Study on the enhanced expression of insecticidal crystal protein in *Bacillus thuringiensis* against the larvae of mosquito

DISSERTATION
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CHAPTER 1

Introduction

1.1 Background of the study

Since the past 15 years in the territory of molecular research, the development and use of entomo-pathogens as classical, conservation, and augmentative biological control agents have incorporated several prosperity and some setbacks [1]. As a consequence, in the domain of molecular field and research employing living organisms to a degree bacteria, viruses, and fungi, or natural products derived from these organisms, can be used auspiciously as biological control agents and more prevalently alternative to chemical insecticide or pesticide. Moreover, it is also well known to everyone that these sorts of microorganisms are playing the key role as paramount or auxiliary pest control measures and symbolize a prized arsenal for the control of pest management in the territory of agro-industry [2]. Bacterial protein toxins are regarded as the biological appliance that has been applied over a long period to illuminate diverse mechanisms in the field of agriculture and medicine. As biological control tool of Gram-positive entomopathogenic bacteria that have paid a significant have received significant consideration owing to have their capability to restrict the insect pests in the sector of agriculture and insect vectors of human diseases. For the advancement of bio insecticides-based *B. thuringiensis*, special scrutiny was directed to restrict the distinct range of insect species encompassed by the orders of Lepidoptera, Diptera, and Coleoptera in conjunction with another form of insect orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) along with other nematodes, mites, and protozoa [3]. *B. thuringiensis* is already grabbed the position as an alternative advantageous to chemical or synthetic insecticide employment as an effective controller in the area of commercial agriculture, woodland management, and restriction of mosquito to play a vital source of genes for transgenic expression for contributing insect resistance in plants [4]. Insects may be the responsible

vectors for the etiological agent for varieties of diseases and be the botheration to humans, carrying the load for health burdens all around the worldwide [5]. Among all the insects, globally, the deadliest of the creatures that carry and cause the most dangerous deadly diseases is the mosquito. Because mosquitoes are capable to kill more humans rather than human murderers do. According to Bill Gates summarizing surveys or statistics like as WHO, each of a near about 725 thousand people passed away owing to this dangerous creature means mosquitoes. In comparison to other creatures like sharks and snakes (Fig.1-1) [6]. (https://www.mosquitomagnet.com/articles/the-economic-cost-of-mosquito-borne-diseases), it is indicating a devastating figure.

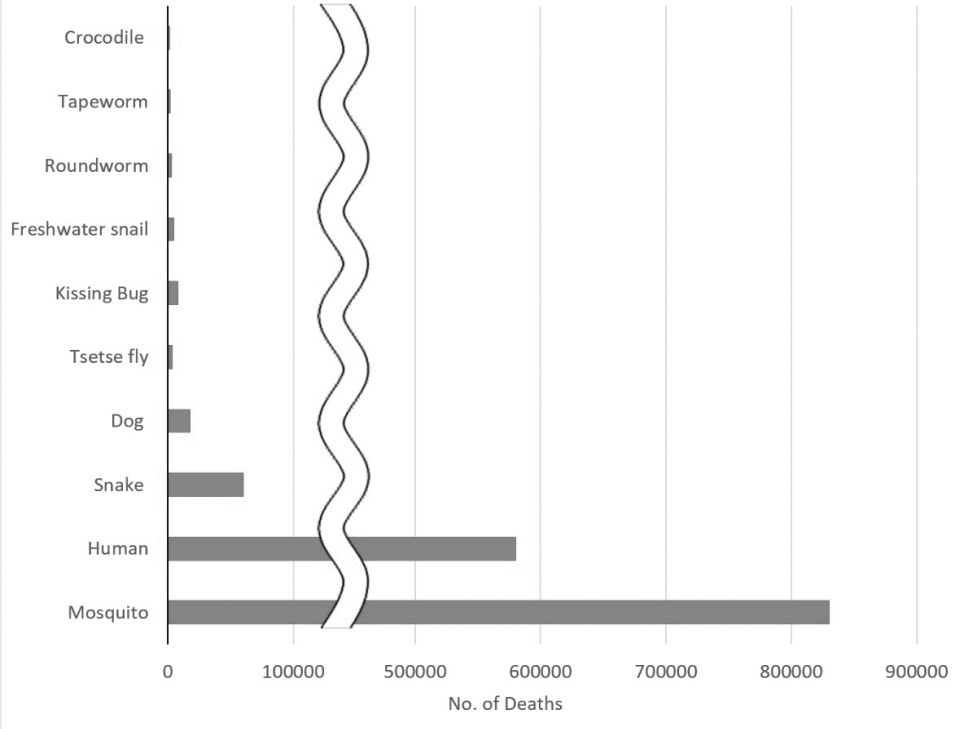

Figure 1-1 Number of people killed in a year by specific organisms

All around the world, mosquito-borne diseases notably, Dengue, chikungunya, and Zika virus isgetting alarming in recent context and at present, there is a lack of alternative or potent vaccines feasible to the greater extent of these diseases [7] *Aedes* (*Stegomyia*) *aegypti* (Linnaeus 1762) acts as a predominant vector in the universal resurgence of epidemic dengue, and also capable of disseminating a variety of other arboviruses which is responsible to contribute detrimental effect on the human populations globally, along with Zika and Chikungunya, that have been more prominent recently into Brazil [8-12].

Indeed, of knowing the negative impact of chemical pesticides or insecticides from the very beginning to date, the employment of the chemical insecticides still is being adopted to restrict insects despite exceedingly toxic to non-target organisms as well as generally mischievous to human and animal health. On account of, *Bacillus thuringiensis* (Bt) might be the option to merge the advancements of chemical pesticides and microbial control agents MCAs: acting to synthesize parasporal crystal inclusion having the capacity to kill mosquito larvae in a way of eco-friendly and environmentally welcoming although the production cost is overpriced than chemical insecticide. So Bt is still the most promising microorganism for its' homicide trait of mosquitoes and how it could be gem selection in the future by the employment of both biological and non-biological approaches.

Table 1-1 Mosquito-Bornediseases

Vectors (Mosquito)	Diseases
Aedes aegypti	Dengue, yellow fever, chikungunya, Zika virus
Aedes albopictus	Chikungunya, dengue, West Nile virus
Culex quinquefasciatus	Lymphatic filariasis
Anopheles (more than 60 known species can transmit diseases)	Malaria, lymphatic filariasis (in Africa)
Haemagogus	Yellow fever
Sandflies	Leishmaniasis
Triatomine bugs	Chagas disease
Ticks	Crimean-Congo haemorrhagic fever, tick-borne encephalitis, typhus, Lyme disease
	Plague, Murine typhus
Flies (various species)	Human African trypanosomiasis, onchocerciasis

(https://apps.who.int/iris/bitstream/handle/10665/111008/WHO_DCO_WHD_2014.1_eng.pdf;jsessionid=6DA9461FCD4806A3FB8A0B4209D6E5FF?sequence=1)

1.2 Basic Biology of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a ubiquitous gram-positive bacterium that belongs to morphological group I in conjunction with *Bacillus cereus*, *Bacillus anthracis,* and *Bacillus laterosporus* [13] characterized by aerobic or facultative anaerobes, rod-shaped, low G + C, and endo spore-forming which have been that has been isolated globally in distinction to a vast diversity of ecosystems together with different varieties living and nonliving sources like soil, water, departed insects, dirt from silos, leaves from short-lives trees, varieties of conifers, and insectivorous mammals, in associate with serious form of necrosis derived human tissues [14–19]. By the employment of ordinary nutrient agar media, it is possible to isolate. The life cycle of Bt is quite ordinary and sufficient growth is aided by nutrients and environmental conditions through spore germination by a-vegetative cell growth and binary fission mechanism for reproduction. For multiplication of cells when it started from one to more there must be required some proper nutrients or like sugars, amino acids, or depletion of oxygen. Bt is capable to show its unique attribute at the onset of sporulation and for the time being of growth phase as parasporal crystalline inclusions to synthesize crystal (Cry) and cytolytic (Cyt) toxins, (also known as δ-endotoxins [20] that is a most distinguishable feature from else species [21]. Crystals are composed of proteins called *δ*-endotoxins, which played a major role in the distinct insecticidal activity [22]. Cry proteins have the tendency to get easily solubilized by the aided of consecutive interactions with the different insect's midgut epithelium to promote the formation of an oligomeric structure for the membrane insertion, pore formation to perceive, and successive binding of the toxin to the membrane receptors to facilitate the killing of midgut cells [23-27]. Behind this reason is for the shake of the most expected biological insecticide that acts as a substitute to chemical insecticide, regarded as the leading bio rational pesticide, eco-friendly, and in conjunction with distinct to a

defined figure of insect species (insect specific toxin) without any toxicity against humans or other organisms [28].

1.2.1 *Bacillus thuringiensis* taxonomy and genetics

The classification of Bt is complicated regularly revisited belongs to a diverse group of opportunistic bacteria *Bacillus cereus sensu lato*, [29]. From the past decades, varieties of taxonomic studies along with different methods have been executed with a view to inaugurating logical or authentic criteria for the separation and analysis of diversity among interspecific and intraspecific for species of the genus [30-32]. The principal method that has been applied for recognition of subspecies particularly for Bt since its description in 1915, is mainly based on the flagellar H antigen reaction [33, 34]. For instance, along with 3,000 strains termed as Bt were assembled in 69 serotypes and 13 subtypes by the improvement of this method in the past few decades [35].

For the management of pest control programs, identification of new strains by the application of molecular tools is extremely essential to make the possible differentiation of the different strains of Bt for detailed studies. Among all the adopted tools, the most predominantly and highlighted are based on 16S rRNA, RAPD, RFLP, REP-PCR, ERIC-PCR, and MLST. For the study of bacterial diversity, the molecular marker regarded is the most prominent tool, 16S rRNA genes. For the studies of phylogenetic analysis and metagenomics, this is the most extensively used marker [35, 38].

But there remain some constraints in the genus of *Bacillus* by the employment of this 16S rRNA particularly for being the high correlation between the sequences of closely related species [39]. The most recently applying the technique is 16S rDNA analysis in conjunction with other markers,

have been developing the scope to differentiate from Bt strains to other species of *Bacillus* to defeat these restrictions [40-45].

1.2.2 Ecological role of *Bacillus thuringiensis*

Bt is regarded to be aboriginal to many environments [46-48] and essentially being considered a soil originated bacterium has the tendency to live as both saprophytic, (employing organic matter form and dead organisms) and parasitic (colonization in living insets) [49]. Globally, Bt strains have been isolated from many habitats, in accordance with soil [48, insects [53] stored-product dust 47, 55, 57], and deciduous and coniferous leaves [53, 58]. Owing to have its insecticidal capability and specificity, Bt is renowned as the most environmentally safe organism without having the distinct aftereffect to non-target organisms like mammals, birds, amphibians, and reptiles [59]. Albeit there is little evidence of toxic effects of Bt demonstrated to useful insects such as bees and *Campoketis chloridae* [13, 60]. The endospore is regarded as the most prominent trait of the *Bacillus* ecology owing to its considerable logic. Firstly, for the rehabilitation of *Bacillus* spp. from the environment, the isolation procedure is regarded as the most frequent approach. Most likely the studies focused on the endospore to the segregation of vegetative cells. Secondly, the spore has a trait with a dormant structure of great longevity, although there are some ecological studies focused on the assessment of aggregation of spores in an environment instead of an estimation of how bacteria can devote to the environment. Even though in a habitat, if there is some presence of the big number of spores of a specific species, it is an active indication of earlier or steady growth and metabolism in that niche [61]. Still, there is some doubt on the true ecological role of Bt [62]. Despite the fact, it is well established that Bt has the capability to

produce the divergent form of toxic protein active against a wide variety of insect orders but still, it is identified some strains without having any toxicity [63].

1.2.3 Pathogenicity and Virulence

The most beautiful and amazing capacity of Bt to make crystal toxins during their sporulation phase is considered to be the major virulence factor to the insect. The insecticidal characteristics of the bacterium are derived from the δ-endotoxin that exists in both the crystalline inclusion and in the spore coat. Pathogenicity and virulence are the two principal indicators that help to figure out the target pest susceptibility to entomopathogen(s) through bioassays. The Cry proteins belonging to the pore-forming proteins (PFPs), where the members of the single largest class are responsible for bacterial virulence factors [64].

During the stationary phase, these bacteria not only produce the specific Cry toxins but also another kind of insecticidal proteins during its vegetative growth phase, designated as Vip proteins (vegetative insecticidal proteins) that have no similarity and disagree significantly with the Cry toxins [65]. However, it is produced during different phases of the life cycle of Bt, where there is no evidence with remarkable detrimental effects on non-target organisms in case of both Cry and Vip proteins contribute a narrow range of target insect pests.

1.2.4 Nomenclature of Bt Toxin

For novel insecticidal proteins, the number of genes has been coded that have expanded frequently, developing the demand for a standard and organized nomenclature system [3], from 1981 when the identification and cloning of the first Bt insecticidal crystal protein [66], For the establishment of first such system, Cry toxins and their corresponding genes were named in

association with a Roman numeral (primary rank distinction) based on the insecticidal activity of the crystal protein, namely: CryI protein toxic against lepidopterans, CryII protein toxic against both in lepidopterans and dipterans, CryIII protein toxic against coleopterans; and CryIV proteins toxic exclusively against dipterans only [67]. Usually, the new system implies while giving a new novel toxin by the indication of a four-rank name conditioning to its degree of couple amino acid identity taken from the earlier named toxins; besides this, similar protein structure, host range, or even mode of action. Does not mention by associating this criterion. For the first and fourth ranks are implied by the Arabic numbers, whereas the uppercase and lowercase letters are designated for the second and third ranks, correspondingly. The most frequently applicable system is nomenclature which is defined the δ-endotoxins (Cry and Cyt) and secret able (Vip and Sip) Bt toxins [3].

1.3 Expression regulation of insecticidal genes

The most obvious basic difference from Bt to its relatives has the amazing capacity to produce crystal inclusions during the stationary phase of growth [67]. The ultimate-products of *cry* gene expression are Cry protein that accumulates to dry weight constitute 20–30 % of the cell gather in mother cell on the onset of stationary phase (started from sporulation stage III and advances through stage VII, each single cell must synthesize 10^6 to 2×10^6 δ-endotoxin molecules to form a crystal) [68]. Various factors that are uniformly manipulated for the high degree synthesis of Cry protein like the transcriptional, posttranscriptional, and posttranslational levels, the copy number of *cry* gene, the stability of *cry* gene mRNA, and the aggregation and crystallization of Cry proteins [69, 28]. In the mother cell, the development and spatial control of *cry* gene expression is generally accomplished by the dint of promoter with its sporulation-specific sigma factors. During the period of sporulation, the factors that are firmly regulated and developed by maintaining a fixed order and the total figure of the sporulation-specific transcription factors are five: σ^H, σ^F, σ^E, σ^G and σ^K. Different sigma factors are active in accordance with a different location like the σ^H factor in the pre-divisional cell; σ^E and σ^K factors in the mother cell; and finally, σ^F and σ^G factors active in the forespore. The identification and determination of different varieties of *cry* genes promoters have already been completed. [18, 70-73].

For instance, the way of expression of the *cry1Aa* gene is initiated by two overlapping promoters (BtI and BtII) [65]. First transcription process is usually started from the downstream promoter, while BtI keeps remains active in between the stages II and VI of sporulation and applies of σ^E factor. The next transcription that is initiated by dint of the upstream promoter, BtII, activation period lasting from stage VI to the end of sporulation and employs σ^K. The σ^A, the sigma factor of vegetative cells, is responsible for the identification of the *cry3A* promoter, and the active

hour is initiated by the end of exponential vegetative growth and continued this active hour through stage III of sporulation.

Initially in the stage of transcriptional level, *cry* genes are classified into two varieties based on the transcriptional regulation mechanisms: the first one is termed as sporulation-dependent *cry* genes that are initiated by virtue of sporulation-specific sigma factors SigK and/or SigE and the rest on is sporulation-independent *cry* genes concealed by the control of the vegetative SigA factor [74]. In addition, there are also some extra factors responsible for the contribution of *cry* gene expression (Table 1-2) particularly in transcriptional regulation:

Table 1-2 Transcription factors involved in *cry* gene expression.

Gene	Factors	Other TFs	References
Cry1A	σ^E, σ^K	Spo0A (+); PDH E2 (+)	[75 49, 76]
Cry2A	σ^E	NA	[54]
Cry2B	σ^E	NA	[54]
Cry3A	σ^A	NA	[77]
Cry4A	σ^E, σ^K σ^H	PPK(+); Hpr/CcpA(-)	[78-81]
Cry4B	σ^E, σ^K	NA	[78]
Cry6Aa2	NA	ORF2(-)	[58]
Cry8Ea1	σ^E, σ^K σ^H	NA	[47]
Cry11A	σ^E, σ^K σ^H	Spo0A	[78]
Cry15A	σ^E,	NA	[82]
Cry18A	σ^E, σ^K		[83]

Notes: TFs: Transcription factors; (−): negative regulation; (+): positive regulation.
NA: undetermined

1.4 Crystal Toxins (δ-endotoxins)

δ-endotoxin is regarded as the most virulence criteria in the case of Bt convey the most pathogenic attribute against insects [16]. δ-endotoxin genes inhabit plasmids [83] in almost Bt toxin genes generally be the part of composite structures in conjunction with mobile genetic elements [85, 86] where a vast of *cry* gene-belonging plasmids to be conjugative in nature [61]. The most widely known are the δ-endotoxins, including Cry and Cyt toxins.

1.4.1. Classification of δ-endotoxins:

Hofte and whitely [61] who took the cardinal attempt to organize the nomenclature and classification scheme considered for delta-endotoxin on the basis of the insecticidal spectra of toxin [61] where the δ-endotoxins [86] is the principal indicator for the insecticidal properties of Bt. They also introduced four leading classes of *cry* genes and other classes *cyt* genes in associate with individual subclasses: *cryI*-specific for Lepidoptera, *cryII*--specific for Lepidoptera and Diptera, *cryIII*-specific for Coleoptera, *cryIV* - specific for Diptera, and *cytA*- specific for Diptera based on sequence analysis and spectrum of toxicity against the insects. Subsequently, other forms of new classes of *cry* genes like *cryV* and *cryVI* were also included where the genes were encoded for the protein toxic against nematodes [87]. Recently, Cry proteins belonged to 75 families in accordance with 800 different *cry* genes [88] on the basis of amino acid sequences. On the other hand, Cyt proteins belong to three families with 38 genes [64]. In addition to crystal proteins, there is another form of a protein called Vip that is synthesized and secreted during the vegetative growth phase and into the medium respectively. In association with Cyt and VIP toxins, there are at best six different families produced by Bt where no similarity to structure, the three-domain (3D) family,

the Bin-family, and the MTX family. From time to time the classification and diversity of Cry, Cyt, and Vip toxins have been updated [89].

1.4.2 Cry toxin

The name of a protein termed Cry toxin originated from the basis owing to synthesizing a parasporal crystal. As a consequence, Cry toxins comprised a number of unrelated lineages where do not exist in a single, homologous family of proteins [90]. Cry toxins belong to the group of δ-endotoxins proteins also termed as parasporal inclusion generated by Bt during the onset of sporulation phases have the capability to destroy the insect and other organisms as well [91].

Cry proteins are also termed as Cry toxins characterized by significant sequence similarity to proteins however the target organism persists unidentified [61, 90]. Presently, the Cry protein produced by the species of *Bacillus* composes the largest group of insecticidal proteins. Up to now, the classification of Cry proteins organized by Bt Toxin Nomenclature Committee [88] has categorized 73 different types of Toxins (Cry1 to Cry73) in association with three-domain Cry (3d-Cry), the Bin-like Cry and ETX_MTX2 family proteins from Bt and Ls, having the toxicity against different insect orders like lepidopterans, coleopterans, hemipterans, dipterans, nematodes (human and animal parasites, and free-living; Rhabditida) some snails [91-99] and/or human-cancer cells based on different origins [100, 101]. Procedures to assess the insecticidal activity of crystal proteins that are correlated with the expression of *E. coli* to obtain pure proteins and have tremendous importance for the Bt community however there might be the possibility of effective antibacterial activity to cradle the cloning and/or efficient expression (e.g., the three-domain toxins Cry13A and Cry14A) [102].

1.4.3 Diversity, structure and evolution of Cry toxins

It is already well established to prove the diversity of Cry proteins by showing their toxic effect against a wide range of insect orders particularly, Lepidoptera, Coleoptera, Hymenoptera, and Diptera, and also to nematodes. Another example for the diversity of Bt to synthesize another category of toxin called Cyt toxins have the capability to show the toxic effect against Diptera. Besides Cry protein, another form of protein also synthesized by Bt called Cyt protein, structurally similar to Cyt toxin possesses the mnemonic Cyt [103]. The nomenclature of Cry and Cyt proteins is based on the primary sequence identity with varieties of gene sequences. There are many insecticidal proteins, phylogenetically not related to the three-domain Cry family have been determined. Amid these, the similarity groups are binary-like toxins and Mtx-like toxins correlated to *B. sphaericus toxins*, and Bt producing parasporins [103].

The specific trait of these members belong to the family is accompanied by the protoxins in conjunction with two distinct lengths. For the large group of protoxins, almost two times longer than the majority of the toxins. The long protoxins of the C-terminal extension are non-essential dispensable for toxicity although it is said to contribute a role in the accumulation of the crystal inclusion bodies into the bacterium [104]. Cyt toxins composed comprise two remarkably similar gene families (Cyt1 and Cyt2) [105]. Protoxins and small portions synthesized by Cyt toxins belonging to the N-terminus and C-terminus are abolished to make it an activated toxin [106]. To date, X-ray crystallography is very indispensable to figure out the tertiary structures of three-domain Cry proteins like Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, and Cry4Ba [91, 116-120], and the structures play a pivotal role to display a large amplitude of similarity to a three-domain organization, indicating the same mechanism of action of the Cry three-domain protein family.

The N-terminal domain or domain I is characterized by a bundle of seven α-helices where the central helix-α5 nature is hydrophobic and enclosed by six different amphipathic helices, which are liable for membrane insertion and pore-formation. Domain II and III are characterized by three anti-parallel β-sheets along with exposed loop regions, and a β-sandwich accordingly [99, 107-111]. Both exposed regions of domain II and domain III are engaged in receptor binding [112]. The structural similarities of Domain I administers with another PFT like colicin Ia, N, and diphtheria toxin are responsible for pore-formation. It has been reported that the structural similarities of Domain II, distribute to different forms of carbohydrate-binding proteins like vitelline, lectin jacalin, and lectin Mpa [91] whereas Domain III, also have the tendency to deliver structural similarity with other carbohydrate-binding proteins like the cellulose binding domain of 1, 4-β-glucanase C, galactose oxidase, sialidase, β-glucuronidase the carbohydrate-binding domain of xylanase U and β-galactosidase [113]. These kinds of similarities are an indication for the carbohydrate moieties might play a pivotal aspect in the action of mechanism for three-domain Cry toxins.

1.4.4 Mode of action of Cry and Cyt toxins in mosquitoes larvae

In the case of lepidopteran insects, the process to digest the crystals in mosquitoes by ingestion and dissolving in the alkaline gut environment by susceptible larvae discharging soluble proteins (Fig. 1-2). Particularly for the 70 kDa Cry11Aa protoxin, the way of proteolytic activation adopting gut extract by elimination of 28 residues from the N-terminal and proceeded to 34 and 32 kDa fragments by intramolecularly cleaving [114] resulted in these two fragments continuing to hold toxicity (Fig. 1-3) [115].

Alternatively, the 130 kDa of Cry4Ba protoxin, where N-terminal, C-terminal, and intra-molecular cleavage proceeded to an active form toxin belonging to two protein fragments of 18 kDa and 46 kDa (Fig. 1-2) [116, 117]. The amino acid residues of 32 and 15 belonging to Cyt2Aa respectively from the N-terminal and the C-terminal ends are eliminated by proteinase K treatment developing a monomeric protein together with hemolytic activity [118].

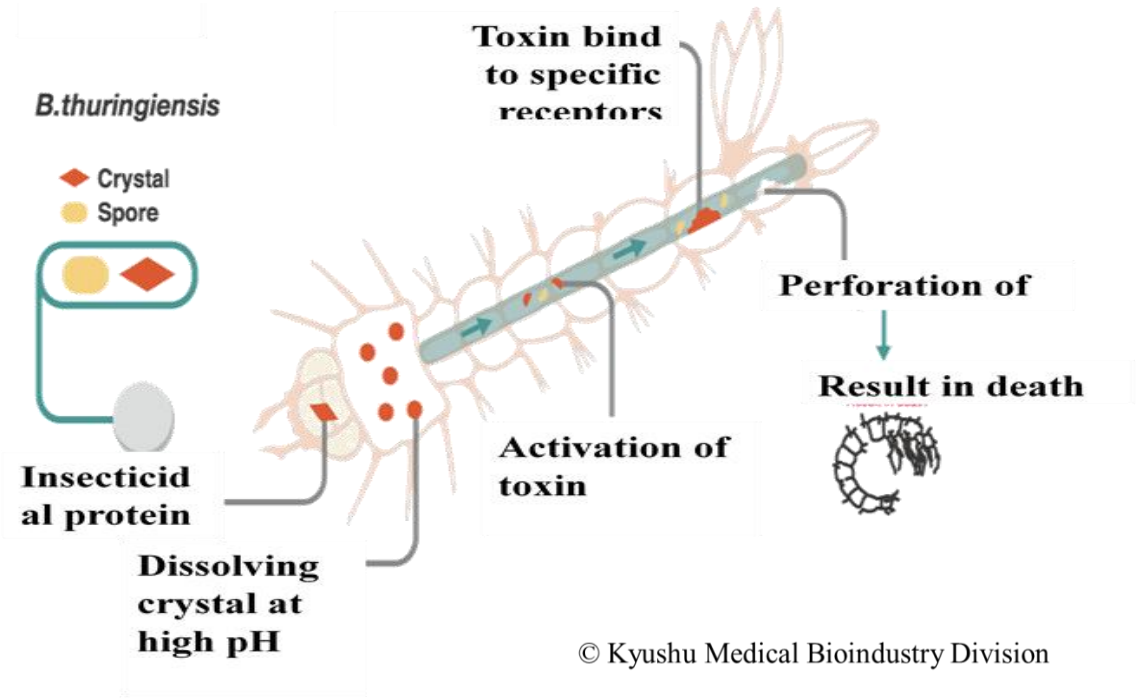

Figure 1-2 Mode of action to kill mosqito larvae by Cry protein from *B. thuringiensis*

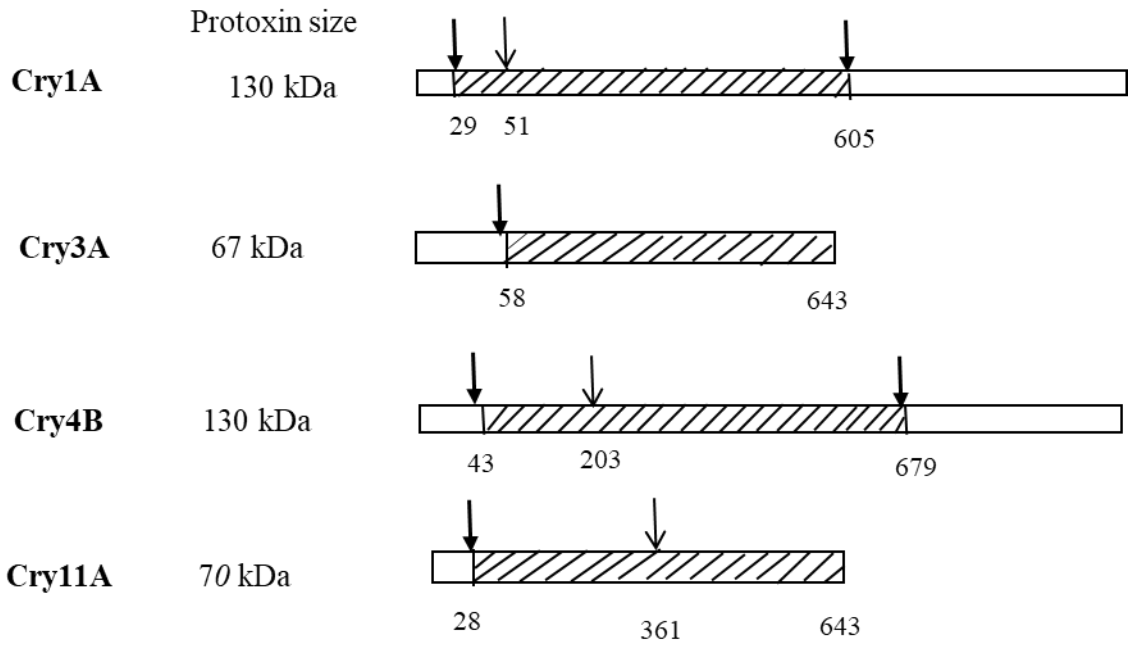

Figure 1-3 N-terminus and C-terminus of Cry protein are removed to activate the toxin

1.4.5 Receptor binding in mosquito midgut membranes

It is well established that the microvilli of the mosquito midgut cells are correlated by the way where Cry toxins bind to specific protein receptors. Alternatively, like Cry toxin, Cyr toxin is not related to the binding of receptor but straightly to do insertion into the membrane and formation of the pore by diffusely synergistic with membrane lipids [119-122] (or damaging the membrane by dint of detergent-like interaction [123].

The mosquitocidal toxin of Cry4A, Cry4B and Cry11Aa, there have been associated with toxin-receptor interaction and specificity in the region of the domain II loop. There is an importance of loop α-8, β-4 region, and loop 3 for binding to lipids of brush border membrane vesicles (BBMV) from *Ae. aegypti* in regard to qualitative binding competition assays with synthetic peptides complementary to predict Cry11Aa domain II exposed regions. For Cry11A toxicity it is

indispensable to interact with *Ae. aegypti* BBMV by confirming the mutagenesis and the region of putative loop α-8 residues [124]. From the overall discussion, there is no doubt on the influence of domain II loop regions for Cry toxin-receptor interaction in mosquitoes.

By the dint of toxin overlay assays, the mosquitocidal toxin of Cry11Aa and Cry4Ba where the binding-proteins of 65 and 62 kDa were determined in BBMV from *Ae. aegypti* larvae [125]. Currently, another three categorized midgut proteins, 200 kDa, 100 kDa, and 65 kDa were identified in *Ae. aegypti* midgut membranes of the mosquitoes where it is impelled with the Cry11Aa toxin confirming the subsistence of distinct receptor particles for Cry toxins. Most of these proteins have the propensity to harbor of the membrane by dint of GPI where the exhibited molecular weight were 100 and 65 kDa [126].

In spite of, GPI-anchored proteins purified by the employment of Cry11Aa affinity chromatography admitted only the protein with 65 kDa designated as a GPI-anchored ALP enzyme [112]. Apart from this, *Ae. aegypti* GPI-ALP protein was also associated with the toxicity of Cry11Aa. There are two peptides displaying phages like P1. BBMV and P8. BBMV is particularly bound to the 65 kDa ALP, by competitive binding of the toxin to BBMV, in association with the interference toxicity of Cry11Aa toxin [121]. Lastly, it is obvious that the GPI-ALP act as a crucial receptor molecule that interferes with the toxicity of Cry11Aa to *Aedes. aegypti* larvae. There is a high affinity between the APN protein bound to Cry11Ba but no tendency to show the interaction with Cry4Ba or Cry11Aa toxins [127].

Cry receptor molecules in mosquito play a major role that is crucial to figure out where the oligomeric structure of a pre-pore intermediate this toxin by the insertion of the membrane. Nonetheless, the mechanism of Cry toxins in association with mosquito might have a similar pattern following to lepidopteran insects suggesting due to the presence of conserved structure of

Cry toxins with multiple receptor molecules, GPI anchored membrane, and the role of domain II in receptor interaction.

1.4.6 Synergism of Cry and Cyt toxins

Recently, a question is arising that is the leading menace or extreme threat regarding insect resistance by using the Bt in the field with lepidopteran insects [128]. Although our principal focus is to consider the resistance fact in the Dipteran order, particularly and undoubtedly, it's a matter of great hope, yet no resistance has been observed in the field in mosquito species controlled with *Bacillus thuringiensis* serovar *israalensis* (Bti) [129]. There is no evidence of resistance in the case of Bti owing to the existence of Cyt1Aa protein in the crystal [130]. However, there is some evidence *of Culex quinquefasciatus* populations resistant to Cry4Aa, Cry4Ba or/and Cry11Aa under the laboratory conditions but the absence of resistance to the Cry protein where the presence of Cyt1Aa protein [131]. Additionally, the insecticidal activity was far better after observing the synergistic reaction between Cyt1Aa and the Cry proteins of Bti [123, 132,133] in comparison to isolated proteins.

Lately, it was established that Cyt1Aa protein by functioning as a receptor molecule is capable to synergizes Cry11Aa toxicity [134]. The binding capability of Cry11Aa to *Ae. aegypti* BBMV was augmented by membrane-bound Cyt1Aa resulted in immense synergistic affection between Cyt1Aa and Cry11Aa in solution ae well as in membrane bound state was identified. The epitopes binding of Cyt1Aa and Cry11A were identified. The heterologous competition assays were helpful by the employment of synthetic peptides related to these regions by site-directed mutagenesis to determine the role of loop β6-αE and part of β7 of Cyt1Aa in binding Cry11Aa [134]. It is already reported that the involvement of the domain II loops α-8 for the interaction of Cry11Aa with ALP

receptor [135]. Mutagenesis of specific amino acid residues plays a vital role in both toxins to indicate the interaction between these two toxins in association with synergistic action [135]. It is obvious from the data that the insertion of Cry11Aa into the mosquito resulted when Cry11Aa interacts with membrane-inserted Cyt1Aa. It has a similar kind of interaction while Cry11Aa binds with its natural receptor. Therefore, it will remain the first instance, of an insect pathogenic bacterium where it's not only generates a toxin but also a functional receptor resulted in improving the binding of toxin and toxicity to target membranes and the mosquito respectively [134].

1.4.7 Applications of Cry toxins

The most three important applications of Bt toxins have been achieved: (i) Controlling of defoliator pests particularly in forestry, (ii) Controlling of the most dangerous deadly diseases (human) producing mosquito vectors, and (iii) Developing transgenic insect-resistant plants. But here, one of our cardinal targets is to kill only the mosquito larvae by the employment of a biological approach.

A proper combination like appropriate timing, weather conditions, and high dosage of spray applications is much essential for application of using Bt. All these factors in combination help to figure out the feasibility of larvae ingesting a lethal dose [136, 137]. The application of Bt for the controlling of mosquitos has resulted in a remarkable decrement in the employment of chemical insecticides to restrict the insects in the forests. As chemical insecticide is harmful to human beings as well as the environment, so it's high time to approach something which should be effective in the control of mosquitoes verse eco-friendly for our future generation.

It's well noted that the effectivity of Bt for the controlling disease vector mosquitoes like *Ae. aegypti* (vector of dengue fever), *Simulium damnosum* (vector of onchocerciasis), and

certain *Anopheles* species (vectors of malaria). The most powerful attribute of Bt with high insecticidal activity cum eco-friendly nature, lack of resistance to toxicity against a non-target organisms and finally the presence of insect-resistant populations to chemical insecticides resulted in accelerated implementation of Bt as an alternative control method of mosquito and black fly populations [129].

At present, by the application Bt protected region is more than 80 % whereas rest of the 20 % region with the chemical larvicide, temephos. This figure will help to successfully control vectors by the application Bt will enhance its operation all around the world. Although there is some limitation for controlling some specific vector-born mosquitoes, particularly Anophelines owing to have the low activity of Bt, demanding the isolation of other strains of Bt along with novel *cry* genes to make it much efficient against these important disease vectors.

For the advancement of microbial-based products with upgraded characteristics, the option is to the consecutive investigation of novel mosquitocidal toxin in conjunction with the high and strategic mechanism of action [138, 139]. Subsequently, after discovering HD 1 isolate resulted in commercial advancement of brand-new products administered to figure out new isolates of Bt as an effective mosquitocidal agent which advances persistant till to date. Bt grabbed the position very promptly as a commercial utilization after its discovery (1977–1982) following a noteworthy instance of a successful biotechnological advancement [140].

From the very beginning, it was always a great concern by using Bti to overcome the resistance that was established by *Simulium* and *Aedes* populations to organophosphates [140-144] and to protect the induction of invasive species, like *Aedes albopictus, Aedes japonicus, and Aedes koreicus* [145-149, 150-153]. For diminishing mosquito proliferation in environmentally restricted areas, Bt is regarded as a danger-free control agent [154-157]. In recent times, the employment of

Bt larvicides has been applied to restrict other species and also in conjunction with other control approaches. For example, Bt is not only used as an *Anopheles* control but also in conjunction with "Long Lasting Insecticide Treated Nets" (LLINS) and "Indoor Residual Spraying" (IRS) [158-164]. The inventive application of Bt along with a unique way like in conjunction with lethal ovitrap to restrict *Aedes* larvae development [175-177] by using "Attractive Toxic Sugar Baits" (ATSB) or sugar patches to target adults [168-170] by the help of spatial spraying to range cryptic breeding sites [138, 171-173]

Nowadays, Bt is one of the most successful formulations that have been applied all around the world in mosquito controlling like Brazil and Germany resulting in minimizing exceeding 90% of the mosquito population without having any detrimental effect on the environment [174]. Even though the employment of recombinant baculoviruses in insect cells for the expression of Cry proteins might be an alternative option for the ample amount of protein production to do functional or structural studies.

1.5 Current development for the expression Cry protein by using a different approach

Bt has been extensively applied in the field of molecular biology and in the agricultural sector for a long period owing to its insecticidal proteins resulting in a valuable environment-friendly bio insecticide. The presence of δ-endotoxins, particularly Cry protein, attributes the insecticidal property to kill the mosquito larvae as an alternative to harmful insecticide and this chemical is responsible for numerous health issues and environmental problems and most remarkably Bt is very species-specific regarding the toxicity. The principal preference of applying Bt as a bio-insecticide of its proven field effectiveness with a long span of time more than four decades without having any report of larval resistance to the insecticidal crystal [138, 175, 176]. So, we should consider the current status of Bt as a microbial insecticide along with the identification of the current problem to make it a more effective bio insecticide. Although Bt acts as an exemplary bio insecticide, there is some complication while storing and stability [177].

Filha *et al.,* (2021) [176] reported it should be more focused on current findings on the mode of action, particularly on the synergistic action of the mechanism of the toxins. Toxin from bacteria like Cyt1Aa with Cry and Bin toxins with synergistic interaction and may lead new insights when it interacts with the midgut cells, can be accomplished in the subsequent time to confer some beneficial effect like broader spectra of action, or to diminish the reduce the chance of resistance selection and to develop the tenacity the persistence covered by field circumstances. In spite of, the outcome of synergistic action has some potential vibe, alternatively, the major hindrance to using of Bt crystals sensitive to abiotic (e.g., photolysis) and biotic factors (e.g., ample amount of organic matter) that interfere with mosquito habitats by shortening their residual impact [178-182].

In the contemporary study, researchers are now stepping ahead by the employment of varieties of Cry protein along with the synergistic action like (Cry and Cyt protein) and also with (Cry and

Vip3 proteins) besides the utilization of only Cry protein or recombinant Cry protein. Cortés *et al*.(2020) [180] reported restricting insect pests by utilizing several groups of Cry and Vip3 proteins with insecticidal properties as well as vectors one and the other in prepared by sprays and in transgenic crops (the so-called Bt crops). However, to restrict the insect pest the application of Cry protein is remarkable, but the mode of action of Cry and Vip3 protein is still unclear.

Bacillus thuringiensis serovar *israelensis* (Bti) is the most extensively used to control the mosquito specifically to restrict the insect vectors of human and animal diseases. Recently genomic study may be the useful tool to investigate the novel insecticidal toxin to boost up or enhancement Cry toxicity or Cry protein expression to develop the potential bio based mosquitocidal agent for killing the mosquito larvae or restrict the insect vectors of human and animal diseases except by the application of Cry protein or the synergism in between Cry and Cyt or Cry and Vip3, It is reported by Alves *et al.,* (2020) [181] that the correlative study of new genomes of Bti driven out in the high integrity of nucleotide sequence indication of an essential outcome for evolutionary studies of this species and remarkable may devote for the development of current scenario or the establishment of an advanced way for the control bio-based that utilize the bacteria, Bt. But at the same time, to further investigate the result and to evaluate the potential differences at transcriptomic/proteomic levels during specific phases (e.g., middle vegetative, early sporulation, and late sporulation) of Bt.

Another essential point must be focused on for the effective expression of Cry toxicity, particularly the domain II loop regions of Cry toxins for the interaction of receptor binding. On the other hand, except Cry toxin, Cyt toxins are also dipteran specific and might play a pivotal part in appearing the Cry toxicity by interaction with membrane lipids. Quintero et al., reported that for mosquitocidal activity along with partial hemolytic, there is evidence influential activity of the

hybrid Cyt1Aa-loop3 proteins was able to show toxicity against mosquitos by doing synergize of Cry11Aa [182]. Bt is the most extensively used biological tool for controlling or killing the mosquito larvae but there is no way to acknowledge some of the constraints owing to have the less residual activity of recent preparation covered by field conditions.[52]. Owing to: (i) declining to the ground of the water body; (ii) impregnation onto silt particles and organic matter; (iii) Converted into nontoxic when depletion by other organisms to0; and (iv) life less by sunlight.

In order to conquer the limitations, the application of δ-endotoxin genes has already been investigated to proper expression in many ways either individually or in association with several Gram-positive and -negative species [183]. Till the most satisfactory outcomes were perceived by the gene expression encoding by the binary toxin of *B*. *sphaericus* in Bti [184]. However, to lines chosen for resistance to the binary toxin, the recombinant bacteria were extremely effective against the fourth instar larvae of *Culex quinquefasciatus* and *Culex Tarsalis*. Recombinant acrystalliferous Bti strain showed the effective toxicity against the fourth-instar larvae of *Cx. quinquefasciatus* that helps to develop the combo effect of *B. sphaericus* binary toxin along with Cyt1Aa and Cry11Ba from Bti and Bt *subsp. jegathesan* respectively [185]

From the past two decades, there are lots of attempts have been applied to improve transgenic mosquito larvicidal cyanobacteria [186]. The most satisfactory outcomes were perceived when *cry4Aa* and *cry11Aa* alone or in combination with Cyt1Aa were expressed by the employment of dual and efficient promoters P_{psbA} and P_{A1} in the filamentous, nitrogen-fixing cyanobacterium *Anabaena* PCC 7120 [187]. For applying Bt with high performance, there is also some evidence of partially inactivate Bti toxicity when the *Anabaena* clone is able to express constitutively *cry4Aa* and *cry11Aa* with *p20* by the irradiation of high doses of UV-B [93].

From the overall discussion, it can be concluded that Bt is an outstanding bio-based insecticidal approach as an alternative to chemical insecticide where there is also some hindrance to proper utilization of Bt as an insecticide. So, addressing of problem can be the key factor to overcome all the limitations to establish the Bt as a potential bio-insecticide to more in depth research for efficient expression of Cry protein as well as Cry toxicity against the larvae of mosquito.

1.6 Action to address the problem

To gain more satisfactory results from Bt, it is more essential to address the problem and accordingly after sorting out the more specific pathway like the efficient expression of Cry protein, stability of Cry protein or mechanism of action and so on should be the main focus according to the research moto. Though Bt as a potential bio insecticidal agent some limitations like storage, stability, inefficient expression of Cry protein particularly in wild type, degradation of Cry protein by UV radiation, excessive sunlight, or heavy rainfall might be the bottleneck to develop the Bt as an unique bio insecticide. Recently it is reported by Derua *et al.,* (2018), the formulation not only will help to stabilize the bio pesticide but also to overcome other trouble like application and handling of the product, prevent from the effect of environment and also the effectivity microbes in the field [188]

But for killing the mosquito larvae the approach should be very goal oriented where the approach should be very handy, efficient expression of Cry protein without any loss of production, without any hassle of purification, and most important alternative to chemical insecticide. So, the present review shortly describes the synopsis of Bt in conjunction with the first-ver approach of LEA (Late embryogenesis abundant) peptide co-expression to develop an effective bio-based mosquitocidal agent for the sustainable environment as well as mankind. So, in this study to improve the efficacy of microbial insecticides by Bt, this study aims to increase the expression of insecticidal proteins in Bt bacteria by LEA peptide co-expression method by the induction of IPTG (isopropyl β-D thiogalactopyranoside) and glucose and without glucose medium with continuous induction of lactose.

Bt is one of the most widely used microorganisms, so from the very beginning till today, many researchers implemented a very useful method to enhance the Cry toxicity to establish such a wonderful mosquitocidal agent as an alternative method to chemical insecticide. LEA peptide co-expression, we have been trying to increase recombinant protein expression in *E. coli* (Ikeno et al, 2013) [189]. In this paper, it was reported that the effect of the increase varied depending on the type of protein. In this study, we challenge for the first time in the world how much the expression of Cry protein by Bt is increased by co-expression of LEA peptide. In addition, it has been reported that there are mutants of LEA peptides that have different effects in increasing expression (Pathak et al., 2017) [190]. In this study, we also determine which mutant peptides have an effect on Cry protein expression. For protein expression and purification, there are so many obstacles to reaching the goal. But in our proposed method, the LEA peptide co-expression system is very simple and handy to operate the system. In this experiment, there is no obstacle to purifying the targeted protein and it is the major bottleneck in the field of protein purification. In the case of Bt it is hard to get an efficient amount of Cry protein from wild-type bacteria to compete with the price of chemical insecticide. LEA peptide co-expression system helps to the efficient expression of Cry protein by the induction of IPTG and lactose to lower down the production cost as a microbial insecticide. So, LEA peptide co-expression system is the first-ever attempt to enhance the Cry protein expression near about 6-fold to act as an alternative to chemical insecticide in the field of biological approached pesticide. It will remarkably be helpful to analyze the deeper concern on the expression of Cry protein associated with the application of LEA peptide co-expression system not only for Cry protein expression but also for another protein expression to input some good output in the field of molecular research.

1.6.1 Strategy and motivation of the study

Proteins are one of the most important biological molecules for life. Choosing an appropriate protein expression system is the key to the success of recombinant protein expression. The expression and production of recombinant proteins by the employment of bacterial expression system always remains the preferred choice as it is a key technology in various fields of research and development. In the field of molecular research and development, co-expression system have developed into a leading scientific interest and focused particularly on desire and the efficiency of production of recombinant protein, and various host cells. With the aim of achieving efficient production of such protein and using the small peptides for the territory of the medical field and diagnostic, health sector with a view to easy access to protein production, cost-benefit ratio, simple technology, and ecofriendly. Recombinant proteins provided important breakthroughs in biomedical biotechnology. Using not only in biomedical research but also in treatment, as drugs and biological science as well. For recombinant protein, high levels of production along with the simple technology that may facilitate the most comprehensive uses both in commercially and the research field. If a technique could be developed to eliminate disturbances in protein expression as well as more specific inherent to the process, the appropriate solution for the large-scale production of recombinant proteins might be conducted. Quality, quantity, and ease of application are some of the principal traits in the case of recombinant co-expression systems. The leading employment of co-expression system and the various fields of biological sciences especially in pharmacology, medical science, food industry, environmental science, drug delivery science, protein engineering, and the study of interaction biomolecules, immunology, agriculture biology, and molecular biology.

For the efficient expression and properties of targeted protein, production of protein, cost-benefit ratio, predetermined operation like crucial points should be considered very carefully. In addition to, for successful or efficient protein expression, still there is prevailing a lot of obstacles, particularly for extensive amount of protein, membrane protein, a nuclear protein, and some other proteins with massive modifications by post-translational process. Different systems have different features and applications that have been used for a long back and undoubtedly, the production of recombinant proteins in microbial systems has revolutionized biochemistry. Still, one of the prominent impediments in the field of protein biology is to yield a satisfactory amount of targeted protein or expression level at a low cost. Every researcher that embarks on a new project that will need a purified protein immediately thinks of how to obtain it in a recombinant form and which organism to use for efficient expression of protein like - bacteria, yeast, filamentous fungi, and unicellular algae or in human cells. In the case of bacterial expression which strain and plasmid should be chosen. Should be the result of multiple combinations of replicons, promoters, and selection markers, multiple cloning sites, and fusion protein/fusion protein removal strategies? Which is the appropriate host or the combination for success for a recombinant co-expression system? What should be the best pathway for efficient expression of the targeted protein? Because from every perspective, the aim and objective of the research are different, and it is very challenging to stipulate any right or exact answer for any of these questions and which is the best strategy that must be worked out to achieve the targeted goal. So, the research project must be started somewhere, and efficient expression level must be followed by the pathway based on the efficacy, probabilities, and special precaution that allows the efficient level of protein expression.

By considering the limitations in the research such as loss of activity during the production of the targeted protein, construction of plasmid, and interference in inefficient expression of the

protein, especially Cry protein expression should be acknowledged for the establishment of peptide-based co-expression system. Cry protein is synthesized by the gram-positive bacteria, Bt during their stationary phase. Naturally, the production of Cry protein is very low in amount and very expensive. Expression of *cry* gene is usually influenced by transcriptional regulation, *cry* gene copy number, the stability of *cry* gene in mRNA, and accumulation and crystallization of Cry protein. Subsequently, obtaining efficient expression of the protein is not an easy task and still, it is competitive with other protein-based commercial products. For efficient expression of Cry protein, different compositions of the medium, temperature, pH, C: N ratio, promoter, Cry toxin modulation are the most used technique that has become the potential pathway by virtue of the ease to manipulate, resilience to practice in various field of research and good biocompatibility. All these pathways enhance the expression of Cry protein but at the same time, it was time-consuming and expensive as well. To compensate for all these difficulties, we should establish in such a technique that will be cost-effective, simple in technique as well as less time consuming for the efficient expression of the protein.

The core purpose of the research is to investigate the efficient expression of Cry protein (Insecticidal protein) by using LEA peptide co-expression system by the employment of a different kind of LEA peptide on the basis of design and characterization. For efficient expression of insecticidal crystal protein, various factors are correlated and also involved. So, it is alluring to postulate that efficient expression of a given *cry* gene in the diverse environmental atmosphere, the growth medium of Bt, the gut environment of the insecticidal larvae like acidic, basic or neutral of its prospective target insects and to some extent one efficient expression system rather than other like sporulation dependent or sporulation independent. In the past, many studies have been made with the aim of developing efficient expression of Cry protein.

Systematic studies on the physiological and nutritional requirements of the organism, especially those needed to obtain a maximum yield of insecticidal crystal protein, are still limited. So, the typical methods which have already been applied belong to the most repeatability or lack of versatility. Even though, in the past, there was no evidence to work on Cry protein in conjugation LEA peptide co-expression system. Apart from this, LEA peptide co-expression system may open a new hope for Cry protein expression as the technique is very simple representing the protein co-expressed with the repetitive sequence of 11 amino acids. Cry protein expression with LEA peptide post period of experiment it is very simple and easy to separate and purify the targeted protein as the co-expressed peptide is very small in size. Hope this research may play a key role in efficient expression of insecticidal Cry protein in Bt by virtue of alternative to the chemical insecticide for mosquito control being an ecofriendly environment.

1.6.2 LEA peptide co-expression system

In the field of protein expression, the most common and conventional approaches are frequently used to boost the quality and quantity of recombinant proteins, such as expression with a hydrophilic protein tag, use of multicopy plasmids, and enhancement of promoter activity. Undoubtedly, the production of recombinant proteins in microbial systems has revolutionized biochemistry. Accordingly, for the extensive amount of heterologous protein production by using several categories of the expression plasmid, several orders of genetically engineered strains, as well as various cultivation approaches could be considered the most extensively used molecular approach. It's already reported by Ikeno *et. al.,* (2013) [189], for an efficient protein expression system by co-expression of LEA peptides to enhance the expression of the recombinant protein

(Ikeno and Haruyama, 2013) [189]. Co-expression of subunits is a valid and immortal technique for the study of protein heterologous complexes.

The selection of host cells is one of the major breakthroughs for protein synthesis machinery that resulted in the high yield of production valuable protein for executing the framework of the total process. *E. coli* is the most widely used host organism owing to have its auspicious traits. (i) The most exceptional rapid growth kinetics. Media including the glucose-salts resulted from the most favorable environmental ambiance to make it twice within 20 minutes [191]. Currently, the most frequently used expression plasmid resulting several combinations of replicons, promoters, selection markers, multiple cloning sites, and fusion protein/fusion protein removal strategies. The reason behind this, the list of most frequently used expression vectors is great in number, and it is hard to figure out or select a suitable one. So, to fulfil our individual desires, it is very essential to take the right decision very sincerely [192].

Previously, the design of LEA peptides sequences was executed for enhancing the expression of target protein through co-expression. According to the trait of LEA peptide is hydrophilic in nature and based on 11-mer residues of group 3 LEA proteins. It's already been investigated by Shinya Ikeno, 2013 that for cellular expression, Green Fluorescent Protein (GFP) is regarded as the targeted protein where the assessment of the expression is very straightforward to determine the fluorescence exclusively without obtaining it from the cell. it is noteworthy that GFP fluorescence based on the time dependent duration in association with co-expression including each variety of LEA peptide having at the interval of one and six recurrences of 11-amino acid units after addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) resulted in the efficient expression of GFP fluorescence exclusively when the co-expression happened only in between11 amino acid with LEA peptide however there is a tendency to reduce the fluorescence intensity

while co-expressing the peptide with longer length. Besides this, the relation between the intensity and the peptide is vice versa and converted into zero because of co-expression in the peptide having six recurrent 11-amino acids. SDS-PAGE is one of the most familiar practices applied to measure the expression level respectively with and without 11amino acids LEA peptide. The expression was efficiently enhanced when GFP is co-expressed with LEA peptide. Finally, the hypotheses admit our investigation that LEA peptides are responsible for the enhancement of transcription action or folding rates of the protein.

By utilizing the knowledge of GFP co-expression system, we attempted to use the LEA peptide co-expression system that can be highly versatile and simple to execute. In this technique, we executed the ease system for the co-expression of protein with only a 13-amino acid peptide. The most difficulties we usually faced in the field of protein purification were exceptionally very handy and straightforward because the co-expressed peptide is minimal. Therefore, it was expected that the outcome of this application should have the appreciable blast not only in the field of bacterial protein expression but also in the field of molecular biology. In this system, our targeted Cry protein was increased exclusively by the employment of LEA peptide co-expression system and is the ever first expression of Cry protein by using this LEA peptide co-expression system.

To resolve this problem, research has been directed towards the enhancement of Cry protein expression by the application of different kinds of LEA peptide that was designed from the original LEA-I: MDAKDGTKEKAGE has described in detail in chapter 2. According to the high advent of LEA peptide co-expression system, although the expression level was satisfactory it was not up to the mark in comparison to the commercial perspective. The 11-mer motif of G3LEA proteins with their high hydrophilicity and high glycine, glutamic acid, lysine, and threonine content at the C-terminal portion was ideal for efficient expression of the target protein. Although the LEA

peptide co-expression system is enhancing the expression level how it is enhancing inside is still remains unclear.

1.7 Organization of present thesis

This research investigates the way of Cry protein expression and Cry toxicity by the conjugation of LEA peptide co-expression system in spite of this Bt has the capability to synthesize the Cry protein but to boost up the expression of Cry protein demand the employment of LEA peptide co-expression system. Through this research, we investigated the characterization of Bt. D142 for efficient expression of Cry protein including the most fundamental trait, spore-forming time, and Cry protein. This attempt had been directed towards the development of new technologies by the employment of (LEA) peptide co-expression system for efficient expression of Cry protein by the induction of IPTG and with glucose medium and lactose as an inducer as well. In the part of this paper, we took the consideration how to pursue it commercially to lower the production cost in comparison to the chemical insecticide that can make sure the alternative to chemical insecticide and eco-friendly environment for the better world. The structure of this paper consists of four chapters. Figure 1-4 shows a diagrammatic representation of the structure of this thesis. The structure and purpose of this paper are shown below.

The first chapter characterized the introduction and background of the study. In the second chapter, we designed two forms of LEA peptide (LEA-II and LEA-K) based on the sequence of group-3 LEA protein, which consists of a repetitive sequence of 11 amino acids. After analyzing the result, Bt-LEA-II mediated co-expression system demonstrated the maximum expression of crystal protein owing to have 3-fold after induction of IPTG with concentration 0.5 mM IPTG at 12^{th} hour. The enhanced expression level of crystal protein was authenticated by bioassay, operating 4th instar larvae of *Aedes albopictus*. Although this unique approach has a great potential to produce -based insecticidal agents by the enhancement of crystal protein it has some limitations. Application of IPTG as an inducer in the commercial purpose of industrialization will have some

difficulties in operational management during the fermentation process and also economical viewpoint will be much overpriced. Then we move forward to the option which should be replacement of IPTG along with more advantageous.

Thereafter, in the third chapter, we designed our experimental framework by utilizing the alternative to IPTG is lactose monohydrate inducer and glucose medium. So, for bacterial fermentation carbon sources can be the key factors to produce spore and crystal proteins as well and finally have a pivotal role in the expression of Cry protein. Without carbon or glucose is there any impact on spore formation, Cry protein expression, and finally for LEA peptide co-expression system. Bacteria would like to utilize only glucose as a priority and then move to lactose when glucose runs out. Since long back, lactose monohydrate has also adopting as an inducer by the trait of the utmost possible and common substitute of IPTG in the development of fermentation process for the recombinant protein expression. In this study, we would like to determine the outcome of lactose monohydrate as an alternative inducer to IPTG to check the optimal protein expression in Bt induced LEA peptide co-expression system where lactose serves either as a carbon source or inducer in opposition to, excessive IPTG has the tendency to become toxic for the cell or aggregation of the cell. So, we designed our experimental strategy in such where it will fulfill the requirement of targeted protein expression along with the consideration of low-cost effects in the industrial operation. In this study, for targeted protein expression we designed three forms of LEA peptide like Bt-LEA-I, Bt-LEA-E & Bt-LEA-K by following the same procedure in chapter 2. Then we analyzed the result with the help of SDS-PAGE and for final confirmation glucose and lactose detection in the future to investigate the targeted protein expression that was amplified by lactose monohydrate based on the composition of media, dosage concentration, addition timing, and amount of addition. Continuous addition by 0.1 mol/L (10 μL with each hour interval till 5

hours) and 50 µL at a time to obtain the best result. Continuous addition of 10 µL was more effective as the bacterial cell can use more efficiently for the cell growth and ultimately for the targeted protein expression. Targeted protein expression of lactose was compared to IPTG where the protein expressed in Bt. D142 synthesized crystal protein.

The final chapter summarizes this paper and provides future perspectives on the future of insecticides based on the insecticidal proteins of Bt.

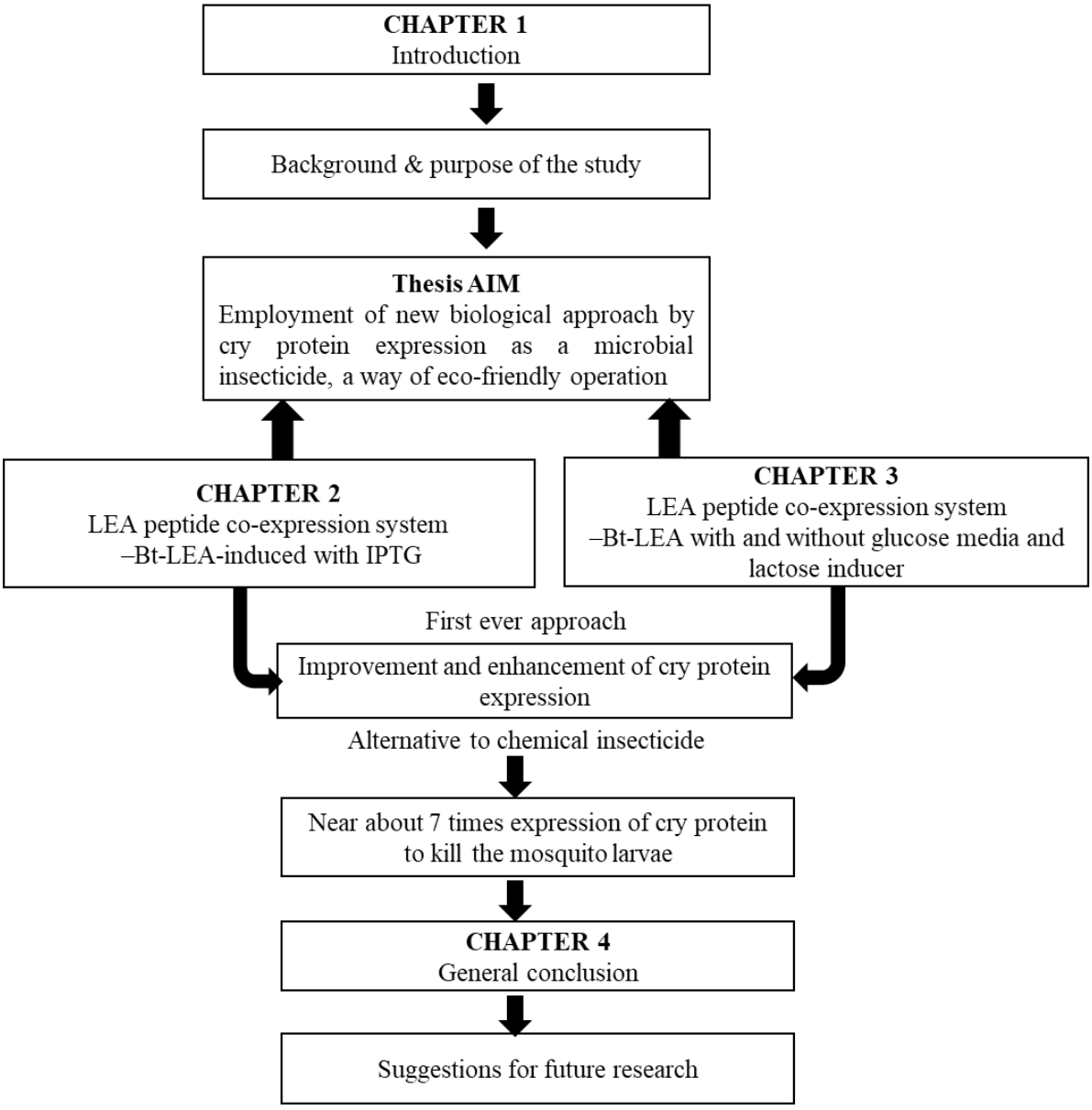

Figure 1-4 Schematic diagram of this Ph.D. thesis

1.8 Purpose of this study:

1. To establish a new system or technologies by using a unique method of LEA peptide co-expression system with Bt (Chapter 2)
2. To check the expression level of Bt synthesized Cry protein where it can perform as an alternative to chemical insecticide by competing for the price. (Chapter 2)
3. To demonstrate that lactose can also be an alternative inducer to IPTG for efficient expression of Cry protein. (Chapter 3)
4. To evaluate the influences of culture conditions such as on different dosage concentration, time interval, and amount of inducer can be considered some key points to enhance the LEA peptide expression. (Chapter 3)

References

1. Gates notes The blog of Bill Gates, “The Deadliest Animal in the World”https://www.gatesnotes.com/Health/Most-Lethal-Animal-Mosquito-Week
2. Knowlton, K., Rotkin-Ellman, M., Soloman, G., (2009) Mosquito-Borne dengue fever threat spreading in the Americas. Natural Resources Defense Council.
3. Bravo, A., Gill, S.S., Soberon, M., (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49, 423-435.
4. Cuervo-Parra, J.A., Cortés, T.R., Ramirez-Lepe, M., (2016) Mosquito-borne diseases, pesticides used for mosquito control, and development of resistance to insecticides. Insecticides resistance. Rijeka: InTechOpen, 111-134.
5. Malaria, R.B., (2005) World malaria report 2005. World Health Organization and UNICEF.
6. Donalisio, M.R., Freitas, A.R.R., (2015) Chikungunya in Brazil: an emerging challenge. Brazilian Journal of Epidemiology 18, 283-285.
7. Gubler, D.J., (1998) Dengue and dengue hemorrhagic fever. Clinical Microbiology 11, 480-496.
8. Gubler, D.J., (1998) Resurgent vector-borne diseases as a global health problem. Emerging Infectious Diseases 4, 442.
9. Honório, N.A., Câmara, D.C.P., Calvet, G.A., (2015) Chikungunya: an arbovirus infection in the process of establishment and expansion in Brazil Chikungunya: an arbovirus in establishment and expansion in Brazil Chikungunya: an arboviruses in establishment and. Public Health Cad 31, 1-3.
10. Camara, T., (2016) Emerging arboviruses, and new challenges for public health in Brazil. Rev Saude Publica 50, 1-7.

11. Vasconcelos, P.d.C., (2015) Zika virus disease: is it a new emerging problem in the Americas. Rev. PanAmaz. Health 6, 9-10.
12. Kotsakiozi, P., Richardson, J.B., Pichler, V., Favia, G., Martins, A.J., Urbanelli, S., Armbruster, P.A., Caccone, A., (2017) Population genomics of the Asian tiger mosquito, *Aedes albopictus*: insights into the recent worldwide invasion. Ecology and Evolution 7, 10143-10157.
13. Parry, J.M., Turnbull, P.C.B., Gibson, J.R., (1983) A color atlas of Bacillus species. Wolfe Medical Publications Ltd.
14. Weaver, S.C., Lecuit, M., (2015) Chikungunya virus and the global spread of a mosquito-borne disease. New England Journal of Medicine 372, 1231-1239.
15. Sneath, P.H., Mair, N.S., Sharpe, M.E., Holt, J.G., (1986) Bergey's Manual of Systematic Bacteriology. Volume 2. Williams & Wilkins.
16. Priest, F.G., (1993) Systematics and ecology of *Bacillus*. *Bacillus subtilis* and other gram-positive bacteria: Biochemistry, Physiology, and Molecular Genetics, 1-16.
17. Bavykin, S.G., Lysov, Y.P., Zakhariev, V., Kelly, J.J., Jackman, J., Stahl, D.A., Cherni, A., (2004) Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. Journal of Clinical Microbiology 42, 3711-3730.
18. Ibrahim, M.A., Griko, N., Junker, M., Bulla, L.A., (2010) *Bacillus thuringiensis*: a genomics and proteomics perspective. Bioengineered Bugs 1, 31-50.
19. Garbeva, P., Van Veen, J., Van Elsas, J., (2003) Predominant *Bacillus spp.* in agricultural soil under different management regimes detected via PCR-DGGE. Microbial Ecology 45, 302-316.

20. Lacey, L., Grzywacz, D., Shapiro-Ilan, D., Frutos, R., Brownbridge, M., Goettel, M., (2015) Insect pathogens as biological control agents: back to the future. Journal of Invertebrate Pathology 132, 1-41.

21. Drobniewski, F., (1993) Endogenous Candida endophthalmitis. Journal of the Royal Society of Medicine 86, 370.

22. Kotiranta, A., Lounatmaa, K., Haapasalo, M., (2000) Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes and Infection 2, 189-198.

23. . Bottone, E.J., (2010) *Bacillus cereus*, a volatile human pathogen. Clinical Microbiology Reviews 23, 382-398.

24. Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., (2003) Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature 423, 87.

25. Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolstø, A.-B., (2000) *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Applied and Environmental Microbiology 66, 2627-2630.

26. Ticknor, L.O., Kolstø, A.-B., Hill, K.K., Keim, P., Laker, M.T., Tonks, M., Jackson, P.J., (2001) Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates Applied and Environmental Microbiology 67, 4863-4873.

27. Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F., Bourguet, D., (2002) Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. Applied and Environmental Microbiology 68, 1414-1424.

28. Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., (2011) *Bacillus thuringiensis*: a story of a successful bio insecticide. Insect Biochemistry and Molecular Biology 41(7), 423-431.
29. Carroll, L.M., Cheng, R.A., Kovac, J., (2020) No Assembly Required: Using btyper3 to assess the congruency of a proposed taxonomic framework for the *Bacillus cereus* group with historical typing methods. Frontiers in Microbiology 11, 2285.
30. Ramisse, V., Patra, G., Garrigue, H., Guesdon, J.-L., Mock, M., (1996) Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiology Letters 145, 9-16.
31. Mock, M., Fouet, A., (2001) Anthrax. Annual Reviews in Microbiology 55, 647-671
32. de Barjac, H., Bonnefoi, A., (1962) Test for biochemical and serological classification of 24 strains of *Bacillus* type *B. thuringiensis*. Entomophaga 7, 5-31.
33. Krieg, A. (1987). Diseases caused by bacteria and other prokaryotes, P. 323-355. In. J.R. Fuxa and Y. Tanada (ed), *Epizootiology* of Insect Disease. John Wiley and Sons, New York.
34. Delécluse, A., Barloy, F., Rosso, M.-L., (1996) Pathogenic bacteria of diptera larvae: structure and specificity of toxins. Annals of the Institute Pasteur / News. Elsevier, pp. Life cycle of *Bacillus thuringiensis*
35. Lecadet, M.M., Frachon, E., Dumanoir, V.C., Ripouteau, H., Hamon, S., Laurent, P., Thiery, I., (1999) Updating the H-antigen classification of *Bacillus thuringiensis*. Journal of Applied Microbiology 86, 660-672.
36. Ishiwata S (1901) On a new type of severe flacherie (sotto disease) (original in Japanese). Dainihon Sansi Kaiho 114:1–5

37. Ishiwata S (1905a) Concerning 'Sotto-Kin' a *Bacillus* of a disease of the silkworm. Report Association Series, Japan 160:1–8

38. Ishiwata S (1905b) About "Sottokin," a *Bacillus* of a disease of the silk-worm. Dainihon Sanshi Kaiho (Report Association Series, Japan) 161:1–5

39. Berliner, E., (1915) On the drowsiness of the meal moth caterpillar (Ephestia kühniella Zell.) And its pathogen *Bacillus thuringiensis* n. Sp. Journal of Applied Entomology 2, 29-56.

40. La Duc, M.T., Satomi, M., Agata, N., Venkateswaran, K., (2004) gyrB as a phylogenetic discriminator for members of the *Bacillus anthracis–cereus–thuringiensis* group. Journal of Microbiological Methods 56, 383-394.

41. Soufiane B, Côté J-C (2009) Discrimination among *Bacillus thuringiensis* H serotypes, serovars and strains based on 16S rRNA, gyrB and aroE gene sequence analyses. Antonie Van Leeuwenhoek 95:33–45

42. Vidal-Quist, J.C., Rogers, H.J., Mahenthiralingam, E., Berry, C., (2013) *Bacillus thuringiensis* colonizes plant roots in a phylogeny-dependent manner. FEMS Microbiology Ecology 86, 474-489.

43. Bhandari, V., Ahmod, N.Z., Shah, H.N., Gupta, R.S., (2013) Molecular signatures for *Bacillus* species: demarcation of the *Bacillus subtilis* and *Bacillus cereus* clades in molecular terms and proposal to limit the placement of new species into the genus *Bacillus*. International Journal of Systematic and Evolutionary Microbiology 63, 2712-2726.

44. Prabhakar, A., Bishop, A., (2014) Comparative studies to assess bacterial communities on the clover phylloplane using MLST, DGGE and T-RFLP. World Journal of Microbiology and Biotechnology 30, 153-161.

45. Caamaño-Antelo, S., Fernández-No, I., Böhme, K., Ezzat-Alnakip, M., Quintela-Baluja, M., Barros-Velázquez, J., Calo-Mata, P., (2015) Genetic discrimination of food borne pathogenic and spoilage *Bacillus spp*. based on three housekeeping genes. Food Microbiology 46, 288-29

46. Milner, R.J., (1994) History of *Bacillus thuringiensis*. Agriculture, Ecosystems & Environment 49, 9-13.

47. Sansinenea, E., (2012) Discovery and description of *Bacillus thuringiensis*. *Bacillus thuringiensis* biotechnology. Springer, pp. 3-18.

48. Mattes, O. (1927) Parasitic diseases of the flour moth larvae and experiments on their use as biological agents. Seat area Total carrier Ges. Naturw. Marburg. 62, 381-417

49. Glazer, A.N., Nikaido, H., (2007) Microbial biotechnology: Fundamentals of Applied Microbiology. Cambridge University Press.

50. Husz, B., (1930) Field Experiments on the Application of *Bacillus thuringiensis* against the Corn Borer. International Corn Borer Invest Scientific Report 1927-28 3.

51. Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P., (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnology Journal 9, 283-300.

52. Margalith Y, Ben-Dov E (2000) Biological control by *Bacillus thuringiensis subp. israelensis.* In: Rechcigl JE, Rechcigl NA (eds) Insect pest management, techniques for environmental protection. Lewis Publishers, Boca Raton

53. Steinhaus EA (1951) Possible use of *Bacillus thuringiensis* Berliner as an aid in the biological control of the alfalfa caterpillar. Hilgardia 20:359–381

54. Delucca, A., AJ II, D., (1979) Two new serovars of *Bacillus thuringiensis*: serovars dakota and indiana (serovars 15 and 16).

55. Dulmage, H.T. and Aizawa, K., (1982). Distribution of *Bacillus thuringiensis* in nature. In: E. Kurstak (Editor), Microbial and Viral Pesticides. Marcel Dekker, New York, pp. 209-237

56. Dulmage, H.T., (1970). Insecticidal activity of HD-I, a new isolate of *Bacillus thuringiensis var. alesti.* Journal of Invertebrate Pathology. 15: 232-239.

57. Goldberg LJ, Margalit J (1977) A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univeritattus*, *Aedes aegypti*, and *Culex pipiens*. Mosq News 37:355–358

58. Kaelin, P., Morel, P., Gadani, F., (1994) Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricorne* (F.). Applied and Environmental Microbiology 60, 19-25.

59. Schnepf, H.E., Whiteley, H., (1981) Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proceedings of the National Academy of Sciences 78, 2893-2897.

60. Raddadi, N., Crotti, E., Rolli, E., Marasco, R., Fava, F., Daffonchio, D., (2012) The most important *Bacillus* species in biotechnology. *Bacillus thuringiensis* Biotechnology. Springer, pp. 329-345.

61. Höfte, H., Whiteley, H., (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiological Reviews 53, 242-255.

62. Raymond, B., Johnston, P.R., Nielsen-LeRoux, C., Lereclus, D., Crickmore, N., (2010) *Bacillus thuringiensis*: an impotent pathogen? Trends in Microbiology 18, 189-194.

63. Roh, J.-Y., Choi, J.-Y., Li, M.-S., Jin, B.-R., Je, Y.-H., (2007) *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. Journal of Microbiology and Biotechnology 17, 547-559.

64. Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., (2014) *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins 6, 3296-3325.

65. Elleuch, J., Jaoua, S., Ginibre, C., Chandre, F., Tounsi, S., Zghal, R.Z., (2016) Toxin stability improvement and toxicity increase against dipteran and lepidopteran larvae of *Bacillus thuringiensis* crystal protein Cry2Aa. Pest Management Science 72, 2240-2246.

66. Bravo, A., Gill, S.S., Soberón, M., (2016) *Bacillus thuringiensis*: mechanisms and use. Insect Biochemistry and Molecular Biology 20, 222-231.

67. Likitvivatanavong, S., Chen, J., Evans, A.M., Bravo, A., Soberon, M., Gill, S.S., (2011) Multiple receptors as targets of Cry toxins in mosquitoes. Journal of Agricultural and Food Chemistry 59, 2829-2838.

68. Pardo-Lopez, L., Soberon, M., Bravo, A., (2013) *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiology 37, 3-22.

69. Vachon, V., Laprade, R., Schwartz, J.-L., (2012) Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. Journal of Invertebrate Pathology 111, 1-12.

70. Bechtel, D.B., Bulla Jr, L.A., (1982) Ultrastructural analysis of membrane development during *Bacillus thuringiensis* sporulation. Journal of Ultrastructure Research 79, 121-132.

71. Young, M., Mandelstam, J., (1980) Early events during bacterial endospore formation. Advances in Microbial Physiology 20, 103-162.

72. Itoua-Apoyolo, C., Drif, L., Vassal, J.-M., Debarjac, H., Bossy, J., Leclant, F., Frutos, R., (1995) Isolation of multiple subspecies of *Bacillus thuringiensis* from a population of the European sunflower moth, *Homoeosoma nebulella*. Applied and Environmental Microbiology 61, 4343-4347.

73. Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D., Roberts, G., Pauli, S., Rodgers, P., Burges, H., (1997) Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests. Journal of Invertebrate Pathology 70, 59-68.

74. Chaufaux, J., Marchal, M., Gilois, N., Jehanno, I., Buisson, C., (1997) Research on natural strains of *Bacillus thuringiensis* in different biotopes throughout the world. Canadian Journal of Microbiology 43, 337-343.

75. Martin, P.A., Travers, R.S., (1989) Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Applied and Environmental Microbiology 55, 2437-2442.

76. Carozzi, N.B., Kramer, V.C., Warren, G.W., Evola, S., Koziel, M.G., (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Applied and Environmental Microbiology 57, 3057-3061.

77. Hastowo, S., Lay, B., Ohba, M., (1992) Naturally occurring *Bacillus thuringiensis* in Indonesia. Journal of Applied Bacteriology 73, 108-113.

78. Smith, R.A., Couche, G.A., (1991) The phylloplane as a source of *Bacillus thuringiensis* variants. Applied and Environmental Microbiology 57, 311-315.

79. Burges, H., Hurst, J., (1977) Ecology of *Bacillus thuringiensis* in storage moths. Journal of Invertebrate Pathology 30, 131-139.

80. Delucca, A., Palmgren, M., Barjac, H.d., (1984) A new serovar of *Bacillus thuringiensis* from grain dust: *Bacillus thuringiensis* serovar colmeri (serovar 21) [Insect pathogen]. Journal of Invertebrate Pathology (USA).

81. Meadows, M.P., Ellis, D.J., Butt, J., Jarrett, P., Burges, H.D., (1992) Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. Applied and Environmental Microbiology 58, 1344-1350.

82. Sun, C., Zhang, Q., Xu, J., Wang, Y., Zhou, M., (2003) Effects of sub-lethal dose of Bt on the parasitization and development of Campoletis chlorideae Uchida. Chinese Journal of Biological Control 19, 110-113.

83. Ramirez-Romero, R., Desneux, N., Decourtye, A., Chaffiol, A., Pham-Delègue, M., (2008) Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (*Hymenoptera, Apidae*)? Ecotoxicology and Environmental Safety 70, 327-333.

84. Maeda, M., Mizuki, E., Nakamura, Y., Hatano, T., Ohba, M., (2000) Recovery of *Bacillus thuringiensis* from marine sediments of Japan. Current Microbiology 40, 418-422.

85. Zhang MY, Lovgren A, Lauden R (1995) Adhesion and cytotoxicity of *Bacillus thuringiensis* to cultured Spodoptera and Drosophila cells. Journal of Invertebrate Pathology 66:4651.

86. Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D., Dean, D., (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews 62, 775-806.

87. Feitelson, J.S., Payne, J., Kim, L., (1992) *Bacillus thuringiensis*: insects and beyond. Bio/Technology 10, 271-275.

88. Adang, M.J., Crickmore, N., Jurat-Fuentes, J.L., 2014. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In: Dhadialla, T.S., Gill, S.S. (Eds.), Advances in Insect Physiology 47. Oxford: Academic Press, p. 39.

89. Keshavareddy, G., Kumar, A., (2016) *Bacillus thuringiensis*. Ecofriendly pest management for food security. Elsevier, pp. 443-473.

90. Crickmore, N., Zeigler, D., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A., Dean, D., (2016) *Bacillus thuringiensis* toxin nomenclature. Available on line http://www. Bt nomenclature. info.

91. de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., Schnepf, H.E., (2003) Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annual Review of Genetics 37, 409-433.

92. Van Frankenhuyzen, K., (2009) Insecticidal activity of *Bacillus thuringiensis* crystal proteins. Journal of Invertebrate Pathology 101, 1-16.

93. Ben-Dov, E., (2014) *Bacillus thuringiensis subsp. israelensis* and its dipteran-specific toxins. Toxins 6, 1222-1243.

94. Chougule, N.P., Bonning, B.C., (2012) Toxins for transgenic resistance to hemipteran pests. Toxins 4, 405-429.

95. van Frankenhuyzen, K., (2013) Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. Journal of Invertebrate Pathology 114, 76-85.

96. Ali, B., Salem, H., Wang, X., Huang, T., Xie, Q., Zhang, X., (2010) Effect of *Bacillus thuringiensis* var. *israelensis* endotoxin on the intermediate snail host of *Schistosoma japonicum.* Current Research in Bacteriology 3, 37-41.

97. Bravo, A., Soberón, M., (2008) How to cope with insect resistance to Bt toxins? Trends in Biotechnology 26, 573-579.

98. Ohba, M., Mizuki, E., Uemori, A., (2009) Parasporin, a new anticancer protein group from *Bacillus thuringiensis*. Anticancer Research 29, 427-433.

99. Ekino, K., Okumura, S., Ishikawa, T., Kitada, S., Saitoh, H., Akao, T., Oka, T., Nomura, Y., Ohba, M., Shin, T., (2014) Cloning and characterization of a unique cytotoxic protein parasporin-5 produced by *Bacillus thuringiensis* A1100 strain. Toxins 6, 1882-1895.

100. Sajid, M., Geng, C., Li, M., Wang, Y., Liu, H., Zheng, J., Peng, D., Sun, M., (2018) Whole-genome analysis of B*acillus thuringiensis* revealing partial genes as a source of novel cry toxins. Applied and Environmental Microbiology 84, e00277-00218.

101. Jouzani, G.S., Valijanian, E., Sharafi, R., (2017) *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. Applied Microbiology and Biotechnology 101, 2691-2711.

102. Yudina, T.G., Brioukhanov, A.L., Zalunin, I.A., Revina, L.P., Shestakov, A.I., Voyushina, N.E., Chestukhina, G.G., Netrusov, A.I., (2007) Antimicrobial activity of different proteins and their fragments from *Bacillus thuringiensis* parasporal crystals against clostridia and archaea. Anaerobe 13, 6-13.

103. Li, J., Koni, P.A., Ellar, D.J., (1996) Structure of the Mosquitocidal δ-Endotoxin CytB from *Bacillus thuringiensis*sp. Kyushuensis and Implications for Membrane Pore Formation. Journal of Molecular Biology 257, 129-152.

104. Li, J., Carroll, J., Ellar, D.J., (1991) Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature 353, 815-821.

105. Crickmore, N., Zeigler, D., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D., (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiology and Molecular Biology Reviews 62, 807-813.

106. Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.-L., Brousseau, R., Cygler, M., (1995) *Bacillus thuringiensis* CrylA (a) insecticidal toxin: crystal structure and channel formation. Journal of Molecular Biology 254, 447-464

107. Morse, R.J., Yamamoto, T., Stroud, R., (2001) Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure 9, 409-417.

108. Galitsky, N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J.R., Pangborn, W., English, L., (2001) Structure of the insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. Acta Crystallographica Section D: Biological Crystallography 57, 1101-1109.

109. Boonserm, P., Davis, P., Ellar, D.J., Li, J., (2005) Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. Journal of Molecular Biology 348, 363-382.

110. Boonserm, P., Mo, M., Angsuthanasombat, C., Lescar, J., (2006) Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. Journal of Bacteriology 188, 3391-3401.

111. Gilbert, L.I., Iatrou, K., Gill, S.S., (2005) Comprehensive molecular insect science. Elsevier. *Bacillus thuringiensis* Mechanisms and Use; pp. 175–206.

112. Griffitts, J.S., Haslam, S.M., Yang, T., Garczynski, S.F., Mulloy, B., Morris, H., Cremer, P.S., Dell, A., Adang, M.J., Aroian, R.V., (2005) Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science 307, 922-925.

113. Dai, S.-m., Gill, S.S., (1993) In vitro and in vivo proteolysis of the *Bacillus thuringiensis subsp. israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. Insect Biochemistry and Molecular Biology 23, 273-283.

114. Angsuthanasombat, C., Crickmore, N., Ellar, D.J., (1993) Effects on toxicity of eliminating a cleavage site in a predicted interhelical loop in *Bacillus thuringiensis* CryIVB δ-endotoxin. FEMS Microbiology Letters 111, 255-261.

115. Komano, T., Yamagiwa, M., Nishimoto, T., Yoshisue, H., Tanabe, K., Sen, K., Sakai, H., (1998) Activation process of the insecticidal proteins cryiva and cryivb produced by *Bacillus thuringiensis subsp. israelensis*. Israel Journal of Entomology 32, 185-198.

116. Armstrong, J.L., Rohrmann, G.F., Beaudreau, G.S., (1985) Delta endotoxin of *Bacillus thuringiensis subsp*. *israelensis.* Journal of Bacteriology 161, 39-46.

117. Gill. Gill, S.S., Singh, G., Hornung, J., (1987) Cell membrane interaction of *Bacillus thuringiensis subsp*. *israelensis* cytolytic toxins. Infection and Immunity 55, 1300-1308.

118. Promdonkoy, B., Ellar, D.J., (2003) Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from *Bacillus thuringiensis*. Biochemical Journal 374, 255-259.

119. Butko, P., (2003) Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. Applied and Environmental Microbiology 69, 2415-2422.

120. Koni, P.A., Ellar, D.J., (1994) Biochemical characterization of *Bacillus thuringiensis* cytolytic δ-endotoxins. Microbiology 140, 1869-1880.

121. Thomas, W., Ellar, D., (1983) Mechanism of action of *Bacillus thuringiensis var israelensis* insecticidal δ-endotoxin. FEBS letters 154, 362-368.

122. Fernández, L.E., Pérez, C., Segovia, L., Rodríguez, M.H., Gill, S.S., Bravo, A., Soberón, M., (2005) Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II. FEBS Letters 579, 3508-3514.

123. Fernandez, L.E., Aimanova, K.G., Gill, S.S., Bravo, A., Soberón, M., (2006) A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochemical Journal 394, 77-84.

124. Abdullah, M.A.F., Valaitis, A.P., Dean, D.H., (2006) Identification of a *Bacillus thuringiensis* Cry11Ba toxin-binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. BMC Biochemistry 7, 1-6.

125. Buzdin, A., Revina, L., Kostina, L., Zalunin, I., Chestukhina, G., (2002) Interaction of 65-and 62-kD proteins from the apical membranes of the *Aedes aegypti* larvae midgut epithelium with Cry4B and Cry11A endotoxins of *Bacillus thuringiensis*. Biochemistry (Moscow) 67, 540-546.

126. Darboux, I., Nielsen-LeRoux, C., Charles, J.-F., Pauron, D., (2001) The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens (*Diptera: Culicidae) midgut: molecular cloning and expression. Insect Biochemistry and Molecular Biology 31, 981-990.

127. Zhang, X., Candas, M., Griko, N.B., Taussig, R., Bulla, L.A., (2006) A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. Proceedings of the National Academy of Sciences 103, 9897-9902.

128. Wirth, M., Georghiou, G., Federici, B., (1997) CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. Proceedings of the National Academy of Sciences 94, 10536-10540.

129. Chilcott, C.N., Ellar, D.J., (1988) Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. Microbiology 134, 2551-2558.

130. Dambach, P., Baernighausen, T., Traoré, I., Ouedraogo, S., Sié, A., Sauerborn, R., Becker, N., Louis, V.R., (2019) Reduction of malaria vector mosquitoes in a large-scale intervention trial in rural Burkina Faso using Bti based larval source management. Malaria Journal 18, 1-9.

131. Angsuthanasombat, C., Crickmore, N., Ellar, D.J., (1992) Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. FEMS Microbiology Letters 94, 63-68.

132. Georghiou, G.P., Wirth, M.C., (1997) Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Applied and Environmental Microbiology 63, 1095-1101.

133. Pérez, C., Fernandez, L.E., Sun, J., Folch, J.L., Gill, S.S., Soberón, M., Bravo, A., (2005) *Bacillus thuringiensis subsp. israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proceedings of the National Academy of Sciences 102, 18303-18308.

134. van Frankenhuyzen, K., (2000) Application of *Bacillus thuringiensis* in forestry. Entomopathogenic bacteria: from laboratory to field application. Springer, pp. 371-382.

135. Bauce, É., Carisey, N., Dupont, A., van Frankenhuyzen, K., (2004) *Bacillus thuringiensis* subsp. *kurstaki* aerial spray prescriptions for balsam fir stand protection against spruce budworm (Lepidoptera: Tortricidae). Journal of Economic Entomology 97, 1624-1634.

136. Buentello Wong, S., (2016) Evaluation of the toxic effect of the parasporal crystal-essential oil set in the "Mexican fruit fly", *Anastrepha ludens* L. (Diptera: tephritidae). Autonomous University of Nuevo León.

137. Berry, C., Crickmore, N., (2017) Structural classification of insecticidal proteins–towards an in silico characterization of novel toxins. Journal of Invertebrate Pathology 142, 16-22.

138. Lacey, L.A., (2007) *Bacillus thuringiensis serovariety israelensis* and *Bacillus sphaericus* for mosquito control. Journal of the American Mosquito Control Association 23, 133-163.

139. de Araujo, A.P., de Melo-Santos, M.A.V., de Oliveira Carlos, S., Rios, E.M.M.M., Regis, L., (2007) Evaluation of an experimental product based on *Bacillus thuringiensis* sorovar. *israelensis* against *Aedes aegypti* larvae (Diptera: Culicidae). Biological Control 41, 339-347.

140. Hougard, J.-M., Sékétéli, A., (1998) Combating onchocerciasis in Africa after 2002: the place of vector control. Annals of Tropical Medicine and Parasitology 92, S165-S166.

141. Pocquet, N., Darriet, F., Zumbo, B., Milesi, P., Thiria, J., Bernard, V., Toty, C., Labbé, P., Chandre, F., (2014) Insecticide resistance in disease vectors from Mayotte: an opportunity for integrated vector management. Parasites & Vectors 7, 1-12.

142. Tissera, H., Samaraweera, P., Jayamanne, B., Janaki, M., U Chulasiri, M., Rodrigo, C., Fernando, S., (2018) Use of *Bacillus thuringiensis israelensis* in integrated vector control of Aedes sp. in Sri Lanka: a prospective controlled effectiveness study. Tropical Medicine & International Health 23, 229-235.

143. Baldacchino, F., Caputo, B., Chandre, F., Drago, A., della Torre, A., Montarsi, F., Rizzoli, A., (2015) Control methods against invasive Aedes mosquitoes in Europe: a review. Pest Management Science 71, 1471-1485.

144. Eritja, R., (2013) Laboratory tests on the efficacy of VBC60035, a combined larvicidal formulation of *Bacillus thuringiensis israelensis* (strain AM65-52) and *Bacillus sphaericus* (strain 2362) against *Aedes albopictus* in simulated catch basins. Journal of the American Mosquito Control Association 29, 280-283.

145. Flacio, E., Engeler, L., Tonolla, M., Lüthy, P., Patocchi, N., (2015) Strategies of a thirteen year surveillance programme on *Aedes albopictus* (*Stegomyia albopicta*) in southern Switzerland. Parasites & vectors 8, 1-18.

146. Guidi, V., Lüthy, P., Tonolla, M., (2013) Comparison between Diflubenzuron and a *Bacillus thuringiensis israelensis* and *Lysinibacillus sphaericus*–Based Formulation for the Control of Mosquito Larvae in Urban Catch Basins in Switzerland. Journal of the American Mosquito Control Association 29, 138-145.

147. Ibañez-Justicia, A., Teekema, S., Den Hartog, W., Jacobs, F., Dik, M., Stroo, A., (2018) The effectiveness of Asian bush mosquito (*Aedes japonicus japonicus*) control actions in colonized peri-urban areas in the Netherlands. Journal of Medical Entomology 55, 673-680.

148. Reuss, F., Kreß, A., Braun, M., Magdeburg, A., Pfenninger, M., Müller, R., Mehring, M., (2020) Knowledge on exotic mosquitoes in Germany, and public acceptance and effectiveness of Bti and two self-prepared insecticides against *Aedes japonicus japonicus*. Scientific Reports 10, 1-14.

149. Williams, G.M., Faraji, A., Unlu, I., Healy, S.P., Farooq, M., Gaugler, R., Hamilton, G., Fonseca, D.M., (2014) Area-wide ground applications of *Bacillus thuringiensis var. israelensis* for the control of *Aedes albopictus* in residential neighborhoods: from optimization to operation. PLoS One 9, e110035.

150. Suter, T., Flacio, E., Fariña, B.F., Engeler, L., Tonolla, M., Müller, P., (2015) First report of the invasive mosquito species *Aedes koreicus* in the Swiss-Italian border region. Parasites & Vectors 8, 1-4.

151. Suter, T.T., Flacio, E., Feijoó Fariña, B., Engeler, L., Tonolla, M., Regis, L.N., de Melo Santos, M.A., Müller, P., (2016) Surveillance and control of *Aedes albopictus* in the Swiss-Italian border region: differences in egg densities between intervention and non-intervention areas. PLoS Neglected Tropical Diseases 10, e0004315.

152. Dritz, D.A., Lawler, S.P., Evkhanian, C., Graham, P., Baracosa, V., Dula, G., (2011) Control of mosquito larvae in seasonal wetlands on a wildlife refuge using Vectomax™ CG. Journal of the American Mosquito Control Association 27, 398-403.

153. Poulin, B., Lefebvre, G., (2018) Perturbation and delayed recovery of the reed invertebrate assemblage in Camargue marshes sprayed with *Bacillus thuringiensis israelensis*. Insect Science 25, 542-548.

154. Merritt, R.W., Lessard, J.L., Wessell, K.J., Hernandez, O., Berg, M.B., Wallace, J.R., Novak, J.A., Ryan, J., Merritt, B.W., (2005) Lack of effects of *Bacillus sphaericus* (Vectolex®) on nontarget organisms in a mosquito-control program in southeastern Wisconsin: a 3-year study. Journal of the American Mosquito Control Association 21, 201-212.

155. Dambach, P., Baernighausen, T., Traoré, I., Ouedraogo, S., Sié, A., Sauerborn, R., Becker, N., Louis, V.R., (2019) Reduction of malaria vector mosquitoes in a large-scale intervention trial in rural Burkina Faso using Bti based larval source management. Malaria Journal 18, 1-9.

156. Dambach, P., Winkler, V., Bärnighausen, T., Traoré, I., Ouedraogo, S., Sié, A., Sauerborn, R., Becker, N., Louis, V.R., (2020) Biological larviciding against malaria vector mosquitoes with *Bacillus thuringiensis israelensis* (Bti)–Long term observations and assessment of

repeatability during an additional intervention year of a large-scale field trial in rural Burkina Faso. Global Health Action 13, 1829828.

157. Fillinger, U., Ndenga, B., Githeko, A., Lindsay, S.W., (2009) Integrated malaria vector control with microbial larvicides and insecticide-treated nets in western Kenya: a controlled trial. Bulletin of the World Health Organization 87, 655-665.

158. Geissbühler, Y., Kannady, K., Chaki, P.P., Emidi, B., Govella, N.J., Mayagaya, V., Kiama, M., Mtasiwa, D., Mshinda, H., Lindsay, S.W., (2009) Microbial larvicide application by a large-scale, community-based program reduces malaria infection prevalence in urban Dar es Salaam, Tanzania. PLoS One 4, e5107.

159. Maheu-Giroux, M., Castro, M.C., (2013) Impact of community-based larviciding on the prevalence of malaria infection in Dar es Salaam, Tanzania. PLoS One 8, e71638.

160. Mpofu, M., Becker, P., Mudambo, K., De Jager, C., (2016) Field effectiveness of microbial larvicides on mosquito larvae in malaria areas of Botswana and Zimbabwe. Malaria Journal 15, 1-8.

161. Nartey, R., Owusu-Dabo, E., Kruppa, T., Baffour-Awuah, S., Annan, A., Oppong, S., Becker, N., Obiri-Danso, K., (2013) Use of *Bacillus thuringiensis var israelensis* as a viable option in an Integrated Malaria Vector Control Programme in the Kumasi Metropolis, Ghana. Parasites & Vectors 6, 1-10.

162. Obopile, M., Segoea, G., Waniwa, K., Ntebela, D., Moakofhi, K., Motlaleng, M., Mosweunyane, T., Edwards, J., Namboze, J., Butt, W., (2018) Did microbial larviciding contribute to a reduction in malaria cases in eastern Botswana in 2012–2013? Public Health Action 8, S50-S54.

163. Derua, Y.A., Kweka, E.J., Kisinza, W.N., Githeko, A.K., Mosha, F.W., (2019) Bacterial larvicides used for malaria vector control in sub-Saharan Africa: review of their effectiveness and operational feasibility. Parasites & Vectors 12, 1-18.

164. Regis, L., Monteiro, A.M., Melo-Santos, M.A.V.d., Silveira Jr, J.C., Furtado, A.F., Acioli, R.V., Santos, G.M., Nakazawa, M., Carvalho, M.S., Ribeiro Jr, P.J., (2008) Developing new approaches for detecting and preventing *Aedes aegypti* population outbreaks: basis for surveillance, alert and control system. Memórias do Instituto Oswaldo Cruz 103, 50-59.

165. Benelli, G., Beier, J.C., (2017) Current vector control challenges in the fight against malaria. Acta Tropica 174, 91-96.

166. Kumar, G., Ojha, V.P., Pasi, S., (2021) Applicability of attractive toxic sugar baits as a mosquito vector control tool in the context of India: a review. Pest Management Science 77, 2626-2634.

167. Dame, Z.T., Rahman, M., Islam, T., (2021) Bacilli as sources of agrobiotechnology: recent advances and future directions. Green Chemistry Letters and Reviews 14, 245-270.

168. Bohari, R., Jin Hin, C., Matusop, A., Abdullah, M.R., Ney, T.G., Benjamin, S., Lim, L.H., (2020) Wide area spray of bacterial larvicide, *Bacillus thuringiensis israelensis* strain AM65-52, integrated in the national vector control program impacts dengue transmission in an urban township in Sibu district, Sarawak, Malaysia. PLoS One 15, e0230910.

169. . De Little, S.C., Williamson, G.J., Bowman, D.M., Whelan, P.I., Brook, B.W., Bradshaw, C.J., (2012) Experimental comparison of aerial larvicides and habitat modification for controlling disease-carrying *Aedes vigilax* mosquitoes. Pest Management Science 68, 709-717.

170. Jacups, S.P., Rapley, L.P., Johnson, P.H., Benjamin, S., Ritchie, S.A., (2013) *Bacillus thuringiensis* var. *israelensis* misting for control of Aedes in cryptic ground containers in North Queensland, Australia. The American Journal of Tropical Medicine and Hygiene 88, 490.

171. Pruszynski, C.A., Hribar, L.J., Mickle, R., Leal, A.L., (2017) A large scale biorational approach using *Bacillus thuringiensis israeliensis* (strain AM65-52) for managing *Aedes aegypti* populations to prevent dengue, chikungunya and Zika transmission. PLoS One 12, e0170079.

172. Lacey, L., Grzywacz, D., Shapiro-Ilan, D., Frutos, R., Brownbridge, M., Goettel, M., (2015) Insect pathogens as biological control agents: back to the future. Journal of Invertebrate Pathology 132, 1-41.

173. Becker, N., (1997) Microbial control of mosquitoes: management of the upper Rhine mosquito population as model programme. Parasitol Today 13:485–487

174. Becker, N., (2000) Bacterial control of vector-mosquitoes and black flies. Entomopathogenic bacteria: from laboratory to field application. Springer, pp. 383-398.

175. Margalit, J., Dean, D., (1985) The story of *Bacillus thuringiensis* var. *israelensis* (Bti). Journal of the American Mosquito Control Association 1, 1-7.

176. Silva-Filha, M.H.N.L., Romão, T.P., Rezende, T.M.T., Carvalho, K.d.S., Gouveia de Menezes, H.S., Alexandre do Nascimento, N., Soberón, M., Bravo, A., (2021) Bacterial toxins active against mosquitoes: Mode of action and resistance. Toxins 13, 523.

177. Kumar, P., Kamle, M., Borah, R., Mahato, D.K., Sharma, B., (2021) *Bacillus thuringiensis* as microbial biopesticide: uses and application for sustainable agriculture. Egyptian Journal of Biological Pest Control 31, 1-7.

178. de Melo-Santos, M.A.V., de Araújo, A.P., Rios, E.M.M., Regis, L., (2009) Long lasting persistence of *Bacillus thuringiensis* serovar. *israelensis* larvicidal activity *in Aedes aegypti* (Diptera: Culicidae) breeding places is associated to bacteria recycling. Biological Control 49, 186-191.

179. Silapanuntakul, S., Pantuwatana, S., Bhumiratana, A., Charoensiri, K., (1983) The comparative persistence of toxicity of *Bacillus sphaericus* strain 1593 and *Bacillus thuringiensis* serotype H-14 against mosquito larvae in different kinds of environments. Journal of Invertebrate Pathology 42, 387-392.

180. Bel Cortés, Y., Ferré Manzanero, J., Hernández Martínez, P., (2020) *Bacillus thuringiensis* Toxins: Functional Characterization and Mechanism of Action. Toxins, 12, 785.

181. Alves, G.B., Melo, F.L., Oliveira, E.E., Haddi, K., Costa, L.T., Dias, M.L., Campos, F.S., Pereira, E.J., Corrêa, R.F., Ascêncio, S.D., (2020) Comparative genomic analysis and mosquito larvicidal activity of four *Bacillus thuringiensis* serovar *israelensis* strains. Scientific Reports 10, 1-12.

182. Torres-Quintero, M.-C., Gómez, I., Pacheco, S., Sánchez, J., Flores, H., Osuna, J., Mendoza, G., Soberón, M., Bravo, A., (2018) Engineering *Bacillus thuringiensis* Cyt1Aa toxin specificity from dipteran to lepidopteran toxicity. Scientific Reports 8, 1-12.

183. Osman, G., Already, R., Assaeedi, A., Organji, S., El-Ghareeb, D., Abulreesh, H., Althubiani, A., (2015) Bio insecticide *Bacillus thuringiensis* a comprehensive review. Egyptian Journal of Biological Pest Control 25, 271.

184. Huang, Y.-J.S., Higgs, S., Vanlandingham, D.L., (2017) Biological control strategies for mosquito vectors of arboviruses. Insects 8, 21.

185. Federici, B.A., Bonning, B.C., St Leger, R.J., (2008) Improvement of insect pathogens as insecticides through genetic engineering. Patho-Biotechnology. Landes Bioscience, Austin, 15-40.

186. Boussiba, S., Wu, X., Ben-Dov, E., Zarka, A., Zaritsky, A., (2000) Nitrogen-fixing cyanobacteria as gene delivery system for expressing mosquitocidal toxins of *Bacillus thuringiensis* ssp. *israelensis.* Journal of Applied Phycology 12, 461-467.

187. Otieno-Ayayo, Z.N., (2008) Emerging Alternative Systems for Mosquito Biocontrol Using Recombinant Bacteria Expressing Genes from *Bacillus thuringiensis* subsp. *israelensis*. Ben-Gurion University of the Negev.

188. Derua, Y.A., Kahindi, S.C., Mosha, F.W., Kweka, E.J., Atieli, H.E., Wang, X., Zhou, G., Lee, M.C., Githeko, A.K., Yan, G., (2018) Microbial larvicides for mosquito control: Impact of long lasting formulations of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* on non-target organisms in western Kenya highlands. Ecology and Evolution 8, 7563-7573.

189. Ikeno, S., Haruyama, T., (2013) Boost protein expression through co-expression of LEA-like peptide in *Escherichia coli*. PLoS One 8, e82824.

190. Pathak, N., Ikeno, S., (2017) *In vivo* expression of a short peptide designed from late embryogenesis abundant protein for enhancing abiotic stress tolerance in *Escherichia coli.* Biochemical and Biophysical Research Communications 492, 386-390.

191. Dure III, L., (2001) Occurrence of a repeating 11-mer amino acid sequence motif in diverse organisms. Protein and Peptide Letters 8, 115-122.

192. Rosano, G.L., Ceccarelli, E.A., (2014) Recombinant protein expression in *Escherichia coli:* advances and challenges. Frontiers in Microbiology 5, 172.

CHAPTER 2

Enhanced insecticidal activity of *Bacillus thuringiensis* using a late embryogenesis abundant peptide co-expression system

2.1. Introduction

Employing living organisms, such as bacteria, viruses, and fungi, or natural products derived from these organisms, can be utilized as biological control agents as an alternative to chemical insecticides or pesticides [1]. *Bacillus thuringiensis* (Bt) is widely used as a biological control agent for pest control management and public health [2-4]. Insecticidal crystal proteins, derived from the sporulating stage of this gram-positive soil bacterium, mainly consist of Cry proteins, most of which are toxic to specific insects [5], and consequently Bt has been widely and successfully used as a biopesticide for more than 50 years in a new era of chemical-free insect control in agricultural systems [5, 6] Bt toxins, the most important insecticidal component in this microorganism, interact with protein receptors on the surface of insect midgut cells, leading to pore formation in the cell membrane and cell death [7]. The Bt crystal protein is being developed as an indispensable tool for the biological control or death of mosquito larvae [8]. This property has given rise not only to scientific inquiry but also commercial interest in Bt. For widespread field use, a large quantity of a spore-crystal preparation with high insecticidal potency is required. For insecticidal activity, spore and crystal formation are the key elements to produce a stable protein.

For efficient expression of Cry protein, various factors are involved. To regulate the high level of crystal protein synthesis, the most crucial factors are transcriptional, post-transcriptional, and post-translational changes, promoter function, mRNA stability, co-expressed peptides, and assistance by accessory proteins [9-12]. Cry protein synthesis is also reported to be driven by strong sporulation-dependent promoters [13]. Apart from the optimization of fermentation medium, different genetic approaches have been applied to enhance the production of Cry proteins. Devidas *et al.,* reported that *B thuringiensis* sv2 strain was highly effective against *Aedes aegypti* larva when conventional and non-conventional sources of carbon and nitrogen in pigeon pea and

soya bean flour, rather than Luria broth media, was employed in preparation [14]. Alternatively, Sansinenea et al. also reported that genetically improved natural Bt strains, particularly Bt recombination, offers a promising way to increase the efficacy and cost-effectiveness of Bt-based bioinsecticide products to develop new biotechnological applications [15]. Although simple yet effective and efficient universal approaches for enhancing protein expression are still being investigated for applications in a wide range of research and development fields, there have been no reports on the efficient expression of Bt crystal proteins using the late embryogenesis abundant (LEA) peptide co-expression system. Therefore, this study aimed to utilize the LEA peptide co-expression system to achieve mass production of parasporal crystal inclusion that has the capacity to kill mosquito larvae in an eco-friendly manner.

LEA proteins belong to a family of hydrophilic proteins [16, 17]. LEA proteins have been classified into seven different groups according to amino acid sequence homology and specific motifs [18]. Some LEA proteins have been shown to function as ion scavengers [19] molecular chaperones [20] or shields of macromolecules to avoid protein aggregation [21] and to restore proper folding to proteins subjected to dehydration [22], heat [23], or freeze-thaw stresses [24]. In our previous study, we found that co-expression of LEA peptide enhanced recombinant protein expression in *Escherichia coli* [25]. Furthermore, we developed a mutant LEA peptide by point mutation of a 13-mer LEA peptide that enhanced protein expression in *E. coli* [26]. Two LEA peptides (LEA-II and LEA-K) were especially effective in enhancing protein expression.

In the current study, we focused on the production of crystal proteins using native Bt as a host. LEA peptides, LEA-II or LEA-K, were expressed in Bt transformants using a pHT01 vector that was introduced in each LEA peptide gene. The expression level and efficacy of crystal protein

killing of mosquito larvae with and without LEA peptide co-expression were compared. We used a new and simple technique to boost protein expression and execute our new approach.

2.2 Materials and Methods

2.2.1Strains and expression vector

E. coli NovaBlue strain was used for gene recombination. *B. thuringiensis* strain D142 was used as a host for expression of the insecticidal protein and LEA peptide. The pHT01 vector (MoBiTec), which is an intracellular *Bacillus* system expression plasmid capable of inducing protein expression with isopropyl-β-thiogalactopyranoside (IPTG), with LEA peptide incorporated, was used in this experiment. This plasmid vector is a shuttle vector that can be used in both *E. coli* and *B. thuringiensis*, with ampicillin and chloramphenicol resistance in *E. coli* and *B. thuringiensis*, respectively.

2.2.2 Design of LEA peptide

In this study, LEA peptides, LEA-II, and LEA-K created from amino acid sequences using the *B. thuringiensis* codon frequency table. The sequences of each LEA peptide are MDAKDGLKEKAGE and MDAKDKTKEKAKE, respectively. The following oligonucleotides were purchased as synthetic DNA from Eurofins (Luxembourg) (Table2-1).

Table 2-1 DNA sequences for construction of each LEA gene

Name	DNA sequence
S-LEA-I	5'-GATCCATGGATGCAAAAGATGGAACAAAAGAAAAAGCAGGTGAATAAT-3'
AS-LEA-I	5'-CTAGATTATTCACCTGCTTTTTCTTTTGTTCCATCTTTTGCATCCATG-3'
S-LEA-K	5′-GATCCATGGATGCAAAAGATAAAACAAAAGAAAAAGCAAAAGAATAAT-3′
AS-LEA-K	5′-CTAGATTATTCTTTTGCTTTTTCTTTTGTTTTATCTTTTGCATCCATG-3′

2.2.3 Introduction of the LEA peptide gene into the pHT01 vector

The pHT01 vector was used to express the LEA peptides LEA-II and LEA-K. The restriction enzymes *B*amHI and *Xba*I (New England Biolabs) were used to cleave the pHT01 vector with a restriction enzyme reaction when placed at 37 °C for 3 hours. The LEA peptide DNAs (sense and antisense) were phosphorylated at 37 °C for 30 minutes using T4 kinase (New England Biolabs) and then slowly heated to 98 °C for 1 minute, then 50 °C for 30 seconds for hybridization. These were mixed with DNA ligase (DNA Ligation Kit Mighty Mix, Takara Bio) and ligated at 16°C to subclone the LEA peptide gene into the pHT01 vector. Thereafter, the *E. coli* NovaBlue strain was transformed and cultured overnight at 37 °C in LB medium. The plasmids were extracted from the expanded *E. coli* using a plasmid extraction kit (NucleoSpin® Plasmid EasyPure, Takara Bio). Finally, the samples were analyzed and confirmed by DNA sequencing.

2.2.4 Construction of Bt-LEA transformants

Construction of transformants expressing LEA peptide (Bt-LEA-II or Bt-LEA-K) was initiated with the following procedure. First, colonies of Bt were collected and pre-cultured in LB medium at 37 °C and 150 rpm. Five hundred microliters of the pre-culture solution and 500 μL of spore-forming solution were added to 50 mL of the LB medium and cultured at 37 °C and 150 rpm until the optical density (OD) value reached 1.5 to 2.0. Thereafter, the cells were harvested by centrifugation and washed several times with sterilized distilled water. Five hundred microliters of 30 % w/v polyethylene glycol (PEG 6000) was added to suspend the cells. For electroporation, 10 μL sterile distilled water, 500 ng pHT01-LEA vector, and 100 μL competent Bt were mixed. The mixture was transferred to an electroporation cuvette (0.2 cm, Bio-Rad) for electroporation (12.5 kV/cm, Gene Pulser Xcell: manufactured by Bio-Rad). After electroporation, the bacterial solution was mixed with Tryptic Soy Broth (TSB) and incubated at 37 °C and 150 rpm for 2 hours.

Thereafter, the cells were harvested by centrifugation (4,000 rpm × 5 min) and suspended in TSB medium. This suspension was cultured overnight at 37 °C on TSB (Cm^+) plate medium to prepare a transformant. For further use, a glycerol stock of each Bt-LEA transformant was prepared after pre-culture. After transformation by electroporation, the colony was harvested and pre-cultured overnight at 27 °C and 150 rpm in TSB (Cm^+) medium. Thereafter, 50 % glycerol (Wako) was mixed with sterilized distilled water and then with the pre-culture solution and dispensed in 500 µL aliquots to prepare a glycerol stock. The prepared glycerol stock was stored at −80 °C.

2.2.5 Cultivation and induction of LEA peptide by IPTG

During the culture of each Bt-LEA transformant, expression levels, and timing of LEA peptides expression were controlled by changing the concentration and time of introducing IPTG as an expression inducer. During the incubation period, the WT (non-transformant) and Bt-LEA transformants were grown in baffled flasks containing TSB with an appropriate antibiotic (5 µg/mL) and 100× spore-forming solution at 27 °C and 150 rpm for 48 hours. Based on incubation conditions, IPTG was added to reach final concentrations of 0.01 mmol/L, 0.1 mmol/L, 0.5 mmol/L, and 1 mmol/L at 4 hours, 8 hours, 12 hours, 16 hours, and 24 hours after the start of incubation. After 48 hours incubation, large crystalized protein was harvested from the cell lysate solution. By comparing the concentration and timing of IPTG, the optimum conditions for incubation for highly efficiently expression of the insecticidal protein were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and insecticidal bioassay.

2.2.6 5SDS-PAGE analysis

The SDS-PAGE analysis was performed with 1 mL of the cultured solution after 48 hours of incubation. To adjust the number of bacteria in each sample, the OD at 600 nm was determined. To extract soluble protein, each culture was centrifuged at 12,000 rpm for 5 minutes. After centrifugation, supernatant fluid was discarded, and 200 µL sterilized distilled water was added to the bacterial pellet, followed by vortexing for 1 minute. The soluble sample solution was mixed with the same volume of 2× SDS-PAGE buffer (2× containing 125 mM Tris-HCl (pH 6.8), 4 % SDS, 20 % glycerol, 0.1 mg/mL of bromophenol blue, 10 % 2- sulfanylethanol, and 2-mercaptoethanol). The mixture was heat-shocked at 95 °C for 5 minutes and then cooled on ice for 5 minutes. Finally, the prepared samples (5 µL each) and Protein Molecular Weight Marker Broad (Takara Bio) were loaded into the wells of a gradient polyacrylamide gel (gel concentration: 5–20 %, ATTO) for 30 minutes at 10.5 mA/gel. After electrophoresis, the separated proteins were visualized by staining with Coomassie Brilliant Blue (EzStain Aqua, ATTO) for approximately 2 hours and de-staining for approximately 1 hour. The results of SDS-PAGE were evaluated using ImageJ [27]. The marker was used as a reference, the average density of the non-transformant bands was considered control 1, and the relative value of bands of approximately 66.4 kDa was quantified. The 66.4 kDa protein was regarded as the insecticidal protein as Cry11Aa in comparison with the non-transformant band [28].

2.2.7 Evaluation of larvicidal activity against *Aedes albopictus*

The susceptibility of the *Aedes albopictus* larvae to the Cry toxin was assessed in the presence of protein treated with the LEA peptide (LEA-II and LEA-K) and WT as a control. The evaluation were performed to determine the activity of the insecticidal protein by adding a diluted culture

solution to *A. albopictus* larvae and counting the number of dead insects after 24 hours. For this bioassay, we used the Bt-LEA (II, K) transformants as a diluted culture solution that was obtained by culturing the Bt-LEA transformant for 48 hours. Induction of each LEA peptide by IPTG was performed at 12 hour of incubation. The diluted solution was used as a tested solution and the 4th instar larvae of *A. albopictus* as an insect and wild type (Bt. D142) as a control. The experiment was executed by using 250 µL of sterilized distilled water and the same volume of the culture solution Bt-LEA (II, K) transformants were added to a microtube to prepare a 50 % (500,000 ppm) culture solution. This solution (10 µL) was collected and mixed with 990 µL of sterilized distilled water to prepare a 5,000 ppm solution as a test solution. Five *A. albopictus* larvae were added into each well of a 12-well microplate, followed by the 4 mL of laboratory tap water. For evaluation, three wells (total of 15 larvae) were used for each concentration of tested samples. 40 µL of diluted test solutions (3,000, 2,000, 1,000, and 500 ppm) was added to wells and the final concentration were (30, 20, 10, and 5 ppm). The bioassay was maintained under controlled conditions at 25°C and 75 % humidity for 48 hours. In each experiment, the tests were repeated three times on different days and the mean mortality rates were obtained in each experiment. Mortality data for each larvicidal concentration were used to determine the LC_{50} using the probit method [29].

2.3 Results and Discussion

2.3.1 Construction, characterization, and determination of the function of the LEA peptide-encoding gene in the Bt.D142 strain

LEA-II and LEA-K peptides were constructed by point mutations in the LEA-I sequence (Table 2-2) using the codon frequency table of Bt, to study the effect of the LEA peptide on the Cry protein of Bt.D142. Point mutation and size and structure of an amino acid is a substantial indicator of the function of the LEA peptide. N. Pathak et al found that point mutation can be beneficial for the enhancement of LEA peptide function and might help in the co-expression of the target protein in cells [26]. In the amino acid sequence of the 13-mer LEA peptide, threonine was substituted by leucine at amino acid position 7 in LEA-II, and glycine was substituted by lysine at amino acid positions 6 and 12 in LEA-K peptide.

Table 2-2 Amino acid sequences of the mutated LEA peptides

Peptides	1	2	3	4	5	6	7	8	9	10	11	12	13
LEA-II	M	D	A	K	D	G	**L***	K	E	K	A	G	E
LEA-K	M	D	A	K	D	**K***	T	K	E	K	A	**K***	E

*The highlighted bold letters indicate the point mutation in each LEA peptide.

2.3.2 Cultivation of Bt-LEA transformants for Cry protein expression

The introduction of genes into cells often affects the expression of endogenous proteins. Therefore, the expression and proliferation of Cry protein in the Bt-LEA transformants (Bt-LEA-II and LEA-K) were evaluated under the same conditions as the non-transformants (WT, Bt. D142;

Fig. 2-1). After induction of IPTG (12 hours), the cell growth curves of the transformants and non-transformants had similar patterns, although there was some change in cell growth. After 48 hours of incubation, cry protein expression in the Bt-LEA transformants were higher in comparison to WT non-transformants and did not inhibit by LEA peptide expression system.

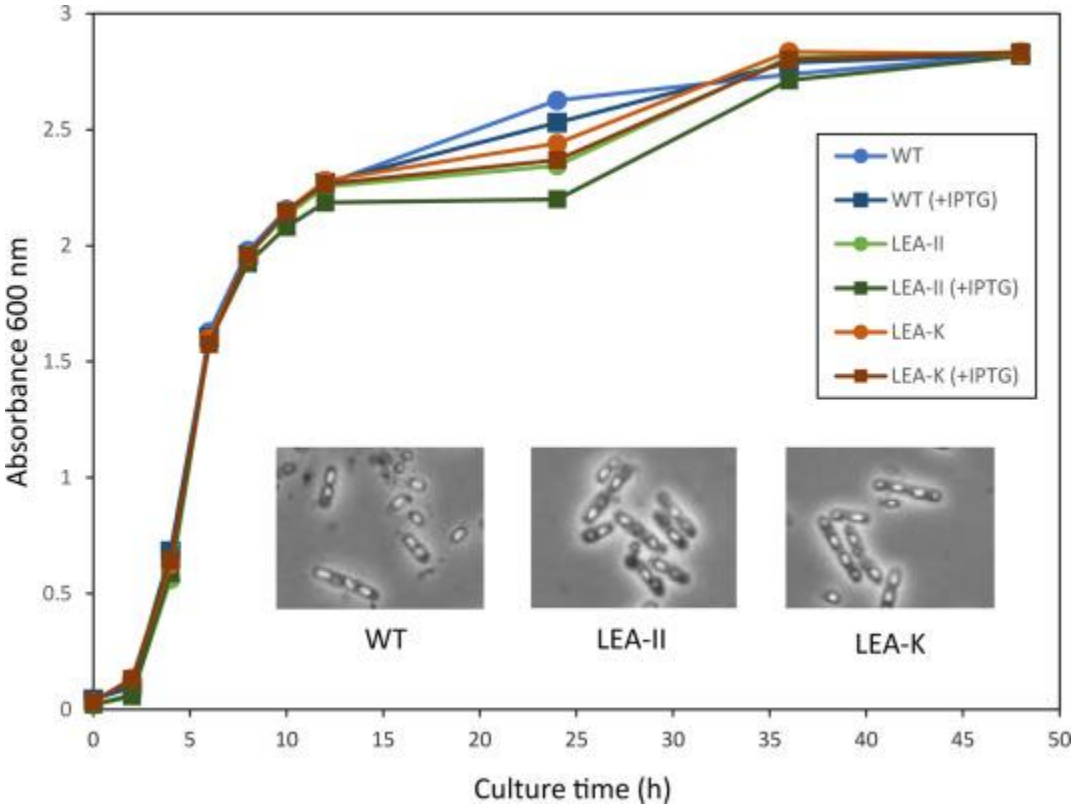

Figure 2-1 Growth curves showing time dependence of cell growth with the co-expression of each mutated LEA peptide before and after IPTG induction.

2.3.3 Enhanced expression of the Cry protein in the Bt-LEA transformant

By employing the LEA peptide co-expression system, the expression level of the Bt Cry protein (Cry11Aa) was confirmed through the analysis of SDS-PAGE. The Bt-LEA transformant (LEA-II), Bt-LEA transformant (LEA-K) and non-transformant (WT) showed the protein band in 66.4 kDa position (Fig 2-2 A, B). The band intensity of the Bt-LEA transformants (LEA-II and LEA-K) was more pronounced than the band intensity of the WT. To determine the highest expression of Cry protein, we varied IPTG concentrations and induction time (Table 2-3 and 2-4). Cry protein expression was induced with 0.01 mmol/L, 0.1 mmol/L, 0.5 mmol/L, and 1 mmol/L concentrations of IPTG for LEA-II and LEA-K. Induction timing of IPTG was examined 4, 8, 12, 16, and 24 hours after initiation. Expression of Cry protein was the highest at a 0.5 mM concentration of IPTG after 12 hours in the LEA-II and LEA-K peptides. These results indicate that the expression level of Cry protein were attributed to co-expression of the LEA-II and LEA-K peptides.

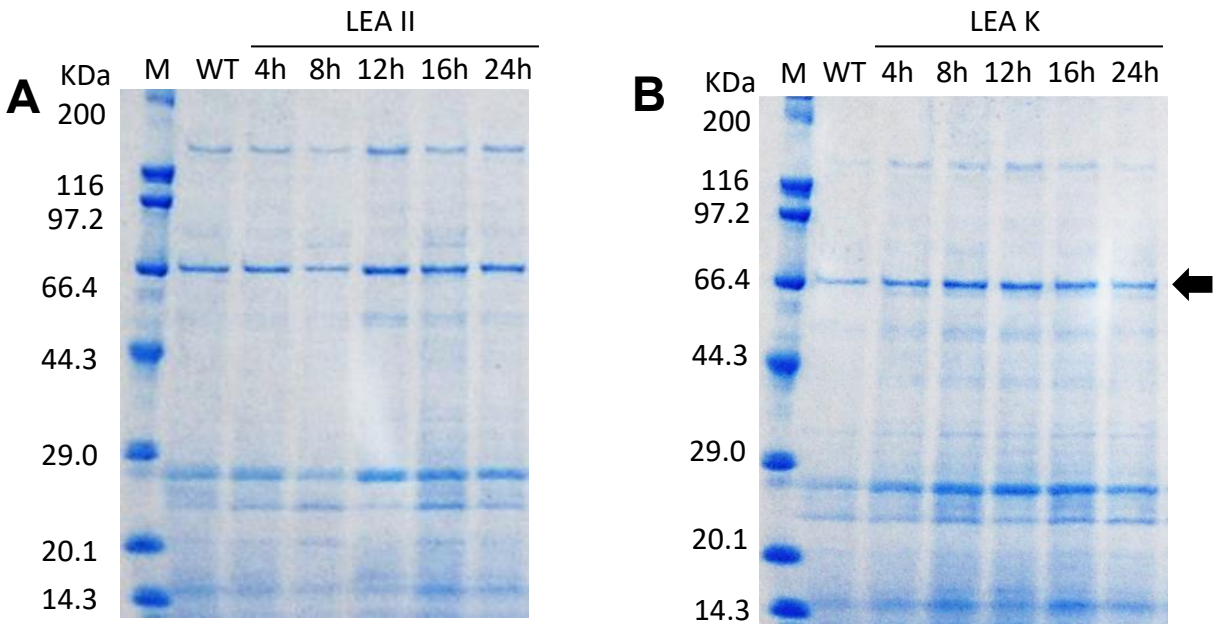

Figure 2-2 SDS-PAGE analysis of insecticidal protein expression in transformant Bt by each IPTG induction time. Bt-LEA-II (A) Lane M: marker; WT: *B. thuringiensis.*D142; induction time: 4 h, 8 h, 12 h, 16 h and 24 h. Bt-LEA-K (B) Lane M: marker; WT: *B. thuringiensis.* D142; induction time: 4 h, 8 h, 12 h, 16 h and 24 h.

Table 2-3 Numerical analysis of insecticidal protein expression levels in Bt-LEA-II transformants using Image J.

IPTG Conc.	Induction Time				
	4 hours	8 hours	12 hours	16 hours	24 hours
0.01 mmol/L	0.89	2.22	1.33	1.66	1.56
0.1 mmol/L	1.43	2.43	1.48	2.30	1.23
0.5 mmol/L	1.69	0.89	2.98	2.03	2.00
1.0 mmol/L	1.25	0.55	1.02	1.24	0.56

Table 2-4 Numerical analysis of insecticidal protein expression levels in Bt-LEA-K transformants using Image J.

IPTG Conc.	Induction Time				
	4 hours	8 hours	12 hours	16 hours	24 hours
0.01 mmol/L	1.85	1.97	1.41	1.91	1.87
0.1 mmol/L	1.92	1.82	1.92	1.69	1.73
0.5 mmol/L	1.10	2.05	2.16	1.88	1.07
1.0 mmol/L	0.76	1.56	1.34	1.18	1.59

Induction time of LEA peptide is critical because the formation of spores and Cry protein expression begins at approximately 24 and 30 hours after the beginning of incubation. This is related to σ factors controlling the formation of spores and σ factors controlling protein expression. The σ factor that regulates expression of the protein is activated in the early stage of spore formation for 2 to 6 hours at the end of the logarithmic growth phase and during the middle to late stage of spore formation 5 hours after the logarithmic growth phase when the promoter starts transcription [30, 31]. Therefore, to increase Cry protein expression, the co-expression of LEA peptide within 24 hours after the start of incubation is considered effective. In contrast, the highest concentration of IPTG (1 mmol/L) tended to suppress Cry protein expression. Therefore, the highest concentration (1 mmol/L) of IPTG might have toxic effects on cells, reducing Cry protein expression. The optimal concentration of IPTG may be system-specific [32], but relatively simple experiments can be executed to guide optimization of its concentration. The concentration of IPTG used to induce lac repressor-regulated promoters can dramatically influence expression [33]. The addition of IPTG is considered especially effective at 12 hours at a concentration of 0.5 mmol/L in this study.

2.3.4 Bioassay for larvicidal activity

To determine the LC_{50} value, bioassays were performed on *A. albopictus* larvae with each being a final concentration of 30, 20, 10, and 5 ppm of the sample, respectively. Analysis was based on the number of dead insects after 24 hours (Table 2-5, Fig.2-3). Relative insecticidal activity to the viable cells was 2.65 times higher for the Bt-LEA transformant than for non-transformant (WT). These results indicate that insecticidal activity was higher in the transformants than the non-transformant because co-expression with LEA peptide enhanced the expression of the

transformants. Toxicity was the highest with 0.5 mM IPTG at 12 hours in LEA-II, as observed by the insecticidal bioassay. This indicates that, unlike in *E. coli*, basic amino acids in the peptide sequence suppress protein expression in Bt [26]

Table 2-5 Bioassay evaluation of Bt-LEA transformants (LEA-II, LEA-K)

IPTG Conc.	Viable bacteria count (CFU/ml)	LC_{50} (ppm)	Relative insecticidal activity
WT	1.8×10^{9}	14.32	1
LEA-II (0.01 mmol/L)	2.1×10^{9}	15.52	0.79
LEA-II (0.1 mmol/L)	9.3×10^{8}	12.40	2.24
LEA-II (0.5 mmol/L)	6.8×10^{8}	14.29	2.65
LEA-K (0.01 mmol/L)	1.5×10^{9}	13.17	1.30
LEA-K (0.1 mmol/L)	1.6×10^{9}	12.06	1.34
LEA-K (0.5 mmol/L)	9.4×10^{8}	12.74	2.15

LC_{50} was expressed as the mean ($n = 3$), Relative toxicity is calculated by LC_{50} value per number of bacteria as against insecticidal activity of WT.

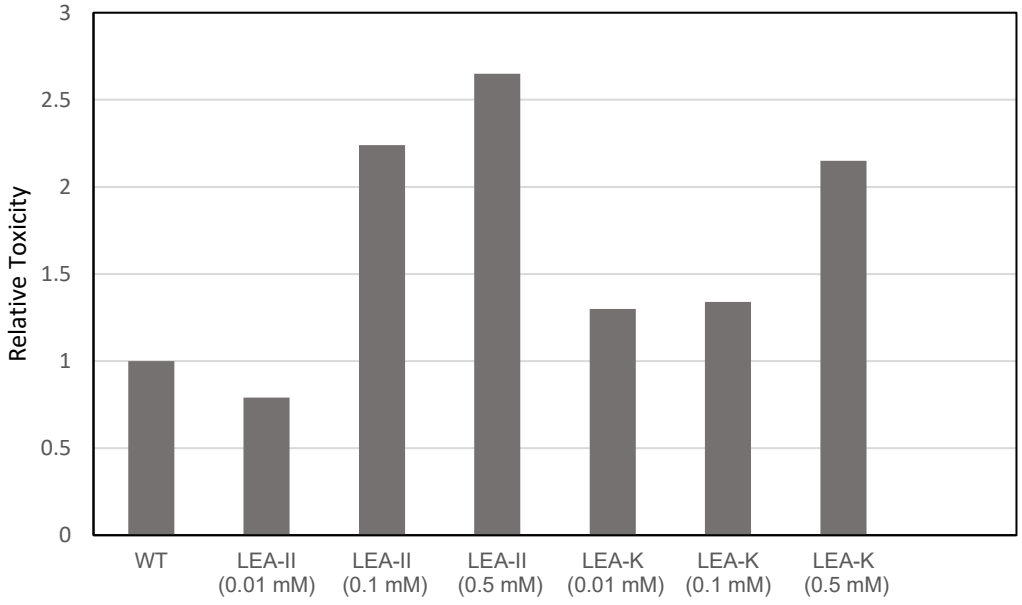

Figure 2-3 Relative toxicity to mosquito larvae of Cry protein expressed by BT co-expressing LEA peptide

2.4 Conclusions

A new, simple, and efficient Cry protein expression method was evaluated using LEA peptide co-expression. Cry protein expression was enhanced in Bt by LEA peptide co-expression compared to Bt without LEA peptide co-expression. We hypothesized that the hydrophobicity of leucine and the positive charge of lysine in LEA peptide played a vital role in the efficient expression of the Cry protein. Stabilization of polar residues and, thus, interactions with the target protein is important for the efficient enhancement of Cry protein expression. LEA peptide may protect a protein from degradation by a protease or may have a molecular shielding effect. The molecular shield function is largely an intermolecular activity implemented by intrinsically disordered proteins specialist, IDPs, distinct from molecular chaperones, but with a role in proteostasis [34]. The innovative design of LEA peptides co-expressed with the Cry protein led to the desired result. We conclude that LEA peptide co-expression is a simple technique that allows a comparison of protein expression.

References

1. Sanchis, V., (2011) From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review, Agronomy for Sustainable Development 31, 217-231.
2. Wang, P., Zhang, C., Guo, M., Guo, S., Zhu, Y., Zheng, J., Zhu, L., Ruan, L., Peng, D., Sun, M., (2014) Complete genome sequence of *Bacillus thuringiensis* YBT-1518, a typical strain with high toxicity to nematodes, Journal of Biotechnolgy. 171, 1-2.
3. Bravo, A., Gill, S.S., Soberon, M., (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control, Toxicon 49, 423-435.
4. Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., (2014) *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins 6, 3296-3325.
5. Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., (2011) *Bacillus thuringiensis*: a story of a successful bioinsecticide, Insect Biochem. Molecular Biology. 41, 423-431.
6. Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P., (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications, Plant Biotechnol. Journal. 9, 283-300.
7. Adang, M.J., Crickmore, N., Jurat-Fuentes, J.L., (2014) Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. Advances in Insect Physiology 47, 39-87.
8. Roh, J.Y., Choi, J.Y., Li, M.S., Jin, B.R., Je, Y.H., (2007) *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control, Journal of Microbiolog and Biotechnology17, 547-559.

9. Agaisse, H., Lereclus, D., (1996) STAB-SD: a Shine–Dalgarno sequence in the 5′ untranslated region is a determinant of mRNA stability, Molecular Microbiology. 20, 633-643.

10. Baum, J.A., Malvar, T., (1995) Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Molecular Microbiology 18, 1-12.

11. Chang, L., Grant, R., Aronson, A., (2001) Regulation of the packaging of *Bacillus thuringiensis* δ-endotoxins into inclusions, Applied and Environmental Microbiology 67, 5032-5036.

12. Sedlak, M., Walter, T., Aronson, A., (2000) Regulation by Overlapping Promoters of the Rate of Synthesis and Deposition into Crystalline Inclusions of *Bacillus thuringiensis* δ-Endotoxins, Journal of Bacteriology 182, 734-741.

13. Park, H.W., Ge, B., Bauer, L.S., Federici, B.A., (1998) Optimization of Cry3A yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence, Applied and Environmental Microbiology 64, 3932-3938.

14. Devidas, P.C., Pandit, B.H., Vitthalrao, P.S., (2014) Evaluation of different culture media for improvement in bioinsecticides production by indigenous *Bacillus thuringiensis* and their application against Larvae of *Aedes aegypti*, Scientific World Journal

15. Sansinenea, E., Vázquez, C., Ortiz, A., (2010) Genetic manipulation in *Bacillus thuringiensis* for strain improvement, Biotechnology Letters 32, 1549-1557.

16. Close, T. J., (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins, Physiologia Plantarum 97, 795-803.

17. Tunnacliffe, A., Wise, M.J., (2007) The continuing conundrum of the LEA proteins Naturwissenschaften 94, 791-812.

18. Wise, M.J., (2003) LEA ping to conclusions: a computational reanalysis of late embryogenesis abundant proteins and their possible roles, BMC Bioinformatics 4, 52.

19. Furuki, T., Shimizu, T., Kikawada, T., Okuda, T., Sakurai, M., (2011) Salt effects on the structural and thermodynamic properties of a group 3 LEA protein model peptide, Biochemistry 50, 7093-7103.

20. Chakrabortee, S., Boschetti, C., Walton, L.J., Sarkar, S., Rubinsztein, D.C., Tunnacliffe, A., (2007) Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function, Proceedings of the National Academy of Sciences of the United States of America USA 104, 18073-18078.

21. Tunnacliffe, A., Hincha, D.K., Leprince, O., Macherel, D., (2010) LEA proteins: Versatility of form and function, in: E. Lubzens, J. Cerda, M. Clark (Eds.), Sleeping Beauties: Dormancy and Resistance in Harsh Environments, Springer, Berlin, 91-108.

22. Babu, R.C., Zhang, J., Blum, A., Ho, T.H.D., Wu, R., Nguyen, H., (2004) HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa L.*) via cell membrane protection, Plant Science 166, 855-862.

23. Kovacs, D., Kalmar, E., Torok, Z., Tompa, P., (2008) Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins, Plant Physiology 147, 381-390.

24. Reyes, J.L., Campos, F., Wei, H., Arora, R., Yang, Y., Karlson, D.T., Covarrubias, A.A., (2008) Functional dissection of hydrophilins during *in vitro* freeze protection, Plant, Cell and Environment 31, 1781-1790.

25. Ikeno, S., Haruyama T., (2013) Boost protein expression through co-expression of LEA-like peptide in *Escherichia coli*, PLoS ONE 8, e82824.

26. Pathak, N., Hamada, H., Ikeno, S., (2018) Construction and characterization of mutated LEA peptides in *Escherichia coli* to develop an efficient protein expression system, Journal of Molecular Recognition 31, e2658.

27. Schneider, C.A., Rasband, W.S., Eliceiri, K.W., (2012) NIH Image to ImageJ: 25 years of image analysis, Nature Methods 9, 671-675.

28. Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyama, H., Sen, K., Sakai, H., (2002) Active form of dipteran-specific insecticidal protein Cry11A produced by *Bacillus thuringiensis* subsp. *Israelensis,* Bioscience, Biotechnology and Biochemistry 66, 516-522.

29. Bliss, C.I., (1934) The method of probits. Science.

30. Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins, Microbiology and Molecular Biology Reviews 62, 775-806.

31. Ichikawa, H., Kroos, L., 2000. Combined action of two transcription factors regulates genes encoding spore coat proteins of *Bacillus subtilis,* Journal of Biological Chemistry 275, 13849-13855.

32. Dvorak, P., Chrast, L., Nikel, P.I., Fedr, R., Soucek, K., Sedlackova, M., Chaloupkova, R., de Lorenzo, V., Prokop, Z., Damborsky, J., (2015) Exacerbation of substrate toxicity by IPTG in *Escherichia coli* BL21 (DE3) carrying a synthetic metabolic pathway, Microbial Cell Factories 14, 201.

33. Sadeghi, H.M., Rabbani, M., Rismani, E., Moazen, F., Khodabakhsh, F., Dormiani, K., Khazaei, Y., (2011) Optimization of the expression of reteplase in *Escherichia coli*, Research in Pharmaceutical Sciences 6, 87-92.

34. Chakrabortee, S., Tripathi, R., Watson, M., Schierle, G.S., Kurniawan, D.P., Kaminski, C.F.; Wise, M.J., Tunnacliffe, A., (2012) Intrinsically disordered proteins as molecular shields, Molecular BioSystems 8, 210-219.

CHAPTER 3

Lactose as a time-dependent promising inducer in response to the efficient expression of insecticidal crystal protein by late embryogenesis abundant peptide co-expression system in *Bacillus thuringiensis*

3.1 Introduction

By utilization of Cry toxins from *Bacillus thuringiensis* (Bt) have been extensively applied in the field of agriculture to substitute the use of chemical insecticides for being controlled of insects. [1]. All around the world, mosquito particularly *Aedes aegypti* is considered one of the utmost dangerous deadly creatures that play a pivotal role as a vector for contributing harmful human diseases, like yellow fever, dengue, and chikungunya in most tropical areas [2]. Bt is utilized to restrict the defoliators and mosquitoes has resulted in a remarkable cutback as an alternative to chemical insecticides for the same purpose [3]. The main attraction of Bt to restrict mosquito and black fly populations with its high insecticidal activity, the lack of resistance and toxicity to non-target organisms, and particularly the presence of insect resistant populations has resulted in tremendous employment as an alternative to chemical insecticides [4]. However, at present times major R&D has started to give priority to agricultural chemicals to grab more lucrative market. Besides this, Bio-insecticides have less attraction owing to have its less sustainability along with pretty penny resulted in less market acceptance in comparison to the chemical compounds [5].

For the production of bio-based insecticide, it is essential to select the medium for the cultivation of bacterial strain particularly for Bt by considering the maximum yields of spore-crystal complexes in association with the cost-benefit ratio. The study declared that a high concentration of glucose (50–90 g/L) sometimes may demonstrate the lower production of bacterial spores, crystal protein, and lower toxicity by cultivating the bacteria in wealthy media while evaluating against *Spodoptera littoralis* and *Anagasta kuehniella* larvae [6]. So, it's the indication that excess glucose or carbon source might play a negative impact on the production of spore, Cry protein, and toxicity as well. The work of Monod J. is one the best admitted prototype that revealed the privileged carbon employment of the glucose-lactose diauxic shift in *Escherichia*

coli [7]. Consequently, some other studies also acknowledged that for many organisms glucose as a carbon source is the preferable selection [8]. So numerous glucose-responsive networks have already been established by bacteria. But there is no authentic evidence or report that how is the effect of non-glucose media for Bt with LEA peptide co-expression system. However, it is reported by Ogura M., where they administered that the *ywlE* disruptant, there was the extensive reduction in glucose induction of *sigX-lacZ* expression and the absence of glucose in the *mcsB* disruptant, a marginal enhancement of basal expression [9].

When the amount of glucose is available, the genes that feel the necessity for the usage of secondary carbon sources are not expressed and in the presence of glucose, the genes required for the utilization of secondary carbon sources are not expressed and to limit the misuse of resources former enzymes are generally inactivated. This incidence is regarded as carbon catabolite repression [8,10]. But the acquirement of carbon catabolite regulation (CCR) is the solely distinct mechanism in varieties of bacteria. If we consider enteric bacteria like *E. coli*, the employment of CCR is driven by the skipping of transcription activation of secondary catabolic genes in the existence of a favored substrate. Contrarily, in the case of Firmicutes, like *Bacillus subtilis*, for CCR, the transcription factor, catabolite control protein A (CcpA) is the leading regulator [10, 11]. In many bacterial species belong to 5-10 % of all genes, where CCR plays a vital role as a central regulatory [12-14]. Protein acetylation in *E. coli* and *B. subtilis* are induced by the addition of glucose culture medium [15, 16].

After the acetylation of CshA, a DEAD-box helicase, from the proteomic study of *B. subtilis* revealed that CshA, a DEAD-box helicase [15, 17] resulted that CshA lysine acetylation [18] and CshA in accordance with RNA polymerase (RNAP) [19] were stimulated by glucose. The inter-relationship between acetylated CshA and RNAP help to augment its SigX affinity,

conveying to induce glucose in *sigX* [18, 20, 21]. Besides, it was also noted the *mcsB* and *clpC* expression [22] is repressed by glucose and induces *ywlE* expression. Many of the enzymes are Arg-phosphorylated originates from the glycolytic and TCA cycle, recommending the McsB/YwlE system is responsible for cell growth belonging to medium including glucose [23-27]. According to the observation of Masri, & Ariff that the effect of glucose feeding from the time being of fermentation resulted in enhancement of cell growth whereas sporulation rate [28]. So, for bacterial fermentation carbon sources can be the key factors for the production of spore and crystal proteins as well and finally have a pivotal role in the expression of Cry protein. So for Cry protein expression, any kind of carbon source is a key indicator and it may come either from the growth media or inducer. Glucose as a media or lactose as an inducer implies the important trait as it is related to the cost benefit ratio for commercial purposes. So as much as if we can optimize the concentration of inducer or reduce the media composition, it would lead to a promising option to use Bt as an alternative biopesticide to compete with chemical insecticide. Because bacteria have the preference to opt for glucose while depleting of glucose and moving forward to lactose [29].

Repression of the synthesis of T7 RNA polymerase is assisted when there is the presence of glucose as the preliminary source of metabolism in the system of glucose/lactose. Lactose has the capability to drive the synthesis of T7 RNA polymerase by aiding the transcription of the target mRNA of the gene cloned in the plasmid vector due to the depleted status of glucose. Although chemical insecticides are low-cost in the rate by considering the detrimental effect of environment and future generation, the world is now moving to get some alternative to chemical, biological based, and effective as well. As biological based products are a bit expensive, so it's high time to adopt some technique that would be effective but will have the capability to fight with the over-

priced of chemical insecticide. Undoubtedly, the development of recombinant proteins in the field of the microbial sector has transformed into a different benchmark of biochemistry [30]. For the protein expression, the inducing expression is the most preferable way where the targeted gene started to the transcript in its exponential phase of growth [31]. The most preferable inducers for microbial protein expression belong to the list of isopropyl β-D-thiogalactopyranoside (IPTG), lactose, galactose, or arabinose, or another variety of sugars or other molecules in the growth medium whose consumption is controlled by the presence of glucose [32]. Yet, lactose is one of the natural inducers of β-galactosidase gene, regarded as the most approved substitute for IPTG owing to its economical trait and non-toxic effect. On the other hand, lactose has also belonged to a carbon source, which is utilized by bacteria has the tendency to invariably change concentration in ongoing fermentation resulted in induction operation more troublesome and challenging to control. However, a few articles reported information regarding the application of lactose as an inducer for the expression of a foreign gene in high density fermentation of *E. coli* [33-36].

By attributing the most beneficial point of lactose as an economical compound in contrast to glucose and IPTG, a unique induction strategy sounds pretty appealing induced by lactose-only feed batch [37]. Permease (lacY) and β-galactosidase (lacZ), are considered the most functional genes for the induction of lactose and lacZ, by importing lactose converted it into allolactose is the original inducer of lac repressor. Usually, induction in the initial state of growth phase by the initiation of auto induction media is followed to normal growth rate before exhaustion of glucose by the effectiveness of glucose and during the period of induction, most of the cultural cells are already in a functional state. For the development of induction expression in *E. coli* diverse group of approaches have been established over the last several decades. Recently, IPTG is the most preferred approach for the induction of promoter expression. Although this system has the

following barriers: (i) Continuous monitoring for the induction of during the optimal cell density. Undoubtedly, the point of induction may differ from one recombinant protein to another resulted in challenging action to automate particularly in which makes the process difficult to automate, notably when numerous proteins are expressed side by side (e.g., for a screen); (ii) narrow range of technical issues (iii) From the commercial viewpoint, less appropriate (iv) Toxic characteristics (Particularly in case of human therapeutic protein production); and (v) Expensive in rate [38]. Normally, one point addition of IPTG is induced by the help of T7 system [39, 40]. Not only that but also some other bottleneck of IPTG including a high metabolic burden on the organism and formation of inclusion body [41-44].

Another attractive criterion of using lactose by the auto induction is the most recently using approach where lactose is involved in the growth medium, and induction started after ingestion of full amount of available glucose [45]. For engineering products, lactose can be considered one of the practical prospects owing to have its economical backup and non-toxic trait. The inducing criteria of lactose differ markedly for various kinds of recombinant protein expression in comparison to IPTG where it is already declared by a large figure of experimental investigations for its maximum working efficiency [46]. Although lactose has appealing criteria, still there is no way to disagree with some of the limitations where special consideration should apply particularly on the induction parameters and timing [47]. To address this problem, the first ever approach of LEA peptide co-expression system by the induction of lactose can be a suitable option to enhance the Cry protein expression. This approach achieves the targeted protein expression by considering the low-cost ratio in industrial operation and also be the alternative option of the most commonly used IPTG. In the current study, we focused on the production of crystal proteins using native Bt

as a host. LEA peptides, LEA-I, LEA-E, and LEA-K, were expressed in Bt transformants using a pHT01 vector that was introduced in each LEA peptide gene.

In this chapter, our purpose is to enhance the Cry protein expression by the employment of Bt induced LEA peptide co-expression system to address the lactose as a promising inducer commercially by optimizing the culture condition, dosage concentration, timing, and also amount of dosage. For recombinant protein expression, *E. coli* is the topmost preferable host whereas an inducer IPTG also belongs to the same priority list. IPTG as an inducer, despite non-metabolizable and overpriced compound, could be taken over by the economical and natural lactose to induce Cry protein expression. In order to do this, we examined the expression of Cry protein expression by the employment of LEA peptide co-expression system using lactose as the inducer.

3.2 Materials and Methods

3.2.1 Strains and expression vector

To conduct this research, strains and particularly for the construction of expression vector we pursued the protocol that have been detailed in our chapter 2. Just we changed the inducer lactose monohydrate instead of IPTG

3.2.2 Design of LEA peptide

In this research, for the construction of several kinds of LEA peptide such as LEA-I, LEA-K, and LEA-E by taking the advantages of Bt codon frequency table in accordance amino acid sequences. The design pattern of LEA peptide where the sequences of each LEA peptide are arranged in an order of MDAKDGTKEKAGE, MDAKDKTKEKAKE, and MDAKDETKEKAEE, subsequently. The synthetic DNAs were acquired from Eurofins (Luxembourg) by specifying the following oligonucleotide sequences (Table 3-1).

Table 3-1 DNA sequences for construction of each LEA gene

Name	DNA sequence
S-LEA-II	5′-GATCCATGGATGCAAAAGATGGATTAAAAGAAAAAGCAGGTGAATAAT-3′
AS-LEA-II	5′-CTAGATTATTCACCTGCTTTTTCTTTTAATCCATCTTTTGCATCCATG-3′
S-LEA-K	5′-GATCCATGGATGCAAAAGATAAAACAAAAGAAAAAGCAAAAGAATAAT-3′
AS-LEA-K	5′-CTAGATTATTCTTTTGCTTTTTCTTTTGTTTTATCTTTTGCATCCATG-3′
S-LEA-E	5’-ATGGATGCAAAAGATGAAACAAAAGAAAAAGCAGAAGAATAAT-3
S-LEA-E	5’-CGTCCCCGGGGCAGCCCGCCTAATGAGCGGGCTTTTTTCACGT-3

3.2.3 Introduction of the LEA peptide gene into the pHT01 vector

For the insertion of LAE peptide gene into the pHT01 vector, we just followed the protocol that have been detailed in our previous chapter 2. Although we generated different kinds of LEA peptides, the vector and protocol were maintained in the same manner.

3.2.4 Construction of Bt-LEA transformants

In this research, the new introduction of the peptide was LEA-E that was generated by following the same instruction that has already been described in Chapter 2, and another two kinds of Bt-LEA-I and Bt-LEA-K were utilized from the stock.

Three different categories of Bt-LEA transformants such as Bt-LEA-I, Bt-LEA-E, and Bt-LEA-K were introduced as a host cell to conduct this research. The construction process of Bt-LEA-I and Bt-LEA-K have been detailed in our previous chapter 2. Whereas, the construction process of Bt-LEA-E was strictly followed in accordance to our previous paper to maintain the equilibrium in order to other peptide.

3.2.5 Cultivation and induction of LEA peptide by IPTG for cell growth and spore counting:

In our first order of experiment, for checking the glucose activity we opted three varieties of groups such as wild type bacteria, empty vector pHT01, and only Bt-LEA-II (Bt-LEA-II showed higher expression). First, we executed the experiment with a duration of 48 hours by culturing the TSB media in the presence and absence of glucose for each group of cultural media respectively. In the experimental protocol, we followed some of the instructions to maintain the equilibrium of

the experimental atmosphere like no antibiotic (chloramphenicol) was added in the case of wild type bacteria (Wt). The experiment was executed by using Wt and, Bt-LEA-II and pHT01 and Bt-LEA transformants to grow in baffled flasks containing tryptic Soya broth (TSB) with an appropriate antibiotic (5 μg/mL) and 100 × spore-forming solutions by maintaining the temperature, rshaking and duration at 27 °C and 150 rpm for 48 hours respectively. Except for Wt, IPTG was added to BT-LEA-II and pHT01 at 12th hour to achieve the final concentration of 0.5 mmol/L. After 48 hours of incubation, first, we harvested the sample by doing heat treatment (65 ºC - 30 minutes) to dilute with 0.85 % NaCl and 0.05 % Tween-80. Thereafter, incubated in agar plate at 27 ºC and finally counting of spore after 16-18 hours. At the sample time, we also harvested the sample in each 4th hour interval till 48 hours to observe the cell growth curve.

3.2.6 Cultivation and induction of LEA peptide by lactose

The experimental condition was conducted by cultivating each of Bt-LEA transformants (Bt-LEA-I, Bt-LEA-E, and Bt-LEA-K) where expression level and timing were manipulated by modifying the same distinct parameters like concentration, induction pattern and induction timing of lactose as an expression inducer. The entire cultivation process was regulated in baffled flasks containing Tryptic soya broth (TSB) supplemented with appropriate antibiotic (chloramphenicol, 5 μg/mL) and 100 × spore-forming solution by maintaining the constant temperature and shaking at 27 °C and 150 respectively with a total duration of 48 hours. Antibiotic (chloramphenicol, 5 μg/mL) was omitted to wild type (non-transformant). To determine the effect of lactose as an inducer the cultures were induced with the final concentrations of 0.0 mmol/L, 0.1 mmol/L, 0.2 mmol/L, 0.5 mmol/L, and 0.1 mmol/L IPTG (for control) respectively for different periods of cultivation in accordance with the requirement of each experiment. The induction time was

attributed in two different slots like 11^{th}, 12^{th}, 13^{th}, 14^{th}, and 15^{th} hour and 16^{th}, 17^{th}, 18^{th}, 19^{th} and 20^{th} hour to figure out the effectivity of induction point of time. Besides this, samples were harvested according to time interval after the induction for analysis. The bacterial cell concentration was quantified by measuring the optical density (OD) with the help of spectrophotometer (AS ONE –ASV11D) 0f A_{600} absorbance. The expression level of cry protein was quantified by the employment of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After confirmation of expression level, the experimental outline was designed to compare induction pattern like intermittent induction (10 µL) or at a time induction (50 µL) at 11^{th}, 12^{th}, 13^{th}, 14^{th}, and 15^{th} hour for each type of LEA transformant. In this regard, except the induction pattern any other parameters were in constant. During this investigation, the samples were also harvested from 0 hour to 12^{th} hour for each two hours interval and thereafter 24^{th}, 30^{th}, $36^{th,}$ and 48^{th} hour respectively to observe the cell growth after induction of lactose.

After 48 hours of incubation, the large crystallized protein was harvested from the cell lysate solution. By comparing the concentration and timing of lactose, the optimum conditions for incubation for highly efficient expression of the insecticidal protein were determined by SDS-PAGE.

3.2.7 SDS-PAGE analysis

After 48^{th} hour of incubation, for each investigation, the sample was collected after measuring the OD at 600 nm and the cells were harvested by centrifugation at 12,000 rpm for 5 minutes by taking 1 mL of culture solution. After centrifugation, the supernatant was removed, and the pellet was dissolved by adding 200 µL by the help of a vortex. To prepare the final sample, the solubilized sample was added to the reagents like 2× SDS-PAGE buffer (2× containing 125 mmol/L Tris-HCl

(pH 6.8), 4 % SDS, 20 % glycerol, 0.1 mg/mL of bromophenol blue, 10 % 2- sulfanylethanol, and 2-mercaptoethanol) by flowing the heat-shock and ice incubation at 95 °C and 5 minutes consequently. Lastly, 5 µL of each processed sample and Protein Molecular Weight Marker Broad (Takara Bio) were loaded into the wells of a gradient polyacrylamide gel (gel concentration: 5–20 %, ATTO) for 30 minutes at 10.5 mA/gel. The separated proteins were observed by staining with Coomassie Brilliant Blue (EzStain Aqua, ATTO) and de-staining by following the same duration 2 hours after electrophoresis. ImageJ was applied to evaluate the results of SDS-PAGE [48] where marker acts as a reference to quantify the average density of the non-transformant bands was considered to control 1, and the relative value of bands of approximately 66.4 kDa. The 66.4 kDa protein was regarded as the insecticidal protein as Cry11Aa in comparison with the non-transformant band [49].

3.2.8 Determination of glucose and lactose in the medium and cell

We determined the glucose and lactose concentration in the medium and cell by using a glucose and lactose detection kit (Boehringer Mannheim/R-Biopharm). We detected the glucose and lactose concentration only in the sample of three categories of Bt-LEA transformants (Bt-LEA-I, Bt-LEA-E, and Bt-LEA-K) with the parameters of without induction, 50 once at 11^{th} hour and finally 10 µL intermittently from 11^{th}, 12^{th}, 13^{th}, 14^{th}, and 15^{th} hour. To determine the glucose and lactose concentration in the medium and cell, the glucose and lactose detection kit were purchased from Boehringer Mannheim/R-Biopharm. The highest expression level was confirmed in case of Bt-LEA-E transformant with the concentration of 0.1 mmol/L (Table 3-3) by the analysis of SDS-PAGE. Based on the expression level, only glucose and lactose concentration were detected in case of Bt-LEA-E transformant in accordance with intermittent induction, (10 µL) at a time

induction, (50 µL), only the Bt-LEA-E (without induction) and from the fresh media of TSB by considering induction time of 11^{th}, 12^{th}, 13^{th}, 14^{th}, and 15^{th} hour.

3.3 Results and Discussion

3.3.1 Construction, characterization, and determination of the function of the LEA peptide-encoding gene in the Bt.D142 strain

From the study of chapter 2, the Bt-LEA-K transformant had a small effect on increasing expression, unlike our previous studies using *E. coli*. Therefore, we designed LEA-E, which was substituted with acidic amino acid instead of basic ones (Table 3-2). By the employment of codon frequency table of Bt, Bt-LEA-E transformant was designed by adding point mutations to the LEA-I sequence to scrutinize whether LEA peptide has any effect to enhance the expression of Cry protein of Bt. D142 or not. Point mutations, size, and structure of amino acids have a major input to the function of LEA peptide. From our previous study [50], it is already well established that point mutations play a vital role in the enhancement of the function of LEA peptides in association with the co-expression of target proteins in cells. In the amino acid sequence of the 13-mer LEA peptides, glycine was replaced with glutamic acid at amino acids 6 and 12 in the LEA-E peptide, and glycine was replaced with lysine at amino acids 6 and 12 in the LEA-K peptide.

Table 3-2 Amino acid sequences of the mutated LEA peptides

Peptides	1	2	3	4	5	6	7	8	9	10	11	12	13
LEA-I	M	D	A	K	D	G	**T**	K	E	K	A	G	E
LEA-K	M	D	A	K	D	**K***	T	K	E	K	A	**K***	E
LEA-E	M	D	A	K	D	**E**	T	K	E	K	A	**E**	E

*The highlighted bold letters indicate the point mutation in each LEA peptide.

3.3.2 Cultivation of Bt-LEA transformants for Cry protein expression

To execute this experiment, one of our major challenges were to introduce the genes into cells whether it has any influence or any prominent change to the expression of endogenous protein, particularly for the expression and proliferation of Cry protein. In this regard, the expression level of Cry protein in the Bt-LEA transformants (Bt-LEA-II) was figured out by providing the same conditions in the case of non-transformants, (WT, Bt. D142-Fig. 2-1) too. The results have already been described in our previous study [40], where the cell growth pattern of the transformants and non-transformants followed the equal patterns, however, a small change was observed.

3.3.3 Cell growth and expression of the Cry protein in the Bt-LEA transformant with and without glucose

Cell growth of the Bt-LEA transformant with and without glucose was shown in Fig.3-1. There was no significant difference in Cry protein expression after 48 hours in the presence or absence of glucose. This means, the LEA peptide co-expression system is functional even in the absence of glucose. For any kind of bacterial cell, the presence of carbon is a key indicator as they utilize it as a growth factor. But it is also reported that generally, bacterial growth, spore formation, and toxin production were much better during cultivation on lower glucose concentrations [6]. So, for bacterial growth glucose as a media composition and how much it is necessary to bacterial cell growth or not. With glucose and without glucose has any direct effect on the expression of Cry protein or not. Glucose concentration has any effect on LEA peptide co-expression system or Cry toxicity. For the expression of Cry protein, there is a report to repress the *cry4Aa* gene by the glucose at the mRNA level in Bti [51] and repression of *cry4A* transcription by the phosphorylated HPr-CcpA complex of Bti when particularly attach to a 15 bp *cre* sequence by overlapping the -

35 element of the *cry4A* promoter [52]. So HPr-CcpA-mediated glucose catabolite repression is responsible for mediating the controlling synthesis of Cry protein in Bti.

When the glucose level is high, no cAMP is made. CAP cannot bind to the DNA without cAMP, so transcription occurs only at a low level. Even though the absence of glucose lac repressor also cannot activate. So, for LEA peptide co-expression system glucose has not any direct effect on bacterial cell growth as well as protein expression. The expression level of the Bt Cry protein (*cry*11Aa) was confirmed through the analysis of SDS-PAGE by employing the LEA peptide co-expression system. The wild type bacteria, pHT01 and Bt-LEA-II in the presence or absence of glucose showed the protein band in 66.4 kDa position (Fig 3-2). After 24^{th} hour both the presence and absence of glucose had less expression whereas 48 hours later the expression level was the same in the presence or absence of glucose. For *Bacillus*, it is a normal process to produce the bacterial spore and ultimate the production of crystal protein, but any factor can initiate more spore formation or early start of initiation. As glucose is the main carbon source for bacterial growth, so during their culture, the bacterial cell always tries to search the carbon source for a normal mechanism. But in the case of LEA peptide co-expression system with Bt, it is clearly an indication that the absence of glucose, bacteria leads to more spores in comparison to normal. Due to lack of carbon sources, the bacteria always try to cope with a normal environment and produce more spores to survive after fighting with the surrounding. So, the absence of glucose in the media leads to a stressed condition to the bacterial for making excess cell growth and. more spore. In this investigation, we tried an overall was hypothesis as it is the first ever try of Cry protein expression with LEA peptide co-expression system with or without glucose.

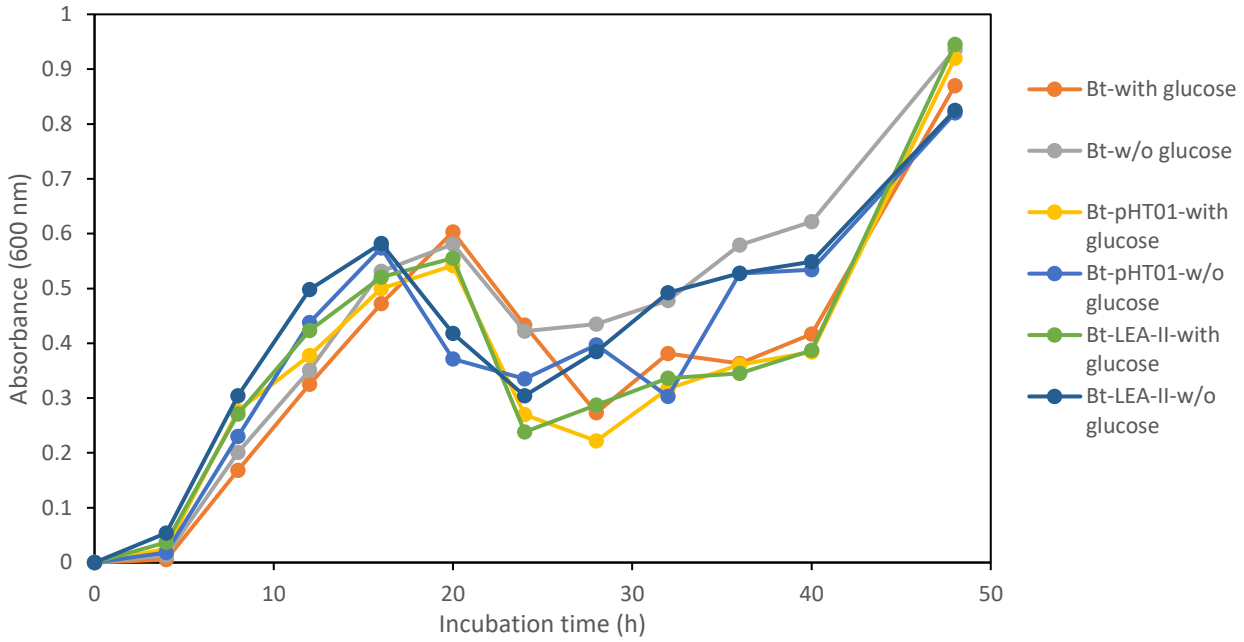

Figure 3-1 Cell growth curve of each recombinant Bt on the basis of glucose and without glucose in culture media

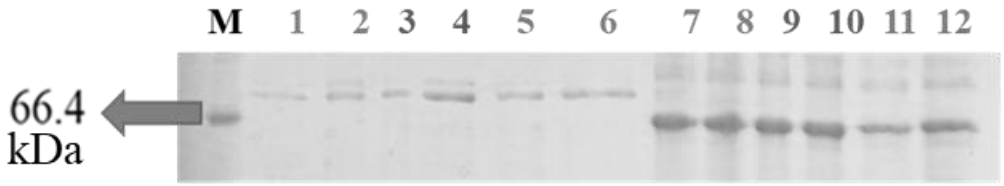

Figure 3-2 SDS-PAGE of Bt, pHT01 and Bt-LEA-II in absence or presence of glucose, 1;24h Bt-with glucose, 2; 24h Bt-w/o glucose, 3; 24h Bt-LEA=II with glucose, 4; 24h Bt-LEA=II w/o glucose, 5; 24h pHT01 with glucose, 6;24h pHT01 w/o glucose, 7; 48h Bt-with glucose, 8; 48h Bt-w/o glucose, 9; 48h Bt-LEA=II with glucose, 10; 48h Bt-LEA=II w/o glucose, 11; 48h pHT01 with glucose, 12=48h pHT01 w/o glucose

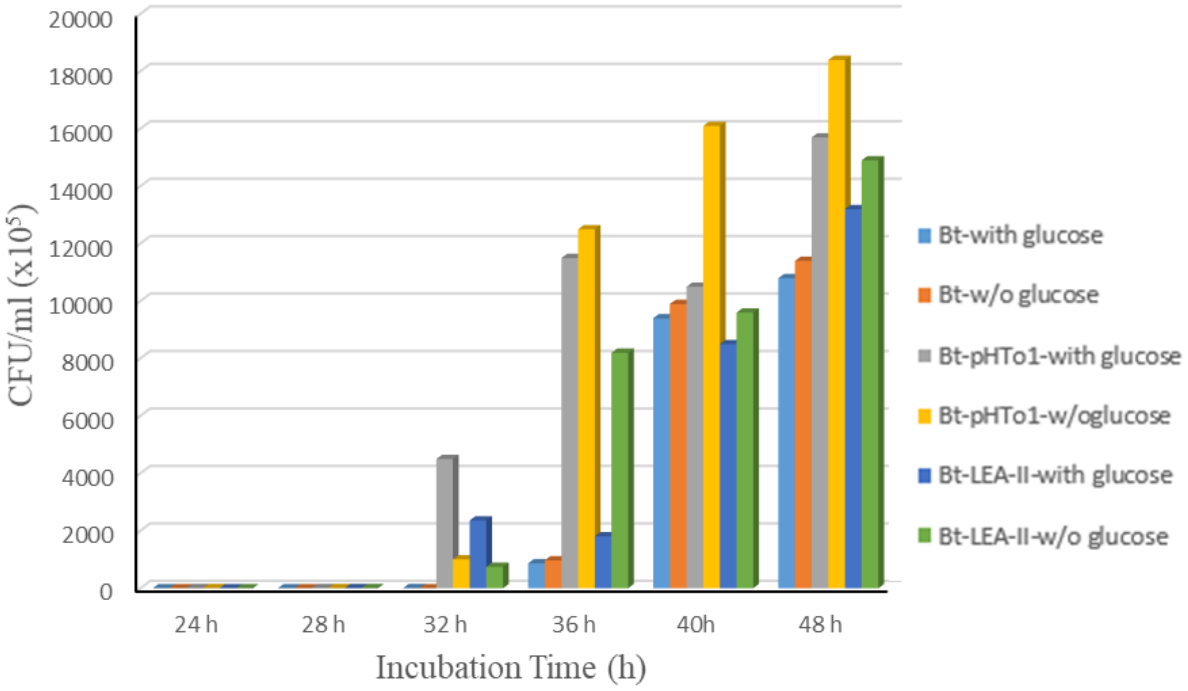

Figure 3-3 Spore counting analysis of each wild type, empty vector and mutated LEA peptide on the basis of time and the presence or absence of glucose after IPTG induction.

3.3.4 Cell growth and expression of the Cry protein in the Bt-LEA transformant with lactose by intermittent and at a time induction.

The cell growth was observed in the Bt-LEA transformant in comparison to intermittent (10 µL) and at a time (50 µL) induction of lactose. No drastic change was observed in the case of three different categories of Bt-LEA transformants (Bt-LEA-I, Bt-LEA-E & Bt-LEA-K). However, among all the Bt-LEA transformants, the highest enhancement was observed in the case of Bt-LEA-E transformants (Fig. 3-4) by the intermittent (10 µL) induction of lactose. In the LEA peptide co-expression system, the reason behind this enhancement might the action of LEA peptide either as a molecular chaperon or molecular shield effect on the cell. On the other hand, the nature and position of Bt-LEA-E transformant such as negatively charged (acidic amino acids), polarity,

and hydrophilicity and also the position (6th and 12th position) could be regarded as key indicator for this efficient expression. In the LEA peptide co-expression, the size and position of amino acid that play a vital role in the co-expression system have been already reported by Pathak et al., [50]. Finally, for the enhancement of Bt-LEA-E transformant the most attractive considering point should be taken or meaningful is the intermittent induction of lactose.

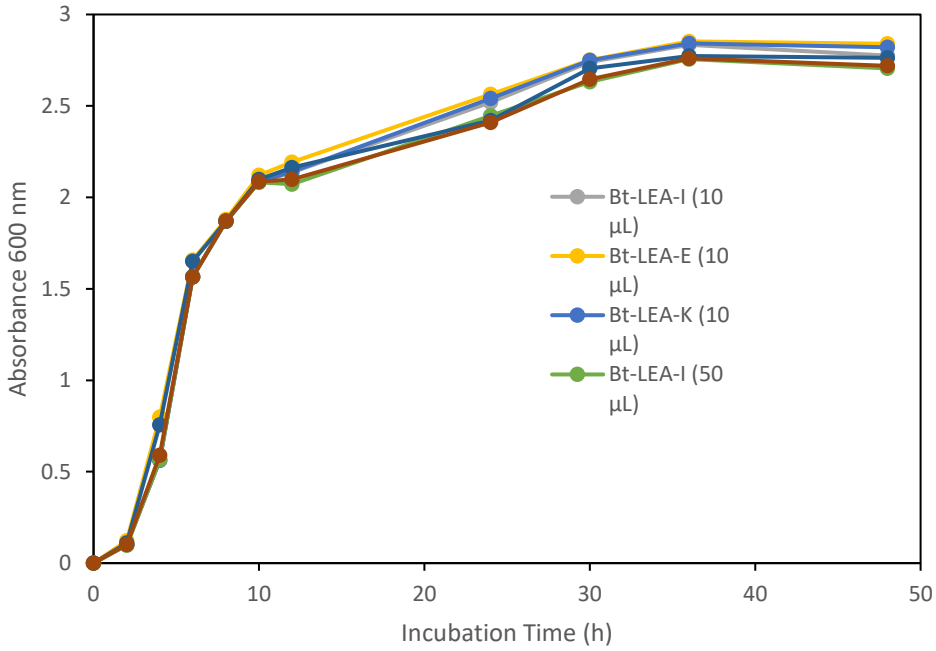

Figure 3-4 Cell growth curves of recombinant Bt which expressed each mutated LEA peptide showing on time dependence by intermittent (10 µL) and at a time (50 µL) induction of lactose. Induction time of intermittent (10 µL) was followed at 11th, 12th, 13th, 14th, and 15th hour, and at a time (50 µL) induction of lactose was 11th hour.

3.3.5 Enhanced expression of the Cry protein in the Bt-LEA transformant

SDS-PAGE data was analyzed by ImageJ, which is the most commonly used method to check protein expression level where the expression level of the Bt Cry protein (*cry*11Aa) was confirmed in conjugate with LEA peptide co-expression system. The Bt-LEA transformant (LEA-I), Bt-LEA transformant (LEA-K), Bt-LEA transformant (LEA-E), and non-transformant (Wt) showed the protein band in the position of 66.4 kDa. The band intensity of the Bt-LEA transformants (LEA-I and LEA-K) was more pronounced than the band intensity of the Wt and IPTG. To determine the highest expression of Cry protein, we varied lactose concentrations and induction time from 11^{th} hour to 15^{th} (Table 3-3) hour and 16^{th} hour to 20^{th} hour (Table 3-4). Cry protein expression was induced with 0.0 mmol/L, 0.1 mmol/L, 0.2 mmol/L, 0.5 mmol/L lactose concentration and 0.1 mmol/L concentrations of IPTG for LEA-I, LEA-E and LEA-K. Induction timing and concentration of lactose were considered to check the intensity of expression. Expression of Cry protein was the highest at a 0.1 mmol/L concentration of lactose at the time interval during 11^{th} hour to 15^{th} hour in the case of LEA-E. It is more obvious that lactose was more effective than IPTG in LEA peptide co-expression system by determining the expression level particularly for 0.1 mmol/L concentration both of lactose and IPTG in the case of Bt-LEA-E transformant. These results indicate that the expression level of Cry protein was attributed to the co-expression of the LEA-I, LEA-E, and LEA-K peptides by the LEA peptide co-expression system. For efficient expression of Cry protein, induction time was considered to be relevant for Bt. D142 as the logarithmic phase is completed about 10 hours after the initiation culture and finally the stationary phase. The continuous addition of lactose has an influence on Cry protein expression in this co-expression system as the bacterial cell can utilize the expression system. Lactose, as an inducer was effective in comparison to IPTG for the enhancement on the expression of *Helicobacter*

pylori where the recombinant genes were able to express the target proteins, was reported by Yan et al., [51]. In this investigation, the dosages and inducing time were 0.8, 50 g/L and 4 hours for rHpa A; 0.8, 100 g/L and 4 hours for rLTKA63; 1.2, 100 g/L and 5 hours for both rUreB and rLTB, respectively [52].

Whereas our first-ever approach was to utilize the induction time and induction pattern like intermittent induction (10 µL) and at a time induction (50 µL. Another approach is also initiated by Tian et al., that the induction of lactose for the optimization of fermentation resulted in 1,382 g of cell mass, representing 84 % enrichment in cell mass in comparison to the induction of IPTG induction [53].

Table 3-3 SDS-PAGE analysis of insecticidal protein expression in transformant Bt by lactose monohydrate and IPTG induction time at 11,12, 13, 14, and 15 hour-each hour 10µL

	0.0 mmol/L			0.1 mmol/L			0.2 mmol/L			0.5 mmol/L			0.1 mmol/L (IPTG)		
Bt	LEA -I	LEA -E	LEA -K	LEA -I	LEA -E	LEA -K	LEA -I	LEA -E	LEA-K	LEA -I	LEA -E	LEA -K	LEA -I	LEA -E	LEA -K
1.00	0.21	2.10	0.85	2.56	2.97	0.67	2.41	1.94	1.19	2.87	2.45	0.85	0.96	1.32	0.72

Table 3-4 SDS-PAGE analysis of insecticidal protein expression in transformant Bt by lactose monohydrate and IPTG induction time at 16, 17, 18, 19, and 20 hour-each hour 10µL

	0.0 mmol/L			0.1 mmol/L			0.2 mmol/L			0.5 mmol/L			0.1 mmol/L (IPTG)		
Bt	LEA -I	LEA -E	LEA -K	LEA -I	LEA -E	LEA -K	LEA -I	LEA -E	LEA-K	LEA -I	LEA -E	LEA -K	LEA -I	LEA -E	LEA -K
1.00	1.19	1.17	1.26	1.22	2.36	1.32	1.56	1.48	1.61	1.55	1.49	1.71	1.28	1.50	1.32

3.3.5 Optimal inducing concentrations and induction pattern of lactose

In this investigation, Lactose with two different concentrations and induction patterns might be the indicator to determine the effective expression of Cry protein. In addition, only lactose is using either an inducer or not. So, in this investigation it is obvious to confer that the induction pattern of lactose (intermittent concentration, 10 µL) might be one of the crucial factor for enhancement of Cry protein expression in comparison to IPTG. This intermittent induction of lactose phenomenon was not formerly illustrated to the expression of protein in associate with LEA peptide co-expression system, particularly for Cry protein expression. So, this may be the hypothesis why intermittent induction (10 µL) is most fruitful in comparison to at a time induction (50 µL) as the bacterial cell can completely utilize it as glucose is getting depletion in accordance with time. Because the presence of glucose often prevents the use of secondary carbon sources [54].

The comparison of the data between Fig. 3-5 and Fig. 3-6 at the 11^{th} hour, the concentration of glucose was lower than lactose. So, it may be another indication that according to time as the concentration of glucose is getting reduced, bacteria prefers to the second carbon source is obvious lactose and utilized as an inducer to enhance the expression of Cry protein. Another purpose of this experiment was to clarify which concentration and the pattern of induction like intermittent (10 µL) or at a time induction (50 µL) were more fruitful. From Fig. 3-5, we can easily determine that the presence of lactose concentration by intermittent induction (10 µL) was higher whereas, at a time induction of lactose concentration (50 µL) was lower at 16^{th} hour. Consequently, if we observe Fig. 3-6, the concentration of glucose (10 µL) was lower than (50 µL) glucose at 16^{th} hour. Even though if we observe Fig. 3-7, there was no presence of galactose at the 16^{th} hour. So finally,

we can confer that intermittent induction of lactose (10 µL) might be a new technique for the enhancement of bacterial protein expression

The lactose in associate with intermittent induction summarized in this study for the very first time to permit the expression of Cry protein by the employment of LEA peptide co-expression system. Lactose-induced culture was able to enhance the more expression in order to grab the position of the most commonly used inducer, IPTG. The efficacy of lactose was checked by intermittent addition to the media. This lactose induced-system was not only effective than IPTG but also presented a new innovative technique of intermittent induction in comparison to at a time induction. By the employment of intermittent induction, the bacterial cell becomes capable to properly utilization of lactose for their growth and lac operon system to enhance the expression of LEA peptide along with protein expression. Remarkably, it could be mentioned this new approach is pretty interesting. How do we say it's interesting? The same amount of lactose by the intermittent induction (10 µL) from 11^{th} hour to 15^{th} hour when the round figure comes 50 µL but at a time induction the total amount is also 50 µL. But only the induction pattern (intermittent induction, 10 µL) influenced the total beauty of this experiment by enhancing the expression of Cry protein 3 fold. Finally, the comprehensive utilization of intermittent induction of lactose in near future may represent a new window to lead a very fruitful operation for efficient expression of the protein in comparison to IPTG.

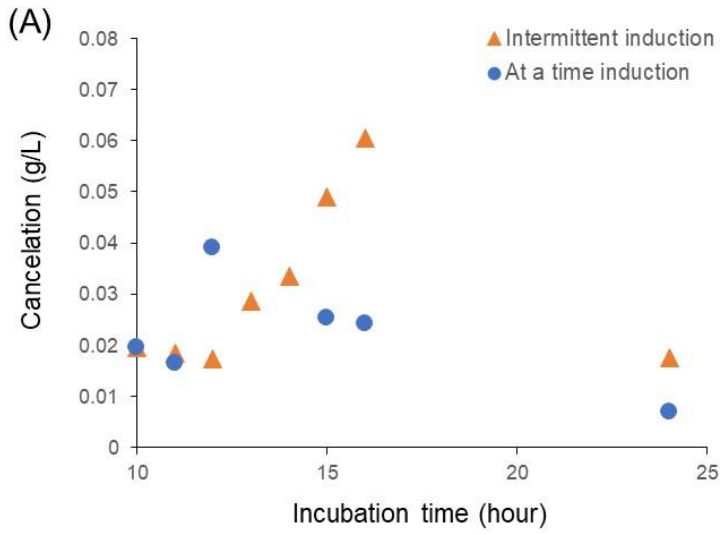

Figure 3-5 Concentration of lactose in culture media incubated recombinant Bt which expressed mutated LEA-E peptide. Sample is showing on time dependence by intermittent (10 µL) and at a time (50 µL) induction by lactose. Induction time of intermittent (10 µL) was followed at 11th, 12th, 13th, 14th, and 15th hour, and at a time (50 µL) induction of lactose was 11th hour.

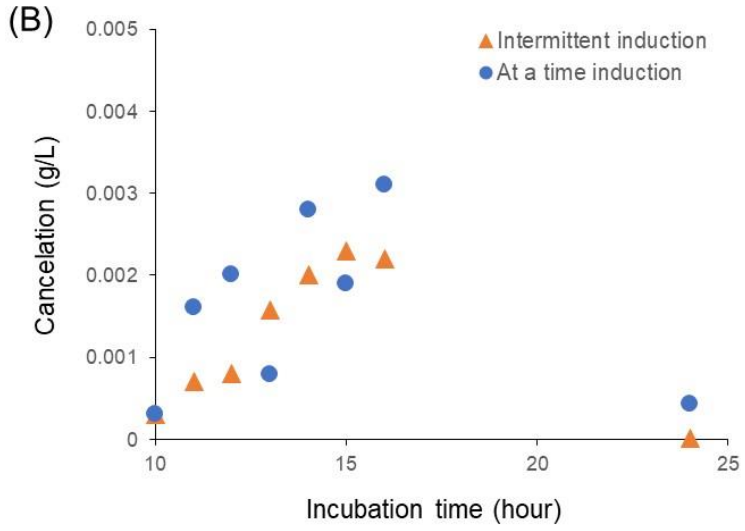

Figure 3-6 Concentration of glucose in culture media incubated recombinant Bt which expressed mutated LEA-E peptide. Sample is showing on time dependence by intermittent (10 µL) and at a time (50 µL) induction by lactose. Induction time of intermittent (10 µL) was followed at 11th, 12th, 13th, 14th, and 15th hour, and at a time (50 µL) induction of lactose was 11th hour.

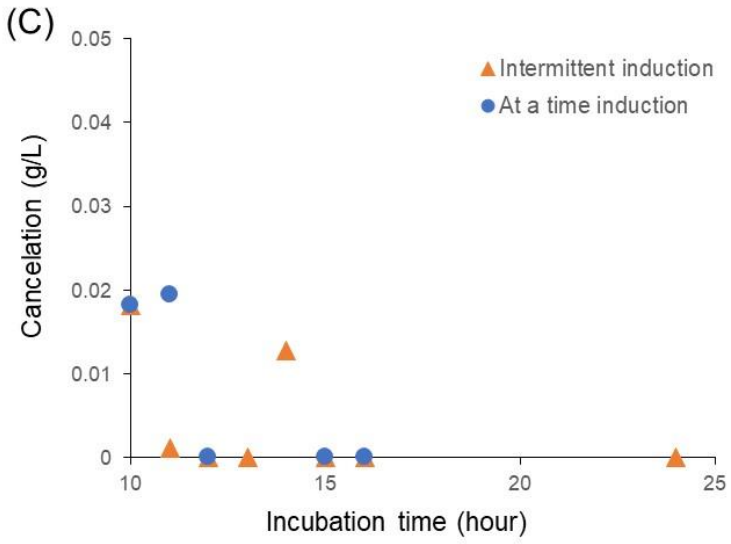

Figure 3-7 Concentration of galactose in culture media incubated recombinant Bt which expressed mutated LEA-E peptide. Sample is showing on time dependence by intermittent (10 µL) and at a time (50 µL) induction using lactose. Induction time of intermittent (10 µL) was followed at 11th, 12th, 13th, 14th, and 15th hour, and at a time (50 µL) induction of

3.4 Conclusion

In the case of the presence or absence of glucose, there is no significant difference in the case of protein expression after 48 hours. So, it's indicating that LEA peptide co-expression system is capable even though in absence of glucose. Also, clearly indicating that an excess amount of glucose can affect the LEA peptide co-expression system. On the other hand, it is also speculating that no glucose in the media, the lac repressor also does not work. So presence or absence of glucose does not have any direct effect to express the Cry protein expression but has a little effect during cell growth. During the cell growth, the absence of glucose, make the bacterial cell more stressed to produce more spore to regulate the normal cell cycle.

On the other hand, here is the alternative inducer lactose was more effective in comparison to the most commonly used IPTG. The efficacy of lactose was checked by continuous addition to the media and to check the effect. Continuous addition was far better than at a time induction. By continuous addition, the bacterial cell can properly utilize for their growth and lac operon system to enhance the expression of LEA peptide, then Cry protein expression. Finally, I would like to mention this new approach is pretty interesting. How do we say it's interesting? The same amount of lactose by the intermittent induction (10 µL) at 15^{th} hour the round figure comes 50 µL but at a time induction (50 µL). But only the induction pattern (intermittent induction, 10 µL) influences the total beauty of this experiment. Finally, the comprehensive utilization of intermittent induction of lactose in near future may represent a new window to lead a very comfortable operation for efficient expression of the protein in comparison to IPTG.

References

1. Heckel, D.G., (2020) How do toxins from *Bacillus thuringiensis* kill insects? An evolutionary perspective. Archives of Insect Biochemistry and Physiology 104, e21673.
2. Patterson, J., Sammon, M., Garg, M., (2016) Dengue, Zika and chikungunya: emerging arboviruses in the New World. Western Journal of Emergency Medicine 17, 671.
3. Bravo, A., Gill, S.S., Soberon, M., (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49, 423-435.
4. Becker, N. (2000) Bacterial control of vector-mosquitoes and black flies in Entomopathogenic bacteria: from laboratory to field application pp. 383-398, Springer.
5. Siegwart, M., Graillot, B., Blachere Lopez, C., Besse, S., Bardin, M., Nicot, P. C. & Lopez-Ferber, M. (2015) Resistance to bio-insecticides or how to enhance their sustainability: a review, Frontiers in Plant Science. 6, 381.
6. Amin, G., Alotaibi, S., Narmen, Y.A., Saleh, W., (2008) Bioinsecticide production by the bacterium *Bacillus thuringiensis*. 1. Pattern of cell growth, toxin production and by-product synthesis. Archives of Agronomy and Soil Science 54, 387-394.
7. Monod, J., (1942) Diauxia and respiration during the growth of *E. coli* cultures. Paper presented at the Annales de L'Institut Pasteur. 68, 548-450.
8. Görke, B., Stülke, J., (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nature Reviews Microbiology 6, 613-624.
9. Ogura, M., (2020) Glucose-Mediated Protein Arginine Phosphorylation/Dephosphorylation Regulates ylxR Encoding Nucleoid-Associated Protein and Cell Growth in *Bacillus subtilis*. Frontiers in Microbiology 11, 2382.

10. Deutscher, J. (2008) The mechanisms of carbon catabolite repression in bacteria. *Current Opinion Microbiology.*11**,** 87-93.

11. Fujita, Y., (2007) Global regulatory networks in *Bacillus subtilis*. Transworld Research Network.

12. Görke, B., Stülke, J., (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nature Reviews Microbiology 6, 613-624.

13. Brückner, R., Titgemeyer, F., (2002) Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiology Letters 209, 141-148.

14. Chubukov, V., Gerosa, L., Kochanowski, K., Sauer, U., (2014) Coordination of microbial metabolism. Nature Reviews Microbiology 12, 327-340.

15. Kosono, S., Tamura, M., Suzuki, S., Kawamura, Y., Yoshida, A., Nishiyama, M., Yoshida, M., (2015) Changes in the acetylome and succinylome of *Bacillus subtilis* in response to carbon source. PLoS ONE 10, e0131169.

16. Schilling, B., Christensen, D., Davis, R., Sahu, A. K., Hu, L. I., Walker-Peddakotla, A., et al. (2015). Protein acetylation dynamics in response to carbon overflow in *Escherichia coli*. Molecular Microbiology 98, 847–863.

17. Lehnik-Habrink, M., Rempeters, L., Kovács, ÁT., Wrede, C., Baierlein, C., Krebber, H., et al. (2013). DEAD-Box RNA helicases in *Bacillus subtilis* have multiple functions and act independently from each other. Journal of Bacteriology 195, 534–544.

18. Ogura, M., and Asai, K. (2016). Glucose induces ECF sigma factor genes, *sigX* and *sigM*, independent of cognate anti-sigma factors through acetylation of CshA in *Bacillus subtilis*. Frontiers in Microbiology, 7,1918.

19. Delumeau, O., Lecointe, F., Muntel, J., Guillot, A., Guédon, E., Monnet, V., et al. (2011). The dynamic protein partnership of RNA polymerase in *Bacillus subtilis*. Proteomics 11, 2992–3001.

20. Shiwa, Y., Yoshikawa, H., Tanaka, T., and Ogura, M. (2015). *Bacillus subtilis degSU* operon is regulated by the ClpXP-Spx regulated proteolysis system. Journal of Biochemistry157, 321–330.

21. Helmann, J. D. (2016). *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. Current Opinion in Microbiology 30, 122–132.

22. Ishii, H., Tanaka, T., and Ogura, M. (2013). The *Bacillus subtilis* response regulator gene *degU* is positively regulated by CcpA and by catabolite-repressed synthesis of ClpC. Journal of Bacteriology195, 193–201.

23. Elsholz, A. K., Turgay, K., Michalik, S., Hessling, B., Gronau, K., Oertel, D., et al. (2012). Global impact of protein arginine phosphorylation on the physiology of *Bacillus subtilis*. Proceedings of the National Academy of Sciences of the United States of America 109, 7451–7456.

24. Schmidt, A., Trentini, D. B., Spiess, S., Fuhrmann, J., Ammerer, G., Mechtler, K., et al. (2014). Quantitative phosphoproteomics reveals the role of protein arginine phosphorylation in the bacterial stress response. Molecular and Cellular Proteomics 13, 537–550

25. Trentini, D. B., Fuhrmann, J., Mechtler, K., and Clausen, T. (2014). Chasing phosphoarginine proteins: development of a selective enrichment method using a phosphatase trap. Molecular and Cellular Proteomics 13, 1953–1964.

26. Junker, S., Maaß, S., Otto, A., Hecker, M., and Becher, D. (2019). Toward the quantitative characterization of arginine phosphorylations in *Staphylococcus aureus*. Journal of Proteome Research18, 265–279.

27. Zhou, B., Semanjski, M., Orlovetskie, N., Bhattacharya, S., Alon, S., Argaman, L., et al. (2019). Arginine dephosphorylation propels spore germination in bacteria. Proceedings of the National Academy of Sciences of the United States of America 116, 14228–14237.

28. Masri, M.M.M., Ariff, A.B., (2020) Effect of different fermentation strategies on *Bacillus thuringiensis* cultivation and its toxicity towards the bagworm, Metisa plana Walker (Lepidoptera: Psychidae). Egyptian Journal of Biological Pest Control 30, 1-10.

29. Aidelberg, G., Towbin, B.D., Rothschild, D., Dekel, E., Bren, A., Alon, U., (2014) Hierarchy of non-glucose sugars in *Escherichia coli*. BMC systems biology 8, 1-12.

30. Rosano, G.L., Ceccarelli, E.A., (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. Frontiers in Microbiology 5, 172.

31. Fernández-Castané, A., Caminal, G. & López-Santín, J. (2012) Direct measurements of IPTG enable analysis of the induction behavior of *E. coli* in high cell density cultures, Microbial cell factories. 11, 1-9.

32. Studier, F.W., (2018) T7 expression systems for inducible production of proteins from cloned genes in *E. coli*. Current Protocols in Molecular Biology 124, e63.

33. Kweon, D.-H., Han, N.S., Park, K.-M., Seo, J.-H., (2001) Overproduction of *Phytolacca insularis* protein in batch and fed-batch culture of recombinant *Escherichia coli*. Process Biochemistry 36, 537-542.

34. Kim M., Elvin C., Brownlee A., Lyons R., (2007) High yield expression of recombinant pro-resilin: lactose-induced fermentation in *E. coli* and facile purification. Protein Expression and Purification 52,230–236.

35. Woyski D, Cupp-Vickery JR., (2001) Enhanced expression of cytochrome P450s from lac-based plasmids using lactose as the inducer. Archives of Biochemistry and Biophysics 388, 276–280.

36. Li Z, Zhang X, Tan T (2006) Lactose-induced production of human soluble B lymphocyte stimulator (hsBLyS) in *E. coli* with different culture strategies. Biotechnology Letter 28, 477–483.

37. Hausjell, J., Weissensteiner, J., Molitor, C., Halbwirth, H., Spadiut, O., (2018) *E. coli* HMS174 (DE3) is a sustainable alternative to BL21 (DE3). Microbial Cell Factories 17, 19.

38. Briand, L., Marcion, G., Kriznik, A., Heydel, J., Artur, Y., Garrido, C., Seigneuric, R., Neiers, F., (2016) A self-inducible heterologous protein expression system in *Escherichia coli*. Scientific Reports 6,1–11.

39. Durani, V., Sullivan, B.J., Magliery, T.J., (2012) Simplifying protein expression with ligation-free, traceless and tag-switching plasmids. Protein Expression and Purification 85, 9-17.

40. Marbach, A., Bettenbrock, K., (2012) Lac operon induction in *Escherichia coli*: systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA. Journal of Biotechnology 157, 82-88.

41. Haddadin, F.a.T., Harcum, S.W., (2005) Transcriptome profiles for high-cell-density recombinant and wild-type *Escherichia coli*. Biotechnology and Bioengineering 90, 127-153.

42. Dvorak, P., Chrast, L., Nikel, P.I., Fedr, R., Soucek, K., Sedlackova, M., Chaloupkova, R., de Lorenzo, V., Prokop, Z., Damborsky, J., (2015) Exacerbation of substrate toxicity by IPTG in *Escherichia coli* BL21 (DE3) carrying a synthetic metabolic pathway. Microbial Cell Factories 14, 1-15.

43. Sina, M., Farajzadeh, D., Dastmalchi, S., (2015) Effects of environmental factors on soluble expression of a humanized anti-TNF-α scFv antibody in *Escherichia coli*. Advanced Pharmaceutical Bulletin 5, 455.

44. Ding, Z., Wen, Y., Yang, B., Zhang, Y., Liu, S., Liu, Z., Han, Z., (2013) Biochemical mechanisms of imidacloprid resistance in *Nilaparvata lugens*: over-expression of cytochrome P450 CYP6AY1. Insect Biochemistry and Molecular Biology 43, 1021-1027.

45. Fox, B.G., Blommel, P.G., (2009) Autoinduction of protein expression. Current Protocols in Protein Science 56, 5.23. 21-25.23. 18.

46. Menzella, H.G., Ceccarelli, E.A., Gramajo, H.C., (2003) Novel *Escherichia coli* strain allows efficient recombinant protein production using lactose as inducer. Biotechnology and Bioengineering 82, 809-817.

47. Gombert, A., Kilikian, B., (1998) Recombinant gene expression in *Escherichia coli* cultivation using lactose as inducer. Journal of biotechnology 60, 47-54.

48. Schneider, C.A., Rasband, W.S., Eliceiri, K.W., (2012) NIH Image to ImageJ: 25 years of image analysis, Nature Methods 9, 671-675.

49. Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyama, H., Sen, K., Sakai, H., (2002) Active form of dipteran-specific insecticidal protein *cry*11A produced by *Bacillus thuringiensis* subsp. *Israelensis,* Bioscience, Biotechnology, and Biochemistry 66, 516-522.

50. Pathak, N., Hamada, H., Ikeno, S., (2018) Construction and characterization of mutated LEA peptides in *Escherichia coli* to develop an efficient protein expression system. Journal of Molecular Recognition 31, e2658.

51. Yan, J., Zhao, S.-F., Mao, Y.-F., Luo, Y.-H., (2004) Effects of lactose as an inducer on expression of *Helicobacter pylori* rUreB and rHpaA, and *Escherichia coli* rLTKA63 and rLTB. World Journal of Gastroenterology 10, 1755

52. Kant, S., Kapoor, R., Banerjee, N., (2009) Identification of a catabolite-responsive element necessary for regulation of the *cry*4A gene of *Bacillus thuringiensis* subsp. *israelensis*. Journal of Bacteriology 191, 4687-4692.

53. Tian, H., Tang, L., Wang, Y., Wang, X., Guan, L., Zhang, J., Wu, X., Li, X., (2011) Lactose induction increases production of recombinant keratinocyte growth factor-2 in *Escherichia coli.* International Journal of Peptide Research and Therapeutics 17, 123-129.

54. Magasanik, B., (1961) Catabolite repression. Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press, 249-256.

CHAPTER 4

General conclusion

4.1 Summary

The production of recombinant proteins is the most desire attraction for any researchers as well as in the field of molecular biology as it has transformed into a new dimension. Undoubtedly, the expression system is the major pathway for recombinant protein or efficient expression of the protein. Although *Escherichia coli* is one of the first preferences for expression, another host cell can also be utilized for the recombinant protein expression where the major bottleneck is associated with efficient expression of the protein. Most common problem for getting the efficient amount of protein, particularly selection of process, duration of time, and extremely considering point of the purification process. In this entire investigation, our approaches how to enhance the efficient expression of Cry protein from *Bacillus thuringiensis* by maintaining the quality and quantity without any tag expression system, without any interference of inclusion bodies, and presence of an unwanted protein that may obtain from the related organism. The purpose of this research is to establish a new system by the first ever interaction of late embryogenesis abundant (LEA) peptide co-expression with Bt synthesized Cry protein for efficient expression as a biological approach to compete with chemical insecticide as well as compensation of the price as an industrial purpose in a way of eco-friendly approach.

In the first chapter, the current problems in combating mosquito-induced infections were pointed out and described the effectiveness of killing mosquito larvae with microbial insecticides by the utilization of Bt synthesized Cry protein. Thereafter, we described how worldwide people are suffering from dangerous deadly diseases like dengue created by the mosquito. So finally we described the current problems, presented our first-ever approach and objectives of this study, and explained the structure of this doctoral thesis.

In the second chapter, for efficient expression of crystal protein against the mosquito larvae, we introduced a very simple but unique technique, LEA peptide co-expression system based on the expression vector pHT01 with a strong σA-dependent promoter to enhance the expression of insecticidal crystal proteins in native Bt. The co-expression of LEA peptide with Cry protein determines exceptional influence for expression of Cry protein without any unexpected change in the expression. One of the most considering points is that insertion of LEA peptide gene in the Bt did not change in the cell growth or other activities. Consequently, we designed two forms of LEA peptides (LEA-II and LEA-K) based on the sequence of group-3 LEA protein, which consists of a repetitive sequence of 11 amino acids. LEA-II and LEA-K were designed from the original peptide LEA-I suggesting that there is a strong interaction between the expression pattern of targeted Cry protein and also the composition, position, and nature of the amino acid. In this study, the amino acid sequence of the 13-mer LEA peptide, threonine was substituted by leucine at amino acid position 7 in LEA-II, and glycine was substituted by lysine at amino acid positions 6 and 12 in LEA-K peptide. It's our hypothesize that the hydrophobic nature of leucine along with the positive charge of lysine in LEA peptide might have an impact on the efficient expression of the Cry protein. Even though, stabilization of polar residues in association with interactions with the target protein is important for the efficient enhancement of Cry protein expression. LEA peptide may protect a protein from degradation by a protease or may have a molecular shielding effect. After analyzing the result, Bt-LEA-II mediated co-expression system demonstrated the maximum expression of crystal protein owing to have 3-fold after induction of isopropyl β-D-thiogalactopyranoside (IPTG) with concentration 0.5 mmol/L IPTG at 12th hour. In this study, it seems by utilizing of LEA peptide co-expression system the Cry protein expression was higher in comparison to wild-type Bt. Even though our previous study indicated the targeted protein will express but how it will

enhance is still questionable. On the other hand, Cry protein expression level was satisfactory but from the commercial point of view, it was not up to the mark. Although this unique approach has a great potential to produce bio based insecticidal agents by the enhancement of crystal protein, still it has some limitations. Another considering point, induction of IPTG in the commercial purpose bit difficult to manipulate as well as economically not welcome. So, in our next step, the approach should be redesigned in such a way where we can get the maximum expression of Cry protein with an alternative inducer of IPTG.

For the third chapter, the experiment was designed by the framework of utilizing the glucose and without glucose in the media and lactose monohydrate inducer as an alternative to IPTG. By using the LEA peptide co-expresion system. Considering the point of glucose, like presence or absence of glucose in the media have any effect on the protein expression and lactose can be an inexpensive and non-toxic inducer for efficient expression of Cry protein. In this study, we also utilized the same LEA peptide co-expression to enhance our targeted protein by gathering the knowledge from the previous chapter. We utilized wild type Bt, empty vector, pHT01, and BT-LEA-II transformant. As glucose is the most preferable carbon source for bacterial growth, spore formation, and Cry protein production as well. But it is reported that excess glucose in the media can suppress the spore formation and also reduce toxicity.

From the results, there was no significant difference in the case of protein expression after 48 hours of incubation with presence or absence of glucose in the cultural media. So it's indicating that LEA peptide co-expression system is capable to express protein even though in absence of glucose. This result is clearly indicating that an excess amount of glucose can affect the LEA peptide co-expression system. On the other hand, it is also speculating that the cultural media

without glucose, the lac repressor does not work. So, presence or absence of glucose does not have any direct effect to express the Cry expression but has a little effect during cell growth. During cell growth, the absence of glucose, make the bacterial cell more stressed to produce more spore to regulate the normal cell cycle. So, it might be the same as both empty vector and Bt-LEA-II transformant when no glucose in the media.

LEA peptide co-expression system was effective to enhance the Cry protein expression by the induction of IPTG. So, by gathering the knowledge from the previous chapter, maybe it is possible to utilize lactose as an inducer to enhance the Cry protein expression in association with LEA peptide co-expression system. For this experiment, we designed three forms of LEA peptides (LEA-I, LEA-E, and LEA-K) like Chapter 2. The presence of lactose in the media either is working as an inducer or carbon source. If lactose works as an inducer, it will bind to the lac repressor and makes it let go to the operator. Then RNA polymerase can transcribe the operon. For more clarification, it is also essential to determine the presence of glucose and lactose. Because in the LEA peptide co-expression system, how is the effect of glucose or lactose can perform either as an inducer or not. Among three forms of LEA peptides, LEA-E was more effective to enhance Cry protein expression. In this study, the amino acid sequence of the 13-mer LEA peptides, glycine was replaced with glutamic acid at amino acids 6 and 12 in the LEA-E peptide, and glycine was replaced with lysine at amino acids 6 and 12 in the LEA-K peptide. LEA-I was the original peptide and it can make a correlation between the expression level of targeted protein and amino acid composition in LEA peptide sequence, which can be exploited to determine the expression level of genes. On the other hand, the nature of hydrophilic glutamic acid in association with negative charge might influence enhancing the Cry protein expression in case of Bt-LEA-E peptide. Not only have that, but also the induction pattern like intermittent induction of lactose (10 μL) was

also more fruitful to bacteria for utilizing expression of Cry protein. Because at the 11^{Th} the amount of glucose already started to depleted status and can effectively utilize lactose as an inducer for the efficient expression of Cry protein.

The overall work follows presented that how LEA peptide can enhance the Cry protein expression where the data indicating the nature, position, and structure of amino acid in the LEA peptide might have the influence to enhance the targeted protein expression through this co-expression system. In association with, the particular role of IPTG and also the time of induction. In addition to IPTG, another alternative inducer of lactose and the pattern of induction have a pivotal role in the efficient expression of Cry protein. Finally, by the employment of intermittent induction of lactose induced LEA peptide co-expression method, Cry protein expression in Bt can be implemented economically and effectively, and is expected to develop into a practical technology.

4.2 Future prospect

The core purpose of our study to enhance the expression of Cry protein by the employment of LEA peptide co-expression system based on the biological approach to compete with the chemical insecticide to kill the mosquito larvae. Based on the diverse kinds of LEA peptide with two different inducers were utilized in this entire resaerch for enhancement of Cry protein expression. We hypothesize that the LEA peptide can work as amolecular chaperone or has the molecular shielding effect on the efficient expression of targeted protein. Besides this, naure and position of amino acid have also the the influence for expression of targeted potein. But still there is some limitation in this investion that how LEA peptide is enhancing the expression of Cry protein in association with the expression of Cry peotein is not highly satisfactory. Although we know that it is hard to gain highest level of expression based on biological approach. So for gaining maximum level of expression, we should focus on othe Bt synthesized other protein like Cyt and VIP protein and how is the effect of this synergistic action of these proteins. Not only synergistic action, but also the study of molecular architecture of Cyt or VIP protein may reveal the way to enhance the expression level and insecticidal activation as well. To focus on the molecular analysis like mutation of Cry protein and recombinant host expression might be another approach for enhancement of Cry protein expression.

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Akhtar Mahmuda

Achievements

Publication:

1. **Mahmuda Akhtar**, Kazurhiro Mizuta, Tomoko Shimokawa, Minoru Maeda, M.M.R.Talukder, Shinya Ikeno, (2021) Enhanced insecticidal activity of *Bacillus thuringiensis* using a late embryogenesis abundant peptide co-expression system. Journal of Microbiological Methods, 106207**. doi.org/10.1016/j.mimet.2021.106207**

Under submission

Mahmuda Akthar, Tomoko Shimokawa, Yinghan Wu, Taichi Arita, Kazuhiro Mizuta, Yuria Isono, Minoru Maeda, and Shinya Ikeno, Lactose as a time-dependent promising inducer in response to the efficient expression of insecticidal crystal protein in *Bacillus thuringiensis* by late embryogenesis abundant peptide co-expression system.

Under Preparations:

Mahmuda Akhtar, Tomoko Shimokawa, Minoru Maeda, Shinya Ikeno. Interaction of Metal oxide based nano particles to *Bacillus thuringiensis* synthesized Cry protein: opportunistic for enhancement of Cry toxicity against the larvae of mosquito. Impact of metal oside based nano partilces

Presentations:

1. **M. Akhtar**, Y. Isono, T. Shimokawa, M. Maeda, S. Ikeno; Lactose as a time-dependent promising inducer in response to the efficient expression of insecticidal crystal protein by late embryogenesis abundant peptide co-expression system in *Bacillus thuringiensis.* The Symposium of Applied Engineering & Sciences 2021 (SAES 2021), UPM, Malaysia on Dec. 5-8, 2021 (Oral Presentation).
2. **M. Akhtar**, K. Mizuta, T. Shimokawa, M. Maeda, S. Ikeno; Late embryogenesis abundant peptide co-expression system, the unique method for adequate expression of insecticidal crystal proteins in *Bacillus thuringiensis*. The Symposium of Applied Engineering & Sciences 2018 (SAES 2018), Tobata Campus, Kyutech on Dec.15-16, 2018 (Oral Presentation)
3. **M. Akhtar**, K. Mizuta, T. Konomi, M.Maeda, S. Ikeno; Improving *cry* gene protein expression in *Bacillus thuringiensis* via engineering, the unique method of late embryogenesis abundant peptide co-expression system. The Eleventh Japan-Korea joint symposium on Bio-micro sensing Technology, International Conference Center on Kitakyushu, Japan on Nov. 11-13, 2018. (Poster Presentation).
4. **M. Akhtar**, K. Mizuta, T. Konomi, M.Maeda, S. Ikeno; Introduction of late embryogenesis abundant peptide co-expression system for adequate expression of insecticidal crystal proteins in *Bacillus thuringiensis.* The 70th Annual Meeting of the Society for Biotechnology, Senriyama Campus, Kansai University Japan on Sep 5-7, 2018. (Oral Presentation)
5. **M. Akhtar**, K. Mizuta, T. Konomi, M.Maeda, S. Ikeno; Application of LEA peptide co-expression system for enhancement of insecticidal crystal protein. The 55th Kyushu Area

Joint Meeting of the Chemistry-Related Societies. Kitakyushu International Conference Center, Kokura, Japan Jun 30, 2018. (Poster Presentation).

6. **M. Akhtar**, K. Mizuta, T. Konomi, M.Maeda, S. Ikeno; Efficient expression of insecticidal proteins from *Bacillus thuringiensis*. The 54th Joint Symposium of Chemistry Related Society in Kyushu Island, on July 1, 2017 (Poster Presentation).
7. **M. Akhtar**, K. Mizuta[1], T. Konomi[1], M.Maeda[2], S. Ikeno[1]; Increase of insecticidal Cry proteins from *Bacillus thuringiensis* by using LEA peptide co-expression system. The Symposium of Applied Engineering & Sciences 2017 (SAES 2017) UPM, Malaysia on Nov. 14-15, 2017 (Poster Presentation).

Appendix

[i] **Culture media and plate**

Tryptic Soya broth (TSB) liquid Media

Reagent	**Adding amount**
BD BactoTM Tryptone	17g/L
BD BactoTM Soytone	3g/L
D+Glucose ($C_6H_{12}O_6$)	2.5g/L
Potassium Dihydrogen Phosphate (KH_2PO_4)	2.5g/L
Sodium Chloride (NaCl)	5g/L

Dissolved 17gm Tryptone, 3 gm Soytone, and 2.5 gm Potassium Dihydrogen Phosphate and 5 gm sodium chloride in 950 mL deionized water. Adjusted the pH of the medium to 7.0 by adding 1mol/L NaOH and finally made the total volume 1 litre. Thereafter, autoclave was done by maintaining 20 minutes at 15 psi. Allowed the medium to make it cool down at 55 ^oC and finally adding of the glucose 2.5 gm to the medium.

100 × spore forming solution

Reagent	**Adding amount**
Calcium chloride Dihydrate ($CaCl_2$. $2H_2O$)	1.02 %/100 mL
Manganese Chloride Tetrahydrate ($MnCl_2$ $4H_2O$)	0.1 %/100 mL
Magnesium Chloride Hexahydrate ($MgCl_2$. And H_2O)	2.03 %/100 mL

First 100 mL distilled water was taken and all the reagents weighed properly. Then all the reagents dissolved into the water and proper mixing by the help of stirrer. Finally filtered and preserved it at 4 ºC.

Nutrient Agar Plate

Reagent	**Adding amount**
Tryptone	10 gm/L
Sodium Chloride (NaCl)	5 gm/L
Yeast Extract (YE)	5 gm/L
Agar Powder	15 gm/L

Dissolved 10 gm tryptone, 5 gm sodium chloride, 5 gm yeast extract and finally 15 gm agar powder to the 1 liter distilled water before autoclave. Thereafter, autoclave was done by maintaining 20 minutes at 15 psi. Allowed the medium to make it cool down at 55 ºC and poured the medial in the agar plate to make it solidified. And finally preserved it at 4 ºC.

Sterile saline

Reagent	**Adding amount**
Sodium Chloride (NaCl)	0.85 %
Tween-80	0.05 %
DW	100 mL

100 ml distilled water was taken in a glass beaker and first added sodium chloride. After that tween-80 was added to solution and mixing properly by the help of stirrer. Finally filtered and preserved it in 4 ºC.

[ii] **Larvicidal activity against *Aedes albopictus***

The susceptibility of the *Aedes albopictus* larvae to the Cry toxin was assessed in the presence of protein treated with the Bt-LEA transformant and wild type (Wt) as a control. The evaluation was performed to determine the activity of the insecticidal protein by adding a diluted culture solution to *Aedes albopictus* larvae and counting the number of dead insects after 24 hours. For this bioassay, a diluted culture solution that was obtained by culturing the Bt-LEA transformant for 48 hours. Induction of each LEA peptide by IPTG was performed at 12th hour of incubation. The diluted solution was used as a tested solution and the 4th instar larvae of *A. albopictus* as an insect and wild type (Bt. D142) as a control. The experiment was executed by using 250 µL of sterilized distilled water and the same volume of the culture solution Bt-LEA transformants were added to a microtube to prepare a 50 % (500,000 ppm) culture solution. This solution (10 µL) was collected and mixed with 990 µL of sterilized distilled water to prepare a 5,000 ppm solution as a test solution. Five *A. albopictus* larvae were added into each well of a 12-well microplate, followed by the 4 mL of laboratory tap water. For evaluation, three wells (total of 15 larvae) were used for each concentration of tested samples. 40 µL of diluted test solutions (3,000, 2,000, 1,000, and 500 ppm) was added to wells and the final concentration were (30, 20, 10, and 5 ppm). The bioassay was maintained under controlled conditions at 25°C and 75% humidity for 48 hours. In each experiment, the tests were repeated three times on

different days and the mean mortality rates were obtained in each experiment. Mortality data for each larvicidal concentration were used to determine the LC_{50} using the probit method by Bliss.

[iii] **Quantification of glucose and lactose**

We determined the glucose and lactose concentration in the medium and cell by using a glucose and lactose detection kit (Boehringer Mannheim/R-Biopharm). We checked the glucose concentration from the fresh media and all the samples as well. To execute this, we prepared two categories of samples like blank and D-glucose samples for checking glucose concentration from each sample. For blank, first solution III (1mL) and distilled water (2.250 mL) were taken and for D –glucose checking 500 µL sample, first solution III (1mL) and 1.750 mL distilled water added, and the absorbance were checked at 340 nm at both of blank and for sample. After that suspension III was added both in blank and sample solution and incubated at room temperature for 20 minutes and finally the absorbance was checked. Every time the absorbance was taken 3 to check the stability of the reaction and before and after addition of solution IV the absorbance was indicated by A1 and A2 consequently. For the detection of lactose and galactose, we prepared three distinct categories of the sample as blank, samples for lactose, and D-galactose. Like glucose detection, first, we checked the lactose and D-galactose concentration from fresh media and finally for all the samples. Here also for blank, the solution I (200 µL), solution II (50 µL), solution III (1 mL), and finally distilled water (2mL). For lactose, the solution I (200 µL), solution II (50 µL), and sample (500 µL) were taken together and incubated at room temperature for 20 minutes. Thereafter, solution III and distilled water (2mL) were added finally. Accordingly, for D-galactose concentrations, except solution II, solution I (200 µL), sample (500 µL), solution III (1 mL), and lastly distilled water (1.55 mL) were added together. All the solutions were checked by using

spectrophotometry of 340 nm absorbance. Consequently, Solution IV was added to each sample and again incubated at room temperature for 20 minutes. Lastly again all the samples were checked by using the same absorbance. In this investigation, every time took absorbance three times and before and after adding solution IV the absorbance was determined by A1 and A2 respectively.

Calculation of concentration

For glucose concentration, if the absorbance A_2 increases constantly, extrapolate to the absorbance to the time of the addition of 4 (HK/G6P-DH). Determination of the absorbance differences (A_2- A_1) for both, blanks and samples. Subtraction of the absorbance difference of the blank form the absorbance difference of the corresponding sample.

$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$

It follows $\Delta A_{D\text{-glucose}}$ (from "D-glucose sample") and

$\Delta A_{lactose+\ D\text{-glucose}}$ (from "lactose sample").

The difference of these values stands for $\Delta A_{lactose}$

For lactose and galactose concentrations, if the absorbance A_2 increases constantly, extrapolate to the absorbance to the time of the addition of 4 (β-galactose dehydrogenase). Determination of the absorbance differences (A_2- A_1) for both, blanks and samples. Subtraction of the absorbance difference of the blank form the absorbance difference of the corresponding sample.

$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$

It follows $\Delta A_{D\text{-galactose}}$ (from "D-galactose sample") and

$\Delta A_{lactose+\ D\text{-galactose}}$ (from "lactose sample").

The difference of these values stands for $\Delta A_{lactose}$

According to the general equation for calculating the concentrations:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 100} \times \Delta A\ [g/l]$$

V=final volume (mL)

v= sample volume (mL)

MW= molecular weight of the substance to be assayed [g/mol]

d= light path (cm)

ε= extinction co-efficient of NADPH at

340 nm=6.3 [Ix $mmol^{-1}$x cm]

For glucose:if the sample has been diluted:

$$\text{Content}_{\text{lactose}} = \frac{C_{\text{lactose}}\ [g/l\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [g/100gm]$$

$$\text{Content}_{\text{D-glucose}} = \frac{C_{\text{D-glucose}}\ [g/l\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [g/100gm]$$

For lactose: if the sample has been diluted

$$\text{Content}_{\text{lactose}} = \frac{C_{\text{lactose}}\ [g/l\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [g/100gm]$$

$$\text{Content}_{\text{D-galactose}} = \frac{C_{\text{D-galactose}}\ [g/l\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [g/100gm]$$