *Bt* toxins in pest control.

2.15 The Vip proteins

In addition to Cry and Cyt toxins, *Bacillus thuringiensis* (*Bt*) produces another class of insecticidal proteins called Vip proteins (vegetative insecticidal proteins). These proteins are secreted during the vegetative growth phase of the bacterium, rather than during sporulation-like Cry or Cyt toxins. Vip proteins represent a separate and complementary mode of action in the *Bt* insecticidal toolbox, making them highly valuable in both biopesticide formulas and genetically modified (GM) crops (Chakroun *et al.*, 2016; Estruch *et al.*, 1996).

Vip proteins are primarily classified into 4 groups based upon their structure and function. The Vip1 and Vip2 proteins act synergistically as binary hazardous compounds, where Vip1 binds to receptors in the insect midgut to help in the entry of Vip2, an ADP-ribosylating enzyme that disrupts cellular procedures; this pair is coleopteran versus generally reputable pests (Barloy *et al.*, 1998). The Vip3 family consists of single-chain contaminants that display powerful activity against Lepidopteran larvae and are structurally distinct from Cry proteins, enabling them to combat bugs that might be resistant to Cry1 or Cry2 (Lee *et al.*, 2003). In contrast, vip4 and vip5 proteins stay less well defined, though ongoing research is examining their potential application in pest control. The specific biological Since Vip proteins are synthesized during the vegetative development phase of *Bt*, they can act previously in the bacterial life cycle, providing more timely security against insect pests (Chakrabarty *et al.*, 2020).

2.16 Cytolytic toxins (Cyt)

Cytolytic toxins (Cyt), which are yielded by *Bacillus thuringiensis*, belong to the δ-endotoxin superfamily and are insecticidal (Butko, 2003). The toxins have the ability to act on insect midgut cells and synergize the activity of some Cry contaminants, and thus might defeat resistance in mosquitoes. Cyt toxins also show structural homology to proteins from other pathogenic microorganisms, suggesting a saved virulence function (Soberón *et al.*, 2013). Gram-negative bacteria also make pore-forming cytolysins, such as the calcium-dependent RTX toxin family and the calcium-independent *Proteus mirabilis* and *Aeromonas* species toxins (Welch, 1991). Exotoxins and endotoxins are both powerful inducers of inflammatory cytokines with important functions in the innate immune response to infection. Overproduction, however, can cause dangerous cytokine storms typical of sepsis and toxic shock syndrome (Cavaillon, 2017). Explanation of the non-lytic inhibitory residential or commercial properties of these toxins can provide a mean their role in pathogenesis.

Cytolytic (Cyt) toxic substances are a group of proteins primarily synthesized by *B. thuringiensis* and other bacteria that are popular for their cell membrane-disrupting residential or commercial property and as effective insecticides. The toxic substances are necessary in the biological control of insect pests and have actually been investigated for their novel mechanisms of action and future use in insect management. Cyt toxins like Cyt1Aa and Cyt2Aa are comprehended to engage with cell membranes through 2 general mechanisms: pore development and detergent-like actions. Pore formation presupposes that Cyt contaminants insert into membranes to form pores, causing cell lysis, particularly in mosquito larvae membranes. On the other hand, the detergent effect is a less deep interaction with membranes, resulting in membrane interruption without deep insertion, as in erythrocytes and small unilamellar blisters (Onofre *et al.*, 2020).

The structure analysis of Cyt toxins reveals that they have a cytolysin fold, which is very important for their membrane-perforating activity. The fold is supportive of conformational change needed for insertion in the membrane and pore formation. The recognition of a lipid-binding pocket in Cyt contaminants such as CytC also prefers a conserved mechanism within the household for interaction with lipid membranes (Cohen *et al.*, 2011). Cyt toxic substances belong to what makes *B. thuringiensis* insecticides effective, specifically versus mosquito genera like *Aedes albopictus*. They synergize with other contaminants like Cry proteins, resulting in increased overall toxicity (Lai *et al.*, 2023). Unique Cyt-like proteins from other organisms, like Dickeya dadantii and Conidiobolus obscurus, have actually also been discovered

to be effective as biopesticides against the pea aphid and the pine wood nematode, respectively, demonstrating their flexibility for use in a wide variety of pest control (Loth *et al.*, 2015; Zhou *et al.*, 2021).

Very effective, the actual molecular systems for Cyt toxic substances are not yet clearly understood, and controversies regarding their mode of action continue to be disputed. More research is required to further clarify these systems in their totality and likewise to clarify the possibilities for engineering Cyt toxic substances to act against a broader variety of insects. More understanding relating to the practical and evolutionary history of Cyt toxins might likewise lead the way for the development of more reliable and long lasting biopesticides (Adang *et al.*, 2014). Cyt1Aa slowly boosts effectiveness of Cry toxin by avoiding their resistance.

2.17 Insecticidal and pesticidal S-layer protein (SLP) of *B. thuringiensis*

Bacillus thuringiensis has emerged as a promising biopesticide due to its production of insecticidal Cry proteins, which are extremely specific to target insects, non-toxic to vertebrates, human beings, and plants, and ecologically benign owing to their total biodegradability. Beyond Cry proteins, *B. thuringiensis* also manufactures numerous extracellular components, including S-layer proteins (SLPs), which are organized into a paracrystalline selection that envelops the bacterial cell surface area. The exact biological function of SLPs stays uncertain, they are assumed to contribute to cell stability and the upkeep of cellular morphology. Due to their area as the external layer of the cell envelope, SLPs might assist in macromolecular exchange with the environment. In many Gram-negative pathogens, SLPs have really been associated with virulence and resistance to complement-mediated lysis. In *Bacillus cereus*, these proteins boost interactions with human leukocytes, hence promoting host colonization and pathogenicity. In *Bacillus anthracis*, a cooperative function between the S-layer and the capsule has really been suggested in moderating host-pathogen interactions (Beveridge *et al.*, 1997; Mignot *et al.*, 2002; Pei & Blaser, 1990).

B. thuringiensis S-layer proteins have ended up being an appealing brand-new group of insecticidal compounds. These proteins form the external layer of many bacteria and are continuously exposed throughout the vegetative phase (Lormendez *et al.*, 2019). S-layer proteins are commonly distributed amongst *B. thuringiensis* strains, with one research study finding them in 25.4% of examined tension (Guo *et al.*, 2008). Their abundance and constant expression make them appealing prospects for mass production and use in bug control. Genetic

adjustment methods supply possible for boosting the efficiency and cost-effectiveness of SLP-based bioinsecticides.

2.18 Other virulence factors of *Bt*

Bacillus thuringiensis (*Bt*) has numerous virulence components beyond its popular Cry proteins. These consist of Vip and Cyt proteins, enterotoxins, hemolysins, proteases, and phospholipases. Numerous of these factors, such as cytk, nhea, and hbla genes, are extensive amongst both *B. cereus* and *B. thuringiensis* strains (Kim *et al.*, 2015). The metalloproteinase ColB, managed by PlcR, plays an important function in *Bt* pathogenesis by helping in digestion destruction and colonization in nematodes and insects (Peng *et al.*, 2016). These virulence elements include to *Bt*'s capability to avert host immune reactions, enhance pollutant activity, and break down extracellular matrices. Understanding these aspects is necessary for examining the eco-friendly specific niche of *Bt* and the biosafety of *Bt*-based items utilized in biological pest control.

B. thuringiensis (*Bt*) produces a variety of secondary virulence elements that add to its general pathogenicity and environmentally friendly success, in addition to Cyt, cry, and vip proteins. These elements may not straight kill insects, they aid in infection by hindering host defenses, encouraging the activation of toxic substances, boosting colonization, and assisting in the destruction of host tissues. They contribute to the whole series of *Bt*'s entomopathogenic action when combined with its significant contaminants (Nielsen-LeRoux *et al.*, 2012; Raymond *et al.*, 2010).

Produced insecticidal proteins (Sip) and mosquitocidal peptide-like proteins (Mpp) represent reasonably recent classes of virulence elements acknowledged in specific *B. thuringiensis* (*Bt*) tension. Sip1Aa1, a representative Sip protein, exhibits toxicity versus coleopteran larvae and is produced throughout the vegetative development phase; it is notably smaller sized than Cry or Vip proteins and is assumed to communicate with unique midgut receptors (Donovan *et al.*, 2019). On the other hand, Mpp proteins, previously referred to as Cry35-like or bin-like proteins, demonstrate activity against mosquito larvae and are presently being explored as synergistic representatives together with other *Bt* toxic substances for the effective management of vector populations (Chauhan *et al.*, 2021).

2.19 Non-insecticidal δ-endotoxins

Bacillus thuringiensis (*Bt*) is a bacterium that produces δ-endotoxins, which are mainly used as bioinsecticides for controlling a variety of insect larvae. While most studies are focused on the

insecticidal activities of these toxins, there is a growing interest in the exploration of the non-insecticidal δ-endotoxins of *Bt* and their uses. Non-insecticidal δ-endotoxins are crystalline proteins that show structural similarities to Cry and Cyt toxins however do not show insecticidal activity under standard bioassay conditions. The lack of such activity might be associated to a number of factors, consisting of the lack of receptor-binding domains necessary for interaction with insect midgut cells, failure to go through activation within the physiological environment of the insect midgut, or expression in low amounts or incomplete kinds that are insufficient to exert toxic effects (Crickmore *et al.*, 1998). These proteins can have multifunctional roles or may have applications outside the arena of insect pest control. Though Cry10Aa and Cyt2Ba are not the primary components of the *Bt* stress, they reveal significant synergy, having significant mosquitocidal activity when used together, thus representing the possibility of managing mosquito populations (Valtierra-De-Luis *et al.*, 2020). The observation shows that even small δ-endotoxins have an essential role in augmenting the efficacy of bioinsecticides through synergistic results.

The introduction of δ-endotoxins like Cry4Aa into protein microcrystals has proven reliable for improving stability against ecological stresses like ultraviolet light. It can likewise be used for non-insecticidal δ-endotoxins as a way of assisting support them and extending their applicability (Ibuki *et al.*, 2022). The structural instability that takes place in some *B. thuringiensis* (*Bt*) strains like QBT220 that lack certain δ-endotoxin genes indicates that the proteins have the possibility of having repressive effects against extra insecticidal proteins. For that reason, this offers an excellent possibility for the expedition of the non-insecticidal functions of δ-endotoxins under the context of *B. thuringiensis* (*Bt*) science and market (Nair *et al.*, 2021). From a biotechnology point of view, non-insecticidal Cry-like proteins represent promising molecular tools with varied applications beyond their standard function in pest control. These proteins can be crafted or customized to target novel biological systems, used as scaffolds for sophisticated protein style, and checked out for potential antimicrobial, nematicidal, or anti-cancer activities (Palma *et al.*, 2014). Thorough characterization and practical analysis of these Cry-like proteins not only expands the scope of *B. thuringiensis* (*Bt*) applications however likewise contributes to the development of ingenious biotechnological methods in health, farming, and industry.

2.20 Structural features of crystal proteins

Crystal proteins have specific structural features that favor their formation and stability. The protein crystal unit cell typically includes several asymmetric molecules, with the exact number determined by the crystal symmetry (Harker, 1957). Protein crystal formation is influenced by a number of factors that include conformational flexibility and the presence of structured solvent at crystal contact interfaces (Salemme *et al.*, 1988). Crystal packing interactions in monomeric proteins are different from those seen in physiological protein-protein interactions in oligomers and have smaller surface areas and amino acid compositions similar to those of solvent-accessible surfaces. These packing interactions are random and are not related to the intrinsic processes of protein-protein recognition. Thermal motion at crystal packing interfaces is intermediate in comparison to that of the solvent-accessible surface and the protein interior (Carugo & Argos, 1997). Some bacteria, like *Bacillus thuringiensis*, produce crystal-forming proteins with characteristic structural features that are responsible for their insecticidal activity (Chestukhina *et al.*, 1985).

Cry proteins, produced by *Bacillus thuringiensis*, are initially synthesized as protoxins with molecular weights typically ranging from 130 to 140 kDa, and exhibit high stability and insolubility under standard environmental conditions due to their compact structure. This structural integrity allows them to resist degradation by proteolytic enzymes, tolerate temperature fluctuations, and withstand exposure to sunlight and ultraviolet radiation to a certain extent. Activation of these protoxins occurs specifically in the alkaline midgut of susceptible insect larvae, where the elevated pH conditions facilitate their solubilization and subsequent proteolytic activation into functional toxins. This targeted mechanism ensures minimal ecological impact by limiting toxicity to non-target organisms (Gill *et al.*, 1992). Minor structural variations, particularly in the loop regions of Domain II, play a critical role in determining the high specificity of Cry proteins. For instance, Cry1 toxins preferentially bind to cadherin-like proteins in Lepidopteran insects, Cry4 and Cry11 exhibit affinity for Dipteran-specific receptors, and Cry3 toxins target Coleopterans (Knowles, 1994). This structural heterogeneity has actually progressed through natural selection and horizontal gene transfer amongst *B. thuringiensis* (*Bt*) strains, making it possible for these proteins to adapt efficiently to a vast array of insect hosts.

B. thuringiensis (*Bt*) often relies on accessory proteins to facilitate the development of parasporal crystals, which are necessary for its insecticidal activity. Notably, the P20 protein

plays a crucial function in improving the proper folding and stability of Cry11 contaminants, thereby promoting efficient formation. In addition, open reading frames (ORFs) situated nearby to cry genes are frequently found to encode crystallization chaperones that help in this process. These auxiliary components are essential to ensuring that the crystals not just form effectively but likewise keep their biological activity. Mutations affecting the formation domains of Cry proteins or the associated assistant proteins can considerably impair contaminant expression and reduce insecticidal efficacy (Sanchis *et al.*, 1996)

2.21 Mode of action

The crystal (Cry) proteins produced by *B. thuringiensis* (*Bt*) exhibit a high degree of host uniqueness, rendering them hazardous to specific insect types while positioning minimal threat to non-target organisms, consisting of advantageous insects, animals, plants, and people. This specificity is attributed to the proteins' distinct mode of action. *Bt* parasporal crystals contain insecticidal crystal proteins (ICPs) in a non-active protoxin form. Upon ingestion by susceptible pests, these crystals liquify under the alkaline conditions (pH 10-12) of the insect midgut, releasing 130-135 kDa protoxins. These protoxins are then proteolytically cleaved by midgut enzymes into active contaminant pieces of 60-65 kDa.

The activated toxins bind to specific receptors on the epithelial cells of the insect's larval midgut, leading to the formation of ion channels or pores in the cell membrane. This pore formation induces osmotic shock, resulting in cell lysis, paralysis, cessation of feeding, and ultimately the death of the insect due to starvation (Adang *et al.*, 1993; Gill *et al.*, 1992; Höfte & Whiteley, 1989; Knowles, 1994).

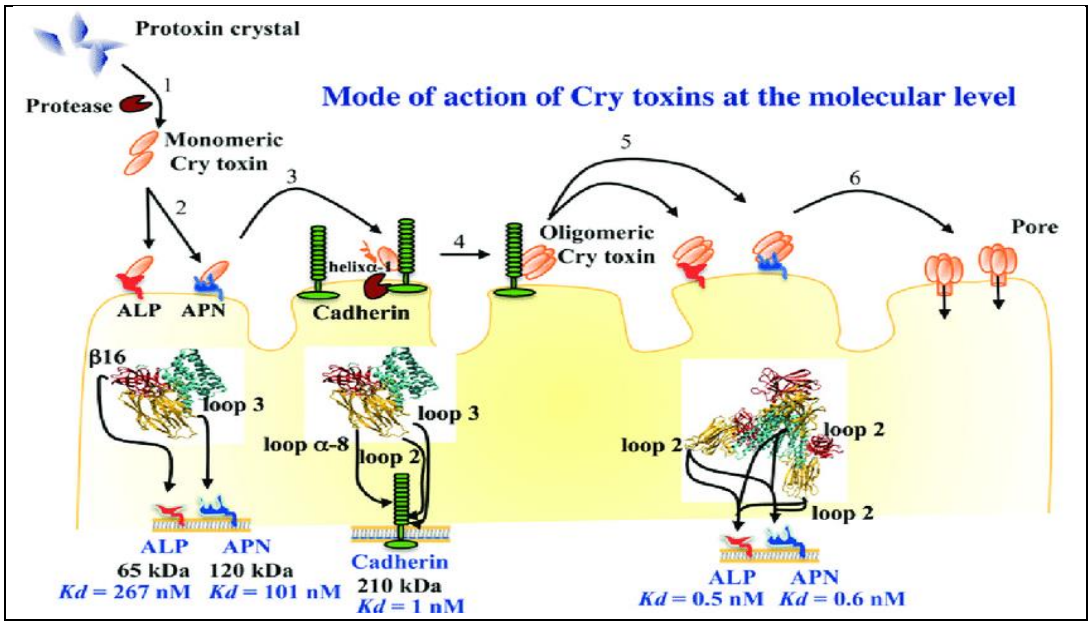


Figure 2.6: The mode of action of *Bacillus thuringiensis* (*Bt*) Cry toxins involves a series of molecular interactions and structural changes within the insect midgut. Upon ingestion by susceptible insects, the crystalline protoxins are solubilized in the alkaline environment of the midgut, where gut proteases cleave the N- and C-terminal regions to produce the activated toxin. Initially, the monomeric toxin binds with low affinity to alkaline phosphatase (ALP) and aminopeptidase-N (APN) receptors on the epithelial cell membrane. Subsequently, it binds to cadherin receptors, which facilitates further proteolytic processing. This cleavage enables the toxin to oligomerize into a pore-forming structure. The oligomeric toxin then binds with high affinity to ALP and APN receptors and integrates into the cell membrane, forming pores that disrupt cellular integrity and ultimately lead to insect death. Figure from (Pardo-López *et al.*, 2013).

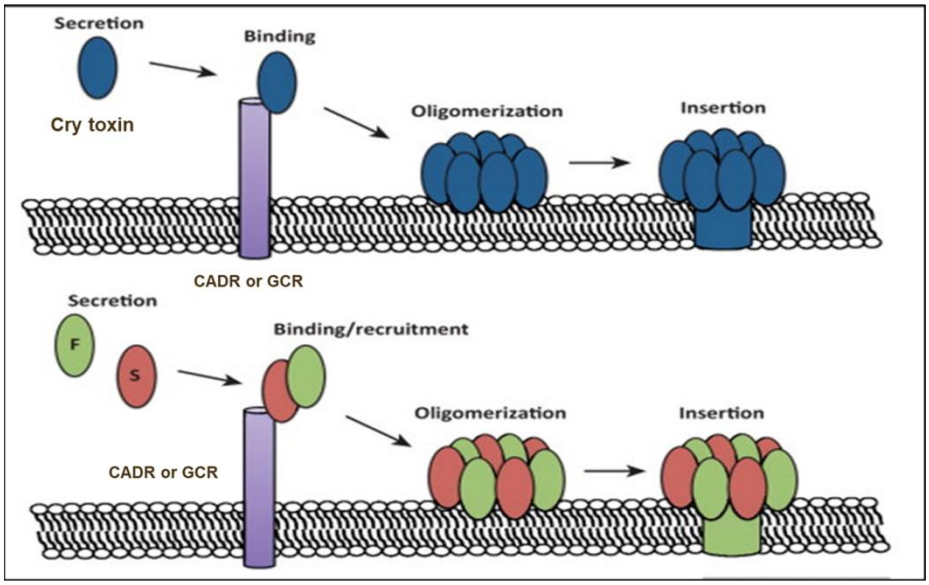


Figure 2.7: Mode of action of Cry toxins against Insect pests

Cry and Cyt toxins that acts on larvae by ingestion, feeding on treated fruit or bait, dissolved in the alkaline midgut of insects and to bind gut receptors by forming pores, *i.e.* forming pores → gut paralysis → septicemia → death.

2.22 Major fruits and vegetable pest population in Bangladesh

The foundation of Bangladesh's economy is agriculture, which provides food security and employs a sizable section of the workforce. However, insect pests, many of which pose significant financial challenges to farmers, continuously threaten crop quality and output. Although Bangladesh's predominant pest populations differ by area, time of year, and crop type, some species are now acknowledged nationally as persistent and harmful. Bangladesh agriculture faces tremendous hurdles from diverse insect infestations across different crops. In rice, green leafhopper (GLH), brown planthopper (BPH), and whitebacked planthopper (WBPH) are important pests, with peak densities seen during the T. Aman season (Afrin *et al.*, 2019). Maize storage is severely damaged by pests, with the maize weevil (Sitophilus zeamais) being the dominant and most destructive, causing up to 85% losses after 5-6 months of storage (Alam *et al.*, 2019). Soybean crops are infected by 39 insect species, with six considered as important pests, including hairy caterpillar, leaf roller, and pod borer, peaking during flowering and pod development phases (Biswas, 2013). Other essential crops, including sugarcane, jute, and citrus, all experience severe pest issues. These findings highlight the relentless and diverse insect concerns in Bangladesh farming, highlighting the requirement for efficient pest management steps throughout numerous crops and seasons.

Farmers greatly depend on chemical insecticides to manage pest populations. For example, 100% of surveyed farmers used insecticides to handle the fall armyworm, with regular treatments at 7- to 15-day periods. Country bean producers usually employ chemical pesticides, typically starting from the seedling (Rahman *et al.*, 2022; Ullah *et al.*, 2023). There is an increasing demand for sustainable insect management methods, such as incorporated bug management (IPM), to decrease dependence on chemical pesticides. This consists of making use of biocontrol chemicals and agroecological approaches to manage insects like the fall armyworm and maize weevil. The extreme usage of pesticides provides substantial dangers to human health, wildlife, and the environment. There is an essential need for better regulatory mechanisms and farmer education to manage these risks effectively (Sarker *et al.*, 2021).

Table 2.1. Major Fruits Pests with their impact in Bangladesh

Major Fruit Pests	Host Fruits & Impact
1. *Bactrocera dorsalis* (Oriental fruit fly) 2. *Bactrocera zonata* (Peach fruit fly) 3. *Zeugodacus cucurbitae* (Melon fruit fly)	Mango, banana, apricot, avocado, bean, radish, citrus, coffee, fig, guava, loquat, mango, roseapple, papaya, passion fruit, peach, pear, persimmon, pineapple, surinam cherry, tomato. However, cucumber, bittergourd, brinjal, sweetgourd, snakegourd, cauliflowers, cabbage, red cabbage, bean most commonly attacked. Impact: Significant economic loss with reduced marketable yield.
4. Idioscopus nitidulus and 5. Idioscopus clypealis (Mango Leaf Hopper)	A significant pest of mango trees, with high infestation rates. Impact: Reduced marketable yield.
Moreover, Several insects, arthropods, fungus, microorganisms and weeds	Guava, papaya etc. Impact: Yield reduction

Lepidopteran, dipteran, coleopteran, and hemipteran pests pose significant challenges to agricultural productivity in Bangladesh due to their broad host range, adaptability, and increasing resistance to conventional control measures. Among the lepidopterans, Spodoptera litura (common armyworm) infests key crops such as tobacco, brinjal, cotton, and pulses, while *Helicoverpa armigera* (cotton bollworm) is a polyphagous pest affecting tomato, chickpea, cotton, and maize. *Plutella xylostell*a (diamondback moth) specifically targets cruciferous vegetables like cabbage and cauliflower, showing notable pesticide resistance, and *Maruca vitrata* (legume pod borer) significantly damages cowpea, pigeon pea, and other pulses. Within the dipteran group, fruit flies of the *Bactrocera* genus, particularly *B. dorsalis*, *B. cucurbitae*, and *B. zonata*, are major threats to fruits such as mango, guava, and papaya, along with cucurbits. Other notable dipterans include the rice gall midge (*Orseolia oryzae*), prevalent during wet seasons in paddy fields, and *Liriomyza* spp. (leaf miners), which compromize the photosynthetic capacity and marketability of leafy vegetables and tomatoes.

Table 2.2. Major Vegetables Pests with their impact in Bangladesh

Major Vegetable Pests	Host Vegetables & Impact
1. *Zeugodacus cucurbitae* (Melon fruit fly) 2. *Zeugodacus tau* (Pumpkin fruit fly)	Cucumber, bittergourd, brinjal, sweetgourd, snakegourd, cucurbits, cowpea, bean, spinach, tomato, bottlegourd, cucumber, bittergourd, brinjal, sweetgourd, cabbage, red cabbage, bean snakegourd, eggplant, carrot, banana, mango etc. Cucurbitaceae and Solanaceae Fruits and Vegetables Impact: Significant yield and quality loss
3. *Leucinodes orbonalis*) (Eggplant Fruit and Shoot Borer) 4. *Epilachna vigintioctopunctata* 5. *E. dodecastigma*, 6. *E. corupta* (Epilachna Beetles) 7. *Empoasca solana* (Delong) (Bean Leafhopper) 8. *Macrosteles fascifrons* (Stal) (Aster Leafhopper) 9. *Empoasca fabae* (Harrris) (Potato Leafhopper) 10. *Circulifer tenellus* (Baker) (Beet Leafhopper) 11. *Tetranychus* sp. (Red Mite)	Eggplant (Brinjal), bean, aster, potato, beet etc. Impact: Losses >86%; up to 140 insecticide applications per season in intensive areas. Significant economic loss and reduced marketable yield.
12. *Spodoptera litura* (Caterpillar) 13. *Plutella xylostella* (Diamond-Back Moth)	Cabbage and Cauliflower Impact: Significant quality loss in broccoli, cabbage, cauliflower and reduced marketable yield.

14. *Aphis craccivora* (Aphids) 15. *Myzus persicae* (Aphids) 16. *Helicoverpa armigera* (Fruit Borer) 17. *Bemisia tabac*i (Whitefly) 18. *Phthorimaea operculella* (Potato Tuber Moth)	Very common pest on various vegetables, including beans and tomatoes, potatoes. Impact: Severe yield reduction with quality
19. *Raphidopalpa foveicollis* (Pumpkin Beetle)	Cucurbits i.e. Pumpkin, Cucumber, Gourd Impact: Reduced vigor and yield
20. *Maruca testulalis*, 21. *Euchrysops cnejus*, 22. *Heliothis armigera* (Pod Borers)	Leguminous Crops e.g. Bean, Yard-Long Bean Impact: Significant yield and quality loss.
23. *Bemisia tabaci* (Whitefly)	Okra Impact: Yield reduction with economic loss.
24. Thrips Order: Thysanoptera	These insects can cause damage to various vegetable crops. Impact: Severe yield reduction with quality loss

In addition, coleopteran pests such as *Callosobruchus* spp. (bruchids) cause post-harvest losses in stored pulses, and *Aulacophora* spp. (cucumber beetles) severely damage cucurbits and may transmit plant pathogens. Hemipteran pests including aphids (e.g., *Myzus persicae*), whiteflies (e.g., *Bemisia tabaci*), and jassids (e.g., *Amrasca biguttula)*, act as phloem feeders and vectors of viral diseases, further compounding the damage across various crop systems (Ali *et al.*, 2021; Angon *et al.*, 2023; Roy *et al.*, 2024). In Bangladesh, a variety of insects and other species predominate as insect pests on fruits and crops. Bangladesh's diversified pest population emphasizes the importance of effective Integrated Pest Management (IPM) solutions in reducing crop losses and ensuring sustainable agricultural practices.

2.23 Pesticides

Early pesticides were chemical compounds valued for their prolonged residual effects and broad-spectrum toxicity against various insects. However, their application has been associated with adverse consequences. Contemporary chemical insecticides are generally regarded as safer than their predecessors, yet concerns persist. Prolonged exposure to these substances may result in severe health issues, including cancer, hepatic damage, immunotoxicity, congenital anomalies, and reproductive disorders in both humans and animals (Weaver, 2000). Additionally, these chemicals contribute to the accumulation and persistence of toxic residues in soil, water, and food; harm beneficial insects; and foster pest resistance (Glazer & Nikaido, 2007; Marrone & MacIntosh, 1992; Van Frankenhuyzen *et al.*, 1993). Despite these issues, chemical insecticides maintain a significant market presence, with global annual sales approximating $5 billion (Glazer & Nikaido, 2007).

Pesticides can be categorized into four major classes based on their origin and chemical composition. The first category includes plant-derived compounds such as pyrethrin and rotenone. The second class comprises pure chemicals, exemplified by substances like Paris green. The third category encompasses synthetic chemicals, which can be further subdivided into chlorinated hydrocarbons (e.g., DDT, dieldrin, HCH), organophosphates (e.g., diazinon, malathion, fenitrothin), carbamates (e.g., propoxur, bendiocarb), and synthetic pyrethroids (e.g., deltamethrin, permethrin). Finally, the fourth class consists of biopesticides, including biological agents such as *Bacillus thuringiensis* (*Bt), Bacillus subtilis* (*Bs*), various fungi, and Chemical pesticides, regardless of their prevalent usage in contemporary farming, present several considerable limitations that impact both ecological balance and human health. Many chemical pesticides are broad-spectrum in nature, leading to damaging effects on non-target arthropods, consisting of beneficial pests. This indiscriminate action frequently interrupts environmental interactions and can result in the break out of secondary pests. Moreover, the consistent nature of these chemicals adds to environmental contamination, with residues regularly identified in water sources, foodstuff, vegetables, milk, and even animal hides. Pesticides also position toxicity threats to mammals and plants, displaying carcinogenic and phytotoxic residential or commercial properties. Another significant issue is the "pesticide treadmill," wherein the repeated and intensifying usage of pesticides ends up being essential as pest populations establish resistance, typically through mechanisms such as natural selection, cross-resistance, and mixed-function oxidase activity. This resistance lowers the efficacy of

chemical control strategies and increases costs every year. Additionally, ecological elements like rains and watering can remove chemicals, even more reducing their efficiency.

The indiscriminate application of pesticides can likewise result in the destruction of natural enemies crucial for pest policy in farming environments. Finally, exposure to these chemicals represents a serious occupational hazard, posing substantial health risks to workers in pesticide manufacturing and to farmers during application. Alternatively, microbial pesticides are considered environmentally safe due to their non-toxic and non-pathogenic nature toward humans and wildlife. Their mode of action is typically highly specific, often targeting a particular insect species or group, thereby minimizing unintended effects on non-target insect populations within treated ecosystems.

Chemical pesticide use in Bangladesh has increased significantly over the past decades, with insecticides comprising over 95% of total pesticides applied to agricultural crops (Rahman *et al.*, 1995). According to the "Institute of Development Policy analysis", Bangladesh imported about 12,000 metric tons of pesticides in 2001. The majority of farmers regularly use chemical pesticides for vegetable cultivation, despite potential negative impacts on the environment and human health (Islam *et al.*, 2023). Pesticide use in rice fields accounts for over 80% of total pesticide application, with some farmers using 1-10 kg of active ingredients per hectare (Parveen & Nakagoshi, 2001). The rapid increase in pesticide use has led to environmental contamination, affecting water resources and ecosystems. While pesticides are seen as necessary for increasing food production to meet the growing population's demands, there is a need for stricter regulations and training programs for farmers and pesticide sellers. Additionally, more research is required to assess the long-term effects and risks associated with increased pesticide use.

2.24 Tephritids pest affecting fruits and vegetables

Tephritid fruit flies are significant vegetable and fruit crop pests globally, resulting in huge economic losses (Ekesi & A, 2011). Ten key species have been identified in China, infesting diverse crops (He *et al.*, 2023). Studies have shown that nine different types of pests can infest mango, citrus, and cucurbits in West Bengal, India. 4 large genera, *Bactrocera*, *Ceratitis*, *Dacus*, and *Trirhithrum*, cover the sub-Saharan fruit flies, and mango is one of their main targets. Tephritid fruit flies cause tremendous financial damage by laying eggs in fruits and veggies, leading to decomposing and decreased marketability (Cha *et al.*, 2023). Tephritid fruit

flies represent a considerable risk to world farming because of their intrusive nature and ease of adaptation to new communities.

Among the vast array of insect pests impacting Bangladesh's horticultural sector, fruit flies from the Tephritidae family stick out as a few of the most financially hazardous. These pests not simply decrease crop yield and quality but similarly limit global trade, as plagued vegetables and fruit generally stops working to fulfill export requirements. Their biology, flexibility, and high reproductive capability make management particularly challenging for farmers and pest control companies alike. Bangladesh is home to a number of species of tephritid fruit flies, amongst which *Bactrocera dorsalis*, *Bactrocera cucurbitae*, *Bactrocera zonata*, and *Bactrocera tau* are especially popular due to their farming significance. *Bactrocera dorsalis*, frequently called the Oriental fruit fly, is the most substantial and devastating types, infesting a variety of tropical fruits such as guava, jackfruit, and mango; it is defined by its polyphagous nature and quick population growth throughout the warm and wet seasons. *Zeugodacus cucurbitae* (*B. cucurbitae*), or the melon fly, generally targets cucurbitaceous veggies like cucumber, pumpkin, bitter gourd, and sponge gourd, cabbage, cauliflowers, with larvae that tunnel through soft tissues, leading to both direct damage and secondary infections induced by fungal and bacterial pathogens. *Bactrocera zonata*, the peach fruit fly, is also polyphagous and commonly infests fruits such as guava, sapodilla, and peach; although it is currently less dominant than *B. dorsalis*, its frequency has been increasing in numerous regions of the country. *Zeugodacus tau* (*B. tau*), understood as the pumpkin fruit fly, also poses a threat to cucurbit crops, although detailed research studies on its distribution and economic impact in Bangladesh remain minimal (Leblanc *et al.*, 2021).

Tephritid fruit flies oviposit within the tissues of host fruits, where the hatching larvae feed internally, leading to soft decaying, premature fruit drop, noticeable exuding or dark puncture marks, and eventually rendering the fruit and vegetables unsalable or unmarketable. These invasions can result in considerable economic losses, ranging from 20% to over 80% in crops such as mango, guava, and various cucurbits during periods of peak invasion (Rahman *et al.*, 2015). The circumstance is particularly critical in smallholder farming systems, where inadequate insect security often leads to underestimation of invasion intensity. Tephritid fruit flies present substantial barriers for insect management due to a number of factors: their puzzling larval stage, which establishes concealed within the fruit flesh; the widespread development of resistance to artificial insecticides, generally occurring from severe and repetitive applications by farmers; and the restricted awareness and application of biological

control techniques and incorporated bug management (IPM) methods. Traditional insecticide-based control measures regularly stop working to accomplish efficient suppression of these pests. The indiscriminate use of chemical pesticides provides considerable threats to non-target organisms, including pollinators and helpful arthropods, as well as to human health and ecological safety.

Tephritid fruit flies cause massive economic damage by laying eggs in vegetables and fruits, resulting in rotting and lessened marketability (Cha *et al.*, 2023). *Bactrocera dorsalis*, usually called the Oriental fruit fly, is the most prevalent and damaging species, infesting a big variety of tropical fruits such as guava, jackfruit, and mango; it is characterized by its polyphagous nature and fast population growth during the warm and moist seasons. Tephritid fruit flies oviposit within the tissues of host fruits, where the hatching larvae feed internally, leading to soft decaying, early fruit drop, visible exuding or dark leak marks, and ultimately rendering the produce unsalable or unmarketable. Tephritid fruit flies present significant challenges for pest management due to numerous factors: their cryptic larval phase, which establishes concealed within the fruit flesh; the widespread development of resistance to synthetic insecticides, mainly resulting from repeated and excessive applications by farmers; and the minimal awareness and application of biological control methods and integrated insect management (IPM) methods. Out 0f 210 species 73 *Bactrocera* and *Zeugodacus* species are economically important, ranked under four categories, based on pest severity, host range, invasiveness and frequency of infestation.

Category A includes widespread invasive polyphagous generalists or highly destructive specialists that have become established outside of their native range

Category B pests are polyphagous fruit pests or destructive specialists more restricted in distribution but at elevated risk of spreading at new location

Category C relatively minor oligophagous or specialist fruit or cucurbit pests and

Category D includes species that have been occasionally bred from commercial/edible fruits or cucurbits.

Table 2.3. Major Tephritids pest in Bangladesh affecting fruits and vegetables

Species	Hosts	Distribution
Bactrocera dorsalis (Oriental fruit fly) **Category A**	Polyphagous fruit pest than 150 kinds of fruit and vegetables, including: banana, mango apricot, avocado, bean, radish, citrus, coffee, fig, guava, loquat, mango, roseapple, papaya, passion fruit, peach, pear, persimmon, pineapple, surinam cherry and tomato. However, avocado, guava and papaya are the most commonly attacked.	Tropical Asia (Widespread), Indian Subcontinents
Bactrocera zonata (Peach fruit fly) **Category A**	Polyphagous fruit pest Cucumber, mango, banana, guava, bittergourd, brinjal, sweetgourd, snakegourd,cauliflowers, cabbage, red cabbage, bean etc All Cucurbitaceae and solanaceae Fruits and Vegetables	Tropical Asia (Widespread), Indian Subcontinents
Zeugodacus cucurbitae (Melon fruit fly) **Category A**	Cucumber, bittergourd, brinjal, sweetgourd, snakegourd, Various cucurbits, cowpea,bean,spinach, tomato,cabbage, eggplant,carrot, banana,mango etc All Cucurbitaceae and Solanaceae Fruits and Vegetables	Tropical Asia (Widespread), Introduced into Africa and Oceania Indian Subcontinents
Zeugodacus tau (Pumpkin fruit fly) **Category B**	Bottlegourd, cucumber, bittergourd, brinjal, sweetgourd, cabbage, red cabbage, bean snakegourd etc. All Cucurbitaceae fruits and vegetables	Tropical Asia (Widespread), Indian Subcontinents

2.25 Advantages of biopesticides as an alternative to chemical pesticides

Biopesticides are an eco-friendly alternative to chemical pesticides for the management of agricultural pests. Biopesticides, which are derived from biological sources such as microorganisms, plants, and minerals, are target-specific, inexpensive, and environmentally harmless. Unlike chemical pesticides, which are toxic to soil biota, aquatic organisms, and human health, biopesticides are free from toxic residues and result in no greenhouse gas emissions (Chowdhury *et al.*, 2024). The biopesticides market in the world is expanding modestly, while in India it has vast potential to be adopted. Biopesticides are readily compatible with Integrated Pest Management (IPM) practices, agricultural modernization, and sustainability. Molecular biology, genetic engineering, and developments in R&D are enhancing the production and efficacy of biopesticides. As there will be more demand for green agriculture, biopesticides will have a pivotal part to play in pest management in the future. *Bt*

biopesticides is not toxic to mammals, birds, and some other beneficial insects i.e. polinators, parasitoids etc. Easily biodegradable containing no harmful residues to the environment, compatible with IPM as well as its Cyt toxins is a low-risk resistance.

Biopesticides are a warm replacement for synthetic chemical pesticides due to their environmental and health benefits. Biopesticides from natural sources such as plants, bacteria, fungi, and minerals offer a myriad of benefits over conventional pesticides. Biopesticides offer a range of advantages that make them a sustainable alternative to chemical pesticides in modern agriculture. Environmentally, they are eco-friendly and biodegradable, significantly reducing the risk of long-term pollution and ecosystem damage (Ayilara *et al.*, 2023). In terms of health, biopesticides exhibit lower toxicity to humans and animals, thereby posing fewer health risks such as cancer and neurological disorders typically associated with synthetic pesticides (Khursheed *et al.*, 2022). Their uniqueness enables them to target bugs specifically while decreasing harm to non-target organisms, including useful microbes and insects, and this targeted action likewise minimizes the likelihood of resistance development amongst pests, a common problem with chemical agents (Reddy & Chowdary, 2021). From a sustainability viewpoint, biopesticides line up with the goals of Integrated Pest Management (IPM) by decreasing reliance on synthetic chemicals (Seiber *et al.*, 2018). In addition, growing customer demand for organic and environmentally friendly agricultural products, together with regulative support, has sped up the market growth and approval of biopesticides (Kumar *et al.*, 2021). Last but not least, biopesticides have several modes of action, i.e., they are attractants, repellents, or anti-feedants, and can hinder pest development and recreation and hence serve as multipurpose chemicals in pest management (Vero *et al.*, 2023).

2.26 Classification of biopesticides

Biopesticides are categorized into three primary classes: microbial pesticides, biochemical pesticides, and plant-incorporated protectants (PIPs). Each category employs distinct mechanisms to manage pest populations, leveraging biological or naturally derived agents to achieve pest control with minimal environmental impact. Microbial pesticides, which include various bacteria, fungi, and viruses, offer targeted and environmentally friendly alternatives to conventional chemical pesticides. *Bacillus thuringiensis* (*Bt*), a Gram-positive bacterium, is widely utilized due to its ability to produce crystal proteins (Cry toxins) that are specifically toxic to certain insect orders such as Lepidoptera and Coleoptera. Additionally, entomopathogenic fungi like *Beauveria bassiana* and insect-specific viruses such as

baculoviruses serve as effective biological control agents by infecting and ultimately eliminating insect hosts. Among the principal benefits of microbial pesticides lies in their high specificity, which substantially lowers the danger to non-target organisms, consisting of helpful insects and mammals. Their naturally low toxicity and the capability for varied formula, make them extremely suitable with Integrated Pest Management (IPM) strategies, promoting sustainable farming practices while decreasing ecological effect.

Biochemical pesticides are a class of pest control agents derived from naturally occurring substances that function through non-toxic mechanisms, often by interfering with pest behavior, growth, or reproduction rather than causing direct mortality. Among these, botanical pesticides such as neem oil (extracted from *Azadirachta indica*) and pyrethrum (sourced from *Chrysanthemum cinerariifolium*) act by disrupting pest physiology or altering behavioral responses. Insect pheromones, which are chemical signals used in intraspecific communication, are employed to manipulate pest populations by luring them into traps or interfering with mating behaviors. Insect growth regulators represent another category, functioning by inhibiting essential developmental processes such as molting and metamorphosis. These biochemical pesticides are generally characterized by low toxicity to non-target organisms and exhibit compatibility with a range of application methods. Moreover, they often display greater environmental persistence compared to microbial pesticides, thereby enhancing the duration and effectiveness of pest control. Biochemical pesticides are a class of bug control agents obtained from naturally taking place compounds that run through non-toxic systems, often by interfering with bug leisure, habits, or development rather than causing direct mortality. These biochemical pesticides are generally specified by low toxicity to non-target organisms and show compatibility with a series of application techniques. Additionally, they frequently show greater environmental persistence compared to microbial pesticides, therefore enhancing the period and efficiency of insect control.

Plant-incorporated protectants (PIPs) are pesticidal substances that are produced by genetically customized plants crafted to express particular genes conferring resistance to pests. A popular example includes crops such as maize and cotton that have actually been modified to express *B. thuringiensis* (*Bt*) proteins, which provide intrinsic protection versus targeted insect pests. Other transgenic plants might be crafted to manufacture alternative pesticidal proteins or secondary metabolites that deter or remove pests. Primary advantage of PIPs lies in their ability to offer continuous, internal protection against pests, thereby reducing the need for external pesticide applications. Their presence within plant tissues enhances the persistence and efficacy of pest

control, potentially lowering the frequency and intensity of pest management interventions (Golijan-Pantović *et al.*, 2022; Mehrotra *et al.*, 2016; Senthil-Nathan, 2015).

2.27 Commercial application of *Bt* based biopesticide

Bacillus thuringiensis (*Bt)* has been utilized for over fifty years in pest and mosquito control. The use of *Bt*-based insecticides in agriculture declined in the 1970s due to the advent of more effective chemical pesticides. However, biotechnological advancements in the 1980s revitalized *Bt* research, notably with the cloning of a crystal toxin gene from *Bt* subsp. *kurstaki* into *E. coli* (Schnepf *et al.*, 1985). Subsequent studies focused on enhancing target specificity and identifying more virulent *Bt* strains. By 1995, global *Bt* sales reached $90 million, reflecting a shift toward safer alternatives to synthetic pesticides (Schnepf *et al.*, 1998). In 1998, the United States registered nearly 200 *Bt* products. Cry-based pesticides are noted for their low development and registration costs, estimated at one-fortieth that of synthetic chemical pesticides. The United States leads in *Bt* applications, particularly in forestry, using the *Bt* subsp. *kurstaki* HD-1 strain, which produces Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa toxins (Dulmage, 1989). *Bt* subsp. *israelensis* is highly effective against mosquitoes and blackflies, with mosquitocidal activity linked to Cry2Aa, Cry1Ab, and Cry1Ca toxins (Haider *et al.*, 1989). Novel isolates with uncharacterized cry genes also exhibit mosquitocidal properties (Ragni *et al.*, 1996).

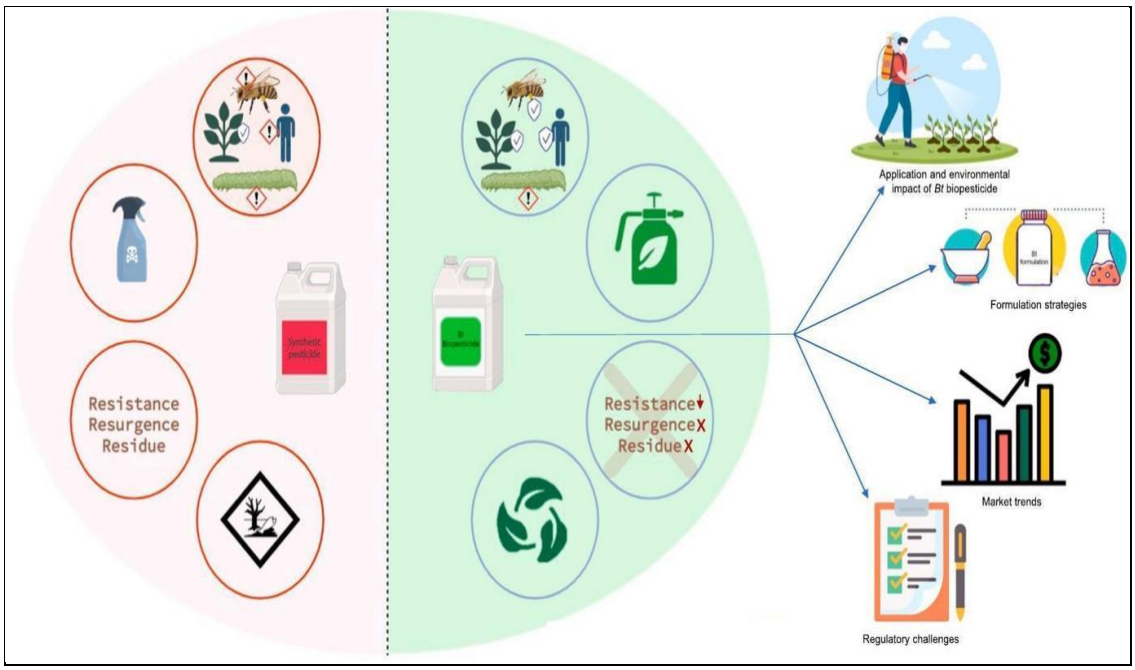


Figure 2.8: *Bacillus thuringiensis* (*Bt*) based biopesticide and synthetic pesticide (Ragasruthi *et al.*, 2024).

In agricultural applications, *Bacillus thuringiensis* (*Bt*) is predominantly applied using ground sprayers, requiring high volumes of aqueous spray for adequate plant coverage, which can be impractical in certain scenarios. Recent advancements, such as aerial spraying from aircraft and air-assisted sleeve booms, have reduced spray volumes, enhanced droplet control, and improved spray penetration while minimizing drift (Navon, 2000). However, the low persistence of *Bt* spore-crystal products, lasting approximately 48 hours on crops like cotton and potatoes, underscores the importance of precise application timing, ideally early in the season during egg hatching and post-sunset to maximize efficacy (FerrÃ *et al.*, 1995; Navon, 2000). Younger larvae exhibit greater susceptibility to *Bt*, making the larval stage optimal for application. The biological activity of *Bt* strains and their insecticidal crystal proteins, toxic to over 3,000 insect species across 16 orders, including Lepidoptera, Diptera, and Coleoptera, as well as non-insect organisms like nematodes and mites, has been extensively patented by commercial entities and research institutions. The proteins' toxicity is particularly pronounced against leaf-feeding Lepidopteran and Coleopteran insects with chewing mouthparts and Dipterans that filter-feed, facilitating *Bt* spore and crystal ingestion (Tavares *et al.*, 2021).

For approximately five decades, *Bacillus thuringiensis* (*Bt)*-based insecticides have been employed commercially to manage specific insect pests without reported adverse effects on human health or the environment (McClintock *et al.*, 1995). Advancements in the 1990s, including natural and recombinant *Bt* products, expanded the insect host range and enhanced pest management strategies through novel formulations, such as toxin encapsulation and feeding stimulants, and improved interactions with plant allelochemicals and natural enemies (Navon, 2000). *Bt* toxins offer high specificity, safety to non-target organisms, short persistence, complete biodegradability, and lower development and registration costs compared to synthetic pesticides (Bohorova *et al.*, 1999). Commercial *Bt* preparations, typically containing spores and δ-endotoxin crystals, are formulated with additives like wetting agents and UV-protective chromophores to mitigate rapid activity loss due to sunlight. Encapsulation in biopolymers further reduces environmental wash-off. Knowledge of insect feeding behavior has informed the development of phagostimulant-enhanced formulations, increasing residual toxicity and selective feeding on *Bt* products (McGuire *et al.*, 2000).

2.28 Production of *Bt* biopesticides

Despite the availability of microbial insecticides derived from *Bacillus thuringiensis* (*Bt)* and *Bacillus sphaericus* (*Bs*), their elevated costs render large-scale application economically unfeasible in developing nations. The commercial viability of *Bt*-based insecticides is contingent upon large-scale fermentation processes that achieve high yields of insecticidal proteins, as the production of *B. thuringiensis* remains relatively costly without substantial output (Zouari *et al.*, 2002). Consequently, research has increasingly focused on regulatory mechanisms to optimize the synthesis of insecticidal proteins. Advances in bioinsecticide production may be realized through the adoption of suitable fermentation technologies, optimization of culture media, mitigation of metabolic constraints, strain enhancement via mutagenesis, and adaptation to abiotic stressors (Azzouz *et al.*, 2014).

Fermentation processes for various *Bt* isolates, irrespective of subspecies, share common characteristics. These include the utilization of sugars (e.g., glucose, molasses, or starch) as carbon sources, acid production during fermentation, and comparable requirements for proteins, protein hydrolysates, ammonium salts, and minerals. However, the efficacy of a given medium varies among isolates, as a formulation optimized for one strain may not support equivalent growth or toxin production in another. Additionally, different *Bt* isolates may produce toxins with distinct insecticidal activity spectra.

The commercial deployment of *Bt* and *Bs* insecticides hinges on several factors, including the cost of raw materials, strain efficiency, fermentation duration, process parameter stability, downstream bioprocessing, and final product formulation. Raw material costs constitute a significant portion, approximately 30-40%, of total production expenses, varying by plant capacity. To enhance affordability in developing countries, local production should leverage cost-effective, locally sourced media, such as agro-industrial by-products. Various agricultural and industrial by-products, including citrus peels, wheat bran, cornmeal, date seeds, beef blood, silkworm pupal skin, groundnut cake, cane molasses, fish meal, cottonseed meal, soybean meal, slaughterhouse residues, fodder yeast, cheese whey, and corn steep liquor, have been employed as raw materials for *Bt* and *Bs* production (El-Bendary, 2006). More recently, waste materials such as sludge and broiler poultry litter have been explored for biopesticide production (Adams & Matthews, 2019). Two primary fermentation methods submerged fermentation and solid-state fermentation are commonly utilized for microbial product synthesis.

The development of diverse formulations based on spore-crystal complexes, designed for ingestion by target insects, reflects decades of research. Innovations in matrices supporting these complexes have led to improvements in toxic activity, palatability to target insects, and extended shelf life. Commercial *Bacillus thuringiensis* (*Bt)* based bioinsecticides encompass a range of products targeting various insect pests. Certis offers several formulations, including Agree WG, Condor, CoStar, Deliver, Jackpot WP, Javelin/Delfin, Lepinox WDG, and Turix WP/Agree WP, all utilizing *Bt* var. *kurstaki* or *aizawai* to target lepidopterans, with Crymax employing genetically engineered strains of *Bt kurstaki* and *aizawai* for the same purpose. AFA Environment Inc. produces Agribac, which uses *Bt* var. *kurstaki* to combat over 30 insect species. Valent Biosciences Corp. provides an extensive portfolio, including DiPel, Biobit, Foray, and Thuricide, all based on *Bt* var. *kurstaki* and targeting lepidopterans, with Thuricide also effective against certain leaf-eating worms. Their XenTari, also *Bt var. kurstaki*-based, is specifically effective against *Spodoptera* spp. and *Plutella xylostella*. Additionally, Valent Biosciences offers Novodor, utilizing *Bt* var. tenebrionis to target coleopterans, and VectoBac, Teknar, and Gnatrol DG, all based on *Bt* var. *israelensis*, which address mosquito and fly larvae, with Gnatrol DG specifically targeting the larval stage of Sciarid mushroom flies. This diverse array of bioinsecticides highlights the specificity of *Bt* strains and their applications in pest management (Rosas-García *et al.*, 2009).

2.29 Pest resistance development and management

The development of resistance in insect populations to a wide range of insecticides is a well-documented and increasingly pressing issue in pest management. This phenomenon primarily arises from the inherent genetic variability within large insect populations, which enables certain individuals to survive insecticidal treatments and subsequently pass on resistant traits to their progeny. Several biological and ecological factors contribute to the acceleration of resistance evolution. Species characterized by rapid reproductive cycles, short generational intervals, high fecundity, and genetically diverse local populations are particularly prone to rapid resistance development (Pimentel & Raven, 2000).

Moreover, the nature and application frequency of insecticides substantially influence the rate at which resistance emerges. Persistent insecticides, which stay active in the environment for extended periods, increase the duration of direct exposure of insect larvae to the hazardous substances, consequently heightening choice pressure and accelerating resistance (Sanahuja *et al.*, 2011). Conversely, regular applications of non-persistent insecticides can likewise boost

choice pressure, specifically if they are applied without appropriate rotation or integration with other insect control methods (Begum *et al.*, 2017).

Insecticide resistance positions extreme difficulties across agricultural, public health, and economic sectors. Because the very first reported case of resistance to artificial chemical insecticides over five decades ago, the problem has intensified into a considerable global environmental issue. This has significant repercussions, including increased crop losses, decreased effectiveness of illness vector control programs, increased health dangers, and environmental degradation arising from the extreme use of chemical agents on resistant insect populations (Pimentel, 1996).

Notably, by the 1990s, there were many recorded cases of resistance amongst different insect pest types to *B. thuringiensis* (*Bt*) based solutions in areas such as Hawaii, Florida, New York, Japan, China, the Philippines, Thailand, and Malaysia. This highlighted resistance as a direct and adverse consequence of the widespread use of insecticides, including biopesticides like *Bt* (Iqbal *et al.*, 2022; Liu *et al.*, 2001). To mitigate the progression of resistance, particularly against *Bt* based insecticides, a range of resistance management strategies has been developed. These strategies primarily aim to delay the onset of resistance and, where possible, restore susceptibility in resistant insect populations. Broadly, three major approaches are recognized in resistance management programs.

The first approach focuses on reducing the selection pressure exerted by insecticides through minimizing exposure and promoting interbreeding between resistant and susceptible individuals. This can be achieved through strategies such as tissue-specific or temporal regulation of toxin expression in plants, the use of toxin mixtures, spatial mosaics, crop rotation, refugia (untreated areas to harbor susceptible insects), and periodic releases of susceptible insect strains.

The second approach emphasizes integrated pest management by combining various pest control methods to enhance the effectiveness of *Bt* formulations. This includes the use of high-dose *Bt* toxins, stacking of multiple toxin genes with different modes of action, and synergistic applications involving sub-lethal toxin concentrations, other entomopathogenic organisms, plant secondary metabolites (allelochemicals), and natural predators or parasitoids (Murray *et al.*, 1991).

The third approach is specific to genetically modified *Bt* crops and involves the deployment of trap crops. These trap crops are designed to attract and divert pest insects away from the primary

cultivated plants, thereby reducing the pressure on main crops and limiting the development of resistance within the target pest population (Andow *et al.,* 2000). Through the careful implementation of these strategies, it is possible to manage insecticide resistance more effectively and sustain the utility of both chemical and biological control agents in modern pest management programs.

2.30 Aims and Objectives

In Bangladeshi agriculture, *Bt* biopesticide can be a helpful substitute for chemical pesticides, which are seriously harming the environment and public health in a silent manner. Transgenic crops would have been a highly alluring choice, but they were less desirable because of immunological side effects with edible crops and rising insect resistance brought on by uncontrolled exposure to *Bt* toxins, which led to selection pressure. However, foliar spraying *Bt* biopesticide has been shown to be safer due to its non-integration into the plant system, which means it has no negative immunological consequences. Once more, farmer-friendly spraying that targets the pests' life cycle without requiring a lot of protection can be carried out, which lowers the likelihood of pest resistance. Therefore, the purpose of this work was to identify the toxic factors, such as cry genes, insecticidal proteins, *etc.*, in order to economically manufacture *Bt* biopesticide from possible native *Bt* strains. For their large-scale production, it is crucial to build an enhanced collection of native *Bt* strains, identify their variety, and separate the possible strains that are efficient against pests of fruits and vegetables. Thus, the basic goals of this research were as follows:

1. To evaluate the entomopathogenic activity of local *Bt* strains against Tephritids fruit flies.
2. To identify, isolate and characterize efficacy of *Bt* strains containing high toxicity against pests.
3. To ensure the environment-friendly control of insect pests cost effectively by potential *Bt* through Bioassay and Biological quality parameters assessment.
4. Production of efficient *Bt* biopesticide on large scale for sustainable delivery to the farmers for field level application.
5. Whole genome sequencing (WGS) of insecticidal protein-synthesizing *B. thuringiensis* strains and data analysis for identification of their insecticidal protein sequence.

CHAPTER 3

BIOASSAY OF POTENTIAL INDIGENOUS *BACILLUS THURINGIENSIS* STRAINS AGAINST SELECTED TEPHRITIDS PESTS IN-VIVO

3.1 Introduction

The subtropical climate of Bangladesh permits growth of a remarkable range of fruits and vegetables (White & Elson-Harris, 1992; White & Evenhuis, 1999), some of which are vulnerable to attack by tephritid fruit flies, serious agricultural pests that are ubiquitous in tropical and subtropical regions (Aluja *et al.*, 1996; Armstrong & Jang, 1997; Hasyim *et al.*, 2008; Vargas *et al.*, 2015). These insects lay eggs in fruit tissue, and larval feeding causes internal damage that renders produce marketable and results in loss of yield 10% to 100% depending on host species and season (Dhillon *et al.*, 2005; Sapkota *et al.*, 2010).

Of the more than 5,000 known species in family Tephritidae (Namin & Korneyev, 2018; Pape *et al.*, 2011), species in tribe Dacini are of major economic importance in Bangladesh. The nation is home to at least 34 recorded species, some of which are new records (Leblanc *et al.*, 2021). *Bactrocera carambolae* and *Zeugodacus madhupuri*, bring Bangladesh's total of Tephritidae species up to 37 (29 from the Dacini tribe and eight from other tribes) (Khan *et al.*, 2017; Leblanc *et al.*, 2013, 2014, 2019; Shishir *et al.*, 2015). Among these, *Bactrocera dorsalis*, which is one of the most polyphagous members with more than 100 hosts that it infests, is significant as one of the most damaging pests (Allwood *et al.*, 1999). The peach fruit fly, *Bactrocera zonata*, in South and Southeast Asia infests soft fruits in wide varieties and is deemed a major quarantine pest. Likewise, the melon fly, *Zeugodacus cucurbitae*, is regarded as a serious pest for cucurbit crops since it inflicts massive damage on vegetables and fruits. The pumpkin fruit fly, *Zeugodacus tau*, is widespread across Southeast Asia and may infest various areas of the host plant. Its larvae are known as latent feeders (Guo *et al.*, 2023), and it serves as a quarantine pest in the majority of countries due to its broad host range, high rate of reproduction, and ease of adaptation (Liu & Ji, 2024).

The ongoing application of chemical pesticides in managing the fruit fly has resulted in a cascade of issues, from environmental pollution to pesticide resistance and possible damage to human and animal health. As a result of this, biological control strategies using insecticidal microbes have become increasingly popular due to their effectiveness and safety to the environment (Bel *et al.*, 1997; Kumar *et al.*, 2008; Martin & Travers, 1989; Meadows *et al.*, 1992; Salehi Jozani *et al.*, 2005). Among these, *Bacillus thuringiensis* (*Bt*), a gram-positive, spore-forming bacterium isolated from soil, is widespread owing to the fact that it secretes upon sporulation insecticidal crystal (Cry) and cytolytic (Cyt) δ-endotoxins. These toxins are very

active against insects belonging to orders Diptera, Lepidoptera, and Coleoptera, and they are environmentally safe since they have species-specific mode of action and produce minimal non-target effects (Ben-Dov *et al.*, 1997; De Meyer *et al.*, 2015; Schnepf *et al.*, 1998).

In all this, *Bt*-based biopesticides are a potential eco-friendly solution for population control of tephritid fruit flies. The present study aims to evaluate the larvicidal efficacy of some of the native *Bt* isolates against four economically important fruit fly pests, namely, oriental fruit fly (*B. dorsalis*), peach fruit fly (*B. zonata*), melon fly (*Z. cucurbitae*), and pumpkin fruit fly (*Z. tau*). By assessing effects of such *Bt* strains on key biological parameters of the target insect pests, the research opens up avenues for the formulation of locally adapted, ecologically compatible *Bt*-formulations. The findings are expected to benefit the integrated pest management (IPM) of Bangladesh, reduce the use of chemical pesticides, promote ecological balance, and enhance the country's crop resilience and export potential.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Media

The Media were prepared by dissolving the ingredients in distilled water and then sterilized by autoclave at 121^{o}C 15 Psi on liquid cycle. Luria bertani (LB) and Modified Youstin media were used. Media compositions were mentioned in Appendix D.

3.2.1.2 Chemicals and reagents

Agar ($C_{14}H_{24}O_9$), ascorbic acid ($C_6H_8O_6$), acetic acid (CH_3COOH), boric acid (H_3BO_3), citric acid ($C_6H_8O_7$), dH_2O, Na_2EDTA ($C_{10}H_{14}N_2O_8.2Na.2H2O$), ethanol ($CH_3CH_2OH$), ethidium bromide ($C_{21}H_{20}BrN_3$), glycerol ($C_3H_8O_3$), HCl (N), methanol ($CH_3OH$), MgCl2, dNTP, Mill feed, Sodium benzoate ($C_7H_5NaO_2$), sugar, soya bran, wheat bran, Taq polymerase, KCl, NaCl, Na2HPO4, KH2PO4, NaOH, peptone, tris-base ($C_4H_{11}NO_3$). The chemicals used in this study were of molecular grade, and their lists and company names are mentioned in Appendix E.

3.2.1.3 Buffers and solutions

Phosphate-buffered saline (PBS), normal saline, plasmid isolation kit (Favorgen), 2X PCR master mix (Invitrogen), PCR purification kit (PureLink™, Invitrogen) TAE, TBE, TE buffer, Tris-HCl, EDTA, etc., buffers and solutions used in this experimental study, and their composition and preparation are mentioned in Appendix F.

3.2.1.4 Equipments

Autoclave machine, biosafety cabinet, centrifuge machine, electronic balance, glassware sterilizer, Insect rearing cages, egging devices, microbiological incubator, magnetic stirrer, micropipettes, nanodrop 2000, orbital shaker incubator, pH meter, agarose gel electrophoresis power supply, -20 ^{o}C fridge, 4 ^{o}C refrigerator, spectrophotometer, thermal cycler, thermo stated shaking water bath, vortex mixture, etc., All equipment used in this study were mentioned in respective methods section and their company and models were mentioned in Appendix G.

3.2.2 Methods

3.2.2.1 Strains of *Bacillus thuringiensis* and growth conditions

A total of 44 *Bt* strains AgS2, ChSd2, CiSa5, DSe1, DSf4, DSh4, FhSb2, FhSb3, JDb1, JDc1, JSd1, KSa2, KSb1, KSe2, MeSa1, MeSb1, MuSa1, MuSc2, MuSc4, MuSe4, NaSc3, NaSd2, NoS2, NoS3, NsSe1, NsSe2, RaSa1, RaSa2, RaSb1, RaSc1, RaSd1, RhSa2, RhSb2, SaS4, SaS6, SaS7/19s, Soi1/li, SpSc1, SSb2, SSe2, TaSa4, TaSb3, TaSc1, and USc3 were previously isolated from the samples that were collected from different locations and environments in Bangladesh (Shishir *et al.*, 2014). These strains were preserved at -80 °C in Fermentation and Enzyme Biotechnology Laboratory (Lab 215), Department of Microbiology, University of Dhaka. In addition, *Bt kurstaki* HD-73, *Bt sotto* T84A1, and *Bt japonensis Buibui* strains were obtained from the Okayama University stock culture collection of *B. thuringiensis* in Japan. For subculturing, maintenance, and spore counting, *Bt* strains were cultivated on LB agar. All cultures were held under constant temperature of 30 °C. Liquid cultures were agitated at 180 rpm in an orbital shaker.

3.2.2.2 Inoculum preparation

We used Luria Bertani (LB) agar to prepare the inoculum of *Bt* strains. Firstly, the strains were streaked on an agar plate and kept in an incubator at 30°C for 24 h. The next day, an isolated colony, aseptically picking from the agar plate was inoculated into a conical flask containing 20 ml LB broth and the Erlenmeyer flask was kept into a shaking incubator at 30°C with180 rpm speed for overnight. Finally, the cell density of the *Bt* strains was measured at OD_{600} using a spectrophotometer after overnight incubation and this was the inoculum culture for all respective experiments, performing in T3 medium. In all sporulation experiments, the inoculum was added to the medium to ensure that the culture medium began with an OD_{600} of 0.1, unless otherwise specified.

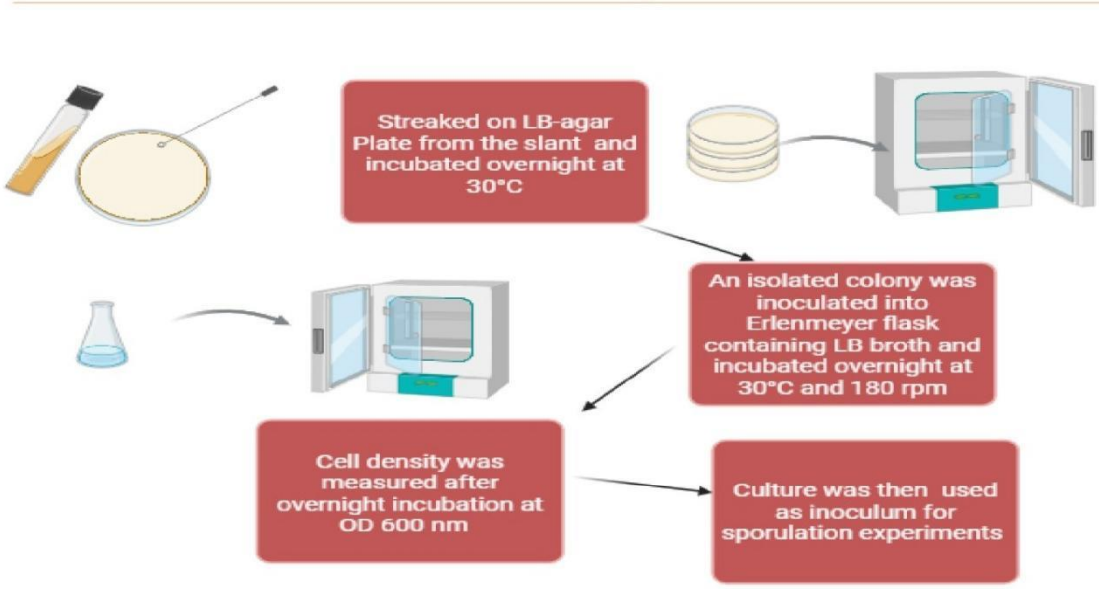


Figure 3.2.1: Preparation of Inoculum

3.2.2.3 Spore crystal mixture preparation

By inoculating the *cry1* gene-positive *Bt* strains and reference strains (Shishir *et al.*, 2015) in 100 ml of T_3 liquid medium (Obeidat *et al.*, 2004) and then culturing each of them for seven days at 30 degrees Celsius with uninterrupted shaking at 250 revolutions per minute, a spore-crystal mixture for bioassay was prepared. This mixture was used for the bioassay. In order to remove the culture from the media, the cultures were centrifuged for fifteen minutes at a speed of 5000 revolutions per minute for 15 minutes. After being cleaned twice with 20 millilitres of cold sterile distilled water, pellets, which consisted of spores and crystal protein combination, were centrifuged at 5000 revolutions per minute for a period of five minutes. After resuspending

the pellets in 20 millilitres of sterile distilled water, they were incubated for an additional two days at a temperature of thirty degrees Celsius with continuous shaking at a speed of two hundred and fifty revolutions per minute.

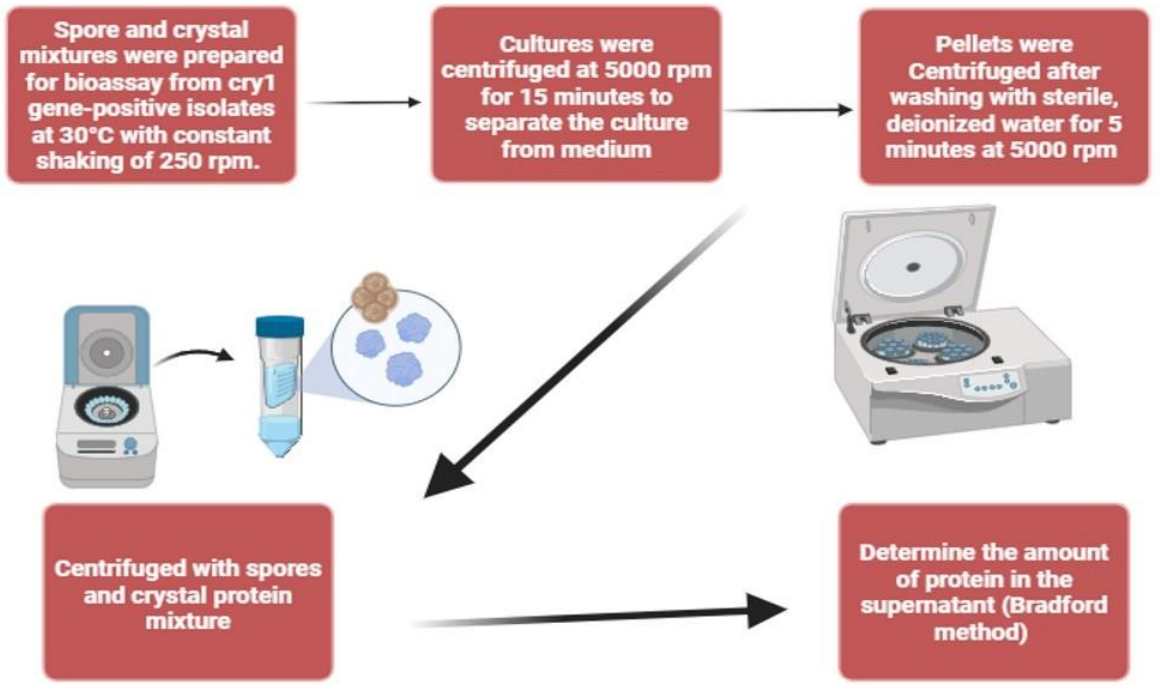


Figure 3.2.2: A flow diagram of preparing the mixture of spore crystal

3.2.2.4 Quantitative estimation of spore load

To count the spores, 1 ml of the spore-crystal solution was heated at 80°C for 10 minutes. The heat-treated suspension was then diluted one step at a time and spread out on LB agar using the spread plate method. After that, the plates were kept at 37°C for 24 hours. The heat had no effect on the spores, but it did kill all the other vegetative cells. Therefore, to calculate actual quantity, the colonies that were germinated from the spores after being incubated were counted and then multiplied by the dilution factor.

3.2.2.5 Protein concentration measurement

The protein concentration was measured using the previously known Bradford technique (Bradford, 1976). The Bradford assay is based on a change in light absorption characteristics of the dye Coomassie Brilliant Blue G-250 caused by protein binding. When dissolved in an acidic solution (85% phosphoric acid), the dye absorbs the lightest at 465 nm. When protein is added, the dye's maximum absorption shifts to 595 nm, and the light absorbance at this wavelength

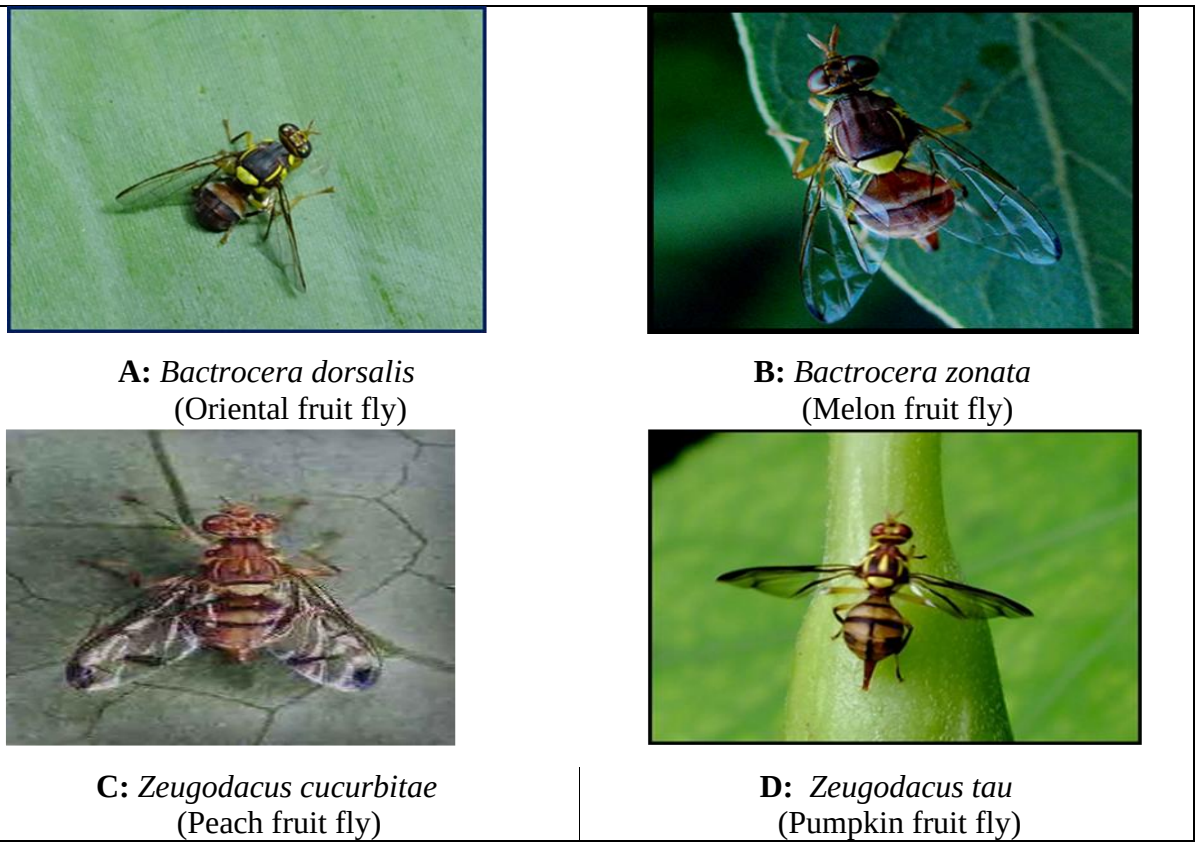
increases linearly with protein concentration. This rise in absorbance can be detected using a spectrophotometer (Genesys 5, Thermospectronic).

3.2.2.6 Tephritid fruit fly species used as test insects with their host range, distribution and economic impact

Table 3.2.2.1: A table of Tephritid fruit fly species

Tephritid flies	**Oriental fruit fly**	**Peach fruit fly**	**Melon fly**	**Pumpkin fruit fly**
Kingdom	Animalia			
Phylum	Arthropoda			
Subphylum	Hexapoda			
Class	Insecta			
Order	Diptera			
Family	Tephritidae			
Subfamily	Dacinae			
Genus	*Bactrocera*		*Zeugodacus*	
Species	*Bactrocera dorsalis*	*Bactrocera zonata*	*Zeugodacus cucurbitae*	*Zeugodacus tau*
Major Hosts	Mango, guava, banana, papaya, citrus, tomato, eggplant	Guava, mango, peach, fig, citrus	Cucumber, bitter gourd, pumpkin, ridge gourd, watermelon, zucchini	Pumpkin, bitter gourd, cucumber, sponge gourd, bottle gourd, snake gourd
Crop Type	Fruits & vegetables	Fruits	Vegetables (Cucurbits)	Vegetables (Cucurbits)
Distribution	Asia, Africa, Pacific Islands	South & Southeast Asia, Middle East, Africa	Southeas Asia, Pacific Islands, Africa	Southeast Asia, Pacific Islands, Africa
Significant Observations	Highly polyphagous; invades diverse fruit and vegetable crops; causes internal feeding and secondary rotting of fruits	Attacks both ripening and ripe fruits; develops resistance to insecticides in some regions.	Infestation causes premature fruit drop and internal tissue damage; pest pressure is highest in warm, humid climates.	Emerging pest in cucurbit crops; often misidentified with similar species.

Economic Impact	Causes multimillion-dollar losses in the mango and guava industries; subject to international quarantine restrictions, which also hampers export trade	Severe post-harvest losses reported, especially in guava; challenges in pest exclusion from export markets	Reduces cucurbit yields by 30–100% in some areas; losses in India and Bangladesh alone are significant in smallholder systems	Moderate to high yield losses in pumpkin and cucumber; threat level rising due to expanding range and limited control options
References	(Clarke *et al.*, 2005)	(Drew & Romig, 2013)	(Dhillon *et al.*, 2005)	(Drew & Romig, 2013)



A: *Bactrocera dorsalis* (Oriental fruit fly)

B: *Bactrocera zonata* (Melon fruit fly)

C: *Zeugodacus cucurbitae* (Peach fruit fly)

D: *Zeugodacus tau* (Pumpkin fruit fly)

Figure 3.2.3: (A-D) Major Tephritids pests tested against indigenous *Bt* Strain

3.2.2.7 Rearing of Insects

Laboratory-developed larval diets were employed to sustain the larvae. A stainless-steel framed cage (120×120×90) cm3 and a (183 X 76 X 38) cm3 cage enclosed with a stainless-steel net were used to adult flies. In general, a stock enclosure was stocked with 5000-7000 and 7000-10000 adult flies, respectively. The larvae commence grazing on their preferred host plant

immediately after hatching, as this supplies them with the nutrients necessary for further growth and development. Typically, two distinct laboratory-developed artificial diets were administered to newly emerged adults: a liquid diet consisting of baking yeast, sugar, and water in a ratio of 1:3:4, and a dry diet consisting of yeast extract, casein, and sugar in a ratio of 1:1:2. For the purpose of humidity, water was stored in a conical receptacle that had been saturated with cotton in the cage. The rearing room was maintained at a temperature of 28±2°C and a relative humidity (RH) of 70-80%. Insect rearing was conducted at the Insect Biotechnology Division (IBD), Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), Savar, Dhaka, Bangladesh.

Figure 3.2.4: Temperature (T) and Relative humidity (RH) control panel in Insectary

3.2.2.8 Collection of eggs from Tephritids flies for bioassay

To obtain an adequate quantity of eggs, mature, ripe, and fresh green bananas were partially husked and positioned within the adult rearing cages of *Bactrocera dorsalis* and *B. zonata* to facilitate oviposition by the flies. Some mature specimens ripened, and fresh pieces of greenish sweet gourd or cucumber were partially husked and placed inside the *Zeugodacus cucurbitae* and *Z. tau* adult rearing cages for oviposition, respectively. After thirty minutes, all infested samples of banana and cucumber were collected and placed into plastic bowls lined with dry sawdust for the further development of *B. dorsalis*, *B. zonata*, *Z. cucurbitae*, and *Z. tau*, separately. Fresh bananas were provided as sustenance for *B. dorsalis* and *B. zonata*, while fresh sweet gourd or cucumber were supplied for *Z. cucurbitae* and *Z. tau* intermittently to facilitate larval development up to early third instars for the bioassay treatment.

On the other hand, the required number of eggs for the bioassay experiment were collected using laboratory-developed egging devices. Egging devices were smashed with banana paste internally and placed inside the *B. dorsalis* and *B. zonata* adult rearing cages for oviposition. Cucumber smashed egg laying devices were placed inside the *Z. cucurbitae* and *Z. tau* adult rearing cages for oviposition to collect the required number of eggs. After half an hour, the egg devices were collected and then were washed through clean running water for the collection of the desired number of eggs for the bioassay experiment.

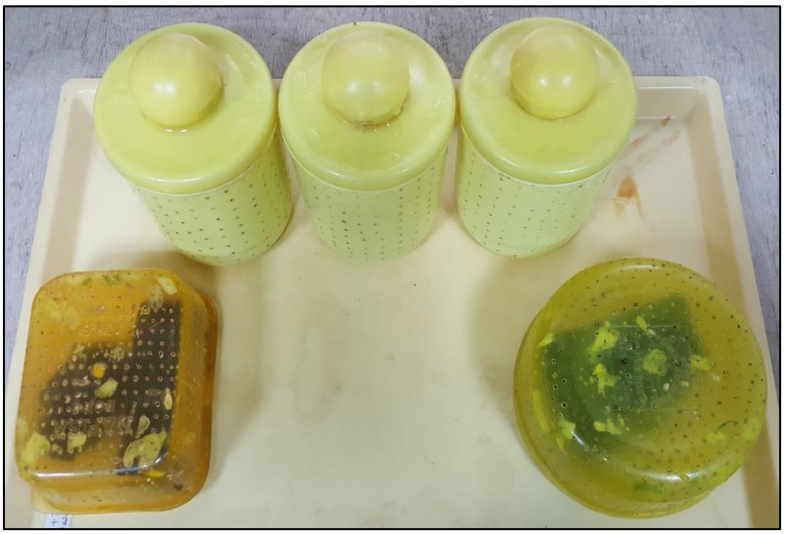


Figure 3.2.5: Different types of egging devices

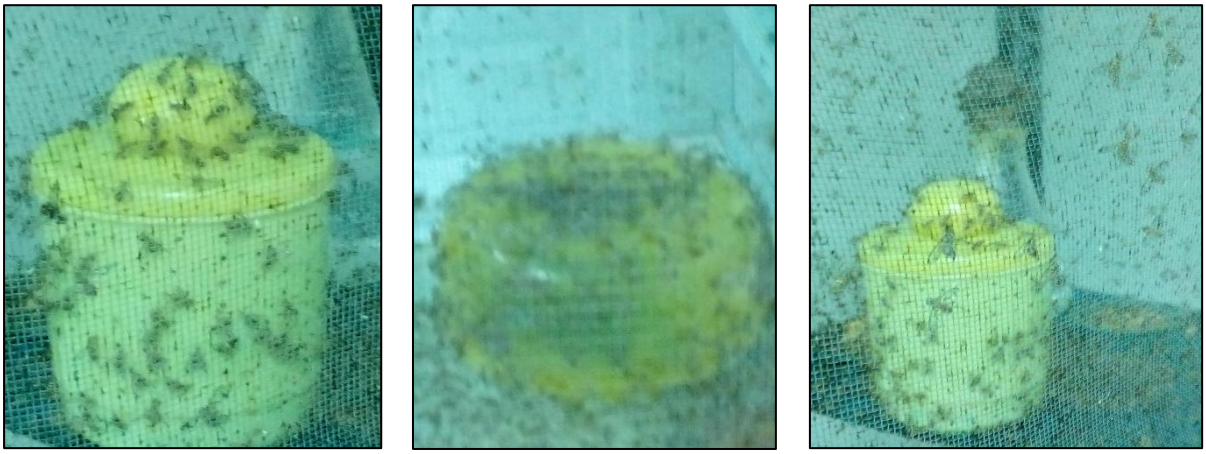
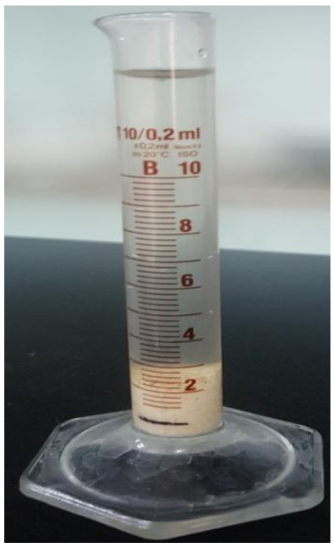


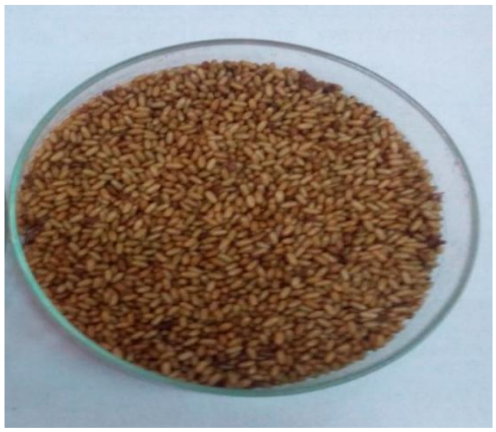
Figure 3.2.6: Devices placed on fruit fly rearing cages for oviposition



A: Eggs

B: Eggs on larval diet

C: Larvae on larval diet



D: Pupae

Figure 3.2.7: (A-D) Different stages of Tephritids fruit fly

3.2.2.9 Bioassay evaluation

The toxicity of the *Bt* strains was analyzed in vivo against the early 3rd instar larvae of oriental fruit fly, *B. dorsalis*, Peach fruit fly, *B. zonata*, melon fruit fly, *Z. cucurbitae* and pumpkin fruit fly, *Z. tau* by bioassay. The spore–crystal suspensions were prepared from *cry1A* gene-positive strains (Obeidat *et al.*, 2004; Shishir *et al.*, 2015). A 1.0 ml aliquot of the spore–crystal suspension was mixed with 10 gm of mashed banana puree for *Bactrocera dorsalis* and *B. zonata*, and with boiled and mashed sweet gourd puree for *Zeugodacus cucurbitae* and *Z. tau*, respectively. Twenty early third-instar larvae of each test insect were placed in the prepared diet in individual Petri dishes.

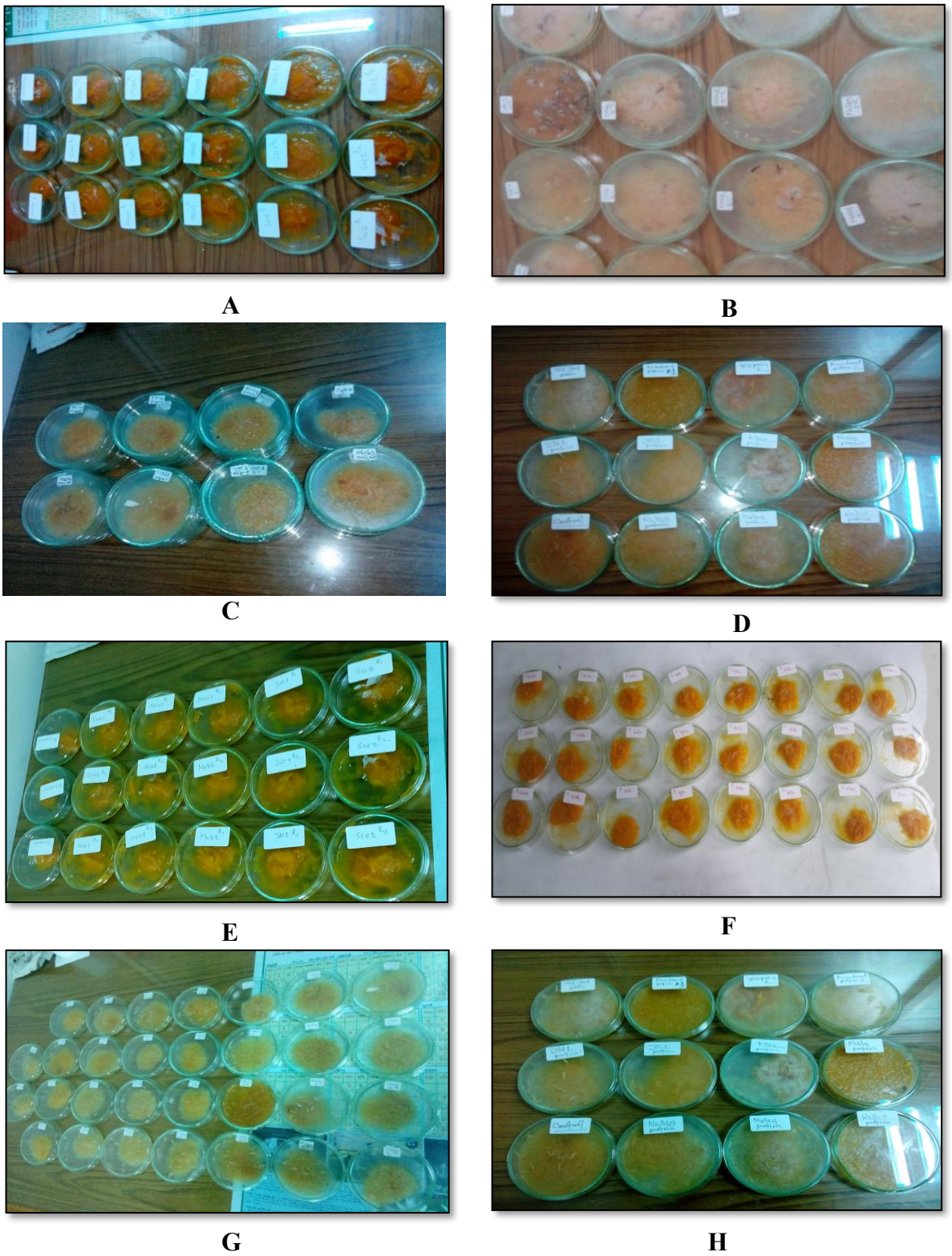
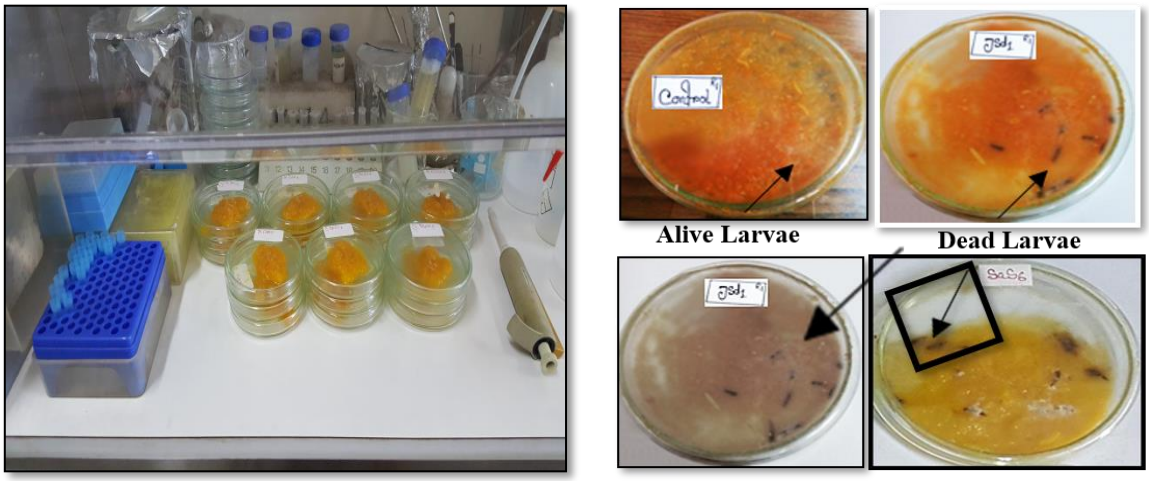


Figure 3.2.8: (A-H) Bioassay preparation of *Bactrocera dorsalis* and *B. zonata*: Mixture of *Bt* spore–crystal suspension with 10 gm banana-paste and filled in each Petri dish. **(E–H)** Bioassay setup of *Zeugodacus cucurbitae* and *Z. tau*: *Bt* spore–crystal suspension was added to 10 gm of priorly boiled mash of sweet gourd at room temperature and weighed into each dish. Third-instar larvae were transferred onto the respective labeled plates to be fed the diet with the incorporation of the *Bt* spore-crystal preparation. The diet was utilized as the treatment medium in the bioassay for *B. dorsalis*, *B. zonata*, *Z. cucurbitae*, and *Z. tau* larvae.

They were maintained at 28 ± 2 °C, 70 ± 10% relative humidity (RH), and photoperiod of 16:8 (light:dark), and were fed under laboratory condition. Mortality was seen in the *Bt*-treated larvae and a corresponding control diet supplemented with sterile distilled water. Observed mortality was Abbott-corrected (Abbott, 1925) to 7 days after treatment. T84A1 *Bt* strain and *B. thuringiensis kurstaki* (*Btk*) HD-73 were the reference strains. All the bioassays were conducted in triplicate, including control treatments.

The mortality rates for the *Bt* strains were recorded alongside a control diet treated with sterile distilled water. This approach also facilitated the adjustment of test mortality using Abbott's formula (Abbott, 1925) over a period of 7 days. *Bts* T84A1 and *Btk* HD-73 were utilized as reference strains. Bioassays were performed in triplicate for all instances concerning the control.



A B

Figure 3.2.9: (A) Bioassay: *Bt* spore-crystal was mixed with 10 gm of banana paste for *B. dorsalis* and *B. zonata* and previously boiled sweet gourd mash for *Z. cucurbitae* and *Z. tau* at room temperature inside a biosafety cabinet, and measured in each plate. *(B)* Observation of live larvae in control and dead larvae (turned into black, indication of death) in *Bt* JSd1 and SaS6 in bioassay experiment

3.2.2.10 Data collection and statistical analysis

Bioassay process involved screening for prospective strains in the first phase and finding lethal concentration values for strains that caused more than 50% mortality in the second step. At the start of the experiment, undiluted spore–crystal suspensions were added in equal amounts to the larval diet for each *Bt* strain. The number of dead larvae was recorded afterward, and mortality data were corrected using Abbott's formula (Abbott, 1925). It was performed in triplicate and the average percentage of mortality was determined for each strain. *Bt* strains that caused more

than 40% mortality were retested in the same way, and those that killed more than 50% of the larvae were selected for the next phase in the process.

The spore–crystal solution of *Bt* strains exhibiting an average mortality rate exceeding 50% was serially diluted with sterile distilled water at 2^{-1}, 2^{-2}, and 2^{-3}-fold dilutions prior to incorporation with the larval meal in the final phase of the bioassay. The LC_{50} and LC_{99} values were determined by counting the number of deceased larvae in triplicate across various concentrations of distinct treatments (a spore-crystal mixture of *Bt* strains).

To estimate the lethal concentration values based on the spore concentration in the suspension, a logarithmic scale was used. By utilizing Probit Analysis-Finney Method [Lognormal Distribution] (Finney, 1952, 1971) on the data of dead larvae from various treatments, the concentrations that killed 50% and/or 99% of the larvae were calculated for the treatments using Stat plus 2009 Windows program and Graphical analysis were done using GraphPad Prism 10 as well as python open source software program. There were three times as many runs of all bioassays, and the means were examined with one-way analysis of variance and compared to see which differences were least significant (LSD). The statistical significance threshold was set at p-values of equal to 0.05.

3.3 RESULTS

3.3.1 Bioassay performed with *Bt* strains against Tephritids fruit flies

Out of the forty-four native *Bt* strains, sixteen caused more than 50% larval mortality against the tested tephritid fruit flies, namely *Bactrocera dorsalis*, *B. zonata*, *Zeugodacus cucurbitae*, and *Z. tau*. From the sixteen strains, eleven were found to cause the highest mortality, exceeding 70% against the *B. dorsalis* and *B. zonata* early 3rd instar larvae, whereas nine exhibited more than 70% larval mortality against the *Z. cucurbitae* and *Z. tua*.

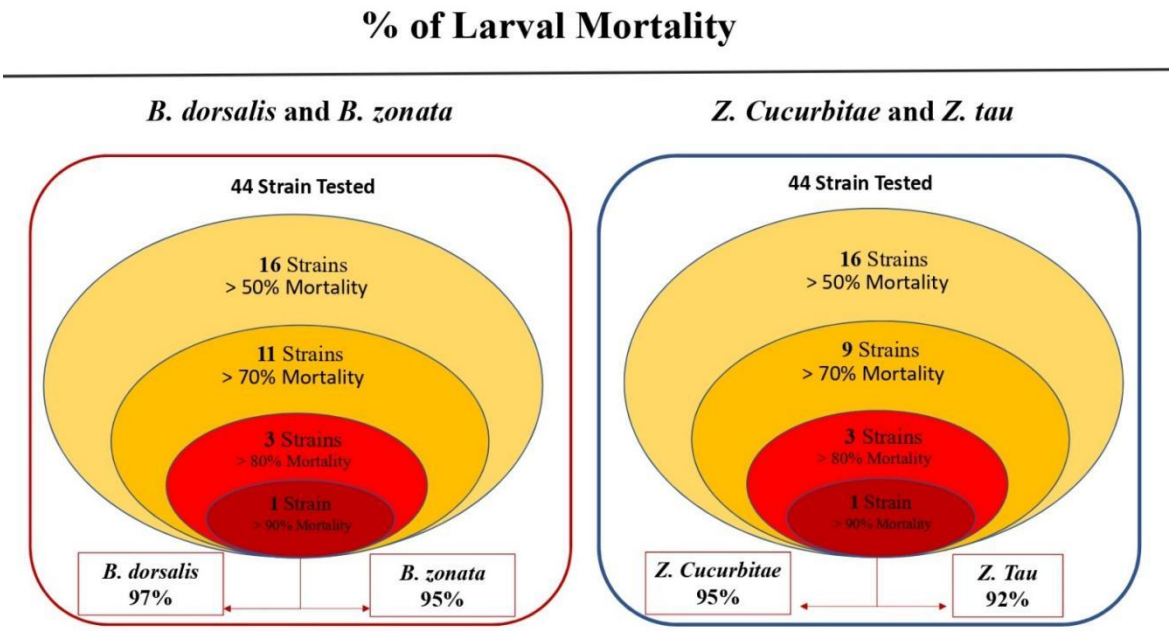


Figure 3.3.1: Bioassay performed with native *Bt* strains against selected Tephritids fruit flies in this study at a glance

3.3.2 Bioassay performed with *Bt* strains against *B. dorsalis* and *B. zonata*

Among the forty-four native *Bt* strains 16 that carried distinct *cry* genes were examined *in vivo* against *Bactrocera dorsalis* and *B. zonata*. These sixteen isolates caused more than 50% larval mortality, with the following mortality rates observed: for *B. dorsalis*, *Bt* DSe1 (55%), DSf4 (72%), FhSb3 (78%), JDc1 (85%), JSd1 (97%), KSa2 (77%), MuSc2 (73%), MuSc4 (58%), NaSc3 (78%), RaSa2 (77%), SaS4 (57%), SaS6 (88%), SaS7/19s (70%), Soi1/li (62%), SSe2 (58%), and TaSa4 (80%). For *B. zonata*, the mortality rates were as follows: *Bt* DSe1 (57%), DSf4 (70%), FhSb3 (78%), JDc1 (87%), JSd1 (95%), KSa2 (73%), MuSc2 (72%), MuSc4 (57%), NaSc3 (75%), RaSa2 (77%), SaS4 (58%), SaS6 (90%), SaS7/19s (70%), Soi1/li (60%), SSe2 (57%), and TaSa4 (79%). Out of the sixteen, eleven strains caused more than 70% larval mortality in early third-instar larvae of both *B. dorsalis* and *B. zonata.*

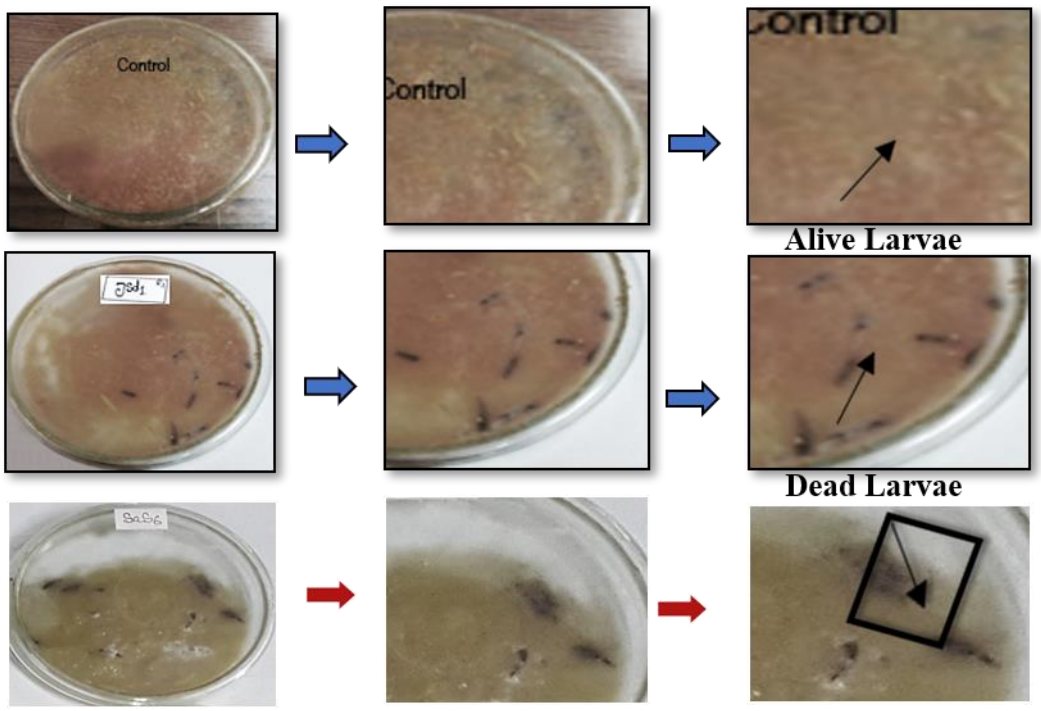


Figure 3.3. 2: Bioefficacy of *Bt* JSd1 & SaS6 against *B. dorsalis* and *B. zonata*

Certain strains showed similar toxicity and larval mortality rates. For *Bactrocera dorsalis*, the following strains exhibited high mortality: *Bt* JSd1 (97%), SaS6 (88%), JDc1 (85%), TaSa4 (80%), FhSb3 (78%), NaSc3 (78%), RaSa2 (77%), KSa2 (77%), MuSc2 (73%), DSf4 (72%), and SaS7/19s (70%). For *Bactrocera zonata*, the strains that caused over 70% mortality were: *Bt* JSd1 (95%), SaS6 (90%), JDc1 (87%), TaSa4 (79%), FhSb3 (78%), RaSa2 (77%), NaSc3 (75%), KSa2 (73%), MuSc2 (72%), SaS7/19s (70%), and DSf4 (70%). Meanwhile, the reference strain *Bts* T84A1 and the control exhibited 95% and 10% mortality, respectively. Some larvae in the control group (without treatment) also died, which were used to correct the test mortality rates.

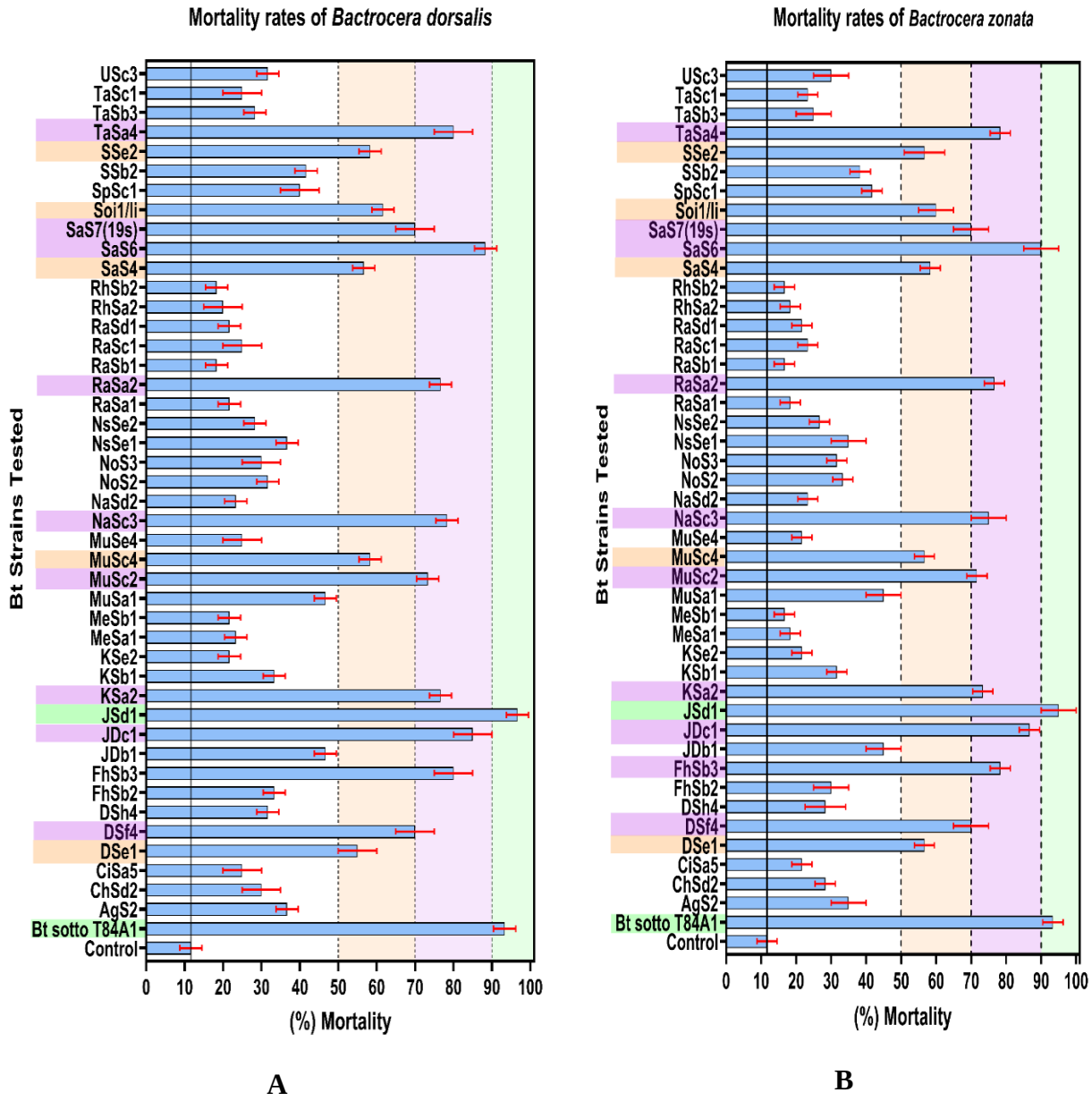


Figure 3.3.3: The spore-crystal protein mixture of native *Bt* strains tested for mortality **(A)** against *B. dorsalis* caused mortality (early 3rd instar larvae); **(B)** against *B. zonata* caused mortality (early 3rd instar larvae).

Bactrocera dorsalis third-instar larvae were tested against all native *Bt* strains, and eleven caused more than 70% mortality, exhibiting toxicity in the following order: JSd1 > SaS6 > JDc1 > TaSa4 > FhSb3 > RaSa2 > NaSc3 > KSa2 > MuSc2 > DSf4 > SaS7/19s. For *Bactrocera zonata* third-instar larvae, ten strains exhibited toxicity in the following order: JSd1 > SaS6 > JDc1 > TaSa4 > FhSb3 > NaSc3 > RaSa2 > KSa2 > MuSc2 > SaS7/19s > DSf4, causing more than 70% mortality. The efficacy of these potential strains was compared with the reference strain *Bts* T84A1 (Fig. 3.3.3 A & B).

3.3.3 Bioassay performed with *Bt* strains against *Z. cucurbitae* and *Z. tau*

Among the 44 tested indigenous *Bacillus thuringiensis* (*Bt*) strains, sixteen were found to cause more than 50% larval mortality against *Zeugodacus cucurbitae* and Z. tau. For *Z. cucurbitae*, the effective strains were: *Bt* DSe1 (53%), DSf4 (68%), FhSb3 (77%), JDc1 (86%), JSd1 (95%), KSa2 (75%), MuSc2 (72%), MuSc4 (55%), NaSc3 (80%), RaSa2 (78%), SaS4 (55%), SaS6 (87%), SaS7/19s (68%), Soi1/li (62%), SSe2 (60%), and TaSa4 (82%). Similarly, for *Z. tau*, the same sixteen strains showed the following mortality rates: *Bt* DSe1 (50%), DSf4 (65%), FhSb3 (70%), JDc1 (83%), JSd1 (92%), KSa2 (72%), MuSc2 (70%), MuSc4 (52%), NaSc3 (72%), RaSa2 (74%), SaS4 (52%), SaS6 (85%), SaS7/19s (65%), Soi1/li (55%), SSe2 (54%), and TaSa4 (75%).

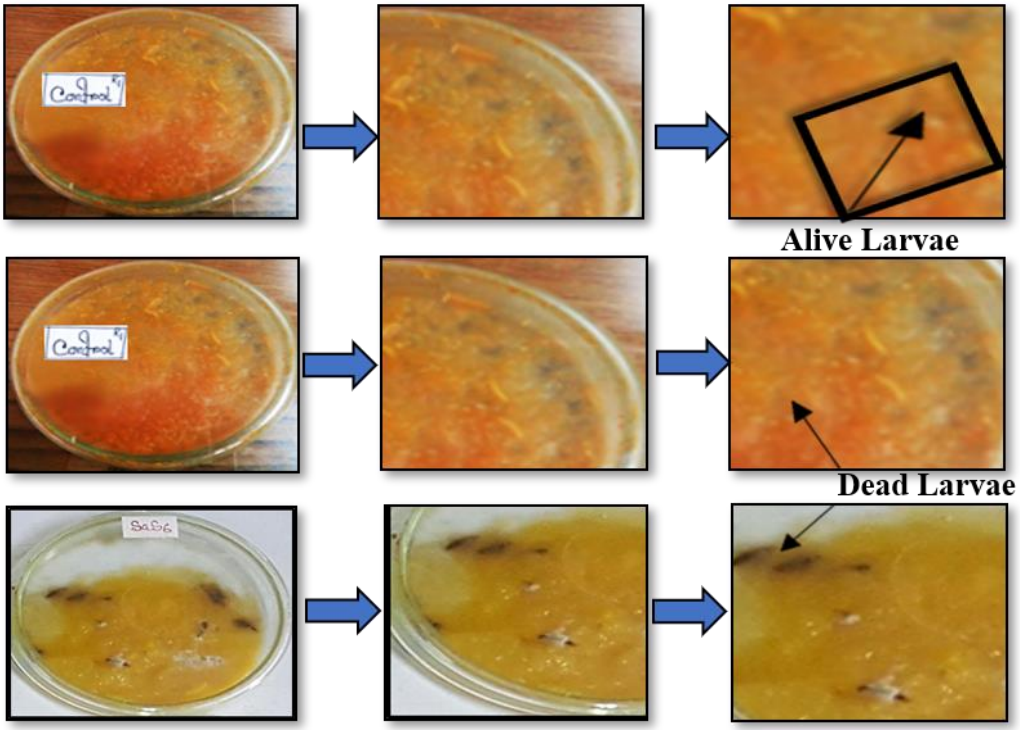


Figure 3.3.4: Bioefficacy of *Bt* JSd1 & SaS6 against *Z. cucurbitae* and *Z. tau*.

Among these, nine *Bt* strains were particularly effective, causing more than 70% mortality in both species. These included JSd1 (95% for *Z. cucurbitae* and 92% for *Z. tau*), SaS6 (87% and 85%), JDc1 (86% and 83%), TaSa4 (82% and 75%), FhSb3 (77% and 70%), NaSc3 (80% and 72%), RaSa2 (78% and 74%), KSa2 (75% and 72%), and MuSc2 (72% and 70%). 10% of the control group died, compared to 94% of the reference strain *Btk* HD-73. Abbott's formula was used to adjust the test mortality when several early third-instar larvae were discovered dead in the untreated control group. Toxicity of the tested potential *Bt* strains were in the following order JSd1> SaS6> JDc1> TaSa4> NaSc3> RaSa2> FhSb3> KSa2> MuSc2 causing more than 70% mortality for *Z. cucurbitae* 3rd instar larvae while the toxicity for *Z. tau* 3rd instar larvae

were in the order JSd1> SaS6> JDc1> TaSa4> RaSa2> NaSc3> KSa2> FhSb3> MuSc2 causing more than 70% mortality respectively and were compared to the efficacy of the potential strains with the reference strain, *Btk* HD73 (Fig. 3.3.5 A & B).

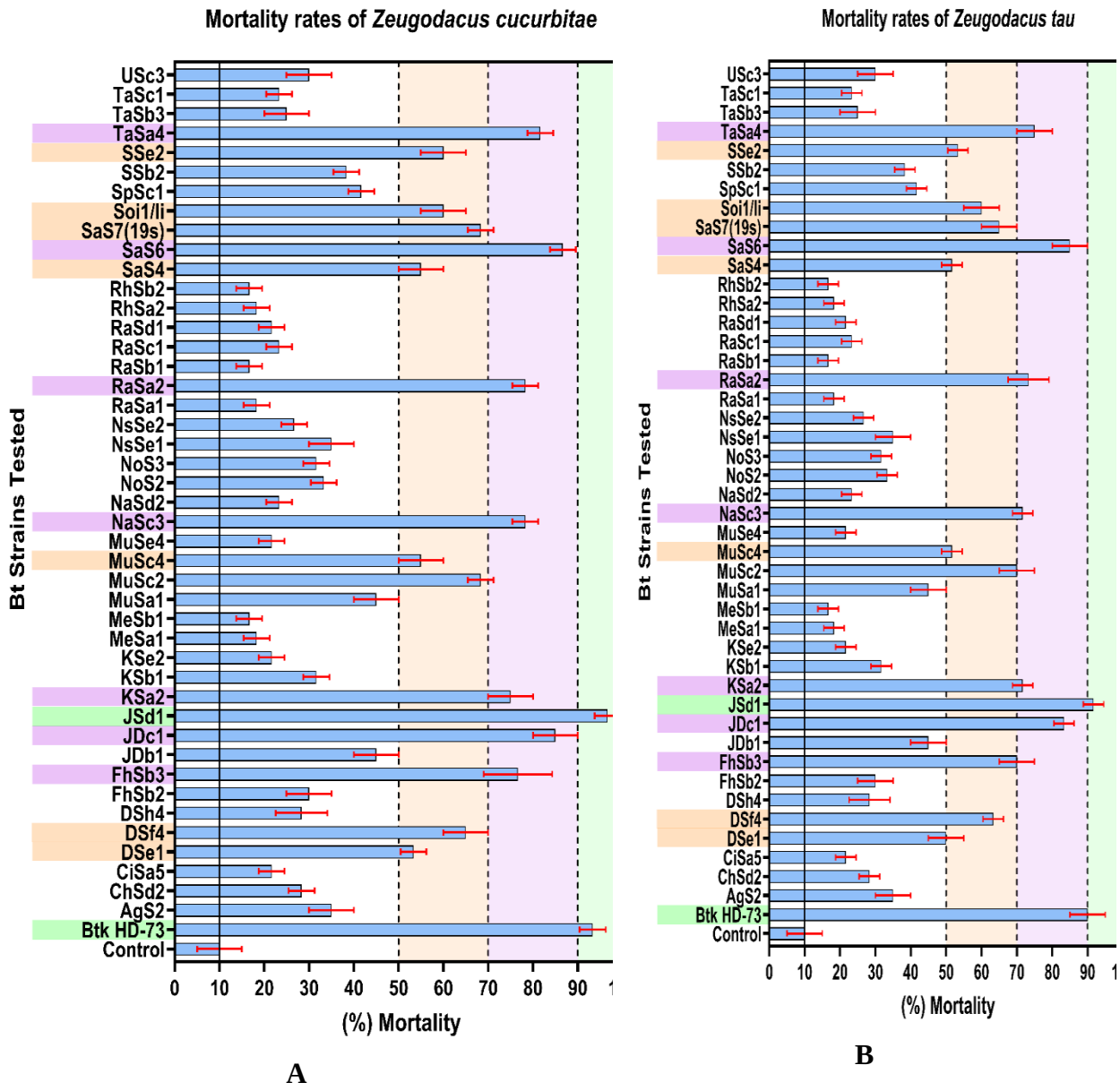


Figure 3.3.5: The spore-crystal protein mixture of native *Bt* strains tested for mortality **(A)** against *Z. cucurbitae*; **(B)** against *Z. tau*

3.3.4 Determinations of Lethal Concentrations (LC) against *B. dorsalis* and *B. zonata*

Bioassays were conducted in triplicate using native *Bacillus thuringiensis (Bt*) strains (spore crystal mixture) that caused more than 70% larval mortality to determine the LC_{50} and LC_{99} values for both test insects. Probit analysis was employed to evaluate the efficacy of each strain based on lethal concentration (LC_{50}, LC_{99}) and lethal

time (LT_{50}, LT_{99}) values. For each isolate, the initial spore–crystal suspension was serially diluted at 2^{-1}, 2^{-2}, and 2^{-3}-fold concentrations.

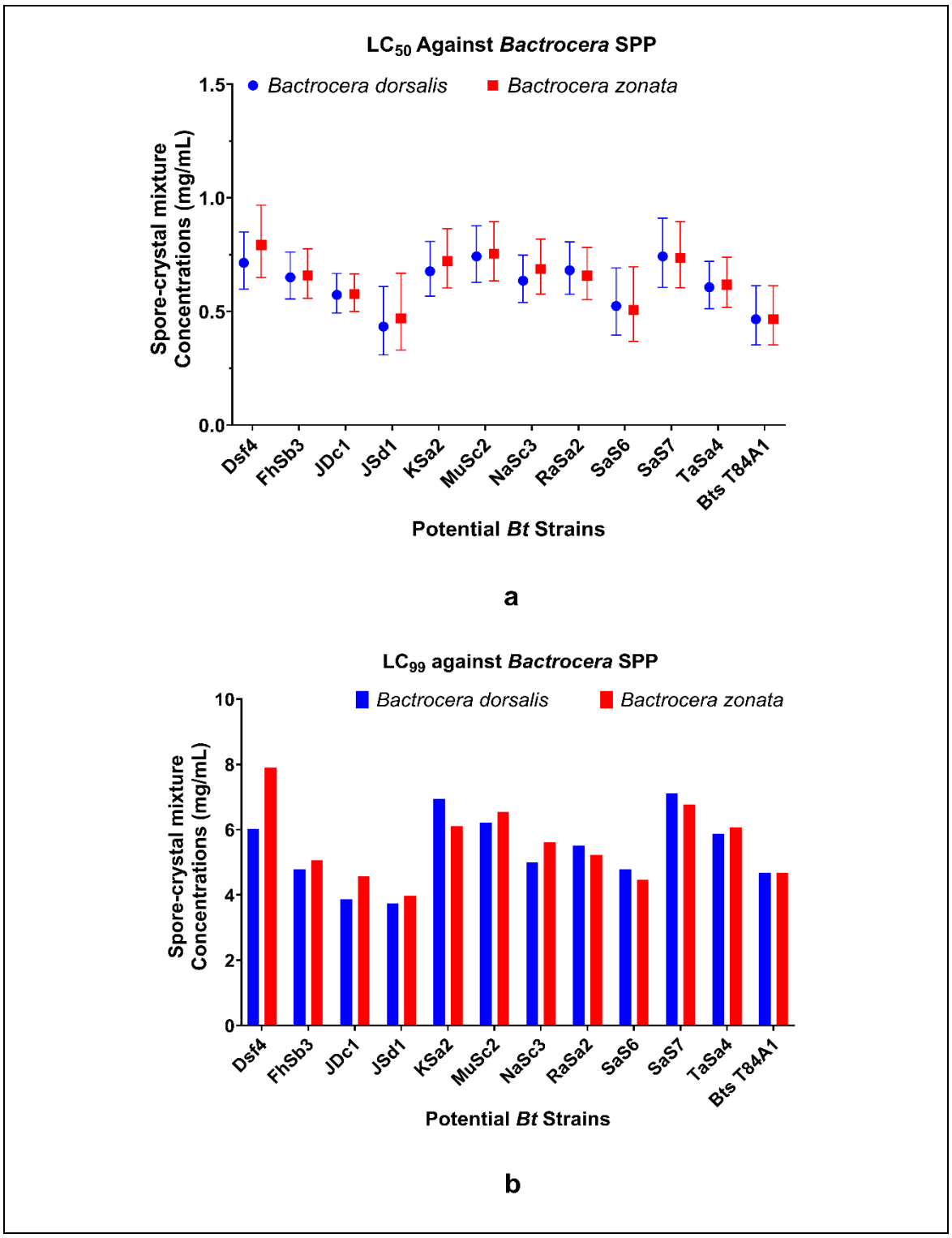


Figure 3.3.6: (a & b) LC_{50} and LC_{99} values of potential *Bt* strains tested against *B. dorsalis* and *B. zonata* (early 3rd instar larvae).

Table 3.3.1: Regression equation, χ^2 values and p values of Lethal Concentrations for potential *Bt* strains against *B. dorsalis and B. zonata* (early 3rd instar larvae).

***Bt* Strains (Potential)**	**Regression equations**	**χ^2 values (df)**	**p-value**
Against the 3rd instar larvae of *Bactrocera dorsalis*			
Dsf4	Y=2.4793 + 2.9521 X	0.4742 (2)	1.66
FhSb3	Y=2.4688 + 3.1118 X	1.2706 (2)	2.27
JDc1	Y=2.6148 + 3.1439 X	3.6793 (2)	1.21
JSd1	Y=2.9887 + 3.155 X	11.7867(2)	4.45
KSa2	Y=2.6676 + 2.8076 X	3.5092 (2)	3.19
MuSc2	Y=2.2207 + 3.1913 X	2.6303 (2)	4.69
NaSc3	Y=2.6143 + 2.9702 X	2.7921 (2)	1.55
RaSa2	Y=2.5374 + 2.9542 X	3.3517 (2)	2.07
SaS6	Y=2.9469 + 2.8530 X	6.2168 (2)	2.37
SaS7	Y=2.7485 + 2.5849 X	2.9487 (2)	6.19
TaSa4	Y=2.8286 + 2.7719 X	3.8807 (2)	1.39
Bts T84A1	Y=2.8346+3.2379 X	7.9240 (2)	2.74
Against the 3rd instar larvae of *Bactrocera zonata*			
Dsf4	Y=2.5193 + 2.7885 X	1.2688 (2)	1.29
FhSb3	Y=2.5290 + 2.0176 X	2.8717 (2)	5.04
JDc1	Y=2.4683 + 3.3253 X	4.4224 (2)	1.71
JSd1	Y=2.9362 + 3.0701 X	11.6693(2)	7.49
KSa2	Y=2.5108 + 2.8987 X	1.9397 (2)	3.08
MuSc2	Y=2.2491 + 3.1351 X	2.3917 (2)	3.27
NaSc3	Y=2.5939 + 2.8744 X	2.0871 (2)	2.33
RaSa2	Y=2.6658 + 2.8522 X	2.2888 (2)	2.07
SaS6	Y=2.8328 + 3.0730 X	9.2420 (2)	3.73
SaS7	Y=2.6823 + 2.6737 X	1.3672 (2)	1.31
TaSa4	Y=2.8882 + 2.6681 X	2.1806 (2)	1.07
Bts T84A1	Y=2.8346+3.2379 X	7.9240 (2)	2.86

The spore concentrations of each strain were estimated using a logarithmic scale, and corresponding larval mortality rates were recorded. Among the sixteen effective strains, eleven were found to cause more than 70% mortality against early third-instar larvae of *Bactrocera*

dorsalis, while ten strains exhibited similar mortality levels against *B. zonata*. These selected strains were subjected to further analysis to determine LC_{50} values with 95% confidence intervals, LC_{99} values (Figure 3.3.6 a & b), along with regression equations, chi-square (χ^2) values, and associated p-values (Table 3.3.1).

For *Bactrocera dorsalis*, LC_{50} values representing the concentration needed to kill 50% of the larvae ranged from 0.433 to 0.742 mg/ml. The LC_{99} values representing the dosage needed to kill 99% of larvae ranged from 3.745 to 7.108 mg/ml. The LC_{50} and LC_{99} values for *B. zonata* ranged from 0.470–0.754 mg/ml and 3.980–6.768 mg/ml, respectively. Most notably, the *Bt* JSd1 strain was differentiated from the rest by recording the lowest LC_{50} values for both species—0.433 mg/ml for *B. dorsalis* and 0.470 mg/ml for *B. zonata*—hence being the most potent among the tested strains in recording 50% larval mortality.

At the other end of the curve was strain SaS7 that recorded the highest LC_{50} (0.742 mg/ml) for *B. dorsalis*, with MuSc2 recording the highest LC_{50} (0.754 mg/ml) for *B. zonata*, and which possessed lower insecticidal activity. When LC_{99} values were used, *Bt* JSd1 again proved to be extremely powerful in employing the lowest concentrations of 3.745 mg/ml for *B. dorsalis* and 3.980 mg/ml for *B. zonata*. SaS7, on the other hand, required the highest concentrations for 99% mortality, 7.108 mg/ml and 6.768 mg/ml for *B. dorsalis* and *B. zonata*, respectively.

For statistical validity, regression equations for all the strains were developed together with their 95% confidence intervals (lower and upper limits), chi-square (χ^2) values, and p-values. The reference strain *Bts* T84A1 yielded the LC_{50} and LC_{99} values of 0.466 mg/ml and 4.679 mg/ml, respectively, for both the target insects. Among all the tested strains, three of them—*Bt* JSd1, SaS6, and JDc1, consistently caused over 80% larval mortality and were thus selected to be further investigated regarding their biological quality parameters on treated and infested preferred host fruits and vegetables against the four most significant and economically important tephritid fruit fly pests studied.

3.3.5 Determinations of Lethal Concentrations (LC) against *Z. cucurbitae* and *Z. tau*

Bioassays were consistently performed in triplicate using indigenous *Bacillus thuringiensis* (*Bt*) strains (spore crystal mixture) that caused more than 70% mortality in early third-instar larvae of *Zeugodacus cucurbitae* and *Z. tau*, with the aim of determining LC_{50} and LC_{99} values for both insect species.

Probit analysis was employed to evaluate the efficacy of each strain based on their lethal concentration (LC_{50}, LC_{99}) and lethal time (LT_{50}, LT_{99}). For each selected strain, the initial spore–crystal mixture was serially diluted to 2^{-1}, 2^{-2}, and 2^{-3} concentrations. The spore

concentrations were estimated on a logarithmic scale, and larval mortality was carefully recorded.

Among the sixteen tested strains, nine exhibited more than 70% mortality in both *Z. cucurbitae* and *Z. tau* and were therefore selected for further toxicity evaluation. For these nine strains, LC_{50} values with 95% confidence limits, LC_{99} values (Figure 3.3.7 a & b), regression equations, chi-square (χ^2) values, and p-values were determined (Table 3.3.2). Probit analysis was repeated to analyze and validate the larvicidal efficacy of these potential strains in terms of both lethal concentration and lethal time.

LC_{50} and LC_{99} values as determined against *Z. cucurbitae* were FhSb3 (0.655 and 4.963), JDc1 (0.565 and 3.792), JSd1 (0.431 and 4.278), Ksa2 (0.689 and 5.458), MuSc2 (0.752 and 6.546), NaSc3 (0.621 and 4.727), RaSa2 (0.658 and 5.075), SaS6 (0.531 and 4.958), TaSa4 (0.573 and 5.447) mg/ml respectively. Meanwhile LC_{50} and LC_{99} values calculated against *Z. tau* were FhSb3 (0.753 and 6.868), JDc1 (0.608 and 4.220), JSd1 (0.472 and 3.992), Ksa2 (0.720 and 6.280), MuSc2 (0.770 and 6.926), NaSc3 (0.726 and 6.567), RaSa2 (0.698 and 6.095), SaS6 (0.557 and 4.798), TaSa4 (0.652 and 5.598) mg/ml respectively. Whereas the LC_{50}; LC_{99} values of reference strain, *Btk* HD-73 were (0.450; 4.478) for both the test insects (Table 3.3.2).

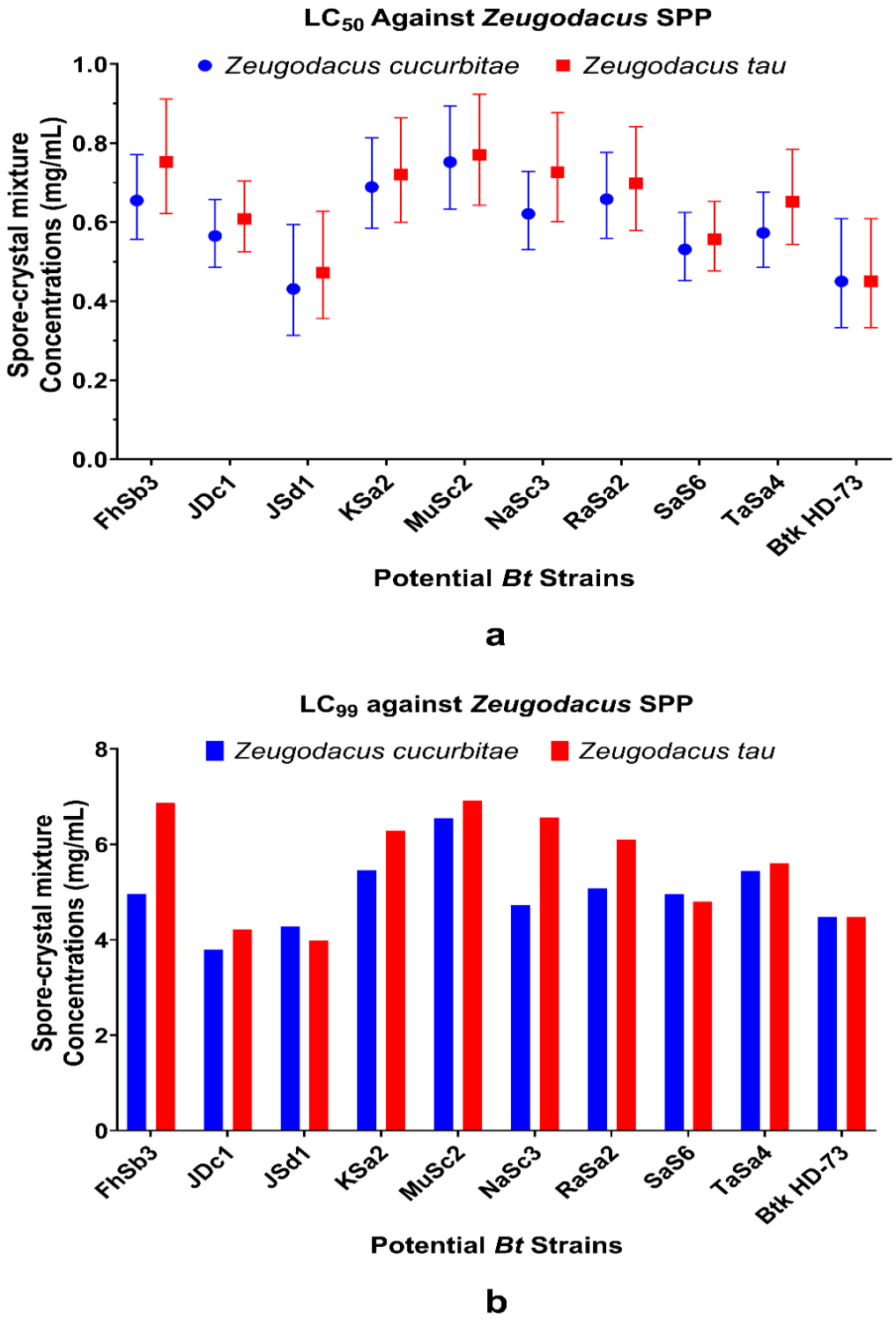


Figure 3.3.7: (a & b) LC_{50} and LC_{99} values of potential *Bt* strains tested against *Z. cucurbitae and Z. tau* (early 3rd instar larvae).

Table 3.3.2: Regression equation, χ^2 values and p values of Lethal Concentrations for potential *Bt* strains against *Z. cucurbitae and Z. tau* (early 3rd instar larvae).

***Bt* Strains (Potential)**	**Regression equations**	**χ^2 values (df)**	**p-value**
Against the 3rd instar larvae of *Zeugodacus cucurbitae*			
FhSb3	Y=2.5239 + 3.0331 X	0.4990 (2)	1.59
JDc1	Y=2.6322 + 3.1481 X	2.8242 (2)	3.63
JSd1	Y=3.2285 + 2.7970 X	8.9852 (2)	2.79
KSa2	Y=2.4270 + 3.0681 X	0.8922 (2)	1.35
MuSc2	Y=2.2933 + 3.0890 X	1.9342 (2)	6.09
NaSc3	Y=2.5761 + 3.0543 X	3.2840 (2)	2.7
RaSa2	Y=2.5290 + 3.0176 X	2.8717 (2)	2.07
SaS6	Y=2.9528 + 2.8104 X	4.2735 (2)	2.95
TaSa4	Y=2.8945 + 2.7755 X	3.9262 (2)	4.13
Btk HD-73	Y=3.0733 + 2.9463 X	8.2041 (2)	1.5
Against the 3rd instar larvae of *Zeugodacus tau*			
FhSb3	Y=2.5663 + 2.7756 X	1.3685 (2)	1.11
JDc1	Y=2.4292 + 3.2792 X	3.4650 (2)	2.81
JSd1	Y=2.9105 + 3.0974 X	7.8263 (2)	4.36
KSa2	Y=2.5500 + 2.8576 X	1.1193 (2)	7.25
MuSc2	Y=2.3340 + 3.0056 X	1.5998 (2)	4.18
NaSc3	Y=2.6222 + 2.7612 X	2.4150 (2)	1.88
RaSa2	Y=2.7185 + 2.7033 X	2.5946 (2)	7.07
SaS6	Y=2.8392 + 2.8961 X	5.3626 (2)	4.68
TaSa4	Y=2.8528 + 2.6351 X	3.4979 (2)	4.05
Btk HD-73	Y=3.0733 + 2.9463 X	8.2041 (2)	1.75

LC_{50} values ranged between 0.431 to 0.752 mg/ml and LC_{99} Values ranged from 4.278 to 10.082 mg/ml for *Z. cucurbitae* respectively whereas for *Z. Tau*, LC_{50} values 0.472 to 0.793 mg/ml and LC_{99} values varied from 3.992 to 8.480 mg/ml respectively. The lowest LC_{50} value was recorded for the indigenous *Bt* strain JSd1 (0.431) for *Z. cucurbitae* and *Bt* JSd1 (0.472) for *Z. tau* indicated the highest potency in causing 50% death of the larvae. On the contrary, MuSc2 exhibited maximum spore requirements LC_{50} values (0.752) for *Z. cucurbitae* and SaS7 showed maximum LC_{50} values (0.793) for *Z. tau* respectively. The LC_{99} values i.e., causing 99% larval mortality, LC_{99} values of *Bt* JSd1 (4.278) for *Z. cucurbitae* and *Bt* JSd1 (3.992) for *Z. tau* was found to be potentials meanwhile *Bt* SaS7 exhibits LC_{99} values (10.082) for *Z. cucurbitae* and SaS7 (8.480) for *Z. tau* was the highest value.

The regression equation, 95% confidence limits (Lower and Upper Value), χ^2 values and p values were derived concerning LC_{50} values. The LC_{50} and LC_{99} values for reference strain *Btk* HD-73 were (0.450) and (4.478) ml/gm for *Z. cucurbitae* and *Z. tau* respectively. LC_{50} and LC_{99} was found to be lowest for JSd1 (0.4317 and 0.4727 mg/ml) and JSd1(4.278 and 3.992 mg/ml) for *Z. cucurbitae and Z. tau* respectively. Similarly, only three potential *Bt* strains shows more than 80% larval mortality *viz.* JSd1, SaS6 and JDc1 were only subjected to observe biological quality parameters assessment from the infested and treated preferred hosts against four tested Tephritids fruit fly pests.

3.3.6 Determination of Lethal Time (LT) against *B. dorsalis* and *B. zonata*

Probit analysis was used to analyse the efficacy of the potential strains based on their lethal concentration (LC_{50}, LC_{99}) and lethal time (LT_{50}, LT_{99}). The calculated LT_{50}; LT_{99} values of potential eleven strains against *B. dorsalis* were DSf4 (135.99; 343.31), FhSb3 (135.73; 327.83), JDc1 (135.09; 342.79), JSd1 (54.09; 328.31), Ksa2 (135.05; 853.14), MuSc2 (147.89; 855.13), NaSc3 (135.89; 465.39), RaSa2 (147.33; 238.67), SaS6 (92.08; 343.71), SaS7/19s (135.39; 419.55), TaSa4 (135.96; 465.39) hrs respectively. Whereas, LT_{50}; LT_{99} values of ten potential strains against *B. zonata* were followed by DSf4 (136.47; 349.56), FhSb3 (135.89; 328.37), JDc1 (135.29; 343.29), JSd1 (54.39; 327.19), Ksa2 (135.55; 852.56), MuSc2 (146.99; 852.87), NaSc3 (135.79; 464.98), RaSa2 (146.56; 237.75), SaS6 (93.36; 344.53), SaS7/19s (136.21; 455.38), TaSa4 (134.93; 464.67) hrs respectively. Meanwhile calculated LT_{50}; LT_{99} values of the reference strain, Bts T84A1 were (55.59; 334.78) hrs. (Figure 3.3.8**)**.

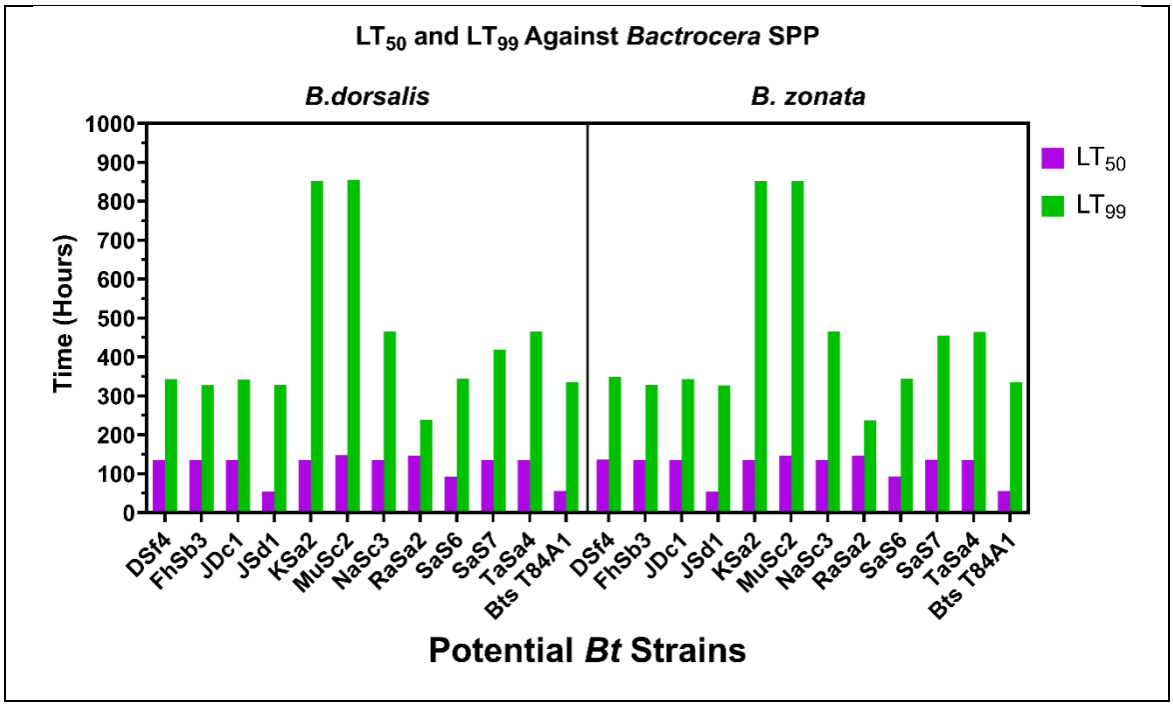


Figure 3.3.8: LT_{50} and LT_{99} values estimated for the potential *Bt* strains against *B. dorsalis* and *B. zonata* (early 3rd instar larvae)

LT_{50} and LT_{99} values for the potential *Bt* strains were ranging from 54.09 to 147.89 hours and from 238.67 to 855.13 hours for *B. dorsalis* while 54.39 to 146.99 hours and from 237.75 to 852.87 hours for *B. zonata* respectively. *Bt* JSd1 seemed to have the lowest LT_{50} values of 54.09 and 54.39 hrs recorded for both the test insect pests which indicated the highest potency in causing the death of 50% of the larvae within four days.

On the other hand, MuSc2 had the highest time requirements (LT_{50}- 147.89 and 146.99 hrs) for both test insect pests. The LT_{99} values i.e., causing 99% larval mortality, Rasa2 (LT_{99}-238.67 and 237.75 hrs) was found to be potentials in both the cases. MuSc2 (LT_{99}- 855.13 and 852.87 hrs) was the higher values against *B. dorsalis* and *B. zonata* respectively. For the reference strain *Bts* T84A1 the LT_{50}- 55.59 hrs and LT_{99}- 334.78 hrs were recorded. Estimated LT_{50} was lowest for *Bt* JSd1 (54.09 and 54.39 hrs) (Figure 3.3.8**)**. Thus, using the dose and time response bioassay, the most effective *Bt* strains are *Bt* JSd1 and secondly *Bt* SaS6 in controlling *B. dorsalis* and *B. zonata* 3rd instar larvae at the lowest dose and the fastest time in the laboratory, allowing for large-scale production and sustainable distribution to farmers.

3.3.7 Determination of Lethal Time (LT) against *Z. cucurbitae* and *Z. tau*

Probit analysis was used to analyse the efficacy of the potential strains based on their lethal concentration (LC_{50}, LC_{99}) and lethal time (LT_{50}, LT_{99}). Calculated LT_{50}; values of nine potential strains against Z. *cucurbitae and Z. tau* were FhSb3 (136.80 and 137.05), JDc1 (136.06 and 136.43), JSd1 (54.16 and 55.17), Ksa2 (136.06 and 137.19), MuSc2 (148.96; 148.13), NaSc3 (136.80 and 136.11), RaSa2 (148.36 and 148.17), SaS6 (93.09 and 94.08), TaSa4 (136.80 and 135.97) hrs respectively. Whereas, estimated LT_{99} values of the nine potential strains against Z. *cucurbitae and Z. tau* were followed by FhSb3 (329.38 and 329.09), JDc1 (344.86; 345.11), JSd1 (329.38 and 330.19), Ksa2 (861.34 and 855.31), MuSc2 (861.34 and 853.09), NaSc3 (466.45 and 470.03), RaSa2 (239.47and 241.73), SaS6 (344.76 and 345.59), TaSa4 (466.45 and 467.17) hrs respectively. Meanwhile calculated LT_{50} and LT_{99} values of the reference strain, Btk HD-73 were (56.19 and 335.41) hrs. (Figure 3.3.9**)**.

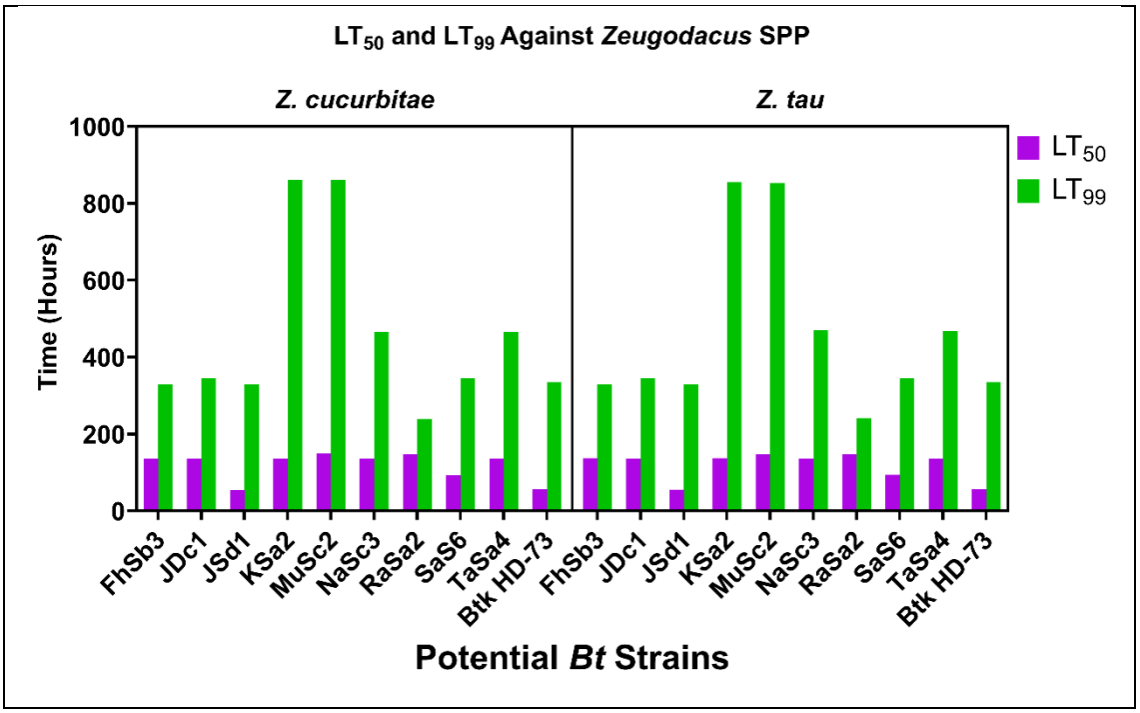


Figure 3.3.9: LT_{50} and LT_{99} values estimated for the potential *Bt* strains against *Z. cucurbitae* and *Z. tau* (early 3rd instar larvae).

The LT_{50} and LT_{99} values for the potential *Bt* strains were ranging from 54.16 to 148.96 hours and from 239.47 to 861.34 hours for *Z. cucurbitae* while 55.17 to 148.17 hours and from 241.73 to 855.31 hours for *Z. tau* respectively. *Bt* JSd1 seemed to have the lowest LT_{50} values of 54.16 and 55.17 hrs recorded for the both test insect pests which indicated the highest potency in causing the death of 50% of the larvae within four days. On the contrary, MuSc2 had the highest

time requirements (LT_{50}- 148.96 hrs) for *Z. cucurbitae* and RaSa2 had the highest time requirements for LT_{50} (148.17) hrs against *Z. tau* respectively.

The LT_{99} values i.e., causing 99% larval mortality, RaSa2 (LT_{99}-239.47 and 241.73 hrs) was found to be potentials in both the cases. MuSc2 and KSa2 exhibits LT_{99} values (861.34) hrs and (855.31) hrs were the higher values against *Z. cucurbitae* and *Z. tau* respectively. For the reference strain *Btk* HD-73 the LT_{50}- 56.19 hrs and LT_{99}- 335.41 hrs were recorded. Calculated LT_{50} values was lowest for *Bt* JSd1 (54.16 and 55.17 hrs) meanwhile lowest LT_{99} values for *Bt* RaSa2 (239.47 and 241.73 hrs) for *Z. cucurbitae* and *Z. tau* respectively (Figure 3.3.9). Thus, the using of dose and time response bioassay, *Bt* JSd1 shows highest potency and *Bt* SaS6 second highest in controlling *Z. cucurbitae* and *Z. tau* 3rd instar larvae at the lowest dose and the fastest time in the laboratory, allowing for large-scale production and sustainable delivery to the farmers.

3.3.8 Phenotypic characterization of potential *Bt* strains

Of the total examined strains, eleven strains were the most lethal to *B. dorsalis* and *B. zonata* larvae meanwhile nine strains exhibited the most lethal against *Z. cucurbitae* and Z. *tau* early third instars larvae. Only eleven of 44 indigenous *Bt* strains tested in this work had their phenotypic features observed with the phase-contrast microscope after sporulation and recorded in Table 3.3.3.

Table 3.3.3: Phenotypic characteristics of the potential *Bt* strains tested against *B. dorsalis, B. zonata, Z. cucurbitae* and *Z. tau* (early 3rd instar larvae)

***Bt* Strains**	**Size**	**Shape**	**Color**	**Margin**	**Consistency**	**Elevation**
DSf4	Medium	Round	Off white	Entire	Opaque	Flat
FhSb3	Medium to large	Round	Off white	Wooly	Opaque	Raised
JDc1	Medium to large	Round	Off white	Wooly	Opaque	Raised
JSd1	Medium to large	Round	Off white	Wooly	Opaque	Raised
KSa2	Medium to large	Round	Off white	Wooly	Opaque	Raised
MuSc2	Medium to large	Round	Off white	Wooly	Opaque	Raised
Nasc3	Medium	Round	Off white	Entire	Opaque	Raised
RaSa2	Large	Round	Off white	Entire	Opaque	Raised
SaS6	Large	Round	Off white	Entire	Opaque	Raised
SaS7	Medium	Round	Off white	Entire	Opaque	flat
TaSa4	Medium	Round	Off white	Entire	Opaque	flat

3.3.9 Spore counting and protein concentration determination

Only eleven of the forty-four evaluated effective indigenous *Bt* strains were utilized in this experiment against selected four Tephritids fruit flies *viz. B. dorsalis, B. zonata, Z. cucurbitae* and *Z. tau* (early 3rd instar larvae) to measure the number of spores/ml suspension and the protein concentration in mg/ml, and the results are reported in Figure 3.3.10 & 3.3.11.

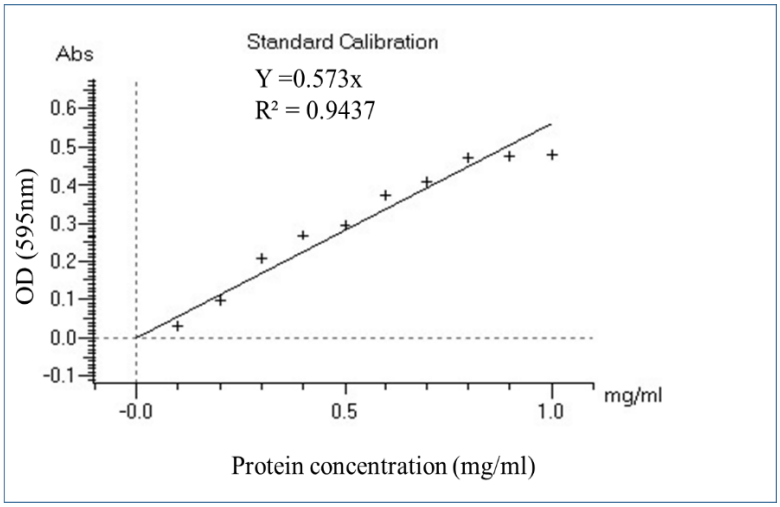


Figure 3.3.10: Standard curve for determining protein concentration by Bradford technique.

The equation y = mx depicts the relationship between absorbance (y) at 595 nm and protein concentration (x) in mg/ml. The slope of the standard curve (m) governs the relationship between absorbance and protein concentration.

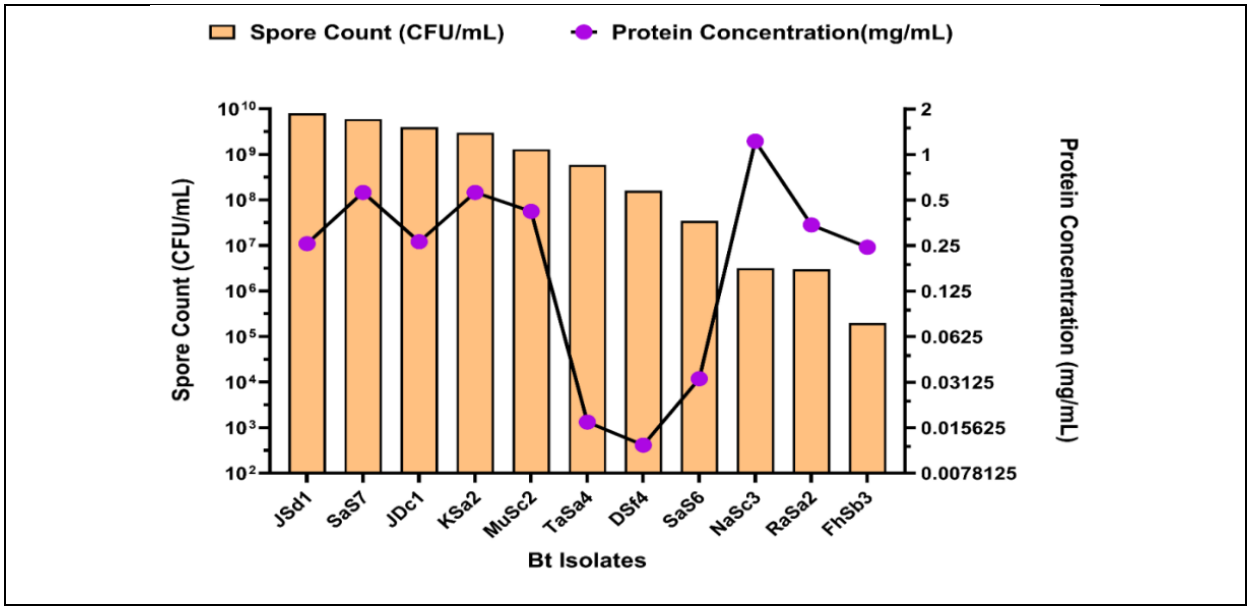


Figure 3.3.11: Spores count and estimation of Protein concentration of the potential *Bt* strains tested against *B. dorsalis, B. zonata, Z. cucurbitae* and *Z. tau* (early 3rd instar larvae).

3.3.10 Analysis of variance (One-Way)

Statistical values of mortality of *B. dorsalis* and *B. zonata* caused by different *Bt* strains at different concentrations. Calculation was excerpted from the ANOVA (one-way).

Table 3.3.4: ANOVA results for *B. dorsalis* and *B. zonata*

Strains	*B. dorsalis*			*B. zonata*		
	F statistic	**P value**	**F critical**	**F statistic**	**P value**	**F critical**
DSf4	163.7779	1.61E-07	4.066180557	218.0002	5.22E-08	3.478049691
FhSb3	133.7	3.57E-07	4.066180557	211.1112	5.93E-08	3.478049691
JDc1	293.0668	1.62E-08	4.066180557	260.6112	2.58E-08	3.478049691
JSd1	327.6668	1.04E-08	4.066180557	285.9446	1.79E-08	3.478049691
KSa2	620.6672	8.23E-10	4.066180557	275.0003	2.09E-08	3.478049691
MuSc2	595.3339	9.71E-10	4.066180557	282	1.89E-08	3.478049691
NaSc3	310.2503	1.29E-08	4.066180557	288.2501	1.73E-08	3.478049691
RaSa2	288.2503	1.73E-08	4.066180557	288.2503	1.73E-08	3.478049691
SaS6	497.2227	1.99E-09	4.066180557	195.4583	8.03E-08	3.478049691
SaS7	109.7917	7.71E-07	4.066180557	175.6667	1.22E-07	3.478049691
TaSa4	146.7917	2.48E-07	4.066180557	159	1.81E-07	3.478049691
Bts T84A1	417.0004	4.00E-09	4.066180557	409.5837	4.30E-09	3.478049691

According to statistical data derived from the one-way ANOVA, the larval mortality does, at α=0.05, substantially differ from the average mortality throughout the bioassay replicates for all four species. Tukey HSD post hoc analysis indicates significant differences between concentrations for each strain, with some exceptions (Appendix A).

Z. cucurbitae and *Z. tau* death statistics resulting from various *Bt* strains at varying concentrations. The computation was taken from the one-way ANOVA (one-way).

Table 3.3. 5: ANOVA results for *Z. cucurbitae* and *Z. tau*

Strains	*Z. cucurbitae*			*Z. tau*		
	F statistic	**P value**	**F critical**	**F statistic**	**P value**	**F critical**
DSf4	284.9	1.81E-08	3.478049691	138.8	3.08E-07	3.478049691
FhSb3	150.8	2.23E-07	3.478049691	95.6	1.32E-06	3.478049691
JDc1	465.2	2.59E-09	3.478049691	237.8	3.71E-08	3.478049691
JSd1	391	5.17E-09	3.478049691	174.8	1.25E-07	3.478049691
KSa2	1177	6.42E-11	3.478049691	525.8	1.59E-09	3.478049691
MuSc2	563.2	1.21E-09	3.478049691	266	2.38E-08	3.478049691
NaSc3	641.8	7.20E-10	3.478049691	343.6	8.63E-09	3.478049691
RaSa2	288.2	1.73E-08	3.478049691	103	9.87E-07	3.478049691
SaS6	473.3	2.42E-09	3.478049691	193.1	8.42E-08	3.478049691
SaS7	274.7	2.10E-08	3.478049691	120.2	5.42E-07	3.478049691
TaSa4	206.4	6.48E-08	3.478049691	238.2	3.68E-08	3.478049691
Btk HD-73	398	4.82E-09	3.478049691	237.9	3.70E-08	3.478049691

Statistical values of mortality of *B. dorsalis*, *B. zonata*, *Z. cucurbitae***,** and *Z. tau* caused by different *Bt* strains at a certain concentration. Calculation was excerpted from the ANOVA (one-way).

Table 3.3.6: ANOVA results for Tephridis flies

Species	F statistic	P-value	F critical
B. dorsalis	14.5248	4.72E-08	2.216308646
B. zonata	19.30735931	2.63E-09	2.216308646
Z. cucurbitae	34.98	4.46E-12	2.216308646
Z. tau	10.94	7.22E-07	2.216308646

According to statistical data derived from the one-way ANOVA, the larval mortality does, at α=0.05, substantially differ from the average mortality throughout the bioassay replicates for all four species and all *Bt* strains. Tukey HSD post hoc analysis reveals significant differences among strains, with strain JSd1 differing significantly from others but showing no significant difference compared to reference strains (Appendixes B and C).

CHAPTER 4

BIOLOGICAL QUALITY PARAMETERS ASSESSMENT OF SELECTED TEPHRITIDS PESTS TREATED WITH POTENTIAL *BACILLUS THURINGIENSIS* STRAINS *IN VIVO*

4.1 Introduction

Tephritid fruit flies (Diptera: Tephritidae) are key pests infesting a vast array of fruits and vegetables across the world and resulting in considerable agricultural and economic losses. Some of the well-known species such as *Bactrocera dorsalis* (Oriental fruit fly), *Bactrocera zonata* (Peach fruit fly), *Zeugodacus cucurbitae* (Melon fly), and *Zeugodacus tau* (Pumpkin fruit fly) infest a variety of crops such as mango, guava, banana, cucumber, bitter gourd, papaya, citrus, tomato, eggplant, pumpkin, peach, fig, ridge gourd, watermelon, zucchini, sponge gourd, bottle gourd, snake gourd. These insects damage crops through oviposition of eggs into fruit tissues, whose larvae feed inside, causing softening, rotting, and premature fall of fruit. The severity of infestations not only reduces the fruit quantity and quality but also hinders marketability and export due to stringent international quarantine requirements (Aluja & Mangan, 2008; Dhillon *et al.*, 2005; White & Elson-Harris, 1992).

B. dorsalis, being a polyphagous, is particularly dreaded due to its broad host range and global distribution. *B. zonata*, native to South Asia, has infested more than 20 countries. *Z. cucurbitae* and *Z. tau*, even though native Asian insects, are present-day quarantine pests with devastating impacts on cucurbit crops. Altogether, about 200 of the 4,000 described Tephritid species are economically significant, yet their biology and ecology are much less documented than other species of fruit flies. As a result of the ignorance, there is an abundance of literature that exists on monitoring and controlling the likes of fruit flies including biological control, mass trapping, chemical baits, male annihilation and sterilization, and mating disruption. This lack of knowledge stifles effective pest control on time and underscores the necessity of improved monitoring and management instruments.

To neutralize this, the environmentally friendly management of such pests is crucial, for which one must possess data on how *Bt* isolates affect their key biological parameters such as. In this article, three strains, *viz. Bt* JSd1, SaS6 and JDc1 with over 80% larval mortality on third-instar larvae of *B. dorsalis*, *B. zonata*, *Z. cucurbitae*, and *Z. tau* were selected for further following biological quality parameters evaluation on the infested preferred hosts treated with most potential *Bt* strains in the laboratory condition.

- **Developmental Periods:** Duration of larvae and pupae that successfully emerge as adults, reflecting rearing efficiency.
- **Pupal Yield:** Number of quality pupae from which quality adults emerged

- **Adult Emergence Rate and Number of Adults:** Measures the percentage of pupae that successfully emerge as adults, reflecting the number of quality adults and rearing efficiency.
- **Flight Ability:** Assesses the proportion of flies capable of sustained flight, indicating overall vigor and suitability for release.
- **Sex Ratio:** Determines the proportion of males to females, crucial for SIT programs that release only sterile males.
- **Longevity:** Evaluates the lifespan of adult flies under controlled conditions, affecting their potential to mate in the field.
- **Mating Competitiveness:** Examines the ability of sterile males to compete with wild males for mates, ensuring effective population suppression.
- **Fecundity and Fertility:** Assesses the number of eggs laid and their hatchability, important for understanding reproductive potential.
- **Pupal Weight, Size and Wing length:** Correlates with adult fitness and flight ability; heavier pupae often yield more robust adults.
- **Morphological Integrity:** Checks for physical deformities *i.e.,* half-emerged, un-emerged, semi-emerged etc. ensuring flies are viable for field release.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Media

Media were used in this study and their compositions were mentioned in section 3.2.1, Chapter 3 (Appendix D)

4.2.1.2 Chemicals

Chemicals were used in this study and their compositions were mentioned in section 3.2.2, Chapter 3 **(**Appendix E**)**.

4.2.1.3 Buffers and solutions

Reagents and buffer solutions were used in this experiment and their compositions were mentioned in section 3.2.4, Chapter 3 **(**Appendix F**).**

4.2.1.4 Equipment

All equipment used in this study were mentioned in respective methods section and their company and models were mentioned in Appendix G.

4.2.2 Methods

4.2.2.1 Bacterial strains and culture maintenance

Three potential *Bt* strains *viz. Bt* JSd1, SaS6 and JDc1 previously isolated from different locations and environment in Bangladesh were maintained in LB agar by sub-culturing and stored at 4°C for further study.

4.2.2.2 Fruits affected by the Tephritids fruit flies

A: Infested Mangoes

B: Infested Mango (On Tree)

C: Infested Guava (On Tree)

D: Infested Banana

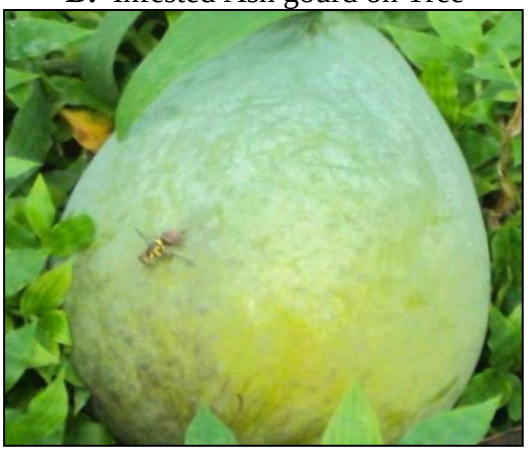
Figure 4.2.1: (A-D) Fruits damaged by the Tephritids fruit flies

4.2.2.3 Vegetables affected by the Tephritids fruit flies

A: Infested Bitter gourd

B: Infested Ash gourd on Tree

C: Infested Bitter gourd



D: Infested bottle gourd

E: Infested Cucumber

F: Infested Brinjal

Figure 4.2.2: (A-F) Vegetables damaged by the Tephritids fruit flies

4.2.2.4 Potential *Bacillus thuringiensis* (*Bt*) strains and culture conditions

Out of 44, only three potential *Bt strains* shows more than 80% larval mortality i.e., JSd1, SaS6 and JDc1 against selected four Tephritids fruit flies. So therefore, *Bt* JSd1, SaS6 and JDc1 were now subjected to observe biological quality parameters assessment of the tested fruit flies from the infested and treated preferred hosts against selected four economically important Tephritids

fruit flies *viz. B. dorsalis, B. zonata, Z. cucurbitae and Z. tau*. These strains were obtained from the Fermentation and Enzymes Biotechnology Laboratory, Department of Microbiology, University of Dhaka. *Bt kurstaki* HD-73, *Bt sotto* T84A1, and *Bt japonensis Buibui* strains were collected from the Okayama University *Bt* stock collection, Japan. The *Bt* strains were cultured on LB agar for maintenance, subculture, and spore count. Incubation temperatures for all types of cultures were kept constant at 30°C, and liquid cultures were shaken at 180 rpm using an orbital shaker.

4.2.2.5 Insect rearing

Rearing of targeted insects was maintained following same and similar method as mentioned in section 3.2.6 of Chapter 3.

A **B**

Figure 4.2.3: (A & B) Insect rearing cages & Rearing rack

4.2.2.6 Preferred host collection for biological quality parameter study

Mature and ripened fruits of the preferred host plants were collected at harvest from local farmers, ensuring uniform size and blemish-free quality. Banana and mango (BARI 4) were collected for *Bactrocera dorsalis*; mango (Amrapali) and guava for *B. zonata*; cucumber and bitter gourd for *Zeugodacus cucurbitae*; and bitter gourd and cucumber for *Z. tau*. After collection, the fruits were gently wiped with a soft cloth to remove any surface debris, packed in polyethylene bags, and transported to the laboratory for further studies on the biological quality

parameters of the four targeted insect pests. The host fruits and vegetables, infested with their specific insect pests, were treated with three potential *Bacillus thuringiensis* (*Bt*) strains, each demonstrating over 80% larval mortality in bioassay experiments shown in Figure 4.2.4 & 4.2.5.

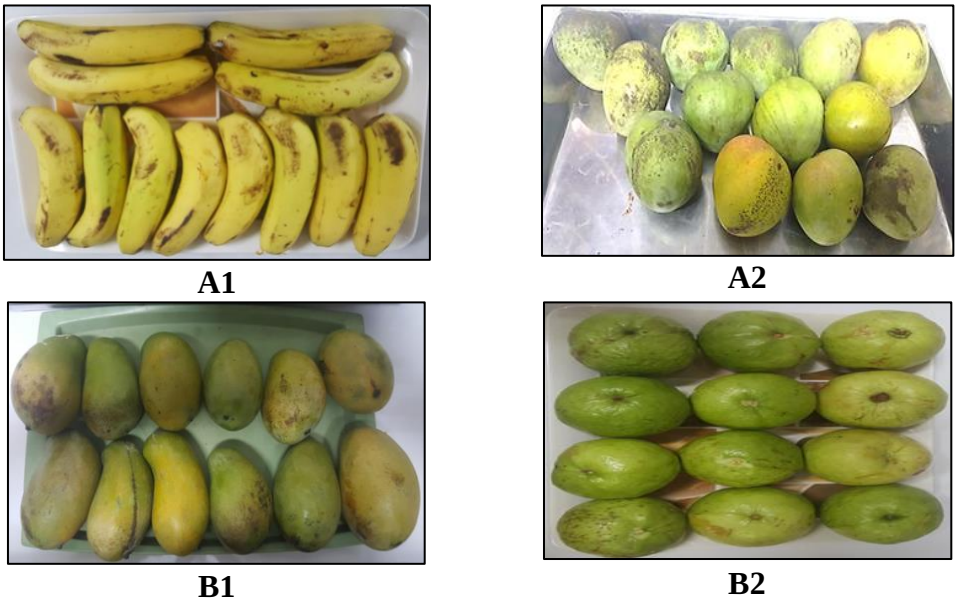


Figure 4.2.4: Collection of preferred hosts for biological quality parameters assessment. **(A1-A2)**: Banana & Mango (BARI4) against *B. dorsalis*; **(B1-B2)**: Mango (Amrapali) & Guava against *B. zonata.*

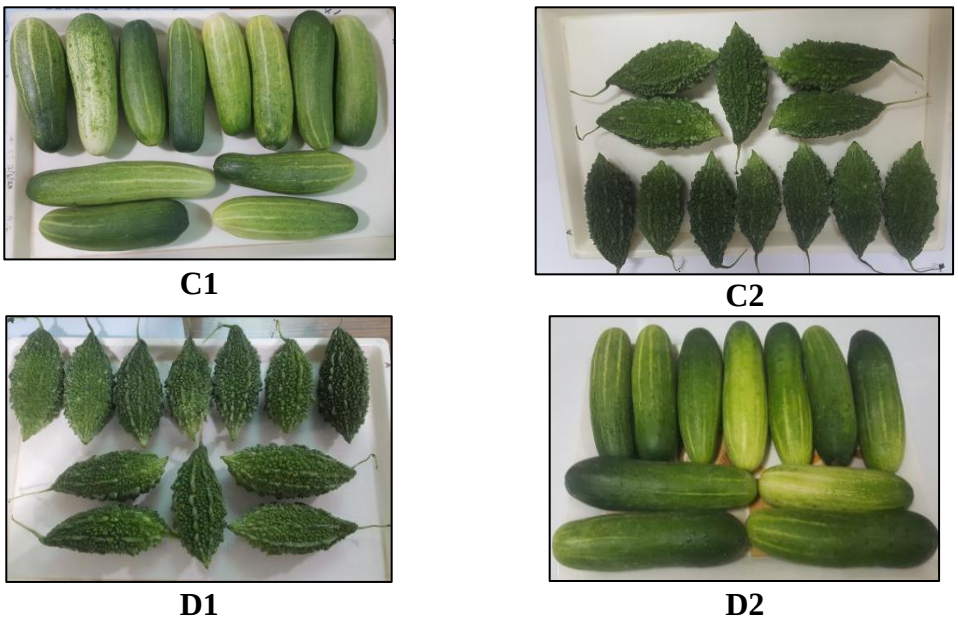


Figure 4.2.5: Collection of preferred hosts for biological quality parameters assessment. **(C1-C2)**: Cucumber & Bitter gourd against *Z. cucurbitae* and **(D1-D2)**: Bitter gourd & Cucumber against Z. *tau.*

4.2.2.7 Hosts placement on adult rearing cages for infestation

Twelve equal-sized and -weighted samples of the most suitable host fruits and vegetables were selected for every target insect species: banana and mango (BARI 4) for *Bactrocera dorsalis*; mango (Amrapali) and guava for *B. zonata*; cucumber and bitter gourd for *Zeugodacus cucurbitae*; and bitter gourd and cucumber for *Z. tau*. These host samples were then introduced into rearing cages of adult fruit flies in the lab for oviposition (Figure 4.2.6) and subsequent observation of biological quality indicators. All the infested host samples were then recovered after 30 minutes and stored in the laboratory for further investigation.

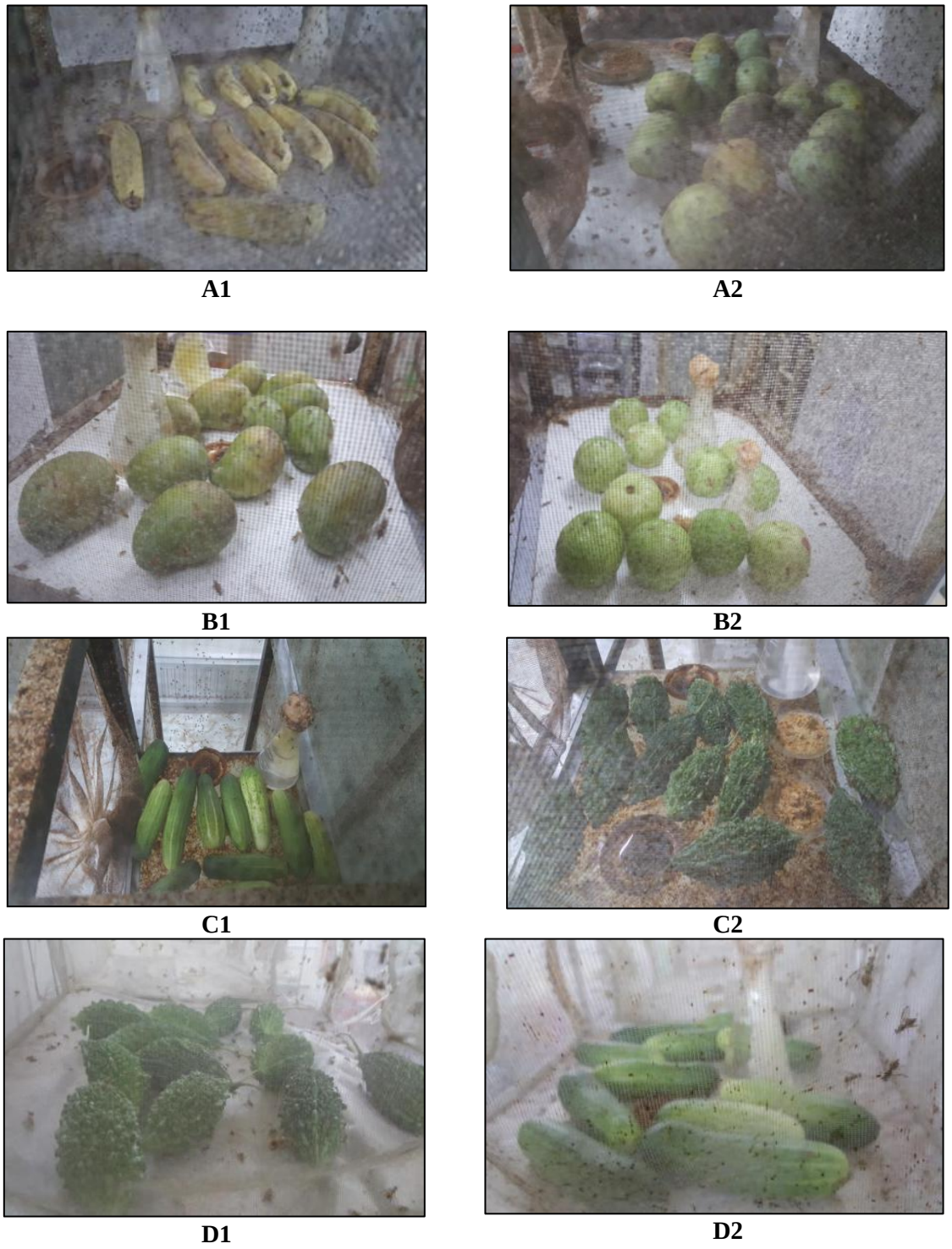


Figure 4.2.6: Hosts placement on adult rearing cages for Infestation **(A1-A2)**: Banana & Mango (BARI4) against *B. dorsalis*; **(B1-B2)**: Mango (Amrapali) & Guava against *B. zonata*; **(C1-C2)**: Cucumber & Bitter gourd against *Z. cucurbitae* and **(D1-D2)**: Bitter gourd & Cucumber against *Z. tau.*

4.2.2.8 Potential *Bt* treatment on preferred hosts for quality parameters assessment

After 24 hours of oviposition, nine infested host fruits were treated with injecting three promising *Bacillus thuringiensis* (*Bt*) strains—JSd1, SaS6, and JDc1—which had previously shown over 80% larval mortality out of eleven strains that were screened. 5 ml of the *Bt* suspension (spore crystal mixture) was injected evenly on each fruit and vegetable on four sides under a biosafety cabinet fruit samples, along with three control samples (infested but untreated), was placed separately in a small plastic bowl. All the small bowls were then placed in twelve larger plastic containers with sawdust and maintained under laboratory conditions for observation. Over the next 6–8 days, sawdust in every bowl was sieved to collect dust-free pupae. These were transferred to twelve Petri dishes, which were marked for intimate observation on biological quality parameters. Observations were recorded on pupation rate (pupal yield), larval and pupal duration, pupal weight, number of adults, adult emergence percentage (%), numbers of males and females emerged (sex ratio, M: F), percentage fliers, wing length, and number of half-emerged and un-emerged. The entire experiment, including the treatments with the three most promising *Bt* strains, was repeated three times for consistency.

Figure 4.2.7: Biological quality parameters assessment on preferred hosts treated with 3 potential *Bt* strains caused more than 80 % larval death i.e., JSd1, SaS6 and JDc1, **(A1-A2)**: Banana & Mango (BARI4) against *B. dorsalis*; **(B1-B2)**: Mango (Amrapali) & Guava against *B. zonata*; **(C1-C2)**: Cucumber & Bitter gourd against *Z. cucurbitae* and **(D1-D2)**: Bitter gourd & Cucumber against *Z. tau*.

4.2.2.9 Biological quality parameters observation

Now all the infested and treated host samples were collected carefully and transferred to plastic bowls for observation continued further biological development of the tested insect pest population on their respective hosts until adult emergence (Figure 4.2.8). This setup enabled the observation of key biological quality parameters, including larval and pupal duration, pupation number (pupal yield), pupal weight, number and percentage of adult emergence, sex ratio (male:female), wing length, flight ability (% fliers), and the number of half-emerged and unemerged flies. Observations were made on Infested banana and mango (BARI 4) for *B. dorsalis*, while infested mango (Amrapali) and guava were used for *B. zonata* treated with most potent three *Bt* strains JSd1, SaS6, and JDc1. In parallel, the biological quality parameters of *Zeugodacus cucurbitae* and *Z. tau* were evaluated using infested and *Bt* treated cucumber and bitter gourd, and bitter gourd and cucumber, respectively. All host fruits were treated with the selected potential *Bacillus thuringiensis* (*Bt)* strains prior to use. The effects of these *Bt* treatments on the regular further biological development and quality of the emerged tephritid fruit flies were recorded and analyzed accordingly.

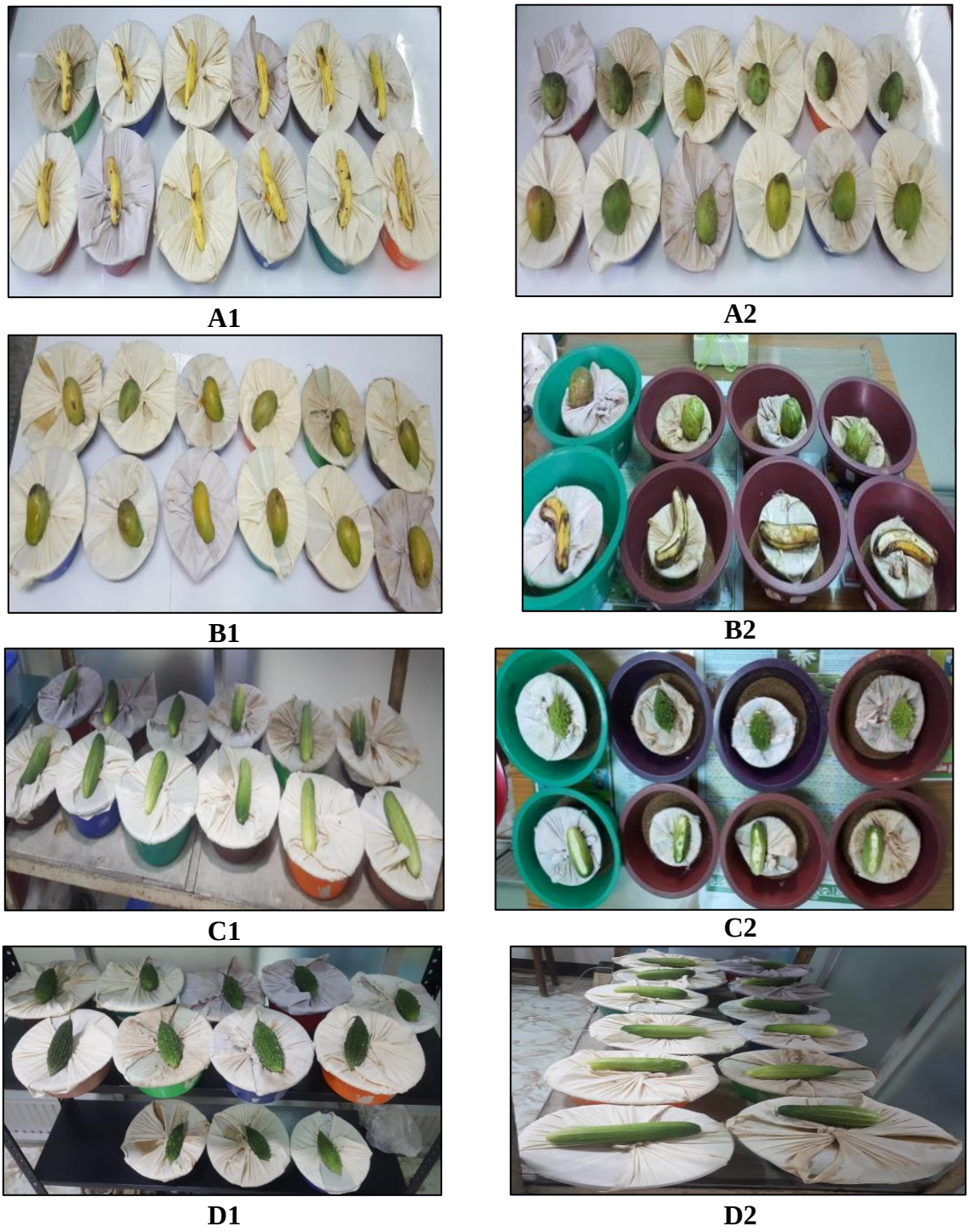


Figure 4.2.8: Observation of biological quality parameters development on preferred hosts treated with 3 potential *Bt* strains, **(A1-A2)**: Banana & Mango (BARI4) against *B. dorsalis*; **(B1-B2)**: Mango (Amrapali) & Guava against *B. zonata*; **(C1-C2)**: Cucumber & Bitter gourd against *Z. cucurbitae* and **(D1-D2)**: Bitter gourd & Cucumber against *Z. tau*.

4.2.2.10 Data collection and analysis

Biological quality parameters i.e., pupal recovery, larval and pupal periods, pupal weight, adult emergence number, % of adult emergence, number of male and female formation (sex ratios), % of fliers, wing length, % of half emerged and % of un-emerged were recorded on treated preferred infested hosts, *viz.* banana, mango (BARI 4) against *Bactrocera dorsalis*, mango (Amrapali), guava against *B. zonata*, cucumber, bitter gourd against *Zeugodacus cucurbitae* and bitter gourd, cucumber against *Z. tau* respectively. All the data's estimated from Biological quality parameters assessment were statistically analyzed and Graphical analysis were computed using GraphPad Prism 10 and python open source software program (https://www.w3schools.com/python/trypython.asp?filename=demo_string_quotes). The statistical significance threshold was set at p-values of equal to 0.05.

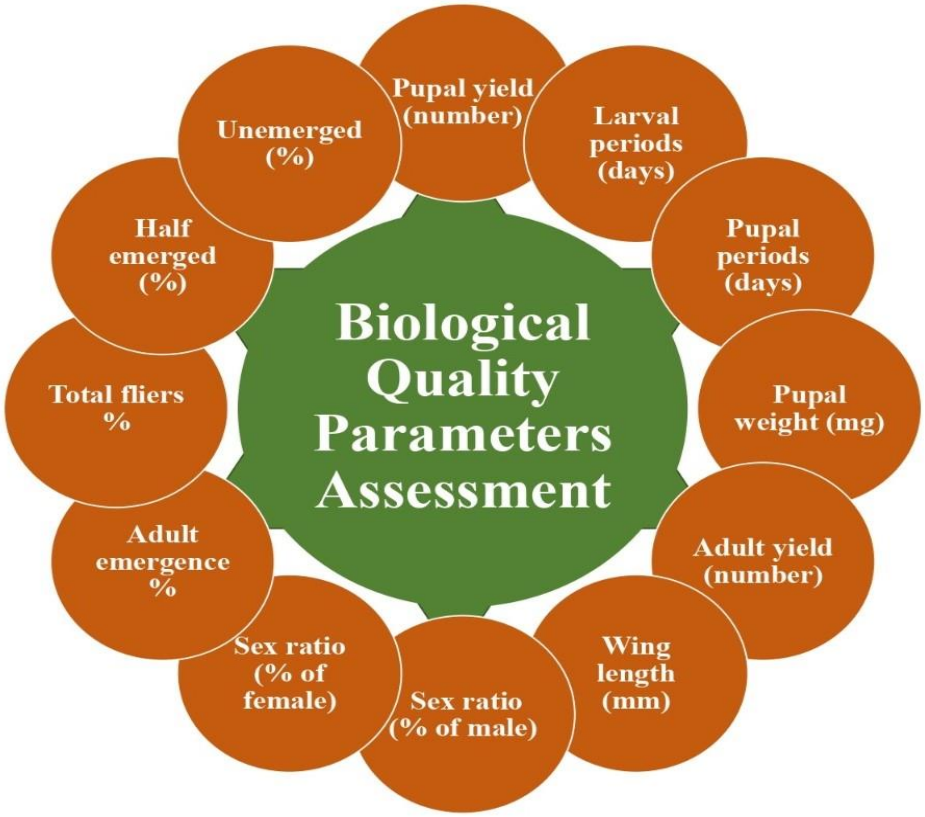


Figure 4.2.9: Biological quality parameters observed in this investigation, preferred hosts were treated with three potential *Bt* strains, JSd1, SaS6, and JDc1, which caused more than 80% larval death against tested Tephritid fruit flies.

4.3 RESULTS

4.3.1 Influence of potential *Bt* biopesticide application on the biological quality parameters of *B. dorsalis* on infested and treated banana and mango (BARI 4)

Pupation number (Pupal yield) and Pupal weight

The number of pupae (107±3.605), (165±6.658), (186±4.618) were recorded from potential *Bt* JSd1, SaS6 and JDc1 treated banana, whereas pupation number (179±6.641), (231±5.291), (254±4.371) were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (BARI 4) against *B. dorsalis*, respectively. The lowest pupation number (107±3.605); (179 ± 6.641), were recorded for potential *Bt* JSd1 treated banana and mango (BARI 4) whereas highest pupation number (275 ± 8.736); (389 ± 5.696) were recorded in control batch for banana and mango (BARI 4) against *B. dorsalis* respectively (Figure 4.3.1 A)

Pupal weight (8.55±0.19), (9.63±0.266) and (9.44±0.121) mg were recorded from potential *Bt* JSd1, SaS6 and JDc1 treated banana while (8.08±0.033), (9.03±0.093) and (9.15±0.1) mg were recorded for the potential *Bt* JSd1, SaS6 and JDc1 treated mango (BARI 4) against *B. dorsalis* respectively. Pupal weight was recorded in control batch (11.73 ± 0.041) and (11.14 ± 0.065) mg for banana and mango (BARI 4) against *B. dorsalis* respectively. (8.55 ± 0.19); (8.08 ± 0.033) mg, were lowest pupal weight from potential *Bt* JSd1 treated banana and mango (BARI 4) while highest pupal weight was recorded in control batch (11.73 ± 0.041); (11.14 ± 0.065) mg for *B. dorsalis* infested banana and mango (BARI 4) respectively (Figure 4.3.1 B)

Adult emergence (Adult yield), Sex ratios and % of adult emergence

Number of adults (50±2.309), (100±3.464), (129±3.214); were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated banana whereas adult number (78±3.215), (139±2.081), (175±3.055); were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (BARI 4) against *B. dorsalis* respectively. In control batch, (267 ± 6.082) and (377 ± 3.214) adults were found for banana and mango (BARI 4) against *B. dorsalis* respectively. Lowest adult emergence number (50 ± 2.309); (78 ± 3.215) were observed on potential *Bt* JSd1 treated banana and mango (BARI 4) whereas highest adult emergence number (267 ± 6.082); (377 ± 3.214) were recorded on control batch for banana and mango (BARI 4) against *B. dorsalis* respectively.

Emergence of male and female (sex ratios, M:F) were (24 ± 2.081: 31 ± 2.221), (51 ± 3.055 : 58 ± 1.154) and (60 ± 3.785: 69 ± 1.527) recorded from potential *Bt* JSd1, SaS6 and JDc1 treated banana meanwhile male and female emergence (sex ratios, M:F) were (34 ± 2.516: 44 ± 2.645),

(65 ± 1.154: 74 ± 2.646) and (84 ± 4.509: 91 ± 3.605) recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (BARI 4) against *B. dorsalis* respectively. The lowest sex ratios (M:F) (24 ± 2.081: 31 ± 2.221); (34 ± 2.516: 44 ± 2.645) and lowest % of adult emergence (46.73 ± 3.15); (43.57 ± 1.535) were recorded on potential *Bt* JSd1 treated banana and mango (BARI 4) while highest sex ratios (130 ± 4.725 : 137 ± 3.055); (186 ± 4.163 : 191 ± 2.645) with highest % of adult emergence (97.09 ± 0.532); (96.91 ± 0.638) were recorded in control batch for banana and mango (BARI 4) against *B. dorsalis* respectively (Figure 4.3.1 C-D)

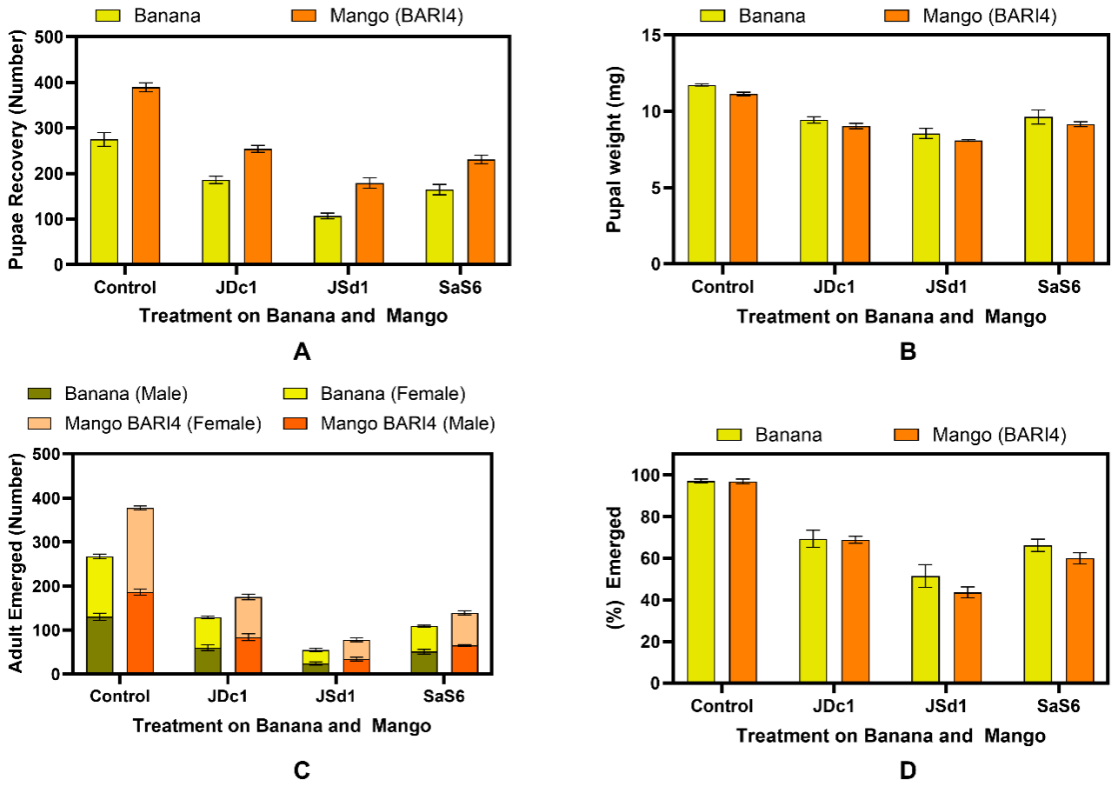


Figure 4.3.1: Biological parameters of *Bactrocera dorsalis* affected by *Bt* treatment, **(A)** Pupal recovery **(B)** Pupal weight **(C)** Adult Emerged (male and female) **(D)** Percentage of Adult emerged. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

Wing length and flight ability

Wing lengths (4.36 ± 0.093), (4.71 ± 0.039) and (4.98 ± 0.05) mm were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated banana whereas (4.13 ± 0.034), (4.42 ± 0.051) and (4.98 ± 0.182) mm were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (BARI 4) against *B. dorsalis* respectively. fliers % (33.03 ± 0.578), (45.49 ± 2.197) and (47.54 ± 0.817); were recorded from banana treated with potential *Bt* JSd1, SaS6 and JDc1, infested with *B. dorsalis* while (43 ± 0.545), (42 ± 0.371), (47.28 ± 0.326) were recorded from infested mango (BARI 4)

treated with potential *Bt* JSd1, SaS6 and JDc1 respectively. Minimum wing length (4.36 ± 0.093); (4.13 ± 0.034) mm, with lowest % of fliers (33.03 ± 0.578); (43 ± 0.371) were observed on potential *Bt* JSd1 treated banana and mango (BARI 4) while maximum wing length (5.74 ± 0.098); (5.15 ± 0.087) mm with highest % of fliers (61.21 ± 0.371); (61.65 ± 0.986) were recorded on control batch for banana and mango (BARI 4) against *B. dorsalis* respectively (Figure 4.3.2 A-B).

Retardation in growth

The % of half emerged (22±0.577); (21.66±4.630); (20.33±0.881) and % of un-emerged (30 ± 1.527); (34.33 ± 1.452); (36.33 ± 1.763) with maximum larval (11 ± 2.905, 10.66 ± 2.516, 10.33 ± 2.081) and pupal period (8.66 ± 0.333, 7.76 ± 0.333, 7.66 ± 0.333) days were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated banana meanwhile % of half emerged (42 ± 1.154); (39 ±1.154); (37 ± 1.527) and % of un-emerged (59 ± 1.527); (53 ± 0.577); (42 ± 1.527) with larval (11.33 ± 0.333, 10.66 ± 0.333, 10.33 ± 0.333) days and pupal period (8.31 ± 0.333, 8.66 ± 0.333, 7.66 ± 0.333) days were recorded from potential *Bt* JSd1, SaS6 and JDc1 treated mango (BARI 4) infested with *B. dorsalis* respectively. For Mango, the difference in pupal period were not significant compared to the control group, and for guava, the difference is not significant for SaS6 strain. Maximum % of half emerged (22 ± 0.577); (42 ± 1.154), maximum % of un-emerged (36.33 ± 1.763, JDc1); (59 ± 1.527) with maximum larval (11 ± 2.905); (11.33 ± 0.333) and pupal (8.66 ± 0.333); (8.31 ± 0.333) days period were recorded for *Bt* JSd1 treated banana and mango (BARI 4) whereas minimum % of half emerged (2.59 ± 0.333); (2.33 ± 0.881), minimum % of un-emerged (5.41 ± 0.333); (3.66 ± 1.452) with minimum larval (8 ± 0.577); (8.66 ± 0.333) and pupal (6.66 ± 0.333); (7.66 ± 0.333) days period were observed on control batch from *B. dorsalis* infested banana and mango (BARI 4) respectively. The toxicidal effect on the biological parameters assessed on potential *Bt* strains treated on banana, mango (BARI 4) exhibited promising results on regular biological development of the test insects, *B. dorsalis* which are in the following order: JSd1>SaS6>JDc1 shown in (Figure 4.3.2 C-E).

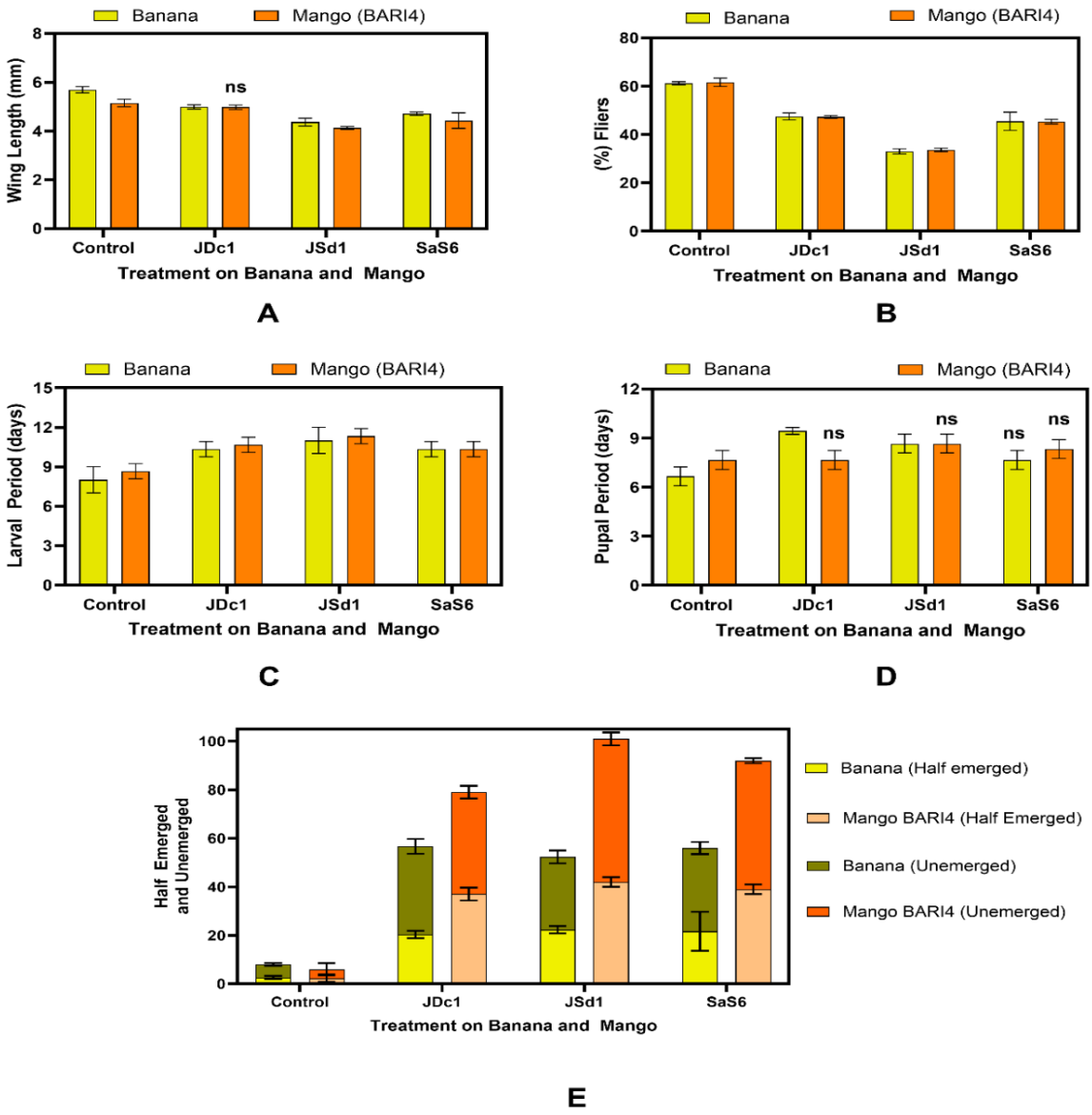


Figure 4.3.2: Biological parameters of *Bactrocera dorsalis* affected by *Bt* treatment, **(A)** Wing length **(B)** Percentage of fliers **(C)** Larval period **(D)** Pupal Period **(E)** Half & Un-emerged adults. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned which indicates 'not significant'.

4.3.2 Influence of potential *Bt* biopesticide application on the biological quality parameters of *B. zonata* on infested and treated mango (Amrapali) and guava

Pupation number (Pupal yield) and Pupal weight

Number of pupae (119 ± 2.309, 189 ± 0.577, 207 ± 6.11) and (135 ± 0.577, 179 ± 3.511, 195 ± 4.509) were recorded from the potential *Bt* JSd1, SaS6 and JDc1 treated banana and mango (Amrapali) infested with *B. zonata* respectively. Lowest pupation number (119 ± 2.309); (135 ±

0.577) were recorded for potential *Bt* JSd1 treated mango (Amrapali) and guava whereas highest pupation number (303 ± 4.582); (281 ± 8.76) were recorded in control for mango (Amrapali) and guava against *B. zonata* respectively (Figure 4.3.3 A).

Pupal weight (8.19 ± 0.034, 9.28 ± 0.186, 9.23 ± 0.08) and (8 ± 0.031, 8.7 ± 0.085, 9 ± 0.029) mg, were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (Amrapali) and guava against *B. zonata* respectively. Pupal weight (8.19 ± 0.034) and (8 ± 0.031) mg, were lowest for potential *Bt* JSd1 treated mango (Amrapali) and guava while highest pupal weight was recorded in control batch (11.43 ± 0.059) and (11 ± 0.067) mg from treated mango (Amrapali) and guava infested with *B. zonata* respectively (Figure 4.3.3 B).

Adult emergence number (Adult yield), Sex ratios and % of adult emergence

Adult emergence number (53 ± 1.527, 123 ± 1.154, 143 ± 4.163) and (58 ± 1.154, 109 ± 0.577, 130 ± 1.732) were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (Amrapali) and guava against *B. zonata* respectively. Lowest adult emergence number (53 ± 1.527); (58 ± 1.154) were observed on potential *Bt* JSd1 treated mango (Amrapali) and guava whereas highest adult emergence number (293 ± 2.886); (271 ± 9.16) were recorded on control batch for mango (Amrapali) and guava against *B. zonata* respectively.

Male and female emergence ratios (M: F) (23 ± 0.577: 30 ± 0.578; 55 ± 0.578: 68 ± 0.577; 67 ± 3.785: 76 ± 3.055) and (26 ± 0.577: 32 ± 0.579; 50 ± 0.579: 59 ± 0.577; 61 ± 1.154: 69 ± 2.309), were recorded from the potential *Bt* JSd1, SaS6 and JDc1 treated mango (Amrapali) and guava infested with *B. zonata* respectively. Meanwhile adult emergence % (44.53 ± 0.904, 65.07 ± 1.154, 69.08 ± 1.086) and (42.96 ± 0.606, 60.89 ± 0.333, 66.66 ± 0.881), were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (Amrapali) and guava against *B. zonata* respectively. The lowest sex ratios (M:F) (23 ± 0.577: 30 ± 0.578); (26 ± 0.577: 32 ± 0.579) and lowest % of adult emergence (44.53 ± 0.904); (42.96 ± 0.606) were recorded on potential *Bt* JSd1 treated mango (Amrapali) and guava whereas highest sex ratios (M: F) (141 ± 2.645: 152 ± 3.214); (134 ± 4.041: 137 ± 4.163) with highest % of adult emergence (96.69 ± 0.435); (96.44 ± 0.262) were recorded in control batch for mango (Amrapali) and guava infested with *B. zonata* respectively (Figure 4.3.3 C-D).

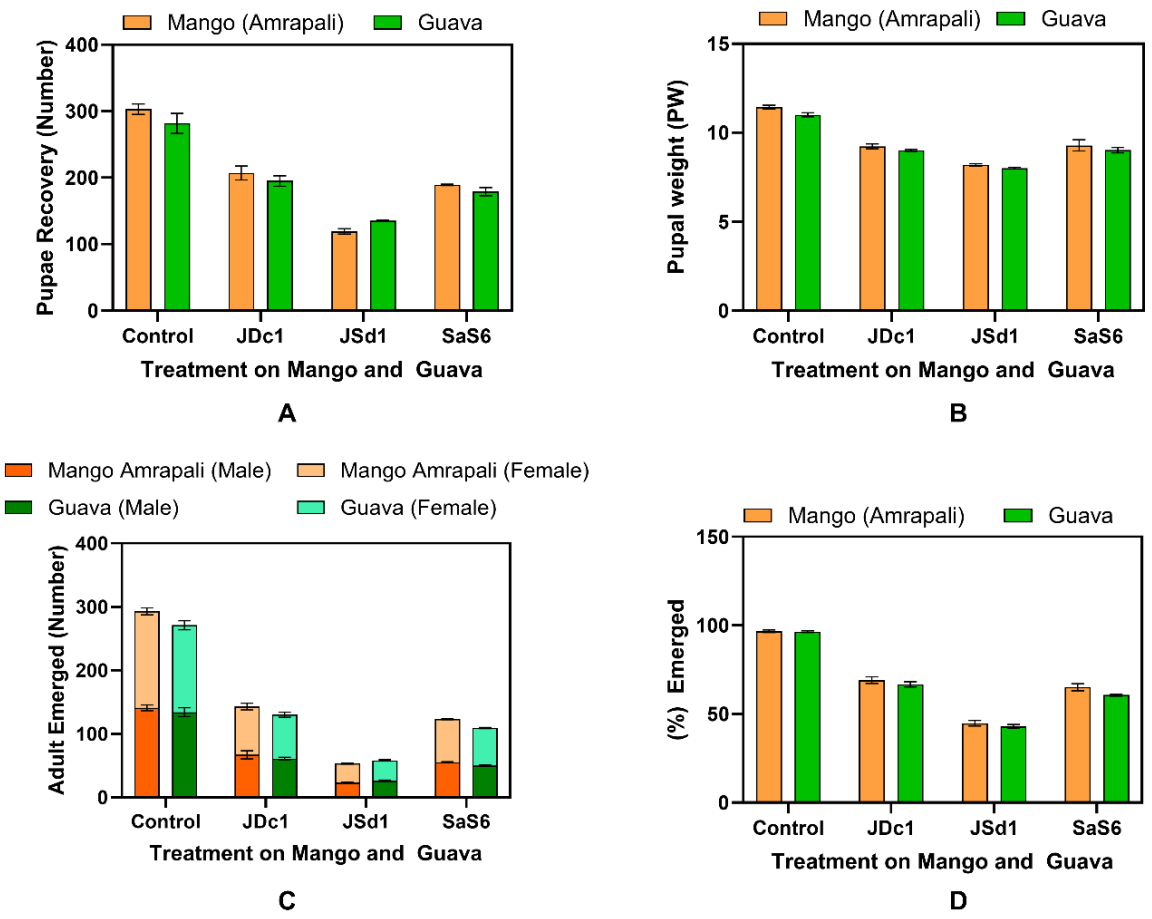


Figure 4.3.3: Biological parameters of *Bactrocera zonata* affected by *Bt* treatment, **(A)** Pupal recovery **(B)** Pupal weight **(C)** Adult Emerged (male and female) **(D)** Percentage of Adult emerged. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

Wing length and flight ability

Length of wing (4.09 ± 0.093, 4.68 ± 0.043, 4.87 ± 0.008); (4.03 ± 0.027, 4.58 ± 0.148, 4.32 ± 0.176) mm and fliers % (35.08 ± 0.251, 48.75 ± 0.287, 47.59 ± 0.835) and (33.45 ± 0.397, 47.19 ± 0.325, 45.16 ± 0.602) were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated mango (Amrapali) and guava against *B. zonata* respectively. Minimum wing length (4.09 ± 0.093); (4.03 ± 0.027) mm, with lowest % of fliers (35.08 ± 0.251); ((33.45 ± 0.397) were observed on potential *Bt* JSd1 treated mango (Amrapali) and guava while maximum wing length (5.57 ± 0.987); (5.05 ± 0.111) mm with highest % of fliers (61.51 ± 0.367); (60.65 ± 0.355) were recorded on control batch for mango (Amrapali) and guava against *B. zonata* respectively (Figure 4.3.4 A-B).

Retardation in growth

Half emerged % (26 ± 1.154, 29 ± 1.155, 28 ± 0.577,) and (34 ± 0.334, 33 ± 1.1554, 30 ± 1.527), % of un-emerged (40 ± 1.155, 37 ± 0.577, 36 ± 1.527) and (41 ± 0.577, 37 ± 1.527, 35 ± 1.154), with larval period (11.33 ± 0.333, 10.33 ± 0.333, 10 ± 0.577); (11.33 ± 0.333, 10.66 ± 0.333, 10.33 ± 0.333) days and pupal period (8.66 ± 0.333, 7.86 ± 0.333, 7.66 ± 0.333); (8.66 ± 0.333, 7.96 ± 0.333, 8.33 ± 0.333) days were observed from the potential *Bt* JSd1, SaS6 and JDc1 treated mango (Amrapali) and guava infested with *B. zonata* respectively. The difference in pupal period were not significant compared to the control group.

Maximum % of half emerged (29 ± 1.155, Sas6); (34 ± 0.334), maximum % of un-emerged (40 ± 1.155); (41 ± 0.577) with maximum larval (11.33 ± 0.333); (11.33 ± 0.333) days and pupal (8.66 ± 0.333); (8.66 ± 0.333) days period were recorded for *Bt* JSd1 treated mango (Amrapali) and guava whereas minimum % of half emerged (3.66 ± 0.881); (3.03 ± 0.578), minimum % of un-emerged (6.34 ± 1.452); (7.33 ± 0.578) with minimum larval (8.66 ± 0.333); (8.33 ± 0.333) days and pupal (6.66 ± 0.333); (7.66 ± 0.333) days period were observed on control batch for mango (Amrapali) and guava against *B. zonata* respectively. The toxicity effect on the biological parameters assessed on potential *Bt* strains treated on mango (Amrapali) and guava exhibited promising results on regular biological development of the test insects, *B. zonata* in the following order: JSd1>SaS6>JDc1 mainly (Fig. 4.3.4 C-E).

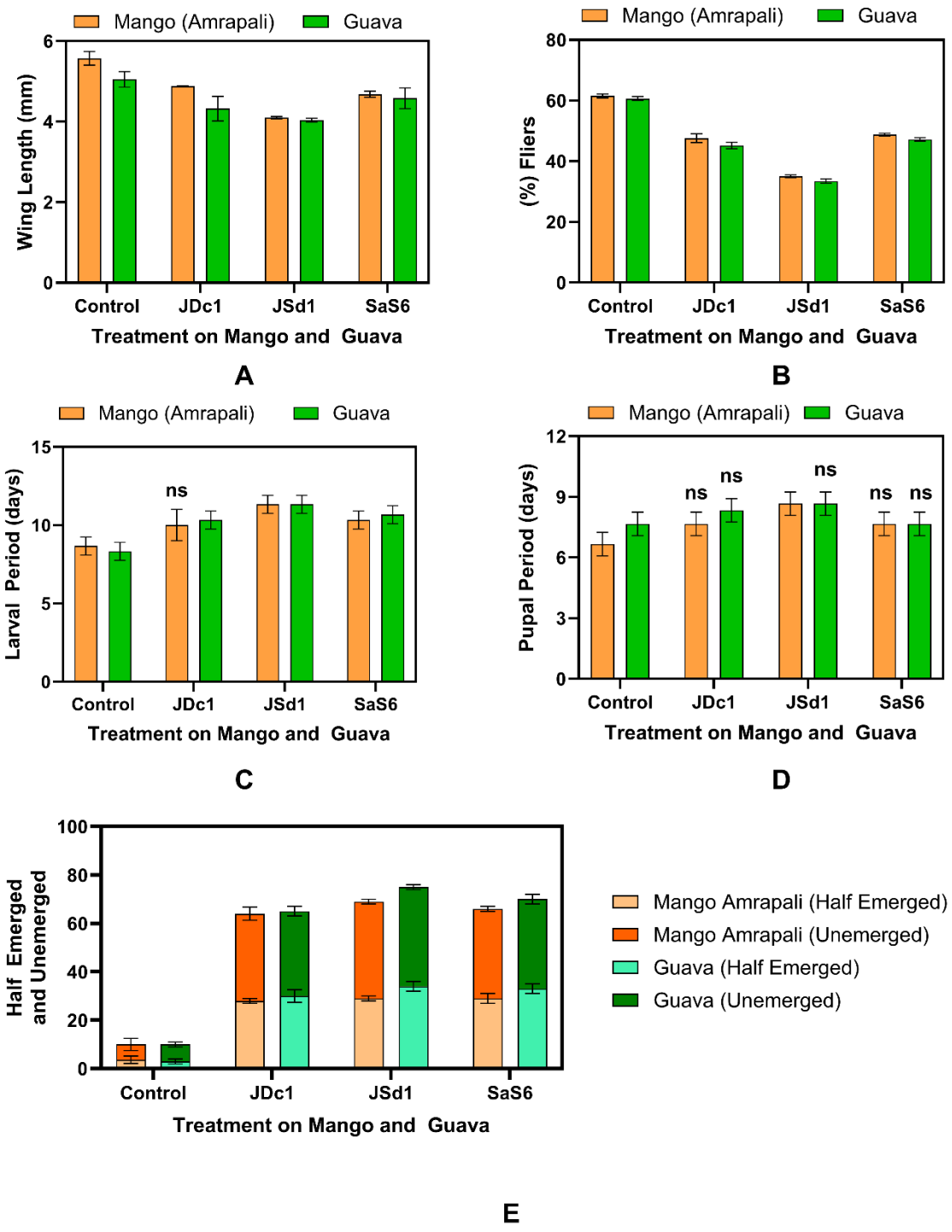


Figure 4.3.4: Biological parameters of *Bactrocera zonata* affected by *Bt* treatment, **(A)** Wing length **(B)** Percentage of fliers **(C)** Larval period **(D)** Pupal Period **(E)** Half & Un-emerged adults. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

4.3.3 Influence of potential *Bt* biopesticide application on the biological quality parameters of *Z. cucurbitae* on infested and treated cucumber and bitter gourd

Pupation number (Pupal yield) and Pupal weight

Pupation number (169.33 ± 8.717, 199 ± 2.082, 248 ± 13.23) and (102 ± 2.081, 153 ± 1.732, 166 ± 5.033) were estimated from the potential *Bt* JSd1, SaS6 and JDc1 treated cucumber and bitter gourd infested with *Z. cucurbitae* respectively. The lowest pupation number (169.33 ± 8.717) and (102 ± 2.081), were recorded for potential *Bt* JSd1 treated cucumber and bitter gourd whereas highest pupation number (340 ± 9.29); (215 ± 1.154) were recorded in control batch for cucumber and bitter gourd against *Z. cucurbitae* respectively (Figure 4.3.5 A).

Weight of pupae (8.62 ± 0.318, 10.84 ± 0.128, 10.09 ± 0.12) and (8.01 ± 0.388, 9.88 ± 0.115, 9.45 ± 0.28) mg, were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated cucumber and bitter gourd infested with *Z. cucurbitae* respectively. Pupal weight (8.62 ± 0.318); (8.01 ± 0.388) mg, were lowest for potential *Bt* JSd1 treated cucumber and bitter gourd while highest pupal weight was recorded in control batch (12.930); (11.86 ± 0.087) mg for cucumber and bitter gourd against *Z. cucurbitae* respectively (Figure 4.3.5 B).

Adult emergence (Adult yield), Sex ratios and % of adult emergence

Number of Adult emergence (79 ± 1.732, 129 ± 1.732, 173 ± 3.055) and (43 ± 2.645, 90 ± 1.154, 106 ± 2.083) were observed from the potential *Bt* JSd1, SaS6 and JDc1strains treated cucumber and bitter gourd infested with *Z. cucurbitae* respectively. Lowest adult emergence number (79 ± 1.732); (43 ± 2.645) were recorded on potential *Bt* JSd1 treated cucumber and bitter gourd whereas highest adult emergence number (332 ± 6.244); (197 ± 2.081) were observed on control batch for cucumber and bitter gourd against *Z. cucurbitae* respectively.

Male and female emergence ratios (M: F) (36 ± 0.577: 43 ± 1.154; 60 ± 1.154: 69 ± 0.577; 84 ± 2.081: 89 ± 0.577) and (19 ± 1.155: 24 ± 1.732; 41 ± 1.156: 49 ± 0.577; 49 ± 1.154: 57 ± 0.881), were recorded from the potential *Bt* JSd1, SaS6 and JDc1 treated cucumber and bitter gourd infested with *Z. cucurbitae* respectively. Meanwhile adult emergence % (46.74 ± 1.15, 64.82 ± 0.612, 69.75 ± 0.395) and (42.15 ± 0.881, 58.82 ± 0.546, 63.85 ± 2.709), were determined for potential *Bt* JSd1, SaS6 and JDc1 treated cucumber and bitter gourd against *Z. cucurbitae* respectively**.** The lowest sex ratios (M:F) (36 ± 0.577: 43 ± 1.154); (19 ± 1.155: 24 ± 1.732) and lowest % of adult emergence (46.74 ± 1.15); (42.15 ± 0.881) were recorded on potential *Bt* JSd1 treated cucumber and bitter gourd while highest sex ratios (M: F) (161 ±

2.645: 171 ± 4.582); (94 ± 3.055: 103 ± 2.081) with highest % of adult emergence (97.64 ± 0.491); (91.62 ± 0.323) were recorded in control batch for cucumber and bitter gourd against *Z. cucurbitae* respectively (Figure 4.3.5 C-D).

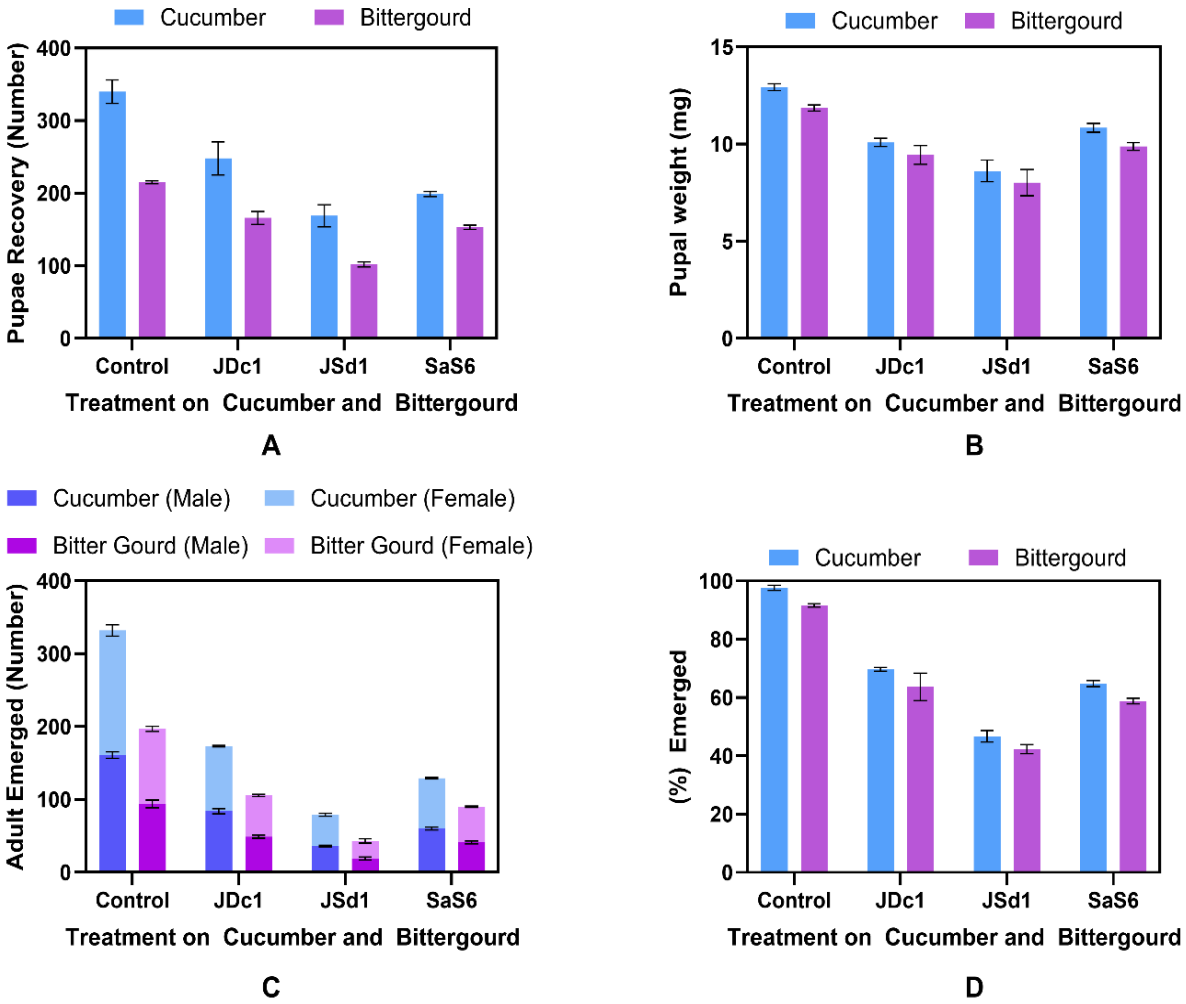


Figure 4.3.5: Biological parameters of *Zeugodacus cucurbitae* affected by *Bt* treatment, **(A)** Pupal recovery **(B)** Pupal weight **(C)** Adult Emerged (male and female) **(D)** Percentage of Adult emerged. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

Wing length and flight ability

Wing length were (4.57 ± 0.165, 5.13 ± 0.04, 5.33 ± 0.06); (4.38 ± 0.26, 5 ± 0.008, 5.05 ± 0.034) mm and % of fliers (36.33 ± 0.45, 51.18 ± 0.203, 55.36 ± 0.196) and (29.63 ± 0.416, 43.84 ± 0.095, 45.28 ± 0.315) calculated from the potential *Bt* JSd1, SaS6 and JDc1 treated cucumber and bitter gourd infested with *Z. cucurbitae* respectively. Minimum wing length (4.57 ± 0.165); (4.38 ± 0.26) mm, with lowest % of fliers (36.33 ± 0.45); (29.63 ± 0.416) were observed on potential *Bt* JSd1 treated cucumber and bitter gourd while maximum wing length

(6.03 ± 0.011); (6.09 ± 0.053) mm with highest % of fliers (66.33 ± 0.037); (66.33 ± 0.268) were recorded on control batch for cucumber and bitter gourd against *Z. cucurbitae* respectively (Figure 4.3.6 A-B).

Retardation in growth

Percent of half emerged (39 ± 1.54, 30 ± 1.154, 36 ± 1.527) and (22 ± 1.154, 28 ± 0.578, 25 ± 1.527), percent of un-emerged (51 ± 1.527, 40 ± 1.528, 39 ± 1.154) and (37 ± 1.158, 35 ± 0.154, 35 ± 2.309), with larval period (11.33 ± 0.333, 10.66 ± 0.333, 9.66 ± 0.577); (11.66 ± 0.333, 10.66 ± 0.333, 10.33 ± 0.333) days and pupal period (9 ± 0.577, 8.33 ± 0.333, 7.66 ± 0.333); (8.66 ± 0.333, 8.33 ± 0.333, 8 ± 0.57) days were recorded to the potential *Bt* JSd1, SaS6 and JDc1 treated cucumber and bitter gourd infested with *Z. cucurbitae* respectively. Compared to the control group, the difference in pupal period were not significant for strain JDc1. Maximum % of half emerged (39 ± 1.54); (28 ± 0.578, SaS6), maximum % of un-emerged (51 ± 1.527); (37 ± 1.158) with maximum larval (11.33 ± 0.33); (11.66 ± 0.33) days and pupal (9 ± 0.577); (8.66 ± 0.33) days period were recorded for *Bt* JSd1 treated cucumber and bitter gourd meanwhile the minimum % of half emerged (3 ± 0.577); (7 ± 0.577), minimum % of un-emerged (5 ± 0.578); (11 ± 0.579) with minimum larval (8.33 ± 0.33); (8.66 ± 0.33) days and pupal (6.66 ± 0.33); (6.66 ± 0.33) days period were observed on control batch for cucumber and bitter gourd infested with *Z. cucurbitae* respectively. The insecticidal effect on the biological parameters determined on the potential *Bt* strains treated on cucumber and bitter gourd revealed promising results on regular biological development of the tested insects, *Z. cucurbitae* in the following order: JSd1>SaS6>JDc1 mainly (Figure 4.3.6 C-E).

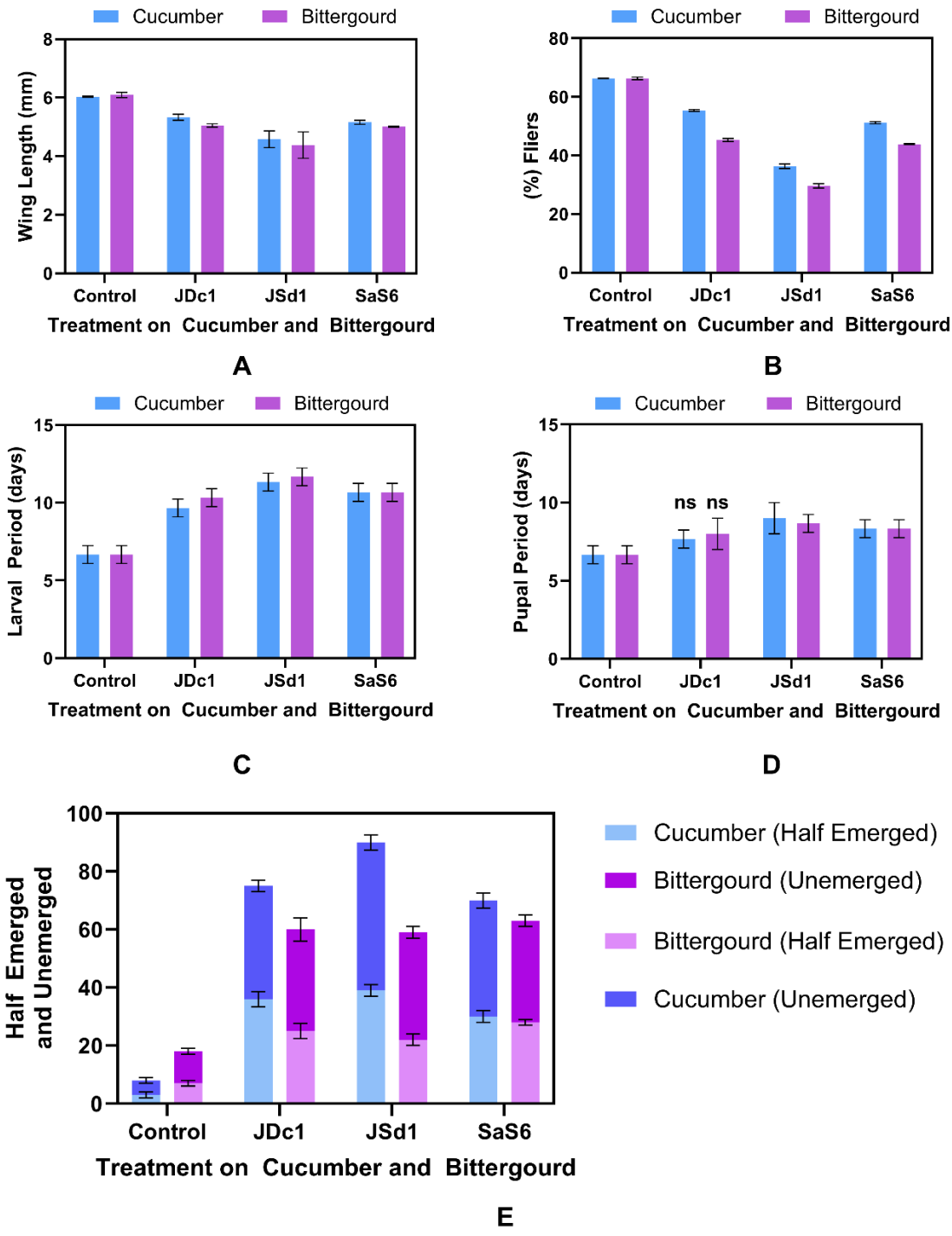


Figure 4.3. 6: Biological parameters of *Zeugodacus cucurbitae* affected by *Bt* treatment, **(A)** Wing length **(B)** Percentage of fliers **(C)** Larval period **(D)** Pupal Period **(E)** Half & Un-emerged adults. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

4.3.4 Influence of potential *Bt* biopesticide application on the biological quality parameters of *Z. tau* on infested and treated bitter gourd and cucumber

Pupation number (Pupal yield) and Pupal weight

Number of pupae (99±2.081, 143±3.511, 166±2.645) and (160±2.081, 196±6.658, 218±3.382) were calculated from the potential *Bt* JSd1, SaS6 and JDc1 treated bitter gourd and cucumber infested with *Z. tau* respectively. Lowest pupation number (99 ± 2.081); (160 ± 2.081), were recorded for potential *Bt* JSd1 treated bitter gourd and cucumber whereas highest pupation number (207 ± 1.527); (319 ± 1.527) were observed in control batch for bitter gourd and cucumber against *Z. tau* respectively (Figure 4.3.7 A).

Pupal Weight (8.25 ± 0.37, 9.89 ± 0.08, 9.19 ± 0.134) and (9.53 ± 0.217, 11.03 ± 0.164, 10.09 ± 0.121) mg, were recorded for potential *Bt* JSd1, SaS6 and JDc1 treated bitter gourd and cucumber infested with *Z. tau* respectively. Pupal weight (8.25 ± 0.37); (9.53 ± 0.217) mg, were lowest for potential *Bt* JSd1 treated bitter gourd and cucumber while highest pupal weight was recorded in control batch (11.25 ± 0.155); (12.19 ± 0.083) mg for bitter gourd and cucumber against *Z. tau* respectively (Figure 4.3.7 B).

Adult emergence (Adult yield), Sex ratios and % of adult emergence

Number of Adult emergence (39 ± 2.083, 81 ± 3.464, 101 ± 1.155) and (71 ± 2.083, 119 ± 1.132, 139 ± 1.154) were observed from the potential *Bt* JSd1, SaS6 and JDc1 strains treated bitter gourd and cucumber infested with *Z. tau* respectively. Lowest adult emergence number (39 ± 2.083); (71 ± 2.083) were observed on the potential *Bt* JSd1 treated bitter gourd and cucumber whereas highest adult emergence number (187 ± 1.154); (303 ± 4.163) were recorded on control batch for bitter gourd and cucumber against *Z. tau* respectively.

Male and female emergence ratios (M: F) (18 ± 0.577: 21 ± 1.154; 38 ± 1.527: 43 ± 2.081; 46 ± 1.154: 55 ± 1.157) and (32 ± 2.081: 39 ± 1.154; 56 ± 1.154: 63 ± 1.527; 65 ± 4.041: 74 ± 2.081), were recorded from the potential *Bt* JSd1, SaS6 and JDc1 treated bitter gourd and cucumber infested with *Z. tau* respectively. Meanwhile percent of adult emergence (39.39 ± 0.874, 56.64 ± 1.533, 60.84 ± 0.635) and (44.37 ± 1.771, 60.71 ± 2.791, 63.76 ± 2.246), were calculated from the potential *Bt* JSd1, SaS6 and JDc1 treated bitter gourd and cucumber infested with *Z. tau* respectively**.** The lowest sex ratios (M:F) (18 ± 0.577: 21 ± 1.154); (32 ± 2.081: 39 ± 1.154) and lowest % of adult emergence (39.39 ± 0.874); (44.37 ± 1.771) were recorded on potential *Bt* JSd1 treated bitter gourd and cucumber while highest sex ratios (89 ± 1.155 : 98 ±

1.154); (148 ± 1.527 : 155 ± 1.732) with highest % of adult emergence (90.33 ± 1.477); (94.98 ± 1.346) were recorded in control batch for bitter gourd and cucumber against *Z. tau* respectively (Figure 4.3.7 C-D).

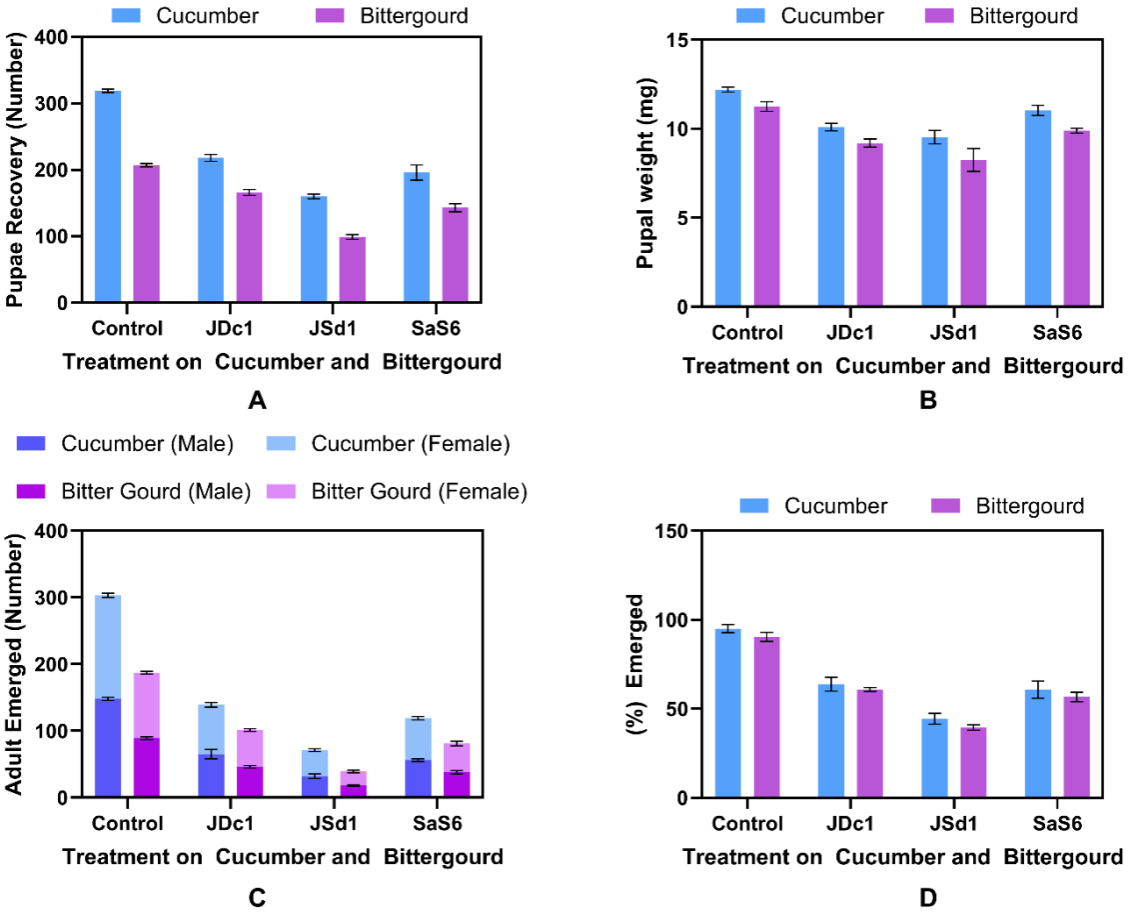


Figure 4.3.7: Biological parameters of *Zeugodacus tau* affected by *Bt* treatment, **(A)** Pupal recovery **(B)** Pupal weight **(C)** Adult Emerged (male and female) **(D)** Percentage of Adult emerged. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

Wing length and flight ability

Length of wing were (4.09 ± 0.046, 5.23 ± 0.222, 5.06 ± 0.051); (4.57 ± 0.165, 5.16 ± 0.04, 5.33 ± 0.06) mm and percent of fliers (29.32 ± 0.584, 43.27 ± 0.216, 44.34 ± 0.145) and (35.79 ± 0.158, 51.34 ± 0.196, 54.52 ± 0.267) recorded from the potential *Bt* JSd1, SaS6 and JDc1 treated bitter gourd and cucumber infested with *Z. tau* respectively. Minimum wing length (4.09 ± 0.046); (4.57 ± 0.165) mm, with lowest % of fliers (29.32 ± 0.584); (35.79 ± 0.158) were observed on potential *Bt* JSd1 treated bitter gourd and cucumber while maximum wing length (6 ± 0.005); (6.03 ± 0.011) mm with highest % of fliers (65.19 ± 0.12); (66.88 ± 0.054) were

recorded on control batch for bitter gourd and cucumber infested with *Z. tau* respectively (Figure 4.3.8 A-B).

Retardation in growth

Half emerged percentage (25 ± 1.155, 29 ± 1.154, 31 ± 0.577) and (42 ± 0.577, 32 ± 1.528, 38 ± 0.578), percent of un-emerged (35 ± 1.154, 33 ± 1.157, 34 ± 2.081) and (47 ± 0.579, 45 ± 1.154, 41 ± 1.154), with larval period (11.66 ± 0.333, 10.66 ± 0.333, 10.33 ± 0.333); (11.33 ± 0.333, 10.66 ± 0.333, 9.66 ± 0.333) days and pupal period (8.66 ± 0.333, 8.33 ± 0.333, 8 ± 0.577); (9 ± 0.573, 8.33 ± 0.333, 7.66 ± 0.333) days were calculated to the potential *Bt* JSd1, SaS6 and JDc1 treated bitter gourd and cucumber against *Z. tau* respectively. Compared to the control group, the difference in pupal period were not significant for strain JDc1.

Maximum percent of half emerged (31 ± 0.577, JDc1) and (42 ± 0.577), maximum percent of un-emerged (35 ± 1.154) and (47 ± 0.579) with maximum larval (11.66 ± 0.333); (11.33 ± 0.333) days and pupal (8.66 ± 0.333); (9 ± 0.573) days period were recorded for *Bt* JSd1 treated bitter gourd and cucumber whereas minimum % of half emerged (9 ± 1.154); (7 ± 1.155), minimum % of un-emerged (11 ± 0.577); (9 ± 0.578) with minimum larval (8.66 ± 0.333); (8.33 ± 0.333) days and pupal (6.66 ± 0.333); (6.66 ± 0.333) days period were observed on control batch for bitter gourd and cucumber against *Z. tau* respectively. Toxicity effect on the biological parameters calculated on the potential *Bt* JSd1 and SaS6 strains treated bitter gourd and cucumber revealed promising results on regular biological development of the tested insects, *Z. tau* shown in Figure 4.3.8 C-E.

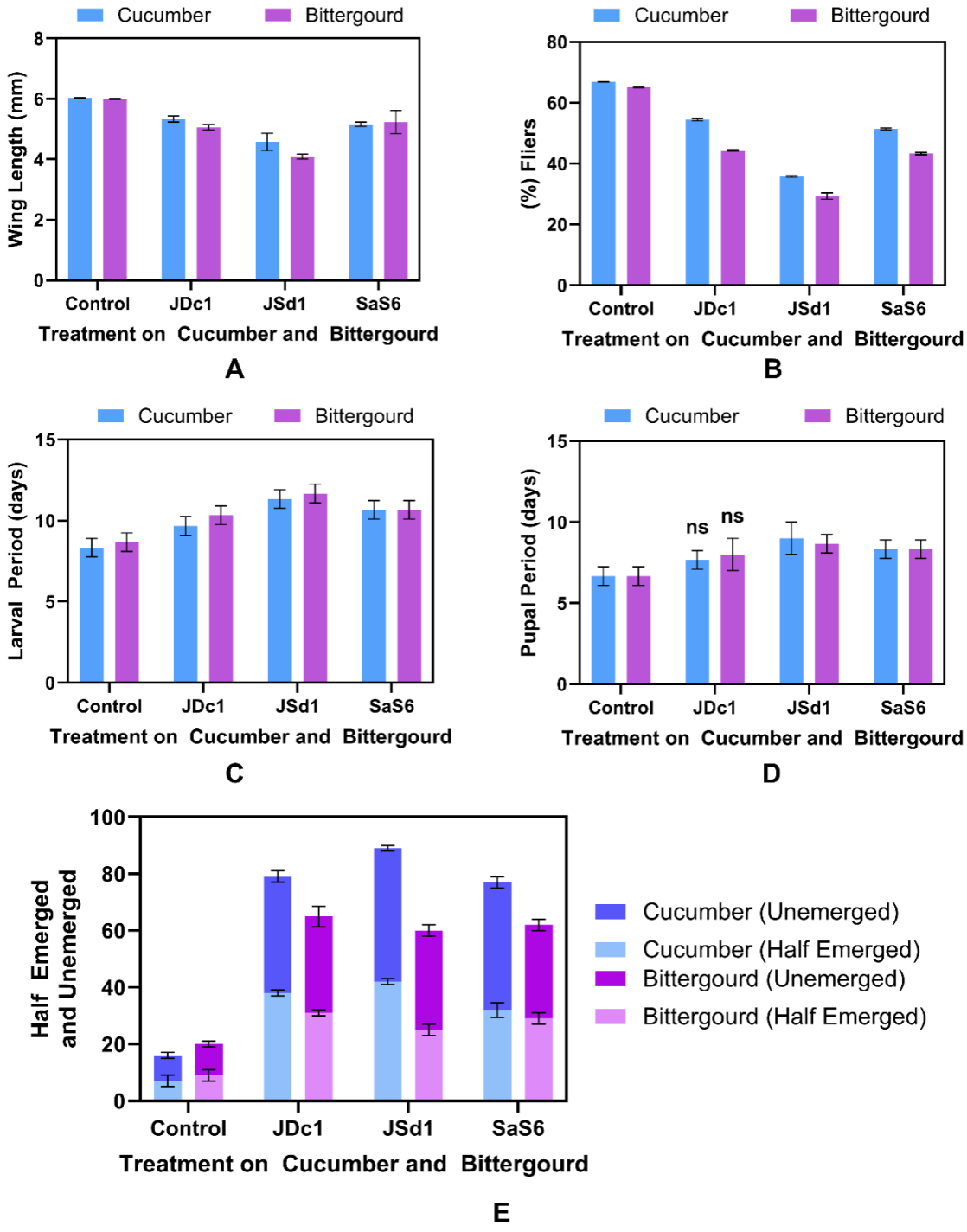


Figure 4.3.8: Biological parameters of *Zeugodacus tau* affected by *Bt* treatment, **(A)** Wing length **(B)** Percentage of fliers **(C)** Larval period **(D)** Pupal Period **(E)** Half & Un-emerged adults. Treatment groups are significant (p-value <0.05) compared to the control groups, unless (ns) is mentioned, which indicates 'not significant'.

CHAPTER 5

WHOLE GENOME SEQUENCING OF INDIGENOUS *BACILLUS THURINGIENSIS* JSd1 AND IDENTIFICATION OF THEIR INSECTICIDAL PROTEIN SEQUENCE

5.1 Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive, endospore-forming soil germs that has actually ended up being a foundation of contemporary biopesticide strategies due to its capacity to produce insecticidal crystal (Cry and Cyt) proteins. These contaminants are synthesized during sporulation and are known for their uniqueness against insect pests, especially within the Lepidoptera, Coleoptera, and Diptera orders, with minimal impacts on non-target organisms such as mammals, birds, and advantageous insects (Palma *et al.*, 2014; Schnepf *et al.*, 1998). The distinct mode of action of Cry proteins-- binding tosecticidal capacity. specific receptors in the insect gut, forming pores, and interrupting cellular stability-- has rendered *Bt* a attractive and ecologically sustainable alternative to chemical pesticides.

Beyond its function in bioinsecticide production, *B. thuringiensis* has actually likewise been instrumental in agricultural biotechnology, particularly in the development of genetically customized (GM) crops revealing Cry proteins for in planta insect resistance. This application has actually resulted in a dramatic reduction in chemical pesticide usage, resulting in enhanced ecological results and economic advantages for farmers (James, 2010; Sanahuja *et al.*, 2011). Genomic studies of *Bt* have actually revealed considerable diversity in cry gene content, which is often housed on large plasmids instead of the chromosome. These plasmids differ commonly amongst pressures and are critical in figuring out the insecticidal spectrum of a given isolate (Day *et al.*, 2014; Zheng *et al.*, 2017).

With advancements in next-generation sequencing (NGS), whole-genome sequencing (WGS) of *B. thuringiensis* has enabled comprehensive characterization of toxin genes, virulence aspects, plasmid structures, and evolutionary relationships. Relative genomic analyses of *Bt* stress such as YBT-1520, hd1, and sbt003 have uncovered a wealth of insecticidal genes and mobile hereditary elements (Liu *et al.*, 2015; Zhong *et al.*, 2011; Zhu *et al.*, 2022). These research studies have lit up the modular company of contaminant operons, horizontal gene transfer occasions, and recombination hotspots that contribute to the evolution and diversification of cry genes. *Bt* strains are now understood to harbor multiple cry genes-- often as numerous as 15-- 20 per genome-- using a complicated arsenal against varied insect targets.

In spite of the worldwide proliferation of *Bt* research study, whole-genome information remains unevenly distributed throughout geographical regions. Bangladesh, a country greatly dependent on farming, faces significant pest burdens that reduce crop yields and demand substantial

pesticide input. While native *Bt* isolates have actually been collected from various agroecological zones in Bangladesh (Shishir *et al.*, 2014), few have actually been genomically identified, and less still have gone through detailed WGS and relative analysis. This gap in genomic information postures a constraint for the development of locally adapted biopesticide items, particularly provided the possibility that native isolates may harbor uncommon or unique cry genes adjusted to local pest fauna.

To bridge this gap, this study presents the whole-genome sequencing and analysis of *Bacillus thuringiensis* strain JSd1, an isolate obtained from Bangladeshi soil. Genome sequencing of *Bt* JSd1 was undertaken to translate its genetic potential and compare it with globally understood strains. *Bt*-based biopesticides provide an environmentally sound alternative, and genomically informed strain choice will be crucial to making sure product stability and performance under local conditions (Chandler *et al.*, 2011; Sanahuja *et al.*, 2011). *Bt* JSd1, with its diverse contaminant arsenal and native origin, is a strong candidate for such applications.

This research study offers the whole-genome sequencing and comparative analysis of *B. thuringiensis* JSd1, valuable insights into the genetic basis of its insecticidal capacity. This work fills a regional knowledge gap and paves the way for the rational design of *Bt*-based pest control strategies suited to the ecological and agricultural contexts of Bangladesh. As *Bt* genomics continues to evolve, regionally grounded efforts such as this will be critical in enhancing global food security and promoting environmentally sustainable farming practices.

5.2 Materials and Method

5.2.1 Materials

5.2.1.1 Media

The media that were used for this research are detailed in Appendix D.

5.2.1.2 Chemicals and reagents

The chemicals and reagents that were used for this research are detailed in Appendix E.

5.2.1.3 Buffers and solutions

Descriptions of the buffers and solutions used in the experiments mentioned in method section, whereas the composition and production methods of these substances detailed in Appendix F.

5.2.1.4 Equipment

Appendix G includes information on the manufacturers and types of equipment utilized in this research, which is detailed in the methods section.

5.2.1.5 Bacterial strains

Indigenous *Bacillus thuringiensis* JSd1 were studied

5.2.2 Methods

5.2.2.1 DNA extraction and sequencing

Genomic DNA was isolated from the pure culture of the *Bt* isolate JSd1 using the QIAamp® DNA Mini Kit (Qiagen, Germany), following the protocol provided by the manufacturer. The quality and quantity of the extracted DNA were evaluated using a Colibri LB 915 microvolume spectrophotometer (Berthold Technologies, Germany). Library preparation for next-generation sequencing (NGS) was conducted with the Illumina DNA Prep Kit, and adapter ligation was performed using the Nextera DNA CD Indexes (Illumina, San Diego, CA, USA), strictly adhering to the manufacturer's guidelines. Sequencing was then executed on the Illumina NextSeq platform, producing 251 bp paired-end reads.

5.2.2.2 Quality assessment, trimming, and assembly

The raw data were analyzed for quality control using the FastQC v0.12.1 (Simon Andrews, 2020). Subsequently, Trimmomatic v0.39 removed the adapters (Bolger *et al.*, 2014). Finally, *de novo* assembly was performed using Unicycler v0.5.1 (Wick *et al.*, 2017). PlasFlow v1.1.0 was used to identify the plasmid contigs and were further confirmed by NCBI Blast searches (Krawczyk *et al.*, 2018). Quality of the assembly, and genome completeness were conducted using QUAST v5.3.0 (Mikheenko *et al.*, 2018), and CheckM v1.0.18 (Parks *et al.*, 2015).

5.2.2.3 Genome functional annotation, subsystem analysis, identification of secondary metabolites and *Bt* toxin related traits

Genome annotation was performed using Prokka v1.14.5, a rapid annotation tool for prokaryotic genomes (Seemann, 2014), and the RAST server (Rapid Annotation using Subsystem Technology) accessed online at http://rast.theseed.org/FIG/rast.cgi on 3 June 2025 (Aziz *et al.*, 2008). For functional gene annotation, EggNOG-mapper v2.1.12 (Cantalapiedra *et al.*, 2021) and the KEGG-based BlastKOALA tool (https://www.kegg.jp/blastkoala/) (Kanehisa *et al.*, 2016) were utilized. The annotated genome was then analyzed to determine genes associated with insecticidal activity. Specifically, *Bacillus thuringiensis* (*Bt*) toxin genes were identified using the *Bt* Toxin_Digger platform (https://bcam.hzau.edu.cn/*Bt*Toxin_Digger/index.php), which employs a combination of BLAST, Hidden Markov Model (HMM), and Support Vector Machine (SVM) algorithms for accurate prediction (Liu *et al.*, 2021).

Circular genome visualization and subsystem classification were conducted using the PATRIC web platform (https://www.bv-brc.org/) as described by (Olson *et al.*, 2023). To identify potential secondary metabolite biosynthetic gene clusters, genome mining was performed using the Antibiotics and Secondary Metabolites Analysis Shell (antiSMASH, version 7.1.0) (Blin *et al.*, 2021), accessible at http://antismash.secondarymetabolites.org/. The assembled genome was submitted to antiSMASH, which produced annotated reports including both known and predicted clusters. Comparative analysis of similar clusters and nonribosomal peptide synthetase (NRP) functions was included. All tools were executed using default settings unless specified otherwise.

5.2.2.4 Phylogenetic analysis

The closest reference and representative genomes to *Bacillus thuringiensis* strain JSd1 were identified using the Mash/MinHash algorithm (Ondov *et al.*, 2016). To infer the phylogenetic position of the strain, PATRIC global protein families (PGFams) were extracted from these genomes (Davis *et al.*, 2016). The corresponding protein sequences were aligned using MUSCLE (Edgar, 2004), and the associated nucleotide sequences were then mapped to these alignments. Both the protein and nucleotide alignments were concatenated to form a combined data matrix. Phylogenetic analysis was conducted using RAxML (Stamatakis, 2014), with support values calculated through rapid bootstrapping as described by (Stamatakis *et al.*, 2008).

5.2.2.5 Comparative analysis of *B. thuringiensis* JSd1 genome

Pangenome analysis was conducted using 11 *Bacillus thuringiensis* genomes (submitted between January and June 2025), selected based on accurate taxonomic classification and contig-level assembly available in the NCBI database (Supplementary Table). The analysis utilized the Roary pipeline v3.13.0 (Page *et al.*, 2015), accessed on 18 June 2025. Genome annotation was carried out using Prokka v1.14.5, and the resulting GFF files were used as input for the Roary pipeline, with a minimum BLASTp identity threshold of 95%. Core genes were aligned using MAFFT v7.526 for multiple sequence alignment (Katoh & Standley, 2013), while clustering was performed through the embedded CD-HIT v4.6.8 algorithm (Fu *et al.*, 2012). The gene clusters were categorized into four classes: 'core' (present in 99–100% of genomes), 'soft core' (95–98%), 'shell' (15–94%), and 'cloud' (0–14%). Additional analysis was conducted on the gene presence/absence matrix generated by Roary. Visualization of pangenome dynamics was carried out using the roary_plots script available at Roary GitHub repository.

5.3 Results

5.3.1 Genome sequencing and assembly of *B. thuringiensis strain* JSd1

A total of 1,806,038 read pairs were retained after filtering and trimming. The draft genome of *Bacillus thuringiensis* strain JSd1 achieved an average coverage of 75× and demonstrated a completeness of 99.26%, distributed across 64 contigs. The annotated genome measured 5,392,540 base pairs in length, with a GC content of 35.28% and an N50 value of 346,644 bp. Additionally, four plasmids were identified within the JSd1 genome (Table 5.3.1).

Table 5.3.1: Genomic features of *B. thuringiensis* strain JSd1

Genome features	***B. thuringiensis* JSd1**	**pBTJSd1A**	**pBTJSd1B**	**pBTJSd1C**	**pBTJSd1D**
Type of sequence	Chromosome	Plasmid	Plasmid	Plasmid	Plasmid
Sequence length	5,392,540	312,365	51,658	4,610	3,448
GC%	35.28	32.5	36.5	38	37.5
CDS	5756	317	85	9	5
Subsystem	294	6	1	-	-
tRNA	68	-	-	-	-
rRNA	9	-	-	-	-
Number of contigs	64	1	1	1	1
CDS Ratio	1.067	1.015	1.645	1.952	1.450
Hypothetical CDS	1406	186	51	8	4
Hypothetical CDS Ratio	0.389	0.662	0.647	0.889	0.8
PLFAM CDS	5609	268	72	6	1
PLFAM CDS Ratio	0.974	0.845	0.847	0.667	0.2
GenBank accession	JBMSUD000000000.1	JBMSUD010000065.1	JBMSUD010000066.1	JBMSUD010000067.1	JBMSUD010000068.1

5.3.2 Genome annotation

Genome annotation identified a total of 5,833 genes, of which 5,756 (98.68%) were protein-coding sequences. The remaining non-coding RNA genes included 68 tRNAs, accounting for approximately 1.17% of the total genes, and 9 rRNA genes, representing 0.15% (Table 5.3.1). A circular representation of the *B. thuringiensis* strain JSd1 genome was generated using the PATRIC web server (https://www.bv-brc.org/) (Figure 5.3.1). In this visualization, forward-strand genes are displayed in blue and reverse-strand genes in green. The GC content and GC skew are also represented to highlight compositional features across the genome.

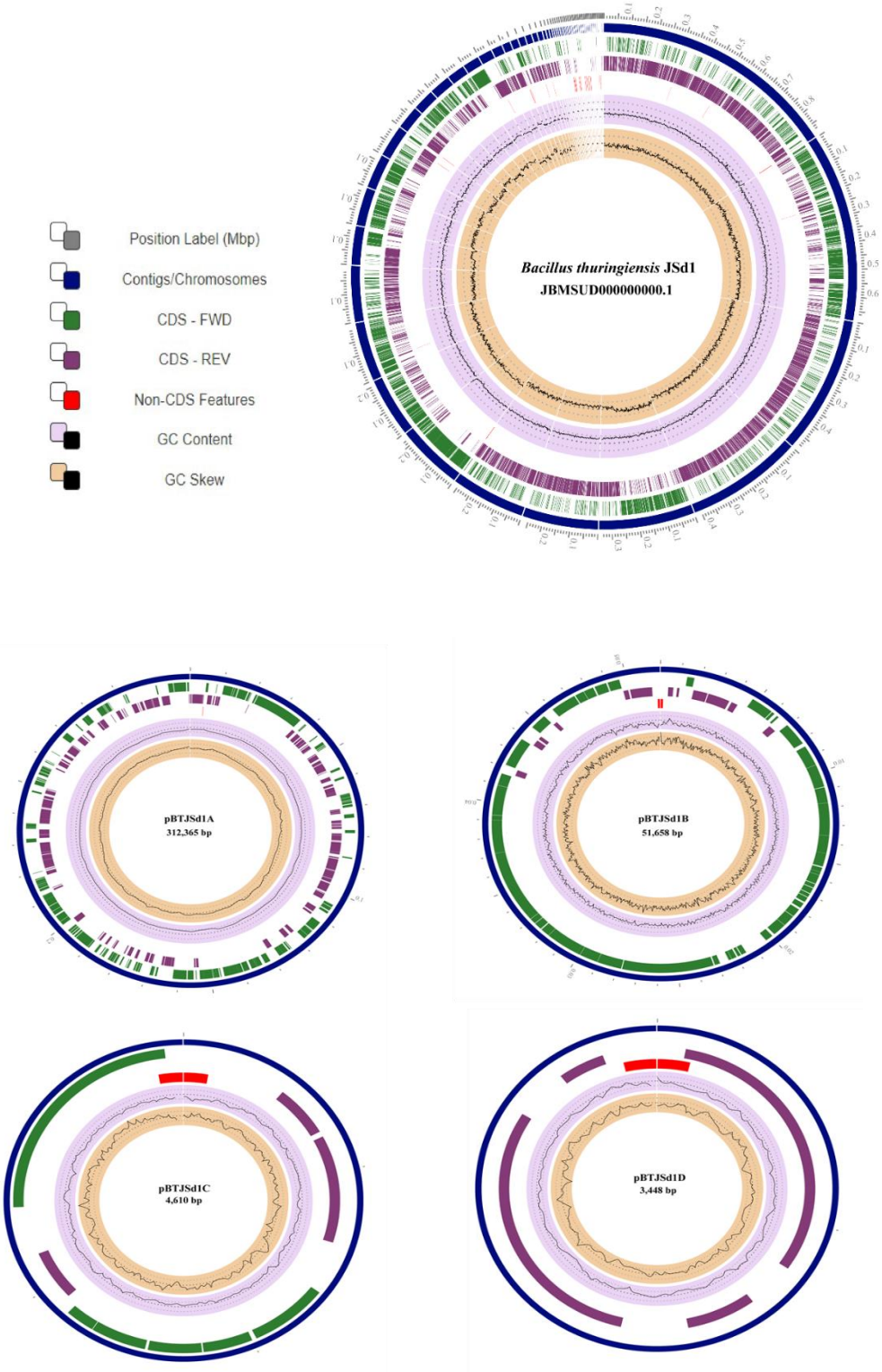


Figure 5.3.1: Circular maps of chromosome and plasmids of *B. thuringiensis* strain JSd1

Out of the total predicted protein-encoding genes (PEGs), 4,350 (75.57%) were functionally annotated, while the remaining 1,406 (24.43%) were categorized as hypothetical proteins. The most enriched subsystem class was "Cofactors, Vitamins, and Prosthetic Groups" with 177

associated genes, followed by "Amino Acids and Derivatives" (166 genes), "Stress Response, Defense, and Virulence" (129 genes), and "Energy and Precursor Metabolite Generation" (127 genes). Other prominent categories included "Protein Synthesis" (124 genes), "Prokaryotic Cell Type Differentiation" (96 genes), "DNA Processing" (87 genes), "Fatty Acids, Lipids, and Isoprenoids" (68 genes), "Cell Cycle, Cell Division, and Death" (60 genes), "Respiration" (57 genes), "Nucleosides and Nucleotides" (54 genes), "Membrane Transport" (53 genes), "RNA Processing" (49 genes), and "Protein Fate" (41 genes), among others (Figure 5.3.2). According to the COG (Clusters of Orthologous Groups) database, 4,212 genes were assigned to various functional categories. These included 347 genes involved in translation, ribosomal structure, and biogenesis; 350 in transcription; 162 in replication, recombination, and repair; 259 in cell cycle control, division, and chromosome partitioning; 217 in energy production and conversion; and 408 genes associated with amino acid transport and metabolism (Figure 5.3.3).

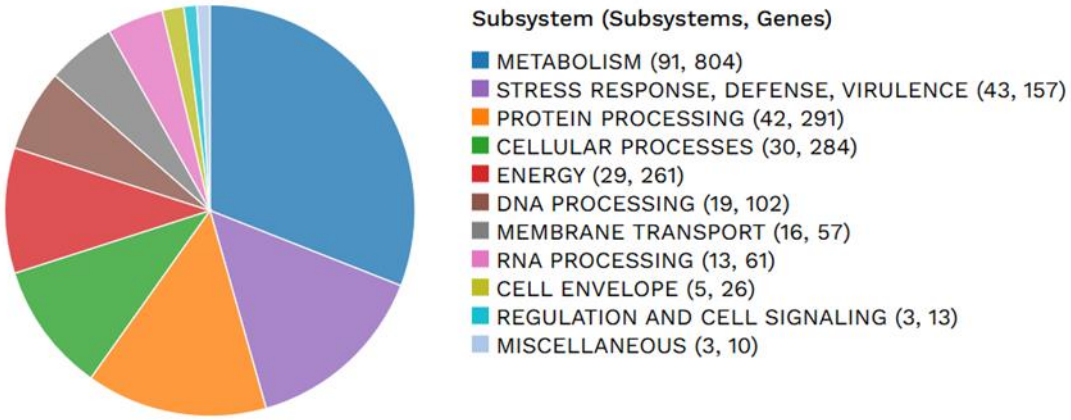


Figure 5.3.2: Distribution of subsystem classes in *B. thuringiensis* JSd1 genome.

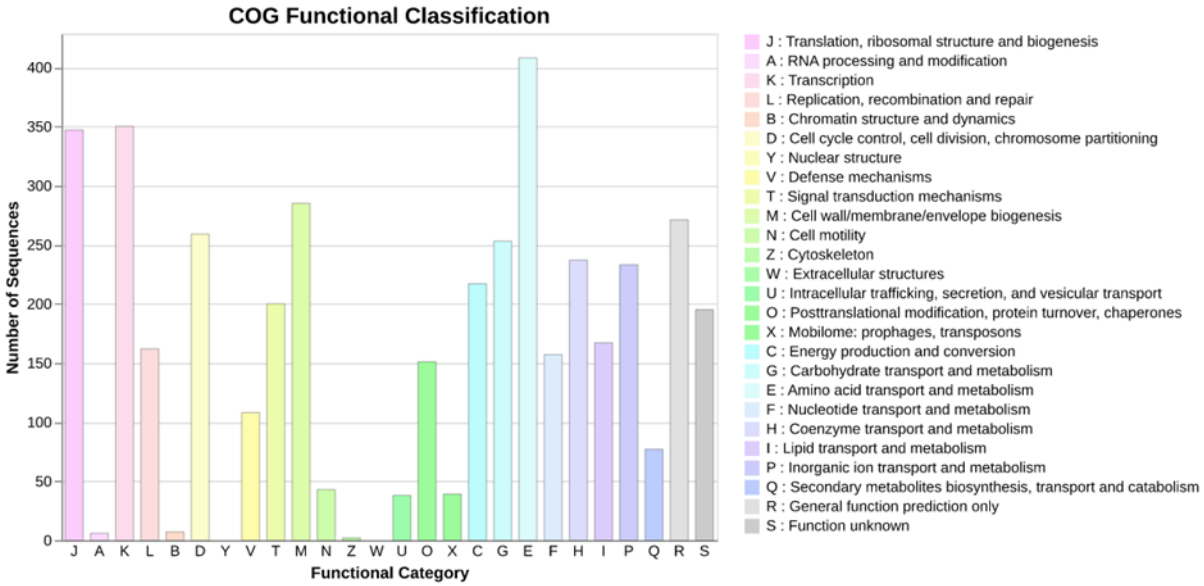


Figure 5.3.3: COG (Cluster of Orthologous Genes) functional classification in *B. thuringiensis* JSd1 genome.

5.3.3 Genomic islands (GIs) of *B. thuringiensis* strain JSd1

A total of 25 genomic islands (GIs) were detected in the genome by comparison with the *Bacillus thuringiensis* serovar konkukian strain 97-27 reference chromosome, using Island Viewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/browse/) (Figure 5.3.4). These islands predominantly contained genes encoding hypothetical proteins (435 in total), organized into 288 gene clusters. Additionally, several genes within the GIs were annotated as mobile genetic elements, including putative transposases such as Tn552 transposase, InsK associated with the IS150 insertion sequence, and transposase for IS1630-like elements. These genomic islands are indicative of horizontal gene transfer events and are considered to contribute significantly to the evolution, adaptability, and diversification of bacterial pathogens. The full list of clustered genes within these islands, along with their genomic loci, is provided in the supplementary data.

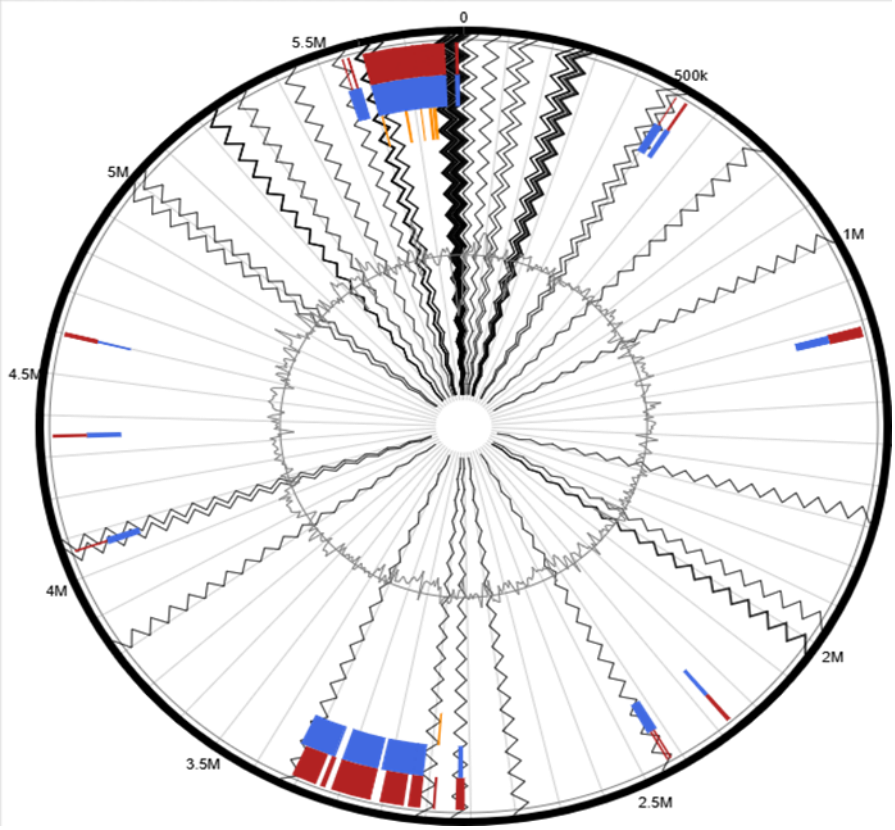


Figure 5.3.4: Genomic Islands prediction and genome visualization of isolates JSd1 against *B. thuringiensis* serovar konkukian str. 97-27.

5.3.4 Genes underlying *B. thuringiensis* toxin related traits

Comprehensive genome annotation and virulence gene screening revealed multiple genes potentially involved in insecticidal activity in the *B. thuringiensis* JSd1 isolate, distributed across both chromosomal and plasmid elements. Notably, several well-characterized insecticidal proteins and related factors were detected.

A low-identity match to the *Cry22A* gene (34.11% identity with AXN69627.1) was identified on plasmid pBTJSd1A. Cry22A is a δ-endotoxin known to act against dipteran and coleopteran larvae, although its low identity in this isolate suggests a possible novel variant. Additionally, a putative *Vip3A* gene (29.38% identity with WP_348638079.1) was annotated on the chromosome with low identity. Vip3A, a vegetative insecticidal protein, is known for its broad-spectrum activity against lepidopteran pests and is typically secreted during the vegetative growth phase. The co-occurrence of *cry* and *vip* gene homologs supports the insecticidal potential of this isolate.

Multiple Bmp1-like proteins were found on the chromosome. These proteins belong to the M4 metalloprotease family, frequently connected with insecticidal and hemolytic activity. Their annotations matched known insecticidal delta-endotoxins (e.g., EEM21720.1, EEM59624.1). A gene encoding Enhancin, understood to break down host peritrophic matrix and boost insecticidal efficacy, was also identified. The genome harbored a sphaericolysin-related gene, homologous to Spp1Aa1/BAS3109/hly, which might function likewise to hemolysins or pore-forming contaminants. In addition, the cytK gene was identified, a widely known cytotoxin connected with cell death and virulence in *B. cereus* group pressures.

Secret metalloprotease genes such as inhA1 and inhA2 were spotted, which contribute to insect virulence by breaking down host immune proteins and boosting toxin penetration. These genes are important virulence consider entomopathogenic germs. A chiA gene was annotated, which helps with the destruction of insect chitin and is typically co-expressed with Cry and Vip contaminants, helping in their effectiveness. A number of chromosomal and plasmid-encoded toxin-antitoxin system genes were detected, including PemK/MazF-like contaminants and MazE antitoxin, which might contribute to stress tolerance or persistence in host environments. These systems are significantly acknowledged for their functions in plasmid upkeep, virulence guideline, and host-pathogen interaction. Other factors such as *sodA1/sodA2* (superoxide dismutases), recA (DNA repair), sigB (general stress sigma factor), and *codY* (nutrient response regulator) were found, indicating a coordinated stress and virulence regulatory network. The presence of internalins (*inlA*) may also suggest involvement in host cell invasion or adhesion.
Besides *Cry22A*, plasmid pBTJSd1A also carried virulence-related genes including *Zwa5A* and *ClpP*, an ATP-dependent protease. These genes, along with additional TA system components on both pBTJSd1A and pBTJSd1B, may contribute to plasmid stability and maintenance of insecticidal gene clusters (Table 5.3.2). The strain was also found to contain five different types of CRISPR sequences (Table 5.3.3).

Table 5.3.2: Genes coding for virulence factors in *Bacillus thuringiensis* JSd1 genome

Protein_id	Virulence factor	Description	Location
fig|1428.2708.peg.3187	asbA	Siderophore biosynthesis protein	Chromosome
fig|1428.2708.peg.1933	Bmp1-other	Bacillolysin / Insecticidal delta-endotoxin protein	Chromosome
fig|1428.2708.peg.2955	Bmp1-other	Bacillolysin	Chromosome
fig|1428.2708.peg.2330	Bmp1-other	Bacillolysin / Insecticidal delta-endotoxin protein	Chromosome
fig|1428.2708.peg.4384	ChitinaseC-	Chitinases	Chromosome

	other/chiA		
fig|1428.2703.peg.1	clpP	chymotrypsin-like activity	pBTJSd1A
fig|1428.2708.peg.557	clpX	ATP-dependent specificity component of the Clp protease.	Chromosome
fig|1428.2708.peg.3744	codY	transcriptional repressor	Chromosome
fig|1428.2703.peg.93	Cry22A	Insecticidaltoxins	pBTJSd1A
fig|1428.2708.peg.5431	cwlD	Cell-wall hydrolases	Chromosome
fig|1428.2708.peg.5529	cwlD	Cell-wall hydrolases	Chromosome
fig|1428.2708.peg.4959	cwlJ	Cell-wall hydrolases	Chromosome
fig|1428.2708.peg.1078	cytK	Cell killing	Chromosome
fig|1428.2708.peg.1934	Enhancin-other	Enhancin family protein	Chromosome
fig|1428.2708.peg.641	GBAA4766	Petrobactin binding proteins	Chromosome
fig|1428.2708.peg.4015	inhA/InhA2-other	Metalloproteases	Chromosome
fig|1428.2708.peg.1272	inhA1-other	Metalloproteases	Chromosome
fig|1428.2708.peg.1318	inlA	Internalins	Chromosome
fig|1428.2708.peg.3897	inlA	Internalins	Chromosome
fig|1428.2709.peg.67	MazE_antitoxin	Type II toxin-antitoxin (TA) system	pBTJSd1B
fig|1428.2708.peg.5064	ndoA	Toxic component of a toxin-antitoxin (TA) module	Chromosome
fig|1428.2708.peg.5624	ndoA	Toxic component of a toxin-antitoxin (TA) module	Chromosome
fig|1428.2708.peg.3276	nheA/Bacillus haemolytic enterotoxin (HBL)	Enterotoxin	Chromosome
fig|1428.2708.peg.3275	nheB/Enterotoxin	Enterotoxin	Chromosome
fig|1428.2708.peg.3274	nheC	Enterotoxin	Chromosome
fig|1428.2708.peg.5013	Nos	Bacterial NOS oxygenase subfamily	Chromosome
fig|1428.2708.peg.3945	Npr	Bacillolysins	Chromosome
fig|1428.2708.peg.4180	nprB	Bacillolysins	Chromosome
fig|1428.2703.peg.156	PemK-like, MazF-like toxin	Type II toxin-antitoxin (TA) system	pBTJSd1A
fig|1428.2708.peg.1313	phnX	Belongs to the HAD-like hydrolase superfamily, PhnX family	Chromosome
fig|1428.2708.peg.4019	plcB	Phospholipase	Chromosome
fig|1428.2708.peg.2239	Pre-toxin TG	-	Chromosome
fig|1428.2708.peg.3365	Pre-toxin TG	-	Chromosome
fig|1428.2708.peg.3367	Pre-toxin TG	-	Chromosome
fig|1428.2708.peg.3791	recA	-	Chromosome
fig|1428.2708.peg.3390	sigB	sigma factor	Chromosome

fig|1428.2708.peg.364	sodA1	radicals which are normally produced within the cells and which are toxic to biological systems	Chromosome
fig|1428.2708.peg.5014	sodA2	superoxide dismutase	Chromosome
fig|1428.2708.peg.5502	sodC	Superoxide dismutase	Chromosome
fig|1428.2708.peg.1848	Spp1Aa1/BAS 3109/alo/hly	Sphaericolysin	Chromosome
fig|1428.2708.peg.2403	Toxin SpoIISA	Type II toxin-antitoxin (TA) system	Chromosome
fig|1428.2708.peg.590	vapC	Toxic component of a toxin-antitoxin (TA) module. An Rnase	Chromosome
fig|1428.2708.peg.1067	Vip3A	vegetative insecticidal protein	Chromosome
fig|1428.2708.peg.269	yhcW	Hydrolase	Chromosome
fig|1428.2708.peg.2590	yokU	YokU-like protein, putative antitoxin	Chromosome
fig|1428.2703.peg.274	Zwa5A-other	Siderophore staphylobactin biosynthesis protein SbnA	pBTJSd1A
fig|1428.2708.peg.3323	Zwa5A-other	Cysteine synthase	Chromosome
fig|1428.2708.peg.459	Zwa5A-other	Cystathionine beta-synthase	Chromosome
fig|1428.2708.peg.5447	Zwa5A-other	Cysteine synthase	Chromosome
fig|1428.2708.peg.196	Zwa6-other	Ornithine carbamoyltransferase	Chromosome
fig|1428.2708.peg.4404	Zwa6-other	Ornithine carbamoyltransferase	Chromosome

Table 5.3.3: Details of CRISPR sequences of JSd1 genome obtained through the CRISPR finder.

CRISPR ID	CRISPR Start Position	CRISPR End Position	CRISPR length	Direct Repeat Consensus Sequences	DR length	No. of Spacers
JBMSUD010000065	130079	130226	147	ATGGCTATAAGCTTTTATTTCAAATTTTATAGC	33	1
JBMSUD010000002	171384	171492	108	TGTATGATTACCTTCCGCATGAGAA	25	1
JBMSUD010000003	289022	289124	102	AAGTTTAGGTTTCTTTTGAGAATGT	25	1
JBMSUD010000014	91796	91893	97	AGAAGAAGTAACGGAAGAGGAAAAGG	26	1
JBMSUD010000030	6387	6514	127	CGTTTTATGATCTTCTAATTGTTTATTTA	29	1

5.3.5 Genes related to the production of secondary metabolites

The antiSMASH analysis identified a total of eight biosynthetic gene clusters (BGCs) in the *B. thuringiensis* JSd1 genome and one additional cluster in the pBTJSd1A plasmid. Among these, three clusters were predicted to encode RiPP-like peptides and linear azole-containing peptides (LAPs), two clusters were associated with non-ribosomal peptide synthetases (NRPs), and the remaining clusters encoded a terpene, a betalactone, and a siderophore. Specific gene clusters—molybdenum cofactor, fengycin, bacillibactin, and petrobactin—showed amino acid sequence similarities of 17%, 40%, 46%, and 100%, respectively, when compared to known clusters. Other clusters displayed no significant similarity to any characterized gene clusters. In the pBTJSd1A plasmid, the sactipeptide and ranthipeptide clusters exhibited 100% sequence homology with the thuricin CD α and thuricin CD β peptides. Notably, the petrobactin cluster includes several biosynthesis-related genes such as *IucA*, *IucC*, an AMP-dependent synthetase/ligase, a sensor histidine kinase, and a polysaccharide deacetylase (Figure 5.3.5).

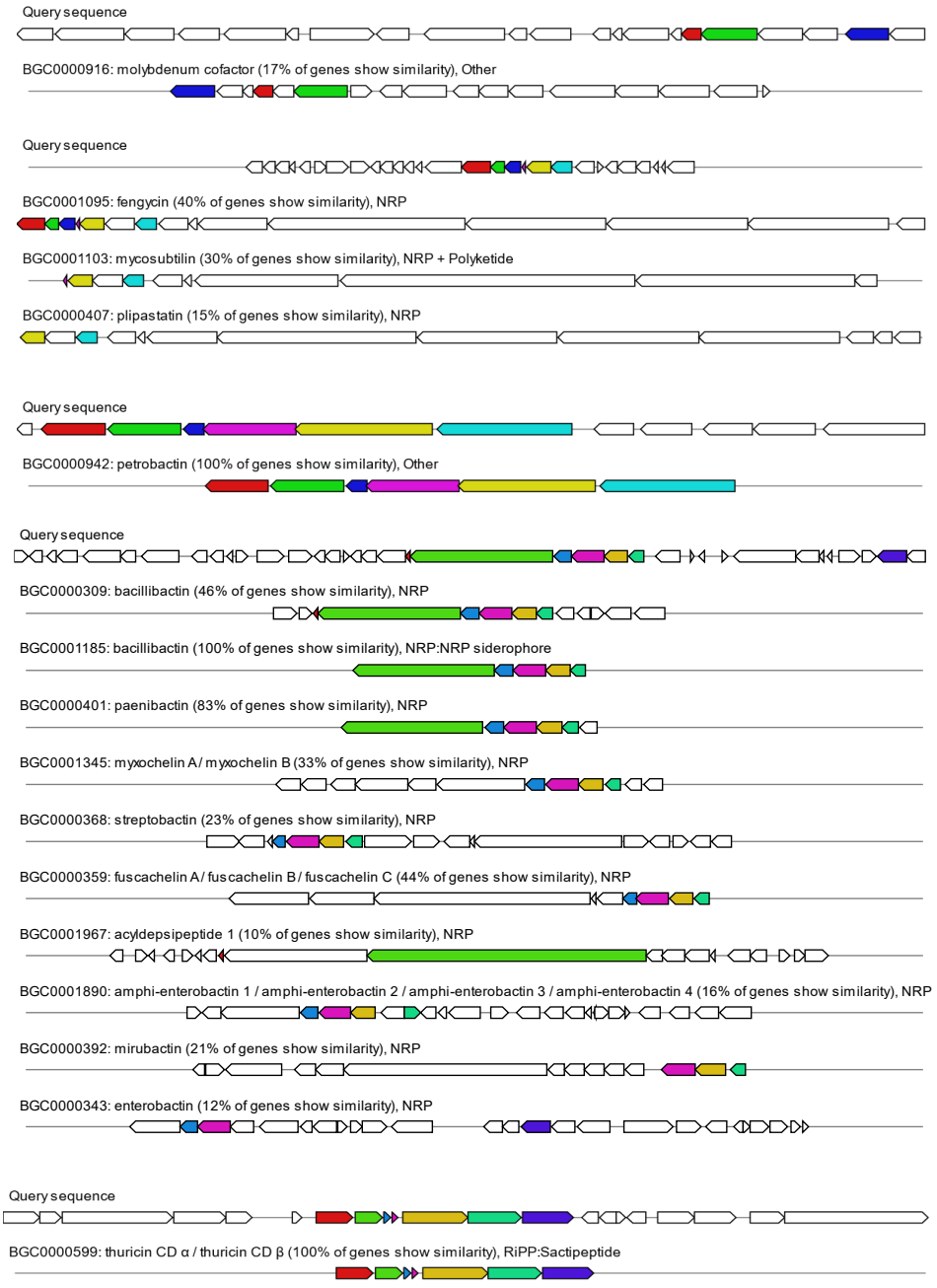


Figure 5.3.5: Secondary metabolite gene clusters in *B. thuringiensis* JSd1 genome identified by antiSMASH

5.3.6 Comparison with closely related *B. thuringiensis* strains

A comparative protein-encoding genome sequence analysis of *Bt* JSd1 strain with two *B. thuringiensis* genomes (*B. thuringiensis* serovar konkukian str. 97-27 and *B. thuringiensis* str. Al Hakam) and a different sub-species of *B. cereus* (*B. cereus ATCC 14579*) revealed that JSd1 genome has a significant variation with the *B. cereus* strains (Fig. x). The color of the blocks within these comparison rings reflects the percent protein sequence identity of corresponding

genes, ranging from 100% (dark blue/purple) to 10% (dark red/orange). Interestingly, noticeable variations were also found between *Bt* JSd1 and *B. cereus* ATCC 14579 (Figure 5.3.6).

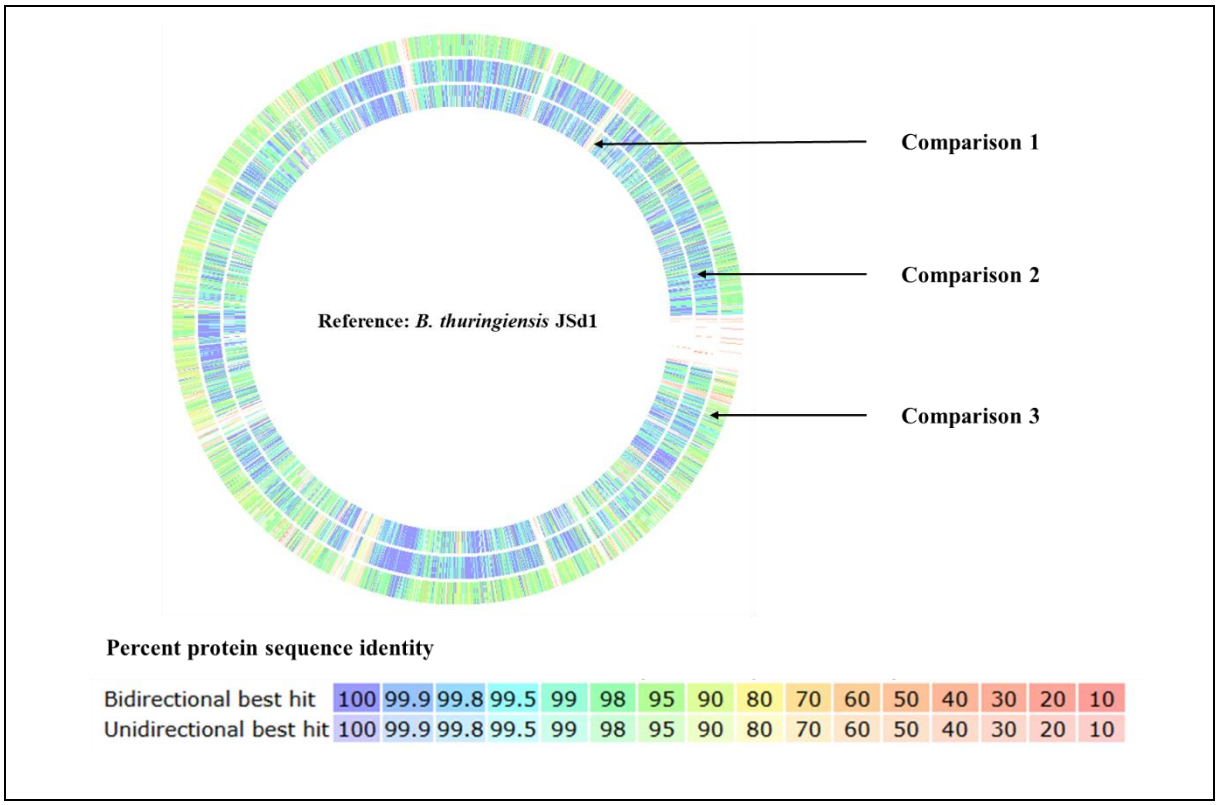


Figure 5.3.6: Comparative genome analysis of isolate JSd1 under RAST server. The first circle Comparison 1 is with *B. thuringiensis* str. Al Hakam (412694.5). The second circle Comparison 2 is with *B. thuringiensis* serovar konkukian str. 97-27 (281309.3) and the third circle Comparison 3 is with *B. cereus* ATCC 14579 (226900.1)

5.3.7 Phylogenetic assessments

The Genome-to-genome distance analyses indicated that the *B. thuringiensis* JSd1 is closely clustered within the *Bacillus cereus* sensu lato group. Specifically, *B. thuringiensis* JSd1 formed a highly supported clade (100% bootstrap support) with *Bacillus thuringiensis* serovar konkukian str. 97-27 and the pathogenic *B. anthracis* strains (str. Ames and str. Sterne). This close relationship is consistent with the well-known phylogenetic proximity among members of the *B. cereus* group. Within the *B. cereus* group, *B. thuringiensis* JSd1 was positioned as a sister clade to *B. thuringiensis* serovar konkukian, and this combined branch was then sister to the *B. anthracis* clade. Another distinct cluster within the *B. cereus* group was observed, comprising *Bacillus thuringiensis* YBT-1518 and *Bacillus thuringiensis* serovar israelensis ATCC 35646, both showing 100% bootstrap support for their internal branching. *B. cereus* ATCC 14579 branched more basally within this main *B. cereus* group clade.

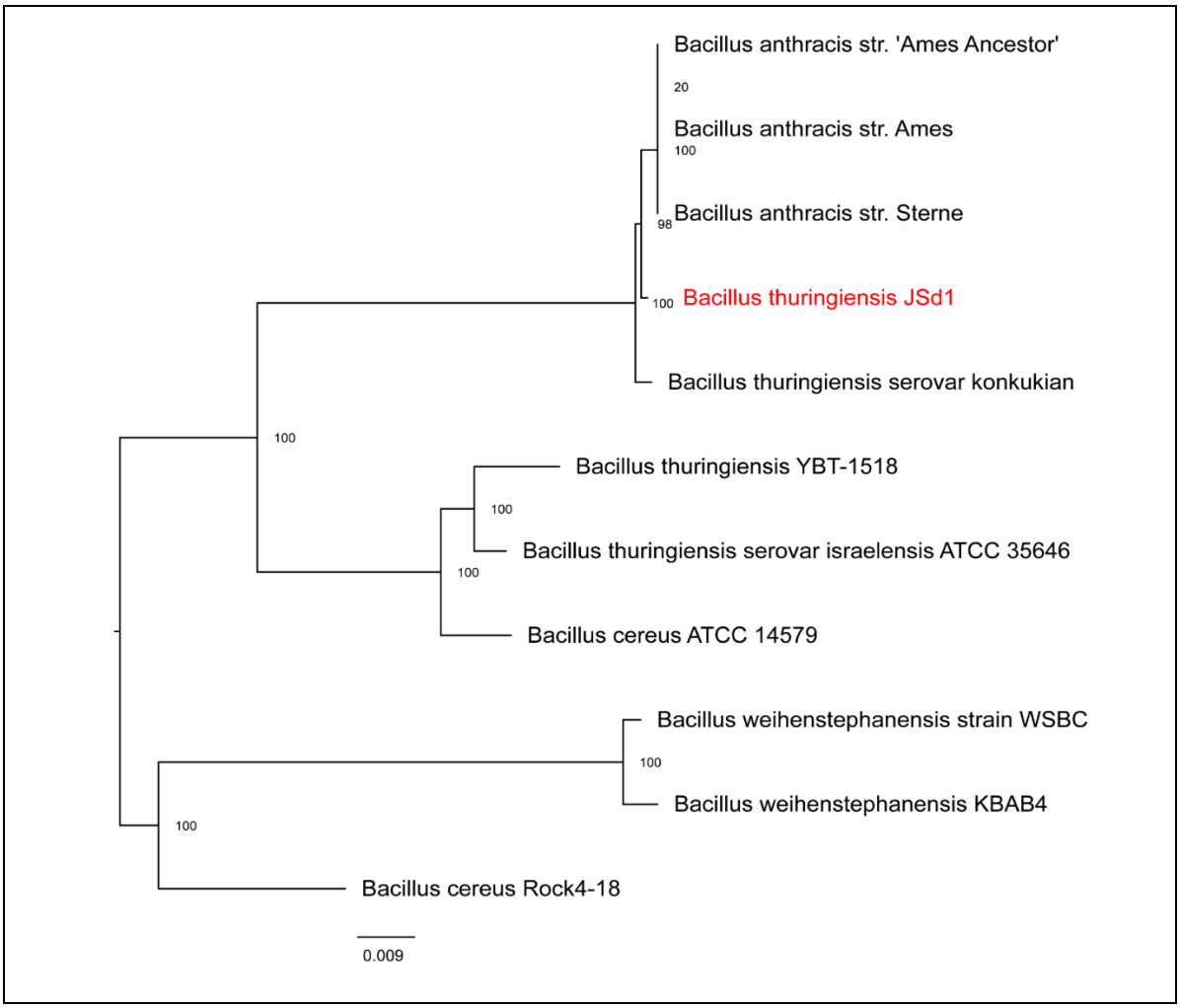


Figure 5.3.7: Phylogenomic relationship of the *B. thuringiensis* JSd1 with other strains.

More distantly related, *B. weihenstephanensis* (strain WSBC 10204 and KBAB4) formed a separate, well-supported clade (100% bootstrap support), indicating its phylogenetic divergence from the *B. cereus* group. *Bacillus cereus* Rock4-18 was positioned as an out group to all other species shown, suggesting it represents an earlier divergence (Figure 5.3.7). This phylogenetic analysis confirms the classification of strain *Bt* JSd1 within the *B. cereus* group and highlights its particularly close genetic relationship to *B. thuringiensis* serovar konkukian and *B. anthracis*.

5.3.8 Pan-genome analysis

In order to infer the genomic similarity or variation among the *B. thuringiensis* isolates, a pan-genome analysis was conducted. A pan-genome, comprising twelve genomes of the *B. thuringiensis* isolates with contig level assembly consisted of 17,742 genes, being distributed into 2,237 core genes (12.6 %) (99% ≤ strains ≤ 100%), 0 soft cores (95% ≤ strains <99%), 7,504 shell (42.3 %) (15% ≤ strains <95%) and 8,001 (45.1 %) cloud genes (0% ≤ strains <15%) (Figure 5.3.8).

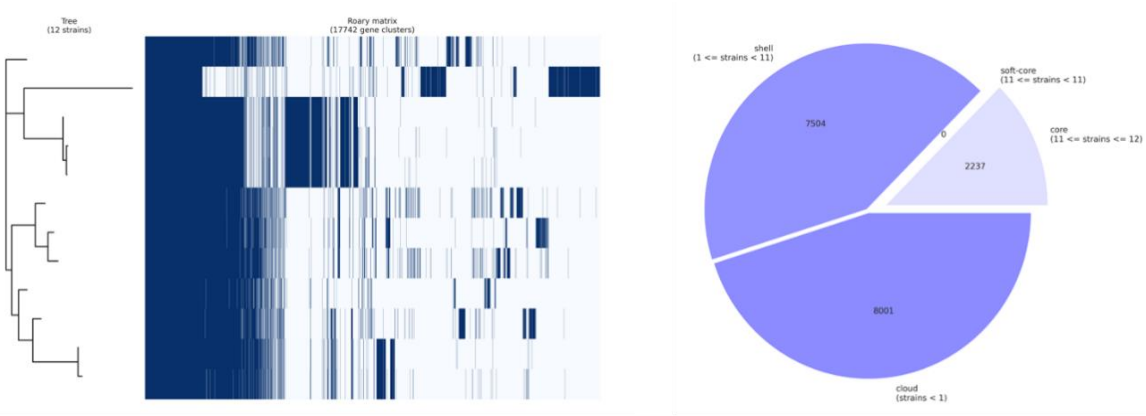


Figure 5.3.8: Pangenome analysis of *B. thuringiensis* JSd1 strains. (a) Pangenome based (gene presence and absence) gene clustering matrix of *B. thuringiensis* JSd1, and 11 different strains of *B. thuringiensis*, (b) breakdown of genes in *B. thuringiensis* genomes

The pan-genome analysis, performed across 12 *Bacillus thuringiensis* genomes including strain *Bt* JSd1, revealed the diverse distribution of a selection of virulence-associated genes (Figure 5.3.9). These genes were broadly categorized into functional groups such as Bacillolysins, Cell-wall hydrolases, Metalloproteases, Chitinases, Collagenases, Internalins, Phospholipase, and Serine proteases. The heatmap visually represents the presence or absence of these genes across the *B. thuringiensis* strains.

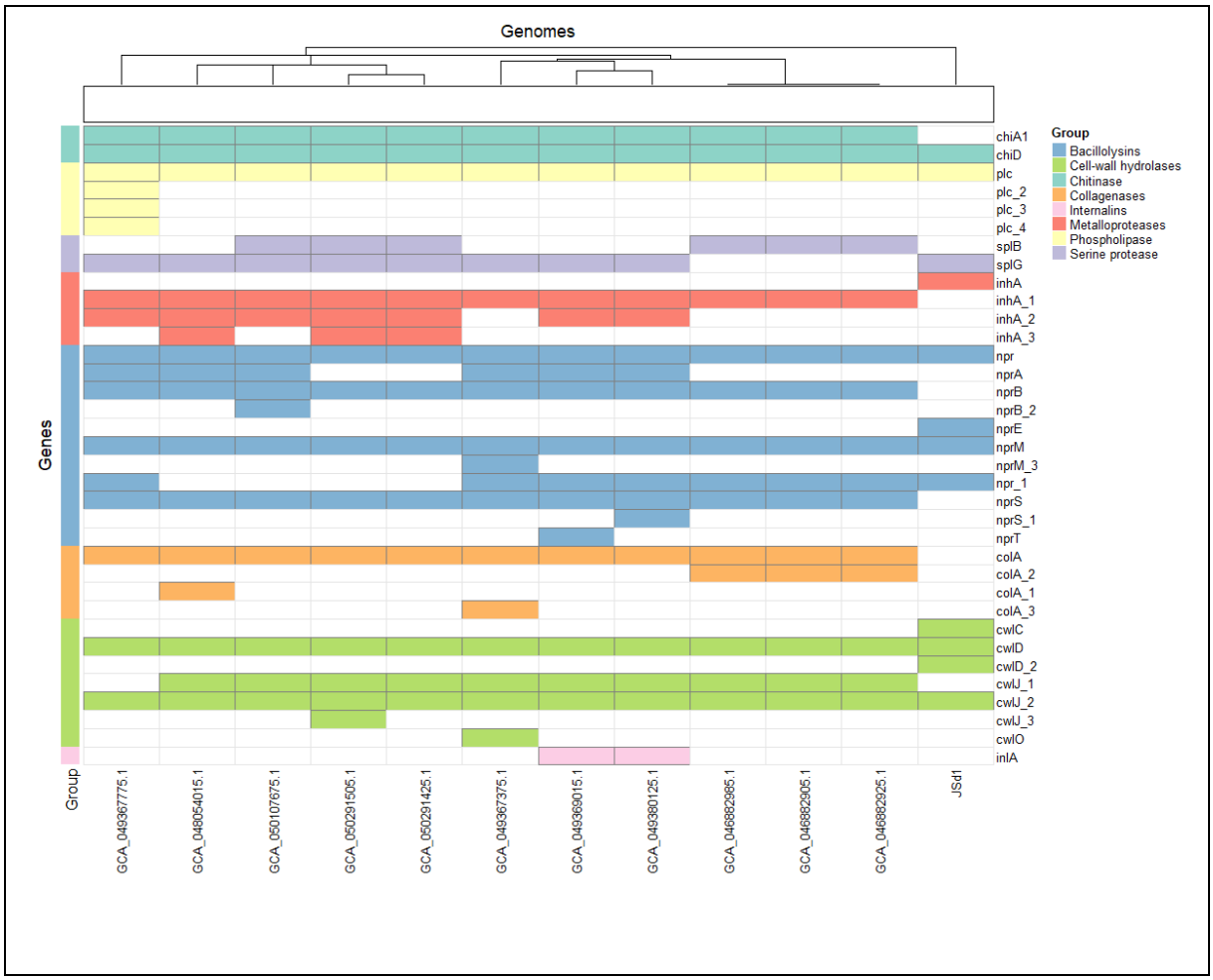


Figure 5.3.9: Heatmap comparison of the distribution of virulence genes among 12 different strains of *B. thuringiensis* strain.

Genes like chiA1 and chiD (Chitinases), and various npr genes (Bacillolysins) were widely distributed among most of the *B. thuringiensis* genomes, indicating their prevalence as common factors within the species. *Bacillus thuringiensis* JSd1 consistently carried a broad repertoire of these widely distributed virulence-associated genes, including chiD, and multiple npr and cwl genes. Its gene presence profile closely mirrored that of other closely related *B. thuringiensis* strains within the dataset. However, the analysis also highlighted significant strain-specific variations within the *B. thuringiensis* pan-genome. Genes such as specific plc types (e.g., plc_2, plc_3, plc_4), inhA genes, and members of the colA family displayed more variable presence/absence patterns across different *B. thuringiensis* strains.

CHAPTER 6

PRODUCTION OF *BACILLUS THURINGIENSIS* BIOPESTICIDE AND FIELD APPLICATION

6.1 Introduction

Effective *Bt* biopesticide deployment hinges on robust formulation, storage, and application strategies. Research highlights the importance of tailored culture conditions and carrier materials to maintain long-term viability and field performance (Iriarte, 1998; Salama, 1983). Indigenous *Bt* strains, adapted to local agroecological conditions, further enhance pest control efficacy. This study evaluates the field efficacy of the developed biopesticide against key insect pests in vegetable and fruit, aiming to contribute to sustainable pest management strategies in resource-limited agricultural contexts in Bangladesh.

6.2 Materials and Methods

6.2.1 Materials

6.2.1.1 Media

Media were used in this study and their compositions were mentioned in Appendix D.

6.2.1.2 Chemicals and reagents

Chemicals and reagents were used in this experiment and their compositions were mentioned in respective method section (Appendix E).

6.2.1.3 Buffers and solutions

Buffers and solutions were used in this experiment were mentioned in respective methods section and their compositions were mentioned in Appendix F.

6.2.1.4 Equipment

All equipment used in this study were mentioned in respective methods section and their company and models were mentioned in Appendix G.

6.2.2 Methods

6.2.2.1 Bacterial strain and culture conditions

Bacillus thuringiensis (*Bt*) strains were routinely maintained on Luria-Bertani (LB) agar to preserve culture purity and viability. The same medium was employed for spore quantification assays. All incubation steps were conducted at a controlled temperature of 30°C to facilitate

optimal bacterial growth. Liquid cultures were cultivated in sterile conical flasks and incubate in an orbital shaker operating at 180 revolutions per minute (rpm), providing sufficient aeration to promote homogeneous cell proliferation and efficient sporulation.

6.2.2.2 Inoculum preparation

To prepare the inoculum, *Bacillus thuringiensis* strains were initially transferred from slants onto LB agar plates under sterile conditions and incubated overnight at 30°C to promote colony development. A single, well-isolated colony was then aseptically selected using a sterile loop and introduced into a 250 mL Erlenmeyer flask containing 50 mL of sterile LB broth. The flask was incubated in a shaking incubator at 30°C and 180 rpm for 16-18 hours to ensure sufficient biomass growth. Following incubation, the optical density (OD) of the culture was measured at 600 nm using a spectrophotometer, with uninoculated LB broth serving as the blank. This overnight culture served as the inoculum for subsequent fermentation in the bioreactor.

6.2.2.3 Sampling and sample analysis

During the fermentation process, samples were aseptically taken from the culture broth at 24-hour intervals, meaning that three samples were taken at 24, 48, and 72 hours in a biosafety cabinet. Samples were taken as aliquots in sterile microfuge tubes for microscopic examination, spore count, and crystal protein concentration measurements.

6.2.2.4 Microscopic studies

The progression of *Bacillus thuringiensis* growth and sporulation during fermentation was monitored using phase contrast microscopy. At various time points, a small volume of culture was placed on a clean microscope slide and gently covered with a coverslip. Any excess liquid at the edges was carefully removed with tissue to ensure a clear view. The prepared slide was then examined under a phase contrast microscope (Appendix D) to observe morphological changes, including vegetative cells, cells undergoing sporulation, and mature spores. This method was effective for assessing developmental stages and confirming the onset and progression of sporulation in the cultures.

6.2.2.5 Determination of spore count

To quantify spore formation, 1.0 mL samples were collected from the fermentation broth at selected time points. Each sample was heated at 80°C for 10 minutes to inactivate vegetative cells while preserving spores. The treated samples were then serially diluted, and 0.1 mL from

the two highest dilutions was plated on LB agar using the spread plate method. Plates were incubated overnight at 37°C to allow spore germination and colony development. Colonyin an orbital shaker operating at 180 revolutions per minute (rpm), providing sufficient aeration to promote homogeneous cell proliferation and efficient sporulation.

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6.2.2.10 Estimation of crystal protein concentration

The concentration of purified crystal proteins was determined using the Bradford assay (Bradford, 1976), which relies on the binding of Coomassie Brilliant Blue G-250 dye to proteins. Bovine serum albumin (BSA) standards ranging from 0 to 100 µg/mL were prepared in 0.1 N NaOH, the same buffer used for resuspending the crystal proteins. For each assay, 20 µL of either the protein sample or standard was mixed with 1.0 mL of Bradford reagent and incubated at room temperature for 5-10 minutes to allow color development. Absorbance was measured at 595 nm using a spectrophotometer, with a blank containing only 0.1 N NaOH and Bradford reagent for calibration. A standard curve was generated by plotting the absorbance values of the BSA standards against their concentrations, and the protein concentration of each crystal protein sample was calculated by interpolating its absorbance from this curve (Ku *et al.*, 2013). All measurements were performed in triplicate to ensure accuracy and reproducibility.

6.2.2.11 Biopesticide production in a 3 L bioreactor

For the scaled-up production of *Bacillus thuringiensis* (*Bt*) biopesticide, fermentation was conducted in a fully automated and controlled 3.0-liter bioreactor system (Appendix D) containing 2.0 liters of the optimized, cost-effective culture medium. The fermentation process was maintained at a constant temperature of 30°C. Aeration was supplied at a rate of 1.0 standard liter per minute (SLPM), and agitation was set at 250 revolutions per minute (rpm), ensuring adequate oxygen transfer and maintaining the dissolved oxygen (DO) concentration at approximately 30%. The DO level was continuously monitored using an integrated oxygen sensor. Although aeration and agitation parameters were stringently controlled, pH regulation was deliberately omitted to permit fluctuations resulting from the metabolic activity of the microorganism. This experimental design was intended to replicate semi-industrial fermentation conditions, thereby providing relevant insights into the feasibility and efficiency of *Bt* biopesticide production at a larger scale.

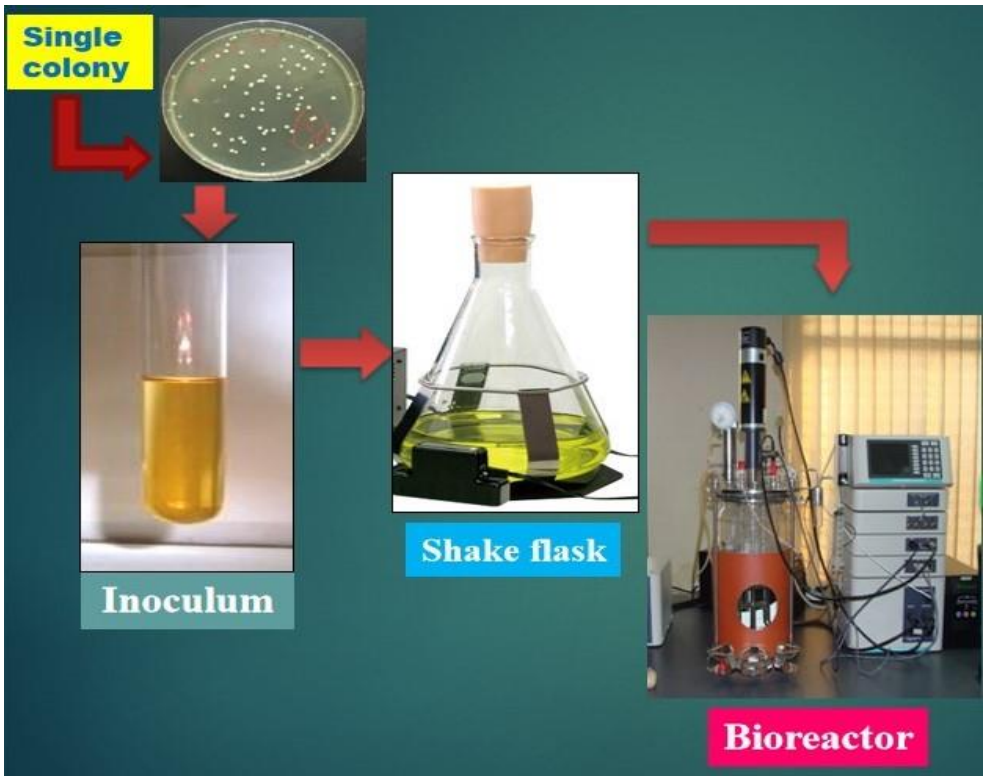


Figure 6.2.1: Production of *Bt* biopesticide at pilot plant facilities in CARS, DU

6.2.2.12 Field evaluation of *Bacillus thuringiensis* formulations on brinjal cultivation

This field study was primarily intended to determine how well *Bacillus thuringiensis* (*Bt*) formulations managed the eggplant fruit and shoot borer (EFSB), *Leucinodes orbonalis*, and how they affected brinjal (*Solanum melongena* L.) yield and quality compared to the chemical pesticide (Cypermethrin: Ripcord 10 EC) and control.

6.2.2.13 Plant material and the experimental site

The trial was carried out at the Olericulture Division of the Horticulture Research Centre (HRC), Bangladesh Agricultural Research Institute (BARI), in Gazipur, in the summer of 2020. Because of its extensive cultivation and its vulnerability to EFSB infestations, the brinjal variety 'BARI begun-8' was chosen.

6.2.2.14 Experimental design and treatments

The study used a randomized complete block design (RCBD), which included five treatments with three replications each. Ten plants in a single row, with a 70 cm plant-to-plant and row-to-row spacing of one meter, made up each plot. Treatments were applied in this study as given bellow:

- T1: Control (pure water sprayed; no *Bt* or chemical treatment)
- T2: Chemical Pesticide (Ripcord 10 EC 30 ml formulation four times at 15 days intervals)
- T3: Treatment A (Applying 100 ml* of the *Bt* formulation four times at intervals of 15 days)
- T4: Treatment B (Three applications of 100 ml* of *Bt* formulation spaced 15 days apart)
- T5: Treatment C (200 ml of the *Bt* formulation was applied twice, separated by 15 days)

*In 100 mL (0.257×100) 25.7 mg spore crystal protein were used

6.2.2.15 Crop management

Thirty-day-old seedlings, raised from seeds sown in early March, were transplanted in early April. Standard agronomic practices, including irrigation, weeding, and pest management, were uniformly applied across all plots. Fertilizers were administered based on the recommended doses: 10,000 kg/ha organic matter, 170 kg/ha nitrogen (N), 50 kg/ha phosphorus (P), 125 kg/ha potassium (K), 18 kg/ha sulfur (S), 4.3 kg/ha zinc (Zn), and 1.70 kg/ha boron (B). Applications were split among final land preparation, basal pit application, and subsequent top-dressings during the growth period.

6.2.2.16 Application of *Bt* biopesticides and chemical pesticide formulations

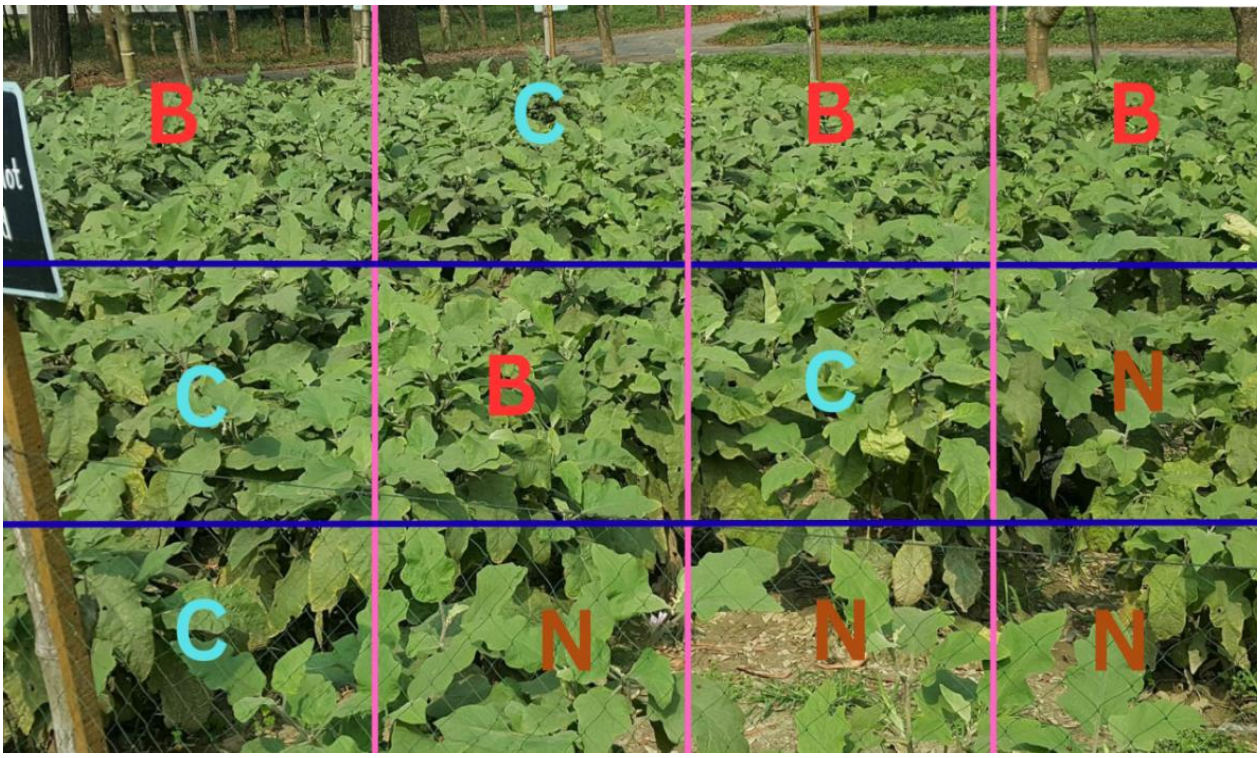
Bt formulations and chemical pesticide were applied as foliar sprays using a hand sprayer. Applications were conducted early in the morning to minimize degradation from ultraviolet light and to enhance efficacy. Control plots received only plain water sprays following the same schedule to maintain consistency.

Throughout the harvesting period, data were collected on various parameters, including:

- Days to first harvest
- Total harvest duration
- Number of marketable fruits per plant
- Average single fruit weight
- Total fruit yield per plant
- Fruit length and diameter
- Percentage of EFSB-infested fruits
- Incidence of bacterial wilt (BW)

EFSB infestation levels were determined through visual inspection of damaged shoots and fruits. Statistical analyses were performed using R software (version 4.5.0), and treatment means were compared to evaluate the efficacy of the different *Bt* application regimes.

This field-based study provided practical insights into the effectiveness of varying *Bt* spray volumes and frequencies in controlling EFSB and their subsequent influence on brinjal yield and quality under real cultivation conditions in comparison to chemical pesticide and control.



A

B

Figure 6.2.2: Application of different treatments **(A)** Randomized Complete Block Design (RCBD) treatment in brinjal farming. C: chemical pesticide; N: no pesticide; B: *Bt* biopesticide. **(B)** *Bt* biopesticide formulation for field application.

6.2.2.17 Data collection and analysis

All the recorded data calculated from field application were analyzed statistically and Graphical analysis were computed using GraphPad Prism 10 and python open-source software program. Statistical significance threshold was set at p-values of equal to 0.05.

6.3 Results

6.3.1 Efficacy of *Bt* preparation in brinjal

The findings revealed notable differences among treatments in terms of yield-contributing traits and pest infestation levels (Table 6.3.1). Regarding days to first harvest, the control group required the longest time (122 days), while the 100 ml four-time spray treatment led to the earliest harvest (103 days), suggesting that *Bt* application may accelerate plant development. Harvest duration was also affected, with the longest period (73.4 days) observed in the 200 ml two-time spray treatment, compared to the shortest (59.3 days) in the control.

A **B** **C**

Figure 6.3.1: Effect on pest infestation and yield of brinjal using different treatments. **(A)** *Bt* biopesticide treatment **(B)** No pesticide treatment **(C)** Chemical pesticide treatment

The number of marketable fruits per plant was highest in the 100 ml four-time spray treatment (32 fruits), followed by the 100 ml three-time spray (27 fruits) and 200 ml two-time spray (24 fruits), while the control had the lowest number (7 fruits). In terms of fruit weight and yield per plant, the 100 ml four-time spray treatment again outperformed others, yielding the heaviest single fruit (104 g) and the highest overall yield (3.85 kg per plant), significantly higher than the control (1.45 kg per plant). Treated plants also showed improved fruit length (9.2 cm) and diameter (6.3 cm) compared to the control (7.2 cm and 5.3 cm, respectively).

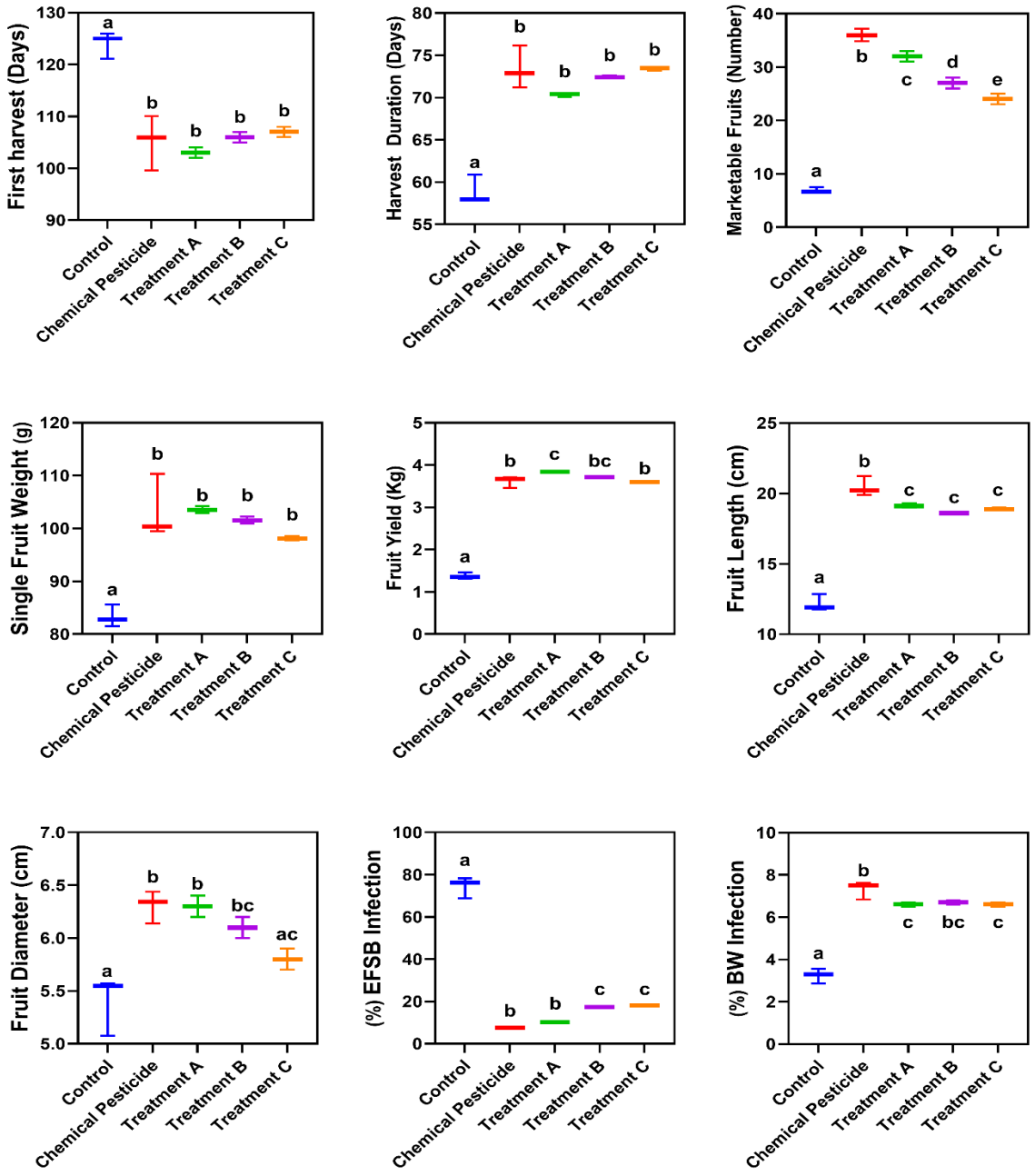


Figure 6.3.2: The yield and yield-contributing characters of brinjal in summer as influenced by *Bt* formulation. (**A**) days to first harvest (**B**) harvest duration. (**C**) number of marketable fruits (**D**) weight of a single fruit (**E**) Fruit Yield (**F**) Fruit Length (**G**) Fruit Diameter (**H**) Eggplant fruit and shoot borer (EFSB) infection (I) Bacterial wilt (BW) infection. Here, "control" = untreated, i.e., no *Bt* application; Treatment A = 100 ml *Bt* formulation applied four times; Treatment B = 100 ml *Bt* formulation applied three times; Treatment C = 200 ml *Bt* formulation applied twice. Bars sharing similar letter do not differ at 5% significance level.

The highest level of EFSB infestation was observed in the control (73.3%), while the lowest was recorded in the 100 ml four-time spray treatment (10.0%). Moderate reductions in infestation were seen in the 100 ml three-time spray (17.4%) and 200 ml two-time spray (18.3%) treatments. However, there was no significant difference among treatments concerning bacterial wilt incidence as shown in Figure 6.3.2.

The effects of control, biopesticide, and chemical pesticide treatments on various plant and fruit parameters were evaluated using one-way ANOVA followed by Tukey HSD post hoc analysis. The ANOVA results indicated significant differences across all measured parameters ($p < 0.05$), with F-statistics exceeding the critical value of 5.14325285, confirming that treatment type had a significant influence on the outcomes. Parameters assessed included days to first harvest, harvest duration, number of marketable fruits per plant, single fruit weight, fruit yield per plant, fruit length, fruit diameter, eggplant fruit and shoot borer (EFSB) infestation, and bacterial wilt (BW) infection. Tukey HSD post hoc tests further elucidated pairwise differences, revealing that both control vs. biopesticide and control vs. chemical pesticide comparisons were statistically significant ($p<0.05$) for most parameters, except for harvest duration in the control vs. biopesticide comparison ($p = 0.05286$), which approached but did not reach significance. Comparisons between biopesticide and chemical pesticide treatments showed no significant differences ($p > 0.05$) for days to first harvest, single fruit weight, fruit diameter, EFSB infestation, and BW infection, suggesting comparable efficacy between these treatments for these parameters. However, significant differences were observed for the number of marketable fruits per plant and fruit yield per plant, indicating distinct treatment effects on these yield-related metrics. ANOVA and Tukey HSD analysis results are presented in Table 6.3.2 and Table 6.3.3 below.

6.3.2 Analysis of Variance

Table 6.3.2.1: Analysis of variance of brinjal in summer influenced by *Bt* formulation

Parameters	ANOVA Results		
	F statistic	**P-value**	**F crit**
Days to 1st harvest	17.71168755	0.003038906281	5.14325285
Harvest duration	17.5501	0.003111	5.14325285
Number of marketable fruits/plants	673.3487	8.73E-08	5.14325285
Single fruit weight (gm)	16.5235	0.003628	5.14325285
Fruit yield/plant (kg)	591.7843	1.28E-07	5.14325285
Fruit length (cm)	79.5431	0.00004801	5.14325285
Fruit diameter (cm)	19.4992	0.002371	5.14325285
EFSB infestation (%)	527.2797	1.81E-07	5.14325285
BW infection (%)	128.2878	0.00001193	5.14325285

6.3.3 Tukey HSD analysis

Table 6.3.3.1: Tukey HSD Analysis of brinjal in summer influenced by *Bt* formulation

Parameters	Tukey HSD results		
	Control vs Biopesticide	**Control vs Chemical pesticide**	**Chemical Pesticide vs Biopesticide**
Days to 1st harvest	0.004818	0.00532	0.9942
Harvest duration	0.05286	0.002496	0.06153
Number of marketable fruits/plants	4.81E-07	1.71E-07	0.002978
Single fruit weight (gm)	0.004566	0.008468	0.8124
Fruit yield/plant (kg)	2.87E-07	5.52E-07	0.02039
Fruit length (cm)	0.0001805	0.00005291	0.1328
Fruit diameter (cm)	0.003158	0.00525	0.8533
EFSB infestation (%)	5.67E-07	4.70E-07	0.5269
BW infection (%)	0.00003952	0.00001371	0.1123

6.3.4 Recommendations for *Bt* biopesticide application

To make the most out of *Bt*-based pest control, certain practical steps should be followed. These simple yet important practices help to improve the effectiveness of *Bt* treatments, leading to healthier crops, better yields, and reduced dependence on chemical pesticides while supporting safe and sustainable farming.

1. **Apply at the right pest stage:** *Bt* works best on pests when they are still young. Spraying should be done when insect larvae are in their early stages, as they are more vulnerable to the toxin at this point.

2. **Cover plants properly:** When spraying *Bt,* make sure the entire plant is well-covered, especially the undersides of leaves. Many pests feed on these hidden areas, and proper coverage helps ensure they ingest the *Bt.*

3. **Choose the right time of day:** Apply *Bt* during cooler hours—early morning or late afternoon—so the product is not quickly broken down by sunlight. Avoid spraying under intense sunlight, as ultraviolet rays can reduce *Bt*'s effectiveness.

4. **Store and mix carefully:** To keep *Bt* products effective, store them in a dry and cool place, away from direct sunlight. Always follow the label instructions for mixing, handling, and applying the product to maintain its quality and performance.

5. **Use alongside other methods:** *Bt* should be part of a broader pest control plan. Combining *Bt* with other techniques—like changing crop types each season, planting pest-tolerant varieties, or using natural predators—can make pest control more successful and long-lasting.

6. **Precautions:** Wear gloves, mask and protective clothing; avoid spraying during heavy rain and high wind.

CHAPTER 7

DISCUSSION AND CONCLUSION

7.1 Discussion

Overusing artificial chemical insecticides in both agricultural and forest communities has not only triggered logging, soil degradation, and the deaths of non-target species, but it has also caused the increase of insect populations that are resistant to pesticides, making them less effective in the long run (Azmi *et al.*, 2015). As a result, there is a rising global concentrate on producing integrated pest management (IPM) solutions that are both great for the environment and affordable. Microbial biopesticides, especially those made from *Bacillus thuringiensis* (*Bt*), have gotten a lot of attention due to the fact that they are extremely particular, safe, and work well against a large range of insect bugs. When *Bt* spores form, they make parasporal crystalline additions that consist of a range of insecticidal proteins, the most important of which being δ-endotoxins such Cry and Cyt proteins (Soberón *et al.*, 2016). When these contaminants are ingested by insects that are sensitive to them, they cause holes to grow in the intestinal epithelium, which kills the larvae (Crickmore, 2006; V. K. Frankenhuyzen, 2009; Palma *et al.*, 2014; Schünemann *et al.*, 2014).

Bt is the most popular microbial biocontrol agent since it is particular and harmless (Jurat-Fuentes & Crickmore, 2017). Some insect species have become resistant to it after duplicated usage, which makes it less effective in the long run (Fiuza *et al.*, 2017; Zago *et al.*, 2014). Scientists are looking into new *Bt* strains with more powerful virulence and unique toxic substance profiles, particularly from habitats that haven't been studied much yet, like forest soils and compost (Reyaz *et al.*, 2017). The soils of Bangladesh, which have a great deal of different agroecological zones and extensive cropping systems, are an appealing place to find native *Bt* strains that may have new and efficient insecticidal residential or commercial properties. In the past, the Enzyme and Fermentation Biotechnology Laboratory has discovered and isolated prospective *Bt* pressures that work versus insects that impact vegetables and fruits (Shishir *et al.*, 2015). The goal of this study was to take a look at native potential *Bt* strains that were gathered from various types of soil in Bangladesh. The focus was on how well they worked against the chosen 4 essential Dipteran pests for the economy: *Bactrocera dorsalis* (the oriental fruit fly) and *B. Zonata* (peach fruit fly), *Zeugodacus cucurbitae* (melon fly), and *Z. tau* (the pumpkin fruit fly)

7.1.1 Efficacy and potential of indigenous *Bacillus thuringiensis* as biocontrol agents against Tephritid fruit flies

Bactrocera is the biggest and most economically harmful genus in the Dacini tribe, posing serious threats to global horticulture (Clarke *et al.*, 2005; Drew, 2004; Fletcher, 1987; White & Elson-Harris, 1992). These frugivorous pests that damaged fruits and vegetables are found all throughout the world, but South-east Asia and Australia are known to have a lot of different kinds of them (Drew, 2004). At least 68 species pose a direct threat to agriculture since they can affect many different types of plants and can infest many fruit and vegetable crops, causing harm to the fruit's insides, lower yields, and severe quarantine rules around the world (Drew, 2004). Bangladesh's subtropical climate provides for a wide range of fruits and vegetables, but it also attracts a lot of fruit flies, including *B. dorsalis*, *B. Zonata*, *Z. cucurbitae* and *Z. tau*, which together cause huge losses in fruit and vegetable yields (30–100%). This study evaluated at how well 44 native *Bacillus thuringiensis* (*Bt*) strains worked against four economically important Tephritid fruit fly species: *Bactrocera dorsalis*, *B. Zonata*, *Zeugodacus cucurbitae*, and *Z. tau*. These pests cause substantial damage to fruits and vegetables in tropical and subtropical areas, leading to significant economic losses.

Of the 44 *Bt* strains tested, 16 showed that more than 50% of the larvae died for all tested four species of fruit flies. Notably, 11 strains killed more than 70% of *B. dorsalis* and *B. zonata* early 3rd instar larvae, although nine strains worked just as well against *Z. cucurbitae* and *Z. tau*. The most efficient strains, *Bt* JSd1, SaS6, and JDc1, always caused death rates of above 80%. *Bt* JSd1 was the most effective, killing 97% of *B. dorsalis* and 95% of *B. zonata* larvae. These results show how useful indigenous *Bt* strains could be focus for controlling pests in a specific way. The study found LC_{50} and LC_{99} values to measure how hazardous *Bt* strains are at different concentrations. *Bt* JSd1 had the lowest LC_{50} values (0.433 mg/ml for *B. dorsalis* and 0.470 mg/ml for *B. zonata*), which means it was quite strong. In the same way, *Bt* JSd1 showed the lowest LC_{50} values for *Z. cucurbitae* and *Z. tau*, which were 0.431 mg/ml and 0.472 mg/ml, respectively. The LC_{99} values showed a similar pattern, with *Bt* JSd1 needing the lowest doses to kill 99% of the larvae. These results suggest that *Bt* JSd1 is the most efficient strain for field applications.

The lethal time (LT) analysis revealed that *Bt* JSd1 also worked the fastest, with LT_{50} values of about 54 hours for all four tested species. In contrast, strains like MuSc2 and Ksa2 took longer to work and needed much longer exposure times. *Bt* JSd1's insecticidal activity starts up

quickly, which makes it a promising candidate for practical pest control, where quick action is important.

The reference strains, *Bts* T84A1 and *Btk* HD-73, showed high efficacy, with death rates similar to those of the best native strains. *Bt* JSd1, on the other hand, has higher LC and LT values than these references, which shows that it is more virulent. This means that *Bt* strains that are only found in certain areas may be better at controlling pests in those areas. This could be a long-term solution for pest control in Bangladesh and other areas with comparable climates and farming conditions. Using chemical pesticides without care has made the environment polluted, made pests resistant, and put people's health at risk. Biopesticides based on *Bt* are a good option because they are target-specific and safe for the environment. Strains like JSd1 and SaS6 work very well against several species of fruit flies, which makes them ideal candidates for IPM methods. These strains could ensure food safety, minimize use of synthetic pesticides, and slow down the development of resistance.

(Aarthi *et al.*, 2024) put fifty *Bt* strains (a combination of spore and crystal proteins) against second instar larvae of *Z. cucurbitae* and discovered that the death rate was between 16 to 92%, while the reference *Bti* showed 95% death. Ten of those strains had a mortality rate of more than 50%, with LC_{50} values ranging from 38.48 to 105.18 (μg/ml) at 120 hours (five days) after treatment. This outcome is in line with what this study found. In this study, though, the lethal concentration was around five times higher, and the lethal time was almost half of what was found in that study. There isn't much information in the literature on how well *Bt* strains work against the other tested three fruit fly species, thus this study adds new information on the efficacy of *Bt* strains against insect pest management.

7.1.2 Impacts of indigenous *Bacillus thuringiensis* on the biological quality parameters of Tephritid fruit flies

Bactrocera dorsalis, *B. Zonata*, *Zeugodacus cucurbitae*, and *Z. tau* are serious pests of crops that cause a lot of damage to fruits and vegetables around the world, causing significant economic losses (Aluja & Mangan, 2008; White & Elson-Harris, 1992). Even though they have an effect on the economy, pest control measures are not very effective since we don't know enough about their biology and ecology. More research is needed on their behavior and population dynamics. Biological control strategies, like the Sterile Insect Technique (SIT), depend on measuring important quality factors including pupal yield, adult emergence, flight capability, and mating competitiveness to make sure that wild populations are successfully kept

in check (Cáceres *et al.*, 2019). Testing *Bacillus thuringiensis* (*Bt*) biopesticides against these pests is a long-term ideal solution. This study looked at how well three native *Bacillus thuringiensis* (*Bt*) strains, JSd1, SaS6, and JDc1, worked against four commercially important Tephritid fruit fly species: *Bactrocera dorsalis, B. Zonata, Zeugodacus cucurbitae,* and *Z. tau*.

All three *Bt* strains caused a big drop in the number of pupae, adults, and flies that could fly, while also making the developmental periods longer and causing flies to be deformed (for example, half-emerged and un-emerged flies). *Bt* JSd1 was always the most poisonous, as shown by the fact that it caused the lowest pupal weight, wing length, and adult emergence rates in all studied fruit fly species on infested host fruits and vegetables. For instance, in *B. dorsalis* infested banana, *Bt* JSd1-treated samples recorded only 107 pupae compared to 275 in the control. The number of adults that came out was also lower, at 46.73% compared to 97.09% in the control group. Similar trends were observed for *B. zonata, Z. cucurbitae*, and *Z. tau*, which shows how these *Bt* strains can work on a wide range of things.

The *Bt* treatments made the larvae and pupae stay in their larval and pupal stages longer, which means their development was slowed down. For example, the larvae of *B. dorsalis* took 11 days to grow on *Bt* JSd1-treated banana, while they took 8 days to grow on the control. Also, the weight of the pupae and the length of their wings, which are important signs of how fit an adult is, were both greatly reduced. These consequences probably happen because of *Bt* toxins mess up the regular processes of nutrition absorption and metabolism, which slows down normal growth and development.

Flight ability, which is very important for sterile insect technique (SIT) programs, was badly hurt. *B. dorsalis* treated with *Bt* JSd1 only had 33.03% fliers, whereas the control group had 61.21%. Sterile males released in SIT programs are less likely to mate since they can't fly as well, which makes population control more effective. Also, skewed sex ratios and lower fecundity make it much harder for pests to reproduce, which helps with long-term pest control.

The effectiveness of *Bt* strains depended on the hosts. For instance, *B. dorsalis* on mango (BARI 4) was more likely susceptible than on banana. *Bt* JSd1 lowered the number of adults that came out to 43.57% in mango (BARI4) and 46.73% in banana. These kinds of variances could be caused by differences in the nutrients in the host or by the fruit fly adapting capacity. This shows how important it is to have host-specific *Bt* application tactics to get the best results.

There isn't a great deal of research on how *Bt* affects fruit flies in a way that doesn't kill them, however there are some studies on other moths. (Erb *et al.*, 2001) showed that *Bt* is proficient at killing moth larvae, however it doesn't have much of an impact on the growth of the larvae and pupae that make it through. This research study reveals that *Bt* can alter how larvae and pupae that survive and grow. According to (Babin *et al.*, 2020), giving non-target Drosophila flies approximately a thousand times the advised *Bt* dose has no considerable effect on them. This implies that *Bt* pressures can be utilized against harmful fruit flies without hurting the environment.

The results showed that these *Bt* stress had a big impact on the biological quality criteria of the fruit flies. *Bt* JSd1 had the most appealing effect, followed by SaS6 and JDc1. The outcomes show that *Bt* biopesticides might be a safe method to manage these harmful fruit flies pests and support making use of *Bt* biopesticides in incorporated integrated pest management (IPM) programs eco-friendly.

7.1.3 Genomic insights into the insecticidal potential and adaptive traits of *Bacillus thuringiensis* JSd1

Whole-genome sequencing (WGS) has actually greatly improved our understanding of the hereditary structure of germs's ability to trigger illness, adapt to brand-new environments, and have biotechnological potential (Gangmei *et al.*, 2025; Rabha *et al.*, 2023). We do a complete genome analysis of *Bacillus thuringiensis* strains JSd1, which was discovered in Bangladesh, in this research study. The *Bt* JSd1 genome was sequenced 75 times, which led to a top-quality assembly with a GC content of 35.28%. The genome assembly and annotation revealed that the pressure had a great deal of coding possible (98.68% of its genes code for proteins). It also revealed that the pressure has a great deal of insecticidal genes, secondary metabolite clusters, mobile hereditary elements, and adaptive functions. All of these characteristics suggest that *Bt* JSd1 might be beneficial in long-term pest control strategies.

The *Bt* JSd1 genome has 5,833 genes, 5,756 of which code for proteins. The functional profile indicates that a large part of the genome is involved in basic metabolic and biosynthetic processes, such as making cofactors, breaking down amino acids, responding to stress, and making energy. The fact that *Bt* JSd1 has many genes associated to stress response and defense that it is highly adapted to changing and possibly hostile environments, which is a quality of *B. thuringiensis* strains come from a wide range of ecological origins (Ali *et al.*, 2022).

One interesting thing is that there are a lot of hypothetical proteins (around 24.43%), which means that the genetic potential is not well understood. This is something that happens a lot in the genomes of *Bacillus cereus* group (Juhas *et al.,* 2009). It shows both horizontal gene acquisition and the changing evolution of secondary metabolic features. The fact that 4,212 genes were put into COG categories adds to the evidence that this genome is functionally complicated, especially when it comes to making proteins, transcribing DNA, digesting DNA, and pathways connected to virulence.

Bt JSd1 has 25 genomic islands, and many of them are full of putative proteins and mobile elements like transposases and insertion sequences. The occurrence of these islands fits with what has been found in other entomopathogenic *B. thuringiensis* species that horizontal gene transfer events shape the genome. Strains (Guerrero *et al.,* 2024). Genomic islands are key places in evolution where genes that are linked to virulence, adapting to the environment, or specializing in a niche can be found (Polz *et al.,* 2013). Finding several transposase-related genes on these islands shows that *Bt* JSd1 may have a greater ability to change its genome, which would make it easier for it to quickly gain adaptive features, *B. thuringiensis* is flexible. The success of genomes as worldwide spread entomopathogens and their amazing capacity to adapt to new habitats (Ceuppens *et al.,* 2013).

One of the main goals of this work was to look at the insecticidal gene repertoire of *Bt* JSd1, as it was taken from soil in Bangladesh where there were a lot of pests. There were several types of virulence factors found, some of which were encoded by chromosomes and others by plasmids.

On plasmid pBTJSd1A, there was a homolog of Cry22A with a low identity. Cry22A is understood to work versus Dipteran larvae, and it might be a brand-new range in this isolate that needs more research study into its function (Zhou *et al.,* 2024). It is especially interesting that there is a possible chromosomal Vip3A gene. Vip3A contaminants deal with Cry proteins to kill Lepidopteran types that are resistant to δ-endotoxins (Khan *et al.,* 2020). Their chromosomal positioning suggests that they may be stable and expressed all the time, that makes *Bt* JSd1 a perfect option for developing recombinant biopesticides.

The discovery of several Bmp1-like metalloproteases, such as Enhancin, Sphaericolysin, and CytK, reinforces the pressure's ability to kill insects. These proteins work together to break down the defences of the host, make toxins more effective, and damage the midgut (Gonzalez *et al.,* 2005). Likewise, discovering chiA chitinase shows a method to break down the exoskeletons of pests, that makes toxins work better (Liu *et al.,* 2002).

These outcomes suggest that *Bt* JSd1 has a large range of insecticidal parts, some of which are various from the normal series. Truth of that chromosomal and plasmid-encoded virulence factors are found together shows that they progressed in modules, which fits with the popular mosaic structure of all studied *B. thuringiensis* genomes (Guerrero *et al.*, 2024; Pacheco *et al.*, 2021; Rabha *et al.*, 2023).

AntiSMASH research study discovered eight biosynthetic gene clusters on the chromosome and one on the plasmid. These clusters code for things like RiPPs, non-ribosomal peptides, siderophores, and terpenes. Some of these, like petrobactin and bacillibactin, are known to help plants and microbes interact with metals and fight against bacteria (Yin *et al.*, 2023). Finding 100% preserved thuricin CD sactipeptide genes on plasmid pBTJSd1A is quite interesting. Thuricins are very good at killing Gram-positive bacteria and may help plants survive in competitive rhizosphere conditions (Rea *et al.*, 2010). Such secondary metabolites can enhance the utility of *B. thuringiensis* strains in integrated pest management (IPM) frameworks by suppressing phytopathogens.

The mosaicism seen the distribution of virulence factors, especially for the phospholipase and collagenase gene families shows the dynamic nature of *B. thuringiensis* pangenome. The accessory genome definitely plays a role in functional variation at the strain level, as shown in earlier studies (Bazinet, 2017).

Phylogenomic analysis puts *Bt* JSd1 in a strong clade with *B. cereus* sensu lato. The cereus sensu lato group is in a strong clade with *B. thuringiensis* serovar konkukian and *B. anthracis*. This fits with what we already know about the *B. cereus* group phylogenetic structure, where differences in species are generally based more on plasmid content and ecological features than on differences in the core genome (Ehling-Schulz *et al.*, 2019). *Bt* JSd1 is very closely related to other pathogenic *B. thuringiensis* strains add to the evidence that it could be useful for insect pest management.

The investigation of the pangenome over 12 *B. thuringiensis* genomes show that there are a lot of extra genes, about 45.1% of which are cloud genes. The core genome is just about 12.6% of the whole genome, which shows how flexible the *B. cereus* group genome is (Alcaraz *et al.*, 2010). The wide range of major virulence factors like chitinases and bacillolysins suggests that they are very important for the lifestyle of entomopathogenic organisms. On the other hand, the strain-specific distribution of phospholipases, internalins, and metalloproteases may help strains become more specialised in their environments or have different host ranges.

Bt JSd1 has a lot of genes that are associated to toxins, which makes it a promising candidate for becoming a next-generation biopesticide. This is especially true because pests are becoming more resistant to the *B. thuringiensis* formulations that is currently being used (Jurat-Fuentes *et al.*, 2021).

7.1.4 Efficacy of *Bt* biopesticide in enhancing brinjal yield and controlling eggplant fruit and shoot borer (EFSB) infestation

The study also looked at how well alternative ways of applying the *Bt* (*Bacillus thuringiensis*) biopesticide worked to control pests and boost yield in brinjal (eggplant). We employed a Randomised Complete Block Design (RCBD) to test the *Bt* preparation on brinjal. The RCBD method grouped diverse treatments, such as *Bt* biopesticide, chemical pesticide, and a negative control, into blocks and repeated them. This means that plots were put into blocks, and treatments were randomly given to each block. This approach works best when you know about variances in the field ahead of time, like how the soil type, drainage, fertility gradients, or insect movement patterns are different. Even when it's not evident what causes differences, you may still use RCBD by making sure that blocks are as square as possible. This helps keep each block the same. The main idea behind RCBD is to make the disparities between blocks as big as possible while making the differences inside each block as little as possible to improve the accuracy of the experiment.

The results showed that there were big disparities between the plants that were treated and the ones that weren't. This shows that *Bt* could be a long-term substitute for chemical pesticides in integrated pest management (IPM). The most interesting thing we found was that *Bt*-treated plants had a lot less Eggplant Fruit and Shoot Borers (EFSB) than the control plants. The untreated plants had a 73.3% infestation rate, whereas the 100 ml four-time *Bt* spray treatment only had a 10.0% infestation rate, which is about the same as the effectiveness of chemical pesticides (no significant difference). This backs up *Bt*'s ability to kill EFSB larvae, which stops them from feeding and keeps crops from being badly damaged. The moderate levels of infection in the three-time (17.4%) and two-time (18.3%) spray treatments show that how often the treatment is applied is very important for keeping pests under control. However, the incidence of bacterial wilt (BW) was still less impacted by *Bt* than by chemical pesticides (p-value > 0.05). This shows that *Bt* is more effective against lepidopteran pests and that further disease control measures are required.

In most situations, using *Bt* has improved the results of the harvest and is about the same as using chemical pesticides, with no big difference. Chemical pesticides, on the other hand, produce more fruit and longer fruit, while the biopesticide group T3 (Treatment A) produces more fruit. This shows how *Bt* helps crops grow better, probably because it lowers the stress that pests cause. The 200 ml two-time spray treatment had a longer harvest period (73.4 days), but it produced less fruits than the four-time spray. This suggests that spraying more often may be more effective than spraying more often with a greater concentration. This could be because *Bt* was always present on the leaves, which kept the larvae exposed for a longer time. The results are identical to those of (Mollah, 2022), who did a similar investigation and discovered that *Bt* spray in Brinjal is just as effective at fighting EFSB infection as chemical pesticides.

In general, these results indicate that using *Bt*, especially the 100 ml four-time spray, is very promising at getting rid of EFSB and improving the yield better. Effectiveness of *Bt* is likely successful since it only kills EFSB larvae, which protects plants and promotes better growth and fruit development.

7.2 Conclusion

The extensive research on indigenous *Bacillus thuringiensis* (*Bt*) strains from Bangladesh demonstrates their significant potential as sustainable biopesticides for integrated pest management (IPM) in agricultural fields and farms. The key findings and their implications are summarized as follows:

1. **High efficacy of indigenous *Bt* strains against Tephritids fruit flies**

- Among 44 tested *Bt* strains, JSd1, SaS6, and JDc1 exhibited the highest insecticidal activity, inducing over 80% mortality in key pests such as *Bactrocera dorsalis*, *B. zonata*, *Zeugodacus cucurbitae*, and *Z. tau*.
- *Bt* JSd1 emerged as the most potent strain, with 97% mortality against *B. dorsalis* and the lowest LC_{50} and LT_{50} values, indicating rapid and concentration-dependent toxicity.
- These strains outperformed reference *Bt* strains (e.g., *Btk* HD-73, *Bts* T84A1), suggesting that locally adapted isolates may be more effective against regional pests.

2. **Disruption of biological quality parameters in fruit flies**

- *Bt* treatments significantly reduced pupal yield, adult emergence, and flight ability, while increasing developmental deformities and prolonging larval-pupal duration.

- *Bt* JSd1-treated flies showed only 33.03% flight ability (vs. 61.21% in controls), impairing their mating competitiveness, critical for Sterile Insect Technique (SIT) integration.

- Host variations influenced efficacy, highlighting the need for host-specific *Bt* application strategies.

3. Genomic insights into *Bt* JSd1's entomopathogenic potential

- Whole-genome sequencing revealed *Bt* JSd1's diverse toxin arsenal, including Cry22A homologs, Vip3A, and accessory virulence factors (e.g., chitinases, metalloproteases).

- The presence of 25 genomic islands suggests active horizontal gene transfer, enhancing adaptability and toxin diversification.

- Secondary metabolite clusters (e.g., thuricin CD) indicate additional plant-growth-promoting and antimicrobial properties, broadening *Bt* JSd1's biotechnological applications.

4. Field application

- Field trials on brinjal confirmed *Bt*'s efficacy, with four-time sprays (100 mL) reducing EFSB infestation to 10% and tripling yield (3.85 kg/plant vs. 1.45 kg/control), comparable to chemical pesticides but without environmental harm.

The native *Bacillus thuringiensis* (*Bt*) strains characterized in this study exhibit promising biocontrol potential, yet require further investigation to elucidate comprehensively their insecticidal properties and practical applications. Future research should systematically evaluate their toxicity spectra against a broader range of economically important pests across different taxonomic orders, while also assessing potential synergistic effects with other biological control agents.

CHAPTER 8

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications:

1. Bari, **M. A.,** Shishir, M. A., Khan, S. A., Khan, S. N., & Hoq, M. M. (2021). Bio-efficacy of Indigenous *Bacillus thuringiensis* JSd1 against Melon Fly, *Zeugodacus cucurbitae* (Coq.) (Diptera:Tephritidae:Dacinae). *International Journal of Entomological Res., 6*(2): 127-134. www.entomologyjournals.com

2. Bari, **M. A.,** Khan, S. N., & Hoq, M. M. (2025). Insecticidal Potential of Indigenous *Bacillus thuringiensis strains* against Pumpkin Fruit Fly. *Bangladesh Journal of Entomology, 33*(1):53-74. http://doi.org/10.63759/bje.33.1.5

Submitted:

1. **Bari, M. A.,** Pal, D. C., Khan, M. A. M., Khan, S. N., Momen, M., Khan, M., Karim, M. M., & Hoq, M. M. (2025)**.** Genomic Insights into *Bacillus thuringiensis* Strain JSd1 from Bangladesh : A Whole Genome Sequencing Approach. (Submitted)

Submission process is On-going:

2. **Bari, M. A., *et al.*** (2025)**.** Biocontrol Potential of Indigenous *Bacillus thuringiensis* JSd1 against Oriental fruit fly, *Bactrocera dorsalis* (Hendel) and Peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera:Tephritidae:Dacinae)

3. **Bari, M. A., *et al.*** (2025)**.** Efficacy of *Bacillus thuringiensis* JSd1 Biopesticides in brinjal yield and controlling of eggplant fruit and shoot borer (EFSB) infestation.

Conference presentations:

1. Bari, **M. A.,** Seheli, K., Khan, M., Khan, S. N., & Hoq, M. M. Insecticidal Activity of Indigenous *Bacillus thuringiensis* strains against Oriental Fly, *Bactrocera Dorsalis* (Diptera:

Tephritidae). 22nd National Conference and AGM 2020, TSC Auditorium, University of Dhaka, Dhaka. 29th October, 2021**. (Oral Presentation)**

2. Bari, **M. A.,** Islam, L., Alim, M. A., Seheli, K., Khan, S. N., & Hoq, M. M. Toxicity of Indigenous *Bacillus thuringiensis* Strains Against the Oriental Fly, *Bactrocera Dorsalis* (Diptera: Tephritidae). 21st International Biennial Conference and AGM of Zoological Society of Bangladesh (**ZSB),** Nabab Nawab Ali Chowdhury Senate Bhaban, University of Dhaka, Dhaka. December 07-08, 2019**. (Poster Presentation)**

3. Bari, **M. A.,** Bhowmic, P., Alim, M. A., Khan, S. N., Khan, M., & Hoq, M. M. Efficacy of Indigenous *Bacillus thuringiensis* Strains Against the Melon Fly, *Zeugodacus Cucurbitae* (Diptera: Tephritidae). 21st International Biennial Conference and AGM of Zoological Society of Bangladesh (**ZSB),** Nabab Nawab Ali Chowdhury Senate Bhaban, University of Dhaka, Dhaka. 07-08 December, 2019**. (Oral Presentation)**

4. Bari, **M. A.,** Shishir, M. A., Khan, S. A., Khan, S. N., & Hoq**,** M. M. Bio-efficacy of indigenous *Bacillus thuringiensis* strains against Melon fly, *Zeugodacus cucurbitae* (Diptera: Tephritidae) affecting fruits and vegetables. 32nd Annual Conference & General Meeting of Bangladesh Society of Microbiologists (**BSM**), JUST, Jashore. 6th April, 2019. (**Oral Presentation**)

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PhD Seminar:

1st Seminar: Md. Abdul Bari, PhD Candidate. Bioefficacy of indigenous *Bacillus thuringiensis* strains against Tephritids fruit fly pests affecting fruits and vegetables. 1st Seminar**,** Anwarul Azim Chowdhury Lecture Gallery, Dept. of Microbiology, University of Dhaka, Dhaka. 27 July, 2022**.**

2nd Seminar: Md. Abdul Bari, PhD Candidate. Evaluation of *Bacillus thuringiensis* JSd1 as biopesticide against Tephritids fruit fly pests affecting fruits and vegetables. 2nd Seminar, Anwarul Azim Chowdhury Lecture Gallery, Dept. of Microbiology, University of Dhaka, Dhaka. 22 August, 2023**.**

APPENDICES

Appendix A

Tukey HSD

Strains	*B. dorsalis*						*B. zonata*					
	A vs B	A vs C	A vs D	B vs C	B vs D	C vs D	A vs B	A vs C	A vs D	B vs C	B vs D	C vs D
DSf4	0.1748	1.14E-04	1.47E-07	0.000997	4.44E-07	0.00001347	0.08476	0.0000255	4.24E-08	0.0002458	1.28E-07	0.00000646
FhSb3	0.1932	2.89E-04	3.59E-07	0.002983	1.07E-06	0.00002333	0.07806	0.0001137	4.58E-08	0.00187	1.47E-07	0.00000249
JDc1	0.004301	1.50E-05	1.56E-08	0.001025	5.12E-08	9.05E-07	0.01575	0.00004543	2.18E-08	0.00187	6.96E-08	0.00000103
JSd1	0.009491	1.50E-05	1.26E-08	5.36E-04	2.92E-08	4.87E-07	0.01575	4.54E-05	1.76E-08	1.87E-03	4.58E-08	5.89E-07
KSa2	0.001454	1.13E-06	1.59E-09	3.96E-05	5.39E-09	3.48E-08	0.08476	7.36E-05	2.11E-08	9.83E-04	4.24E-08	6.99E-07
MuSc2	0.06651	5.51E-06	2.45E-09	3.96E-05	4.46E-09	2.70E-08	0.2252	1.32E-04	2.11E-08	9.83E-04	3.44E-08	4.95E-07
NaSc3	0.01207	2.55E-05	1.45E-08	9.83E-04	3.44E-08	4.95E-07	0.004917	2.55E-05	1.63E-08	2.13E-03	5.38E-08	6.99E-07
RaSa2	0.004917	2.55E-05	1.63E-08	2.13E-03	5.38E-08	6.99E-07	0.004917	2.55E-05	1.63E-08	2.13E-03	5.38E-08	6.99E-07
SaS6	0.001996	5.31E-06	3.73E-09	3.67E-04	1.05E-08	4.95E-08	0.03297	1.30E-04	5.63E-08	4.63E-03	2.60E-07	0.00000314
SaS7	0.06651	8.53E-04	6.89E-07	3.30E-02	3.15E-06	0.0000275	2.20E-02	1.65E-04	8.31E-08	9.49E-03	4.87E-07	0.00000468
TaSa4	0.008642	2.01E-04	1.62E-07	3.30E-02	1.45E-06	0.00001008	5.84E-03	8.01E-05	1.10E-07	1.16E-02	0.0000011	0.00001174
Ref. *Bt*s T84A1	0.01207	6.46E-06	7.04E-09	1.32E-04	1.45E-08	1.78E-07	0.004917	0.00000646	7.04E-09	0.0002458	1.63E-08	1.78E-07
Strains	***Z. cucurbitae***						***Z. tau***					
	A vs B	A vs C	A vs D	B vs C	B vs D	C vs D	A vs B	A vs C	A vs D	B vs C	B vs D	C vs D
DSf4	0.014915	5.31E-06	1.82E-08	8.73E-05	4.95E-08	3.36E-06	0.3687501	0.0005518 6	3.36E-07	0.0036498	7.82E-07	1.35E-05
FhSb3	0.134416	0.000130	2.05E-07	0.0014542	6.89E-07	1.94E-05	0.1931571	1.10E-03	1.34E-06	1.61E-02	4.34E-06	6.29E-05
JDc1	0.000824	2.18E-06	4.37E-09	1.74E-04	1.38E-08	1.34E-07	0.0157546	1.87E-04	2.81E-08	1.58E-02	1.13E-07	7.82E-07
JSd1	0.004917	6.46E-06	7.98E-09	2.46E-04	1.84E-08	2.49E-07	1.67E-02	5.78E-05	8.32E-08	2.55E-03	5.40E-07	1.01E-05
KSa2	0.000131	5.38E-08	2.93E-11	2.92E-06	3.21E-10	7.04E-09	1.45E-03	3.15E-06	3.01E-09	2.01E-04	9.18E-09	4.73E-08
MuSc2	6.65E-02	5.51E-06	3.01E-09	3.96E-05	5.39E-09	3.48E-08	2.25E-01	7.36E-05	2.43E-08	0.0004793	4.24E-08	9.90E-07
NaSc3	0.000513	1.86E-06	1.27E-09	2.01E-04	5.39E-09	2.17E-08	2.00E-03	1.45E-05	1.05E-08	0.0019961	3.05E-08	2.90E-07
RaSa2	4.92E-03	2.55E-05	1.63E-08	2.13E-03	5.38E-08	6.99E-07	5.53E-02	6.88E-04	8.61E-07	2.96E-02	4.34E-06	4.46E-05
SaS6	2.00E-03	5.31E-06	4.37E-09	3.67E-04	1.20E-08	6.68E-08	2.36E-02	1.98E-04	5.73E-08	1.15E-02	3.00E-07	2.50E-06
SaS7	5.23E-03	2.54E-05	1.82E-08	2.00E-03	6.68E-08	9.58E-07	3.47E-02	5.52E-04	4.44E-07	3.47E-02	2.50E-06	1.98E-05
TaSa4	3.65E-03	7.10E-05	3.82E-08	1.58E-02	3.36E-07	2.50E-06	2.13E-03	2.55E-05	2.43E-08	4.92E-03	1.78E-07	2.01E-06
Ref. *Bt*k HD-73	1.21E-02	9.95E-06	7.98E-09	2.46E-04	1.63E-08	1.78E-07	1.87E-03	1.14E-04	2.46E-08	0.0780561	1.93E-07	7.82E-07

Appendix B

ANOVA analysis of *B. dorsalis* and *B. zonata*

Strains	*B. dorsalis*				*B. zonata*			
	diff	**Lwr**	**upr**	**p adj**	**diff**	**lwr**	**upr**	**p adj**
DSf4-*Bts*_T84A1	-4.666666667	-6.96808479	-2.365248543	8.27E-06	-5.333333333	-7.58183822	-3.084828447	5.43E-07
FhSb3-*Bts*_T84A1	-2.666666667	-4.96808479	-0.365248543	0.013838179	-3	-5.248504886	-0.751495114	0.003096137
FhSb3-DSf4	2	-0.301418123	4.301418123	0.131463954	2.333333333	0.084828447	4.58183822	0.037174497
JDc1-*Bts*_T84A1	-1.666666667	-3.96808479	0.634751457	0.326451872	-1.333333333	-3.58183822	0.915171553	0.60346711
JDc1-DSf4	3	0.698581877	5.301418123	0.004035478	4	1.751495114	6.248504886	6.60E-05
JDc1-FhSb3	1	-1.301418123	3.301418123	0.904938156	1.666666667	-0.58183822	3.915171553	0.296595376
JSd1-*Bts*_T84A1	0.666666667	-1.634751457	2.96808479	0.994546593	0.666666667	-1.58183822	2.915171553	0.993413177
JSd1-DSf4	5.333333333	3.03191521	7.634751457	8.25E-07	6	3.751495114	8.248504886	6.05E-08
JSd1-FhSb3	3.333333333	1.03191521	5.634751457	0.001148541	3.666666667	1.41816178	5.915171553	0.000235611
JSd1-JDc1	2.333333333	0.03191521	4.634751457	4.49E-02	2	-0.248504886	4.248504886	0.113898289
KSa2-*Bts*_T84A1	-3.333333333	-5.634751457	-1.03191521	1.15E-03	-4	-6.248504886	-1.751495114	6.60E-05
KSa2-DSf4	1.333333333	-0.96808479	3.634751457	0.634352676	1.333333333	-0.915171553	3.58183822	0.60346711
KSa2-FhSb3	-0.666666667	-2.96808479	1.634751457	0.994546593	-1	-3.248504886	1.248504886	0.891524245
KSa2-JDc1	-1.666666667	-3.96808479	0.634751457	0.326451872	-2.666666667	-4.915171553	-0.41816178	0.011007586
KSa2-JSd1	-4	-6.301418123	-1.698581877	9.36E-05	-4.666666667	-6.915171553	-2.41816178	5.61E-06
MuSc2-*Bts*_T84A1	-4	-6.301418123	-1.698581877	9.36E-05	-4.333333333	-6.58183822	-2.084828447	1.90E-05
MuSc2-DSf4	0.666666667	-1.634751457	2.96808479	0.994546593	1	-1.248504886	3.248504886	0.891524245
MuSc2-FhSb3	-1.333333333	-3.634751457	0.96808479	0.634352676	-1.333333333	-3.58183822	0.915171553	0.60346711
MuSc2-JDc1	-2.333333333	-4.634751457	-0.03191521	4.49E-02	-3	-5.248504886	-0.751495114	3.10E-03
MuSc2-JSd1	-4.666666667	-6.96808479	-2.365248543	8.27E-06	-5	-7.248504886	-2.751495114	1.71E-06
MuSc2-KSa2	-0.666666667	-2.96808479	1.634751457	0.994546593	-0.333333333	-2.58183822	1.915171553	0.99998952
NaSc3-*Bts*_T84A1	-3	-5.301418123	-0.698581877	0.004035478	-3.666666667	-5.915171553	-1.41816178	0.000235611
NaSc3-DSf4	1.666666667	-0.634751457	3.96808479	0.326451872	1.666666667	-0.58183822	3.915171553	0.296595376
NaSc3-FhSb3	-0.333333333	-2.634751457	1.96808479	0.999991728	-0.666666667	-2.915171553	1.58183822	0.993413177
NaSc3-JDc1	-1.333333333	-3.634751457	0.96808479	0.634352676	-2.333333333	-4.58183822	-0.084828447	0.037174497
NaSc3-JSd1	-3.666666667	-5.96808479	-1.365248543	0.00032596	-4.333333333	-6.58183822	-2.084828447	1.90E-05
NaSc3-KSa2	0.333333333	-1.96808479	2.634751457	0.999991728	0.333333333	-1.915171553	2.58183822	0.99998952
NaSc3-MuSc2	1	-1.301418123	3.301418123	0.904938156	0.666666667	-1.58183822	2.915171553	0.993413177
RaSa2-*Bts*_T84A1	-3.333333333	-5.634751457	-1.03191521	0.001148541	-3.333333333	-5.58183822	-1.084828447	0.000853619
RaSa2-DSf4	1.333333333	-0.96808479	3.634751457	0.634352676	2	-0.248504886	4.248504886	0.113898289
RaSa2-FhSb3	-0.666666667	-2.96808479	1.634751457	0.994546593	-0.333333333	-2.58183822	1.915171553	0.99998952
RaSa2-JDc1	-1.666666667	-3.96808479	0.634751457	0.326451872	-2	-4.248504886	0.248504886	0.113898289
RaSa2-JSd1	-4	-6.301418123	-1.698581877	9.36E-05	-4	-6.248504886	-1.751495114	6.60E-05
RaSa2-KSa2	0	-2.301418123	2.301418123	1	0.666666667	-1.58183822	2.915171553	0.993413177
RaSa2-MuSc2	0.666666667	-1.634751457	2.96808479	0.994546593	1	-1.248504886	3.248504886	0.891524245
RaSa2-NaSc3	-0.333333333	-2.634751457	1.96808479	0.999991728	0.333333333	-1.915171553	2.58183822	0.99998952
SaS6-*Bts*_T84A1	-1	-3.301418123	1.301418123	9.05E-01	-0.666666667	-2.915171553	1.58183822	9.93E-01
SaS6-DSf4	3.666666667	1.365248543	5.96808479	0.00032596	4.67E+00	2.41816178	6.915171553	5.61E-06
SaS6-FhSb3	1.666666667	-0.634751457	3.96808479	3.26E-01	2.333333333	0.084828447	4.58183822	3.72E-02
SaS6-JDc1	0.666666667	-1.634751457	2.96808479	0.994546593	0.666666667	-1.58183822	2.915171553	0.993413177
SaS6-JSd1	-1.666666667	-3.96808479	0.634751457	0.326451872	-1.333333333	-3.58183822	0.915171553	0.60346711
SaS6-KSa2	2.333333333	0.03191521	4.634751457	0.044873645	3.333333333	1.084828447	5.58183822	0.000853619
SaS6-MuSc2	3	0.698581877	5.301418123	0.004035478	3.666666667	1.41816178	5.915171553	0.000235611
SaS6-NaSc3	2	-0.301418123	4.301418123	0.131463954	3	0.751495114	5.248504886	0.003096137
SaS6-RaSa2	2.333333333	0.03191521	4.634751457	0.044873645	2.666666667	0.41816178	4.915171553	0.011007586
SaS7-*Bts*_T84A1	-4.666666667	-6.96808479	-2.365248543	8.27E-06	-4.666666667	-6.915171553	-2.41816178	5.61E-06
SaS7-DSf4	-7.11E-15	-2.301418123	2.301418123	1	0.666666667	-1.58183822	2.915171553	0.993413177
SaS7-FhSb3	-2	-4.301418123	0.301418123	1.31E-01	-1.666666667	-3.915171553	0.58183822	2.97E-01
SaS7-JDc1	-3.00E+00	-5.301418123	-0.698581877	0.004035478	-3.333333333	-5.58183822	-1.084828447	0.000853619
SaS7-JSd1	-5.333333333	-7.634751457	-3.03191521	8.25E-07	-5.333333333	-7.58183822	-3.084828447	5.43E-07
SaS7-KSa2	-1.333333333	-3.634751457	0.96808479	0.634352676	-0.666666667	-2.915171553	1.58183822	0.993413177
SaS7-MuSc2	-0.666666667	-2.96808479	1.634751457	0.994546593	-0.333333333	-2.58183822	1.915171553	0.99998952
SaS7-NaSc3	-1.666666667	-3.96808479	0.634751457	0.326451872	-1	-3.248504886	1.248504886	0.891524245
SaS7-RaSa2	-1.333333333	-3.634751457	0.96808479	0.634352676	-1.333333333	-3.58183822	0.915171553	0.60346711
SaS7-SaS6	-3.666666667	-5.96808479	-1.365248543	0.00032596	-4	-6.248504886	-1.751495114	6.60E-05
TaSa4-*Bts*_T84A1	-2.666666667	-4.96808479	-0.365248543	0.013838179	-3	-5.248504886	-0.751495114	0.003096137
TaSa4-DSf4	2	-0.301418123	4.301418123	0.131463954	2.333333333	0.084828447	4.58183822	0.037174497
TaSa4-FhSb3	0	-2.301418123	2.301418123	1	-1.78E-15	-2.248504886	2.248504886	1.00E+00
TaSa4-JDc1	-1	-3.301418123	1.301418123	0.904938156	-1.666666667	-3.915171553	0.58183822	0.296595376
TaSa4-JSd1	-3.333333333	-5.634751457	-1.03191521	0.001148541	-3.666666667	-5.915171553	-1.41816178	0.000235611
TaSa4-KSa2	0.666666667	-1.634751457	2.96808479	0.994546593	1	-1.248504886	3.248504886	0.891524245
TaSa4-MuSc2	1.333333333	-0.96808479	3.634751457	0.634352676	1.333333333	-0.915171553	3.58183822	0.60346711
TaSa4-NaSc3	0.333333333	-1.96808479	2.634751457	0.999991728	0.666666667	-1.58183822	2.915171553	0.993413177
TaSa4-RaSa2	0.666666667	-1.634751457	2.96808479	0.994546593	0.333333333	-1.915171553	2.58183822	0.99998952
TaSa4-SaS6	-1.666666667	-3.96808479	0.634751457	0.326451872	-2.333333333	-4.58183822	-0.084828447	0.037174497
TaSa4-SaS7	2	-0.301418123	4.301418123	0.131463954	1.666666667	-0.58183822	3.915171553	0.296595376

Appendix C

ANOVA analysis of *Z. cucurbitae* and *Z. tau*

Strains	*Z. cucurbitae*				*Z. tau*			
	diff	**Lwr**	**upr**	**p adj**	**diff**	**lwr**	**upr**	**p adj**
DSf4-*Btk*_HD-73	-5.333333333	-6.884949115	-3.781717551	3.83E-10	-5	-7.775614691	-2.224385309	5.46E-05
FhSb3-*Btk*_HD-73	-3.333333333	-4.884949115	-1.781717551	3.12E-06	-4	-6.775614691	-1.224385309	0.001223476
FhSb3-DSf4	2	0.448384218	3.551615782	0.004574879	1	-1.775614691	3.775614691	0.971313892
JDc1-*Btk*_HD-73	-2	-3.551615782	-0.448384218	0.004574879	-1.333333333	-4.108948024	1.442281358	0.836688676
JDc1-DSf4	3.333333333	1.781717551	4.884949115	3.12E-06	3.666666667	0.891051976	6.442281358	0.003470197
JDc1-FhSb3	1.333333333	-0.218282449	2.884949115	0.140569632	2.666666667	-0.108948024	5.442281358	0.067545817
JSd1-*Btk*_HD-73	0.333333333	-1.218282449	1.884949115	0.999605593	-3.55E-15	-2.775614691	2.775614691	1
JSd1-DSf4	5.666666667	4.115050885	7.218282449	1.07E-10	5	2.224385309	7.775614691	5.46E-05
JSd1-FhSb3	3.666666667	2.115050885	5.218282449	5.81E-07	4	1.224385309	6.775614691	0.001223476
JSd1-JDc1	2.333333333	0.781717551	3.884949115	0.000708887	1.333333333	-1.442281358	4.108948024	0.836688676
KSa2-*Btk*_HD-73	-3.666666667	-5.218282449	-2.115050885	5.81E-07	-3.666666667	-6.442281358	-0.891051976	0.003470197
KSa2-DSf4	1.666666667	0.115050885	3.218282449	0.027759314	1.333333333	-1.442281358	4.108948024	0.836688676
KSa2-FhSb3	-0.333333333	-1.884949115	1.218282449	0.999605593	0.333333333	-2.442281358	3.108948024	0.999998803
KSa2-JDc1	-1.666666667	-3.218282449	-0.115050885	0.027759314	-2.333333333	-5.108948024	0.442281358	0.159538043
KSa2-JSd1	-4	-5.551615782	-2.448384218	1.16E-07	-3.666666667	-6.442281358	-0.891051976	0.003470197
MuSc2-*Btk*_HD-73	-4.333333333	-5.884949115	-2.781717551	2.51E-08	-4	-6.775614691	-1.224385309	0.001223476
MuSc2-DSf4	1	-0.551615782	2.551615782	0.487575206	1	-1.775614691	3.775614691	0.971313892
MuSc2-FhSb3	-1	-2.551615782	0.551615782	0.487575206	-5.33E-15	-2.775614691	2.775614691	1
MuSc2-JDc1	-2.333333333	-3.884949115	-0.781717551	0.000708887	-2.666666667	-5.442281358	0.108948024	0.067545817
MuSc2-JSd1	-4.666666667	-6.218282449	-3.115050885	5.83E-09	-4	-6.775614691	-1.224385309	0.001223476
MuSc2-KSa2	-0.666666667	-2.218282449	0.884949115	0.910934095	-0.333333333	-3.108948024	2.442281358	0.999998803
NaSc3-*Btk*_HD-73	-3	-4.551615782	-1.448384218	1.80E-05	-3.666666667	-6.442281358	-0.891051976	0.003470197
NaSc3-DSf4	2.333333333	0.781717551	3.884949115	0.000708887	1.333333333	-1.442281358	4.108948024	0.836688676
NaSc3-FhSb3	0.333333333	-1.218282449	1.884949115	0.999605593	0.333333333	-2.442281358	3.108948024	0.999998803
NaSc3-JDc1	-1	-2.551615782	0.551615782	0.487575206	-2.333333333	-5.108948024	0.442281358	0.159538043
NaSc3-JSd1	-3.333333333	-4.884949115	-1.781717551	3.12E-06	-3.666666667	-6.442281358	-0.891051976	0.003470197
NaSc3-KSa2	0.666666667	-0.884949115	2.218282449	0.910934095	0	-2.775614691	2.775614691	1
NaSc3-MuSc2	1.333333333	-0.218282449	2.884949115	0.140569632	0.333333333	-2.442281358	3.108948024	0.999998803
RaSa2-*Btk*_HD-73	-3.333333333	-4.884949115	-1.781717551	3.12E-06	-3.333333333	-6.108948024	-0.557718642	0.009701468
RaSa2-DSf4	2	0.448384218	3.551615782	0.004574879	1.666666667	-1.108948024	4.442281358	0.58646848
RaSa2-FhSb3	-1.78E-15	-1.551615782	1.551615782	1	0.666666667	-2.108948024	3.442281358	0.998906719
RaSa2-JDc1	-1.333333333	-2.884949115	0.218282449	0.140569632	-2	-4.775614691	0.775614691	0.333045135
RaSa2-JSd1	-3.666666667	-5.218282449	-2.115050885	5.81E-07	-3.333333333	-6.108948024	-0.557718642	0.009701468
RaSa2-KSa2	0.333333333	-1.218282449	1.884949115	0.999605593	0.333333333	-2.442281358	3.108948024	0.999998803
RaSa2-MuSc2	1	-0.551615782	2.551615782	0.487575206	0.666666667	-2.108948024	3.442281358	0.998906719
RaSa2-NaSc3	-0.333333333	-1.884949115	1.218282449	0.999605593	0.333333333	-2.442281358	3.108948024	0.999998803
SaS6-*Btk*_HD-73	-1.333333333	-2.884949115	0.218282449	0.140569632	-1	-3.775614691	1.775614691	0.971313892
SaS6-DSf4	4	2.448384218	5.551615782	1.16E-07	4	1.224385309	6.775614691	0.001223476
SaS6-FhSb3	2	0.448384218	3.551615782	0.004574879	3	0.224385309	5.775614691	0.026293997
SaS6-JDc1	0.666666667	-0.884949115	2.218282449	0.910934095	0.333333333	-2.442281358	3.108948024	0.999998803
SaS6-JSd1	-1.666666667	-3.218282449	-0.115050885	0.027759314	-1	-3.775614691	1.775614691	0.971313892
SaS6-KSa2	2.333333333	0.781717551	3.884949115	0.000708887	2.666666667	-0.108948024	5.442281358	0.067545817
SaS6-MuSc2	3	1.448384218	4.551615782	1.80E-05	3	0.224385309	5.775614691	0.026293997
SaS6-NaSc3	1.666666667	0.115050885	3.218282449	0.027759314	2.666666667	-0.108948024	5.442281358	0.067545817
SaS6-RaSa2	2	0.448384218	3.551615782	0.004574879	2.333333333	-0.442281358	5.108948024	0.159538043
SaS7-*Btk*_HD-73	-5	-6.551615782	-3.448384218	1.45E-09	-5	-7.775614691	-2.224385309	5.46E-05
SaS7-DSf4	0.333333333	-1.218282449	1.884949115	0.999605593	-7.11E-15	-2.775614691	2.775614691	1
SaS7-FhSb3	-1.666666667	-3.218282449	-0.115050885	0.027759314	-1	-3.775614691	1.775614691	0.971313892
SaS7-JDc1	-3	-4.551615782	-1.448384218	1.80E-05	-3.666666667	-6.442281358	-0.891051976	0.003470197
SaS7-JSd1	-5.333333333	-6.884949115	-3.781717551	3.83E-10	-5	-7.775614691	-2.224385309	5.46E-05
SaS7-KSa2	-1.333333333	-2.884949115	0.218282449	0.140569632	-1.333333333	-4.108948024	1.442281358	0.836688676
SaS7-MuSc2	-0.666666667	-2.218282449	0.884949115	0.910934095	-1	-3.775614691	1.775614691	0.971313892
SaS7-NaSc3	-2	-3.551615782	-0.448384218	0.004574879	-1.333333333	-4.108948024	1.442281358	0.836688676
SaS7-RaSa2	-1.666666667	-3.218282449	-0.115050885	0.027759314	-1.666666667	-4.442281358	1.108948024	0.58646848
SaS7-SaS6	-3.666666667	-5.218282449	-2.115050885	5.81E-07	-4	-6.775614691	-1.224385309	0.001223476
TaSa4-*Btk*_HD-73	-2.333333333	-3.884949115	-0.781717551	0.000708887	-3.333333333	-6.108948024	-0.557718642	0.009701468
TaSa4-DSf4	3	1.448384218	4.551615782	1.80E-05	1.666666667	-1.108948024	4.442281358	0.58646848
TaSa4-FhSb3	1	-0.551615782	2.551615782	0.487575206	0.666666667	-2.108948024	3.442281358	0.998906719
TaSa4-JDc1	-0.333333333	-1.884949115	1.218282449	0.999605593	-2	-4.775614691	0.775614691	0.333045135
TaSa4-JSd1	-2.666666667	-4.218282449	-1.115050885	0.000110515	-3.333333333	-6.108948024	-0.557718642	0.009701468
TaSa4-KSa2	1.333333333	-0.218282449	2.884949115	0.140569632	0.333333333	-2.442281358	3.108948024	0.999998803
TaSa4-MuSc2	2	0.448384218	3.551615782	0.004574879	0.666666667	-2.108948024	3.442281358	0.998906719
TaSa4-NaSc3	0.666666667	-0.884949115	2.218282449	0.910934095	0.333333333	-2.442281358	3.108948024	0.999998803
TaSa4-RaSa2	1	-0.551615782	2.551615782	0.487575206	1.78E-15	-2.775614691	2.775614691	1
TaSa4-SaS6	-1	-2.551615782	0.551615782	0.487575206	-2.333333333	-5.108948024	0.442281358	0.159538043

Appendix D

Microbiological Growth Media: Luria Bertani Agar & Broth

Ingredients	Quantity (g/L)
Yeast extract	5
Tryptone	10
NaCl	10
Distilled water	Up to 1000 mL
pH	7
Agar* (for LB agar)	15

Procedure: The ingredients are dissolved in distilled water by the process of stirring while applying mild heat. The medium is sterilized using autoclaving at a temperature of 121 °C for a duration of 15 minutes.

* In LB broth no agar was added

Microbiological Growth Media: Nutrient Agar

Ingredients	Quantity (g/L)
Beef extract	3
Peptone	5
NaCl	5
Distilled water	Upto 1000 mL
pH	7
Agar	15

Procedure: The ingredients are dissolved in distilled water by the process of stirring while applying mild heat. The medium is sterilized using autoclaving at a temperature of 121 °C for a duration of 15 minutes.

Microbiological Growth Media: Sheep Blood Agar

Ingredient	Quantity (g/L)
Liver extract	2.5
Yeast extract	5
Proteose peptone	15
NaCl	5
Agar	15
Distilled water	Up to 1000 mL

Instructions: Bring to a boil in order to fully dissolve the medium. Perform sterilization by subjecting to autoclaving at a temperature of 121 °C for a duration of 15 minutes. Cool the mixture to a temperature range of 40-50 °C and carefully add 7% v/v of sterile defibrinated blood using aseptic techniques.

Microbiological Growth Media: T3 Broth

Ingredient	Quantity (g/L)
Bacto-tryptone	3
Bacto-tryptose	2
Yeast extract	1.5
MnCl2	0.005
Phosphate buffer	50 mM
pH	6.8
Distilled water	Up to 1000 mL

Procedure: The ingredients are dissolved in distilled water by gently swirling with the application of heat. The medium is sterilized using autoclaving at a temperature of 121°C for a duration of 15 minutes**.**

Microbiological Growth Media: Chromogenic agar for *Bacillus cereus* group

Instructions: Dissolve 49.22 g in 1 liter of distilled water. Utilize an autoclave apparatus to achieve sterilization of things by subjecting them to a temperature of 121°C for a duration of 15 minutes, while maintaining a pressure of 15 pounds. Lower the temperature to a range of 45 to 50 °C. Aseptically introduce the rehydrated contents of 1 vial of the *Bacillus* Selective Supplement to selectively isolate *B. cereus* and *B. thuringiensis* (FD324).

Appendix E

Chemicals and Reagents

Name of chemicals/reagents/substrates	Brand/Country of Origin
Acrylamide	Carl Roth, Germany
Agar	Sigma, USA
Agarose	Promega, USA; Carl Roth, Germany
Aluminium sulfate	Merck, India
Ammonium persulphate	Wako, USA
Ammonium sulfate	Merck, India
Bis-acrylamide	Carl Roth, Germany
Boric acid	Merck, India
Bromophenol Blue	Wako, USA
Calcium chloride ($CaCl_2$)	Sigma, USA
Coomassie Brilliant Blue G250	Thermo Scientific, USA
Di potassium hydrogen phosphate	Merck, Germany
Di sodium hydrogen phosphate	Merck, Germany
Dithiothreitol (DTT)	American Bio analytical, USA
EDTA	BDH, England
Ethanol (EtOH)	Merck, Germany
Ethidium bromide (EtBr)	Sigma, USA
Glacial acetic acid	Merck, Germany
Glycerol	Sigma, USA
Glycine	Wako, USA
HiCrome™ Bacillus Agar Base	HiMedia, India
Hydrochloric acid (HCl)	Merck, Germany
Immersion oil	Merck, Germany
Potassium chloride	Sigma, USA
Potassium dihydrogen orthophosphate (KH_2PO_4)	Merck, Germany
Lysozyme	Wako, USA
Methanol	Sigma, USA
Magnesium chloride	Sigma, USA
Magnesium sulfate	Sigma-Aldrich
Manganese chloride	Merck, Germany
n-Hexane	Merck, Germany
Sodium chloride (NaCl)	Sigma, USA
Sodium carbonate ($NaCO_3$)	Sigma, USA
Sodium bicarbonate ($NaHCO_3$)	Merck, Germany

Disodium phosphate (Na_2HPO_4)	Merck, Germany
Monosodium phosphate (NaH_2PO_4)	Merck, Germany
Sodium Hydroxide (NaOH)	Merck, Germany
Sodium sulfide	Sigma-Aldrich
Peptone	Oxoid, England
Phosphoric acid	Merck, Germany
Phenol red	Sigma, USA
PMSF (phenylmethylsulfonyl fluoride)	Thermo Scientific™
Potassium hydrogen phosphate (K2HPO4)	Sigma, USA
Protein marker	NEB,England
Proteinase K	MP Biomedicals LLC, France
Sodium acetate	Merck, Germany
Sodium dodecyl sulphate (SDS)	Wako, USA
Sodium hydroxide (NaOH)	Sigma, USA
Gibco Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific
Tris-base	Sigma, USA
Tryptone	BD, USA
Tryptose	BD, USA

Appendix F

Buffers and Solutions

Phosphate buffer	
A. 0.5L of 1M K2HPO4	at 174.18 g/mol (solution A)
B. 0.5L of 1M KH2PO4	at 136.09 g/mol (solution B)
Parasporal Protein Solubilizing Buffer (pH 11.0)	
Na2CO3	0.53 g
DTT	0.17 g
Sterilized dH2O	up to 100 mL
The Bradford Reagent	
The Bradford reagent was prepared by dissolving 100 mg of coomassie blue G-250 in 50 mL of 95% ethanol. To this solution, 100 mL of 85% (w/v) phosphoric acid was added, and the combination was further diluted to a total volume of 1 liter using water.	
Proteinase K dissolving solution (10 mL storage buffer)	
Glycerol	5 mL
1M Tris-HCL	100 µL
CaCl2	0.029 g
Sterilized dH2O	up to 100 mL
0.1M PMSF	
PMSF	0.0174 g
Ethanol (95%)	1 mL
30% Acrylamide-bisacrylamide Solution	
Acrylamide	29.0 g
Bisacrylamide	1.0 g
Distilled water	100 mL
Ammonium-persulphate (APS) at a concentration of 10%	
APS	1.0 g
Distilled water	10 mL
Buffer solution with a pH of 6.8 for use in the upper gel	
Tris-base	6.05 g
SDS	0.4 g
pH to	6.8
Distilled water	Up to 100 mL
Buffer solution with a pH of 8.8 for use in the lower gel	
Tris-base	36.4 g
SDS	0.8
pH to	8.8
Distilled water	Up to 100 mL

Protein sample preparation buffer	
0.5 M tris-Cl (Upper gel buffer)	10 mL
10% SDS	10 mL
1.0 M DTT	5 mL
Glycerol	10 mL
Distilled water	14 mL
Solution containing 0.1% Bromophenol blue (BMB) or tracking dye	
dH2O	100 mL
Bromophenol blue	0.1 g
SDS Electrophoresis Buffer (10X)	
Tris-base	30.3 g
Glycine	144.1 g
SDS	10 g
pH	8.3
Distilled water	Up to 1000 mL
Staining solution (1L)	
Coomassie blue	2 g
Methanol	450 mL
dH2O	450 mL
Glacial Acetic Acid	100 mL
De-staining solution (1L)	
Methanol	50 mL
Glacial Acetic Acid	70 mL
dH2O	880 mL
KOH solution (16%)	
KOH	16 g
Distilled water	100 mL
Naphthol solution (5%)	
Naphthol	5 g
Ethanol	100 mL

Phosphate-buffered saline (1X PBS)

A solution of 1X PBS was created by dissolving 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of sodium dihydrogen phosphate (Na2HPO4), and 0.24 g of potassium dihydrogen phosphate (KH2PO4) in 800 mL of distilled water (H2O). The pH was neutralized to 7.4 using hydrochloric acid (HCl) and then sterilized using autoclaving.

Normal saline

A solution of normal saline was created by dissolving 0.85 g of sodium chloride (NaCl) in 100 mL of distilled water and then sterilized using autoclaving.

Bromophenol Blue (1%)

Mix 10 mg Bromophenol Blue with 1 mL dH_2O and mix well. Store at room temperature.

Sodium dodecyl sulphate (10%)

A solution was prepared by dissolving 10 g of Sodium dodecyl sulphate (SDS) in 80 mL of distilled water. The mixture was stirred gently on a magnetic stirrer to prevent the formation of foam. The ultimate volume was modified to 100 mL using distilled water and kept at room temperature.

Tris-HCl solution with a concentration of 1.0 M

A solution was prepared by dissolving 121.1 g of tris-base in 800 mL of distilled water. The appropriate pH was achieved by adding concentrated HCl, and the final volume was adjusted to 1 L using distilled water. The solution underwent sterilization using autoclaving and was thereafter kept at room temperature (RT).

Ethylenediaminetetraacetic acid (EDTA) at a concentration of 0.5 M

The amount of Na_2EDTA (disodium ethylene diamine tetra-acetic acid) is 186.1 g and 20 g of NaOH pellets were introduced into 800 mL of distilled water and dissolved by agitation on a magnetic stirrer. The pH was modified to 8.0 by adding a small amount of 10 M NaOH and then the total volume was brought up to 1 L using distilled water. The solution was sterilized using the process of autoclaving and thereafter kept at room temperature.

Buffer solution for Tris-Borate-EDTA (TBE) with a concentration of 10 times the standard strength.

A solution was prepared by dissolving 108.8 g of Tris base and 55 g of boric acid in 800 mL of deionized water. Additionally, 40 mL of 0.5 M EDTA (pH 8.0) were added to the solution. The capacity was modified to 1000 mL using deionized water.

Buffer solution with a pH of 8.0, often known as TE buffer.

A solution of 10 millimolar tris-Cl (pH 8.0) and 1 millimolar EDTA was made by diluting concentrated stocks of 1 molar tris-Cl (pH 8.0) and 0.5 molar EDTA. The buffer was held at a temperature of 4 ^oC

Gram's Iodine Solution

The ingredients, consisting of 1.0 g of iodine, 2.0 g of potassium iodide, and 300 mL of distilled water, are ground in a mortar and dissolved by gradually adding water. The prepared solution is thoroughly combined by agitating.

Appendix G

List of Equipment

Name of Equipment	Model/ Brand/Country of Origin
Autoclave machine	Hirayama model HL-42, AE, Japan
Biosafety cabinet	ESCO Class II BSC, USA
Bioreactor, 3.0 L	New Brunswick, USA
Centrifuge machine	MX-305 High Speed Refrigerated Micro Centrifuge
DNA Ladder	Invitrogen, USA
Electronic balance	SHIMADZU, ELB200
Glassware sterilizer	Redline, Binder, Germany
Microbiological incubator	Redline, Binder, Germany
Magnetic stirrer	CIMAREC, Barnsted Thermolyne
Micropipettes	Eppendorf research and Nichiryo
Nanodrop spectrophotometer	Nanodrop 2000, Thermoscientific; NanoDrop One, Thermoscientific
Sonication machine	Omni-Ruptor 4000 Ultrasonic Homogenizer, OMNI International. USA
Orbital shaker incubator	Excella E25 Incubator Shaker series, New Burnswick Scientific
pH meter	INOLAB WTW series, pH 720
Power supply	BIORAD, USA
Refrigerator (4^{o} C)	Royal Frestech
Spectrophotometer	Genesys 5, Thermospectronic
Thermal cycler	ProFlex, Applied Biosystems
Thermo stated shaking water bath	Water Bath 1083, GFL
Vortex mixture	VM-2000, DIGISYSTEM