

Development of a cost effective medium for enhanced production of *Bacillus thuringiensis* -endotoxin



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Dedicated
To
My beloved parents

DECLARATION

I hereby declare that the dissertation entitled “**Development of a cost effective medium for enhanced production of *Bacillus thuringiensis* -endotoxin**” prepared by me under the joint supervision and able guidance of Professor Dr. Naiyyum Choudhury, Coordinator, Biotechnology Program, BRAC university and Professor Dr. Md. Mozammel Hoq, Department of Microbiology, University of Dhaka in partial fulfillment of MS in Biotechnology, BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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Abstract

The control of pest populations by using bacteria as insect pathogens has been an attractive alternative to the application of chemical pesticides. As chemicals cause damage to the environment, biological control is preferable and *Bacillus thuringiensis* (*Bt*) which produces insecticidal δ -endotoxin (crystal protein) have been most widely used as biopesticide in agriculture. But to date no broad based target oriented work on *Bt* mass production has been initiated in Bangladesh. The present study was aimed at enhancing the δ -endotoxin synthesis in a suitable medium by *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) HD-73 harboring potential *cry* genes active against lepidoptera insects by regulating some key components of medium such as carbon and nitrogen sources, amino acid (cystine) and basal salts affecting fermentation. In this regard, the growth, sporulation and δ -endotoxin synthesis by *Btk* HD-73 were examined in the culture of different media at 30°C in shake and bioreactor culture conditions. The experiment was carried out under both monophasic submerged fermentation (SmF) and then biphasic solid state fermentation (SSF) so as to which facilitates sporulation under its stressed conditions. In SmF condition, the conventional Luria-Bertani (LB) medium which was enriched with nitrogen source (10% defatted soybean meal) supported 28.57% sporulation and 125% endotoxin increase over LB (alone). In biphasic SSF condition although the sporulation increased but the endotoxin yield was decreased when compared with monophasic SmF condition. The effect of cystine on sporulation and endotoxin synthesis was highly pronounced in LB-soybean medium (LBS) with a range of 19.54% and 131.35% higher endotoxin yield respectively in SmF condition. The basal salts-soybean-cystine (SMc) medium resulted in 7.65% decrease in endotoxin production than in LB-soybean-cystine (LBSc) medium, but it is comparable. Addition of molasses balanced the C:N ratio in the SMc medium thus helping 84.85% higher endotoxin synthesis after 24 hours fermentation. Substitution of basal salts with cost effective sea water yielded about 21% less endotoxin. For large scale production, use of soybean extract than that of soybean mass in the culture medium supported better performance. The optimum medium thus obtained consisting of soybean extract-molasses-cystine with sea water was used in 3L bioreactor cultivation for endotoxin synthesis by *Btk* HD-73 under 30% saturation of dO_2 through cascade of agitation and aeration. The production rate obtained was 1.67 fold higher in bioreactor than in shake culture. The present results may successfully be used for large scale production of biopesticide in Bangladesh.

Contents

Chapter 1 Introduction and Literature Review		Page no. (1-31)
1.1	Introduction	1
1.2	Literature review	4
1.2.1	<i>Bacillus thuringiensis</i>	4
1.2.2	History of <i>Bacillus thuringiensis</i>	5
1.2.3	Ecology and prevalence of <i>B. thuringiensis</i>	6
1.2.4	Genetic Diversity of <i>B. thuringiensis</i>	7
1.2.4.1	<i>B. thuringiensis</i> genome	7
1.2.4.2	The <i>cry</i> genes	8
1.2.4.3	Cry protein structure	9
1.2.5	Mechanism of action of <i>Bt</i> formulations	11
1.2.6	Other pathogenic factors of <i>Bacillus thuringiensis</i>	12
1.2.7	Pesticides	13
1.2.7.1	Classifications of pesticides	13
1.2.7.2	<i>Bacillus thuringiensis</i> : As a biopesticide	14
1.2.7.2.1	Advantages of <i>Bt</i>	14
1.2.7.2.2	Disadvantages of <i>Bt</i>	14
1.2.8	Insects controlled by <i>Bt</i>	15
1.2.9	Applications	16
1.2.10	Factors affecting growth, sporulation and toxin production by <i>Bt</i>	17
1.2.10.1	Effects of different substrate (Carbon and Nitrogen as nutrient source)	18
1.2.10.2	Effects of cysteine	20
1.2.10.2.1	Spore and crystal formation in relation to <i>cys/cysSH</i> concentration	20
1.2.10.2.2	Effect of addition of <i>cys/cysSH</i> at the stationary phase	21
1.2.10.2.3	Effect of addition of <i>cys/cysSH</i> after the stationary phase	21
1.2.10.2.4	Microscopic observation	21

Contents

1.2.10.3	Effects of molasses	22
1.2.10.4	Effects of metal ions	22
1.2.10.5	Effects of pH	23
1.2.10.6	Effects of temperature	23
1.2.10.7	Effects of aeration	24
1.2.11	Mass production of <i>Bt</i>	24
1.2.11.1	Submerged Fermentation (SmF)/ Liquid Fermentation (LF)	25
1.2.11.2	Solid State Fermentation (SSF)	27
1.3	Aims and Objectives	30

Chapter 2 Materials and Methods **Page no. (32-43)**

2.1	Handling of laboratory apparatus and glassware	32
2.2	Solutions and reagents	32
2.3	Bacterial strain and culture conditions	32
2.4	Media preparation	33
2.4.1	Name and factors	33
2.4.2	Media preparation for submerged fermentation	33
2.4.2.1	Luria-Bertani Broth (Commercial media)	33
2.4.2.2	Defatted soybean mill supplemented LB (LBS)	33
2.4.2.3	Cystine supplemented LBS (LBSc)	34
2.4.2.4	Cystine supplemented soybean-basal salts medium (SMc)	34
2.4.2.5	Molasses supplemented soybean-cystine medium (SMmc)	34
2.4.2.6	Sea water in soybean-molasses-cystine medium (SSWmc)	35
2.4.2.7	Sea water in soybean extract-molasses-cystine medium (SeSWmc)	35
2.4.3	Media preparation for Biphasic Solid state fermentation	35
2.5	Inoculum preparation	35
2.6	Fermentation procedure for biopesticide production	36

Contents

2.6.1	Submerged fermentation	36
2.6.2	Biphasic Solid state fermentation	36
2.7	Sampling	36
2.8	Analysis of sample	37
2.8.1	Microscopic studies	37
2.8.2	Estimation of spore count	37
2.8.3	Estimation of crystal protein concentration	38
2.8.3.1	Partial purification of Cry proteins	38
2.8.3.2	Preparation of diluted bovine serum albumin (BSA) standard	38
2.8.3.3	Preparation for determination of α -endotoxin concentration	39
2.9	SDS-PAGE analysis of α -endotoxin <i>Bt kurstaki</i> HD-73	39
2.9.1	Sample preparation	40
2.9.2	Preparation of separating gel	40
2.9.3	Preparation of stacking gel	40
2.9.4	Sample application and gel run	41
2.9.5	Staining and destaining of the gel	41
2.10	Biopesticide production in 3L Bioreactor	41
2.10.1	Inoculum development	41
2.10.2	Growth conditions in Bioreactor	42
2.10.3	Fermentation in Bioreactor	42

Chapter 3 Results

Page no. (44-61)

3.1	Phenotypic characterization of bacterial strains	44
3.1.1	Colony characteristics	44
3.1.2	Crystal protein morphology of <i>Bacillus thuringiensis</i>	45

Contents

3.2	Construction of standard curve for estimation of protein by Bradford method	46
3.3	Determination of factors affecting sporulation and -endotoxin synthesis by <i>Btk</i> HD-73	47
3.3.1	Effects of defatted soybean mill with LB on sporulation and -endotoxin formation by submerged fermentation	47
3.3.2	Evaluation of biphasic fermentation on sporulation and -endotoxin formation	49
3.3.3	Role of cystine in defatted soybean mill with LB	50
3.3.4	Replacement of LB with basal salts	52
3.3.5	Performance of molasses in soybean-cystine medium	53
3.3.6	Replacement of basal salts with sea water in soybean-molasses-cystine medium	55
3.3.7	Finally formulated medium (soybean extract in place of soybean mill in soybean-molasses-cystine medium)	56
3.4	Evaluation of different media, at a glance, on sporulation	58
3.5	Evaluation of different media, at a glance, on -endotoxin synthesis	58
3.6	Production kinetics of <i>Btk</i> HD-73 under controlled condition in a 3L bioreactor	59
3.7	SDS-PAGE analysis of crystal protein	61

Chapter 4 Discussion

Page no. (62-67)

Conclusion and Recommendations

Page no. (68-69)

Chapter 5 References

Page no. (70-85)

Appendices

Page no. (i-viii)

List of Figures

Figure no.	Figure Name	Page no.
Figure 1.1	Formation of the toxic parasporal crystal in <i>B. thuringiensis</i>	4
Figure 1.2	The structure of Cry 3A	10
Figure 1.3	The structure of Crt 2A	10
Figure 1.4	Mechanism of toxicity of <i>Bt</i> δ -endotoxin toward insect	12
Figure 1.5	Alfaalfa webworms killed by <i>Bacillus thuringiensis</i>	16
Figure 2.1	BIO FLO 110 Fermentor / Bioreactor; company: NEW BRUNSWICK SCIENTIFIC	43
Figure 3.1	Colony characteristics on LB agar medium	45
Figure 3.2	Crystal protein morphology of <i>Bacillus thuringiensis</i> under phase contract microscope	45
Figure 3.3	Standard curve for estimation of protein concentration by Bradford method	46
Figure 3.4	Evaluation of soybean mill & LB medium on sporulation & endotoxin synthesis by <i>Btk</i> HD 73 in submerged fermentation	48
Figure 3.5	Sporulation and δ -endotoxin synthesis under submerged and biphasic solid state fermentation	50
Figure 3.6	Role of cystine in defatted soybean mill with LB	51
Figure 3.7	Replacement of LB with basal salts in soybean-cystine medium	53
Figure 3.8	Performance of molasses in soybean-cystine medium	54
Figure 3.9	Replacement of basal salt with sea water in soybean-molasses-cystine medium	56
Figure 3.10	Effect of soybean extracts in place of soybean mill in the soybean-molasses-cystine medium	57
Figure 3.11	Evaluation of different media, at a glance, on sporulation	58
Figure 3.12	Evaluation of different media, at a glance, on δ -endotoxin synthesis	58
Figure 3.13	Total spore count and δ -endotoxin concentration in 3L bioreactor	60
Figure 3.14	SDS-PAGE analysis of partially purified Cry protein obtained from the culture performed in SeSWmc agar	61

List of Tables

Table no.	Table Name	Page no.
Table 3.1	Evaluation of soybean mill & LB medium on sporulation & endotoxin synthesis by <i>Btk</i> HD 73 in submerged fermentation	48
Table 3.2	Sporulation and -endotoxin synthesis under submerged and biphasic solid state fermentation	49
Table 3.3	Role of cystine in defatted soybean mill with LB	51
Table 3.4	Replacement of LB with basal salts in soybean-cystine medium	52
Table 3.5	Performance of molasses in soybean-cystine medium	54
Table 3.6	Replacement of basal salts with sea water in soybean-molasses-cystine medium	55
Table 3.7	Effect of Soybean extract in place of soybean mill in the soybean-molasses-cystine medium	57
Table 3.8	Total spore count and -endotoxin concentration in 3L bioreactor	59

List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
conc.	Concentration
DDT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
<i>Et al.</i>	And others
etc.	And the rest
G/Gm	Gram
Hr	Hour
i.e.	That is
kD	Kilo Dalton
L	Liter
M	Molar
Mg	Miligram
Min	Minute
ml	Mililiter
mM	Milimole
Nm	Nanometer
N	Normal
OD	Optical density
pH	Negative logarithm of hydrogen ion concentration
Psi	Pound per square inch
Rpm	Rotation per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sec	Second
SLPM	Standard Liter Per Minute
Subsp.	Sub species
TCA	Trichloroacetic acid
U	Unit
v/v	Volume per volume
vol.	Volume
w/v	Weight per volume

CHAPTER

1

INTRODUCTION

AND

LITERATURE REVIEW

1.1 Introduction

The competition for crops between human and insects is as old as agriculture. Use of chemical substances to control pests was started in the mid-1800s. Early insecticides consisted of some inorganic chemicals and organic arsenic compounds. Organochloride compounds, organophosphates, carbamates, pyrethroids and formamides followed these compounds. Many of these chemicals are also being used today. Certain properties made these chemicals useful, such as long residual action and toxicity to a wide spectrum of organisms. However, chemical pesticide applications have caused many environmental problems including insect resistance, toxicity to humans and to beneficial insects (Glazer and Nikaido, 1995).

One of the practical means of increasing crop production is to minimize the pest associated crop losses. Insect pest control by toxic chemicals has brought about considerable protection to crop yields over decades. Unfortunately, extensive and indiscriminate uses of these conventional insecticides have resulted in environmental pollution, risks to human and animal health, adverse effect on the non-target beneficial insects, resistance to chemicals and resurgence of minor pests (Rao *et al.*, 1999).

Bangladesh is an agricultural country facing many problems of noxious insects that cause significant reduction of major agricultural products. Chemical control is the main solution for insect pest problem in Bangladesh as well as in many countries in the world. The extensive and indiscriminate uses of chemical pesticides have created many problems. Chemical insecticides affect both beneficial insects and pest species. Consequently, biological agents can be used as alternative strategies for insect control.

Biological pesticides are becoming an important component in crop and forest protection and in insect vector control. These pesticides are natural, disease causing microorganisms that infect or intoxicate specific pest groups (Carlton, 1988; Spear, 1987). Like all organisms, insects are susceptible to infection by pathogenic microorganisms.

Many of these infectious agents have a narrow host range and, therefore, do not cause uncontrolled destruction of beneficial insects and are not toxic to vertebrates. The greatest successes in microbial pesticides have come from the uses of *Bacillus thuringiensis* (*Bt*). *Bacillus thuringiensis* is a major microorganism, which shows entomopathogenic activity (Glazer and Nikaido, 1995; Schnepf *et al.*, 1998). The organism is a ubiquitous, gram-positive and spore-forming bacterium that forms parasporal crystals during the stationary phase of its growth cycle. Commercial preparations of *Bt* have been shown to be the most successful biological control products worldwide (Carlton, 1988). At present, *Bacillus thuringiensis* is the only "microbial insecticide" in widespread use.

Bt is a naturally occurring bacterium common in soils throughout the world. Several strains can infect and kill insects. Because of this property, *Bt* has been developed for insect control. In recent years, there has been tremendous renewed interest in *Bt*. Several new products have been developed, largely because of the safety associated with *Bt* based insecticides. Its insecticidal activity depends on parasporal crystals encoded by *cry* genes and this insecticidal activity varies according to insect type. Natural isolates of *B. thuringiensis* have been used as a biological pesticide since the 1950s for the control of certain insect species among the orders Lepidoptera, Coleoptera and Diptera. The genes of *B. thuringiensis* coding parasporal crystals are also a key source for transgenic expression which provides pest resistance in plants (Schnepf *et al.*, 1998). This feature makes *B. thuringiensis* the most important biopesticide on the world market (Bernhard *et al.*, 1997). In 1995, worldwide sales of *B. thuringiensis* based insecticides were estimated at \$90 million representing about 2% of the total global insecticide market (Lambert and Peferoen, 1992; Schnepf *et al.*, 1998).

Because of the economic importance of *Bt* as powerful biological control agents against harmful insect pests, special attention was paid to elucidate and optimize growth conditions of *Bt* that leading to the highest yields of their toxins. Salama *et al.*, (1983) and Sachdeva *et al.*, (1999) were reported that the commercial application of the organism depends on the cost of raw materials, strain efficiency, fermentation cycle, maintenance of process parameters, bioprocessing of fermentation fluid, and formulation of the final product.

The cost of raw materials is one of the principal factors influencing the overall *Bt* production. In the conventional *Bt* production process, the cost of raw materials varied between 30% and 40% of the total cost depending on the plant production capacity (Ejiofor 1991; Lisansky *et al.*, 1993). Therefore, local production of this insecticide in countries like Bangladesh should focus on the use of media containing cheap, locally available sources including agro-industrial by products.

In the current study, attempts were made to use defatted soybean meal, one of the most common and cheap by products of edible oil industry in Bangladesh, as a raw material for production of *Bt* toxin by the reference strain *Bacillus thuringiensis* subsp. *kurstaki* HD-73. An attempt was made to determine the effect of various factors such as different carbon and nitrogen sources, amino acid such as cystine and basal salts on sporulation and δ -endotoxin synthesis by *Bacillus thuringiensis* subsp. *kurstaki* HD-73. Moreover, sea water was used in place of basal salts for development of cost effective medium for large scale production of *Bt* biopesticide. The pattern of sporulation and toxin production of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 in media formulated with defatted soybean meal and various factors were investigated.

1.2 Literature Review

1.2.1 *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is a gram-positive, spore-forming bacterium that is well known for the production of proteinaceous parasporal crystalline inclusion during sporulation which is toxic to insect upon ingestion. This inclusion is commonly called crystal, parasporal body, delta endotoxin or insecticidal crystal protein (ICP) (Hannay, 1953; Heimpel, 1967; Hickel and Fitch, 1990). Cry proteins constitute a family of related proteins that can kill insects of agricultural and health importance belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, and Mallophaga orders as well as some invertebrates.

B. thuringiensis is a member of the genus *Bacillus* and like the other members of the taxon has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvents. The most distinguishing feature of *Bacillus thuringiensis* from closely related *Bacillus* species (e.g. *B. cereus*, *B. anthracis*) is the presence of a parasporal crystal body that is near to spore, outside the exosporangium during the endospore formation, which is shown in Figure 1.1 (Andrews *et al.*, 1985).

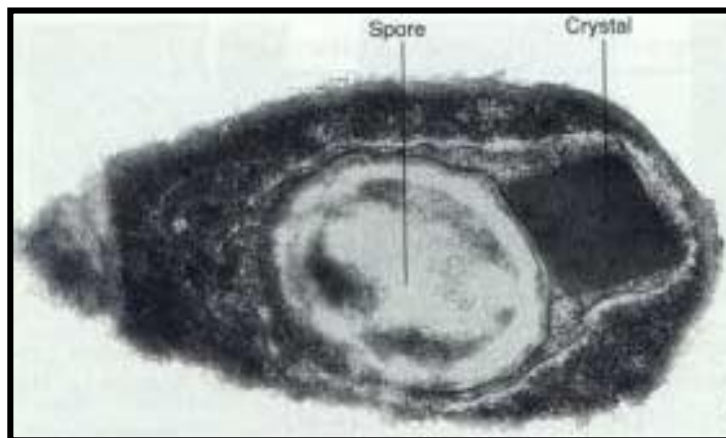


Figure 1.1: Formation of the toxic parasporal crystal in *B. thuringiensis* (Madigan *et al.*, 2000; Brock Biology of Microorganisms, Chapter 12, pp 509)

The spore formation of the organism varies from terminal to sub terminal in sporangia that are not swollen, therefore, *B. thuringiensis* resembles other *Bacillus* species in morphology and shape (Stahly *et al.*, 1991). This bacterium has filamentous appendages (or pili) on the spores (Des Rosier and Lara, 1981; Smirnova *et al.*, 1991; Zelansky *et al.*, 1994). Colonies have a dull appearance and often an undulate margin from which extensive outgrowths do not develop (Sneath, 1986). *Bt* has been used as a successful biological control agent for more than 40 years. *Bt* biopesticide have inherent advantages in certain pest control applications. They are used as a resistance management tools in insect control. Due to their distinct mode of action they are alternated or combined with chemical pesticides.

1.2.2 History of *Bacillus thuringiensis*

In 1901 a spore forming bacterium was isolated in Japan by Ishiwata from diseased larvae of the silkworm, *Bombyx mori*. Ishiwata described this bacterium as sotto disease Bacillus. The Japanese word “sotto” means limp, the typical condition of insect with this disease (Dulmage and Aizawa, 1982). In 1911, without the knowledge of the work done by Ishiwata, Berliner isolated this bacterium from disease larvae of Mediterranean flour moth, *Anagasta kuhniella* and rediscovered *Bt*. He named it *Bacillus thuringiensis*, after the German town Thuringia where the moth was found. In 1915, Berliner reported the existence of a crystal within *Bt*, but the activity of this crystal was not discovered until much later (De Barjac and Bennefoi, 1968).

Farmers started to use *Bt* as a pesticide in 1920. The first commercial product of the bacterium, called ‘Sporeine’, was available in France in 1930s (Jacobs, 1951). Sporeine, at that time was used primarily to kill flour moths. However, it was not commercially available until the 1950s. Subsequent isolation of an efficient variant strain (*B. thuringiensis kurstaki* strain HD1) by Dulmage (1970) made it the most widely employed biological pest control agent. Development of *B. thuringiensis* as a microbial insecticide followed from better strains, increased efficiency in production and quality control leading to the development of formulations with high activity and improved spray characteristics (Van Frankenhuyzen, 1993).

However, some constraints limited their penetration into major crop markets (Gelernter and Evans, 1999), *viz.*, their specificity, narrow host range, low persistence on the plant, high cost of production, *etc.* In spite of these drawbacks, *B. thuringiensis* formulations are some of the most eco-friendly insecticides ever used.

1.2.3 Ecology and prevalence of *B. thuringiensis*

Martin and Travers (1989) isolated *B. thuringiensis* from soil samples in five continents (Africa, Asia, Europe, North and South America) and their associated islands. They found that the frequency of this bacterium is higher in East Asia than in other areas of the world. Their findings suggested that soil is the primary habitat of *B. thuringiensis* in nature. *B. thuringiensis* is indigenous to many environments including soil (Martin and Travers, 1989; Bernard *et al.*, 1997), insect cadavers (Corazzi *et al.*, 1991; Kaelin *et al.*, 1994; Itaqou-Apoyolo *et al.*, 1995; Lopez-Meza and Ibarra, 1996; Cadavos *et al.*, 2001), stored product dust (Chambers *et al.*, 1991; Meadows *et al.*, 1992; Hongyu *et al.*, 2000), leaves of plants (Smith and Couche, 1991; Bel *et al.*, 1997; Mizuki *et al.*, 1999), and aquatic environments (Iriarte *et al.*, 2000; Ichimatsu *et al.*, 2000). Moreover, *B. thuringiensis* has recently been isolated from marine sediments (Maeda *et al.*, 2000), and also from the soils of Antarctica (Forsty and Logan, 2000). *B. thuringiensis* strains show genetic diversity with different toxic potential mostly due to plasmid exchange between strains (Thomas *et al.*, 2001).

Arrieta *et al.*, 2004 isolated a total of 202 *B. thuringiensis* isolates from Costa Rican coffee plantations infested with *Hypothenemus hampei*. These were analyzed through morphology of the crystal inclusions and SDS-PAGE profile of delta endotoxins. Evaluation of 105 isolates showed diverse crystal morphologies and presence of many *cry* genes per strain.

There is a long history of recording of *B. thuringiensis* in soil (Addison, 1993). There are many methods to isolate this bacterium from different habitats, which include shaken flask technique, leaf lift technique and leaf scrub technique coupled with sodium acetate selection (Smith and Couch, 1991, Travers *et al.*, 1987) or penicillin cycling (Johnson and Bishop, 1996).

Different methods are used for the characterization of *B. thuringiensis* isolates such as observation of crystal morphology, biochemical testing, crystal protein (parasporin) protein and rapid technique for screening of a large number of isolates (Juarez-Perez *et al.* 1997; Porcar & Juarez-Perez 2002).

1.2.4 Genetic Diversity of *B. thuringiensis*

1.2.4.1 *B. thuringiensis* Genome

B. thuringiensis strains have a genome size of 2.4 to 5.7 million base pairs (Carlson *et al.*,1994). Most *B. thuringiensis* strains contain several circular and linearextrachromosomal elements (plasmid DNA) ranging from 2 kb to greater than 200 kb (Carlton and Gonzalez, 1985). They make up to 20% of the total DNA (Aronson, 2002). The genes (*cry* genes) encoding crystal proteins are mostly carried on large plasmids (Li *et al.*,1991). Sequence hybridization studies have shown that these genes are also found in the *B. thuringiensis* chromosome (Carlson *et al.*,1994).

B. thuringiensis and its subspecies also contain a large variety of transposable elements including insertion sequences and transposons (Mahillon *et al.*,1994). Insertion sequences (IS) are especially found in large plasmids and many of these sequences carry protoxin genes. Plasmids that do not include protoxin genes also play a role in the regulation of protoxin synthesis. Plasmids also enhance and provide supplementary growth factors when nutrients are limited. If protoxin gene is found on a transposable element, it can move into and out of the chromosome. Because of this movement, protoxin sequences may sometimes be present in the chromosome of some subspecies (Aronson *et al.*, 1986). It is postulated that they are involved in the amplification of the *cry* genes in the cell. A second possibility for their role is mediating the transfer of plasmid between self-conjugative plasmids and chromosomal DNA or non-conjugative plasmids (Schnepf *et al.*, 1998).

1.2.4.2 The *cry* Genes

The genes coding for the insecticidal crystal proteins are normally associated with plasmid of large molecular mass (Gonzales and Carlton, 1980). Many Cry protein genes have been cloned, sequenced, and named *cry* and *cyt* genes. To date, over 100 *cry* gene sequences have been organized into 32 groups and different subgroups on the basis of their nucleotide similarities and range of specificity (Crickmore *et al.*, 1998; Bravo *et al.*, 1998). For example, the proteins toxic for lepidopteran insects belong to the Cry 1, Cry 9, and Cry 2 groups. The toxins against coleopteran insects are the Cry 3, Cry 7, and Cry 8 proteins and Cry1Ia1, which is a subgroup of Cry 1 proteins. The Cry 5, Cry12, Cry 13 and Cry 14 proteins are nematocidal, and the Cry 2Aa1, which is a subgroup of Cry 2 proteins, Cry 4, Cry 10, Cry 11, Cry 16, Cry 17, Cry 19, and Cyt proteins are toxic to dipteran insects (Zeigler, 1999). Each of the *B. thuringiensis* strains can carry one or more crystal toxin genes, and therefore, strains of the organism may synthesize one or more crystal protein. Transfer of plasmids among *B. thuringiensis* strains is the main mechanism for generating diversity in toxin genes (Thomas *et al.*, 2001).

According to Rowe and Margaritis *et al.*, (1987) and WHO (1999), there have been nine different toxins described in *Bt* strains. These toxins are α -exotoxin (phospholipase C), β -exotoxin (thermostable exotoxin), γ -exotoxin (toxic to sawflies), δ -endotoxin (protein parasporal crystal), louse factor exotoxin (active only against lice), mouse factor exotoxin (toxic to mice and Lepidoptera), water-soluble toxin, Vip3A (*Bt* vegetative insecticidal protein) and enterotoxin (produced by vegetative cells). Out of these several toxins produced by *Bt* strains, δ -endotoxin received much attention and have been exploited commercially for production of bioinsecticides. *Bt* crystals have various forms (bipyramidal, cuboidal, flat rhomboid, or a composite with two or more crystal types). The crystal toxins (δ -endotoxin) are belonging to two structurally different groups:

1. Cry family, with specific cytolytic activity as Cry1Aa1, Cry1Ba1, Cry2Aa1, etc.
2. Cyt family, which is a nonspecific cytolytic and hemolytic as Cyt1Aa1, Cyt2Aa1, etc. (WHO 1999 and Delecluse *et al.*, 2000).

1.2.4.3 Cry Protein Structure

Several terminologies are used for the crystalline inclusion bodies, for example, insecticidal crystal proteins (ICP), cry toxins or δ -endotoxin. These parasporal crystals consists of proteins, which exhibit highly toxic insecticidal activity. On the otherhand actively growing cells are not toxic because they lack the crystalline inclusions. The primary structure of *Bt* δ -endotoxins varies with the gene that encodes the protein. Numerous *Bt* toxin genes have now been identified and are classified according to a designation proposed by Hofte and Whitely (1989). Briefly, the toxins identified to date mostly exist in three size designations, namely 125-128, 65-75, and 25-28 kilodaltons (kd).

The three dimensional structures of the four δ -endotoxins (Cry 1, Cry 2, Cry 3 and Cyt 2A) have been resolved by X-ray crystallography (Grachulski *et al.*, 1995; Li *et al.*, 1991; Liu *et al.*, 1996). The Cry 1, Cry 2, and Cry 3 are remarkably similar, each of them consisting of three domains, which is shown in Figure 1.2. The N-terminal Domain I consists of seven α -helices. These are six amphipathic helices which around a central core helix. Domain II consists of three β -sheets with three-fold symmetry. This conformation is called 'Greek Key'. The C-terminal, domain III, consists of two antiparallel of β -sheets in a 'jelly-roll' formation. Each domain has a role in the mode of action of the toxin. Domain I is involved in membrane insertion and pore formation. Domains II and III are both involved in receptor reorganization and binding. Additionally, a role for domain III in pore function has been found (De Maagd *et al.*, 1996, 2001).

The activated cry toxins have two functions: receptor binding and ion channel activity. The activated toxin binds to the specific receptors on the mid-gut epithelia of susceptible insect (Hofman *et al.*, 1988). Binding is a two stage process involving reversible and irreversible binding (Van-Rie *et al.*, 1989). These steps may include toxin binding to the receptor, insertion of the toxin into apical membrane or both. On the other hand, the Cyt toxins have no specific receptor recognition, although, they cause pore formation. Many, if not most, inclusions contain more than one protein; for example, the inclusion body of *B. thuringiensis* subsp. *kurstaki* (*Btk*) HD-3 comprises five different polypeptides, three Cry1 and two Cry2 protoxins (Hofte and Whiteley, 1989).

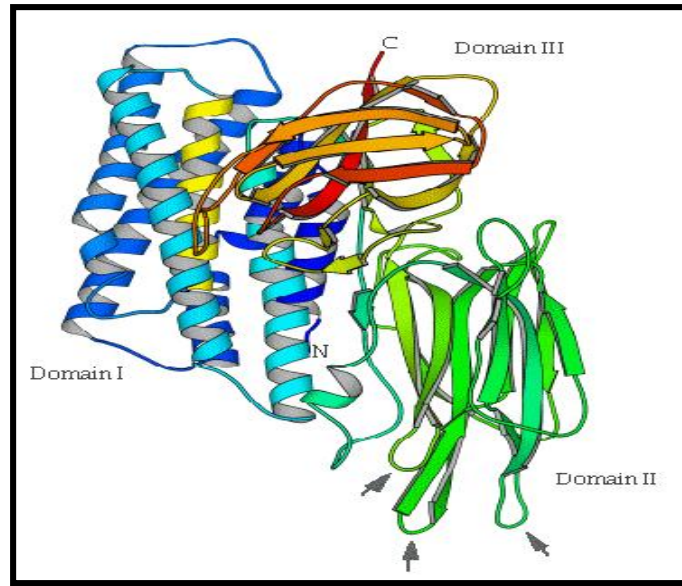


Figure 1.2: The structure of Cry 3A (<http://www.bioc.cam.ac.uk/UTOs/Ellar.html>)

However, Cyt 2A structure is radically different from the other three structures (Crickmore *et al.*, 1998). It consists of a single domain, which is shown in Figure 1.4. The structure of the domain is composed of alpha helix outer layers wrapped around a mixed beta-sheet (Schnepf *et al.*, 1998).

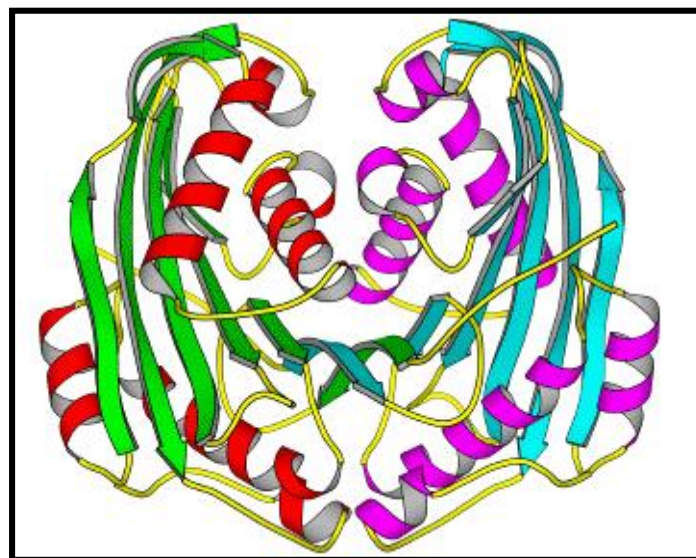


Figure 1.3: The structure of Cyt 2A (<http://www.bioc.cam.ac.uk/UTOs/Ellar.html>)

1.2.5 Mechanism of action of *Bt* formulations

Crystal proteins of a given strain of *B. thuringiensis* are highly specific to certain groups of insects. When a susceptible insect ingests these crystalline proteins, known as delta endotoxins (130 kDa), they are solubilized and proteolytically digested to yield the active polypeptide (60–70 kDa), which specifically binds to protein receptors in the epithelial cells of the insect midgut (Hofte and Whitely, 1989; Luthy and Wolfersberger, 2000) and produce pores, leading to the loss of normal membrane function. As a result epithelial cells lyse, larvae stop feeding, get paralyzed and die of starvation, septicemia or a combination of both (Schwart and Laprade, 2000). The process of solubilization, proteolysis and receptor binding of Domain II and III followed by insertion and pore formation by arrangement of Domain I of the toxic protein (De Maagd *et al.*, 2001).

The mode of action of *B. thuringiensis* has been reviewed by Schnepf *et al.*, (1998) and can be summarized in the following stages: 1) ingestion of sporulated *B. thuringiensis* and insecticidal crystal protein (ICP) by an insect larva; 2) solubilization of the crystalline ICP in the midgut; 3) activation of the ICP by proteases; 4) binding of the activated ICP to specific receptors in the midgut cell membrane; 5) insertion of the toxin in the cell membrane and formation of pores and channels in the gut cell membrane, followed by destruction of the epithelial cells (Cooksey, 1971; Fast, 1981; Huber *et al.*, 1981) and 6) subsequent *B. thuringiensis* spore germination in the hemocoel and septicemia may enhance mortality (Figure.1.4).

The intact toxin crystal proteins must undergo solubilization and proteolysis to remove the N- and C-terminal amino acids before they become active in the gut of a target insect. However, in case of transgenic plants, *cry* gene coding for a truncated form of the toxin is employed with most part of the C-terminal end removed, thus requiring only minimal proteolytic processing for activation. Elucidation and visualization of three dimensional structures has helped understanding its processing and conformation to initiate binding to the receptors at the midgut cell membrane (Hodgman and Ellar, 1990).

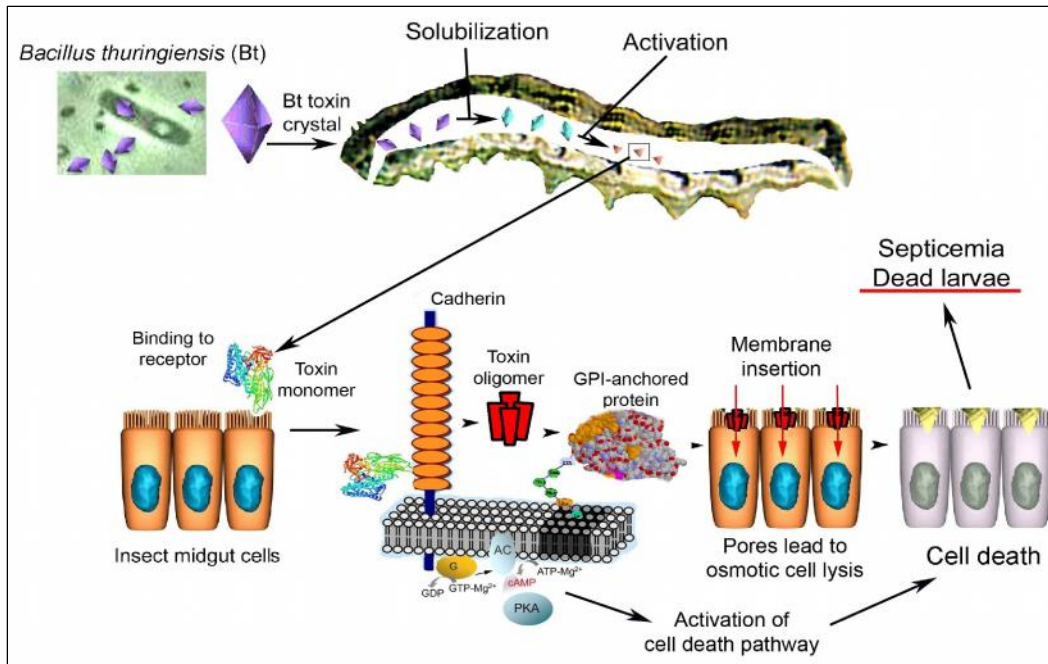


Figure 1.4: Mechanism of toxicity of *Bacillus thuringiensis* -endotoxin toward insect.

(<http://web.utk.edu/jurat/Btresearchtable.html>)

1.2.6 Other Pathogenic Factors of *Bacillus thuringiensis*

During the active growth cycle, certain strains of *B. thuringiensis* produce extracellular compounds, which might contribute to virulence. These extracellular compounds include phospholipases, -exotoxins, proteases, chitinases and vegetative insecticidal proteins (VIPs) (Zhang *et al.*, 1993; Estruch, 1996; Schnepf *et al.*, 1998). *B. thuringiensis* also produces antibiotic compounds having antifungal activity (Stabb *et al.*, 1994). However, the cry toxins are more effective than these extracellular compounds and allow the development of the bacteria in dead or weakened insect larvae.

Some strains of *B. thuringiensis* produce a low molecular weight, heat stable toxin called -exotoxin, which has a nucleotide-like structure. Because of its nucleotide like structure it inhibits the activity of DNA-dependent RNA polymerase of both bacterial and mammalian cells (Glazer and Nikaido, 1995). *B. thuringiensis* strains also produce a protease, which is called inhibitor A.

This protein attacks and selectively destroys cecropins and attacins which are antibacterial proteins in insect. As a result, the defense response of the insects collapses. The protease activity is specific because it attacks an open hydrophobic region near the C-terminus of the cecropin and it does not attack the globular proteins (Dalhambar and Steiner, 1984).

Other important insecticidal proteins, unrelated to Cry proteins, are vegetative insecticidal proteins (VIPs). These proteins are produced by some strains of *B. thuringiensis* during vegetative growth. These VIPs do not form parasporal crystals and are secreted from the cell. For this reason, they are not included in the Cry protein nomenclature. For example, the VIP 1A gene encodes a 100 kD, a protein which is processed from its N-terminus. This processing produces an 80 kDa product, which has been shown to be toxic to western corn root warp larvae (Schnepf, 1998).

1.2.7 Pesticides

A pesticide is a substance or mixture of substances used to kill a pest. FAO has defined the term of pesticide as: “Any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies.”

The term pesticide also includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport.

1.2.7.1 Classification of pesticide

According to the nature of the pesticides they are grouped into four classes:

- a) Plant derivatives e.g. Pyrethrin, Rotenone.
- b) Pure chemicals e.g. Paris green.

c) Synthetic chemicals:

- Chlorinated hydrocarbon compounds e.g. DDT, Dieldrin, and HCH.
- Organophosphate compounds e.g. Diazinon, Malathion, Fenitrothin.
- Carbamates e.g. Propoxur, Bendiocarb.
- Synthetic Pyrinoids e.g. Detamethrin, Permethrin.

d) Biopesticides.

1.2.7.2 *Bacillus thuringiensis*: As a biopesticide

1.2.7.2.1 Advantages of *Bt*

The specific activity of *Bt* generally is considered highly beneficial. Unlike most insecticides, *Bt* insecticides do not have a broad spectrum of activity, so they do not kill beneficial insects. This includes the natural enemies of insects (predators and parasites), as well as beneficial pollinators, such as honeybees. Therefore, *Bt* integrates well with other natural controls. For example, in Colorado, *Bt* to control corn borers in field corn has been stimulated by its ability to often avoid later spider mite problems. Mite outbreaks commonly result following destruction of their natural enemies by less selective treatments (Bernard R. Glick and Jack J. Pasternak, 2010). They also reported that, perhaps the major advantage is that *Bt* is essentially nontoxic to people, pets and wildlife. This high margin of safety recommends its use on food crops or in other sensitive sites where pesticide use can cause adverse effects.

1.2.7.2.2 Disadvantages of *Bt*

Bt is susceptible to degradation by sunlight. Most formulations persist on foliage less than a week following application. Some of the newer strains developed for leaf beetle control become ineffective in about 24 hours (Andrews *et al.*, 1987).

The highly specific activity of *Bt* insecticides might limit their use on crops where problems with several pests occur, including no susceptible insects (aphids, grasshoppers, etc.). As strictly a stomach poison insecticide, *Bt* must be eaten to be effective, and application coverage must be thorough. This further limits its usefulness against pests that are susceptible to *Bt* but rarely have an opportunity to eat it in field use, such as codling moth or corn earworm that tunnel into plants

(Cannon R.J.C., 1993). Additives (sticking or wetting agents) often are useful in a *Bt* application to improve performance, allowing it to cover and resist washing.

Since *Bt* does not kill rapidly, users may incorrectly assume that it is ineffective a day or two after treatment. This, however, is merely a perceptual problem, because *Bt*-affected insects eat little or nothing before they die.

Bt based products tend to have a shorter shelf life than other insecticides. Manufacturers generally indicate reduced effectiveness after two to three years of storage. Liquid formulations are more perishable than dry formulations. Shelf life is greatest when storage conditions are cool, dry and out of direct sunlight.

1.2.8 Insects controlled by *Bt*

Kurstaki strain (Biobit, Dipel, MVP, Steward, Thuricide, etc.):

- **Vegetable insects**
 - Cabbage worm (cabbage looper, imported cabbageworm, diamondback moth, etc.).
 - Tomato and tobacco hornworm.
- **Field and forage crop insects**
 - European corn borer (granular formulations have given good control of first generation corn borers).
 - Alfalfa caterpillar, alfalfa webworm.
- **Fruit crop insects**
 - Leaf roller.
 - Achemon sphinx.
- **Tree and shrub insects**
 - Tent caterpillar.
 - Fall webworm.
 - Leaf roller
 - Red-humped caterpillar.
 - Spiny elm caterpillar.

- Western spruce budworm.
- Pine budworm.
- Pine butterfly.

Israelensis strains (Vectobac, Mosquito Dunks, Gnatrol, Bactimos, etc.)

- Mosquito.
- Black fly.
- Fungus gnat.

San diego/tenebrionis strains (Trident, M-One, M-Trak, Foil, Novodor, etc.)

- Colorado potato beetle.
- Elm leaf beetle.
- Cottonwood leaf beetle.

1.2.9 Applications of *Bt*

The greatest use of *Bt* involves the *kurstaki* strain used as a spray to control caterpillars on vegetable crops.

In addition, *Bt* is used in agriculture as a liquid applied through overhead irrigation systems or in a granular form for control of European corn borer. The treatments funnel down the corn whorl to where the feeding larvae occur.



Figure 1.5: Alfalfa webworms killed by *Bacillus thuringiensis*.

To control mosquito larvae, formulations containing the *Bt israelensis* strain are placed into the standing water of mosquito breeding sites.

For these applications, *Bt* usually is formulated as granules or solid, slow-release rings or briquettes to increase persistence. Rates of use are determined by the size of the water body. Make applications shortly after insect eggs are expected to hatch, such as after flooding due to rain or irrigation. *Bt* persistence in water is longer than on sun-exposed leaf surfaces, but reapply if favorable mosquito breeding conditions last for several weeks. Although the *israelensis* strain is quite specific in its activity, some types of nonbiting midges, which serve as food for fish and wildlife, also are susceptible and may be affected.

Use of *Bt (israelensis)* for control of fungus gnat larvae involves drenching the soil. *Bt* applied for control of elm leaf beetle or Colorado potato beetle (*san diego/tenebrionis* strain) is sprayed onto leaves in a manner similar to the formulations used for caterpillars. *Bt* does not control shore flies, another common fly found in greenhouses (Edwards D.L. *et al.*, 1988).

1.2.10 Factors affecting growth, sporulation and toxin production by *Bacillus thuringiensis*

The nutritional aspects of *Bacillus thuringiensis* have been studied extensively by a number of workers (Nickerson & Bulla 1975; Rogoff & Yousten 1969; Singer *et al.*, 1966). It is well established that various strains of *B. thuringiensis* would not grow in so-called mineral salt medium unless certain growth factors such as glutamic acid and either aspartate or citrate are added to the medium. Further, the addition of a known mixture of amino acids or casein hydrolysate allowed rapid growth of *B. thuringiensis* but sporulation was poor unless glucose was added (Singer *et al.*, 1966). Cysteine or cystine, when added to a mineral salt medium, promoted vegetative growth of *B. thuringiensis* (Nickerson & Bulla, 1975). They also reported the role of various amino acids on lipid metabolism (Rajalakshmi & Shethna, 1977). Gangurde and Shethna (1995) have demonstrated the effect of defatted mustard meal on *B. thuringiensis* subsp. *israelensis* and *B. sphaericus*.

1.2.10.1 Effects of different substrate (Carbon and Nitrogen nutrient source)

The media used for industrial production of *B. thuringiensis* are composed of complex nitrogen and carbon sources. Production of *B. thuringiensis* has been found to vary drastically in media derived from various nutrient sources. Prabakaran *et al.*, (2008) used locally available raw materials such as soybean flour, ground nut cake powder and wheat bran extract to improve the yield of cell mass and sporulation of *B. thuringiensis israelensis*. Whey and molasses, which can be used as low-cost and available substrates at industrial scale, were potential carbon substrates for delta-endotoxin production (Icgen *et al.*, 2002).

Bt uses sugars, usually glucose, fructose, maltose, ribose, molasses, starch, dextrin, wheat flour and insulin, producing acid during the fermentation (Nickerson and Bulla, 1974; Saalma *et al.* 1983; Arcas *et al.*, 1984; Zamola *et al.*, 1981; Foda *et al.*, 1985; El-Bendary, 1994; Sadek, 2000; Icgen *et al.*, 2002 and Ozkan, Dilek *et al.*, 2003). Ozkan *et al.*, (2003) studied various nutritional and cultural parameters influencing dipteran-specific δ -endotoxin synthesis by *Bacillus thuringiensis israelensis* (*Bti*) HD500. They reported that insulin, dextrin, maltose, lactose, sucrose, whey and glycerol were stimulatory, while glucose, starch, and molasses were suppressive.

With respect to the nitrogen source suitable for *Bt* production, the overwhelming majority of literatures revealed the inability of most of the *Bt* varieties to utilize inorganic nitrogen source as a sole nitrogen source in the growth medium. Instead, at least one amino acid particularly glutamate, aspartate, valine, leucine, serine or threonine has to be added in order to allow growth of the organism in a minimal medium (Nickerson and Bulla, 1974; Normansell *et al.*, 1980; El-Bendary, 1994; Avignone-Rossa and Mignone, 1995 and Sadek, 2000). However, cysteine and cystine amino acids showed clear inhibitory effect on growth, sporulation and toxin formation by *Bt* (Rajalakshmi and Shethna, 1980; El-Bendary, 1994 and Sadek, 2000). Icgen *et al.* (2002a) found that pentone was the best organic nitrogen source supporting sporulation and toxin production by *Bt*.

B. thuringiensis grows in culture media containing sources of nitrogen, carbon and mineral salts. Various agricultural and industrial by-products, such as maize glucose, soybean flour, peanuts, cane molasses and liquid swine manure, are carbon and nitrogen rich and may be used as raw materials in biopesticide production. Tirado-Montiel *et al.* (2001) first tested the use of wastewater sludge for biopesticide production, although the entomotoxicity level reported was low.

Various researchers have explored alternatives to the preparation of several less expensive culture media for *Bti* biopesticide production. Often, locally available, cost-effective substrates have been used and have been shown to achieve comparable or better results than those obtained using conventional medium. Prabakaran and Balaraman (2006) attempted to develop a medium based on raw materials including soybean flour (*Glycine max*), groundnut cake powder (*Arachis hypogea*), and wheat bran extract (*Triticum aestivum*) in a 100-L fermentor. Prabakaran *et al.*, (2008) made a cost-effective medium with coconut water, which is a raw material that is abundantly available as a waste product from the coconut oil industry. Yezza *et al.* (2006) conducted the bioconversion of industrial wastewater and wastewater sludge into abiopesticide in a pilot fermentor.

Poopathi and Kumar (2003) used potato, common sugar, and Bengal gram substrates. Poopathi and Abidha (2007, 2008) made feather extract and feather powder, and explored the possibility of degrading chicken feathers discarded from the poultry processing industry. Obeta and Okafor (1984) assessed the production of insecticidal properties using shake flasks, making five variations of the basal medium by adding different types of legume seeds, including groundnut cake (*Arachis hypogea*), cow pea (*Vigna unguiculata*, white variety), cow pea (*Vigna unguiculata*, black variety), soya beans (*Glycine soja*), and bambara beans (*Voandzeia subterranean*). Ghribi *et al.*, (2007) applied a new medium composed of only starch, soya bean and diluted sea water. Ozkan *et al.*, (2003) studied various nutritional and cultural parameters influencing delta-endotoxin synthesis and found that, among carbon sources, insulin, dextrin, maltose, lactose, sucrose, whey and glycerol were all stimulatory, while glucose, starch and some molasses were suppressive.

1.2.10.2 Effects of cysteine

Since sporulation and germination in bacilli are dependent on the nutritional status of the organism (Hardwick and Foster, 1952), a study of the nutritional requirement of *Bacillus thuringiensis* is important for delineating the control mechanisms which regulate spore and parasporal crystal formation. Certain amino acids support growth, sporulation and crystal formation of *B. thuringiensis*, while others inhibit the growth (Singer *et al.*, 1966; Singer and Rogoff, 1968; Bulla *et al.*, 1975; Nickerson and Bulla, 1975; Rajalakshmi and Shethna, 1977). A lower concentration of cystine (Nickerson and Bulla, 1975) or cysteine (Rajalakshmi and Shethna, 1977) promotes growth; sporulation and crystal formation in *B. thuringiensis*, while at a higher concentration of cys/cysSH, only the vegetative growth was observed (Rajalakshmi and Shethna, 1977).

Dipok Vora and Y. I. Shethna (1999) reported the effect of cysteine on the growth, sporulation and toxin production by *B. thuringiensis* subsp *kurstaki*. A mineral salts medium supplemented with peptone and 40mg% cystine supported enhanced sporulation (10^{11} spores/ml) and high yields of insecticidal crystal protein (17.2 mg/ml) in *Bacillus thuringiensis* subsp. *kurstaki* (3a3b) in 2.0L baffled aerated cylinders. They showed that these high yields could also be achieved with defatted soybean and ground nut seed meal extracts when supplemented with cystine. A lower concentration of cystine (Nickerson and Bulla, 1975) or cysteine (Rajalakshmi and Shethna, 1977) promotes growth, sporulation and crystal formation in *B. thuringiensis*, while at a higher concentration of cys/cysSH, only the vegetative growth was observed, (Rajalakshmi and Shethna, 1977).

1.2.10.2.1 Spore and crystal formation in relation to cys/cysSH concentration

The efficiency of spore and crystal formation was studied in relation to cys/cysSH concentration. It was noticed that in the control, as well as in the presence of cys/cysSH (0.05%), the efficiency of spore and crystal formation was 100 percent. At a concentration of 0.1% of cys/cysSH, the efficiency was drastically reduced. At 0.15 and 0.2% of cys/cysSH, only heat-labile spores were formed with complete inhibition of crystal formation.

At 0.25% cys/cysSH, there was complete inhibition of spore and crystal formation in *B. thuringiensis* var. *thuringiensis* (Rajalakshmi and Shethna, 1980).

1.2.10.2.2 Effect of addition of cys/cysSH at the stationary phase

It was noticed that when 0.05% of cys/cysSH was added at 0 h of the stationary phase, heat-stable spores and toxic crystals were formed. At 0.1 and 0.15%, only heat-labile spores were produced with no crystal formation. Whereas, when 0.2% of cys/cysSH was added, spore and crystals were not formed microscopically, and the bioassay also showed no toxicity (Rajalakshmi and Shethna, 1980).

1.2.10.2.3 Effect of addition of cys/cysSH after the stationary phase

Sporulation and parasporal crystal formation were found to be inhibited even at the concentration of 0.15% of cys/cysSH when the addition was made 1 h after the onset of the stationary phase. The efficiency of sporulation decreased as the time of addition of excess cys/cysSH was increased (Rajalakshmi and Shethna, 1980).

1.2.10.2.4 Microscopic observation

The cells grown on cys/cysSH medium were thinner and longer than the control cells. Photomicrography revealed that the control cells grown with 0.05% cys/cysSH produced spores and crystals (figure 3). At moderate concentration of cys/cysSH (0.15%) only heat-labile spores were formed (figure 4). At high concentration (0.25%), both spore and crystal formation were found to be completely inhibited; besides, there was a change in the morphology of the experimental cells, about 15 to 29 min before lysis (Rajalakshmi and Shethna, 1980).

1.2.10.3 Effects of molasses

Improvement of bioinsecticides production could be achieved by application of an adequate fermentation technology, essentially with the use of appropriate media, by overcoming metabolic (Zouari, N., S. Ben and S. Jaoua, 2002) limitations, and by the improvement of *B. thuringiensis* strains through mutagenesis. At present the cost of *B. thuringiensis* production through existing

fermentations technology is high because of the high cost of the production medium (Ghribi, D., N. Zouari and S. Jaoua, 2004). A less expensive medium for culturing of *B. thuringiensis* will facilitate the production of biopesticides in a cost-effective manner. *B. thuringiensis* was produced in different media using the seeds of legumes, dried cow blood, fishmeal and corn steep liquor, powders of edible leguminous seeds and cane sugar molasses, corn extract and corn steep liquor, or potato starch (Kumar *et al.*, 2000; Dregval O.A. *et al.*, 2002).

Molasses consists of water, sucrose, proteins, vitamins, amino acids, organic acids and heavy metals such as iron, zinc, copper, manganese, magnesium, calcium, etc. Heavy metals, when contained in high concentrations in the medium, cause critical problems during the fermentation. They inhibit the microbial growth; influence the pH of the substrate, beside to be involved in the inactivation of the enzymes associated with biosynthesis of the product (Roukas, 1998).

1.2.10.4 Effects of metal ions

Metal ions such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Cu^{2+} and Fe^{2+} are essential for the production of the highest sporulation and δ -endotoxin formation by *Bt* (Faloci *et al.*, 1986; Sadek, 2000 and Içgen *et al.*, 2002b). Ozkan *et al.* (2003) stated that Mn^{2+} was the most critical element for the biosynthesis of Cry4Ba and Cry11Aa by *Bti* HD500 at 10^{-6} M concentration. However Mg^{2+} and Ca^{2+} favored toxin production when provided at 8×10^{-3} M concentrations, respectively, while Fe^{2+} , Zn^{2+} , and Cu^{2+} negatively influenced toxin biosynthesis.

In contrast, Sikdar *et al.* (1991) have recommended the addition of Fe^{2+} and Cu^{2+} for stimulation of Cry toxin production by the same subspecies (*Bti* HD500). Arcas *et al.*, (1984) have proposed a medium (hereafter designated as Arcas' medium) for the cultivations of *Bt*. This medium produces more spores and insecticidal protoxins than any other published medium and contains (in g/l) glucose(10), yeast extract (4), $(\text{NH}_4)_2\text{SO}_4$ (1), KH_2PO_4 (3), K_2HPO_4 (3), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (4), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.041), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.03) in their study, they showed that mineral salts in this medium have important role for the growth, sporulation and toxin production by *Bt*. In 1995, Wei-Ming Liu and Rakesh K. Bajpai reported a modified version of this medium; the modified media were shown superior in terms of productions of cells mass and protoxins, and potency of the protoxins.

The high cost of *B. thuringiensis* products is due to production being located in the developed countries where production costs are higher, and also due to expenses paid in transportation to the operational sites. Thus, local production should significantly reduce costs of pest control and could help the development of local fermentation industries and their improvement, besides the utilization of agro-industrial byproducts (Ghribi D. *et al.*, 2007). In order to reduce the production costs, cheap byproducts were used. Recently, more attention has been given to the improvement of bioinsecticide production by application of an adequate fermentation technology (Zouari N. *et al.*, 2002).

1.2.10.5 Effects of pH

The growth of *Bt* occurs in the pH range of 5.5–8.5 (Rowe and Margaritis 1987, Içgen *et al.*, 2002b and Ozkan *et al.*, 2003). The usual initial pH is 6.8–7.2; decreasing to 5.8 as acetate is released, then rising to 7.5–8 as it is consumed.

1.2.10.6 Effects of temperature

The normal temperature for growth and toxin production of *Bt* is 30 °C. Ozkan *et al.*, (2003) found that Cry4Ba synthesis by *Bti* HD500 was the best when the organism was grown at 25 °C, whereas Cry11Aa synthesis was optimal at 30 °C.

1.2.10.7 Effects of aeration

Aeration is very important for *Bt* fermentation. Foda *et al.* (1985) noted the failure of the organism to survive or sporulate under low aeration levels. Most submerged fermentation of *Bt* is done using aeration rates approximately one air volume/volume of medium/minute. Recent studies on metabolism of *Bt* during growth and sporulation have employed higher aeration level e.g. 1.4 air volume/volume of medium/minute (Rowe, 1990).

1.2.11 Mass production of *Bt*

Due to the economic importance of *Bt* as powerful biological controls agents against harmful insect pests, special attention was paid to elucidate and optimize growth conditions of *Bt* that leading to the highest yields of their toxins. Saalma *et al.*, (1983) and Sachdeva *et al.*, (1999) reported that the commercial application of the organism depends on the cost of raw materials, strain efficiency, fermentation cycle, maintenance of process parameters, bioprocessing of fermentation fluid, and formulation of the final product. The cost of raw materials is one of the principal costs involved in overall *Bt* production. In the conventional *Bt* production process, the cost of raw materials varied between 30 and 40% of the total cost depending on the plant production capacity (Ejiofor, 1991 and Lisansky *et al.*, 1993). Therefore, local production of this insecticide in developing countries should depend on the use of production media made of cheap, locally available source including agro-industrial by-products (Ampofo, 1995).

For large scale production of *Bt*, different approaches were investigated to contrast media that could support good production of spores and toxins at reasonable costs. Various agricultural and industrial by-products used as raw material in *Bt* production were citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupil skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal ,residues from chicken slaughter house, fodder yeast, cheese whey and corn steep liquor (Saalma *et al.*, 1983; Obeta and Okafor, 1983; Mummigatti and Raghunathan, 1990; Abdel-Hameed *et al.*,, 1990; Lee and Seleena, 1991; El-Bendary, 1994; Sachdeva *et al.*, 1999; Foda *et al.*, 2002 and 2003).Recently other wastes such as sludge and broiler poultry litter were utilized for biopesticide (Adams *et al.*, 2002 and Vidyarthi *et al.*, 2002).

In general, two methods of fermentation are used for production of microbial products, submerged fermentation and solid state fermentation.

1.2.11.1 Submerged Fermentation (SmF)/ Liquid Fermentation (LF)

SmF utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An additional advantage of this technique is that purification of products is easier. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form (Subramaniam, R. and Vimala, R., 2012)

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

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

Saalma *et al.*, (1983) investigated several agro-industrial by-products for *Bt* α -endotoxin production. They found the fodder yeast, beef blood and chicken slaughter residues were among the byproducts that produced high sporulation and potent α -endotoxin preparations.

Widjaya *et al.*, (1992) reported a defined medium containing 1.5% yeast extract for growing *Bti*. A final *Bti* spore yield in this medium was 2.9×10^9 CFU/ml. Replacement of yeast extract with fish meal extended growth phase with similar final values for cell and spore counts. Kang *et al.*,

(1992) carried out fed-batch culture to increase cell mass followed by batch culture for spore production of *Bt* in GYS medium, which contains glucose, yeast extract and some mineral salts. They found that high cell mass obtained by increasing the feeding of glucose in constant fed-batch culture did not proceed to spore formation. However, intermittent fed-batch culture did not proceed to spore formation.

However, intermittent fed-batch culture supported fast cell growth and resulted in good sporulation during subsequent batch culture (1.258×10^{10} CFU/ml). Liu and Tzeng (1998) reported that the optimum medium composition for production of high level of spores by *Bti* (8.658×10^8 CFU/ml) was 5.01% tapioca; 5.46% fish meal and 0.06% $(\text{NH}_4)_2\text{SO}_4$.

Montiel *et al.*, (2001) used sludge as a raw material for the production of *Bt* based bioinsecticides using *Bt kurstaki*. The sludge samples were used under three different preparations: without pre-treatment (hydrolyzed sludge) and the supernatant obtained after centrifugation of the hydrolyzed sludge. The highest viable cell, spore counts and δ -endotoxin production were when the organism was grown in hydrolyzed sludge, while the liquid phase (supernatant) showed the lowest sporulation and toxicity.

Vidyarthi *et al.*, (2002) compared the growth and δ -endotoxin production by *Bt kurstaki* in tryptic soy yeast extract (TSY) medium; soybean based commercial medium and wastewater sludge medium. They found that the highest toxicity was obtained in a sludge medium and was comparable to that of the concentrated commercial *Bt* formulation available in the market (FORAY 48B). They also found that the optimum value of C: N ratio in combined sludge for *Bt* production was 7.9-9.9.

Zouari *et al.*, (2002) investigated the production of several *Bt* strains active against Lepidoptera and Diptera in gruel (a cheap and abundant byproduct of semolina factories) and fish meal media.

They observed that Diptera-specific strains produced less δ -endotoxin (1246-1998 mg/l) than Lepidoptera-specific ones (3060-3301 mg/l). However, addition of 10g/l sodium acetate

increased 38-79% α -endotoxin production by Diptera-specific strains in shake flask cultures. Similar α -endotoxin production was obtained with or without 10g/l sodium acetate with an excess of aeration in a 2 L fermenter.

1.2.11.2 Solid State fermentation (SSF)

SSF utilizes solid substrates, like bran, bagasse, and paper pulp. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high a_w (water activity), such as bacteria (Babu and Satyanarayana, 1996).

Under the circumstances of the developing countries, the use of submerged fermentation for *Bt* production may not be economically feasible due to the high cost of submerged fermentation equipments such as the cost of the well-equipped deep-tank fermenter, high-speed cooling centrifuge as well as drying facilities e.g. spray dryer.

Accordingly, the SSF methodology offers an alternative approach. Advantages of solid state fermentations are: 1. Low cost methodology, 2 low wastewater output, 3. low capital investments, 4. some spore-forming microorganisms only sporulate when grown on a solid substrate (Mudgett 1984, Walter and Oaau 1992 and Capalbo 1995).

Although the extensive application of SSF technology in production of different microbial products, little information have so far been published on the possible use of SSF methodology in the production of *Bt* and other microbial control agents. The earliest report on possible application on SSF in production of *Bt* appeared in a form of US patent by Mechalas (1963) followed by reports by Dulmage and Rhodes (1971) and Sitting (1977). In China, Wang (1988) reported that stable high quality *Bt* products were easily obtained through certain simple and economic SSF process. The medium used was wheat bran, husk of rice and lime powder.

He claimed that this process proved to be power saving with low cost and popular in provinces in China.

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

Yang *et al.*, (1994) optimized a method advocated for the production of *Bt* by SSF process. They praised the advantages of low cost, high insecticidal activity and convenience of storage of the products. In this method, several agricultural wastes were used as solid culture media for production of δ -endotoxin by *Bt*. They investigated the effects of several culture conditions e.g. inoculum size, pH, seed age, initial moisture content, amount of plant ash used, and fermentation temperature. Bioassays against fourth instars larvae of *Seaiiothisaci nereasra* and *Pieris rapae* proved the high potency of the product. Capalbo *et al.*, (1994) devised two column bioreactors namely an aerated fixed bed and a fluidized bed fermenters for SSF of *Bt*.

They claimed that these two column bioreactors could be used to solve the questions addressed and encountered in SSF methodology including heat and mass transfer, aeration extent, sterility level as well as productivity of this approach.

Capalbo (1995) reviewed the aspects of the fermentation process and risk assessment of *Bt* production in developing countries. She concluded that the local production of bioinsecticides is highly appropriate for pest control in developing countries. She also reported that *Bt* could be cheaply produced on a wide variety of low cost organic substrates under SSF conditions.

More recently, Foda *et al.*, (2002) produced *Bt* through SSF technology using ground soybean seeds as a substrate in the presence of talcum powder and wheat bran as carrier materials. The highest growth and sporulation were obtained at 10% (w/w) of ground soybean.

Adams *et al.*, (2002) used several varieties of heat-sterilized broiler litter as substrates in solid state fermentations to produce biocontrol agents. They studied litter produced by one flock of broilers from medicated and non medicated controlled rations and litters produced by two flocks and four flocks on a single application of bedding material from medicated commercial sources for production of *Bt japonensis*, a pathogen of Japanese beetle larvae. *Bt japonensis* could not grow in unextracted 1-flock litter nor in water extracted litter, but grew in methanol extracted litter to 5×10^{10} CFU/g litter and a spore count of 1×10^{10} CFU/g litter.

It also grew in unprocessed 2-flock and 4-flock litters, achieving cell counts of 3×10^9 and 1×10^9 CFU/g litter, respectively and spore counts of 1×10^9 CFU/g litter. Bioassays of soil containing over 0.5% (db) litter fermented with *Bt* resulted in over 90% mortality in 21 days for first instars of Japanese beetle. They concluded that the *Bt* produced via solid state fermentation using broiler poultry litter have potential in biocontrol applications in soil environment.

1.3 Aims and Objectives

The present study is a part of the core research program on the biotechnological production of *Bacillus thuringiensis* (*Bt*) biopesticide for control of major vegetable pests in Bangladesh. In this connection, the program includes a nationwide screening program for suitable *Bt* strains effective against the pests prevailing in vegetables and other crops in our country. Culture collection of about 300 *Bt* strains which have been isolated from different eco-regions of Bangladesh has already been made. These *Bt* isolates were characterized also with respect to their Cry protein and *cry* gene profile, revealing its abundance and diversity in Bangladesh (Asaduzzaman *et al.*, 2014). Suitable strains have been selected based on larvicidal action through bioassay and application in vegetable field. Finally with a view to developing bioprocess for the large scale production of *Bt* biopesticide, factors affecting cultivation conditions for maximum sporulation and protein synthesis needs to be studied.

It is well established that various strains of *Bacillus thuringiensis* require certain growth factors such as protein rich medium for growth. Moreover, carbon and nitrogen ratio balance itself is directly important for the crystal protein production. So, proper optimization of nutrients and other factors will enable to produce a *Bt* formulation with high spore count and high toxin titer.

A study was performed in the working laboratory previously where concentration was given only on the nitrogen source. Mustard seed meal based medium was used for biopesticide production whereas the optimization of other critical factors enhancing sporulation and endotoxin formation by *Bt* strains in particular is essential.

The use of *Bt* based biopesticides is limited because of the high production costs. However it may become feasible and cheap, if affordable ways for mass production of the entomopathogen was developed. As large amount of soybean and molasses are produced annually in our country, these can be used for a cost effective medium formulation for the suitable production of biopesticide in our country.

Moreover, the study aimed at determination of other factors such as cystine, mineral salts affecting sporulation and δ -endotoxin synthesis by *Bacillus thuringiensis* and finally to formulate an optimized medium for biopesticide production with the use of cheap substrates such as soybean, molasses and sea water as substitutes of basal salts in combination of factors enhancing δ -endotoxin synthesis by *Bt*.

The general objective of this study was to evaluate the use of low cost raw materials rich in carbon and nitrogen as a nutrient source to produce *Bt* based biopesticides. In present study, an attempt was made to use inexpensive substrate such as molasses and soybean as carbon and nitrogen sources for biopesticide production instead of relatively expensive glucose and peptone known to be used for *Bt* biopesticide production (Dipak Vora and Shethna, 1999).

Development of efficient *Bt* biopesticide with enhanced δ -endotoxin production in a cost effective manner is the main target of this work and to that end the specific objectives of the study were as follows: -

- To observe the efficiency of locally available and cheap raw substrate i.e. defatted soybean meal as nitrogen source for δ -endotoxin production.
- Production of δ -endotoxin (Cry protein) in shake flask by monophasic/ submerged and biphasic solid state fermentation on formulated media.
- To investigate the effect of cystine as growth and sporulation factors on the above production medium.
- To compare the suitability of molasses as carbon source in basal medium containing various trace elements by *Btk* HD-73 on growth, sporulation and δ -endotoxin synthesis.
- Use of sea water in place of basal salts in the culture medium.
- Use of soybean extract as nitrogen source as replacement of soybean mass to optimize the culture conditions that supported endotoxin production.
- To examine the production of *Bt* biopesticide (cells and endotoxin) in lab scale bioreactor cultivation using the optimized media formulation and environmental factors in the shake culture.

CHAPTER

2

MATERIALS

AND

METHODS

2. Materials and Methods

The present study was carried out in the Enzyme and Fermentation Biotechnology Laboratory, Department of Microbiology, University of Dhaka. The materials and procedures employed in the present study are described below.

2.1 Handling of laboratory apparatus and glassware

All glassware were washed with mild detergents, rinsed 4-5 times in tap water and finally rinsed twice in distilled water before use and dried in oven. When needed, glassware like Petri plates were heat sterilized at 180°C for 1h in hot air oven (Binder ED23, Germany) before use. Micropipette tips, glass pipette, falcon tubes and microfuge tubes were sterilized by autoclaving at 121°C for 15 min at 15 psi (Hirayama, Model HA-300M, Japan).

2.2 Solutions and reagents

Required solutions and reagents were available in the laboratory and in the media room of the department and were used without further purification as these were of reagent grade. List of the chemicals is given in the appendix-III.

2.3 Bacterial strain and culture conditions

Bacillus thuringiensis subsp. *kurstaki* HD-73 (*Btk* HD-73), kindly provided from *Bt* stock collection of Okayama University, Japan was used as reference strain. *Btk* HD-73 was sub-cultured from main stock on LB agar medium and a single colony was picked every time to avoid further contamination. Culture was maintained in LB agar (per litre: tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g) slants and plates as working stock and 15% glycerol stock (15% glycerol+ 85% fresh culture in LB broth) for long term storage at -80°C freeze. Phase contrast microscopy was used to observe the δ -endotoxin synthesis during spore formation. Incubation temperature was maintained at or below 30°C for all types of culture conditions.

2.4 Media Preparation

2.4.1. Name and factors

Name of media for trial: 1. Luria Bertani (LB) broth, 2. Defatted soybean meal supplemented LB (LBS), 3. Cystine supplemented LBS (LBSc), 4. Cystine supplemented soybean-basal salts (SMc), 5. Molasses supplemented soybean-cystine (SMmc), 6. Sea water in soybean-molasses-cystine (SSWmc), 7. Sea water in soybean extract-molasses-cystine (SeSWmc)

Factors considered: The combinations of different carbon and nitrogen sources as well as other nutritional factors were evaluated for developing most efficient medium for biopesticide production. Defatted soybean meal (Source: local market of Savar) was used as carbon and nitrogen sources. Cystine (Sigma, USA) was used to enhance the sporulation. Molasses (Source: local market of Savar) was used to increase the growth rate. Basal salts were used to replace the ingredients of LB medium. Sea water was used as the cost effective source of trace elements other than the reagent grade costly basal salts.

Fermentation type: Two types of fermentation method were applied for cultivation of the *Btk* HD-73 in the formulated media. These are: submerged fermentation and biphasic solid state fermentation.

2.4.2 Media preparation for submerged fermentation

2.4.2.1 Luria-Bertani broth (Commercial media)

The composition of Luria-Bertani broth were tryptone 10(g/L), yeast extract 5(g/L) and NaCl 10 (g/L) (Sambrook and Russell, 2001; Gerhardt *et al.*, 1994). The components were dissolved in 1 liter of distilled or deionized water. After preparation, media was distributed to 500 ml Erlenmeyer flasks and autoclaved at 121°C for 20 min.

2.4.2.2 Defatted soybean meal supplemented LB (LBS)

The LB broth was prepared as described in section 2.4.2.1. The broth was then supplemented with 10 g of defatted soybean meal. Defatted soybean meal used throughout the study was obtained from the local market of Savar, Dhaka. The soybean was finely grinded before using.

10 g defatted soybean meal was added to 90 ml LB broth in a 500 ml conical flask and autoclaved.

2.4.2.3 Cystine supplemented LBS (LBSc)

The LBS was prepared as described in section 2.4.2.2 and autoclaved. The broth was then supplemented with 300 µl of cystine. 10% cystine stock was prepared by suspending 6 g cystine in 60.0 ml of phosphate buffer and autoclaved. This suspension was vortexed and added to the medium. It was observed that the high α -endotoxin production was achieved using 300µl cystine on soybean meal media (Dipak Vora & Y. I. Shethna, 1999).

2.4.2.4 Cystine supplemented soybean-basal salts medium (SMc)

In this experiment LB was replaced with basal salts. 10 g defatted SM was added to 90 ml basal salt solution in a 500 ml conical flask. The growth medium or the basal salt solution was prepared by the published method of Gangurde and Shethna, 1995. The composition of the basal salt solution were $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5(g/L); $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1(g/L); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02(g/L); $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02(g/L); CaCl_2 , 0.01(g/L) & KH_2PO_4 1.0(g/L) (Gangurde and Shethna 1995).

The pH was adjusted to 7.2 before sterilization at 121°C for 20 min. Cystine (300 µl) was added after autoclaving the media. Cystine stock was prepared as the described method on section 2.4.2.3. The flasks were then allowed to cool down to room temperature before inoculation.

2.4.2.5 Molasses supplemented soybean-cystine medium (SMmc)

For SMmc preparation, 10 g defatted SM was added to 90 ml basal salt solution in a 500 ml conical flask. 0.5 g molasses was added to the 100 ml of prepared media. The pH was adjusted to 7.0 before sterilization at 121°C for 20 min. Cystine (300 µl) was added after autoclaving the media. Then the flasks were allowed to cool down to room temperature before inoculation.

2.4.2.6 Sea water in soybean-molasses-cystine medium (SSWmc)

Brine or sea water was used in SM as a substituent of basal salts. Sea water was collected from sea coast of Potuakhali. It contains (g/L): Na⁺ 12; Cl⁻ 22; K⁺ 0.4; Ca⁺ 0.14; HCO³⁻ 0.40; Mg⁺ 1.3; SO₄²⁻ 2.640; Fe²⁺ (Ghribi N. *et al.*, 2007). 10 g defatted soybean meal was added to 20 ml sea water (8:2) in a 500 ml Erlenmeyer flask and then made up to 100 ml with distilled water. 0.5 g molasses was added before autoclaving the media. Cystine (300µl) was added after autoclaving the media.

2.4.2.7 Sea water in soybean extract-molasses-cystine medium (SeSWmc)

100 ml of 10% soybean suspension was boiled for 10 min and the aqueous part was separated from the solid mass. 0.5 g molasses and 20 ml of seawater were mixed with the prepared soybean extract and the volume was adjusted to 100 ml in an Erlenmeyer flask by adding distilled water. 0.3 ml of 10% cystine suspension in phosphate buffer was added into the mixture to make the final cystine concentration 300 mg/L. It was then autoclaved and ready for inoculation upon cooling down at room temperature.

2.4.3 Media preparation for biphasic solid state fermentation

10 g finely grinded defatted soybean meal was added with 90 ml of LB (Luria-Bertani) broth. The pH was adjusted to 7.0 before sterilization at 121°C for 20 min. The flasks were allowed to cool before inoculation. The medium was grown above up to 7-10 hours. It was then centrifuged aseptically (1000×g for 10min, 4°C) to separate the liquid and the solid matter. The solid matter was then incubated up to 72 h (including initial 7-10 h).

2.5 Inoculum preparation

Btk HD-73 was streaked on LB-agar plate from the slant and incubated overnight at 30°C. An isolated colony was picked from the LB- agar plate aseptically with a loop following overnight incubation and was inoculated into 50ml of LB broth in a 250 ml Erlenmeyer flask.

It was then incubated overnight at 30°C in an orbital shaker at 150 rpm. The cell density of the culture medium was measured following overnight incubation by taking the absorbance at a wavelength of 600 nm using sterile LB broth as blank. This overnight culture was then used as inoculum for all media assessment studies and the fermentation was started with an $OD_{600} = 0.1$ for all if not otherwise stated.

2.6 Fermentation procedure for biopesticide production

2.6.1 Submerged fermentation

Batch type fermentation was carried out for the study. The *B. thuringiensis kurstaki* HD-73 was grown in the 500 ml Erlenmeyer flasks containing sterilized medium prepared before. Each flask containing 100 ml of different media were inoculated with bacterial strain HD-73 grown in the seed culture. A 5% (v/v) inoculum was used to inoculate 100 ml of sterilized media.

The shaking flasks were incubated at a stirrer speed of 150 rpm for 72 hours at 30°C. Two flasks were used as duplicate set of study and were examined time to time up to 72 hours. Culture samples were withdrawn from the flasks at different intervals to determine viable spore (VS) and -endotoxin concentration.

2.6.2 Biphasic Solid state fermentation

To achieve biphasic solid state fermentation, after 7hr fermentation of defatted soybean meal supplemented LB (LBS) medium at 150 rpm and 30°C, the supernatant in it was completely removed (1000×g, 10 min) aseptically for harvesting extracellular enzymes as byproduct and the resultant wet solid mass with embedded *Btk* was incubated 65 hours more for enhancing - endotoxin production at static conditions (30°C).

2.7 Sampling

Samples from each culture medium were drawn aseptically in a laminar air flow at every 24 h, up to 72 h of bacterial growth in a laminar air flow. For various analytical purposes, samples were collected separately.

2.8 Analysis of samples

Collected samples were used for the microscopic observation, estimation of spore count and estimation of crystal protein concentration.

2.8.1 Microscopic studies

Phase contrast microscopy: The presence of spore and crystal protein was observed by phase contrast microscopy. Bacterial strains were cultured on LB agar medium and incubated at 30°C for 3 days. A single colony was then mixed with a drop of distilled water that was placed on a clean slide and then covered with a cover slip. Excess liquid from edge of the cover slip was blotted by a tissue paper. Then the slide was placed under a phase contract microscope (Primo Star, Carl Zeiss, Germany) and the presence of spore and crystal protein was observed. Photomicrographs of every sample were recorded for analysis.

2.8.2 Estimation of spore count

The progress of bio-pesticide production was monitored by measuring the spore count at 24h intervals. 1 ml sample was collected in sterile microfuge tube and was heat treated at 80°C for 15 min, serially diluted, then plated on the LB agar plates and incubated at 30°C for 24h to form developed colonies.

To determine the spore count from solid medium, the culture samples were accurately weighed (10.00 g in a 250 ml Erlenmeyer flask), dissolved by 90 ml sterile distilled water, and then agitated in a shaker at 150 rpm for 30 min (Zhang *et al.*, 2013). The prepared samples were heat treated at 80°C for 15 min, serially diluted, then plated on the Luria-Bertani (LB) agar plates and incubated at 30°C for 24h to form developed colonies

For all counts, an average of at least three replicate plates was used for each tested dilution. For enumeration, the colonies counted on the plates were between 30 and 300 CFU (Colony Forming Units).

2.8.3 Estimation of crystal protein concentration

The purification of crystal protein was done by the modified method of Fatma Öztürk *et al.*, 2009 & Liu *et al.*, 1994. The total crystal protein was determined by Bradford method (Bradford M.M. 1976).

2.8.3.1 Partial purification of Cry proteins

Preparation of the samples for crystal protein content was carried out by first centrifuging the samples (1 ml sample, in a 1.5 ml microfuge tube) at 10,000 rpm for 10 min. 1 g sample was collected from solid state fermented media and was suspended in 10 ml sterile dH₂O, stirred well and centrifuged. For solid state fermented media, the pellet was discarded and the supernatant again was centrifuged at the same condition. The sedimented pellet containing spore-crystal complex was washed twice with 1 ml cold sterile distilled water and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was treated with 250µl 1.0M NaCl and 5mM EDTA and mixed thoroughly by vortexing. Again centrifugation was carried out at 10,000 rpm for 10 min.

Again the supernatant was discarded and pellet was treated with 400µl 5mM EDTA and mixed thoroughly by vortexing. Then again centrifugation was carried out at 10,000 rpm for 10 min. The sedimentated pellet was then redispersed in 1ml 0.1N NaOH solution (Wu, Lei *et al.*, 2011).

The last step took advantage of the fact that crystals are soluble in alkaline solution but not in neutral or acidic ones. The final solution was centrifuges at 10,000 rpm for 10min. the supernatant containing dissolved crystal protein was used in total protein analysis; dilutions of the supernatant were made with 0.1N NaOH solution, if necessary (Liu, Bajpai *et al.*, 1994).

2.8.3.2 Preparation of diluted bovine serum albumin (BSA) standard

The protein in the culture supernatant was estimated according to the Bradford method (Bradford M.M., 1976). For the construction of standard curve 1 gm of Bovine Serum Albumin (BSA) was taken in 100 ml volumetric flask and the volume was adjusted to 100 ml by distilled water.

The solution was then diluted to 1.0, 0.8, 0.6, 0.5, 0.4, 0.2 and 0.1 mg/ml concentrations in different volumetric flask. 5 ml Bradford test solution was taken in 9 test tubes. 0.1 ml standard solution of different dilutions was added in 7 different tubes and was well mixed. In the control 0.1 ml distilled water was added instead of sample. After 5 minutes the absorbance was taken at 595 nm.

100 μ l of culture supernatant was mixed well with 5 ml of Bradford test solution and the absorbance was taken at 595 nm after 5 minutes. A standard curve of absorbance versus protein concentration was prepared by using the data of diluted standard protein concentrations and their corresponding absorbance. The amount of soluble protein was determined from that standard curve using the following equation and expressed as mg per ml of test sample.

$$y = mx$$

Where, y = absorbance at 595 nm

x = protein concentration in mg/ml

m= slope of the standard curve

2.8.3.3 Procedure for determination of Δ -endotoxin concentration

For determination of Δ endotoxin concentration 1ml of Bradford reagent was aliquoted in the microfuge tube. Then 20 μ l of distilled water was added in a microfuge tube containing 1 ml of Bradford reagent as control. And then the test samples were added in the microfuge tube. The tubes were allowed to rest for five minutes in room temperature. Then they were mixed by turning the microfuge tube up and down for several times. Then the absorbance of the control was taken. This was made auto zero. Then the protein concentration of the test samples was measured.

2.9 SDS-PAGE analysis of Δ -endotoxin of *Bt* subsp. *kurstaki* HD-73

SDS-PAGE analysis was carried out according to the procedure described by Sambrook *et al.*, 1989.

2.9.1 Sample preparation

Bt subsp. *kurstaki* HD-73 was cultured in sea water in soybean extract-molasses-cystine (SeSWmc) which were prepared according to the procedure described in section 2.4.2.7 and 15 g/L agar (Merck, Germany) was added to give solid texture.

The culture was incubated at 30°C for 3 days. Colonies were scrapped off from the solid medium after 3 days of incubation using sterile loop in laminar flow and suspended in 100 µl sterile distilled water in microfuge tube. Then -endotoxin concentration of each sample was determined by Bradford method (procedure mentioned in section 2.8.3.3)

2.9.2 Preparation of separating gel

For preparation of separating gel glass plates were cleaned and dried and placed on the gel casting stands (BIO-RAD™ Instruments). Then 10% separating gel was prepared gently by mixing the distilled water (2.0 ml), lower gel buffer (1.25 ml) and 30% acrylamide-bisacrylamide (1.70 ml). This step was followed by rapid addition of TEMED (4 µl) and freshly prepared 10% ammonium sulfate (APS) (20µl).

The freshly mixed solution was poured into glass plate chamber using a 1ml micropipette, without generating air bubbles. The gel mixture was poured to a level of about 5cm below the top edge of the glass plates and was then overlaid with distilled water. It was then left for about 45 min for polymerization.

2.9.3 Preparation of stacking gel

For preparation of 5% stacking gel solution, distilled water (1.4 ml), upper gel buffer (600 µl), and 30% acrylamide-bisacrylamide (400 µl) were mixed first. Before pouring the stacking gel solution, water layer on top of separating gel was poured off. Then 10% APS (10 µl) and TEMED (2.5 µl) were added to the beforehand prepared 5% stacking gel solution.

The stacking gel solution was then poured on the top of the separating gel using a 1 ml micropipette. A 10-well comb was inserted in between the glass plates carefully so that bubble was not generated. Then the gel was allowed to settle for at least 60 min for polymerization.

2.9.4 Sample application and gel run

20 µl of previously prepared sample protein was mixed with 100 µl sample loading buffer (composition mentioned in appendix I) in microfuge tube and was boiled at 100°C for 15 min. Then centrifugation was carried out at 10,000 rpm for 10 min 20 µl supernatant. The comb was removed and the whole apparatus was placed in the gel cassette which was then placed into the BIO-RAD™ Mini-Protein™ Tetra cell. The cell as well as the gel cassette was filled with electrophoresis buffer (composition mentioned in appendix I). Using a micropipette, 20 µl of the previously prepared sample was mixed with 1µl tracking dye (0.1% bromo-phenol blue) and loaded into the wells. Then the cell was connected to the BIO-RAD™ PowerPack™ basic. A constant voltage of 100 V was adjusted. Finally electrophoresis was carried out until the tracking dye reached the bottom level of the gel.

2.9.5 Staining and destaining of the gel

The glass plates were immersed in distilled water and by using the plate separator, the gel was released from the glass plates. Then the gel was placed into staining solution (0.02% Coomassie Brilliant Blue,G-250 in 2% (w/v) phosphoric acid,5% aluminum sulfate and 10% ethanol) for a period of 2 hours on a rotary shaker. The gel was then transferred to a container containing distilled water and was rinsed with distilled water repeatedly to visualize the protein bands.

2.10 Biopesticide production in 3L Bioreactor

2.10.1 Inoculum Development

For production of 5% inoculum for fermentation in 2 liter of sea water in soybean extract-molasses-cystine (SeSWmc), one loop-full of *Btk* HD-73 was transferred from the stock culture to 5 ml of Luria-Bertani (LB) broth and was incubated for 12 hours.

This was then added to 75 ml of the above mentioned medium and incubated for 12 hours and then finally added to the bioreactor containing 1900 ml of the above mentioned medium. The SeSWmc medium was prepared by the method mentioned in section 2.4.2.7

2.10.2 Growth conditions in Bioreactor

Sea water in soybean extract-molasses-cystine (SeSWmc) was used for the cultivation of *Btk* HD-73 under controlled condition of bioreactor. Temperature was controlled at 30°C and pH was kept uncontrolled. Aeration and agitation were controlled at cascading mode to maintain the dO₂ at 30%. Agitation high was set at 250 rpm while the aeration high was set at 1 SLPM.

2.10.3 Fermentation in Bioreactor

For this experiment bioreactor facilities in the Enzyme and Fermentation Biotechnology Laboratory at Department of Microbiology in the University of Dhaka were used. The vessel volume of that stirred tank bioreactor (model: BIO FLO 110 Fermentor / Bioreactor; company: NEW BRUNSWICK SCIENTIFIC) was 3 liter and the working volume was 2 liter.

The bioreactor was equipped with instrumentation in order to measure and control the agitation, pH, temperature, foam, dissolve oxygen (dO₂) and exit gases. The medium was aerated by a pump (MTH) through a membrane filter. The agitator was equipped with four bladed impellers. These impellers homogenized air and the bio-controller displayed the dissolve oxygen concentration by sensing with an electrode. For temperature control, there was an outer jacket wrapping the vessel and a chiller was connected to supply cool water through a ring inside the vessel.

Before autoclaving, the vessel was washed carefully. All the screw-able nuts were fastened tightly and the vessel was equipped with a pH probe. This probe was calibrated by dipping it in a pH 7 buffer and this value was set at zero.

The probe was then rinsed with distilled water and dipped again in pH 4 buffer and set span. All the connection except air outlet was closed by tying with rubber. In this experiment the pH and foam were not controlled but dO₂ was controlled by cascading mode. However, the soybean oil added acted as the anti-foam and pH probe detected the pH. The vessel was then filled with SeSWmc medium, which was prepared by the method mentioned in section 2.4.2.7 and was then placed in autoclave machine and was sterilized by autoclaving at 121°C for 15 min at 15 psi (Hirayama, Model HA-300M, Japan).

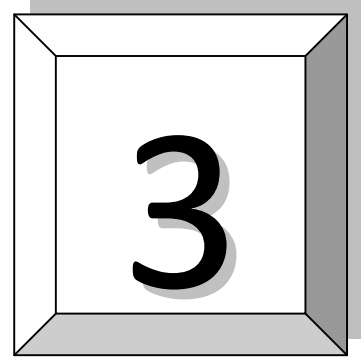


Figure 2.1: BIO FLO 110 Fermentor / Bioreactor; company: NEW BRUNSWICK SCIENTIFIC

After autoclaving the vessel was placed near the bio-controller and all the connections such as pH electrode, dO_2 electrode, agitator, chiller inlet and outlet were connected carefully. When the temperature came down to 30°C , then aeration was continued to saturate the medium with dissolved oxygen. The dO_2 set point and set span were set at 30% and 100% respectively. Agitation and aeration were controlled at cascading mode to maintain dO_2 at 30%. Agitation and aeration were set at 250 rpm and 1 SLPM respectively. Inoculum was added aseptically. Fermentation was carried out continuously for 36 hours.

Sampling was done at different time intervals and -endotoxin was purified and concentration was measured by the procedure mentioned in section 2.8.3.1.

CHAPTER



RESULTS

3. Results

Bacillus thuringiensis acts as an important biological control agent against various pests. The nutritional requirements affecting the growth, sporulation and δ -endotoxin synthesis by *Bt* deserves critical study, among them carbon and nitrogen sources play a vital role. Moreover there are amino acids, trace elements that affect their physiology of δ -endotoxin synthesis.

The present study was carried out to determine the sporulation and δ -endotoxin production in submerged and biphasic solid state fermentation by *Bacillus thuringiensis* strains. To develop a cost effective medium for large scale production of biopesticide locally available cheap raw materials such as defatted soybean meal as nitrogen, and molasses as carbon source were used. The effects of different carbon and nitrogen sources, amino acid such as cystine, basal salts on growth, sporulation and δ -endotoxin synthesis by *B. thuringiensis kurstaki* (*Btk*) HD-73 was also considered. Sea water was used as substitute of basal salts to make the production medium more cost effective. Moreover, biphasic fermentation conditions i.e. growth in liquid culture followed by transfer of the media into solid culture particularly were conducted to observe the effect of stress on sporulation enhancement and endotoxin synthesis.

3.1 Phenotypic characterization of bacterial strains

3.1.1 Colony characteristics

Btk HD-73 was inoculated on to LB agar for 24 hours at 30°C to observe the colony characteristics. The colonies were white in color, opaque, slightly raised elevation and regularly outlined.



Figure 3.1: Colony characteristics of *Btk* HD-73 on LB agar medium.

3.1.2 Crystal protein morphology of *Bacillus thuringiensis*

The phase contrast microscopy results showed the presence of visible crystal proteins and spore position. Parasporal inclusions were produced outside of the endospore and were distinctly separated from it. Crystal structure of *Btk* HD-73 is shown in Figure 3.2.

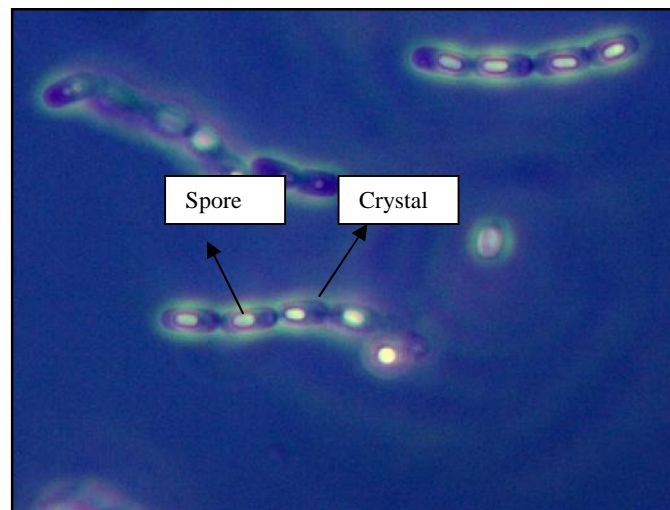


Figure 3.2: Crystal protein morphology of *Bacillus thuringiensis* under phase contract microscope

3.2 Construction of standard curve for estimation of protein by Bradford method

For determination of -endotoxin concentration by Bradford method a standard curve with BSA (Bovine Serum Albumin) was prepared. A range of standard solutions of BSA was prepared according to the procedure described in section 2.8.3.2. Standard curve for estimation of protein in unknown samples was constructed and showed in Figure 3.3. The best fit linear equation intercepting zero is derived. The equation $y = 0.059x$ was used for estimation of protein in unknown sample where 'y' is the absorbance at 595 nm and 'x' is the concentration of protein in unknown sample in mg/ml.

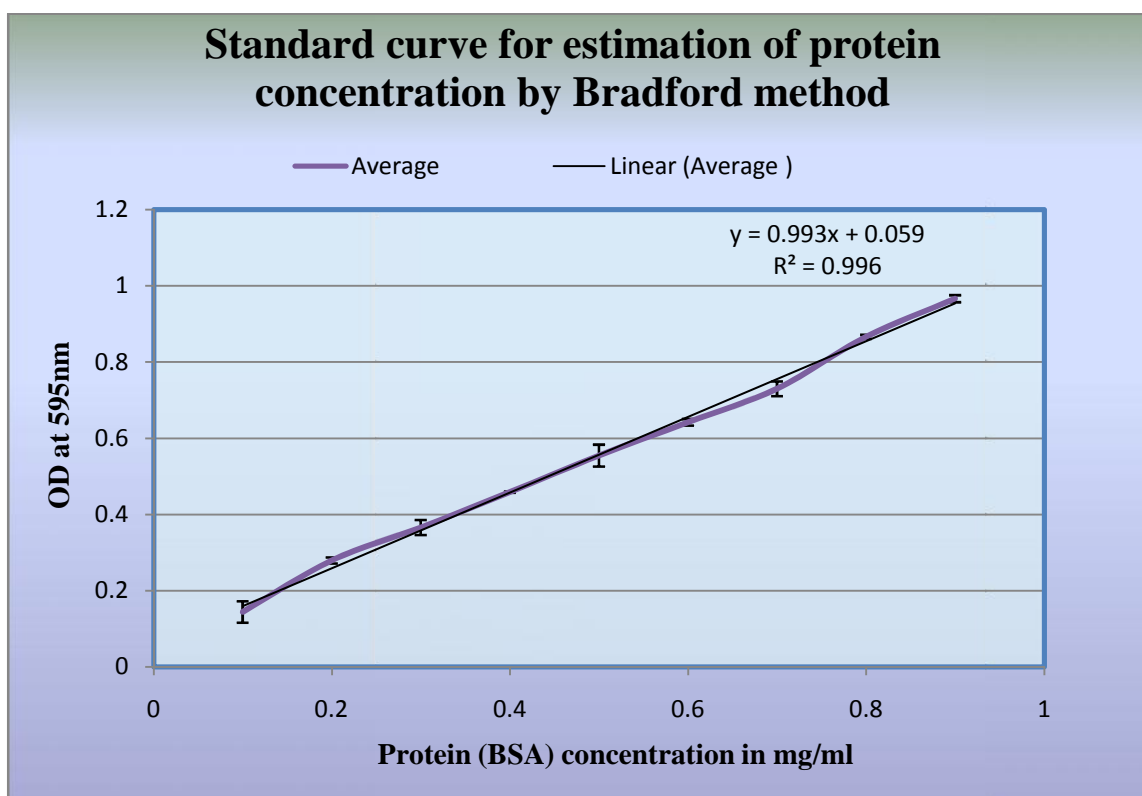


Figure 3.3: Standard curve for estimation of protein concentration by Bradford method

The strong linear relationship ($R^2 > 0.99$) between the absorbance of protein and concentration demonstrates exceptional reliability in estimating protein content of unknown samples.

3.3 Determination of factors affecting sporulation and α -endotoxin synthesis by *Bacillus thuringiensis kurstaki* HD-73

The factors that affect the sporulation enhancement and α -endotoxin synthesis by *Bacillus thuringiensis kurstaki* HD-73 were determined. The effect of locally available defatted soybean meal as nitrogen source, molasses as carbon source, and effect of cystine and effect of sea water as replacement of basal salts was observed.

The spore count and the concentration of α -endotoxin in the fermentation were determined according to the described methods (section 2.8.2-2.8.3). Two sets of experiments were performed and results were recorded. In the following sections, data and their graphical representations are given.

3.3.1 Effect of defatted soybean meal with LB on sporulation and α -endotoxin formation by submerged fermentation

The effect of LB alone and with defatted soybean meal to support sporulation and α -endotoxin synthesis by *Btk* HD-73 was studied. In this regard, 10 g ground defatted soybean meal was added to 90 ml of LB. Fermentation was carried out for 72 hours at 30°C under shake culture. Maximum sporulation and α -endotoxin concentration were obtained at 72 hours for LB medium (control) which were 7.217 log CFU/ml and 0.149 mg/ml respectively. Whereas, maximum sporulation and α -endotoxin concentration were 7.447 log CFU/ml and 0.371 mg/ml at 48 hours in defatted soybean meal with LB (LBS) medium. The effect of defatted soybean meal with LB medium is shown in Table 3.1. A graphical representation is also given in Figure 3.4 for clear observation of the results.

Table 3.1: Evaluation of soybean meal & LB medium on sporulation & endotoxin synthesis by *Btk* HD 73 in submerged fermentation

Time (hr)	Spore count (log CFU/ml)		Difference rate (%)	-endotoxin concentration (mg/ml)		Difference rate (%)
	LB	LBS		LB	LBS	
24	4.041±0.056	7.371±0.092	58.35	0.047±0.004	0.236±0.060	133.85
48	5.585±0.039	7.447±0.044	28.57	0.085±0.019	0.371±0.028	125.43
72	7.217±0.093	7.273±0.041	0.77	0.149±0.029	0.341±0.013	78.36

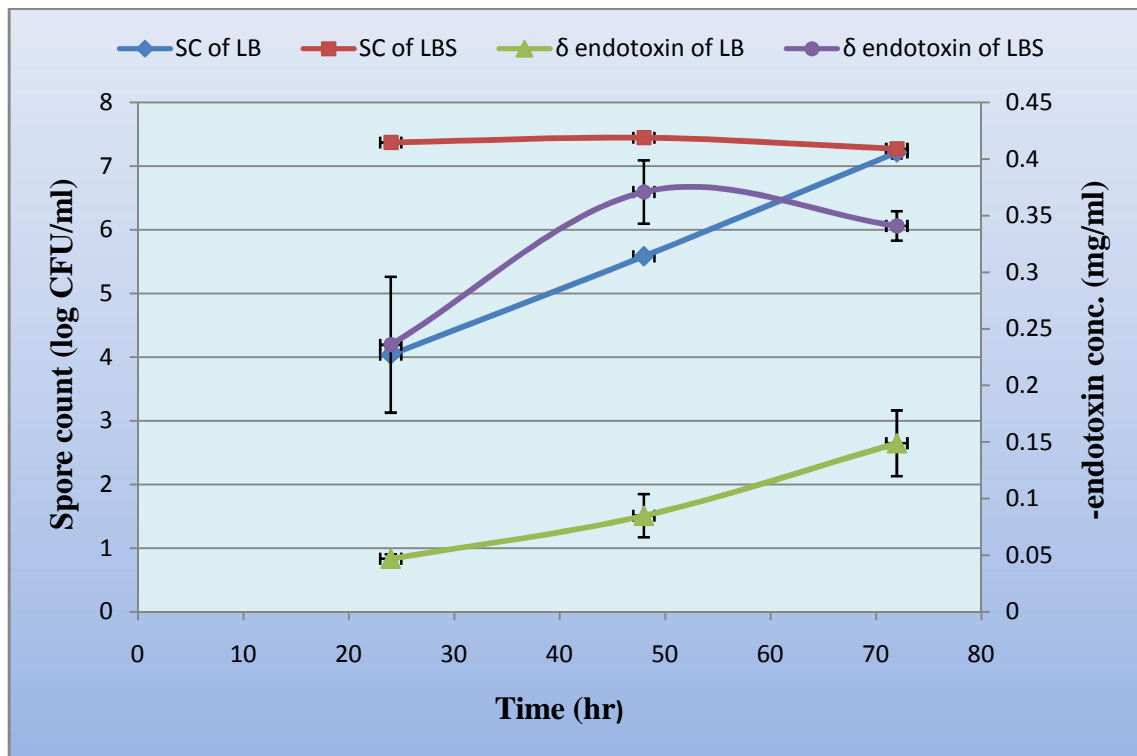


Figure 3.4: Evaluation of soybean meal & LB medium on sporulation & endotoxin synthesis by *Btk* HD 73 in submerged fermentation

3.3.2 Evaluation of biphasic fermentation on sporulation and α -endotoxin formation

In this experiment, the performance of biphasic fermentation in defatted soybean meal supplemented with the conventional LB medium for enhancing the growth, sporulation and overproduction of α -endotoxin by *Btk* was evaluated. Of different concentrations and combinations of media tested, 10% (w/v) grinded soybean meal supplemented with LB was found as the best composition for toxin production. To achieve biphasic solid state fermentation (SSF), after 7 h submerged fermentation of LBS (150 rpm, 30°C), the supernatant it was completely removed (1000×g, 10 min) by centrifugation aseptically and the resultant wet solid matter with grown *Btk* cells was incubated 65 h more at static condition (30°C). In comparison to submerged fermentation of LBS (monophasic), yield of sporulation and α -endotoxin production was 2.72% higher and 93.86% lower respectively in solid state fermentation of LBS (biphasic) at 48 hours fermentation.

Table 3.2: Sporulation and α -endotoxin synthesis under submerged and biphasic solid state fermentation

Time (hr)	Spore count (log CFU/ml)		Difference rate (%)	α -endotoxin concentration (mg/ml)		Difference rate (%)
	Submerged condition	Biphasic condition		Submerged condition	Biphasic condition	
24	7.371±0.092	7.414±0.024	0.58	0.236±0.060	0.102±0.004	79.28
48	7.447±0.044	7.653±0	2.72	0.371±0.028	0.134±0.011	93.86
72	7.273±0.041	8.866±0.054	19.74	0.341±0.013	0.151±0.003	84

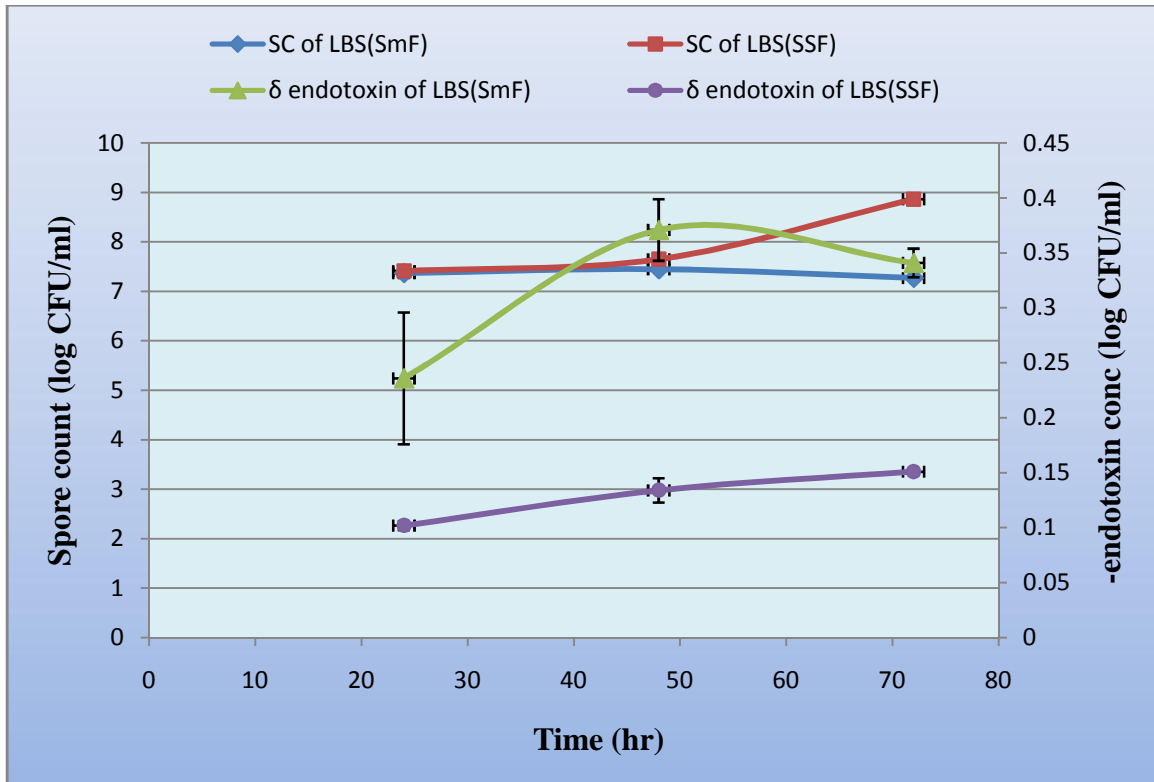


Figure 3.5: Sporulation and δ -endotoxin synthesis under submerged and biphasic solid state condition

3.3.3 Role of cystine in defatted soybean meal with LB

The effect of cystine on sporulation and δ -endotoxin formation by *Btk* HD-73 was observed in LB soybean medium (LBS). This medium contained 10 g soybean along with 90 ml LB media supplemented with 300 μ l cystine (LBSc).

300 mg/L cystine displayed the highest sporulation and toxin concentration as shown in Table 3.3 and Figure 3.6. A lower concentration of cystine promoted growth, sporulation and crystal formation in *B. thuringiensis* (Rajalakshmi and Shethna, 1977). Fermentation was carried out for 72 hours. Maximum sporulation and δ -endotoxin concentration were obtained at 48 hours. The sporulation and δ -endotoxin increased 19.54% and 131.35% respectively in cystine added LBS medium. A significant change was observed on the δ -endotoxin concentration when cystine was added.

Table 3.3: Effect of cystine in defatted soybean meal with LB on sporulation and endotoxin production

Time (hr) / Media	Spore count (log CFU/ml)		Difference rate (%)	-endotoxin concentration (mg/ml)		Difference rate (%)
	LBS	LBS _c		LBS	LBS _c	
24	7.371±0.092	7.096±0.124	3.80	0.236±0.060	0.726±0.058	101.87
48	7.447±0.044	9.06±0.08	19.54	0.371±0.028	1.791±0.086	131.35
72	7.273±0.041	7.431±0.045	2.15	0.341±0.013	1.433±0.034	123.11

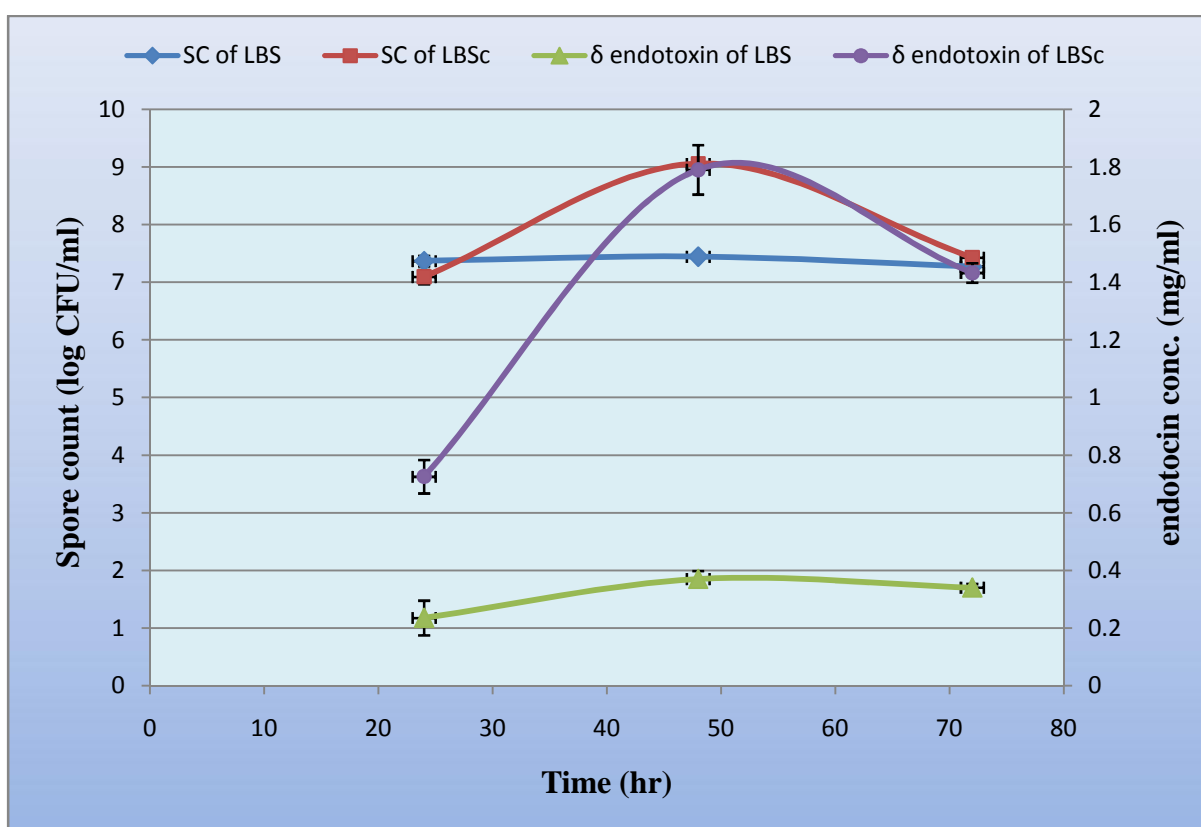


Figure 3.6: Role of cystine in defatted soybean meal with LB

3.3.4 Replacement of LB with basal salts in soybean-cystine medium

A positive effect was observed on the LBS medium supplemented with cystine in previous experiment (Figure 3.6). In this experiment, basal salt solution was used instead of LB for its cost effectiveness. The composition of the basal salt solution were $MgSO_4 \cdot 7H_2O$, 0.5(g/l); $MnSO_4 \cdot H_2O$, 0.1(g/l); $FeSO_4 \cdot 7H_2O$, 0.02(g/l); $ZnSO_4 \cdot 7H_2O$, 0.02(g/l); $CaCl_2$, 0.01(g/l) & KH_2PO_4 1.0(g/l) (Gangurde and Shethna, 1995). Fermentation was carried out for 72 hours. Maximum sporulation and α -endotoxin concentration were obtained at 48 hours which were 9.352 log CFU/ml and 1.659 mg/ml respectively on defatted soybean meal with cystine (SMc). Though the endotoxin was 7.65% less than that of LBSc, which is comparable and it is acceptable because of its cost effectiveness.

Table 3.4: Effect of replacement of LB with basal salts in soybean-cystine medium on sporulation and endotoxin production.

Time (hr) / Media	Spore count (logCFU/ml)		Difference rate (%)	α -endotoxin concentration (mg/ml)		Difference rate (%)
	LBSc	SMc		LBSc	SMc	
24	7.096±0.124	7.681±0.025	7.9	0.726±0.058	0.831±0.001	13.5
48	9.06±0.08	9.352±0.017	3.17	1.791±0.086	1.659±0.003	7.65
72	7.431±0.045	8.568±0.026	14.2	1.433±0.034	1.326±0.050	7.75

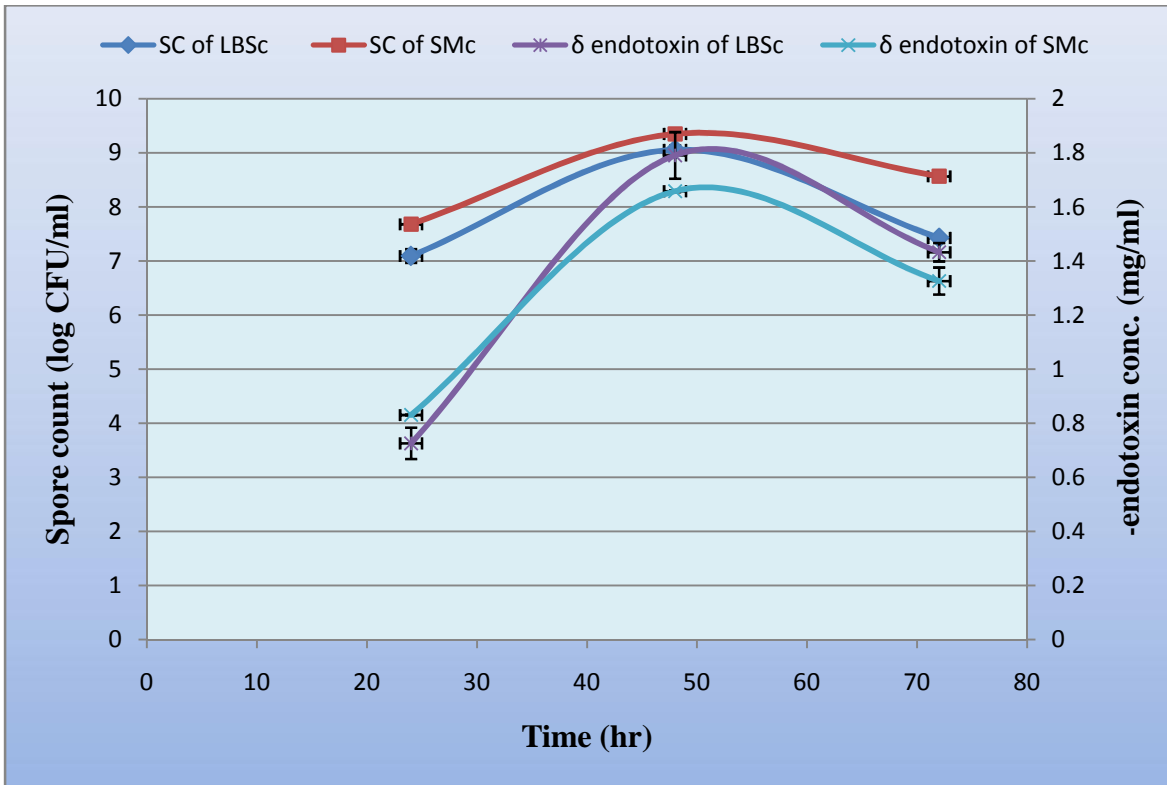


Figure 3.7: Replacement of LB with basal salts in soybean-cystine medium

3.3.5 Performance of molasses in soybean-cystine medium

The effect of locally available defatted oil-seed-derived nitrogen source supplemented with cystine was studied with a view to enhancing the commercial potential of the biopesticide production by *Bt* strains. For this purpose a soybean-cystine medium with molasses (SMmc) was designed for trial. In this medium, soybean and molasses were used as nitrogen and carbon source. This medium also contained basal salt solutions at a concentration of g/l in distilled water: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.0005, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0005, and CaCl_2 0.1. 0.5 g molasses was used in 100 ml of the media to check the substrate efficiency of the media due to molasses. The sporulation and toxin formation was 9.114 log CFU/ml and 2.376 mg/ml after 48 hours of fermentation which was higher in the present medium composition (SMmc) than that in soybean-cystine medium (SMc) after 72 hours.

Table 3.5: Effect of molasses in soybean-cystine medium on sporulation and endotoxin production

Time (hr)	Spore count (log CFU/ml)		Difference rate (%)	-endotoxin concentration (mg/ml)		Difference rate (%)
	SMc	SMmc		SMc	SMmc	
24	7.681±0.025	7.857±0.017	2.26	0.726±0.058	1.796±0.072	84.85
48	9.352±0.017	9.114±0.144	2.57	1.659±0.003	2.376±0.171	35.53
72	8.568±0.026	6.041±0.055	34.59	1.326±0.050	1.246±0.053	6.22

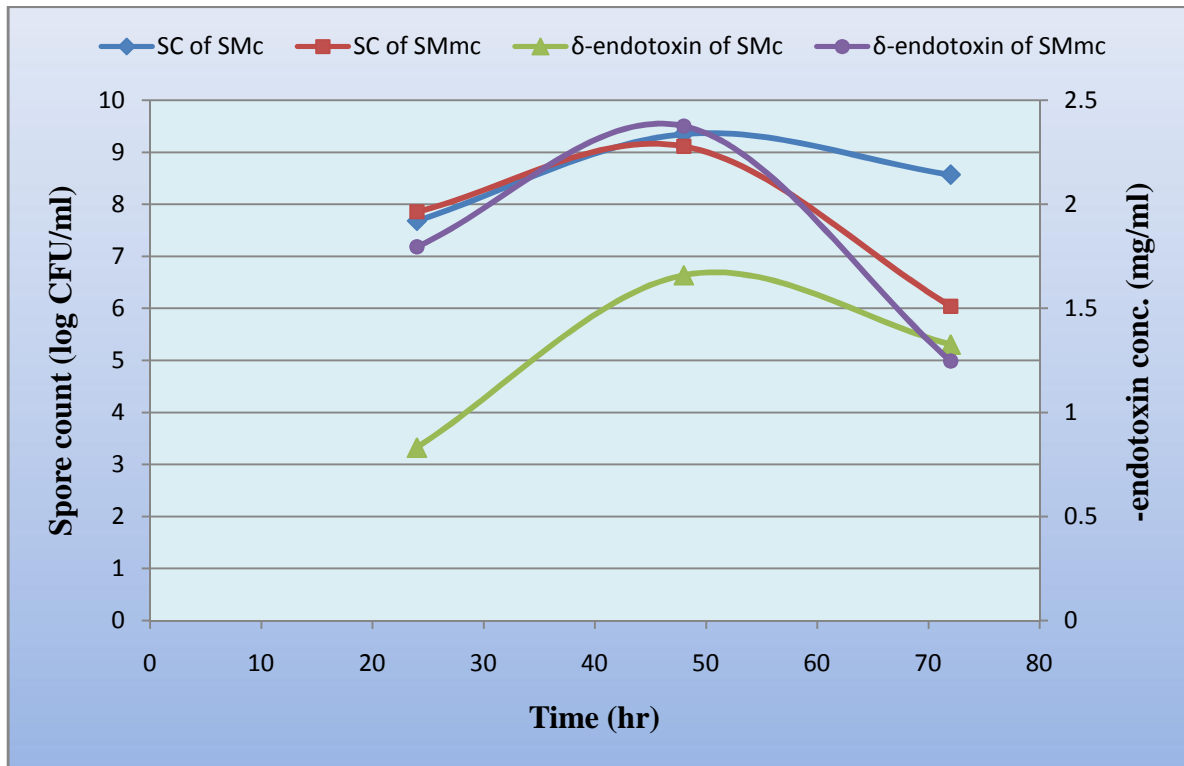


Figure 3.8: Effect of molasses in soybean-cystine medium

3.3.6 Replacement of basal salts with sea water in soybean-molasses-cystine medium

In order to reduce the cost of bioinsecticide production, sea water was used for possible supply of the minerals required for α -endotoxin production in the culture medium by *Btk*. So, an experiment was performed to observe the effect of substitution of basal salt solutions ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and CaCl_2) in soybean medium with sea water. In this experiment, maximum sporulation and α -endotoxin were 8.176 log CFU/ml and 1.918 mg/ml at 48 hours as compare to 9.114 log CFU/ml and 2.376 mg/ml in the media with basal salts (previous experiment) (Table 3.6). Though the medium containing basal salt solutions gave higher α -endotoxin yield in comparison to medium containing sea water, medium formulated with sea water is more cost effective and suitable for large scale production of biopesticide.

Table 3.6: Effect of replacement of basal salts with sea water in soybean-molasses-cystine medium on sporulation and endotoxin production

Time (hr)	Spore count (log CFU/ml)		Difference rate (%)	α -endotoxin concentration (mg/ml)		Difference rate (%)
	SMmc	SSWmc		SMmc	SSWmc	
24	7.857±0.017	7.249±0.008	8.049	1.796±0.072	1.296±0.072	32.34
48	9.114±0.144	8.176±0.212	10.85	2.376±0.171	1.918±0.171	21.33
72	6.041±0.055	8.512±0.047	33.95	1.246±0.053	1.649±0.053	27.84

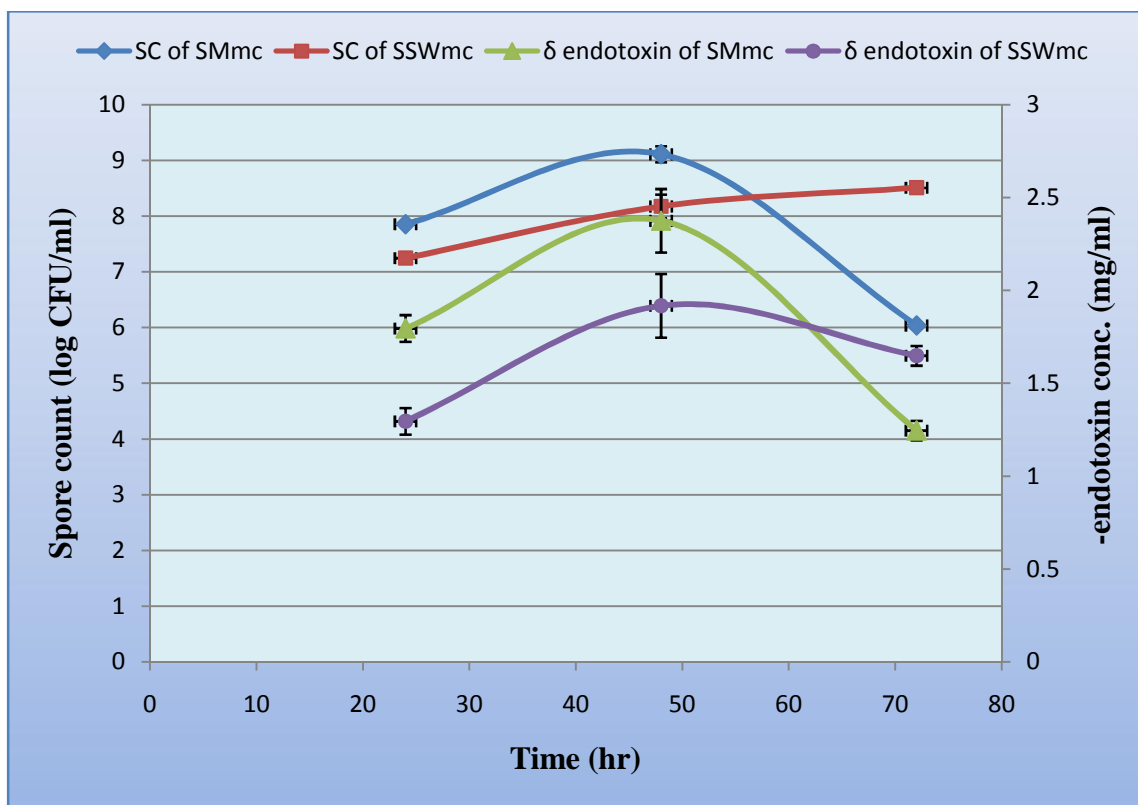


Figure 3.9: Replacement of basal salts with sea water in soybean-molasses-cystine medium

3.3.7 Evaluation of formulated medium (Soybean extract in place of soybean meal in soybean-molasses-cystine medium) on sporulation and endotoxin production.

In the previous experiment, the positive effect of sea water has been showed. For large scale production there are few drawbacks for the medium with soybean mass. For this purpose a defatted soybean extract-molasses-cystine medium was designed for trial. The sporulation and -endotoxin concentration was 8.596 log CFU/ml and 1.386 mg/ml at 24 hours. But -endotoxin concentration decreased from 1.359 mg/ml to 0.471 after 72 hours.

Table 3.7: Effect of soybean extract in place of soybean meal in the soybean-molasses-cystine medium on sporulation and endotoxin production

Time (hr)	Spore count (log CFU/ml)		Difference rate (%)	-endotoxin concentration (mg/ml)		Difference rate (%)
	SSWmc	SeSWmc		SSWmc	SeSWmc	
24	7.249±0.008	8.596±0.007	16.99	1.296±0.072	1.386±0.072	6.71
48	8.176±0.212	7.512±0.047	8.46	1.918±0.171	1.359±0.212	34.11
72	8.512±0.047	6.352±0.068	29.06	1.649±0.053	0.471±0.108	111.13

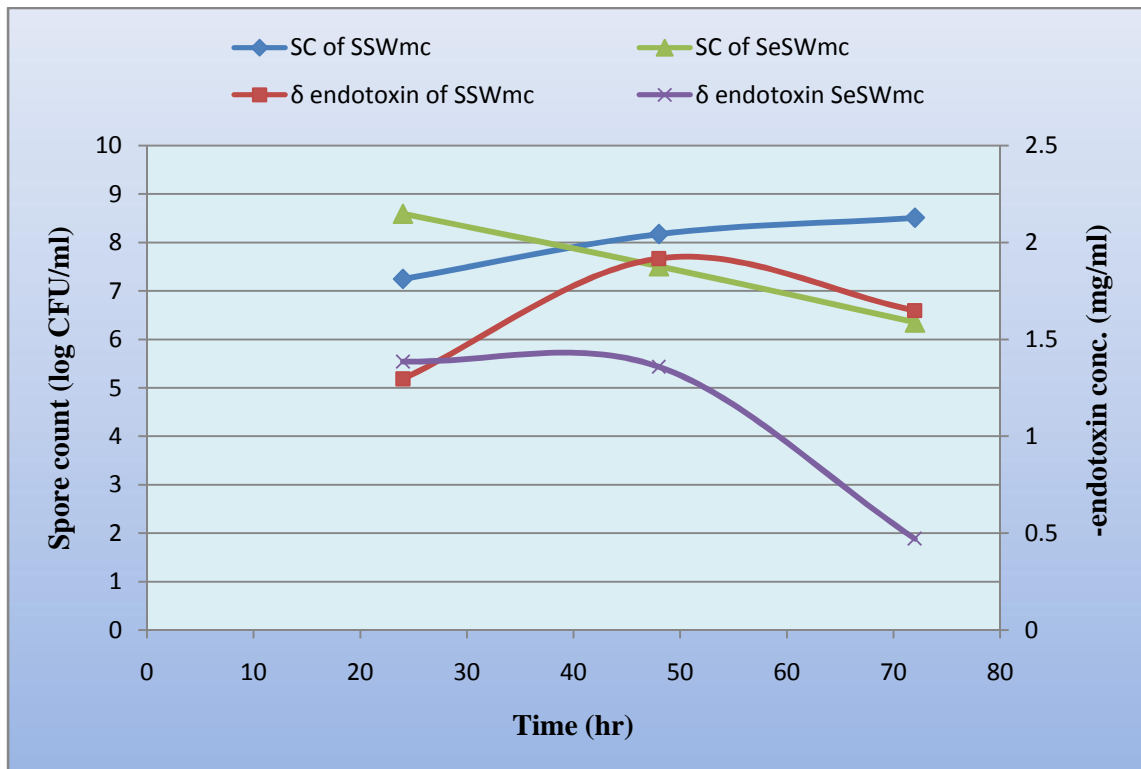


Figure3.10: Effect of soybean extract in place of soybean meal in the soybean-molasses-cystine medium

3.4 Evaluation of different media on sporulation

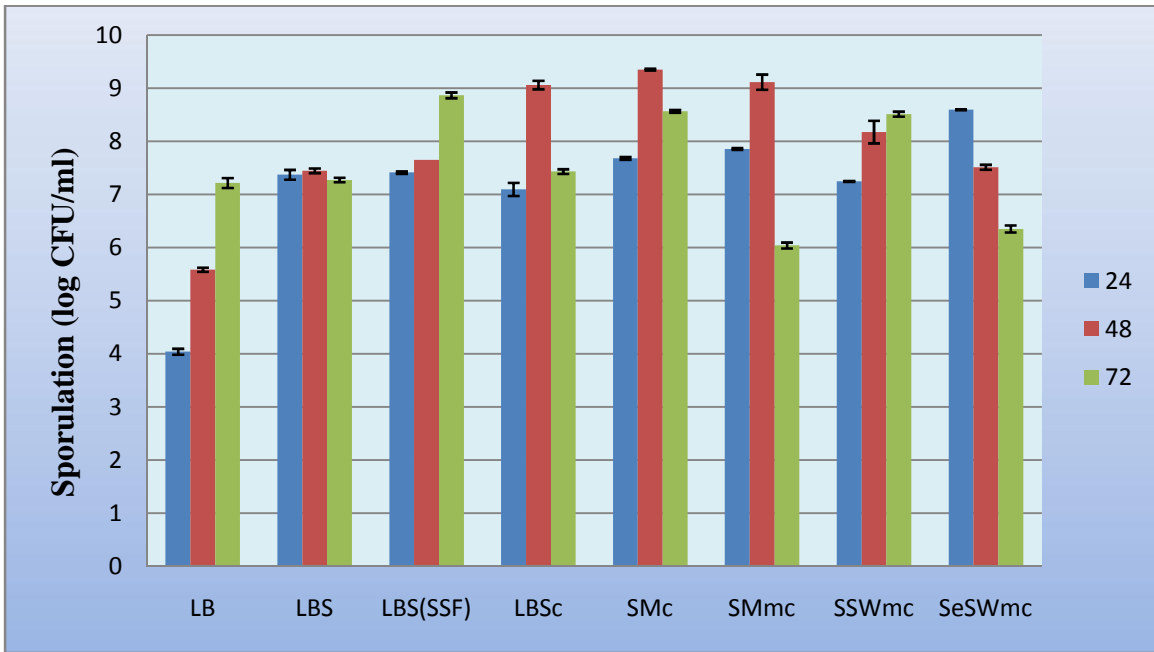


Figure 3.11: Evaluation of different media, at a glance, on sporulation

3.5 Evaluation of different media on LPS -endotoxin synthesis

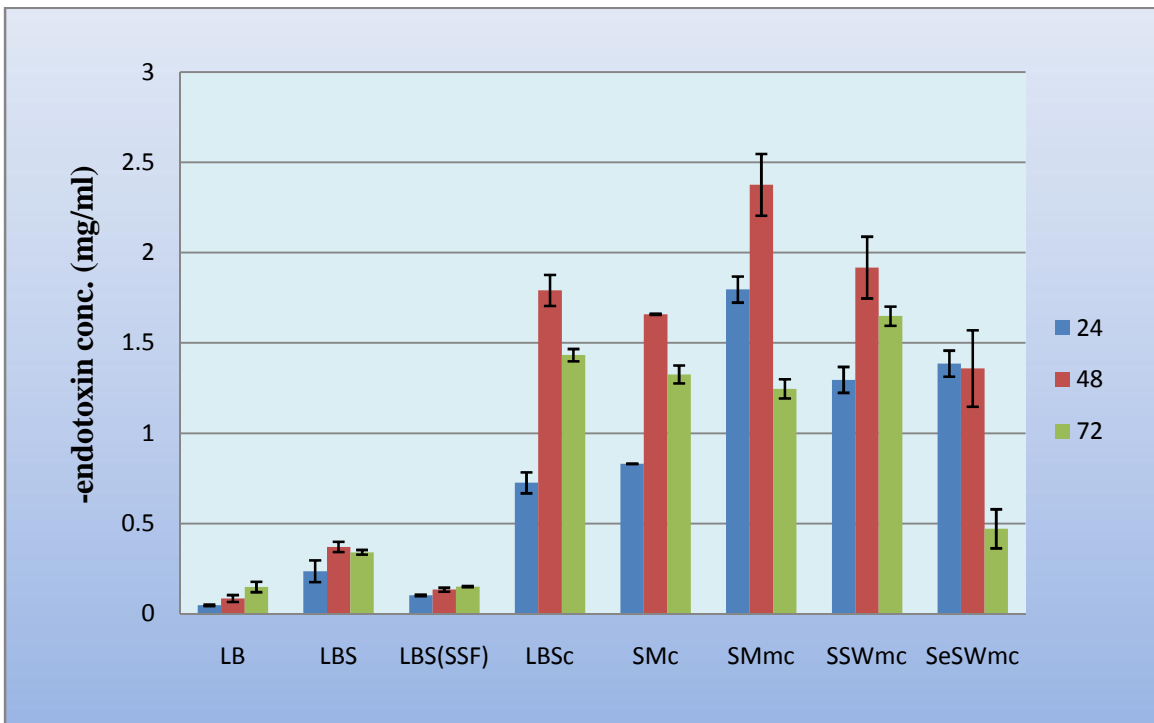


Figure 3.12: Evaluation of different media, at a glance, on LPS -endotoxin synthesis

3.6 Scale up of production kinetics of *Btk* HD-73 under controlled condition in a 3L bioreactor

Defatted soybean extract medium with sea water, cystine, and molasses was used for the cultivation of *Btk* HD-73 under controlled condition of bioreactor. 5% inoculum of *Btk* HD-73 was used for fermentation. Temperature was controlled at 30°C and pH was kept uncontrolled. Aeration and agitation were controlled at cascading mode to maintain the dO₂ at 30%. Agitation rate was set at 250 rpm while the aeration high rate set at 1 SLPM. Fermentation was carried out for 36 hours. Microscopic observation was carried at different time intervals and α -endotoxin concentration was observed at 12, 16, 20, 24 and 36 hours. All data are summarized in Table 3.8 and a graphical representation is also given in Figure 3.14. Maximum α -endotoxin yield was 2.3 mg/ml after 24 hours. An increase in 1.67 fold of endotoxin production rate was obtained in bioreactor than in shake culture.

Table 3.8: Total spore count and α -endotoxin concentration in 3L bioreactor

Time(hours)	Total spore count(log CFU/ml)	α -endotoxin concentration (mg/ml)
0	5.643±0.051	0.009±0.001
12	6.380±0.018	0.610±0.025
16	7.716±0.032	1.3±0.022
20	8.225±0.007	2.1±0.046
24	9.321±0.004	2.317±0.006
36	8.315±0.043	1.816±0.059

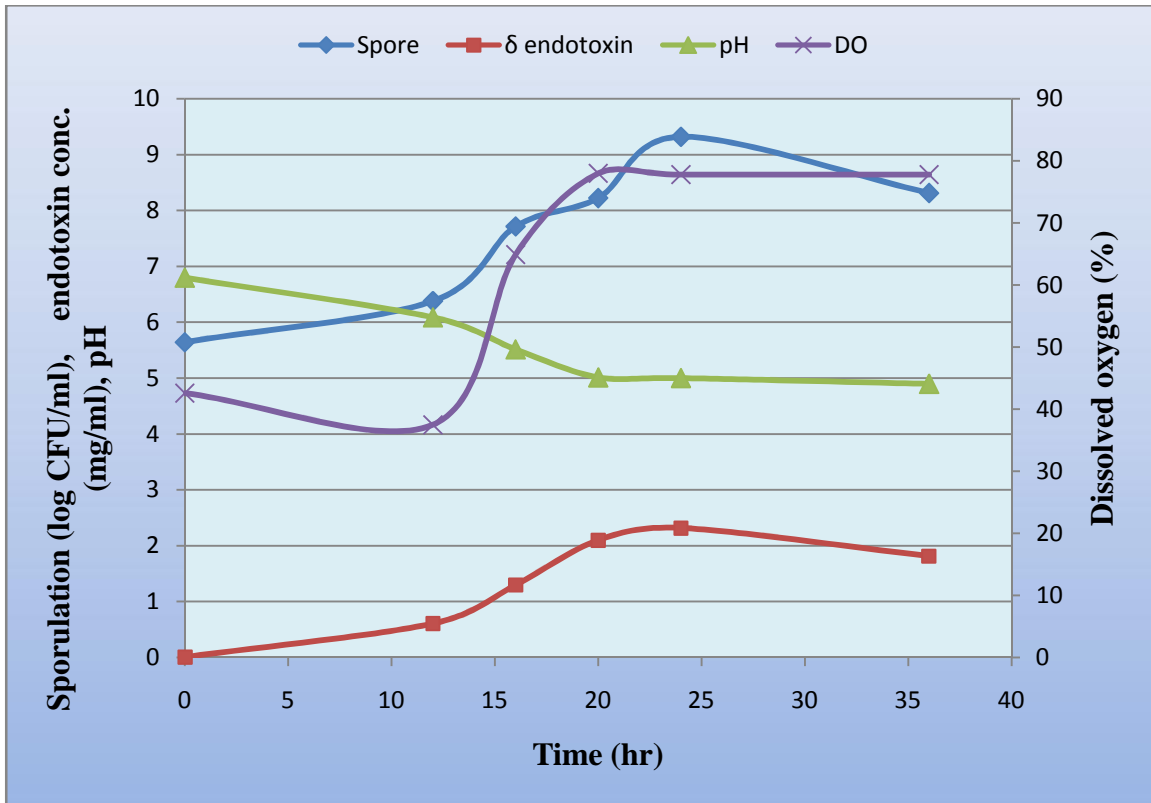


Figure 3.13: Total spore count and δ -endotoxin concentration in 3L bioreactor

3.7 SDS-PAGE analysis of crystal protein

The SDS-PAGE analysis of Cry proteins recovered from *Btk* HD-73 revealed the presence of Cry1Ac protein (Figure 3.14). The molecular weight was determined by using Alphaview SA (version 3.4.0.0). A thicker Cry1Ac protein band (133 kD) from *Btk* HD-73 for sea water in soybean extract-molasses-cystine agar media was visible.

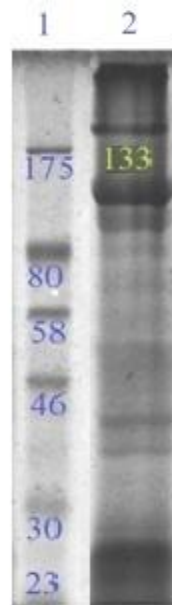


Figure 3.14: SDS-PAGE analysis of partially purified Cry protein obtained from the culture performed in SeSWmc agar. **Lane 1:** Marker (ColorPlus Prestained protein marker, Broad range, NEB), **Lane 2:** *Btk* HD-73

CHAPTER

4



DISCUSSION

4. Discussion

Bacillus thuringiensis (*Bt*) is a gram-positive, spore-forming soil bacterium that produces insecticidal crystal proteins during sporulation. *Bt* is the most widely used microbial control agent all over the world. Biological pesticides based on *Bt* are becoming increasingly important in pest management programs. For large scale production of biopesticide from strains of *Bt*, different approaches were investigated to design media that could support good production of spores as well as toxins at reasonable cost. As sporulation and toxin production are a simultaneous process, it is very important to optimize the factors including nutrients and culture conditions to attain maximum biomass yield leading to maximum sporulation and high endotoxin titer.

The nutritional aspects of *Bt* have also been studied extensively by a number of workers (Nickerson and Bulla, 1975; Rogoff and Yousten, 1969; Singer *et al.*, 1966). It is well established that various strains of *Bt* would not grow in so-called mineral salt medium unless certain growth factors such as glutamic acid and either aspartate or citrate are added to the medium and it also requires protein rich medium for growth. Carbon and nitrogen sources and their critical concentrations also not only affect the growth rate of microbes in general but also sporulation and rate of production. This aspect is rather more important with *Bt* as the endotoxin (cry protein) synthesis is critically related with sporulation rate. So, proper optimization of nutritional and other factors are essential for large scale production of *Bt* biopesticide with selective indigenous *Bt* strains.

In the present working laboratory at DU, about 300 *Bt* isolates have been identified and characterized from different eco-regions of Bangladesh. The abundance and diversity of *Bt* strains with their *cry* genes and cry protein profiles have also been studied (Asaduzzaman *et al.*, 2014). This study is particularly aimed at screening out potential *Bt* strain active against different pests affecting vegetables and crops in order to produce biopesticide in large scale at effective cost for field application by the farmers of Bangladesh.

The present study established the potential of defatted soybean meal to support growth and toxin production by *Bacillus thuringiensis* subsp. *kurstaki* HD-73. The defatted soybean meal has high protein content (Gangurde & Shethna, 1995) and low cost, is readily available in Bangladesh and can be handled easily. In addition the effects of various factors such as cystine, molasses, and sea water on the growth, sporulation and toxin production of *Bt* was also concerned. The pattern of growth and toxin production of the HD-73 in the media prepared from defatted soybean meal was observed.

Particularly, differentiation of the cell into sporulation from vegetative phase has made its influence complex (Anderson R.K.I., 2003). In present study the effect of different carbon and nitrogen sources on sporulation and α -endotoxin synthesis by *Btk* HD-73 was observed. Production of biomass as well as its endotoxin level by *Btk* HD-73 has been found to vary drastically in media derived from various nutrient sources. Two media were used for this purpose which was Luria-Bertani (LB) broth and defatted soybean meal in the LB broth. In defatted soybean meal with LB (LBS) medium the sporulation and α -endotoxin formation increased 58.35% and 133.85% respectively after 24 hours as compared to the commercial medium, LB (Table 3.1). The increase in sporulation and α -endotoxin yield may be due to complex nitrogen source. Soybean meal present in the medium contained 91.52% dry matter, 43.30% crude protein (D. A. Jahan, L. Hussain *et al.*, 2013). Other than that the condition of the medium was submerged fermentation which is favorable for increasing sporulation and α -endotoxin yield.

For the next experiment the biphasic solid state fermentation (SSF) was applied with a view to rapid growth of cells in liquid culture followed by their transfer to solid culture. SSF is best defined as the cultivation of microorganisms on solid substrates devoid or deficient in free water; however, the substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey, 2003). In comparison to submerged fermentation of LBS (monophasic), yield of sporulation and α -endotoxin production was 2.72% higher and 93.86% lower respectively in solid state fermentation of LBS (biphasic) at 48 hours fermentation. This condition remains same till 72 hours where the sporulation was higher in the solid state fermented medium than submerged fermented medium but α -endotoxin yield was lower in the

biphasic solid state condition. Gangurde and Shethna (1995) showed that toxicity (α -endotoxin formation) of *Bt* is proportional to the degree of sporulation. In this case this theory did not hold good. This may be due to the single step extraction process of the cry protein purification method followed in the present work. The major factors that affect microbial synthesis in a biphasic SSF system include: selection of a suitable substrate and microorganism, substrate pre-treatment, particle size, water activity (a_w), size and type of the inoculum, temperature and fermentation time (Pandey *et al.*, 1999). So, further trials are needed to evaluate the unexpected behavior of the microorganism in the solid state fermentation.

There are some complexity of SSF scale up, lack of devices to measure relevant operating variables inside the reactor (i.e. pH, DO, a_w , biomass) and difficulty in metabolic heat removal are factors that impede the technological development of SSF. So for endotoxin production of *Bt* submerged fermentation rather than solid state fermentation was applied later on for this study.

Certain amino acids help in growth, sporulation and α -endotoxin formation. Dipok Vora and Y. I. Shethna, 1999 reported the effect of cystine on the growth, sporulation and toxin production by *Bt* subsp *kurstaki*. In the present study the effect of 300 mg/L cystine was observed in cystine supplemented LB-soybean medium (LBSc) for 72 hours. It could be clearly observed from Figure 3.6 that the presence of cystine in LBS medium enhanced growth, sporulation and α -endotoxin synthesis. It was observed from Table 3.3 that maximum sporulation and endotoxin yield was obtained at 48 hours which was 19.54% and 131.35% higher than the control medium (LBS with no cystine). This increase may be due to the fact that cystine might have interfered with some of the macromolecular changes during sporulation and parasporal crystal formation (Rajalakshmi and Shenthna Y.I., 1980). Lower cystine concentration facilitates sporulation related specific events such as dipicolinic acid synthesis by *Bt* whereas higher concentration inhibits this phenomena. They have showed that maximum sporulation and α -endotoxin concentration were obtained at 300 mg/L of cystine on Cystine Basal Media (CBM) and it was 1523 $\mu\text{g/ml}$.

Substitution of LB with basal salts in defatted soybean meal-cystine medium will lower the cost of the medium. For this purpose the medium was designed accordingly. The medium

supplemented with defatted soybean meal, cystine and basal salts (SMc) showed 9.352 log CFU/ml sporulation whereas in LB-soybean-cystine (LBSc) medium it was 9.06 log CFU/ml. The endotoxin formation was 1.791 mg/ml for LBSc and 1.659 mg/ml for SMc. Though the endotoxin concentration was 7.65% less than that of LB-soybean-cystine medium (LBSc) but it was quite comparable. Basal salt media contains all the necessary salts ($MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CaCl_2$, & KH_2PO_4) which help to promote the growth of *Bacillus thuringiensis*. In the commercial LB media yeast extract is used. Yeast extracts are rich in nitrogen, vitamins and other growth stimulating compounds and therefore are used as an ingredient in media for the cultivation of microorganisms. The nitrogen source of yeast extract-based medium was progressively substituted by soybean meal. With the idea of finding cheap medium for bioinsecticide production soybean could be used cheaply in fermentation industry for insects control programs.

Role of molasses on sporulation and δ -endotoxin synthesis by *Btk* HD-73 was also observed in defatted soybean meal-cystine (SMc) medium for 72 hours (Table 3.5). It has been seen that after 48 hours the spore count and δ -endotoxin concentration both were increased in medium with soybean-molasses-cystine when compared with medium with soybean-cystine. The sporulation and δ -endotoxin formation was 7.857 log CFU/ml and 1.796 mg/ml in soybean-molasses-cystine medium (SMmc). On the other hand, sporulation and δ -endotoxin formation was 7.681 log CFU/ml and 0.726 mg/ml in soybean-cystine medium (SMc) after 24 hours of fermentation. The toxin production was 84.85% higher in SMmc when compared with SMc. This increase in δ -endotoxin yield may be due to balances of C: N ratio and growth factor present in the molasses. Molasses aided the earlier growth of the organism. Moreover, the use of molasses in the production of *Bt* has many advantages. Molasses contains no protein or dietary fibre but contains sucrose, glucose, fructose and carbohydrates. It is available throughout the year, permits high sporulation and biomass production and also it is easy to prepare and to store.

In order to reduce the cost of bioinsecticide production, sea water was used to replace the basal salts (Table 3.6) required for δ -endotoxin production in the culture medium of *Bt*. The δ -endotoxin formation was 2.376 mg/ml and 1.918 mg/ml at 48 hour in the medium with basal salts (SMmc) and in medium with sea water respectively. Though the medium containing basal

salt solutions gave 21% higher α -endotoxin yield in comparison to medium containing sea water, it is more cost effective and suitable for large scale production of biopesticide. D. Ghribi and N, Zouari (2007) formulated a medium with 30 g/l starch and 25 g/l soya bean and diluted sea water. Using four folds diluted sea water; they have found highest (3274 mg/l) α -endotoxin concentration. They had performed the experiment with *Btk* strain BNS3 and they showed 7% improvement of α -endotoxin production with sea water. However, in present study 21% decrease of α -endotoxin occurred with sea water than that with basal salts. It may be due to the composition or source of sea water or due to any seasonal variations or unknown regulatory mechanisms. The α -endotoxin concentration decreased from 1.918 mg/ml to 1.649 mg/ml and from 2.376 mg/ml to 1.246 mg/ml in SSWmc and SMmc media respectively after 48 hour. This may be due to the reason that several proteolytic enzymes were synthesized by *Bacillus* species during growth and sporulation as has been reported (Doi 1972). Ennouri *et al.*, 2013 showed that there is a relationship between α -endotoxin and proteases. Decreasing proteolytic activities in the fermentation medium might increase the accumulation of α -endotoxin in the insecticidal crystal proteins, which deserves further critical investigation.

For the next experiment, defatted soybean meal extract with sea water, molasses and cystine (SeSWmc) was formulated due to the fact that in large scale production there are few drawbacks when soybean mass are used. It produces more foam and thus antifoam is needed to control that. This increases the production cost. The generation of foam during the course of a bioprocess remains a major technological challenge. Soybean extract is advantageous as compared to soybean mass because the latter poses difficulty in mixing and dO_2 availability in the bioreactor cultivation. The SeSWmc medium showed 8.596 log CFU/ml sporulation and 1.386 mg/ml endotoxin concentration after 24 hours whereas SSWmc showed 7.249 log CFU/ml and 1.296 mg/ml endotoxin concentration after 24 hours. Though this concentration remains quite same after 48 hours in SeSWmc medium but it increases 34.11% in SSWmc medium. The soybean extract medium gave highest toxicity within 24 hours of fermentation. Thus it is desirable for large scale production because of the fact that it will reduce the fermentation time at the same time reduce the production cost.

This formulated medium was then used for production of biopesticide by *Btk* HD-73 in a 3L fermenter under comparable controlled conditions. Temperature was controlled at 30°C and pH was kept uncontrolled. Initially the pH was 6.8 and later it became acidic and after 36 hours the pH drop to 4.98 (Figure 3.14). This might be due to acid production by carbohydrate fermentation. For *Btk* HD-73 δ -endotoxin concentration increased after 16 hours as vegetative cells entered into sporulation phase and maximum δ -endotoxin yield was 2317 mg/L at 24 hours. There was a 1.67 fold increase of endotoxin concentration in bioreactor then that of shake flask fermentation. This characteristic of the bacterium suggests that it can be a potential and efficient *Bt* toxin producer and the results of present bioprocess conditions can successfully be applied with indigenous *Bt* strains in the working laboratory.

SDS-PAGE analysis of partially purified Cry protein of the reference strain (*Btk* HD-73) cultured in sea water in soybean extract-molasses-cystine (SeSWmc) broth showed expected protein band of 133 KD (Figure 3.14). According to Hofte and Whiteley, 1989 δ -endotoxin of *Bt kurstaki* HD-73 Cry1Ac has a molecular weight around 125-138 KD. Thicker protein band of 133 KD was observed in the formulated medium.

CONCLUSION

AND

RECOMMENDATIONS

5. Conclusion and Recommendations

The production cost of *Bt* toxin is still a crucial factor in third world countries like Bangladesh. Normally, the raw *Bt* toxin mixed with a suitable surfactant is being formulated for application in to the agricultural field. This would further increase the total cost. The farmers expect the availability of low cost *Bt* toxin without compromising its entomotoxicity values. Here comes the significance of the present findings. The present study was successful to compare the suitability of soybean extract and molasses as nitrogen and carbon sources respectively and to formulate a cost effective medium using these locally available cheap raw materials for large scale production of biopesticide. Sea water that was used as substituent of basal salts was found to have comparable effect on δ -endotoxin synthesis. Moreover, pronounced enhancement of sporulation and δ -endotoxin synthesis by *Bt* strains occurred in presence of cystine in the culture medium. The δ -endotoxin yield in the medium consisting of soybean extract, molasses, and cystine with sea water was maximum after 24 hours fermentation in bioreactor condition using the reference strain (*Btk* HD-73). So the results obtained from this study are very promising and will be very useful to develop efficient *Bt* biopesticides on large scale in Bangladesh. This will also help to reduce hazards in food chain and thereby enhance the food safety which will consequently decrease the health risk.

Future studies will focus on optimization of scale up parameters for maximizing production of *Bt* biopesticides in a cost effective manner. Every possible substrate, starting from household waste to agro-industrial byproducts, could be checked for the feasibility of *Bt* biopesticide production to make it cost effective. After that, indigenous *Bt* strains with higher insecticidal activity will be used for large scale production of crystal protein. Continuous searching for more and more *Bt* with diversities keeps utmost importance in resistance management against *Bt* formulations. So, screening for potential *Bt* strains should be an ongoing process. Bioactivity of crystal proteins purified from the *B. thuringiensis* isolates will be examined on different insect groups. It is imperative to gain a complete understanding of toxin mode of action and the role that receptors play in this mechanism. Because, crystalline genes are mostly carried on the plasmids, plasmid profiles will also be prepared and the *cry* genes, they contain, will be cloned.

CHAPTER

5

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APPENDICES

Appendix-I

Media and Reagents

LB Agar

Ingredients	Amount (g/L)
Tryptone	10
NaCl	10
Yeast extract	5
Distilled water	1000 (ml)
pH	7.0
Agar	15

Directions: Ingredients are dissolved in distilled water by stirring with gentle heating. Medium is sterilized by autoclaving at 121°C for 15 min.

LB Broth

Ingredients	Amount (g/L)
Tryptone	10
NaCl	10
Yeast extract	5
Distilled water	1000 (ml)
pH	7.0

Directions: Ingredients are dissolved in distilled water by stirring. Medium is sterilized by autoclaving at 121°C for 15 min.

Bradford Reagent

A. Stock solution:

300 mg serva blue G (Comassie G-20) was dissolved in 300 ml methanol (BDH, England). 600.0 ml of 85% phosphoric acid (BDH, England) was then added and stirred well.

B. Test solution:

50.0 ml solution was dissolved with 850 ml distilled water and stirred well. The solution was then filtered with whatman filter paper.

Tris-HCL buffer (0.5 M)

Tris (Hydroxymethyl-aminomethane) was dissolved in distilled water to a 0.5 M solution and the pH was adjusted to the appropriate value with conc. HCl.

Phosphate buffer

A. 0.5L of 1M K_2HPO_4 at 174.18g/mol=87.09g (solution A)

B. 0.5L of 1M KH_2PO_4 at 136.09g/mol=68.045g (solution B)

Directions: Mixing of appropriate amount of solution A&B for the desired pH value.

30% acrylamide-bisacrylamide solution

Ingredients	Amount (g/mL)
Acrylamide	29
Bisacrylamide	1
Distilled water	100 (ml)

10% ammonium persulphate (APS)

Ingredients	Amount (g/mL)
APS	1.0 g
Distilled water	10 ml

0.1% BMB (Bromophenol blue solution) or tracking dye

Ingredients	Amount (g/mL)
Bromophenol blue	0.1 g
Distilled water	100 ml

Directions: Stored at 4°C

Staining solution

Ingredients	Amount (g/mL)
Coomassie brilliant blue G-20	0.20 g
10% acetic acid	100 ml

Sample loading buffer

Ingredients	Amount (g/mL)
0.5 M tris-Cl (Upper gel buffer)	10 ml
10% SDS	10 ml
2-mercaptoethanol	1 ml
Glycerol	10 ml
Distilled water	19 ml

Electrophoresis buffer (pH 8.3)

Ingredients	Amount (g/mL)
Tris-base	3.0 g
Glycine	14.4 g
10% SDS	10 ml
Distilled water	1000 ml

Upper gel buffer (pH 8.8)

Ingredients	Amount (g/mL)
Tris-base	18.17 g
SDS	0.4 g
Distilled water	Up to 11 ml

Directions: pH adjusted to 8.8 by adding HCl

Lower gel buffer (pH 8.8)

Ingredients	Amount (g/mL)
Tris-base	18.17 g
SDS	0.4 g
Distilled water	Up to 11 ml

Directions: pH adjusted to 8.8 by adding HCl

Appendix-II

Nutritional value of soybean

Ingredients	Composition
Dry Matter	89%
Crude Protein	48%
Crude Fiber	03.0%
Neutral Detergent Fiber	07.1%

Nutritional value of molasses

Molasses contains no protein or dietary fibre and close to no fat. Each tablespoon (20 g) contains 58 kcal (240 kJ) and other ingredients are:

Ingredients	Composition
Sucrose	5.88 g
Glucose	2.38 g
Fructose	2.56 g
Carbohydrates	14.95 g

Seawater composition (by mass)

Element	Percent
Oxygen	85.84
Sulfur	0.091
Hydrogen	10.82
Calcium	0.04
Chloride	1.94
Potassium	0.04
Sodium	1.08
Bromine	0.0067
Magnesium	0.1292
Carbon	0.0028

Total Molar Composition of Seawater (Salinity = 35)

Component	Concentration (mol/kg)
H ₂ O	53.6
Cl ⁻	0.546
Na ⁺	0.469
Mg ²⁺	0.0528
SO ₄ ⁻²	0.0282
Ca ²⁺	0.0103
K ⁺	0.0102
<u>C_T</u>	0.00206
Br ⁻	0.000844
<u>B_T</u>	0.000416
Sr ²⁺	0.000091
F ⁻	0.000068

Appendix-III

List of Apparatus

Apparatus	Model/ Company
Autoclave	Hirayama model HL-42, AE, Japan
Autoclave for bioreactor	TOUCHCLAVE
Centrifugation	Biofuge Primo (Heraeus) and Hittch-Mikro-Rapid (Zrntrifugen D-72002, Japan)
Electronic balance	DENVER Instrument
Glassware sterilizer	Heraeus model 0042, W. Germany
Incubator	Heraeus model D-6072, W. Germany
Laminar airflow	ESCO vertical Laminar flow cabinet
Magnetic stirrer	CIMAREC
Micropipettes	Eppendorf research and Nichiryo
Orbital shaker	N-BIOTEK incubator
Pumps for aeration	MTH PUMPS
pH meter	INOLAB WTW series
Refrigerator (4°C)	Royal Frestech
Spectrophotometer, DR 4000U	HACH, USA.
Thermo stated shaking water bath	N-BIOTEK and MEMMERT
3 liter bioreactor	NEW BRUNSWICK SCIENTIFIC

Appendix-III

List of Chemicals

Name of chemicals	Source
Acrylamide	Merck, Germany
Agar	Merck, Germany
Ammonium sulfate	BDH, England
Bis-acrylamide	Merck, Germany
BSA	Sigma, USA
CaCl ₂	Merck, Germany
Coomassie blue	Merck, Germany
CuSO ₄	Merck, Germany
Cystine	Sigma, USA
EDTA	BDH, England
Ethanol	Merck, Germany
FeSO ₄	Merck, Germany
HCl	Merck, Germany
K ₂ HPO ₄	Merck, Germany
KH ₂ PO ₄	Merck, Germany
MnSO ₄	Merck, Germany
NaCl	Merck, Germany
NaOH	Merck, Germany
Peptone	Oxoid, England
Phosphoric acid	Merck, Germany
SDS	Wako, Japan
Trizma base	Sigma, USA
Tryptone	Merck, Germany
Yeast Extract	Oxoid, England
ZnSO ₄	Merck, Germany